

# **Pathogenesis of Rett syndrome and study of the role of MeCP2 protein in neuronal function**

Mónica Joana Pinto dos Santos

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Universidade do Porto

Orientadora – Prof. Doutora Patrícia Espinheira de Sá Maciel  
Professora Auxiliar  
ICVS/ECS, Universidade do Minho

Co-orientador – Professor Doutor António Jorge dos Santos Pereira de Sequeiros  
Professor Catedrático  
Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto

Co-orientadora – Professora Doutora Maria Amélia Duarte Ferreira  
Professora Catedrática  
Faculdade de Medicina, Universidade do Porto



Aos meus pais

“A lua anda devagar, mas atravessa o mundo”  
(Provérbio Africano)



## Preceitos Legais

De acordo com o disposto no nº 2 do artigo 8º do Decreto-lei nº 388/70, nesta dissertação foram utilizados os resultados dos trabalhos publicados ou em preparação abaixo indicados. No cumprimento do disposto no referido Decreto-lei, a autora desta dissertação declara que interveio na concepção e execução do trabalho experimental, a interpretação dos resultados e na redacção dos resultados publicados ou em preparação, sob o nome de Santos M:

Based on the nº 2 do artigo 8º do Decreto-lei nº 388/70, in this dissertation were used experimental results published or under preparation stated below. The author of this dissertation declares that participated in the planification and execution of the experimental work, in the data interpretation and in the preparation in the work stated below, under the name Santos M:

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## Resumo

A síndrome de Rett (RTT) é uma doença do neurodesenvolvimento que afecta quase exclusivamente meninas. Depois de um período de aparente desenvolvimento normal entre 6-18 meses segue-se uma paragem no desenvolvimento seguida de uma deterioração das capacidades motora, autonómica, social e intelectual. As pacientes com RTT apresentam doença do movimento (ataxia e apraxia), comportamento autista, estereotipias manuais e atraso mental. Além desta apresentação dita clássica da síndrome, as formas variantes incluem fenótipos mais suaves e outros mais graves, assim como uma forma variante que afecta meninos, geralmente mais grave devido à hemizigotia do cromossoma X.

Mutações no gene que codifica uma proteína de ligação aos metil-CpG (*MECP2*) são a causa primária de RTT (>90% nos casos clássicos e 30% nos atípicos). No entanto, mutações no *MECP2* também foram encontradas, com uma frequência mais baixa, em indivíduos com outras doenças do neurodesenvolvimento parcialmente sobrepostas a RTT, como por exemplo autismo, atraso mental não sindrómico e síndrome de Angelman. As mutações no *MECP2* ocorrem por todo o gene e são de vários tipos. Apesar disto, uma proporção significativa de casos com RTT permanece sem uma causa genética identificada, o que sugere o envolvimento de regiões não codificantes do *MECP2* ou de outros genes nesta patologia.

A principal função da proteína MeCP2 é a de repressora da transcrição. A MeCP2 liga-se ao DNA metilado e actua recrutando as proteínas Sin3A e histonas desacetilases formando-se um complexo que vai desacetilar as histonas e assim reprimir a transcrição. Mutações na MeCP2 vão assim causar uma desregulação da transcrição de genes alvo. No entanto, outras funções da MeCP2 podem também ser afectadas, uma vez que certas mutações na MeCP2 que não afectam a sua capacidade de repressão ocorrem em locais de ligação da MeCP2 a outras proteínas.

O nosso objectivo neste estudo é “mapear” a ocorrência de certas mutações no gene *MECP2* fazendo-as corresponder a determinados fenótipos nos humanos e no ratinho para assim melhor compreender o mecanismo patogénico subjacente à variabilidade fenotípica de RTT, em particular à disfunção motora.

No nosso estudo Genético da população Portuguesa com RTT ou com doenças do neurodesenvolvimento relacionadas identificámos diferentes tipos de mutações no *MECP2*, distribuídas por todo o gene. Dada a ausência de uma correlação genótipo-fenótipo significativa em estudos anteriores, tentámos uma abordagem original a este

problema baseada no efeito funcional previsto e observado das diferentes mutações no *MECP2*. Encontramos uma correlação interessante entre a ocorrência de mutações que anulam a expressão da proteína e mutações que eliminam a capacidade de repressão da MeCP2 com formas mais graves da doença, em que predominam sinais extrapiramidais. Por outro lado, mutações com um efeito mais suave, como a R133C, predominam em formas da doença onde o atraso mental é o sintoma cardinal.

Modelos animais de RTT foram criados em ratinho que mitigam a doença em muitos aspectos como a disfunção motora, problemas intelectuais e anomalias do comportamento emocional e social; tal como as doentes, os ratinhos mutantes nascem aparentemente normais e os sintomas evidenciam-se mais tarde. A correlação genótipo-fenótipo que encontrámos dos doentes com RTT também parece aplicar-se nos modelos mutantes da *Mecp2* no ratinho.

Apesar da descrição clássica de RTT, certos investigadores sempre se questionaram se as doentes com RTT não apresentariam manifestações subtis logo após o nascimento; de facto, recentemente foram descritas anomalias no desenvolvimento inicial das doentes com RTT, desde os primeiros dias após o nascimento. De forma a averiguar se nos ratinhos mutantes *Mecp2*, tal como nas doentes, o período do neurodesenvolvimento inicial era anormal, realizámos um estudo do neurodesenvolvimento pós-natal nestes ratinhos mutantes. Encontrámos diferenças subtis, mas significativas que eram dependentes do sexo, entre ratinhos mutantes *Mecp2* e controlos na aquisição e/ou estabelecimento de reflexos neurológicos. Os reflexos neurológicos são indicadores da maturação normal do cérebro e as alterações que nós encontrámos nos ratinhos mutantes *Mecp2* podem ser manifestações precoces de sintomas neurológicos posteriores. Estes dados levaram-nos de seguida a caracterizar o perfil locomotor dos ratinhos nulizigóticos para *Mecp2*, que aparentemente está já comprometido em estadios precoces. Assim, explorámos o estabelecimento e a progressão do défice motor e tentámos dissecar a sua origem. A performance dos ratinhos KO para *Mecp2* foi avaliada em diferentes paradigmas que avaliam a função motora e verificámos que já desde as três semanas de idade, os ratinhos KO para *Mecp2* apresentam problemas na marcha, dadas as anomalias no estabelecimento do início da marcha e no tipo de marcha. Às quatro semanas de idade, os ratinhos mutantes apresentavam-se hipoactivos provavelmente devido aos défices motores. Finalmente, às cinco semanas de idade, descoordenação motora foi também identificada. Estes défices motores sugerem potencialmente um envolvimento do tronco cerebral, do cerebelo, do estriado e do córtex na patogénese de RTT.

O envolvimento de múltiplos sistemas poderá sugerir uma disfunção dos sistemas modulatórios monoaminérgicos do cérebro na patogénese de RTT. De facto, a desregulação de neurotransmissores como a norepinefrina, a dopamina e a serotonina foi por várias vezes, mas nem sempre consistentemente, descrita nos cérebros e no líquido cerebroespinal de doentes com RTT. Uma redução global nos níveis de monoaminas foi também encontrada nos ratinhos KO para *Mecp2*. De modo a clarificar a contribuição destes sistemas para a diferente sintomatologia apresentada em RTT, realizámos um estudo neuroquímico de diferentes regiões cerebrais do ratinho KO potencialmente envolvidas na patogénese tipo-RTT, em dois momentos diferentes, antes (três semanas de idade) e depois (8 semanas de idade) do estabelecimento de sintomas mais graves. Verificámos que tanto os sistemas serotoninérgico como o noradrenérgico estavam afectados, mostrando uma redução nos níveis de neurotransmissores desde as três semanas de idade. Verificámos também que o córtex pré-frontal e o córtex motor eram as regiões primariamente afectadas, enquanto o hipocampo e o cerebelo poderão estar envolvidos em fases mais tardias da doença.

O atraso mental é um dos sintomas cardinais em RTT, com a maioria das pacientes apresentando défices intelectuais moderados a profundos. Adicionalmente, factores que se sabe estarem envolvidos na regulação da neurogénese pós-natal no hipocampo, como neurotransmissores, neurotrofinas, hormonas esteróides e actividade neuronal, estão também alterados nas doentes com RTT e nos modelos em ratinho da doença. Neste trabalho avaliámos a neurogénese pós-natal no giro denteado do hipocampo em ratinhos KO para *Mecp2* e verificámos que esta estava aumentada nos ratinhos mutantes em comparação com os controlos. Na nossa interpretação dos dados, isto pode ser uma consequência de uma redução global da actividade neuronal nesta região. Um aumento da neurogénese pós-natal não é necessariamente benéfico, sendo necessários estudos adicionais para se concluir acerca das consequências deste achado.

Os dados resultantes deste trabalho contribuíram para uma maior compreensão dos substratos neuronais subjacentes aos primeiros défices motores exibidos pelos ratinhos KO para *Mecp2*, um dos modelos de estudo de RTT, e poderão contribuir para uma melhor compreensão desta doença.



## Abstract

Rett syndrome (RTT) is a neurodevelopmental disorder that affects mainly girls. It features a period of apparently normal development during 6-18 months followed by an arrest in development with further deterioration of motor, autonomic and social and cognitive skills. RTT females present with a movement disorder (ataxia and apraxia), autistic behaviour, hand stereotypies and mental retardation. Besides this classical form of the syndrome, variant forms may comprise milder or more severe presentations, as well as the male phenotype, usually more severe due to hemizyosity of the X chromosome. Mutations in the methyl-CpG binding protein 2 gene (*MECP2*) are the primary cause of RTT (>90% in classical and 30% in atypical RTT cases). However, to a lower extent, mutations in *MECP2* have also been identified in patients with other, partially overlapping neurodevelopmental disorders, such as autism, non-syndromic mental retardation and Angelman syndrome. *MECP2* mutations occur throughout the entire gene and are of all types. Nevertheless, a significant proportion of RTT cases remain without a genetic explanation, which suggests the involvement of non-coding *MECP2* regions or other genes in this pathology.

The major role of MeCP2 protein is as a transcriptional repressor. MeCP2 binds to methylated DNA and acts through the recruitment of Sin3A and histone deacetylases to form a complex that will deacetylate histones in order to repress transcription. Mutations in the MeCP2 will cause a dysregulation in transcription of target genes. Nevertheless, other function(s) of MeCP2 may also be affected as some mutations in the MeCP2 that do not impair its repression capacity, occur in sites of MeCP2 binding to other proteins.

Our goal in this study is to “map” specific mutations in the *MECP2* gene with a specific phenotype in human and mice and to understand the pathogenic mechanism underneath this phenotypic variability, in particular in the motor impairment.

In our Genetic study of Portuguese patients with RTT or with related neurodevelopmental disorders we identified different types of mutations in the *MECP2* gene, distributed throughout the entire gene. Given the lack of a significant phenotype-genotype correlation in previous studies, we attempted an original approach to this question based on the predicted and observed functional effect of the different *MECP2* mutations. We found an interestingly correlation between null alleles and mutations that completely abolish the repression capacity of MeCP2 with a more severe form of the disorder, where extrapyramidal signs predominate. On the other hand, mutations with a milder effect, such

as R133C, seem to predominate in the forms of the disease where mental retardation is the cardinal feature.

Animal models of RTT were created in mice that mimic the disorder in many aspects such as motor dysfunction, cognitive defects and abnormalities of the emotional and social behaviour; as patients, mutants are born apparently normal and the symptoms become evident later. Impressively, the genotype-phenotype correlation that we found in the RTT patients also seem to apply in the *Mecp2*-mutant models.

Despite the classical RTT description, researchers always questioned whether RTT patients did have subtle manifestations soon after birth; in fact abnormalities in the early development of RTT patients were recently described to be present from the first days after birth. In order to address whether in the *Mecp2*-mutant mouse model, as in patients, the early neurodevelopmental period was abnormal, we performed a postnatal neurodevelopmental study in these mutant mice. We found subtle but significant sex-dependent differences between *Mecp2*-mutant and wild type animals in the acquisition and/or establishment of neurological reflexes. Neurological reflexes are good indicators of normal brain maturation and the impairments we found in the *Mecp2*-mutant mice could be early manifestations of later neurological symptoms. This led us to further characterize the locomotor profile of the *Mecp2*-null mice, which apparently is already compromised at a precocious stage. Hence, we explored further the onset and progression of the motor impairment and attempt to dissect its nature. We assessed the *Mecp2*-null mice performance in different paradigms that assess motor function and we found that already from the three-weeks of age *Mecp2*-null mice exhibited an impaired gait, as given by abnormalities in gait onset and gait pattern. At four-weeks of age hypoactivity was noticed that was probably due to the motor impairments. Finally, at five weeks of age motor de-coordination was also detected. These behavioural motor impairments suggested a potential involvement of the brainstem, cerebellum, striatum and cortex in the RTT pathology.

The involvement of a range of systems may suggest that a dysfunction of the modulatory monoaminergic brain systems of the brain in RTT pathophysiology. In fact, a deregulation of neurotransmitters such as norepinephrine, dopamine and serotonin have repeatedly, although not always consistently, been shown to be altered in the brain and cerebrospinal fluid of RTT patients. A global reduction in the monoamine levels was also found in the *Mecp2*-null mice. In order to clarify the contribution of monoamines to the different clinical components of the RTT phenotype, we performed a neurochemical study of different brain regions of the *Mecp2*-null mouse potentially playing a role in RTT-like pathophysiology, at



two different timepoints: before ( three-weeks of age) and after (eight-weeks of age) the establishment of overt symptoms.

We found that both the serotonergic and noradrenergic systems are affected, showing a reduction in the levels of the neurotransmitters already at three weeks of age. Additionally, we verified that the prefrontal and motor cortices were the primarily affected regions, whereas the hippocampus and cerebellum may play a role in later stages of the disorder.

Mental retardation is one of the cardinal features in RTT, with most of the affected patients presenting moderate to profound cognitive impairments. Additionally, factors known to regulate postnatal hippocampal neurogenesis, such as neurotransmitters, brain-derived neurotrophic factor, steroid hormones and neuronal activity, were found to be altered, both in RTT patients and in mouse models of the disorder. We assessed dentate gyrus hippocampal neurogenesis in four-week-old *Mecp2*-null mice, and found it to be increased in the *Mecp2*-null mice as compared to wt controls. In our interpretation, this may be a consequence of a globally reduced neuronal activity in this brain region. Increased neurogenesis may not necessarily be beneficial and further studies are needed in order to elucidate on the consequences of this finding.

The evidence produced with this work improved our understanding of the neural basis of the first motor impairments present in the *Mecp2*-null mouse, a model of RTT, and may contribute to a better understanding of this disorder.



## Resumé

Le syndrome de Rett (RTT) est une maladie du neurodéveloppement qui affecte presque exclusivement les filles. Après une période de développement apparemment normal, vers les 6-18 mois apparaît un arrêt du développement suivi d'une détérioration des capacités motrice, autonome, sociale et intellectuelle. Les patients avec RTT présentent une maladie du mouvement (ataxie et apraxie), comportement autiste, stéréotypies manuelles et retard mental. Hors cette présentation dite classique du syndrome, les formes variantes incluent des phénotypes plus légers et d'autres plus graves, ainsi qu'une variante qui affecte des garçons, et qui est en général plus grave, à cause de l'hémizygotie du chromosome X.

Des mutations chez le gène qui codifie une protéine de liaison aux methyl-CpG (MECP2) sont la cause primaire de RTT (>90% chez les cas classiques et 30% chez les atypiques). Toutefois, des mutations chez le MECP2 ont aussi été trouvées, moins fréquemment, chez des malades avec d'autres maladies du neurodéveloppement partiellement superposées à RTT, comme l'autisme, le retard mental non syndromique et le syndrome d'Angelman. Les mutations dans MECP2 se produisent partout dans le gène et sont de plusieurs types. Cependant, une proportion significative de cas avec RTT reste sans cause génétique identifiable, ce qui suggère l'engagement de régions non codantes de MECP2 ou d'autres gènes chez cette pathologie.

La principale fonction de la protéine MeCP2 est celle de répresseur de la transcription. La MeCP2 se lie au ADN méthylé et agit en recrutant les protéines Sin3A et les désacétylases des histones, formant un complexe qui va désacétyler les histones, de façon à réprimer la transcription. Des mutations chez la MeCP2 vont ainsi causer une dérégulation de la transcription des gènes cibles. Cependant, d'autres fonctions de la MeCP2 peuvent aussi être affectées, une fois que certaines mutations chez la MeCP2 qui n'affectent pas sa capacité de répression se produisent en sites de liaison de la MeCP2 à d'autres protéines.

Notre objectif dans cette étude était de faire correspondre certaines mutations dans le gène MECP2 à un certain phénotype chez les humains et chez la souris, et aussi comprendre le(s) mécanisme(s) pathogénique(s) sous-jacent(s) à la variabilité phénotypique de RTT.

Dans notre étude génétique de la population portugaise avec RTT ou avec d'autres maladies du neurodéveloppement semblables à RTT, nous avons identifié plusieurs types de mutation dans MECP2, distribués par tout le gène. Vue l'absence d'une corrélation

genotype-phénotype significative chez des études préalables, nous avons essayé un abordage original à ce problème, basé à l'effet fonctionnel prévu et observé des différentes mutations dans MECP2. Nous avons trouvé une association intéressante entre la présence de mutations qui anulent l'expression la protéine et de mutations qui détruisent complètement la capacité de répression de la MeCP2 et les formes les plus graves de la maladie, où les signes extrapyramidaux prédominent. Par contre, des mutations avec un effet plus léger, comme R133C, prédominent chez les formes de la maladie où le retard mental est le symptôme cardinal.

Des modèles animaux de RTT ont été créés chez la souris qui imitent la maladie en plusieurs aspects, tels que la dysfonction motrice, problèmes intellectuels et anomalies de la conduite émotive et sociale; comme les malades, les souris mutantes sont nées apparemment normales et les symptômes se rendent évidents plus tard. La corrélation génotype-phénotype que nous avons trouvé chez les patients avec RTT apparaît s'appliquer aussi aux modèles mutants du MeCP2 chez la souris.

Malgré la description classique de RTT, certains investigateurs se sont toujours questionnés si les malades avec RTT ne présenteraient-elles pas des manifestations subtiles dès qu'elles sont nées; en fait, des anomalies pendant le développement initial des malades avec RTT, dès les premiers jours après la naissance, ont été décrites récemment. Pour vérifier si chez les souris mutantes MeCP2, comme chez les malades, la période de neurodéveloppement initial était anormal, on a fait une étude du neurodéveloppement postnatal chez les souris mutantes.

Nous avons trouvé des différences dépendantes du genre, subtiles mais significatives, entre animaux mutants MeCP2 et animaux sauvages, à l'acquisition et/ou établissement de réflexes neurologiques. Les réflexes sont de bons indicateurs d'une maturation cérébrale normale, et les déficiences observées chez la souris mutante MeCP2 peuvent être manifestations précoces de futurs symptômes neurologiques. Ceci nous a mené à caractériser davantage le profil locomoteur de la souris KO pour MeCP2, qui apparemment est déjà troublé à un stade précoce. Ainsi, nous avons exploré davantage le début et la progression de l'affaiblissement moteur et les efforts pour disséquer leur nature. Nous avons évalué la performance des souris sans MeCP2 en différents paradigmes qui évaluent la fonction motrice et nous avons observé que déjà dès l'âge de trois semaines les souris sans MeCP2 présentaient une marche handicapée, marquée par les anomalies du début de la marche et la configuration de la marche. À l'âge de quatre semaines, une hypoactivité a été observée, probablement originée par des déficiences motrices. Finalement, à l'âge de cinq semaines, on a aussi détecté une

décoordination motrice. Ces déficiences motrices comportementales ont suggéré un potentiel engagement du tronc cérébral, du cervelet, du striatum et du cortex chez la pathologie RTT.

L'engagement d'une série de systèmes peut suggérer une dysfonction des systèmes modulateurs monoaminergiques du cerveau chez la pathophysiologie de la RTT. En fait, la dérégulation des neurotransmetteurs tels que la noradrénaline, la dopamine et la sérotonine ont plusieurs fois, si bien que pas toujours de façon consistante, présenté altération dans le cerveau et dans le liquide céphalo-rachidien chez les malades avec RTT. Une réduction globale aux niveaux de monoamine a aussi été observée chez les souris sans MeCP2. Pour éclaircir la contribution des monoamines pour les différents composants cliniques du phénotype RTT, une étude neurochimique a été faite sur les plusieurs régions cérébrales de la souris KO pour MeCP2, qui potentiellement jouent un rôle dans la pathophysiologie de RTT, en deux moments différents: avant (âge de trois semaines) et après (âge de huit semaines) l'établissement de symptômes évidents.

On a observé que les systèmes sérotoninergique et noradrénergique sont affectés, montrant une réduction des niveaux des neurotransmetteurs, déjà à l'âge de trois semaines. On a aussi vérifié que le cortex préfrontal et moteur étaient les régions principalement affectées, tandis que l'hippocampe et le cervelet peuvent jouer un rôle aux stades plus tardifs de la maladie.

Le retard mental est une des caractéristiques cardinales de RTT, la plupart des malades présentant des handicaps cognitifs modérés à profonds. Aussi, des facteurs régulateurs de la neurogénèse de l'hippocampe, comme la sérotonine, la noradrénaline, le facteur neurotrophique dérivé du cerveau, les hormones stéroïdes et l'activité neuronale, ont présenté des modifications, chez des malades avec RTT et chez les modèles souris de la maladie. Une évaluation de la neurogénèse au dentate gyrus de l'hippocampe à l'âge de quatre semaines chez les souris KO pour MeCP2, par comparaison avec les contrôles de type sauvage. Selon notre interprétation, cela peut être conséquence d'une activité neuronale globalement réduite dans cette région cérébrale. La neurogénèse augmentée peut ne pas être nécessairement bénéfique et il faut d'autres études pour éclaircir sur les conséquences de cette découverte.

Les nouvelles données produites avec ce travail ont amélioré notre compréhension de la base neuronale des premiers handicaps moteurs présents chez la souris KO pour MeCP2, un modèle de RTT, et peuvent contribuer pour une meilleure compréhension de cette maladie.



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## Appendix II – Published articles

**Article 1** - Santos M, Coelho PA, Maciel P. “Chromatin remodelling and neuronal function: exciting links”. *Genes Brain and Behavior* **2006** 5(suppl. 2): 80-91.

**Article 2** - Shi J, Shibayama A, Liu Q, Nguyen VQ, Feng J, Santos M, Temudo T, Maciel P, Sommer SS. “Detection of heterozygous deletions and duplications in the *MECP2* gene in Rett syndrome by Robust Dosage PCR (RD-PCR)”. *Hum Mutat* **2005** May; 25(5):505.

**Article 3** - Venâncio M, Santos M, Pereira SA, Maciel P, Saraiva. "An explanation for another familial case of Rett syndrome: maternal germline mosaicism". *Eur J Hum Genet.* **2007** Aug 15(8):902-4.

**Article 4** - Temudo T, Oliveira P, Santos M, Dias K, Vieira JP, Moreira A, Calado E, Carrilho I, Oliveira G, Levy A, Barbot C, Fonseca MJ, Cabral A, Dias A, Lobo Antunes N, Cabral P, Monteiro JP, Borges L, Gomes R, Barbosa C, Santos M, Mira G, Andrada G, Freitas P, Figueiroa S, Sequeiros J and Maciel P. "Stereotypies in Rett Syndrome: analysis of 83 patients with and without detected *MECP2* mutations". *Neurology* **2007** April 10; 60(15):1183-7.

**Article 5** - Coutinho AM, Oliveira G, Katz C, Feng J, Yan J, Yang C, Marques C, Ataíde A, Miguel TS, Temudo T, Santos M, Maciel P, Sommer SS and Vicente AM. "*MECP2* coding sequence and 3'UTR variation in 172 unrelated autistic patients". *Am J Med Genet – Part B Neuropsychiatr Genet* **2007** Jun 5, 144(4): 475-83.

**Article 6** - Santos M, Silva-Fernandes A, Oliveira P, Sousa N and Maciel P. "Evidence for abnormal early development in a mouse model of Rett syndrome". *Genes Brain & Behavior*, **2007** Apr 6(3): 277-86.

## Abbreviations

|          |                                                       |
|----------|-------------------------------------------------------|
| 3'UTR    | 3' untranslated region                                |
| 5-HIAA   | 5-Hydroxyindoleacetic acid                            |
| 5-HT     | 5-hydroxytryptophan (serotonin)                       |
| μL       | microlitter                                           |
| Adra2a   | adrenergic receptor α, subunit 2a                     |
| Adrb2    | adrenergic receptor β, subunit 2                      |
| AMPA     | α-amino-3-hydroxy-5-methylisoxazole-4- propionic acid |
| AS       | Angelman syndrome                                     |
| ATRX     | α-thalassemia, mental retardation syndrome, X-linked  |
| BDNF     | brain derived neurotrophic factor                     |
| BrdU     | 5-bromodeoxyuridine                                   |
| CA1      | Cornus Ammon                                          |
| CPu –    | caudate-putamen                                       |
| CpG      | cytosine-phosphodiester-guanine                       |
| Crh      | corticotrophin-releasing hormone gene                 |
| CSF      | cerebrospinal fluid                                   |
| CNS      | central nervous system                                |
| DA       | dopamine                                              |
| DAB      | diaminobenzidine                                      |
| DG       | dentate gyrus                                         |
| DLX5/6   | distal-less homeobox 5/6                              |
| D/MRN    | dorsal/medial raphe nuclei                            |
| DNMT1    | DNA methyl transferase 1                              |
| DNMT3A/B | DNA methyl transferase 3 alpha/beta                   |
| DOPAC    | 3,4-Dihydroxyphenylacetic acid                        |
| DOVAM-S  | detection of virtually all mutations by SSCP          |
| EDTA     | ethylenediaminetetracetic acid                        |
| EEG      | electroencephalogram                                  |
| EPM      | elevated plus maze                                    |
| GABA     | gamma-aminobutyric acid                               |
| GABRB3   | GABA A receptor β3 subunit                            |
| GFAP     | glial fibrillary acidic protein                       |
| HDAC     | histone deacetylase                                   |

|         |                                                                 |
|---------|-----------------------------------------------------------------|
| HPLC-EC | high-pressure liquid chromatography – electrochemical detection |
| Htr1a   | serotonin receptor, subunit 1a                                  |
| Htr2a   | serotonin receptor, subunit 2a                                  |
| Htr2b   | serotonin receptor, subunit 2b                                  |
| Htr3a   | serotonin receptor, subunit 3a                                  |
| HPA     | hypothalamus-pituitary-adrenal                                  |
| Hprt    | hypoxanthine guanine phosphoribosyl transferase                 |
| HVA     | 4-hydroxy-3-methoxy-phenylacetic acid; homovanillic acid        |
| KA      | kainate                                                         |
| Kb      | kilobase                                                        |
| Ko      | knock out                                                       |
| LTD     | long-term depression                                            |
| LTP     | long-term potentiation                                          |
| MBD     | methyl-CpG binding domain                                       |
| MBD1    | methyl-CpG binding protein 1                                    |
| MCx     | motor cortex                                                    |
| MECP2   | methyl-CpG binding protein 2 gene                               |
| MeCP2   | methyl CpG-binding protein 2                                    |
| MRI     | magnetic resonance imaging                                      |
| NE      | norepinephrine                                                  |
| NET     | norepinephrine transporter                                      |
| NeuN    | neuronal specific marker                                        |
| NLGN3/4 | neuroligin 3/4 gene                                             |
| NLS     | nuclear localization signal                                     |
| mEPSCs  | miniature excitatory postsynaptic currents                      |
| NMDA    | n-methyl d-aspartate receptor                                   |
| OF      | open field                                                      |
| PAGE    | polyacrylamide gel electrophoresis                              |
| PBS     | phosphate buffered saline                                       |
| PCR     | polymerase chain reaction                                       |
| PFA     | paraformaldehyde                                                |
| PFCx    | prefrontal cortex                                               |
| PND     | postnatal day                                                   |
| PWS     | Prader-Willi syndrome                                           |
| qRT-PCR | quantitative real-time PCR                                      |

|        |                                                                   |
|--------|-------------------------------------------------------------------|
| RD-PCR | robust dosage PCR                                                 |
| RG     | Arginine-glycine stretch                                          |
| RTT    | Rett syndrome                                                     |
| SEM    | standard error mean                                               |
| SERT   | serotonin transporter                                             |
| SN-VTA | ventral mesencephalon (substantia nigra - ventral tegmental area) |
| SGI    | subgranular zone infrapyramidal                                   |
| SGS    | subgranular zone suprapyramidal                                   |
| SGZ    | subgranular zone                                                  |
| SSCP   | single strand conformation polymorphism                           |
| SVZ    | sub-ventricular zone                                              |
| TBS    | tris buffered saline                                              |
| TdT    | terminal deoxynucleotidyl transferase                             |
| TE     | Tris-EDTA                                                         |
| TSR    | template suppression reagent                                      |
| TRD    | transcription repression domain                                   |
| UV     | ultraviolet                                                       |
| UBE3A  | ubiquitin protein ligase E3A                                      |
| VEGF   | vascular endothelial growth factor                                |
| Wt     | wild type                                                         |
| WW     | group II WW binding domain                                        |
| XCI    | X-chromosome inactivation                                         |
| YB-1   | Y-box binding protein 1                                           |





## CHAPTER 1

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### GENERAL INTRODUCTION

Part of this chapter is included in the following peer reviewed article:

Mónica Santos, Paula Coelho and Patrícia Maciel. "Chromatin remodelling and neuronal function: exciting links". *Genes Brain & Behavior*, **2006** 5(suppl. 2): 80-91.

Another manuscript is also in preparation, an invited review to be included in a special issue of *Genes Brain & Behavior* on the theme "Behaviour pathologies: biological approaches":

Mónica Santos and Patrícia Maciel. "Mouse models of RTT: how well do they mimic the disorder?"



Brain development begins during foetal life and proceeds until childhood, through a series of well-orchestrated events of cell proliferation, migration and maturation. However, the brain is a dynamic structure, in which structural/functional adaptation (plasticity) also occurs throughout the lifetime in response to the surrounding environment. In humans, brain development starts after conception and it is completed postnatally, as young adults (for a review see Toga et al. 2006). The first two decades of life are critical and can have a major impact in the mature human brain function.

Neurodevelopmental disorders are a group of diseases that result from an injury to the developing brain, which can be of genetic, environmental or multifactorial origin. Children with developmental disabilities frequently arrest their maturation at a given stage, from which they do not proceed to a higher level. One of the main features that patients with neurodevelopmental disorders share is cognitive impairment, mostly due to the perturbation of cortical development.

### **1.1. Rett syndrome**

Over forty years have passed since the first description of Rett syndrome (RTT; OMIM #312750) by Andreas Rett (Rett 1966), but it was only twenty years later that the disorder was internationally recognized through the work of Hagberg and colleagues (1983), who described a group of 35 affected girls from Sweden, Portugal and France. RTT is a pervasive developmental disorder that affects mainly girls, and is distributed worldwide; it is a predominantly neurological disorder, yet the phenotype also includes somatic growth failure. RTT is a major cause of inherited mental retardation in females, affecting 1/10,000 to 1/22,000 girls (Hagberg 1985; Kozinetz et al. 1993). Most of the RTT cases are sporadic; however, some familial cases have also been described (about 1%) (Zoghbi 1988).

#### **1.1.1. Clinical presentation**

RTT is characterized by cognitive and behavioural disturbances (mental retardation with notable deficits in language, autism and the characteristic stereotypic hand movements), motor impairment (apraxia, dyspraxia and ataxia) and autonomic dysfunction (breathing irregularities, sleep and gastrointestinal disturbances). Today, the

---

diagnosis of RTT has to rely on a battery of characteristic and co-existing clinical criteria, and a sequence of stages (Hagberg et al. 1983; Hagberg et al. 2002), combined with a procedure of differential diagnostic exclusions and molecular testing. In addition to the necessary criteria (table 1.1), there are a number of main, supportive and exclusion criteria (tables 1.2 and 1.3), that must be taken into account when considering RTT as a diagnosis.

**Table 1.1.** Necessary criteria in the diagnosis of classical Rett syndrome (adapted from Hagberg et al. 2002).

| Manifestation                        | Age                  | Comments                                                                                   |
|--------------------------------------|----------------------|--------------------------------------------------------------------------------------------|
| Infant apparently normal initially   |                      | - Pre-/perinatal period as well as first 6 months of life or longer                        |
| Head circumference stagnation        | 3 months - 4 year    | - Normal at birth, then a decelerating growth rate                                         |
| Purposeful hand skill loss           | 9 months - 2.5 years | - Communicative dysfunction, social withdrawal, mental deficiency, loss of speech/babbling |
| Classical stereotypic hand movements | after 1 - 3 years    | - Hand washing/wringing or clapping/tapping                                                |
| Gait/ posture dyspraxia              | 2 - 4 years          | - Gait "ataxia"/more or less jerky truncal "ataxia"                                        |

**Table 1.2.** Main (A) and supportive (B) criteria in the diagnosis of atypical Rett syndrome (adapted from Hagberg et al. 2002).

|                                                                          |                                                                                             |
|--------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|
| The child has to present at least 3 of the 6 main manifestations         |                                                                                             |
| A1                                                                       | Loss of (partial or subtotal) acquired fine-finger skill in late infancy/early childhood    |
| A2                                                                       | Loss of acquired single words/phrases/ nuance babble                                        |
| A3                                                                       | RTT hand stereotypies, hands together or apart                                              |
| A4                                                                       | Early deviant communicative ability                                                         |
| A5                                                                       | Deceleration in head growth of 2 standard deviations (even when still within normal limits) |
| A6                                                                       | Follow the RTT syndrome disease profile                                                     |
| The child has to present at least 6 of the 11 supportive manifestations. |                                                                                             |
| B1                                                                       | Breathing irregularities (hyperventilation and/or breath-holding)                           |
| B2                                                                       | Bloating/marked air swallowing                                                              |
| B3                                                                       | Characteristic RTT teeth grinding                                                           |
| B4                                                                       | Gait dyspraxia                                                                              |
| B5                                                                       | Neurogenic scoliosis or high kyphosis (ambulant girls)                                      |
| B6                                                                       | Developmental of abnormal lower limb neurology                                              |
| B7                                                                       | Small blue/cold impaired feet, autonomic/trophic dysfunction                                |
| B8                                                                       | Characteristic RTT EEG development                                                          |
| B9                                                                       | Unprompted sudden laughing/screaming spells                                                 |
| B10                                                                      | Impaired/delayed nociception                                                                |
| B11                                                                      | Intensive eye communication - "eye pointing"                                                |

**Table 1.3.** Exclusion criteria in the diagnosis of Rett syndrome (adapted from Hagberg et al. 1985).

|                                                                                 |
|---------------------------------------------------------------------------------|
| Evidence of intrauterine growth retardation                                     |
| Organomegaly or other signs of storage disease                                  |
| Retinopathy or optic atrophy                                                    |
| Evidence of perinatally acquired brain damage                                   |
| Existence of identifiable metabolic or other progressive neurological disorder  |
| Acquired neurological disorders resulting from severe infections or head trauma |

The “classical” progression of RTT develops in four stages (figure 1.1), following an apparently normal development with uneventful pre- and perinatal periods (around 6 to 18 months), where some of the patients learn some words and some are able to walk and feed themselves (Kerr and Engerstrom 2001). In stage I, a deceleration/arrest in the psychomotor development is noticed, after an initial “normal” development; in stage II, there is a loss of previously acquired skills, establishment of autistic behaviour and signs of intellectual dysfunction; hand skilful abilities are lost and replaced by stereotypical hand movements, a hallmark of RTT. The pre-school/school years correspond to stage III (pseudo-stationary stage), when some improvement may be appreciated, with partial recovery of previously acquired skills. This is later followed by the progressively incapacitating stage IV, which can last for years (Hagberg et al. 2002); at this final stage, patients develop trunk and gait ataxia, dystonia, autonomic dysfunction and many of them have a sudden unexplained death, in adulthood.

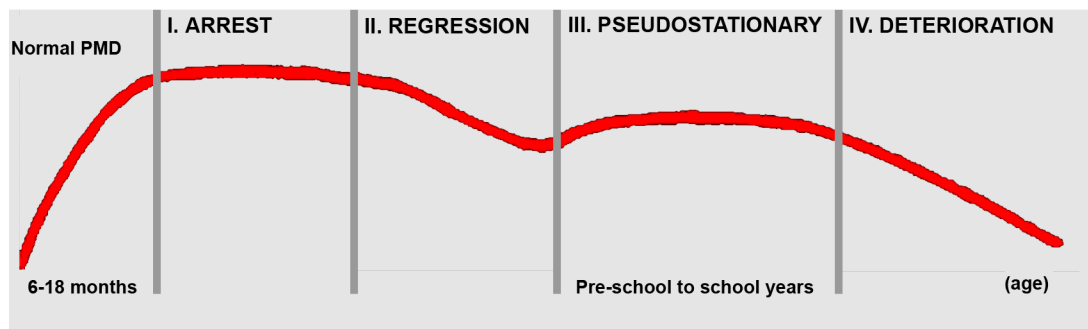


**Figure 1.1. Temporal profile of Rett syndrome disorder.** After an initial apparently normal developmental period, the disorder progresses in four stages (I – IV). (PMD – psychomotor development).

One of the hallmarks of RTT is the presence of hand stereotypies, which include wringing, twisting and clapping (Hagberg et al. 2002). In addition to these there is an enormous variety of different hand stereotypies, in most cases in the midline and also stereotypies involving other parts of the body (Temudo et al. 2007); all are absent during

sleep. In the majority of RTT patients, the appearance of hand stereotypies coincides with or precedes the loss of purposeful hand use (Temudo et al. 2007).

Breathing anomalies are also present in RTT patients and occur only during the awake state, with episodes of hyperventilation, apnoeas, breath holding and air swallowing, that leads to considerable distension of the abdomen (Hagberg et al. 2002).

Sleep disturbances were reported in the majority of the RTT girls, suggestive of an altered circadian rhythm. RTT girls present an immature pattern of sleep, with more daytime sleep than age-matched controls, for subjects older than 15 years. Particularly the more severe patients, such as patients with a seizure disorder or who were never able to walk, had significantly more daytime sleep than normal children (Ellaway et al. 2001). It is also frequently described that RTT girls wake up in the middle of the night screaming or have night laughter (Hagberg et al. 2002). Additionally, rapid eye movement sleep was noticed to be impaired in RTT patients, who show an elevation of the phasic inhibition index without disturbance of the tonic inhibition index (Kohyama et al. 2001; for a review see Nomura 2005).

Seizures are an important problem in RTT, with a high frequency, varying between 58% and 94% in different patient series (Steffenburg et al. 2001; Huppke et al. 2007). The electroencephalogram (EEG) profile of RTT is very well defined and is invariably abnormal at some time during the course of RTT, with the presence of focal, multifocal, and generalized epileptiform abnormalities (Glaze 2002; Glaze 2005); however, the occurrence of seizures may be misestimated if evaluated only clinically, as many of the events described as clinical seizures were not associated with EEG seizure discharges, and vice-versa (Glaze et al. 1998; Moser et al. 2007). The mean age for seizure onset was 4 years (later part of clinical stage II and early stage III); after adolescence, the severity of epilepsy tends to decrease (Steffenburg et al. 2001).

Autism is a transient feature in RTT patients, most characteristic of stage II. In the pseudo stationary stage (III), RTT girls do not exhibit the autistic behaviour anymore, and, instead, they present intense eye communication, sometimes using this feature as a technique to communicate, in the absence of speech.

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In addition to the classical presentation of the syndrome described above, atypical forms of the disorder, that do not completely meet the accepted diagnostic criteria, have also been frequently recognized. These atypical forms deviate from classical RTT in age of onset, evolution of the clinical profile and severity. The atypical presentations of the syndrome might be either milder forms, such as the forme fruste (most common group – 11.5%) and the preserved speech variant, or more severe forms, such as the early epileptogenic encephalopathy and the congenital forms (which are rare, around 7.0% together) (Percy 2001; Hagberg et al. 2002). The existence of RTT in males is also considered a variant form of the disorder.

### **1.1.2. Neuropathology**

RTT females have, in general, short stature, but, remarkably, their brain shows a reduction in size and weight, in relation to the height of the child (Armstrong et al. 1999; Hagberg et al. 2001; Huppke et al. 2003).

When RTT brains were studied by magnetic resonance imaging (MRI), a selective regional reduction in brain volumes was observed. The volume of grey and white matter was reduced, particularly in the prefrontal, posterior frontal and anterior temporal regions; a reduction in the volume of caudate nucleus and midbrain was also reported (reviewed in Armstrong 2001). The cerebellum has also been shown to present a progressive atrophy and loss of specific neurons, such as Purkinje cells (Oldfors et al. 1990; Armstrong 2002).

Gross abnormalities such as hypoplasia or ectopias are not seen. The reduction in brain size appears to result mainly from a reduction of cortical thickness, which in turn corresponded to a markedly reduced neuronal size and increased cell packing density (reviewed in Armstrong 2002). In addition, post-mortem studies of RTT brains showed that the dendritic arborisation pattern of pyramidal neurons was simplified in layers III and V of frontal, motor and inferior temporal cortices (reviewed in Armstrong 2001). Also, the number of dendritic spines and the synaptic density are decreased in the frontal lobe (reviewed in Armstrong 2002).

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### **1.1.3. Neurochemistry/biochemical data**

Studies of brains and cerebrospinal fluid (CSF) from RTT patients have revealed alterations in the levels of neurotransmitters and their metabolites, receptors and trophic factors (summarized in table 1.4). Abnormalities have mostly been reported in the biogenic amines, such as the noradrenergic, dopaminergic and serotonergic systems, although these findings were not consistent across all different studies. The excitatory glutamatergic and the inhibitory GABAergic transmissions were also studied and shown to be elevated in RTT girls, during the first decade of life, and then reduced, when compared to controls (reviewed in Armstrong 2005).

However, the overall results are still conflicting and it is difficult to draw a clear conclusion from the human data, due to the fact that only a small number of cases were studied (some of them without molecular confirmation, as they were performed before the cloning of the gene), and at different ages and, thus, different stages of the disease. We also have to bear in mind the limitations of post-mortem studies, as well as of extrapolating from the CSF data.

### **1.1.4. Genetics of RTT**

The genetic basis and mode of inheritance of RTT were initially difficult to establish, since 99% of the cases are sporadic. However, the identification of RTT segregating in a few families (Ellison et al. 1992; Miyamoto et al. 1997; Schanen et al. 1997; Sirianni et al. 1998) and the concordance rate in monozygotic twins (Tariverdian et al. 1987; Bruck et al. 1991; Ogawa et al. 1997); suggested that RTT was a dominant disorder linked to the X chromosome, which affected only girls and was mostly fatal in boys. Genetic exclusion mapping in the few families described allowed researchers to exclude the RTT *locus* from the regions Xp21.2 to Xq21-q23 (Ellison et al. 1992), and later from Xp22.2 to Xq22.3 (Schanen et al. 1997). The identification of a family with three affected RTT siblings allowed the localization of the gene to the Xq28 *locus* (Sirianni et al. 1998), a very gene-rich region. In 1999, Amir and colleagues (1999) identified, by positional cloning, mutations in the methyl-CpG binding protein 2 gene - *MECP2* - as being responsible for the RTT phenotype.

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**Table 1.4.** Neurochemical studies in Rett syndrome.

| Reference                       | Specimens                  | Method                                      | RTT group                                      | Control group                                                          | Finding                                                                                                                                                                                                                                           |
|---------------------------------|----------------------------|---------------------------------------------|------------------------------------------------|------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| (Ormazabal et al. 2005)         | CSF                        | HPLC                                        | n=16 (2-23yrs)<br>RTT                          | n=38 (2-18yrs)                                                         | ↓ levels of 5-HIAA in 2 out of 8 RTT with low levels of 5-Methyltetrahydrofolate<br>normal HVA concentrations                                                                                                                                     |
| (Ramaekers et al. 2003)         | CSF                        | HPLC                                        | n=4<br>RTT                                     | group of similar age to RTT                                            | ↓ levels of 5-HIAA                                                                                                                                                                                                                                |
| (Blue et al. 1999b)             | postmortem<br>brain tissue | receptor binding                            | n=9 (2-30 yrs)                                 | n=10 (2-20 yrs)                                                        | density of glutamate receptors:<br>putamen - ↓ AMPA and NMDA in older RTT patients<br>caudate - ↓ KA receptors in older RTT patients<br>mGluR not altered in basal ganglia<br><br>density of GABA receptors:<br>caudate - ↑ in young RTT patients |
| (Blue et al. 1999a)             | postmortem<br>brain tissue | receptor binding                            | n=9 (2-30 yrs)<br>classical RTT                | n=10 (2-20 yrs)<br>no neurological disorder                            | density glutamate receptors (NMDA, AMPA, mGluR):<br>frontal cortex - ↑ in young and ↓ in older RTT patients<br>KA not altered in frontal cortex                                                                                                   |
| (Lappalainen and Riikonen 1996) | CSF                        |                                             | n=11 (2-17 yrs)<br>diagnostic criteria for RTT | n=11 (3-15 yrs)<br>mild neurological disorder<br>no mental retardation | ↑ glutamate                                                                                                                                                                                                                                       |
| (Wenk and Mobley 1996)          | postmortem<br>brain tissue |                                             | n=12 (4-30 yrs)<br>RTT                         | n=14 ♀ (2,5-20yrs)<br>non-neurological disease                         | no differences were found in DA, HVA, D2 receptor                                                                                                                                                                                                 |
| (Lekman et al. 1990)            | CSF and urine              |                                             | n=38 (CSF)<br>n=36 (urine)<br>typical RTT      | group of similar age to RTT                                            | CSF:<br>no differences in HVA, MHPG and 5-HIAA<br>urine:<br>no differences in HVA, MHPG and 5-HIAA                                                                                                                                                |
| (Lekman et al. 1989)            | postmortem<br>brain tissue |                                             | n=4 (12-30 yrs)<br>typical RTT                 |                                                                        | substantia nigra of the 2 older patients:<br>50% ↓ in DA and HVA, 5HT and 5-HIAA, NE<br>normal levels of MHPG<br><br>no differences in the younger patients                                                                                       |
| (Zoghbi et al. 1989)            | CSF                        | gas chromatography<br>and mass spectrometry | n=32 (2-16 yrs)<br>RTT                         | group of similar age                                                   | ↓ levels in the metabolites MHPG, HVA and 5-HIAA<br>in classical RTT patients                                                                                                                                                                     |
| (Perry et al. 1988)             | CSF                        |                                             | n=5<br>RTT                                     |                                                                        | no differences in HVA, MHPG and 5-HIAA<br>normal levels of GABA and other amino acids                                                                                                                                                             |
| (Percy et al. 1987)             | CSF                        |                                             | n=18<br>15 typical RTT; 3 variants             |                                                                        | ↓ biogenic amine metabolites in typical RTT                                                                                                                                                                                                       |
| (Zoghbi et al. 1985)            | CSF                        | gas chromatography<br>and mass spectrometry | n=6 (2-15 yrs)                                 | group of similar age                                                   | ↓ levels of MHPG and HVA<br>no differences in the levels of 5-HIAA                                                                                                                                                                                |

Legend: AMPA, α-amino-3-hydroxy-5-methylisoxazole-4- propionic acid; CSF, cerebrospinal fluid; DA, dopamine HPLC, high performance liquid chromatography; GABA, gamma-aminobutyric acid; HVA, 4-hydroxy-3-methoxy-phenylacetic acid; homovanillic acid; KA, kainate; MHPG, 3-methoxy-4-hydroxyphenylglycol; NE, norepinephrine; NMDA, N-methyl d-aspartate receptor; 5-HIAA, 5-Hydroxyindoleacetic acid; 5-HT, serotonin; (↑), increase; (↓), decrease;

### 1.1.5. *MECP2* mutations in RTT

More than 200 different mutations have been described in the *MECP2* gene (<http://mecp2.chw.edu.au/mecp2/>). Mutations can be either missense or nonsense, a significant percentage (7-10%) (Bienvenu et al. 2000; Schanen et al. 2004) of the mutations being rearrangements of the gene (deletions and duplications). Most mutations, however, result in a C>T transition, which is in agreement with the hypermutability of methylated CpG dinucleotides (Nielsen et al. 2001; Trappe et al. 2001).

Most of the *MECP2* mutations arise “*de novo*” (Amir et al. 2000). Around 90% of the sporadic cases of classic RTT and 30% of the atypical RTT cases present a mutation in the *MECP2* gene (Amir and Zoghbi 2000; Buyse et al. 2000; Dragich et al. 2000; Huppke and Gartner 2005). A considerable percentage of (typical but mostly atypical) RTT cases are still without a proven genetic cause. It is possible that *MECP2* mutations in regions other than the coding one exist, such as the introns, or the 5’ and 3’ regulatory regions. It is also possible that other genes might lead to a RTT phenotype.

Several reasons may explain the lack of males with a RTT phenotype. One of the reasons may be the severity of the *MECP2* mutation, since males do not have an extra normal *MECP2* copy for dosage compensation, as females do. Also, the mutation bias may contribute to this gender difference. A *MECP2* mutation may occur in oogenesis, in spermatogenesis, or at the somatic level, i.e., post-zygotically. Mutations in RTT females, however, appear to derive mostly from mutations in the paternal germline (Amir et al. 2000; Girard et al. 2001; Trappe et al. 2001). As fathers contribute with a Y chromosome to their sons, this would reduce the frequency of RTT males. Thus, the low frequency of affected males would not be due to a lethal effect of the mutation in the embryo, which is consistent with the fact that higher miscarriage rates have not been reported in families with a RTT patient (Killian 1986; Fyfe et al. 1999).

Given the large number of different mutations in the *MECP2* gene, it is not unexpected that a significant clinical diversity is found. To date, however, there is no convincing evidence relating genotype and phenotype. Attempts to establish a genotype-phenotype correlation in RTT have not been very conclusive. In fact, authors draw different conclusions across the different studies reported. Several reasons have been put forward to explain this apparent lack of correlation. First, the *MECP2* gene is located in the X-chromosome, and thus is subjected to inactivation (lyonization in females); the fact

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that, in different patients, a different proportion of cells in different brain regions may be expressing the mutant allele would contribute to the highly variable clinical presentation of this syndrome. Second, the high variability of mutation types and locations within the *MECP2* gene, which would undoubtedly contribute to a variable phenotypic expression, leads to small numbers of patients with any given mutation, making it difficult to achieve the statistical power needed to detect a correlation.

In relation to the type of mutation, in general, truncating mutations are correlated with an overall higher severity score (Cheadle et al. 2000; Monros et al. 2001; Huppke et al. 2002; Schanen et al. 2004). Others, however, did not achieve the same results (Amir et al. 2000; Huppke et al. 2000; Weaving et al. 2003).

A correlation was found between the type of mutation (missense *versus* truncating) and different RTT clinical characteristics. However, different studies achieve different correlations. For example, patients with respiratory dysfunction more frequently carry truncating mutations, and missense mutations are more likely to occur in patients with scoliosis (Amir et al. 2000). In another study, a positive correlation was found between missense mutations and the ability to alone, ambulation and a later age of onset of stereotypies (Monros et al. 2001). Other studies reported a positive correlation between truncating mutations and a decelerated head growth and inability to walk (Huppke et al. 2002), a worse language performance (Cheadle et al. 2000; Schanen et al. 2004). Nevertheless, others were not able to find a correlation between type of mutation and clinical signs (Bienvenu et al. 2000; Auranen et al. 2001; Yamada et al. 2001; Weaving et al. 2003).

In relation to the position of mutation, (methyl-CpG binding domain (MBD) *versus* transcription repression domain (TRD)), no differences were found (Huppke, 2002; Schanen 2004). However, in one study mutations in the MBD were correlated with a more severe phenotype (Amano et al. 2000).

Combining the type and position of the mutation and correlating with clinical signs, a correlation was found with a higher deceleration of head growth in missense MBD *versus* TRD and early *versus* late truncating mutations (Hoffbuhr et al. 2001; Schanen et al. 2004). Early truncating mutations were also associated with a higher clinical severity in

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relation to late truncating mutations (Cheadle et al. 2000; Hoffbuhr et al. 2001; Pan et al. 2002; Schanen et al. 2004).

The conclusion is that, overall, most studies did not detect an unequivocal genotype-phenotype correlation.

### 1.1.6. *MECP2* mutations in other neurodevelopmental disorders

In addition to classical and atypical RTT, mutations in the *MECP2* gene have been identified in patients with a wide spectrum of neurological phenotypes; from autism and mental retardation to Angelman syndrome (AS)\*, and affecting both males and females (figure 1.2). This led to the idea that *MECP2* mutations could underlie a large number of these disorders all of which shared part of the RTT clinical phenotype. Another idea was that these clinical presentations could possibly be associated with specific types of mutation in *MECP2*, i.e., that mutations of different functional effects in the MeCP2 protein could give rise different phenotypes, according to the affected functional domains.

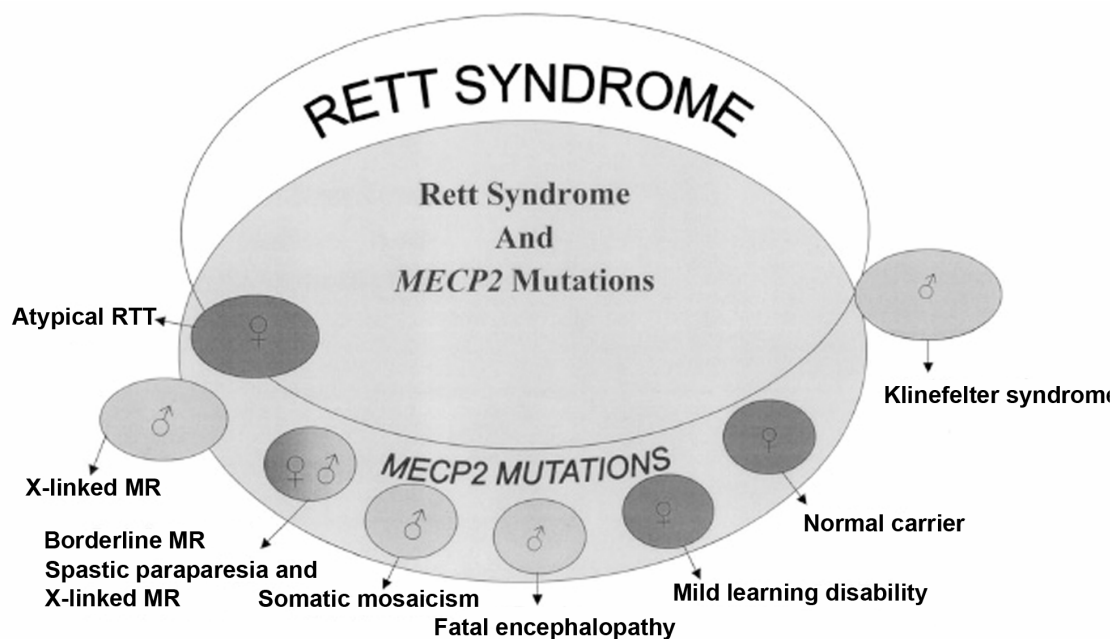


Figure 1.2. *MECP2* mutations in RTT related disorders. (Adapted from Percy 2001)

\* Angelman syndrome (OMIM, #105830). Characterized by mental retardation, movement or balance disorder, characteristic abnormal behaviours, and severe limitations in speech and language.

## 1.2. The methyl-CpG binding protein 2

### 1.2.1. The *MECP2* gene

The mouse *Mecp2* was first mapped between L1 cell adhesion molecule (*L1cam*) and *Rsvp* in the X-chromosome, a region which is syntenic to the human Xq28 locus (Quaderi et al. 1994). Physical mapping studies further located the human *MECP2* to the Xq28 locus, between *L1CAM* and *RCP/GCP*, oriented from the telomere to the centromere (D'Esposito et al. 1996). The mouse *Mecp2* sequence was compared to the human and rat sequences, and 90.7% and 95.5% similarity (respectively) was found at DNA level (Coy et al. 1999).

The *MECP2* gene (figure 1.3) has four coding exons and one of the largest known 3'-untranslated regions (3'UTR), spanning 8.5 kb in length (Reichwald et al. 2000). Three known protein domains were identified in the corresponding protein product; the methyl-MBD, partially encoded in exons 3 and 4, and the TRD and group II WW binding domain (WW) (Buschdorf and Stratling 2004) encoded in in exon 4. In addition, one nuclear localization signal (NLS) was identified. Different transcript variants are produced from this gene, four of which originating from different polyadenylation signals in the 3'UTR (1.8-, 5-, 7.2- and 10.1-kb) (Coy et al. 1999), and other from two alternative splicing sites (Kriaucionis and Bird 2004; Mnatzakanian et al. 2004); these alternative mRNAs are differentially expressed in the various tissues.

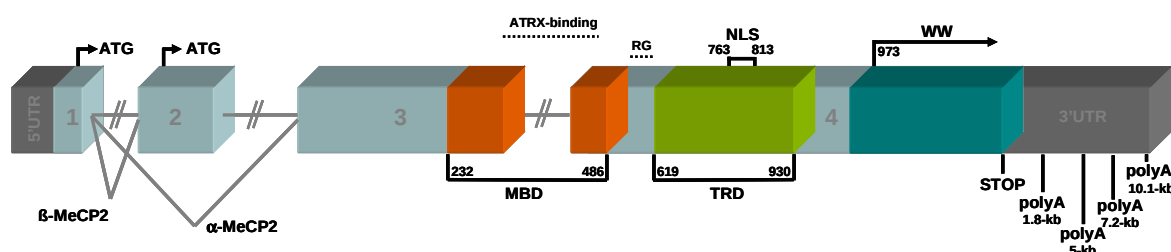


**Figure 1.3. Schematic representation of the structure of the *MECP2* gene.** MBD – methyl-CpG binding domain, TRD – transcription repression domain, NLS – nuclear localization signal, WW - group II WW domain, polyA – polyadenylation site, kb – kilobase, 3'/5'UTR – 3'/5'-untranslated region, ATG – start codon.

### 1.2.2. The MeCP2 protein

MeCP2-like proteins were detected in different species such as human, mouse, rat, chicken, pig, cow, rabbit and frog, but not in *Drosophila* (Meehan et al. 1992), which is in

accordance with the importance of DNA methylation in all vertebrates, but not in invertebrates.

*In vitro* and *in vivo* studies showed that MeCP2 protein is a nuclear chromatin-associated protein (75-100 kDa depending on the human isoform and around 80 kDa in rodents), that binds selectively to symmetrically methylated CpG dinucleotides (at least one methylated CpG pair), through its MBD; the NLS consists of amino acids 255-271 (RKAEADPQAIPKKRGRK), and the MBD of amino acids 78-162 (Meehan et al. 1992; Nan et al. 1993; Nan et al. 1996). MeCP2 possesses in the C-terminal region a TRD (amino acids 207-310); it was shown, *in vitro*, that MeCP2 represses the transcription of methylated reporter genes, but not of unmethylated ones. MeCP2 has a genome-wide binding distribution and its repression capacity is dependent on the distance of the methyl-CpG from the promoter (the higher the distance the weaker the repression capacity) and on the density of the methyl-CpGs (the higher the methylation the stronger the repression capacity) (Nan et al. 1997). Recently a group II WW binding domain (from amino acid 325 to the C-terminus of the protein) was also identified; it allows MeCP2 to specifically bind to group II WW domains of splicing factors (Buschdorf and Stratling 2004). Another potential protein functional domain could be an arginine-glycine repeat stretch (RG, amino acids 185-190), located after the MBD, which was proposed to mediate the binding of MeCP2 to RNA (Jeffery and Nakielny 2004).

The function of a protein can be sometimes be elucidated by the identification of its interacting partners. One of the first partners of MeCP2 to be identified was the *Xenopus* homologue of the co-repressor Sin3A, which is associated with MeCP2 in a complex with histone deacetylase activity (HDAC1 and HDAC2) (Jones et al. 1998). MeCP2 interacts with Sin3A through its TRD, and the histone deacetylase activity of the formed complex represses the transcription of target genes (figure 1.4).

Furthermore, MeCP2 also directly binds to two other co-repressors: c-Ski and N-CoR. c-Ski binds to the TRD of MeCP2 and seems to be necessary for methyl CpG-mediated transcriptional repression (Kokura et al. 2001). The entire MBD and TRD region of MeCP2, however, is necessary for the binding of N-CoR (Kokura et al. 2001). Until now, three different co-repressor molecules have been described to be involved in MeCP2-mediated transcriptional repression. It is unlikely, however, that these three co-

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repressors assemble together, but rather as individual co-repressor complexes, at the same time or sequentially.

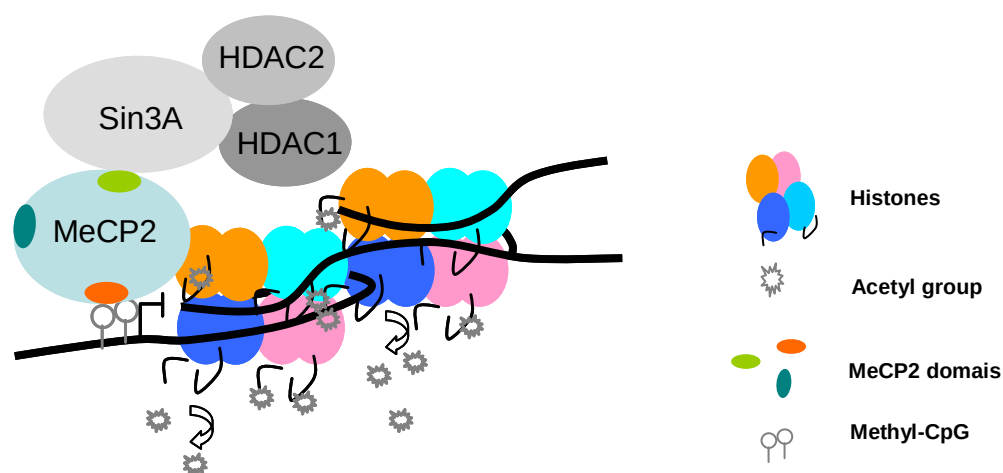


**Figure 1.4. Schematic representation of the major function of MeCP2 protein.** MeCP2 binds to methylated DNA through its MBD and recruits co-repressor complexes with histone deacetylase activity by binding, through its TRD to the co-repressor Sin3A.

Several other roles have been proposed for the MeCP2 protein, which are involved in histone deacetylase-independent silencing. The chromatin structure is associated with regulation of gene expression. We already discussed the role of MeCP2 in chromatin remodelling, through binding to methylated DNA and deacetylation of histones. Besides histone deacetylation, histone methylation is another epigenetic modification involved in the organization of chromatin structure and regulation of gene expression. MeCP2 was reported to be associated with a histone methyltransferase activity, specifically involved in the methylation of lysine 9 of histone H3, strengthening a repressive chromatin state by bridging DNA methylation to histone methylation (Fuks et al. 2003). The association of MeCP2 with histone H3 methyltransferase is primarily mediated by its MBD.

It is still an open question whether MeCP2 always recruits these two activities (deacetylation and methylation of histones) simultaneously, or whether there is a functional specification depending on the event that MeCP2 is regulating, such as imprinting, X chromosome inactivation (permanent) or embryonic development and activity-dependent gene transcription (transient).

There are essentially two classes of DNA methyltransferases, the *de novo* DNA methyltransferases (DNMT3A and DNMT3B) which define new methylation patterns, and

the maintenance DNA methyltransferases (such as DNMT1). DNMT1 uses as substrate hemi-methylated DNA and copies the pattern already established during DNA replication; it thus is responsible for the maintenance of the primitive/basal DNA methylation status. It was shown that MeCP2 associates with DNMT1, through its TRD domain, and both play a role in the maintenance of the DNA methylation pattern during DNA replication (Kimura and Shiota 2003). MeCP2 binds to the template hemi-methylated double stranded DNA and recruits DNMT1, which will add a methyl group to cytosines in the newly synthesized strands.

Experimental data suggests that MeCP2 might be involved in local or higher order chromatin reorganization. In this respect, it has been shown that MeCP2 mediates the formation of a silent chromatin loop in the distal less homeobox 5 (*Dlx5*)-distal less homeobox 6 (*Dlx6*) locus, associated with methylation of lysine 9 of histone H3 (Horike et al. 2005). Additionally, the spatial organization of the heterochromatin in the nucleus has also a role in transcriptional silencing and is involved in the maintenance of cellular differentiation (Kosak and Groudine 2004). Heterochromatin aggregates in clusters that lead to the formation of large chromocenters and the levels of MeCP2 have been correlated with this process (Brero et al. 2005). The MBD of MeCP2 is necessary for this interaction, and is independent of the pathway that involves methylation of lysine 9 of histone H3.

Proteins that interact with RNA or are components of RNA-protein complexes (RNP) commonly have a RG repeat region. The MeCP2 protein has a small stretch of RG repeats following the MBD (figure 1.3). As mentioned above, the MeCP2 was shown to be able to bind mRNA and double-stranded siRNA and form a RNP *in vitro* (Jeffery and Nakielnny 2004). The interaction occurs through the RG domain and independently of the MBD of MeCP2. Additionally, the binding of MeCP2 to methylated DNA or siRNA occurs in a mutually exclusive manner.

What would be the advantage of MeCP2 binding to specific RNAs? This could provide specificity of transcription regulation by driving the binding of MePC2 to specific methylated chromatin regions. This feature of MeCP2 was not yet demonstrated *in vivo*, but if this is the case, this fact could eventually link RNA to chromatin regulation, through DNA-methylation. But, it is also possible that the MeCP2-RNP complex has a function different from its well characterized role in the regulation of gene expression. Could

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MeCP2 also be involved in the post-transcriptional regulation of gene expression, controlling mRNA stability, localization and translation, which regulate many important events in development and plasticity? Identification of which specific RNA molecules are targets of the MeCP2 protein will help the scientific community to unveil the link between RNA and MeCP2.

In accordance with the previous finding, another function has been attributed to MeCP2, as a splicing regulator. MeCP2 was described to interact with Y-box binding protein 1 (YB-1), which is a nuclei acid binding protein involved in many cellular functions, including alternative splicing (Stickeler et al. 2001). MeCP2-YB1 binding requires RNA for its formation and stabilization. The proposed idea is that MeCP2 regulates alternative splicing through its WW C-terminal domain (amino acids 195-329), promoting exon inclusion (Young et al. 2005). It was hypothesized that the posttranscriptional modulation of alternative splicing could represent an epigenetic control of gene expression (Young et al. 2005). Alternative splicing allows for the existence of several transcripts from the very same gene. In this way, a dysfunction in alternative splicing may have more drastic consequences on the expression of one gene than a dysfunction in its transcription.

Very recently, another partner of MeCP2 has been identified. The alpha-thalassemia, mental retardation syndrome, X-linked (ATRX) is described to be a SWI2/SNF2 DNA helicase/ATPase, which has been shown to alter the structure of chromatin (Berube et al. 2000). Mutations in the *ATRX* gene are responsible for the neurological syndrome ATR-X<sup>†</sup>. MeCP2 interacts with ATRX (both *in vitro* and *in vivo*), through a domain (ATRX-domain) that partially overlaps the MBD of MeCP2; it was described that certain human MeCP2 mutations (such as R133C, which causes a mild RTT phenotype, and A140V, present in males with X-Linked Mental Retardation) disrupt this interaction (Nan et al. 2007).

MeCP2 was initially proposed to be a “global silencer” acting at the chromatin structure level. Accumulating evidence seems to suggest that the way MeCP2 plays its role(s) might depend on the cellular and molecular context. The identification of the molecular targets of MeCP2 will help in elucidating the contribution of MeCP2 in those pathways.

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<sup>†</sup> ATR-X (OMIM, #301040). Present with severe psychomotor retardation, characteristic facial features,  $\alpha$ -thalassemia and genital abnormalities.

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### 1.2.3. MeCP2 mRNA and protein expression pattern

The *MECP2/Mecp2* mRNA has a ubiquitous expression in embryonic neural and non-neural tissues with different spatial and temporal patterns. However, through development the highest mRNA levels are found in the brain structures. Different transcripts were detected (~1.8-, ~5-, ~7- and ~10-kb), the *MECP2* ~10-kb transcript being generally described as the predominant form in the brain (table 1.5). At late embryonic stages, the expression of *MECP2* is pronounced in the cortex, the corpus striatum, the hippocampus and the dorsal thalamus. Postnatally, in the early postnatal brain, a uniform distribution of expression is found through most brain regions, whereas in the adult brain the highest levels of expression were found in regions such as the olfactory bulb, the cerebral cortex, the caudate-putamen, the hippocampal formation and the cerebellum.

The two *Mecp2* splice variants also presented a particular pattern of expression. At birth, both splice transcripts (*Mecp2ε1* and *Mecp2ε2*) were widely distributed throughout the nervous system. At postnatal day 21, a developmental shift in the expression pattern of both transcripts occurred, which was maintained at postnatal day 60, and the expression became brain-region specific: the *Mecp2ε2* transcript was expressed predominantly in the dorsal thalamus and cortical layer V, whereas *Mecp2ε1* remained widely expressed throughout the brain (Dragich J, 2007). The physiological meaning of this expression pattern remains unknown.

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**Table 1.5.** Spatial and temporal expression of the different *MECP2/Mecp2* transcripts.

|           | Species/ method                                         | <i>MECP2/ Mecp2</i> expression profile                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | Reference                 |
|-----------|---------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------|
| PRENATAL  | human/ mouse<br>northern blot/<br>in situ hybridization | - three transcripts were detected, ~1.8-, ~5- and ~10-kb, in heart, brain, lung, liver, kidney<br>- the ~10-kb transcript was the most abundant<br>- E10.5-E12.5 ubiquitous low level of expression of the ~10-kb transcript                                                                                                                                                                                                                                                                                                                                                                                                                                       | (Coy et al. 1999)         |
|           | rat<br>in situ hybridization                            | - E14-E19 mRNA Mecp2 expression was more pronounced in neural structures than in peripheral organs                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | (Jung et al. 2003)        |
|           | human<br>microarray<br>IF+LSC, RT-PCR                   | → inverse correlation between ~10-kb transcript usage and age<br>- in fetal brain: ~93% of cells express the ~10-kb transcript<br>- in adult cerebrum: ~40% of cells express the ~10-kb transcript                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | (Balmer et al. 2003)      |
|           | mouse<br>in situ hybridization                          | - all transcripts (~1.8-, ~5-, ~7- and ~10-kb) are ubiquitously and equally expressed in neural and non-neural tissues<br>- from E16.5 predominant expression in the CNS: cortex, corpus striatum, hippocampus and dorsal thalamus                                                                                                                                                                                                                                                                                                                                                                                                                                 | (Pelka et al. 2006)       |
| POSTNATAL | human<br>northern blot                                  | - three transcripts were detected, ~1.8-, ~7- and ~10-kb, in heart, brain, placenta, lung, liver skeletal muscle, kidney, pancreas<br>- the transcript ~1.8-kb is the most abundant<br>- in the brain: the ~10-kb is the most abundant transcript                                                                                                                                                                                                                                                                                                                                                                                                                  | (D'Esposito et al. 1996)  |
|           | human/ mouse<br>northern blot/<br>ISH                   | - three transcripts detected, ~1.8-, ~5- and ~10-kb, in heart, brain, pancreas, lung, liver, muscle, kidney, pancreas<br>- the ~5-kb transcript is the most abundant<br>- at PNW1: expression of the ~10-kb transcript is seen in all parts of the brain<br>- in the fully differentiated brain: ↑↑ expression of the ~10-kb transcript in the olfactory bulb and in the hippocampal formation                                                                                                                                                                                                                                                                     | (Coy et al. 1999)         |
|           | human/ mouse/ rat<br>northern blot                      | - three transcripts were ubiquitously detected, ~1.8-, ~7- and ~10-kb, in neural and several non-neural tissues, with different expression patterns<br>- in the brain: highest expression of the ~10-kb transcript                                                                                                                                                                                                                                                                                                                                                                                                                                                 | (Reichwald et al. 2000)   |
|           | human/ mouse<br>northern blot/<br>immunoblot            | - two transcripts were detected, ~1.8- and ~10-kb, with varying levels in most tissues<br>- the most abundant transcript in the brain is the ~10-kb                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | (Shahbazian et al. 2002b) |
|           | rat<br>ISH                                              | mRNA Mecp2 expression:<br>- in the early postnatal brain: fairly uniform distribution through most brain regions<br>- in the adult brain: strongest expression in the cerebral cortex, the hippocampus, the cerebellum and the olfactory bulb                                                                                                                                                                                                                                                                                                                                                                                                                      | (Jung et al. 2003)        |
|           | Human<br>microarray<br>IF+LSC, RT-PCR                   | → inverse correlation between ~10-kb transcript usage and age<br>- in fetal brain: ~93% of cells express the ~10-kb transcript<br>- in adult cerebrum: ~40% of cells express the ~10-kb transcript                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | (Balmer et al. 2003)      |
|           | Mouse<br>ISH<br>RT-PCR                                  | - all transcripts (~1.8-, ~5-, ~7- and ~10-kb) are ubiquitously transcribed from PNW2-20<br>- pronounced regionalization at PNW20: hippocampal formation, layers II/III of cerebral cortex, caudate-putamen, amygdala, piriform cortex, hypothalamus, granular cells of olfactory bulb<br>- levels of all transcripts dropped from E16.5 to PNW12, specially the ~10-kb transcript<br>- levels of the ~10-kb raised from PNW12 to PNW60 and all the others remained relatively stable (substantia nigra, basal ganglia, cerebellum and occipital cortex)<br>- the most abundant transcript in the brain is the ~10-kb and in the peripheral tissues in the ~1.8-kb | (Pelka et al. 2006)       |

Legend: CNS, central nervous system; E, embryonic day; IF, immunofluorescence; ISH, *in situ* hybridization; LSC, laser scanning cytometry; PNW, postnatal week; RT-PCR, real time PCR.

At the protein level, the highest MeCP2 expression levels were found in the brain, but strong expression was also seen in other organs, such as the lung and heart. In the brain MeCP2 was present in most regions and the protein levels increased during the central nervous system (CNS) development. The areas that presented the highest expression levels were the olfactory epithelium, the several cerebral cortices, the dentate gyrus, the brainstem and the molecular layer of the cerebellum (table 1.6). Using developmental stage markers, it was possible to map the MeCP2 protein in the neuronal lineage, but not in glial cells. In fact, expression studies of MeCP2 in the glial cells gave conflicting results (LaSalle et al. 2001; Shahbazian et al. 2002b; Kishi and Macklis 2004; Nagai et al. 2005).

Subpopulations of MeCP2-expressing cells were identified. The subpopulation of cells that expressed high levels of MeCP2 (MeCP2<sup>hi</sup>) increased with age, in contrast with the subpopulation that expressed low levels of MeCP2 (MeCP2<sup>lo</sup>), which was more represented in the younger brain.

The highest number of MeCP2 positive cells was found within mature neurons, rather than neural progenitor cells and immature neurons. In accordance, MeCP2 was firstly detected in regions that were primarily formed, such as the Cajal-Retzius cell layer and the deeper layers of the cerebral cortex and multiple brainstem nuclei. Also, as the CNS matured, the levels of the protein were increased (figure 1.5). All these data suggest that expression of MeCP2 correlates with neuronal maturity and functional synaptogenesis.

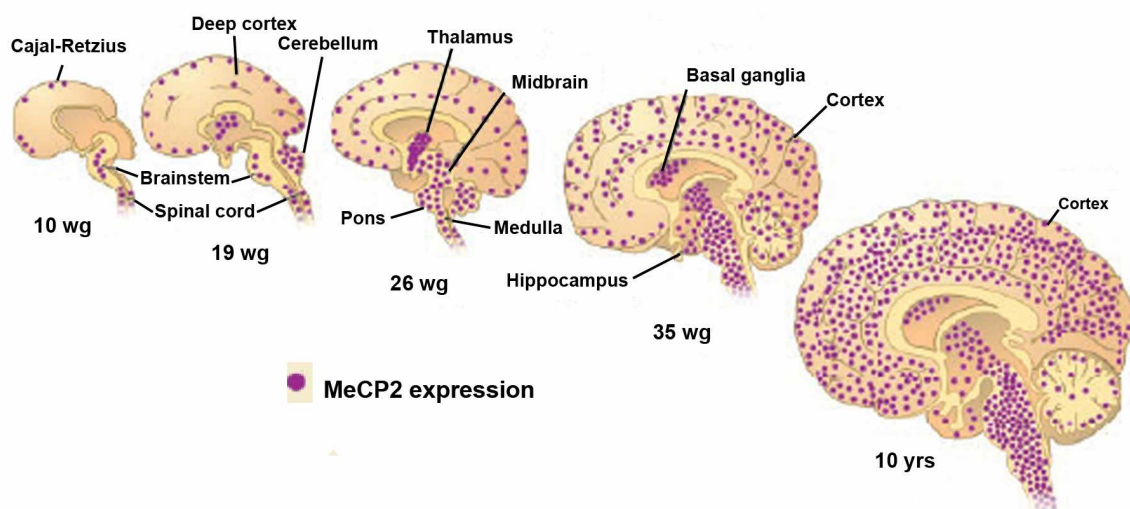


**Figure 1.5. Temporal and spatial distribution of MeCP2 protein during human brain development.** (Adapted from Zoghbi 2003)

**Table 1.6.** Spatial and temporal MeCP2 protein expression.

| Species  | Method | Day/ week        | MeCP2 expression profile                                                                                                                                                                                                         | Reference                 |
|----------|--------|------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------|
| PRENATAL | IHC    | Mouse<br>E10.5   | - ↓ in the marginal zone of brain (Cajal-Retzius cells)<br>- diffuse nuclear staining                                                                                                                                            | (Shahbazian et al. 2002b) |
|          |        | E11.5            | - ↑ in the marginal zone of the brain (Cajal-Retzius cells) and also in spinal cord, pons and medulla                                                                                                                            |                           |
|          |        | E14.5            | - thalamus, caudate/putamen, cerebellum                                                                                                                                                                                          |                           |
|          |        | E14.5 - E16.5    | - deeper layers of cerebral cortex<br>- punctate nuclear staining                                                                                                                                                                |                           |
|          |        | E16.5 - E18.5    | - hypothalamus, hippocampus and deep cerebellar nuclei and all cortical layers                                                                                                                                                   |                           |
|          | IHC    | human<br>10-14wg | - detected in multiple brainstem nuclei, Cajal-Retzius cells of the cerebral cortex                                                                                                                                              | (Cohen et al. 2003)       |
|          |        | 19wg             | - ↓ in deeper cortical layers, thalamus, caudate, substantia nigra, globus pallidus, hippocampus and cerebellum                                                                                                                  |                           |
|          |        | 26wg             | - ↑↑ in Cajal-Retzius neurons and in the deep cortical layers (10%)<br>- in ependyma, choroid plexus and spinal cord                                                                                                             |                           |
|          |        | 29wg             | - in locus ceruleus, basis pontis and colliculi                                                                                                                                                                                  |                           |
|          |        | 35wg             | - in putamen and reticular formation of brainstem (80%)                                                                                                                                                                          |                           |
|          | IHC    | rat<br>E14       | - expression is not ubiquitous in the developing cortex: MeCP2+<br>in 25% of Nestin+ cells<br>in 75% of β-III-tubulin+ cells                                                                                                     | (Jung et al. 2003)        |
|          |        | mouse<br>E12.5   | Olfactory epithelium<br>- a few MeCP2+ nuclei were visible, which increased with embryonic age<br>- MeCP2 staining was punctate                                                                                                  |                           |
|          | IHC    | mouse<br>E16.5   | Olfactory bulb<br>- increased expression with age<br>↑ MeCP2 expression in Cajal-Retzius cells layer<br>↓ MeCP2 expression in cortical plate<br>↓↓ MeCP2 expression in deep layers of the ventricular zone and intermediate zone | (Kishi and Macklis 2004)  |
|          |        |                  |                                                                                                                                                                                                                                  |                           |

Table 1.6 (continued)

|           | Species         | Method                      | Day/ week                  | MeCP2 expression profile                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | Reference                 |
|-----------|-----------------|-----------------------------|----------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------|
| POSTNATAL | rat/<br>mouse   | southwestern                |                            | - different tissue expression pattern:<br>>kidney >spleen >liver >testis<br>- the highest levels are found in brain extracts                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | (Meehan et al. 1992)      |
|           | human/<br>mouse | IF+LSC<br>tissue microarray |                            | - subpopulations of MeCP2(hi) and MeCP2(lo) phenotype<br>- the highest MeCP2 expression was found in the CNS tissues, which also exhibited the highest % of MeCP2(hi) cells, as compared with non-CNS tissues<br><br>histologic distribution of MeCP2(hi) and MeCP2(lo) in CNS<br>- higher proportion of MeCP2(hi) in layer IV of cerebral cortex and in the molecular layers of the cerebellum<br>- highest percentage of MeCP2(lo) in the granular layer of the cerebellum<br>- MeCP2(hi) and MeCP2(lo) mosaics are found in the glial cells of the cerebral and of the cerebellar white matter | (LaSalle et al. 2001)     |
|           | Mouse           | immunoblot<br>IHC           | PNW19                      | - MeCP2 tissue-specific levels:<br>↑↑↑ in brain, lung and spleen<br>↑↑ in kidney and heart<br>↓ in liver, stomach, small intestine                                                                                                                                                                                                                                                                                                                                                                                                                                                                | (Shahbazian et al. 2002b) |
|           |                 |                             | PNW1-28                    | - MeCP2 cell-specific levels<br>- spatial distribution in brain<br>present in most regions of the brain<br>- temporal distribution in brain<br>relatively uniform levels preserved after birth<br>- Ø in glia                                                                                                                                                                                                                                                                                                                                                                                     |                           |
|           | human           |                             | 10yrs                      | - 80% MeCP2 cortical expression<br>- 100% expression in the reticular formation of the brainstem                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                           |
|           | rat             | IHC                         | adult                      | - ↑↑↑ in layer III and deep layers of the cortex and in the pyramidal and granule cells of the hippocampal formation.<br>- Ø in GFAP+ astrocytes of the cortex or hippocampus                                                                                                                                                                                                                                                                                                                                                                                                                     | (Jung et al. 2003)        |
|           | mouse           | IHC<br>WB                   |                            | Olfactory system<br>- MeCP2 expression increased from PND0 to PNW20<br>- at PNW7: staining was reduced<br>- MeCP2 is expressed in a laminar fashion: in the regions containing the sustentacular cells and mature ORNs<br>- MeCP2 staining uniformly distributed in nuclei<br>- populations of cells with high versus light staining<br>- At PNW2: very few MeCP2+ cells co-localize with NST+ cells and the majority of MeCP2+ cells are associated with OMP+ cells<br>Olfactory bulb<br>- increased expression with age                                                                         | (Cohen et al. 2003)       |
|           | human           | microarray<br>IF+LSC        | young<br>juvenile<br>adult | - in cerebral cortex (layers III-V):<br>MeCP2(hi) more represented in juvenile and adult cerebrum<br>MeCP2(lo) more represented in young cerebrum                                                                                                                                                                                                                                                                                                                                                                                                                                                 | (Balmer et al. 2003)      |

Table 1.6  
(continued)

|           | Species | Method               | Day/ week                 | MeCP2 expression profile                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | Reference                |
|-----------|---------|----------------------|---------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------|
| POSTNATAL | mouse   | IHC                  | PND0-21                   | <ul style="list-style-type: none"> <li>- neocortex</li> <li>↑ MeCP2 expression in superficial and deep layers</li> <li>↑↑ MeCP2 expression in Cajal-Retzius cells layer</li> <li>MeCP2 nuclear staining becomes progressively more punctate through development</li> <li>- MeCP2 is expressed in mature neurons (MeCP2+/NeuN+), but not in glia (MeCP2+/GFAP-; MeCP2+/S100β- and MeCP2+/CNPase-)</li> <li>- MeCP2 expression levels increase as neuronal differentiation progresses</li> </ul>                                                                                                                                                                                                                             | (Kishi and Macklis 2004) |
|           | human   | microarray<br>IF+LSC | infant<br>juvenile, adult | <ul style="list-style-type: none"> <li>- in frontal cortex:</li> <li>increase in MeCP2 mean and MeCP2(hi) population with age</li> </ul>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | (Samaco et al. 2004)     |
|           | rat     | IHC<br>immunoblot    |                           | <ul style="list-style-type: none"> <li>- ↑↑ MeCP2+ cells in olfactory bulb (60% at birth), which remained at constant levels throughout life</li> <li>- ↑ MeCP2+ cells in several cortices (10% at birth). All cortices have a peak at PNW1; the frontal cortex exhibit the highest expression levels</li> <li>- ↓ MeCP2+ cells in the cingulate cortex. No differences between birth and PNW112</li> <li>- ↓↓↓ low level in dorsal hippocampus throughout entire life span</li> <li>- in dentate gyrus ↑ high level throughout life, peaking at 12 weeks</li> <li>- ↑↑ in the first week of life in the shell of nucleus accumbens (30%)</li> <li>- ↑ increase at 4 weeks in caudate-putamen and septal nuclei</li> </ul> | (Cassel et al. 2004)     |

Legend: CNPase, oligodendrocyte marker; E, embryonic day; GFAP, glial fibrillary acidic protein (astrocyte marker); IF, immunofluorescence; IHC, immunohistochemistry; ISH, *in situ* hybridization; LSC, laser scanning cytometry; NeuN, neuron nuclei specific marker; NST, bed nucleus of stria terminalis; OMP, olfactory neuron marker protein; ORN, olfactory receptor neurons; PND, postnatal day; PNW, postnatal week; RT-PCR, real time PCR; S100β, glial marker; WB, western blot; wg, week of gestation; yrs, years; (hi), high expression; (lo), low expression; (↑), increase; (↓), decrease

#### 1.2.4. Other methyl-CpG binding proteins

The levels of CpG (cytosine-phosphodiester-guanine) methylation and the state of chromatin structure are two major features that have been associated with the potential of gene activity in mammals (reviewed in Razin and Cedar 1991; Razin 1998; Ng and Bird 1999; Sharma et al. 2005). In fact, heterochromatic regions in the genome were shown to present the highest levels of CpG methylation (Razin and Cedar 1977). One mechanism by which DNA methylation can cause transcriptional repression is through the binding to methylated DNA of a group of proteins that constitute the methyl-CpG binding proteins family (MBD family). MeCP2 was the first member of the MBD family to be recognized (Meehan et al. 1992), following which four additional proteins were identified in human and rodent: MBD1, MBD2, MBD3 and MBD4 (Hendrich and Bird 1998). All these proteins share a similar MBD-like motif, through which they bind to methylated DNA, but all of them have individual specificities. MeCP2 was shown to be able to bind selectively to at least one pair of symmetrically methylated CpG (Nan et al. 1993), and both MBD1 and MBD4 also bind hemimethylated DNA. Binding of MBD3, however, is not specific to methylated DNA. MBD1, MBD2 and MBD3 are, like MeCP2, associated with the recruitment of histone deacetylase activity. The MBD4 protein, however, seems to be involved in a different class of processes, namely in DNA repair at m5CpG sites (Hendrich et al. 1999).

In embryonic stem (ES) cells, in which methylation is not required, MBD1 was not detected, and MBD2 and MBD4 expression levels were significantly reduced. MBD3 was shown to be present in ES cells, further suggesting a role for this protein that is independent of methylation (Hendrich and Bird 1998). MeCP2 is present in ES cells, but its localization is disrupted in DNMT-deficient cells (Nan et al. 1996).

One interesting aspect for the purpose of understanding RTT pathology is that null mutations in several of the MBD family proteins lead to nervous system or behavioural phenotypes. Mice knock-out (ko) for the *Mbd1* gene display reduced neurogenesis in the hippocampus, perform worse than wild-type (wt) animals when tested in the Morris water maze (a test for hippocampal-dependent cognitive performance), and have a reduction in dentate-gyrus LTP (Zhao et al. 2003). MBD1 is expressed in neurons throughout the brain, with highest concentration in the hippocampus (CA1 and dentate gyrus regions), and is not expressed in glia. *Mbd2* ko mothers do not present a proper nurturing behaviour towards their offspring (Hendrich et al. 2001). *Mbd3* ko animals die before birth, suggesting an essential role of this protein during development (Hendrich et al. 2001). The

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different phenotypes of these two last mutants might be explained, in part, by the expression pattern of the corresponding proteins. Expression profiles of MBD2 and MBD3 in the developing brain are not parallel: during development and in adulthood, MBD3 is expressed in ontogenetically younger brain regions, in contrast with MBD2 expression, that is weak in embryonic brain, but pronounced in the adult brain (Jung et al. 2003) (reviewed in Ballestar and Wolffe 2001).

Why the function of the methylated DNA-binding proteins is of particular relevance for the nervous system is still an open question. One hypothesis is that neurons require an effective machinery for appropriate chromatin remodelling (and consequent transcriptional regulation) in response to environmental stimuli (Santos et al. 2006)

#### **1.2.5. Targets of MeCP2**

The identification of neuronal targets of MeCP2 transcriptional regulation is one avenue of research that may provide important clues to RTT pathogenesis, and possibly also lead to an increased understanding of other pervasive developmental disorders, such as autism and AS, in which MeCP2 levels appear to be low (Samaco et al. 2004).

Most initial microarray studies have failed to identify any substantial and consistent changes in transcription levels in *Mecp2*-null mice (Tudor et al. 2002), clonal cell cultures from individuals with RTT (Traynor et al. 2002), or in postmortem RTT brains (Colantuoni et al. 2001). These results might suggest functional redundancy between the different methyl-binding proteins or a more focused action of MeCP2 as a selective regulator. This could occur through time or region-specific actions of the protein in the brain, involvement of MeCP2 in specific epigenetic events (such as imprinting of certain genes), action at a specific developmental stage, or through its involvement in activity-dependent transcription. In any of these scenarios, important differences in the transcription levels of certain genes may exist in the absence of MeCP2, but their detection will only be possible if suitable experimental designs are used.

A recent study by Ballestar and collaborators (2005) combining microarray studies, chromatin immunoprecipitation analysis, bisulfite genomic sequencing and treatment with demethylating agents, in lymphoblastoid cell lines derived from RTT patients, revealed the deregulation of the expression of a number of genes, which were also shown to have methylated promoters, directly bound by MeCP2. Approximately half of these target genes

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presented high expression levels in RTT cells when compared to *wt* cells, whereas the remaining half were downregulated, most likely because of an indirect effect of MeCP2 on genes that are, in turn, regulating these. The role of the deregulated genes in the pathogenesis of RTT remains to be clarified.

MeCP2 was shown to be involved in the imprinting control region of the *H19* gene (Drewell et al. 2002). *H19* is an example of a gene for which imprinting occurs for the paternal allele. The promoter region of the paternal allele is highly methylated and the silencing was shown to be methylation-dependent and mediated by MeCP2 (Drewell et al. 2002). However, the analysis of different imprinted genes, including the *H19* gene, in cultured T-cell clones from blood and in brains from patients with mutations in the *MECP2* gene revealed normal monoallelic expression in all clones and brain samples (Balmer et al. 2002), which might suggest *in vivo* redundancy amongst the MBD family of proteins.

Horike and collaborators (2005) recently found that the *DLX5*, a gene that is involved in the synthesis of gamma aminobutyric acid (GABA), is upregulated in RTT. In humans, *DLX5* has an imprinted pattern with expression of the maternal allele only, while in mice *Dlx5* is biallelically transcribed, but preferentially from the maternal allele. The authors found that, in the cortex of *Mecp2*-null mice and in human lymphoblastoid cells from individuals with RTT, (1) transcription levels were higher than normal, and (2) there was an altered parental imprinting of the gene, which was not due to methylation status, as CpGs at this region were unmethylated, and the extent of this effect was dependent on the type of mutation. MeCP2 is able to form a silent chromatin loop in the *Dlx5-Dlx6* locus (Horike et al. 2005). Although the region through which MeCP2 regulates *Dlx5* expression is not known yet, this strengthens the possible link between MeCP2 and imprinting and, for the first time, connects RTT to this epigenetic mechanism. It also provides useful clues to RTT pathogenesis, since affected GABA neurotransmission could explain some of the cognitive symptoms of RTT.

Two other candidate targets of MeCP2 are the ubiquitin protein ligase E3A (*UBE3A*) and *GABRB3* genes. These are particularly interesting, since *UBE3A* is linked to AS, and *GABRB3* (which encodes the protein GABA receptor  $\beta 3$  subunit), has been consistently implicated in autism, in association studies, both disorders presenting some phenotypic overlap with RTT. In contrast to *DLX5*, *UBE3A* and *GABRB3* levels were found to be decreased in RTT, AS and autism brains. *Mecp2*-deficient mice also display decreased

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levels of *Ube3a* and *Gabrb3*, in spite of the lack of alterations in the imprinting pattern of the *Ube3a* gene (Samaco et al. 2005). A possible mechanism through which MeCP2 regulates the expression of *UBE3A* has recently been proposed: MeCP2 apparently binds to the methylated Prader-Willi syndrome<sup>‡</sup> (PWS)-imprinting centre (mutated in AS and PWS) at the maternal allele, where the antisense *UBE3A* gene resides. Mutant MeCP2, if unable to bind this region would cause an epimutation at this imprinting centre, affecting the expression of *UBE3A* (Makedonski et al. 2005).

Experiments performed in *Xenopus* embryos showed that MeCP2 targets the gene *xHairy2a* during development. In the absence (or presence of a mutant form) of MeCP2 the expression of the *xHairy2a* gene was misregulated, with consequences in neuronal differentiation. This study showed that also MeCP2 interacts with the silencing mediator of retinoid and thyroid receptor (SMRT) complex, via Sin3A, and that mutant MeCP2 had defective binding to the SMRT corepressor complex. It was thus proposed that DNA methylation and MeCP2 binding could modulate the levels of *xHairy2a* expression and have an essential role in early neurogenesis (Stancheva et al. 2003).

The most interesting target of MeCP2 identified so far is doubtlessly the gene encoding brain derived neurotrophic factor (*BDNF*), one of the genes for which transcription is regulated in a neuronal activity-dependent manner. Data from two different studies showed that MeCP2 is involved in *Bdnf* gene silencing, in the absence of neuronal activation. MeCP2 was shown to bind to the methylated rat *Bdnf* promoter III (equivalent to promoter IV in the mouse), and, upon membrane depolarization of cultured cortical neurons, to dissociate from the promoter and lead to a higher transcription level of the *Bdnf* gene (Chen et al. 2003; Martinowich et al. 2003). Chen and collaborators (2003) also showed that the release of MeCP2 protein was due to calcium influx, that caused a phosphorylation of MeCP2. Given the role of *BDNF* in development and neuronal plasticity (McAllister et al. 1999; Binder and Scharfman 2004) and the timing when MeCP2 demand becomes crucial, which coincides with moments of synapse development and maturation, the aforementioned evidence easily fits a model in which MeCP2-regulated chromatin remodelling underlies neuronal plasticity, which could explain some symptoms of the RTT phenotype, such as reduced dendritic arborization and complexity in some

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<sup>‡</sup> Prader-Willi syndrome (OMIM, #176270) is characterized by diminished foetal activity, obesity, muscular hypotonia, mental retardation, short stature, hypogonadotropic hypogonadism, and small hands and feet.

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areas of the brain (see review by Armstrong 2001), and the clinical finding of mental retardation.

Recently another target of MeCP2 was identified – the corticotropin-releasing hormone gene (*Crh*) (McGill et al. 2006). MeCP2 binds to the methylated promoter of the *Crh* gene, which encodes the CRH protein, that is involved in the behavioural and physiologic response to stress. Briefly, during a stress response CRH activates the hypothalamus-pituitary-adrenal (HPA) axis, acting at receptors on anterior pituitary to stimulate the release of adrenocorticotrophic hormone (ACTH), which then enters the bloodstream and acts at receptors in the adrenal gland cortex to stimulate the synthesis and release of glucocorticoids (for a review see Bale and Vale 2004). *Mecp2*<sup>308/Y</sup> mice were found to have increased anxiety levels and, after restraint stress, presented higher levels of corticosterone than their wt controls. The enhanced physiologic response to stress (increased HPA axis activity) is due to overexpression of the *Crh* transcript in the brain of the *Mecp2*<sup>308/Y</sup> mice. Some of the consequences of chronic stress are deficits in cognition and reduced synaptic plasticity, including reduced dendritic branching and impaired LTP and LTD (Cerqueira et al. 2007). The overlap of these features with the RTT phenotype suggests that the higher levels of CRH found in the *Mecp2*-mutant brains upon a stressful experience can contribute to the clinical manifestations of this disorder.

### **1.3. Knock out and transgenic mouse models of RTT: do they mirror the human disorder?**

Human and rodents diverged at some timepoint of their evolution and particularly at the high functioning level. In this way, it is reasonable to think that a similar mutated gene can have a differential display in the behaviour of humans and rodents. Instead of capturing an entire clinical syndrome in an animal model, we often have models of certain endophenotypes, and it is common that the different heritable components can be better modelled in one mutant than in another.

Motor dysfunction, cognitive and social impairments are the main features of RTT pathology. Several models of RTT were created in mice that mimic the disorder in these different aspects and have had a valuable role in the study of the basis of its pathogenesis. Three of the models are ubiquitously null for the *Mecp2* gene (Chen et al.

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2001; Guy et al. 2001; Pelka et al. 2006); in two other models, the *Mecp2*-null mutation is restricted to the CNS or postmitotic neurons in the forebrain, hippocampus and brainstem, (Chen et al. 2001; Guy et al. 2001). A transgenic mouse model of RTT was also created with a hypomorphic *Mecp2* allele that truncates the protein prematurely at codon 308 (Shahbazian et al. 2002a).

A battery of behavioural, physiological, biochemical and anatomical tests were used to assess these RTT models, by different research groups, in order to validate them as useful tools in the study of RTT. The etiologic basis of RTT was already identified as the mutation of the *MECP2* gene (Amir et al. 1999). However, given the hypothesized function of the encoded protein, a repressor of several target genes that are involved in different biological functions, the mechanisms by which *MECP2* mutations cause the RTT phenotype are not yet fully understood. It is necessary to characterize the pathways involved in a given endophenotype (such as cognitive impairment), and for this it is wise to use the mouse model in which the endophenotype is best modelled.

How well do mouse models replicate RTT? In the next section, we will go through the diagnostic criteria of RTT one by one and see how well each clinical feature is replicated in the different mouse models.

One of the most remarkable features that is modelled in every one of the RTT mouse models is the uneventful prenatal history and the apparently normal perinatal periods of development, with the appearance of the first symptoms later in life; a few weeks or a few months after birth in the *Mecp2*-null and *Mecp2*-mutant animals, respectively. This concordance in real-time and not in developmental time makes us consider cautiously the role of the MeCP2 protein in CNS development *versus* in the consolidation of CNS maturation.

### **1.3.1. Neurological symptoms**

Three of the diagnostic criteria for RTT are (1) manual stereotypies, (2) an impairment of locomotion, abnormal gait, both ataxic and apraxic, with a wide base, (3) neurogenic scoliosis/kyphosis and (4) dystonia.

In the different mouse models of RTT several motor impairments such as hindlimb claspings, unusual/stiff gait and uncoordinated gait were described (Chen et al. 2001; Guy

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et al. 2001; Shahbazian et al. 2002a; Pelka et al. 2006). When they were assessed for a motor coordination task in the rotarod apparatus, which evaluates the function of the cerebellum, all *Mecp2* mutants showed a lower latency to fall off the rotating rod, suggesting that all presented motor coordination deficits (Shahbazian et al. 2002a; Gemelli et al. 2005; Pelka et al. 2006). In addition, the motor phenotype of the *Mecp2*<sup>308/Y</sup> model was progressive since at 10 weeks of age no differences were found between mutants and wt animals, but 5-month-old mutant animals showed a gait abnormality, as shown by their impaired performance in this and other functionally related motor tests (wire suspension, dowel and vertical pole tests) (Shahbazian et al. 2002a; Moretti et al. 2005). In this last comparison it should be taken into account that the animals tested at 10 weeks and at 5 months were in a different genetic background (129SvEv *versus* a mixed 129SvEv x C57Bl/6), which could also be the cause of the differences found.

Moreover, the *Mecp2*<sup>308/Y</sup> model showed kyphosis later in life (Shahbazian et al. 2002a) as described in RTT patients. Assessment of muscle weakness in the grip strength meter did not show any difference between mutant *Mecp2*<sup>308/Y</sup> model and wt animals (Shahbazian et al. 2002a). The fact that both wt and ko's were able to grip the bar normally suggests that dystonia is not present in these animals.

Spontaneous motor activity was assessed in the *Mecp2* ko and *Mecp2* transgenic models, which exhibited a decreased spontaneous locomotor activity (Chen et al. 2001; Guy et al. 2001; Shahbazian et al. 2002a; Pelka et al. 2006; Stearns et al. 2007). In two studies, the deficits in motor activity occurred mostly in the dark phase of the day, the more active period for rodents (Chen et al. 2001; Moretti et al. 2005). To our knowledge it has never been reported that RTT girls are less active than normal individuals, but this may be difficult to evaluate given their inability to walk in most cases. Nevertheless, this is a feature exhibited by all RTT mouse models, which probably reflects a general motor impairment.

Another remarkable RTT feature that is modelled are the hand stereotypies of the RTT patients, paralleled by the forepaw stereotypies exhibited by the *Mecp2*<sup>308</sup> mouse model. Behavioural stereotypies are often exhibited by animals and frequently attributable to their stress status, for example animals kept in cages or in a zoo and, in this case, the stress is caused by an environmental factor. In this way, it is possible that the forepaw stereotypies exhibited by the mouse could be a signal of altered stress response,

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suggesting that MeCP2 protein play a role in the regulation of this process. Another possibility, however, is that, as for the hand stereotypies in RTT patients, these stereotypic movements originate from a dysfunction of the cortex and striatum brain regions.

Despite the fact that the major implications of the MeCP2 dysfunction are neurological, the estatoponderal growth is also reduced/retarded in girls with RTT, which present a reduced height and hypotrophic small hand and/or feet than normal for the age. In mice, the body weight is altered in the *Mecp2*-mutant animals when compared to wt controls, and it depends on the genetic background of the RTT model. However, animal height and paw size have not been assessed, as far as we know; in RTT patients small hand and size appears to be a striking feature

Evaluation of emotional status and of intellectual and cognitive abilities is a complex task and disturbances in one of these capacities can cause an impaired performance in the other. In this way, each one of these features might be a confounding factor to the assessment of the other and should be taken into account when considering RTT disorder in an individual. Confounding factors in the psychological assessment of RTT patients include autistic behaviour, anxiety, memory disorder and impairments in language. The task of correlating human to mouse impairment is thus quite demanding in this case.

### **1.3.2. Autism**

RTT children are characterized, at least during one phase of the disorder, by the presence of autistic features, which tend to disappear as the disease progresses. Knowledge of the basis of this endophenotype will be helpful not only for the management of RTT patients, but possibly also to a large group of children affected by disorders of the autistic spectrum. The identification of a causal mutation for an autistic spectrum disorder, as is the case of RTT, provides the first molecular pathways to be addressed by researchers in this area. Autism in children manifests by an isolation from the surrounding world, avoidance of social relationships and closure into their own world. Parents of a RTT child, in contrast, often claim that the problem is not in the willingness to communicate, but more in an inability to do so. This could be true but it could also be a myth and final scientific proof is missing.

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In rodents, “autistic-like” behaviour is assessed through an indirect analysis of social behaviour through several tests: the tube test, the social interaction and the partition tests, the intruder-resident test and the nest building test (Moretti et al. 2005). Social behaviour is a highly complex function involving multiple neural systems. This behaviour was analysed in the *CamKII-Mecp2* ko (Gemelli et al. 2005) and in the *Mecp2*<sup>308/Y</sup> transgenic mouse models (Shahbazian et al. 2002a; Moretti et al. 2005). The data obtained from these two studies suggested that mutant animals were not very interested in the surrounding world, including new inanimate objects, or in conspecific animals. It should be taken into account, however, that their low social status could have had implications in the interpretation of the emotional behaviour.

### **1.3.3. Anxiety**

There are very few reports in the literature referring to anxiety status in RTT children (Sansom et al. 1993; Mount et al. 2002; Robertson et al. 2006). The fact is that, given the psychological tests usually employed to assess anxiety, it is difficult to do so in patients with a moderate to profound degree of mental retardation, such as RTT patients. Nevertheless, this feature has been described as present by clinicians, researchers and parents of RTT patients.

When anxiety-like behaviour was assessed in the different mouse models of RTT, the data from the different mutants gave conflicting results. The *CamKII-Mecp2* ko and the *Mecp2*<sup>308/Y</sup> mutants presented heightened levels of anxiety when compared to wt controls, as assessed in the traditionally used elevated plus maze (EPM) and open field (OF) paradigms (Shahbazian et al. 2002a; Gemelli et al. 2005; McGill et al. 2006). However, in the *Mecp2* ubiquitous ko models created by Bird laboratory and by Tam laboratory the levels of anxiety exhibited by the mutants were not different or were lower than those exhibited by their wt controls, using the same paradigms (Guy et al. 2001; Pelka et al. 2006). Very recently, another study assessed the anxiety-like behaviour in the other *Mecp2*-null mice (created by the Jaenisch laboratory). In this study, the authors found that, depending on the paradigm employed, the *Mecp2* mutant animals presented higher (thigmotaxis on swim maze and freezing in a new context in the absence of the cue) or lower levels (EPM and zero maze) of anxiety when compared to wt control mice (Stearns et al. 2007).

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The role of anxiety in RTT is not yet clear and it is difficult to draw a conclusion from the above data obtained in mouse models of the disorder. One factor that might account for the differences achieved in the several studies is the different genetic background of the RTT mouse models studied, which should be taken into account, specially in respect to the evaluation of the anxiety status and cognitive abilities since different strains are known to display different behaviour in these respects (Wolff et al. 2002; Brooks et al. 2005; Tang and Sanford 2005). However, the background of the strain did not seem to influence the results obtained in the different RTT mouse models. For example, *Mecp2* ko animals under 3 months of age presented no differences or lower levels of anxiety than age-matched controls, whether in a pure (C57BL6 and 129SvEv) or in a mixed (129/C57BL6) background (Guy et al. 2001; Pelka et al. 2006; Stearns et al. 2007). Over 4 months of age mutant animals presented heightened anxiety levels, again irrespectively of the genetic background (Shahbazian et al. 2002a; Gemelli et al. 2005; McGill et al. 2006; Stearns et al. 2007). Nevertheless, further studies in the mouse models and RTT patients should be performed in order to establish whether anxiety is an important component of the RTT phenotype.

#### **1.3.4. Mental Retardation**

Another important feature in RTT is mental retardation, with most of the patients presenting learning disabilities. The level of mental retardation presented by the RTT patients and the specificity of their cognitive defects are sometimes difficult to evaluate because of the absence of speech, and of behavioural features such as social avoidance, thus a detailed picture of the cognition impairments is not available. It becomes therefore complicated to establish a parallel between the human disorder and the mouse phenotype concerning cognition.

The Morris water maze task and the conditioned fear test (context and cued) are two widely used paradigms to assess cognition in rodents (for a review see Sousa et al. 2006). The Morris water maze test is useful in assessing spatial learning and reflects the function of the hippocampus, and the fear conditioning test in assessing emotional learning and memory, reflecting both the hippocampal and the amygdalar functions. Both the *CamKII-Mecp2* ko (Gemelli et al. 2005) and the *Mecp2*<sup>tm1.1Tam</sup> ko (Pelka et al. 2006) presented deficits in the fear conditioning test, as given by a reduction in the amount of

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freezing behaviour of the mutants. The *Mecp2*<sup>308/Y</sup> mutant mice also presented abnormalities in the spatial and emotional cognition tasks (Moretti et al. 2006).

### **1.3.5. Sleep**

The sleep pattern of RTT girls is impaired as they do not present the reduction in daytime sleep with age as it normally happens in normal individuals (Ellaway et al. 2001). In rodents, is possible to study circadian activity/sleep-wake cycle using two different paradigms: the infrared beam system to detect movement and the wheel-running paradigm, to detect usage of a running wheel, installed in their home cage. Activity can then be (automatically) analysed under constant dark and after entrainment of animals to the 12:12h light dark-cycle. In order to evaluate whether the circadian rhythm was altered also in a mouse model of RTT, circadian response has been assessed in the *Mecp2*<sup>308/Y</sup> mutant, and no differences were found between mutant and wt control animals (Moretti et al. 2005). This feature was not reported in any other model of RTT and thus further and more detailed studies should be considered regarding this component of the syndrome, that is of major importance for the quality of life of the families of RTT patients.

### **1.3.6. Autonomic dysfunction**

Respiratory dysfunction is another very important feature in the RTT phenotype, the underlying pathological mechanisms of which are not yet known. Three scenarios have been proposed: (1) an underlying cortical dysfunction, since the problems occur only during the awake period and thus could be a “conscious” behavioural manifestation, (2) brainstem immaturity; or (3) disturbance of neuromodulatory regulation within the ponto-medullary respiratory network. With the availability of mutant models, which also mimic the breathing problems exhibited by RTT patients (Guy et al. 2001; Chen et al. 2001), the study of the primary lesion became possible. Viemari and colleagues (2005) showed, both *in vivo* and *in vitro*, that the *Mecp2*-null animals developed a progressive respiratory dysfunction from age 4-weeks, with a highly variable cycle period (respiratory frequencies and apnoeas) when using a medullary preparation. The breathing disturbances presented by the *Mecp2*-null animals were mapped to a deficiency in the noradrenergic and serotonergic modulation of the medullary respiratory circuitry. Norepinephrine (NE) levels were already significantly reduced at 1 month of age, as referred above, before the establishment of breathing dysfunction. In another study, using an *in vitro* working heart-

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brainstem preparation (WHBP), the postinspiratory (early expiration) stage of the respiratory cycle of the *Mecp2*-null mouse was shown to be impaired, due to an hyperexcitability of the pontine-medullary neurones (Stettner et al. 2007). Postinspiration is particularly important for the control of laryngeal adductors, which control breathing movements (apnoeas, air swallowing and ventilation) and speech, both affected in RTT patients.

Different brain areas have been pointed as the cause of the breathing problems. Stettner and colleagues (2007) suggested the Kolliker-Fuse region of the pons, Viemari and colleagues (2005) data pointed to the PreBotzinger complex in the medulla. In RTT patients additional regions have been suggested to be involved in the respiratory rhythmogenesis disturbance, such as the striatal motor system, locus coeruleus and also the cortex. These data are in favour of a deregulation of a modulatory system, such as the noradrenergic system, that projects extensively to several brain regions. Cortical dysfunction does not seem to be involved in the breathing impairment since Stettner and colleagues (2007) used a WHBP, which lack cortical inputs, and recorded similar disturbances as those found by others in RTT and intact *Mecp2*-null mouse brains (Julu and Witt Engerstrom 2005; Viemari et al. 2005). NE released from pontine (A5 and A6) and medullary (A1/C1) neurons was shown to modulate the respiratory rhythm generator located in the medulla (Hilaire et al. 2004; Zanella et al. 2006). Therefore, a deregulation in these neurotransmitters might be responsible for the hyperexcitability verified in these neurons of the *Mecp2*-null mice (Viemari et al. 2005). The precise mechanisms responsible for this modulation are still elusive, but one possibility is through the *N*-methyl-d-aspartate (NMDA) or GABA receptors.

Sudden death is a potential cause of death in RTT patients (20-26%) and autonomic dysfunction (respiratory disorder, severe seizure and cardiac arrhythmia) may contribute to this occurrence. Cardiac instability is a prime suspect cause and electrocardiogram revealed a prolongation of QT intervals and T-wave abnormalities. These parameters were never reported in mouse models of RTT; thus and this being one of the leading causes of death in RTT, the study of cardiac function in these animals is imperative.

### **1.3.7. Pathology**

Regional differences were found in the brains of RTT patients that affect the grey matter, caudate-putamen, midbrain and also cerebellum. MRI studies showed that *Mecp2*

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ko mice present, overall, a reduction in the size of the brain when compared to age-matched controls. When compared to the volume of the cerebrum, the caudate-putamen, hippocampus and thalamus did not show any noticeable variation. However, regional variations were noticed in the thickness of the motor cortex, the corpus calosum and, although not significantly different, the cerebellum also exhibited a trend to be of reduced size (Saywell et al. 2006).

Further, it was found that the neocortex, a region that plays an important role in cognition and motor-sensory integration (Dalley et al. 2004; Arnsten and Li 2005), of symptomatic *Mecp2* ko mice presented thinner layers with an increased cell density. Also, the pyramidal cells of layers II/III in these null mice were smaller and with a less complex dendritic arborisation (Kishi and Macklis 2004). Behavioural abnormalities that reflect the function of the neocortex and hippocampus have been described in the *Mecp2*<sup>308/Y</sup> mouse model (Moretti et al. 2006). However, the morphology of the neurons and dendrites was unaltered in these two brain areas of symptomatic and asymptomatic *Mecp2*<sup>308/Y</sup> mice (Moretti et al. 2006).

### **1.3.8. Electrophysiology**

LTP and LTD are the electrophysiological correlates of neuronal plasticity that are thought to underlie cognitive abilities (Levenson et al. 2002). In this regard, electrophysiological abnormalities were described in all the RTT mouse models. It was shown that, in the absence of MeCP2 protein, ko animals exhibited hippocampal (CA1) impairment of LTP and absence of LTD in an age-dependent manner, i.e, presented by the symptomatic but not by the asymptomatic mice (Asaka et al. 2006). Also, the transgenic *Mecp2*<sup>308/Y</sup> RTT model exhibited a dysfunction in the neocortex and hippocampal LTP (18-22 weeks of age) and LTD (already at 4-6 weeks of age) (Moretti et al. 2006). Additionally, it was shown that cortical pyramidal and hippocampal neurons of *Mecp2*-null mice had a reduced spontaneous activity (Dani et al. 2005; Nelson et al. 2006).

The overexpression of MeCP2 in mice also caused a progressive neurological phenotype, with an electrophysiological outcome as in MeCP2 deficiency, but in the opposite direction. In this model, mutant animals presented an enhanced basal synaptic plasticity and LTP at the hippocampus (Collins et al. 2004). These findings suggest that

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the levels of MeCP2 must be tightly regulated in order to maintain normal electrophysiological balance and proper functioning of the neuron.

### **1.3.9. Neurochemistry**

As discussed above, several studies addressed the neurochemical alterations in the RTT patients but, due to several factors, it was never possible to clearly establish the role of neurochemical dysfunction in the RTT pathology. The availability of mouse models of the disorder should now allow the determination of the role of neurotransmitters in the disease. In this context, a neurochemical study was performed in the total brain of *Mecp2* hemizygous males and their wt littermates, revealing that the concentration of the biogenic amines NE, serotonin (5-HT) and dopamine (DA) in *Mecp2* hemizygous males was lower than in their wt control animals, and that the differences were stronger with increasing age (Ide et al. 2005). In another study, it was shown that, at two months of age, *Mecp2*-null mice presented deficits in NE and 5-HT levels in the medulla, but not in the pons or forebrain (Viemari et al. 2005). NE levels were already significantly reduced by 1 month of age. These findings support some of the hypotheses put forward regarding the primary neurochemical imbalance in RTT patients, but need to be further dissected.

### **1.3.10. Final remarks**

The ultimate goal of all the research in RTT is to find a cure/therapy to the RTT disorder or at least to ameliorate the symptoms and recover some function in these patients. Experiments were performed in order to evaluate the possible rescue of the RTT phenotype in *Mecp2*-mutant animals. RTT phenotype was rescued in *Mecp2*-null mice by expression of either a human or mouse *MECP2/Mecp2* transgene (Collins et al. 2004; Luikenhuis et al. 2004). However, the levels of the MeCP2 protein were shown to be critical; excess MeCP2 was as detrimental as was its deficiency, causing a progressive neurological phenotype, different from RTT.

Several therapeutic approaches are being tested in the mouse models of RTT (such as desipramine, BDNF supplementation, re-introduction of MeCP2 bound to TAT peptides) with very exciting and promising preliminary results. Interesting and surprising were the results recently achieved by the groups of Adrian Bird and Rudolf Jaenisch (Giacometti et al. 2007; Guy et al. 2007). They created mouse models with a conditional *Mecp2* rescue transgene, and showed that activation of the expression of MeCP2 protein later in life, when mice were already presenting RTT-like symptoms, was sufficient for the

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animals to recover from the overt symptoms. This evidence points to a role of MeCP2 in the maintenance of the function of the adult/mature neuron, acting in the lifelong later phase of the brain maturation.

Eight years after the discovery of the gene mutated in RTT new light is now shed into RTT research. The implications of these findings are quite enthusiastic as RTT neurons are not “damaged for life” and the question of RTT as a neurodevelopmental versus neurodegenerative disorder rises. If this is the case, then a generalized optimism may be put forward for several therapies.

In summary, RTT has now been quite well modelled in mouse models, which are extensively characterized. Although these models do not mirror the entire RTT phenotype, they do mirror particular and clinically (relevant) components of it. In the testing of any scientific hypothesis, either envisaging a therapeutic approach or elucidation of the pathways underlying RTT pathogenesis, the choice of the appropriate RTT mouse model, may be crucial for the possibility to obtain an answer.

#### **1.4. Aims of the work**

The subject of this thesis is the manner in which loss of MeCP2 function leads to RTT. The thesis is divided into two main parts: (A) study of the *MECP2* gene in patients with RTT and other neurodevelopmental disorders and (B) study of a mouse model *knock out* for the *Mecp2* gene as a model of RTT.

The specific aims of this work were:

1. To determine the contribution of the different functional groups of *MECP2* gene mutations to the wide range of clinical phenotypes exhibited by RTT patients and patients with related neurodevelopmental disorders.
  2. To understand the onset (early stages) of the RTT pathology – at the behavioural, neuroanatomical and neurochemical levels – caused by lack of the MeCP2 protein, using a mouse model of this disorder.
  3. To explore the role of MeCP2 protein in adult hippocampal neurogenesis, using a mouse model of RTT.
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## CHAPTER 2

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### MeCP2 AND THE HUMAN NERVOUS SYSTEM: EXPLORING THE *MECP2* GENE IN PATIENTS WITH NEURODEVELOPMENTAL DISORDERS

Part of the work presented in this chapter is included in the following peer-reviewed publications (see appendix II):

- Shi J, Shibayama A, Liu Q, Nguyen VQ, Feng J, **Santos M**, Temudo T, Maciel P, Sommer SS: "Detection of heterozygous deletions and duplications in the *MECP2* gene in Rett syndrome by Robust Dosage PCR (RD-PCR)". *Hum Mutat* **2005** May; 25(5):505.

- Temudo T, Oliveira P, **Santos M**, Dias K, Vieira JP, Moreira A, Calado E, Carrilho I, Oliveira G, Levy A, Barbot C, Fonseca MJ, Cabral A, Dias A, Lobo Antunes N, Cabral P, Monteiro JP, Borges L, Gomes R, Barbosa C, Santos M, Mira G, Andrada G, Freitas P, Figueiroa S, Sequeiros J and Maciel P. "Stereotypies in Rett Syndrome: analysis of 83 patients with and without detected *MECP2* mutations". *Neurology* **2007** April 10; 68(15):1183-7.

- Coutinho AM, Oliveira G, Katz C, Feng J, Yan J, Yang C, Marques C, Ataíde A, Miguel TS, Temudo T, **Santos M**, Maciel P, Sommer SS and Vicente AM. "*MECP2* coding sequence and 3'UTR variation in 172 unrelated autistic patients". *Am J Med Genet – Part B Neuropsychiatr Genet* **2007** Jun 5, 144(4): 475-83.

- Venâncio M, **Santos M**, Pereira SA, Maciel P, Saraiva MJ. "An explanation for another familial case of Rett syndrome: maternal germline mosaicism". *Eur J Hum Genet*. **2007** Aug 15(8):902-4.

- **Santos M**, Temudo T, Carrilho I, Gaspar I, Barbot C, Medeira A, Cabral H, Oliveira G, Gomes R, Lourenço MT, Venâncio M, Calado E, Moreira A, Maciel P. "Mutations in the *MECP2* gene are not a major cause of Rett-like phenotype in male patients". (Submitted to Genetic Testing).

- **Santos M**, Jin Yan, Temudo T, Jinong F, Sommer S, Maciel P. "Analysis of highly conserved regions of the 3'UTR of the *MECP2* gene in patients with clinical diagnosis of Rett syndrome and mental retardation". (Submitted to Disease Markers).

- Temudo T, **Santos M**, Ramos E, Dias K, Vieira JP, Moreira A, Calado E, Carrilho I, Oliveira G, Levy A, Barbot C, Fonseca MJ, Cabral A, Cabral P, Monteiro JP, Borges L, Gomes R, Mira G, Pereira AS, Santos M, Epplen JT, Sequeiros J and Maciel P. "Rett syndrome and Rett disorder: an attempt to redefine the phenotypes". (*in preparation*).



## 2.1 Abstract

Mutations in the *MECP2* are responsible for the majority of the classical RTT cases and for a considerable proportion of atypical RTT cases. Still, a considerable number of RTT cases remains without a genetic explanation and for these must contribute either mutations in non-coding regions of the gene, or mutations in other genes. Additionally, the phenotypic spectrum of *MECP2* mutations was proposed to be considerably broader than initially thought.

Most of the *MECP2* mutations are sporadic, occurring through the entire gene and of all types. This allelic heterogeneity constitutes a difficulty in the molecular diagnosis process and also hampers a proper genotype-phenotype correlation.

In this work, we established a DNA bank of 250 Portuguese patients with RTT and related neurodevelopmental phenotypes. Additionally, we contributed to the establishment of different molecular methods for the detection of *MECP2* mutations, and present the most suitable strategy for the molecular diagnostic test of RTT and related neurodevelopmental disorders.

Mutations in the *MECP2* gene were found, in agreement with other studies, in 96.2% of classical RTT and 27.9% of atypical RTT Portuguese patients. In our *MECP2*-mutation positive RTT population, we considered three clinical subtypes: predominantly mental retardation, ataxia and extrapyramidal forms. In the ataxia group we observed predominance of the missense mutations (75%) and in the extrapyramidal group (the more severe form) of the truncating mutations (81.5%). In the mental retardation form of the disease missense and truncating mutations were equally distributed. A further genotype-phenotype correlation of (1) mutations predictably affecting different *MECP2* domains and (2) mutations with different observed effects upon the function(s) or expression of the protein, with the clinical presentation of the disease showed that specific groups of mutations are associated with the clinical subtype. In spite of the small numbers used in this correlation, the results are interesting, suggesting that this approach to correlation analysis could be useful in large series of patients and/or in a meta-analysis of previous studies.

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In respect to the *MECP2* mutation-negative cases, we searched for mutations in the 3'UTR, but no pathogenic variants were found, suggesting that the involvement of this region in RTT must be rare.

This opens the door to other genes as causative agents, and the few direct targets of MeCP2 protein seem to be the most interesting candidates. However, at this point their analysis should not yet constitute a routine in the diagnosis of RTT, more research being needed before an integration can be achieved from clinical outcome and the function of these new genes.

## 2.2. Introduction

Mutations in the *MECP2* gene lead to the neurodevelopmental disorder Rett syndrome (Amir et al. 1999).

For many years the diagnosis of RTT was entirely based on a patient's clinical presentation. From 1999, the identification of mutations in the *MECP2* gene in patients with a clinical diagnosis of RTT (Amir et al. 1999), introduced the possibility of using this biological marker to further support the clinical diagnosis of RTT. After this initial publication, several other studies have been published, reporting mutations in this gene in large series of sporadic and familial, classical and atypical RTT patients of European, Asian and American origin (Wan et al. 1999; Amano et al. 2000; Amir et al. 2000; Bienvenu et al. 2000; Cheadle et al. 2000; Hampson et al. 2000; Huppke et al. 2000; Auranen et al. 2001; Giunti et al. 2001; Hoffbuhr et al. 2001; Monros et al. 2001; Vacca et al. 2001; Yamada et al. 2001; Huppke et al. 2002; Pan et al. 2002)

Surprisingly, the following characterization of the phenotype(s) associated with *MECP2* mutations showed that these were not limited to the RTT phenotype, but apparently led to a much broader spectrum of manifestations than initially thought. Mutations in the *MECP2* gene could be found in patients with classical and atypical RTT, in males with severe neonatal encephalopathy (Imessaoudene et al. 2001; Lynch et al. 2003; Leuzzi et al. 2004), males with classical RTT-associated with a Klinefelter syndrome or a somatic mosaicism (reviewed in Schanen 2001), females with only minor learning impairments (Lesca et al. 2007), males and females with mental retardation (Couvert et al.

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2001; Van Esch et al. 2005), males and females with autism (Carney et al. 2003), Angelman and Prader-Willi syndromes (Kleefstra et al. 2002; Hitchins et al. 2004; Kleefstra et al. 2004), schizophrenia and psychosis (Cohen et al. 2002; Klauck et al. 2002) or even (in the case of some missense mutations) no phenotype at all (Dayer et al. 2007).

The highest frequency of *MECP2* mutations is found in classical RTT (>80%), followed by atypical RTT (30%) (Amir and Zoghbi 2000; Huppke and Gartner 2005). The frequency of mutations in this gene is now known to be much lower in all other phenotypes (Couvert et al. 2001; Imessaoudene et al. 2001; Yntema et al. 2002; Hitchins et al. 2004; Campos et al. 2007; Lesca et al. 2007).

Concerning mutation-negative patients, the non-coding regions, especially the long and conserved 3' untranslated region (3'UTR) of the *MECP2* gene also constitute good candidates to screen for mutations, but they have not been extensively explored. These could account for the remaining (20% in classical and 70 % in atypical) RTT cases without a known genetic cause. The large frequency of genetically unexplained cases may suggest, instead, that other gene/s might be involved, but this still remains to be determined (Amir and Zoghbi 2000).

### **Foreground to the work presented in this chapter**

The advantage of having a molecular diagnostic marker prompted us to perform the epidemiological study of the Portuguese RTT patients and other patients with closely related neurodevelopmental disorders. The determination of the spectrum of *MECP2* mutations and their associated phenotypes is important in clinical terms for a molecular diagnosis strategy, in the field of child neurology and psychiatry; it is also interesting from the functional genomics perspective, since the correlation between the loss of MeCP2 function(s) and the resulting phenotype(s) in humans may help to elucidate the function of this protein and of the pathways that it integrates in the normal development, maturation and function of the nervous system.

Mutations in the *MECP2* gene are usually sporadic and distributed along the whole gene, comprehending all mutation types: missense, nonsense, small and large, complex genomic rearrangements and mutations affecting the splice mechanisms (Laccone et al. 2001; Lee et al. 2001; Bienvenu et al. 2002; Miltenberger-Miltenyi and Laccone 2003). Allelic heterogeneity is a difficulty in the molecular diagnosis of this kind of disorders. In

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order to detect virtually all mutations, efficiently, rapidly and at a low cost, a suitable strategy should be specifically designed for each gene. We attempted to do this for the *MECP2* gene.

Another challenging point in the molecular diagnosis of allelically heterogeneous diseases is the difficulty in distinguishing a pathogenic mutation from a polymorphism, and predicting the effect of each mutation in the function of the protein. In order to distinguish a pathogenic mutation from a polymorphism several steps can be followed: (1) study of the parents, and eventually other sibs, for the presence of the variant; (2) analysis of the frequency of the variant in a control population; (3) checking whether the nucleotide change affects a splice site; (4) evaluation of the nature of the amino acid change, in terms of charge and hydrophobicity; and (5) evaluation of the conservation of the affected amino acid across different species and protein family members; (6) further functional studies, at the protein level should be performed to try to conclude about the consequences of that mutation. We describe our strategy in dealing with these questions.

Finally, a genotype-phenotype correlation in RTT has not been clearly established yet, due (1) to the diversity of mutations (type and location) that may affect differently the function of the protein, (2) the phenomenon of X-chromosome inactivation (XCI), and also (3) the RTT clinical profile, with evolving phases. These facts hamper genuine prognosis and the prediction of a response to therapy. We attempted to approach this question in an original manner in order to achieve a more effective correlation.

### **Specific aims**

The general goal of this work was to determine the contribution of the different functional groups of *MECP2* gene mutations to the wide range of clinical phenotypes exhibited by RTT patients, and patients with related neurodevelopmental disorders.

For this purpose, our specific goals in this part of the work were:

1. To establish a DNA bank of Portuguese patients with RTT and with related neurodevelopmental disorders.
  2. To optimize different molecular methods for the detection of all sporadic *MECP2* mutations, and propose the most suitable strategy for the molecular diagnostic test of RTT and related neurodevelopmental disorders.
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3. To define the spectrum of phenotypes associated with *MECP2* mutations in the Portuguese population, by studying patients with RTT and related neurodevelopmental disorders.

4. To determine the clinical outcome of mutations in different domains of the MeCP2 protein.

5. To assess the contribution of mutations in a non-coding region of the *MECP2* gene (the 3'UTR) for RTT pathogenesis.

## 2.3. Material and Methods

### 2.3.1. Subjects

We have collected a total of 250 patients (210 girls and 40 boys) with a clinical classification of classical or atypical RTT (Hagberg et al. 1983; Hagberg et al. 2002), or with a related neurodevelopmental disorder (mental retardation, autism, Angelman syndrome (AS) and West syndrome\*) (figure 2.1). Patients were recruited through all the country, but most of them came from the north region of Portugal. In this study, 105 patients were observed at least once by the same neuropsychiatrician (Dr Teresa Temudo), thus reducing the inter-subjective variation in the diagnosis of RTT. The remaining patients in the study have been sent for diagnostic purposes to our laboratory by general practitioners or paediatricians, from several hospitals in the country, with a clinical classification of RTT or a related neurodevelopmental disorder. For these, the extent and quality of clinical information available to us was highly variable.

The sample included a total of 40 boys with some kind of unexplained neurodevelopmental disease. Of these, we have obtained more detailed clinical information for 29 boys, with phenotypes that ranged from Asperger syndrome<sup>†</sup> and mental retardation to encephalopathy and the atypical RTT-like clinical picture previously described in boys (reviewed in Schanen 2001) (figure 2.2). For 11 of the male patients tested, no clinical information was available.

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\* West syndrome (OMIM, #308350). Also known as X-linked infantile spasm syndrome is characterized by early-onset generalized seizures, hypsarrhythmia, and mental retardation.

<sup>†</sup> Asperger syndrome (OMIM, #608638). Is considered to be a form of childhood autism, primarily distinguished from autism by the higher cognitive abilities and a more normal and timely development of language and communicative phrases.

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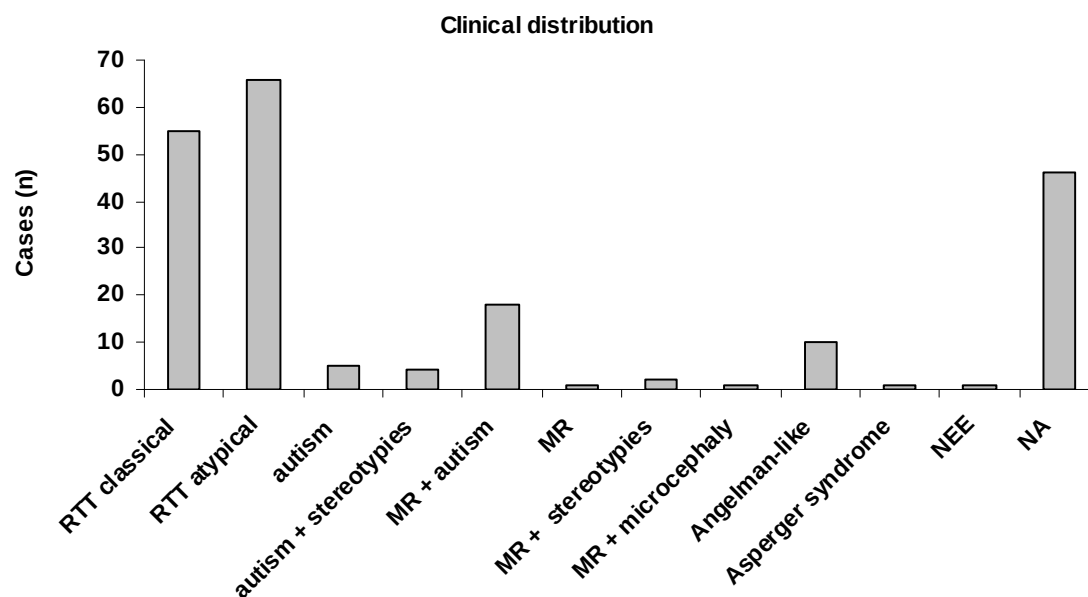


**Figure 2.1. Clinical classification of 240 girl patients analysed for the *MECP2* gene.** RTT, Rett syndrome; MR, mental retardation; NEE, neonatal epileptic encephalopathy; NA, not available.

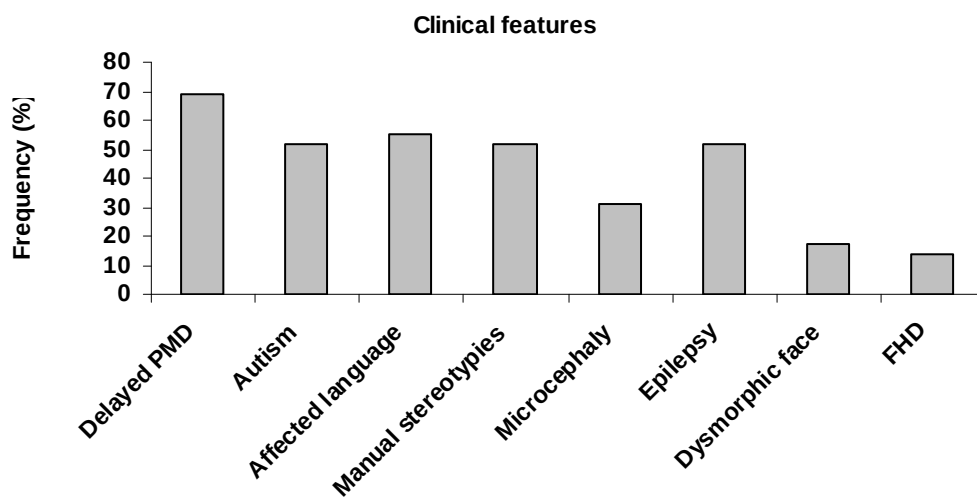


**Figure 2.2. Clinical features exhibited by 29 male patients analysed for the *MECP2* gene.** PMD, psychomotor development; FHD, familial history of disease.

Whenever possible, parents of the patients were also collected and included in the study. We have studied a total of 80 trios; for 15 patients, only one of the parents was collected (13 mothers and 2 fathers).

As a control population we used DNA from 134 unrelated healthy individuals (males, n=40 and females, n=93) of Portuguese origin.

After receiving the material in the laboratory, each sample was attributed a unique number, and all the available clinical information was processed in a protected database, separate from the personal and the genetic information; crossing of information was only allowed to 2 users (PM and MS).

### **2.3.2. Methods**

#### **DNA extraction**

We received peripheral blood samples (5 to 10 mL) from patients and, whenever possible, their parents. Genomic DNA was extracted from leucocytes, using the Puregene DNA isolation system (Gentra, Minneapolis, MN).

#### **Single strand conformation polymorphism (SSCP) and sequencing**

The coding region and exon/intron boundaries of the *MECP2* gene (RefSeq ID: NM\_004992) were amplified by PCR, using one pair of primers for exon 2, three pairs of primers for exon 3, and five pairs of primers for exon 4 (table S2.1 in appendix I). DNA amplification was performed in a final volume of 25  $\mu$ l of a PCR mixture, which consisted of 1X enzyme buffer, 0.5 mM  $MgCl_2$ , 0.2 mM dNTP, 0.8  $\mu$ M of each primer, 10% DMSO, 1.5U Taq DNA Polymerase (Fermentas), and approximately 100 ng of genomic DNA. The thermal cycling profile (My Cycler, BioRad) consisted of an initial denaturation step for 5 min, at 95°C, followed by 35 cycles of denaturation, for 1 min, at 95°C, annealing for 1 min, at Ta°C (specific for each pair of primers, table 2.1.1 in appendix I), elongation for 1 min, at 72°C, and a final extension step for 5 min, at 72°C.

The total PCR product was denaturated for 5 min at 95°C, chilled on ice and loaded in a non-denaturing 15% polyacrylamide gel electrophoresis (PAGE), and fragments

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were allowed to migrate in a DCODE system (BioRad), at 20°C and 300V, over approximately 14 hours.

The pattern of migration was visualized by silver staining (0.2%), and the gel was transferred to a 3MM Whatman paper and allowed to dry, at 80°C, for 30 min.

The samples in which a different pattern of migration was detected were re-amplified by PCR, using the same primers and conditions described above (table S2.1 in appendix I). The sequencing reaction consisted of 1X enzyme buffer, 1  $\mu$ M of primer forward/reverse, 4  $\mu$ L of Pre-mix Big dye (Applied Biosystems), and 3  $\mu$ L of the PCR product, in a final volume of 10  $\mu$ L. The thermal cycling profile (My Cycler, BioRad) consisted of an initial denaturation step for 3 min, at 94°C, followed by 25 cycles of denaturation for 10 sec, at 96°C, annealing for 5 sec, at 58°C, and elongation for 4 sec, at 60°C.

After purification of the sequencing reaction, the pellet was resuspended in template sample resuspension, and ran in an ABI model 377 automatic sequencer (Perkin-Elmer, Norwalk, CT), for the identification of the possible variants.

### **Detection of small deletions and insertions**

PCR mixtures and conditions were used as described under “single strand conformation polymorphism (SSCP) and sequencing”. The total PCR product was denatured for 5 min, at 95°C, chilled on ice, and loaded in a denaturing 8% sequencing PAGE; fragments were allowed to migrate at 1200/1500V, over approximately 3 hours.

### **Allele-specific PCR**

Allele-specific PCR was optimized for the detection of each of the five recurrent mutations, and for one variant of unknown function (K305R) identified in the *MECP2* gene (table S2.2 in appendix I).

Two PCR mixtures for each variant were prepared in order to amplify either the normal or the mutated allele, using three primers: one that is common to both reactions; and the other two, one for each PCR mixture, specific either for the normal or for the

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mutated allele. In a final volume of 25  $\mu$ l: 1X enzyme buffer, 0.5 mM  $\text{MgCl}_2$ , 0.2 mM dNTP, 0.8  $\mu$ M of each primer pair (either for the normal allele or for the mutated allele), 10% DMSO and 1.5U Taq DNA Polymerase (Fermentas), and 100 ng DNA. The thermal cycling profile (My Cycler, BioRad) consisted of an initial denaturation step for 5 min, at 95°C, followed by 35 cycles of denaturation for 1 min, at 95°C, annealing for 1 min, at  $T_a$ °C (specific for each pair of primers, table S2.2 in appendix I), elongation for 1 min, at 72°C, and then a final extension step for 5 min, at 72°C.

PCR products were electrophoresed in a 2% agarose gel, and visualized under UV light.

### **Direct sequencing**

The coding region and exon-intron boundaries of the *MECP2* gene were amplified by PCR, using one pair of primers for exons 1, 2 and 3, and three pairs of primers for exon 4 (table S2.3 in appendix I). DNA amplification of exon 1 was performed in a final volume of 25  $\mu$ l. After an initial denaturation step of the DNA eluted in TE buffer (60 ng) for 10 min, at 97°C; the PCR mixture was added (0.5 mM  $\text{MgCl}_2$ , 0.133 mM dNTP, 0.2  $\mu$ M of each primer, 5% DMSO and 2.5U Taq DNA Polymerase (Fermentas)). The thermal cycling profile (My Cycler, BioRad) consisted of 40 cycles of a denaturation for 1 min, at 95°C, annealing for 2 min, at 63°C, elongation for 3 min, at 72°C, and then a final extension step for 5 min, at 72°C.

For the DNA amplification of exons 2 to 4, a final volume of 30  $\mu$ l PCR mixture (0.8 mM  $\text{MgCl}_2$ , 0.133 mM dNTP, 0.2  $\mu$ M of each primer and 2U of Taq DNA Polymerase (Fermentas)) was used. The thermal cycling profile (My Cycler, BioRad) consisted of an initial denaturation for 5 min, at 95°C, 35 cycles of a denaturation for 1 min, at 95°C, annealing for 1 min, at  $T_a$ °C (specific for each pair of primers, see table S2.3 in appendix I), extension for 1 min, at 72°C, and a final extension for 5 min, at 72°C.

After PCR amplification the different fragments were automatically sequenced, using the same primers used for the PCR, in a ABI 377 model (Perkin Elmer), and the sequences analysed for point mutations or small rearrangements (deletions and duplications). Sequence changes were confirmed by re-amplification of genomic DNA and sequencing in the opposite direction.

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### **Detection Of Virtually All Mutations – SSCP (DOVAM-S)**

Several non-coding regions of the *MECP2* 3'UTR were selected, based on their conservation among human and mouse species (almost 100% conservation at the nucleotide level), in a total of 9 blocks (NM\_004992: c.1607-c.1956, c.2561-c.2891, c.3551-c.3805, c.3768-c.4128, c.6851-c.7029, c.7116-c.7436, c.8372-c.8645, c.8607-c.8872 and c.9844-c.10182), which also included the regulatory regions around three of the four polyadenylation signals. These 3'UTR blocks were scanned for mutations with DOVAM-S (Shibayama et al. 2004). The different blocks were first amplified robotically, pooled, denatured and electrophoresed, under five nondenaturing conditions, varying in gel matrix, buffer, temperature, and additive: (1) 10% PAGE+/30 mM Tricine/Triethanolamine, at 20°C, (2) 10% HR1000/30mM Tricine/Triethanolamine, at 4°C, (3) 10% PAGE+/TBE/5% glycerol, at 20°C, (4) 10 % HR1000/TBE/2.5% glycerol, at 4°C, and (5) 10% PAGE+/30mM Capso, at 4°C. PCR products with mobility shifts were sequenced with the ABI 377 (Perkin-Elmer, Norwalk, CT), and nucleotide alterations were analyzed. Sequence changes were confirmed by re-amplification with genomic DNA and sequencing in the opposite direction.

For the DNA amplification of the different 3'UTR blocks, a final volume of 25 µl PCR mixture (2.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 2.5 µM of each primer and 2U of AmpliTaq Gold (Gibco)) was used. The thermal cycling profile (My Cycler, BioRad) consisted of an initial denaturation for 10 min, at 94°C, 35 cycles of a denaturation for 15 sec, at 94°C, annealing for 30 sec, at 55 °C, extension for 1 min, at 72°C, and a final extension for 10 min, at 72°C (table S2.4 in appendix I).

### **Detection of large rearrangements by robust dosage-PCR (RD-PCR)**

The DNA concentrations were measured in a UV spectrophotometer, at 260 nm, and adjusted to a working concentration of 30 ng/µL in TE buffer. Genomic DNA samples were incubated at 90°C in TE buffer, for 10 minutes. Four RD-PCR assays for three coding exons of *MECP2* gene were designed according to (Shi et al. 2005) (table S2.5 in appendix I). These assays were divided into two groups: group I, which included amplification of exons 2 and 3 of *MECP2* and exon 12 of ataxia telangiectasia mutated (*ATM*) gene, was used as an autosomal internal control segment; group II included the two other assays for exon 4, subdivided in two fragments (4I and 4II) of *MECP2*, and was used as the internal autosomal control segment the fucosyltransferase 2 (*FUT*) gene.

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The RD-PCR conditions were slightly modified from the original report (Shi et al. 2005), and adapted to our laboratory conditions.

For group I, 60 ng of DNA were used in a PCR mixture, which consisted of 1X buffer #3 (Roche), 4.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 0.2 μM of each pair of primers, 0.5 μg BSA, 1U of Platinum Taq DNA Polymerase (Invitrogen), 1U of Platinum Taq DNA polymerase HiFi (Invitrogen), in a final volume of 25 μl. For Group II, 60 ng of DNA were used in a PCR mixture consisting of 1X buffer #3 (Roche), 3 mM MgCl<sub>2</sub>, 0.2 mM dNTP-G, 0.05 mM/0.15 mM of 7-deaza GTP/dGTP, 0.2 μM of each pair of primers, 10% DMSO, 0.5 μg BSA, 1U of Platinum Taq DNA Polymerase (Invitrogen), 1U of Platinum Taq DNA polymerase HiFi (Invitrogen), in a final volume of 25 μl. The thermal cycling profile (My Cycler, BioRad) consisted of 25 or 30 cycles (for groups I and II, respectively) of denaturation of 15 sec, at 94°C, annealing for 30 sec, at Ta°C (specific for each pair of primers, see table S2.5 in appendix I), and elongation for 1 min, at 72°C.

For validation of the four assays covering the coding region of the *MECP2* gene, a blinded analysis was performed with 48 blinded genomic DNA samples, where either the sex status, or the number of each status, was unknown. The male sample was functionally equivalent to a RTT patient with a large heterozygous deletion.

In order to characterize the deletion junction of patient P3, ten more RD-PCR assays were developed, in the 3' and 5' flanking regions of the *MECP2* gene (Shi et al. 2005).

The PCR product (12 μl) was electrophoresed in a 2% agarose gel (0.2 μg/mL ethidium bromide) for 2 hours, at 120V, and scanned in a *Alphamager* (BioRad). *Spotdenso* software was used to quantify the PCR yield. The ratio of yields (ROY) was calculated by dividing the target net signal by the internal control net signal. For normalization, the ROY of patient samples were divided by the average ROY of control normal females.

### **Southern blotting analysis**

Southern blotting was performed using probes RTT2, RTT3 and p(A)10, hybridizing with exon 2, exon 3 and the end of the 3'UTR (figure 2.3, and table S2.6 in appendix I).

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Probes were generated by PCR from genomic DNA, purified from 1% agarose gel by QIAEX II (QIAGEN, Valencia, CA), and labeled with  $^{32}\text{P}$  dCTP by Prime-It II Random primer (Stratagene, Cedar Creek, TX). The genomic DNAs (8  $\mu\text{g}$ ) of female control, male control and patient P3 were digested with *Hind* III and *Pst* I for probe RTT2, *Sac* I for probe RTT3 and *Hind* III and *Sac* I for probe p(A)10. Digested DNA fragments were separated in a 1.5% agarose gel, and blotted into a nylon membrane (Hybond H-N+; Amersham Pharmacia Biotech, Buckinghamshire, England). Hybridization was performed overnight, at 65°C, and washings were carried out in a series of SSC/SDS solutions (0.1%SDS, 2%-0.1% SSC). Membranes were exposed to storage phosphor screen, scanned by Typhoon 9410 Imager (Amersham, Molecular Dynamics, Sunnyvale, CA). ImageQuant™ software was used to quantify the signals.

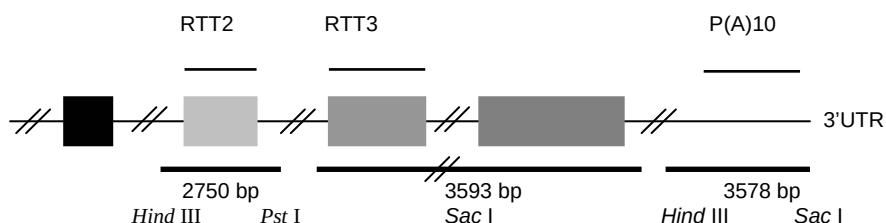


**Figure 2.3. Schematic representation of the *MECP2* gene.** Regions analyzed by Southern blotting and probes used in the assay (figure is not to scale).

### Determination of X chromosome inactivation (XCI) pattern

XCI assays were performed in genomic DNA isolated from leukocytes of peripheral blood, to assess the pattern of XCI. The assay was based on a previously described method (Allen et al. 1992), which allows the determination of the X-inactivation status, using a polymorphic trinucleotide repeat polymorphism in the androgen receptor gene (*AR*, RefSeq ID: NM\_000044.2), flanked by two methylation-sensitive restriction enzyme sites.

Two  $\mu\text{g}$  of genomic DNA were digested with the endonuclease *Hha* I (1x enzyme buffer #4, 1XBSA and 2U *Hha* I (NEB), in a final volume of 20  $\mu\text{L}$ ), at 37°C, overnight. The restriction enzyme hydrolyzed only the unmethylated alleles.

Two  $\mu\text{L}$  of the digestion mixture were used for the amplification of exon 1 of *AR* gene (table S2.1.7 in appendix I). A final volume of 30  $\mu\text{L}$  PCR mixture (1X enzyme buffer,

0.625 mM MgCl<sub>2</sub>, 0.2 mM dNTP-A, 0.032 mM <sup>35</sup>S dATP, 0.8 μM of each primer, 2% formamide and 1.8U of Taq DNA Polymerase (Fermentas)) was used. The thermal cycling profile (My Cycler, BioRad) consisted of an initial denaturation step for 5 min, at 95°C, 34 cycles of a denaturation for 45 sec, at 95°C, annealing for 30 sec, at 60°C, extension for 30 sec, at 72°C, and a final extension for 5 min, at 72°C.

After PCR amplification, 6 μL of each sample were loaded in a denaturing 6% PAGE, and the fragments were allowed to separate at 1200/1700V. The gel was transferred to a 3MM Whatman paper, allowed to dry, and then exposed to an X-ray film (Kodak) for 3 days, at room temperature.

Films were scanned by Typhoon 9410 Imager (Amersham); scoring of the XCI pattern was made by densitometry of the amplified DNA bands, with the ImageQuant software.

#### **Identification of reported mutations in neuroligin 3 (*NLGN3*) and neuroligin 4 (*NLGN4*) genes**

Exon 6 of the *NLGN3* and exon 5 of *NLGN4* genes were amplified by PCR (primers in table S2.8 in appendix I). For the DNA amplification, a final volume of 30 μl PCR mixture (0.8 mM MgCl<sub>2</sub>, 0.133 mM dNTP, 0.2 μM of each primer and 2U of Taq DNA Polymerase (Fermentas)) was used. The thermal cycling profile (My Cycler, BioRad) consisted of an initial denaturation step for 5 min, at 95°C, 35 cycles of a denaturation for 1 min, at 95°C, annealing for 1 min, at T<sub>a</sub> °C (specific for each pair of primers, see table S2.1.8 in appendix I), extension for 1 min, at 72°C, and a final extension for 5 min, at 72°C.

After PCR amplification the different fragments were automatically sequenced in an ABI 377 model (Perkin Elmer) and the sequences analysed for the reported mutations (*NLGN3*: c.1186insT and R451C; *NLGN4*: c.1253delAG) or others, eventually. Sequence changes were confirmed by re-amplification of genomic DNA and sequencing in the opposite direction.

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## 2.4. Results

### Optimization of the molecular diagnostic method

We analysed a total of 84 patients, with a clinical diagnosis of classical or atypical RTT by SSCP. The analysis of the SSCP pattern of migration of the different fragments of exons 2, 3 and 4 of the *MECP2* gene revealed several alterations (figure 2.4).

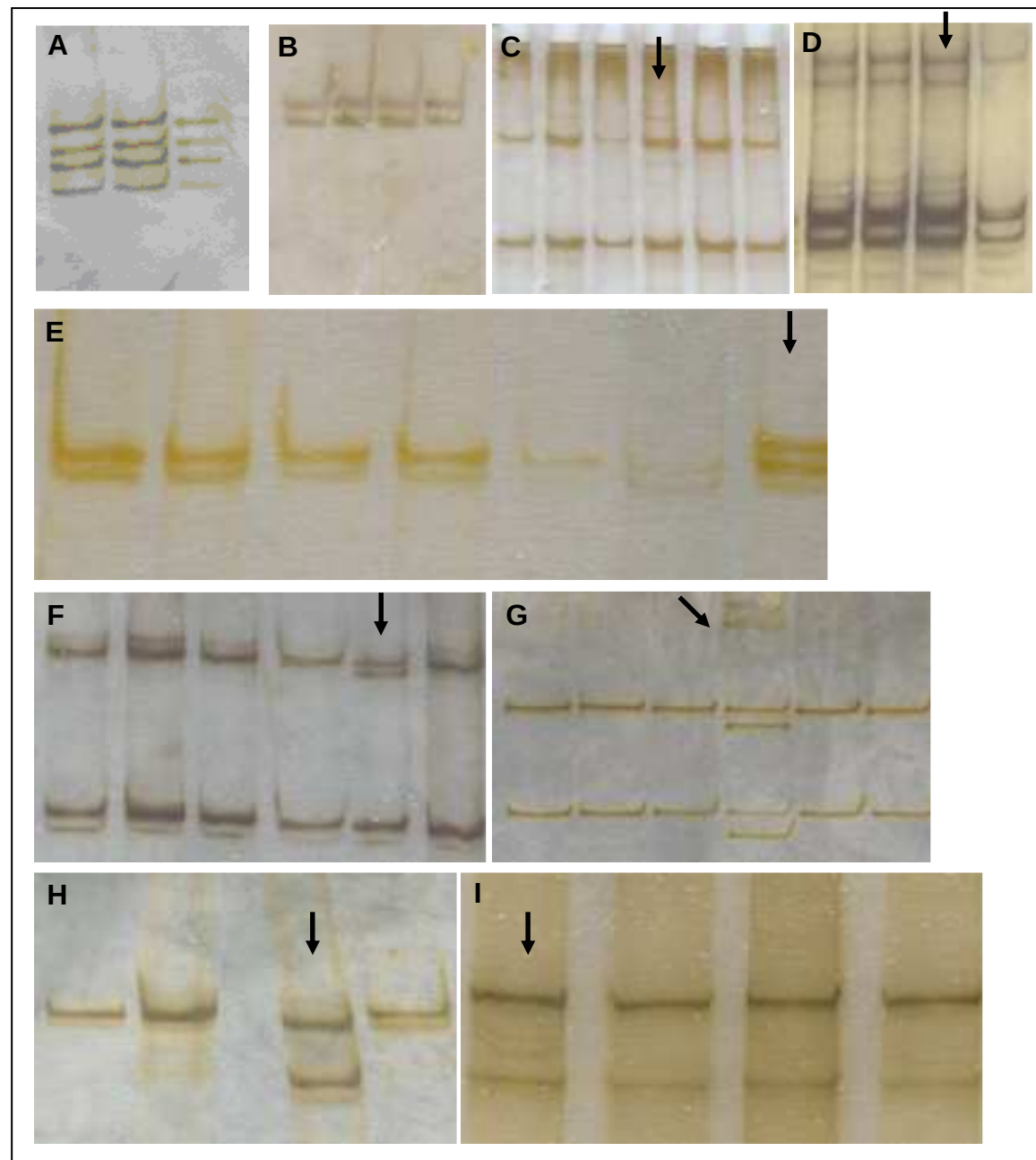


**Figure 2.4. Single strand conformation polymorphism (SSCP) of the *MECP2* gene.** Different patterns of migration were found for each of the *MECP2* fragments analysed. A – exon 2; B, C and D – exon 3, fragments 3.1 to 3.3 respectively; E, F, G, H and I – exon 4, fragments 4.1 to 4.5 respectively.

In exon 2, no SSCP variants were found. In exons 3 and 4, several variants were detected; however, most of them were concentrated within exon 4 (73%) (table 2.1).

After sequencing of the SSCP variants we identified: two mutations in exon 3 and 24 mutations in exon 4 (table 2.1), distributed through the different fragments. For fragment 4.1, 78.6% of the SSCP variants proved to be true alterations, but for all the other fragments the percentage of sequence variants confirmed as mutations was much lower, ranging between 16.7% and 55.6%. These values revealed a high percentage of false positives in the SSCP technique and its low specificity (table 2.1).

**Table 2.1.** *MECP2* variants found by SSCP and identified by direct sequencing of the gene.

| Gene  | Exon | Fragment | SSCP variants<br>(n) | Mutations<br>(%(n)) | False positives<br>(%(n)) | False negatives<br>(%(n)) |
|-------|------|----------|----------------------|---------------------|---------------------------|---------------------------|
| MECP2 | 2    | 2        | 0                    | -                   | -                         | -                         |
|       |      | 3.1      | 4                    | 0                   | 100.0 (4)                 | 100.0 (1)                 |
|       | 3    | 3.2      | 7                    | 0                   | 100.0 (7)                 | 0                         |
|       |      | 3.3      | 12                   | 16.7 (2)            | 83.3 (10)                 | 60.0 (3)                  |
|       | 4    | 4.1      | 14                   | 78.6 (11)           | 21.4 (3)                  | 42.1 (8)                  |
|       |      | 4.2      | 21                   | 28.6 (6)            | 71.4 (15)                 | 0                         |
|       |      | 4.3      | 9                    | 55.6 (5)            | 44.4 (4)                  | 28.6 (2)                  |
|       |      | 4.4      | 7                    | 28.6 (2)            | 71.4 (5)                  | 0                         |
|       |      | 4.5      | 13                   | 0                   | 100.0 (13)                | 0                         |

Legend: SSCP, single strand conformation polymorphism; n, number of occurrences.

The coding region and exon/intron boundaries of all of the 84 patients included in this study were also entirely sequenced. By direct sequencing, several other mutations that had been missed by the SSCP analysis were identified (false negatives). We detected four additional mutations in exon 3 and ten more in exon 4 (table 2.1 and table 2.2); only 35.7% (5/14) of these mutations had already been identified by SSCP, which suggests a low sensitivity of the SSCP technique.

**Table 2.2.** *MECP2* mutations identified by SSCP and direct sequencing.

| Exon | Variant:<br>SSCP (n) | Direct sequencing (n) |
|------|----------------------|-----------------------|
| 3    |                      | K39fsX43 (1)          |
|      | R106W (1)            | R106W (2)             |
|      | I125I (1)            | Q110X (1)             |
| 4    |                      | R133C (3)             |
|      | P152R (1)            |                       |
|      | T158M (1)            | T158M (3)             |
|      | R168X (9)            |                       |
|      |                      | T184fsX185 (1)        |
|      |                      | R211S (1)             |
|      | R255X (4)            |                       |
|      | G269fsX288 (1)       |                       |
|      | R270X (1)            |                       |
|      |                      | R294X (1)             |
|      | T299T (1)            |                       |
|      |                      | P302L (1)             |
|      | I303fsX477 (1)       |                       |
|      | P322A (1)            |                       |
|      | V380M (1)            |                       |
|      | L386fsX389 (1)       |                       |
|      | L386fsX390 (1)       |                       |
|      | L386fsX399 (1)       |                       |

Legend: SSCP, single strand conformation polymorphism; n, number of occurrences.

### Mutations and polymorphisms in the *MECP2* gene

Analysis of the *MECP2* sequence changes in exons 1 to 4, exon/intron boundaries and (for some cases) the 3'UTR regions was performed in a total sample of 250 patients (210 girls and 40 boys). Several variations in the *MECP2* gene were identified in this study (figure 2.5). Most of them were already described in the literature and in the *MECP2* mutation database (<http://mecp2.chw.edu.au/>); however, others were identified here for the first time. The alterations were distributed through the entire gene, in coding (exons 1, 3 and 4), as well as non-coding (intron 3 and 3'UTR) regions, with different frequencies of all types of variants (figure 2.6).



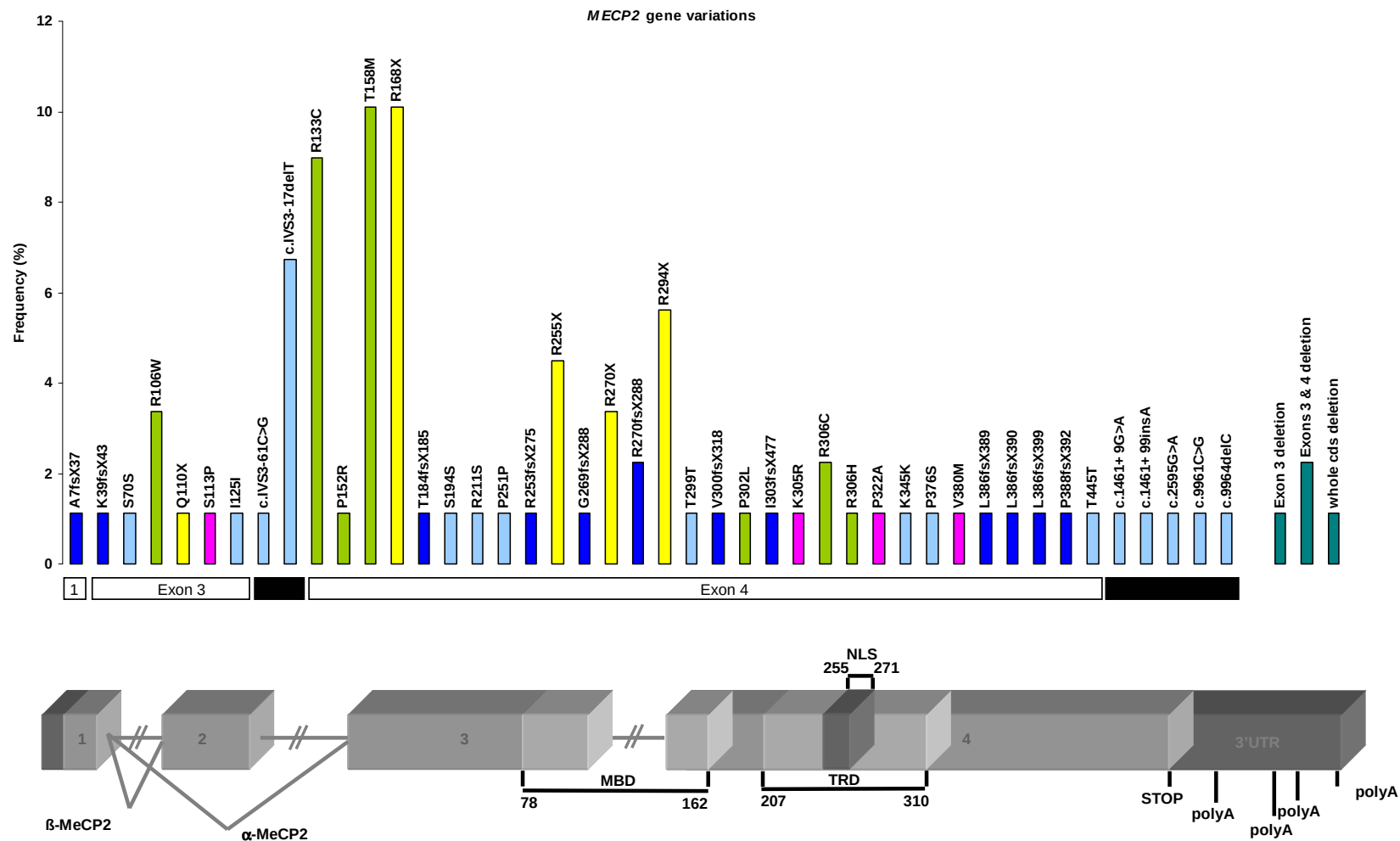


**Figure 2.5. *MECP2* gene variants identified in the Portuguese population with Rett syndrome and other neurodevelopmental disorders.** **A.** Number of occurrence of each variant and their localization in the *MECP2* gene. White boxes: coding region of the gene, Black boxes: non-coding regions of the gene. **B.** Schematic representation of the *MECP2* gene. MBD, methyl CpG binding domain; TRD, transcription repression domain; NLS, nuclear localization signal. Numbers represent amino acid positions. Figure is not to scale.

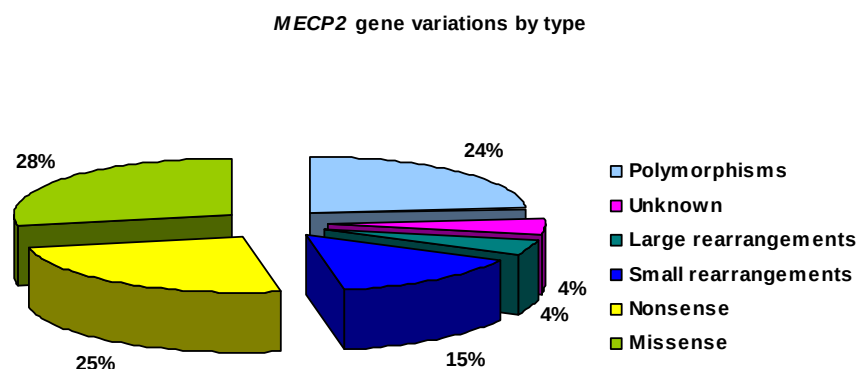


**Figure 2.6. Types of variants found in the *MECP2* gene.** Frequency of each type of mutation (missense, nonsense and rearrangements), polymorphisms and variants of unknown significance, identified by direct sequencing of the *MECP2* gene.

### Polymorphisms and variants of unknown significance

We detected a total of 25 variants (20 different) in 23 patients that were silent polymorphisms, synonymous changes or variants of unknown biological significance (table 2.3).

Seven nucleotide changes were detected in our sample of patients that do not result in an amino acid change. Six of these silent changes were already described as polymorphisms in the literature (c.210C>T, S70; c.373A>C, I125; c.582C>T, S194; c.897C>T, T299; c.1035A>G, K345 and c.1335G>A, T445), and one was described in this study for the first time (c.753C>T, P251). Polymorphisms S194, T299 and T445 had already been identified in unaffected individuals (<http://mecp2.chw.edu.au/>).

We searched for exonic splice enhancers (ESE) in the exons of *MECP2*, where these variants were found, but the alterations were not localized in any known ESE site (ESEfinder 3.0) (Cartegni et al. 2003; Smith et al. 2006).

Additionally, we identified 6 sequence alterations that result in an amino acid change (S113P, R211S, K305R, P322A, P376S and V380M). In these cases, the pathogenic value of the alteration had to be carefully considered. The R211S and P376S alterations were already described in other populations as polymorphisms and are thought not to have consequences in the function of the protein (<http://mecp2.chw.edu.au/mecp2/>). The

consequences of the S113P and V380M alterations, described here for the first time, and of the K305R and P322A substitutions, already reported in the literature, are unknown. In an attempt to characterize the pathogenic value of these amino acid changes, we assessed, for each one of these alterations: (1) its presence in the parents of the affected patient, when available; (2) the conservation of the amino acid changed in paralogs (figure 2.7) and orthologs of the *MECP2* (figure 2.8); (3) the nature of both amino acids involved; and (4) the presence of that alteration in a control population.

The S113P alteration was not present in the parents of this patient. The change of a serine (S) by a proline (P) occurs between amino acids of different groups, one hydrophilic, usually located in the surface of proteins, the other a special amino acid. The serine might form hydrogen bonds with other polar molecules, through its hydroxyl group, and is a potential site of phosphorylation or other post-transcriptional modifications. Proline is a very particular amino acid: it has a rigid cyclic ring and it sometimes is found at points where the polypeptide chain loops back into the protein, having an important role in the folding of the protein. The serine at position 113 is highly conserved, both between members of the same family (MBD2, MBD3 and MBD4); and across species (*M. fascicularis*, *R. norvegicus*, *M. musculus* and *X. laevis*); it is also localized in an important domain, the MBD. We are currently assessing the frequency of this variant in the Portuguese population by AS-PCR in order to clarify its role as a polymorphism or a causative mutation.

The K305R substitution was not present in the parents of the patient. The lysine (K) amino acid at position 305 of MeCP2 is not conserved among the proteins of the MBD family, but it is highly conserved across species (*M. fascicularis*, *R. norvegicus*, *M. musculus* and *X. laevis*). Both K and R are positively charged hydrophilic amino acids that contribute to the overall charge of the protein; hence, this is a conservative substitution. We did not find this variant by AS-PCR in 226 X chromosomes of a Portuguese control population.

The P322A alteration was not present in the parents of the patient. The change of a proline (P) by an alanine (A) is between amino acids of different groups (a special amino acid, with particular features as referred above) by a hydrophobic amino acid. Alanine, as a hydrophobic amino acid, tends to be localized in the core of the protein. The P at position 322 is conserved in MBD1 and highly conserved across different species (*M.*

*fascicularis*, *R. norvegicus*, *M. musculus* and *X. laevis*). Position 322 of the MeCP2 is located in the C-terminal region, which was described to be involved in facilitating the binding of the protein to nucleosomal DNA (Chandler et al. 1999). Its presence was previously tested in a control population of more than 100 X-chromosomes in a population of European ancestry, but it was not found (Bienvenu et al. 2000).

The alteration V380M was also present in the healthy mother of the female patient, which had a random XCI pattern. The valine (V) at position 380 of MeCP2 is conserved in the MBD1, a protein of the methyl binding domain (MBD) family, and in *M. fascicularis*. The change of a valine by a metionine is a conservative substitution, as both amino acids are hydrophobic. This alteration might affect the potential group II WW domain of MeCP2 (localized from amino acid 325 to C-terminal region), which is involved in splicing (Buschdorf and Stratling 2004). The C-terminal region was described to be involved in facilitating the binding of the protein to nucleosomal DNA (Chandler et al. 1999). We are currently assessing the frequency of this variant in the Portuguese population by AS-PCR in order to clarify its role as a polymorphism or a causative mutation.

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**Table 2.3.** Polymorphisms and variants of unknown significance in the *MECP2* gene.

| Pathogenicity  | Exon | NT change      | CpG site | AA change | Type       | Ts/Tv | Conservation   | Domain      | F/M | Reference            |
|----------------|------|----------------|----------|-----------|------------|-------|----------------|-------------|-----|----------------------|
| Non pathogenic |      | c.1461+ 9G>A   |          |           | non-coding |       |                | 3'UTR       |     | @                    |
|                |      | c.1461+ 99insA |          |           | non-coding |       |                | 3'UTR       | F   | @                    |
|                |      | c.2595G>A      |          |           | non-coding | G>A   |                | 3'UTR       | NA  | This study           |
|                |      | c.9961C>G      |          |           | non-coding | C>G   |                | 3'UTR       | F   | This study           |
|                |      | c.9964delC     |          |           | non-coding |       |                | 3'UTR       | M   | Coutinho et al, 2007 |
|                |      | c.IVS3-17delT  |          |           | non-coding |       |                | intron      |     | Couvert et al, 2001  |
|                |      | c.IVS3-61C>G   | N        |           | non-coding | C>G   |                | intron      | F   | Orrico et al, 2000   |
|                | 3    | c.210C>T       | Y        | S70S      | silent     | C>T   | M, Mus, Rat    | N-terminal  |     | @                    |
|                | 3    | c.373A>C       |          | I125I     | silent     | A>C   | M, Mus, Rat, X | MBD         |     | @                    |
|                | 4    | c.582C>T       | Y        | S194S     | silent     | C>T   | M, Mus, Rat, X | interdomain |     | @/                   |
|                | 4    | c.753C>T       | Y        | P251P     | silent     | C>T   | M, Mus, Rat    | TRD         | F   | This study           |
|                | 4    | c.897C>T       | Y        | T299T     | silent     | C>T   | M, Mus, Rat, X | TRD         |     | @                    |
|                | 4    | c.1035A>G      |          | K345K     | silent     | A>G   | M, Mus, Rat    | C-terminal  |     | @                    |
|                | 4    | c.1335G>A      | Y        | T445T     | silent     | G>A   | M, Mus, Rat    | C-terminal  |     | @                    |
| Unknown        | 4    | c.633G>C       | N        | R211S     | missense   | G>C   | M, Mus, Rat, X | TRD         |     | @                    |
|                | 4    | c.1126C>T      | N        | P376S     | missense   | C>T   | M, Rat         | C-terminal  |     | @                    |
|                | 3    | c.338C>T       | N        | S113P     | missense   | C>T   | M, Mus, Rat, X | MBD         | Ø   | This study           |
|                | 4    | c.915A>G       |          | K305R     | missense   | A>G   | M, Mus, Rat, X | TRD         | Ø   | @                    |
|                | 4    | c.964C>G       | Y        | P322A     | missense   | C>G   | M, Mus, Rat, X | C-terminal  | Ø   | Bienvenu et al, 2000 |
|                | 4    | c.1138G>A      | Y        | V380M     | missense   | G>A   | M              | C-terminal  | M   | This study           |

Legend: NT, nucleotide; AA, amino acid; Ts, transition; TV, transversion; Y, yes; N, no; M, *Macaca fascicularis*; Mus, *Mus musculus*; Rat, *Rattus norvegicus*; X, *Xenopus laevis*; TRD, transcription repression domain; MBD, methyl-CpG binding domain; 3'UTR, 3' untranslated region; @, [http://mecp2.chw.edu.au/mutation\\_database](http://mecp2.chw.edu.au/mutation_database)

|                 |     |                                                               |
|-----------------|-----|---------------------------------------------------------------|
| H.sapiens_MBD2  | 1   | ---MRAHPGGGRCCPEQEEGESAAGGSGAGGDSAIEQGGQSALAPSPVSGVRREGARGG   |
| H.sapiens_MBD3  | 1   | -----                                                         |
| H.sapiens_MeCP2 | 1   | -----MVAGMLGLREEKSEDQLQGLKDKPLKFKKVKDKD----                   |
| H.sapiens_MBD1  | 1   | ---MAEDWLDCPALGPGWKRREVFRKSGATCGRSPTYQSPGDRIRSKVELTRYLGPACDL  |
| H.sapiens_MBD4  | 1   | MGTTGLESLSLGDRAAPTVTSSERLVPDPPNDRKEDVAMELERVGEDEEQMMIKRSSE    |
| consensus       | 1   | q g k                                                         |
| H.sapiens_MBD2  | 58  | GRGRGRWKQAGRGGGVCGRGRGRGRGRGRGRGRGRGRPPSGGSLGGDGGGCGGGGSG     |
| H.sapiens_MBD3  | 1   | -----                                                         |
| H.sapiens_MeCP2 | 36  | -----KEEKEGKHEPVQPSAHSAEPAEAGKAETSEG                          |
| H.sapiens_MBD1  | 60  | TLDFDKQILCYPAKPAHVAVASKRRKPSRAKTRKRQVGPQSGEVKEAPRDETKAD       |
| H.sapiens_MBD4  | 61  | CNPLLQEPIASAQFGATAGTECRKSVPCGWERVVKQRLFGKTAGRFDVYFISPQGLKFRS  |
| consensus       | 61  | r k gk r sg g g                                               |
| H.sapiens_MBD2  | 118 | GGGAPRREPVPFPGSGAGPGPRGRATESGKRMDPCALPPGWKKEEVIRKSGLSAGK---   |
| H.sapiens_MBD3  | 1   | -----MERKRWECPALPQGWEREVEVPRRSGLSAGH---                       |
| H.sapiens_MeCP2 | 68  | SGSAP-----AVPEASAPKQRRSIIRDGRPMYDDPTLPEGWTRKLKQKSGRSAGK---    |
| H.sapiens_MBD1  | 120 | TDTPASFAPAGCCENCISFSGDTQQRRLKTLCKDCRAQRIAFNREQRMFKRVGCGEC     |
| H.sapiens_MBD4  | 121 | KSSLANYLHKNGETSLKPEDFDFTVLSKRGIKSRYKDCSMAALTSHLQNOQNNNSWN---  |
| consensus       | 121 | sap g r dcp lp gw k v rk sg sagk                              |
| H.sapiens_MBD2  | 175 | -----SDVYFSPSGKKFRSKPQLARYLGNTVDLSS---FDFRT                   |
| H.sapiens_MBD3  | 31  | -----RDVYFSPSGKKFRSKPQLARYLGGSMDLST---FDFRT                   |
| H.sapiens_MeCP2 | 120 | -----YDVYLINPQGKAFRSKVELIAYFEKVGDTSLDPNDFDFTV                 |
| H.sapiens_MBD1  | 180 | AACQVTEDCGACSTCLLQLPHDVASGLFCKCERRCLRIVERSRGCGVCRGCTQEDCGH    |
| H.sapiens_MBD4  | 178 | -----LRTSRCKCKDVFMPSPSSSELQESRGLSNFTSTHLLKDEEGVDDVNF          |
| consensus       | 181 | rdVy psgk f sk l y v dls fdf                                  |
| H.sapiens_MBD2  | 211 | G-----KMMPSKLQKNKQRLRNDPLNQNGKPDNLNTLPIRQTASIF                |
| H.sapiens_MBD3  | 67  | G-----KMLMSKMNSRQVRVYDSSNQVGKPDNLNTALPVRQTASIF                |
| H.sapiens_MeCP2 | 160 | TGRGSPS-----RREQPKPKPKSPKAPGTGRGRGRPKGSGTTRPKAATSEGVQKRVLE    |
| H.sapiens_MBD1  | 240 | CPICLRPPRPGLRRQWKVCQRRCLRGKHARRKGGCDKMAARRRPGAQPLPPPPPSQSPE   |
| H.sapiens_MBD4  | 226 | RKVRKPKGKVITILKGIPIKTKKGRKSCSGFVQSDSKRESVCNKADAESPEVAQKSQLD   |
| consensus       | 241 | r k sk k k rlr dsk k kp ntt pv qt sie                         |
| H.sapiens_MBD2  | 253 | KQP-----VTKVTNHPSN-----KVKSDPQRMNE                            |
| H.sapiens_MBD3  | 109 | KQP-----VTKITNHPSN-----KVKSDPQKAVD                            |
| H.sapiens_MeCP2 | 215 | KSPGKLLVKMPFQTSPPGKAEGGGATTSTQVMVIKRPGR-----KRKAEADPQAI       |
| H.sapiens_MBD1  | 300 | PTEPHPRALAPSPAEIFIYCVDEDELQPYTNRRQRNKC-----GACAACLRRMD        |
| H.sapiens_MBD4  | 286 | RTVCISDAGACGETLSVTSEENSLVKKKERSLSGSGNFCSEQKTSGIINKFCSAKDSEHN  |
| consensus       | 301 | ksp t vtnhp k ksd r e                                         |
| H.sapiens_MBD2  | 277 | QPRQLFWEKRLQGLSASDVTEQIIKTMELPKGLQGVGPG-----                  |
| H.sapiens_MBD3  | 133 | QPRQLFWEKKLSGLNAFDIAEELVKTMDLPKGLQGVGPG-----                  |
| H.sapiens_MeCP2 | 265 | PKKRGRKPGSVVAAAAAEAKKAVKESSIRSVQETVLPVIRKTR-----              |
| H.sapiens_MBD1  | 350 | CGRCDFCCDKPKFGGSGNQKRQKCRWRQCLQFAMKRLLPSVSESEDGAGSPPPYRRRRKP  |
| H.sapiens_MBD4  | 346 | EKYEDTFLESEEIGTKVEVVERKEHLHTDILKRGSEMDNNSPTRKDFTG-----        |
| consensus       | 361 | r fw rl ga a dv ek ik l gl vlp t                              |
| H.sapiens_MBD2  | 316 | -----SNDETLLSAVASALHTSSAPITGQVSAAVEKNPAVWLNTSQP-LCKAFIV       |
| H.sapiens_MBD3  | 172 | -----CTDETLLSAIASALHTSTMPITGQLSAAVEKNPGVWLNTTQP-LCKAFMV       |
| H.sapiens_MeCP2 | 310 | -----ETVSEVKEVVKLLVSTLGEKSGKGLKTCKSPGRKSKESP-KGRSSSA          |
| H.sapiens_MBD1  | 410 | SSARRHHLGPTLKPTLATRTAQDHTQAPTQKEAGGGFVLPPTGDLVFLREGASSPVQV    |
| H.sapiens_MBD4  | 396 | ---EKIFQEDTIPRTQIERKTSLYFSSKYNKEALSPRRKAFKKWTTPRSPFNVLQETL    |
| consensus       | 421 | s tlls vas lhtss gvsa v k pg wl s p k v                       |
| H.sapiens_MBD2  | 365 | TDEDIRKQEERVQVRKK-LEEALMADILSRAADTEEMDIEMDSGDEA-----          |
| H.sapiens_MBD3  | 221 | TDEDIRKQEELVQVRKK-LEEALMADMLAHVEELARDGEAPLDKACAEDDDEDEEEEEE   |
| H.sapiens_MeCP2 | 359 | SSPPKKEHHHHHHHSESP-KAPVPLLPPLPPPPPEPESSEDPTSPPEPQDLSSSVCKEEK  |
| H.sapiens_MBD1  | 470 | PGPVAASTEALLQEAQCSGLSWVALPVQVQKEADTQDEWTPGTAVLTSPVLVPGCPSKA   |
| H.sapiens_MBD4  | 453 | FHDPWKLLIATIFLNRSTGKMAIPVLWKFLKYPYSAEVARTADWRDVSSELLKPLGLYDLR |
| consensus       | 481 | t e r e vq r l vml l e p s l e                                |

**Figure 2.7. Sequence comparison of the paralogs of the MeCP2 protein.** In blue are represented the missense mutations and in pink the variants of unknown function found in the current study. The alignment was performed using the Multiple Sequence Alignment – Clustal W.

|                      |     |                                                                                                                 |
|----------------------|-----|-----------------------------------------------------------------------------------------------------------------|
| H.sapiens_MeCP2      | 1   | --MVAGMLGLREEKSEDQDLQGLKDKPLKFKVKKKDKKEEGKHEPVQPSAHHSAEPAE                                                      |
| M.fascicularis_MeCP2 | 1   | --MVAGMLGLREEKSEDQDLQGLKDKPLKFKVKKKDKKEDKEGKHEPVQPSAHHSAEPAE                                                    |
| M.musculus_MeCP2     | 1   | --MVAGMLGLREEKSEDQDLQGLRDKPLKFKKAKKDKKEDKEGKHEPLQPSAHHSAEPAE                                                    |
| R.novergicus_MeCP2   | 1   | --MVAGMLGLREEKSEDQDLQGLKEKPLKFKVKKKDKKEDKEGKHEPLQPSAHHSAEPAE                                                    |
| X.laevis_MeCP2       | 1   | MAAAPSGEERLEEKSEDQDLQGLQKDKPPKLRKVKKKDKKDEEE--KQEPFHSSEHQPGEPAD                                                 |
| consensus            | 1   | EEKSEDQDLQG kdKP K kK KKDKKee E K EP S H aEPae                                                                  |
| H.sapiens_MeCP2      | 59  | AGKAETSESGSAPAVPEASASPQRRSIIRDGPYDDPTLPEGWT <sup>R106W</sup> KLKQRK <sup>S113P</sup> SGRSAG                     |
| M.fascicularis_MeCP2 | 59  | AGKAETSESGSAPAVPEASASPQRRSIIRDGPYDDPTLPEGWT <sup>R106W</sup> KLKQRK <sup>S113P</sup> SGRSAG                     |
| M.musculus_MeCP2     | 59  | AGKAETSESGSAPAVPEASASPQRRSIIRDGPYDDPTLPEGWT <sup>R106W</sup> KLKQRK <sup>S113P</sup> SGRSAG                     |
| R.novergicus_MeCP2   | 59  | AGKAETSESGSAPAVPEASASPQRRSIIRDGPYDDPTLPEGWT <sup>R106W</sup> KLKQRK <sup>S113P</sup> SGRSAG                     |
| X.laevis_MeCP2       | 60  | EGKADMSAEENLAVPESSASPQRRSVIRDGPYEDPTLPEGWT <sup>R106W</sup> KLKQRK <sup>S113P</sup> SGRSAG                      |
| consensus            | 61  | GKAe SE AVPE SASPKQRRSIIRDGPYDDPTLPEGWT <sup>R106W</sup> KLKQRK <sup>S113P</sup> SGRSAG                         |
| H.sapiens_MeCP2      | 119 | KYDVYLINPQGKAF <sup>R133C</sup> SKVELIAYFEKVGDTSLD <sup>P152R</sup> <sup>T158M</sup> PNDFDFTVTGRGSPSRREQPPKKPKS |
| M.fascicularis_MeCP2 | 119 | KYDVYLINPQGKAF <sup>R133C</sup> SKVELIAYFEKVGDTSLD <sup>P152R</sup> <sup>T158M</sup> PNDFDFTVTGRGSPSRREQPPKKPKS |
| M.musculus_MeCP2     | 119 | KYDVYLINPQGKAF <sup>R133C</sup> SKVELIAYFEKVGDTSLD <sup>P152R</sup> <sup>T158M</sup> PNDFDFTVTGRGSPSRREQPPKKPKS |
| R.novergicus_MeCP2   | 119 | KYDVYLINPQGKAF <sup>R133C</sup> SKVELIAYFEKVGDTSLD <sup>P152R</sup> <sup>T158M</sup> PNDFDFTVTGRGSPSRREQPPKKPKS |
| X.laevis_MeCP2       | 120 | KFDVYLINPQKAF <sup>R133C</sup> SKVELIAYFQKVGDTSLD <sup>P152R</sup> <sup>T158M</sup> PNDFDFTVTGRGSPSRREQPPKKPKA  |
| consensus            | 121 | KyDVYLINPqGKAF <sup>R133C</sup> SKVELIAYF KVGDTSLD <sup>P152R</sup> <sup>T158M</sup> PNDFDFTVTGRGSPSRREQ PPKKPK |
| H.sapiens_MeCP2      | 179 | PKAPGTGRGRGRPKSGTTRPKAATSEGQVQKRVLEKSPGKLLVKMPFQTSPPGGKAEGGG                                                    |
| M.fascicularis_MeCP2 | 179 | PKAPGTGRGRGRPKSGTTRPKAATSEGQVQKRVLEKSPGKLLVKMPFQTSPPGGKAEGGG                                                    |
| M.musculus_MeCP2     | 179 | PKAPGTGRGRGRPKSGTGRPKAAASEGQVQKRVLEKSPGKLLVKMPFQASPPGGKGEVGG                                                    |
| R.novergicus_MeCP2   | 179 | PKAPGTGRGRGRPKSGTGRPKAAASEGQVQKRVLEKSPGKLLVKMPFQASPPGGKGEVGG                                                    |
| X.laevis_MeCP2       | 180 | PKSSVSGRGRGRPKSGIKKVKPPVKSEGQVQKRVIEKSPGKLLVKMPYSG---TKEASD                                                     |
| consensus            | 181 | PK tGRGRGRPKGS SEGQVQKRVLEKSPGKLLVKMPf Eg                                                                       |
| H.sapiens_MeCP2      | 239 | ATTSTQVMVIKRPGRKRKAEDPQAIPKKRGRKPG--SVVAAAAAEAKKKAVKESSIRSV                                                     |
| M.fascicularis_MeCP2 | 239 | ATTSTQVMVIKRPGRKRKAEDPQAIPKKRGRKPG--SVVAAAAAEAKKKAVKESSIRSV                                                     |
| M.musculus_MeCP2     | 239 | ATTSAQVMVIKRPGRKRKAEDPQAIPKKRGRKPG--SVVAAAAAEAKKKAVKESSIRSV                                                     |
| R.novergicus_MeCP2   | 239 | ATTSAQVMVIKRPGRKRKAEDPQAIPKKRGRKPG--SVVAAAAAEAKKKAVKESSIRSV                                                     |
| X.laevis_MeCP2       | 236 | ATTSQVLVIKRGGRKRKSETDPSAAPKKRGRKPSNVSLAAAAAEAKKKAIKESSIKPL                                                      |
| consensus            | 241 | ATTS QvMVIK RGRKR E DP A PKRGRKP Sv AAAA AKKKAVKESSIr v                                                         |
| H.sapiens_MeCP2      | 297 | QETVL <sup>P302L</sup> IK <sup>K305R</sup> KTRETVSIEVKEVVK <sup>P322A</sup> LLVSTLGEKSGKGLKTKSPGRKSKESSPKGRSS   |
| M.fascicularis_MeCP2 | 297 | QETVL <sup>P302L</sup> IK <sup>K305R</sup> KTRETVSIEVKEVVK <sup>P322A</sup> LLVSTLGEKSGKGLKTKSPGRKSKESSPKGRSS   |
| M.musculus_MeCP2     | 297 | HETVL <sup>P302L</sup> IK <sup>K305R</sup> KTRETVSIEVKEVVK <sup>P322A</sup> LLVSTLGEKSGKGLKTKSPGRKSKESSPKGRSS   |
| R.novergicus_MeCP2   | 297 | QETVL <sup>P302L</sup> IK <sup>K305R</sup> KTRETVSIEVKEVVK <sup>P322A</sup> LLVSTLGEKSGKGLKTKSPGRKSKESSPKGRSS   |
| X.laevis_MeCP2       | 296 | LETVL <sup>P302L</sup> IK <sup>K305R</sup> KTRETISVDVKDTIK <sup>P322A</sup> PELTPVIEKVMKGQNPAKSPESRSTEGSPKIKTG  |
| consensus            | 301 | ETVL <sup>P302L</sup> IK <sup>K305R</sup> KTRETvSievKe vK <sup>P322A</sup> vs l EK KG KSP KS E SPK rs           |
| H.sapiens_MeCP2      | 357 | SASSPPKKEHHHHHHHSESPKAPV <sup>R306C</sup> LLPPLPPPPPEPESSEDPTSPPEPQDLSSSVCKEE                                   |
| M.fascicularis_MeCP2 | 357 | SASSPPKKEHHHHHHHSESPKAPV <sup>R306H</sup> LLPPLPPPPPEPESSEDPTSPPEPQDLSSSVCKEE                                   |
| M.musculus_MeCP2     | 357 | SASSPPKKEHHHHHHHSESTKAP <sup>V380M</sup> LLP--SPPPPPEPESSEDPTSPPEPQDLSSSICKEE                                   |
| R.novergicus_MeCP2   | 357 | SASSPPKKEHHHHHHHHAESPAP <sup>V380M</sup> LLP--SPPPPPEPQSSDPTISPPEPQDLSSSICKEE                                   |
| X.laevis_MeCP2       | 356 | LPKKELQHHHHHHHHHHHHHSES-----KASATSPEPETS <sup>R306C</sup> SKDNIGVQEPQDL <sup>V380M</sup> SVKMKCEE               |
| consensus            | 361 | HHHHHHH k PEP ss D EPQDLS vCKEE                                                                                 |
| H.sapiens_MeCP2      | 417 | KMPRGGSLESDGCPKEPAKTQPAVAT-----AATAAEKYKHRGEGERKDIVSSSMR                                                        |
| M.fascicularis_MeCP2 | 417 | KMPRGGSLESDGCPKEPAKTQPAVAT-----AATAAEKYKHRGEGERKDIVSSSMR                                                        |
| M.musculus_MeCP2     | 415 | KMPRGGSLESDGCPKEPAKTQPMVAT-----TTTVAEKYKHRGEGERKDIVSSSMR                                                        |
| R.novergicus_MeCP2   | 415 | KMPRAGSLESDGCPKEPAKTQPMVAAAATTTTTTTTVAEKYKHRGEGERKDIVSSSMR                                                      |
| X.laevis_MeCP2       | 412 | KLP-----ESDGCQEPAKTQPADKCR-----NRAEGERKDIVSS-VPR                                                                |
| consensus            | 421 | KmP ESDGC EPAKTQP RgEGERKDIVSS mPR                                                                              |
| H.sapiens_MeCP2      | 469 | PNREEPVDSRTPVTERVS                                                                                              |
| M.fascicularis_MeCP2 | 469 | PNREEPVDSRTPVTERVS                                                                                              |
| M.musculus_MeCP2     | 467 | PNREEPVDSRTPVTERVS                                                                                              |
| R.novergicus_MeCP2   | 475 | PNREEPVDSRTPVTERVS                                                                                              |
| X.laevis_MeCP2       | 450 | PTREEPVDTRTTVTERVS                                                                                              |
| consensus            | 481 | P REEPVDSRT VTERVS                                                                                              |

**Figure 2.8. Sequence comparison of the orthologs of the MeCP2 protein.** In blue are represented the missense mutations and in pink the variants of unknown function found in the current study. The alignment was performed using the Multiple Sequence Alignment – Clustal W

Two alterations were identified in intron 3: IVS3-17delT and IVS3-61C>G, were both already described in the literature as polymorphisms. The IVS3-17delT alteration was the most frequent polymorphism in our patient population (28.6%, 6/21), with all the other polymorphisms present only once. The effect of the variant IVS3-17delT on mRNA splicing was evaluated by RT-PCR, and no abnormal transcript was produced. The variant IVS3-61C>G was also present in the father of the patient.

We also identified alterations in the 3'UTR of *MECP2* gene. In the 3'UTR, five alterations (c.1461+9G>A, c.1461+99insA, c.2595G>A, c.9961C>G and c.9964delC) were detected. The 1461+9G>A and the 1461+99insA (identified by the direct sequencing of exon/intron boundaries) were already described in the literature as polymorphisms. The c.9964delC variant was described in a Portuguese control population, with a frequency of 5.2% (5/96) (Coutinho et al. 2007), while the c.2595G>A and c.9961C>G variants were identified by us, for the first time. Two variants, c.9961C>G and c.9964delC, were present in the same patient: the c.9961C>G variant was also present in the (unaffected) father of the patient (hence this variation should not be a pathogenic mutation) while the variant c.9964delC was present in her mother. We could not test the c.2595G>A variant in the parents of the patient, since their DNA was not available; however, the patient was later shown to have another causal mutation (whole coding sequence deletion), and so this variant was most likely not the relevant pathogenic alteration.

### **Mutations in the *MECP2* gene**

Mutations in *MECP2* were found in 25.6% of our total patient sample (64/250). A mutation was found in 78.6% (44/56) of the patients classified as classical RTT, and in 19.4% (13/67) of the atypical RTT cases.

The *MECP2* mutations identified (n=64) span the entire gene (figure 2.5 and table 2.4). Missense mutations were the most common type, representing 39.1% of patients (n=25, 7 different) of all mutations, followed by nonsense mutations with 34.4% of patients (n=22, 5 different), and the small and large rearrangements with 20.3% (n=13) and 6.2% (n=4) of patients, respectively (figure 2.9).

Most mutations were concentrated in the functional MBD (36.7%, n=22) and TRD domains (36.7%, n=22). In the other regions of the gene, the percentage of mutations

---



identified was lower: 3.3% (n=2) in the N-terminal region, 16.7% (n=10) in the interdomain region, and 6.7% (n= 4) in the C-terminal region.

Most of the missense mutations were located in the MBD (32.8%), while the nonsense mutations were more dispersed over the gene, but prevalently located in the TRD (18.8%). The small rearrangements were located mainly in exon 4, in the TRD and the C-terminal region of the gene (9.4% and 6.2%, respectively) (figure 2.9).

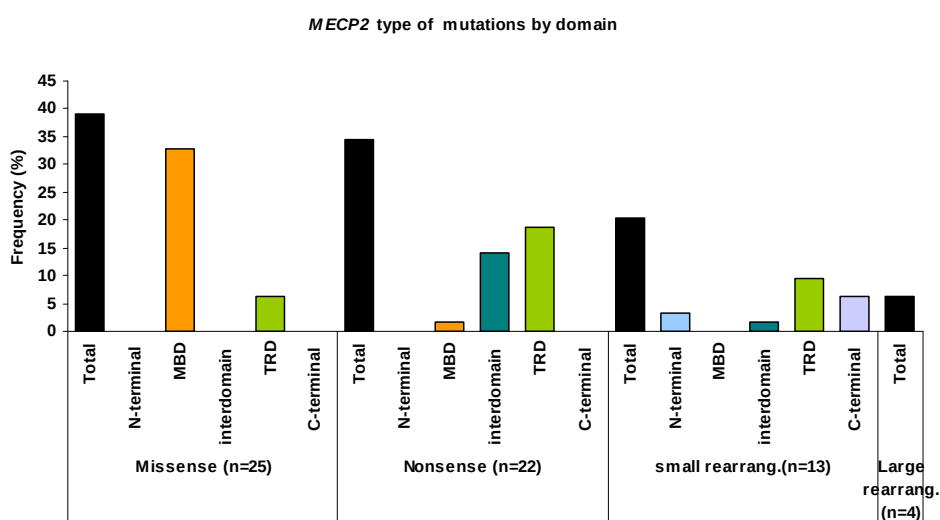


**Figure 2.9. Types of mutations identified in the *MECP2* gene and their distribution by domain.** Percentage of types of mutations (missense, nonsense and rearrangements) identified by direct sequencing or RD-PCR of the *MECP2* gene in the Portuguese population with RTT syndrome or related phenotype. MBD, methyl CpG-binding domain; TRD, transcription repression domain.

One mutation is located in exon 1 (1.7%), five in exon 3 (8.3%) and 54 in exon 4 (90.0%), which encodes part of the MBD and the TRD (figure 2.10).

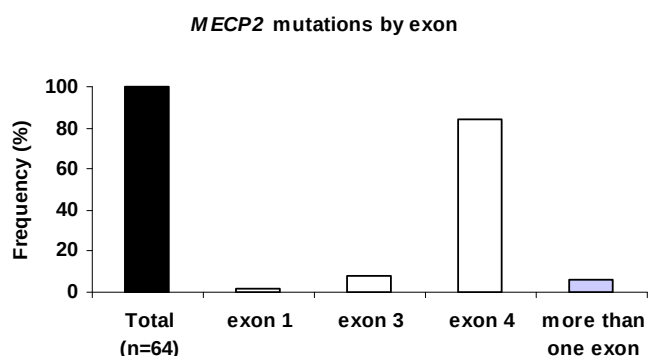


**Figure 2.10. Distribution of *MECP2* mutations in the coding region.** Frequency of the *MECP2* mutations per exon.

**Table 2.4.** *MECP2* mutations identified in coding region and exon/intron boundaries.

| Exon | Nucleotide change                           | Occur. | CpG site | AA change           | Type            | Ts/Tv | Domain      | NLS affected | Reference  |
|------|---------------------------------------------|--------|----------|---------------------|-----------------|-------|-------------|--------------|------------|
| 3    | c.316C>T                                    | 3      | Y        | R106W               | missense        | C>T   | MBD         | N            | @          |
| 4    | c.397C>T                                    | 8      | Y        | R133C               |                 | C>T   | MBD         | N            |            |
| 4    | c.455C>G                                    | 1      | N        | P152R               |                 | C>G   | MBD         | N            |            |
| 4    | c.473C>T                                    | 9      | Y        | T158M               |                 | C>T   | MBD         | N            |            |
| 4    | c.905C>T                                    | 1      | N        | P302L               |                 | C>T   | TRD         | N            |            |
| 4    | c.916C>T                                    | 2      | Y        | R306C               |                 | C>T   | TRD         | N            |            |
| 4    | c.917G>A                                    | 1      | Y        | R306H               |                 | G>A   | TRD         | N            |            |
| 3    | c.328C>T                                    | 1      | Y        | Q110X               | nonsense        | C>T   | MBD         | Y            | This study |
| 4    | c.502C>T                                    | 9      | Y        | R168X               |                 | C>T   | interdomain | Y            | @          |
| 4    | c.763C>T                                    | 4      | Y        | R255X               |                 | C>T   | TRD         | Y            | @          |
| 4    | c.808C>T                                    | 3      | Y        | R270X               |                 | C>T   | TRD         | Y            | @          |
| 4    | c.880C>T                                    | 5      | Y        | R294X               |                 | C>T   | TRD         | N            | @          |
| 1    | c.14-21del (8bp)                            | 1      |          | A7fsX37             | frameshift      |       | N-terminal  | Y            | This study |
| 3    | c.116-117delAA                              | 1      |          | K39fsX43            |                 |       | N-terminal  | Y            | This study |
| 4    | c.512-548dup (37bp)                         | 1      |          | T184fsX185          |                 |       | interdomain | Y            | This study |
| 4    | c.757-793del (37bp)                         | 1      |          | R253fsX275          |                 |       | TRD         | Y            | This study |
| 4    | c.808delC                                   | 1      |          | G269fsX288          |                 |       | TRD         | Y            | @          |
| 4    | c.808delC                                   | 2      |          | R270fsX288          |                 |       | TRD         | Y            | @          |
| 4    | c.898-904del (7bp)                          | 1      |          | V300fsX318          |                 |       | TRD         | N            | @          |
| 4    | c.908-914del + ins agaaggacc + 1068-1097del | 1      |          | I303fsX477          |                 |       | TRD         | N            | This study |
| 4    | c.1157-1200del (44bp)                       | 1      |          | L386fsX389          |                 |       | C-terminal  | N            | @          |
| 4    | c.1157-1197del (41bp)                       | 1      |          | L386fsX390          |                 |       | C-terminal  | N            | @          |
| 4    | c.1156-1175del (20bp) + insCTTT             | 1      |          | L386fsX399          |                 |       | C-terminal  | N            | This study |
| 4    | c.1163-1197del (35bp)                       | 1      |          | P388fsX392          |                 |       | C-terminal  | N            | @          |
| 3, 4 | exons 3 and 4                               | 1      |          | large rearrangement | exon deletion   |       |             | Y            | This study |
| 3, 4 | exons 3 and 4                               | 1      |          | large rearrangement |                 |       |             | Y            |            |
| 4    | exon 4                                      | 1      |          | large rearrangement |                 |       |             | Y            |            |
| all  | large deletion                              | 1      |          | large rearrangement | allele deletion |       |             | Y            | This study |

Legend: NT, nucleotide; Occur., number of occurrences; AA, amino acid; Ts, transition; TV, transversion; Y, yes; N, no; TRD, transcription repression domain; MBD, methyl-CpG binding domain; NLS, nuclear localization signal; @, <http://mecp2.chw.edu.au/mutation> database

Despite the fact that mutations in the *MECP2* gene are sporadic and occur throughout the entire gene, a number of recurrent mutations were identified (figure 2.11). In our population, the most frequent mutations were T158M and R168X, with 9 occurrences each, and R133C with 8 occurrences. This suggests that hotspots of mutation must exist. Among all the point and small deletion/insertion mutations, 63.3% (38/60) affect an arginine (R) amino acid a predominance that is most likely due to the nature of its codon (R, putative codons: CGU, CGC, CGA, CGG, AGA and AGG). When checked at the DNA level, in general, most point mutations were due to a C>T transition (95.7%) at CpG sites (95.7%), as described (Laccone et al. 2001). Specifically in our population, the recurrent mutations were all also due to C>T transitions at CpG sites.

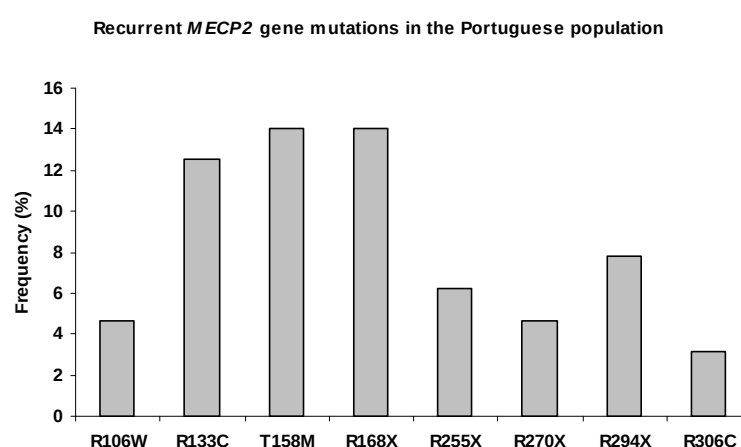


**Figure 2.11.** Recurrent mutations in the *MECP2* gene in the Portuguese population with RTT or other neurodevelopmental disorder.

We found 7 different missense mutations in our study; four in the MBD (R106W, R133C, P152R and T158M) and three in the TRD (P302L, R306C and R306H). Except for the R306H substitution, all the changes were between amino acids of different groups. The changed amino acids were all highly conserved through different species (*M. fascicularis*, *R. norvegicus*, *M. musculus* and *X. laevis*) (figure 2.8) and located in functional domains.

### Large rearrangements

The RD-PCR method, as described by Shi in collaboration with our group (Shi et al. 2005), was used for the detection of large rearrangements in exons 2, 3 and 4 of the *MECP2* gene.

Initially, we included in the study a group of 65 Portuguese female patients and, later, we added a second group of 152 Portuguese patients (females and males) in whom point mutations in the *MECP2* gene had previously been excluded by us.

In total, we have identified four large rearrangements (all deletions) of the *MECP2* gene. One deletion (patient P3) was found in the first group of 65 patients analysed. The deletion junction of patient P3 was characterized by the development of other RD-PCR assays, and it was located within a region of 37,2 kb upstream from the 5' end of exon 1 and 18,1 kb downstream from 3' end of exon 4 (Shi et al. 2005).

Southern blotting analysis was used to confirm the deletion identified in patient P3 by RD-PCR. The signal intensity of patient P3 was similar to that of the male control with probes RTT2, RTT3 and p(A)10 (figure 2.12), indicating that only one copy of the *MECP2* gene was present in patient P3. Southern blot confirmed in this way the results obtained by the RD-PCR method.

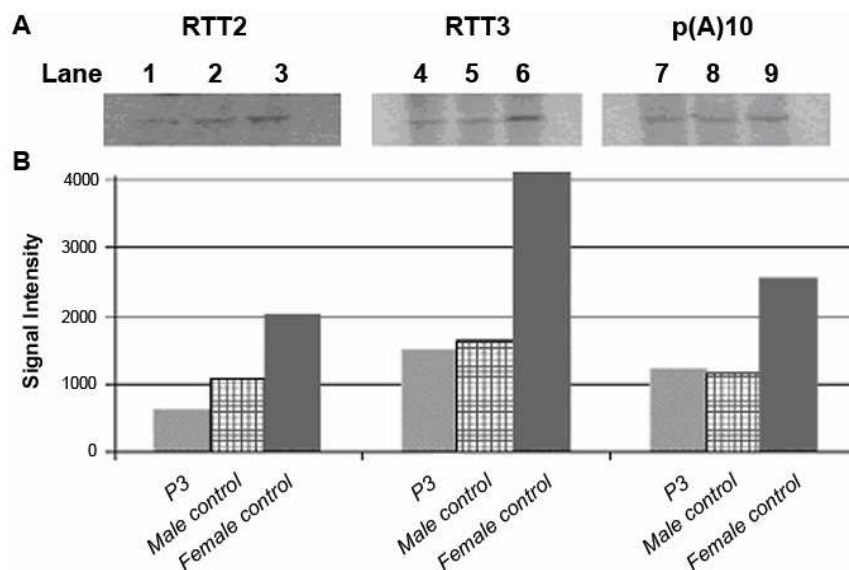


**Figure 2.12. Southern blotting analysis.** A – Images of Southern blotting with probes RTT2, RTT3 and p(A)10. Lanes 1, 4 and 7 are patient P3; lanes 2, 5 and 8 are male control; and lanes 3, 6 and 9 are female control. B – Quantification of each individual signal intensity.

In the second group of patients, which had previously been excluded for point mutations, we identified three additional large deletions (figure 2.13). According to the RD-PCR profile, patient P1 has a deletion of exon 4, and patients P2 and P4 presented a deletion of exons 3 and 4.

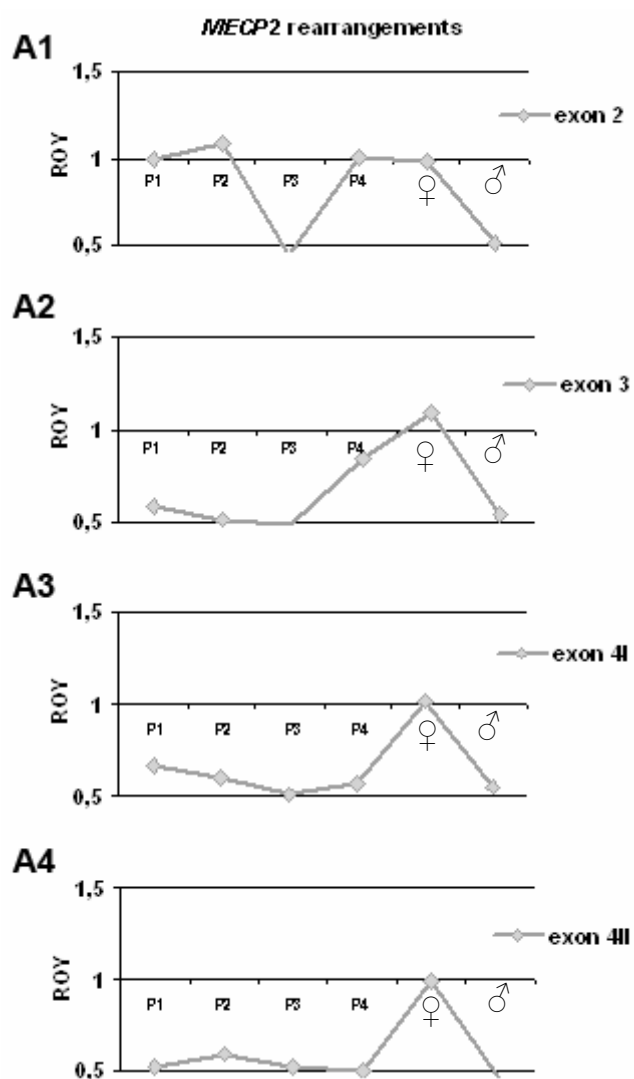


**Figure 2.13.** Analysis of the copy number of the coding region (exons 2, 3 and 4) of the *MECP2* gene. A - RD-PCR profile of *MECP2* gene for exons (A1) 2, (A2) 3, (A3) 4I and (A4) 4II. P1 to P4 are girl patients with a large deletion, ♀ is a female control and ♂ a male control. Values are the mean of 3 independent experiments.

### Prenatal diagnosis

We received five requests for prenatal diagnosis. The probands were first diagnosed as RTT, and a mutation in the *MECP2* gene further confirmed the clinical diagnosis. The mutations identified in the five probands were: T158M (in two probands), T184fsX185, R294X and L386fsX389.

DNA extracted from peripheral blood of both parents and from a new sample of the proband, and DNA extracted from the amniocytes was tested for the mutation previously identified in the proband by direct sequencing and, when possible, other supportive technique, as is the case of detection of small rearrangements (in the case of T184fsX185 and L386fsX389) and allele-specific PCR (in the case of T158M).

Each mutation was confirmed in the new sample of the probands but none was found in the parents, or in the foetus.

### ***MECP2* mutation-positive patients and their phenotypes**

A genotype-phenotype correlation was attempted in a RTT group, observed by Dr Teresa Temudo. In this group, in the patients classified with the classical RTT form the frequency of *MECP2* gene mutations was 96.2% and in the atypical RTT form, a mutation was found in 29.7% of the patients.

We sub-divided our *MECP2* mutation-positive RTT population in three clinical subtypes: predominantly mental retardation (MR), a mildest form with few neurological signs except mental retardation and autistic features; ataxia (AT), an intermediary form in which ataxia predominated, the majority of the patients acquired independent gait but it was ataxic and rigid, and an extrapyramidal presentation (EP), with major axial hypotonia, in which dystonia and rigidity present after few years of evolution of the disease (Temudo et al, in preparation).

We attempted to perform a genotype-phenotype correlation bearing in mind this proposed clinical classification. The frequency of missense *versus* truncating mutations was significantly different between the three clinical subtypes of RTT (Fisher's exact test,  $p=0.001$ ) (figure 2.14). In the MR group, 52.3% of the patients had missense mutations. In the AT group, 75% of the patients had missense mutations; and in the more severe EP group 81.5% of the cases had a truncating mutation. Globally the distribution of mutation types was significantly different between the clinical groups.

The majority of truncating mutations in the MR group do not affect the NLS (29.4%). Additionally, contributing to this group is the R270X mutation that affects only the last aminoacid of the NLS, and so it may not impair its function or have a milder effect. The missense mutations in this group were all described to have a milder effect, if any effect at

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all. It would be interesting to study the XCI patterns of patients with the R106W and R255X mutations included in this group, given its predictable severe effect.

Interestingly, the T158M mutation was predominant in the AT group, which could suggest some specificity of the effect of this mutation upon MeCP2 function. In the AT group all truncating mutations disrupted the NLS.

In the more severe EP group, the majority of truncating mutations affect the NLS (66.7%) and the R168X is predominant in this group of patients. In two patients, with the mutations P152R and R294X, and three patients with very late truncating mutations, such as L386fsX389, L386fsX399 and P388fsX392, it should be interesting to analyse the pattern of XCI, as these mutations would be predicted to have milder effects.

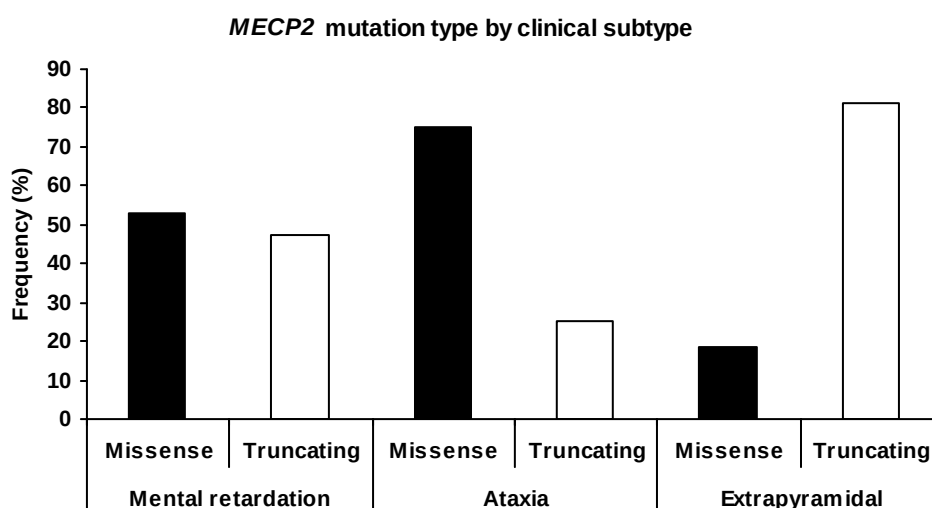


**Figure 2.14.** Frequency of *MECP2* mutation type by RTT clinical subtypes.

In an attempt to establish more detailed correlations between genotype and phenotype in RTT, we classified the mutations present in our series of patients according (I) to the predicted effect upon the function of MeCP2 or (II) to the observed effect upon the expression levels (mRNA or protein) and/or protein function, considering information obtained in different experimental systems (Yusufzai and Wolffe 2000; Kudo et al. 2001; Georgel et al. 2003; Kudo et al. 2003; Petel-Galil et al. 2006) (table 2.5). We then compared the frequency of these mutation classes among the three clinical subtypes.

Some interesting differences are observed, although the number of patients within each group is not large enough to perform statistical analysis. For example, missense mutations in the TRD were predominantly present in the MR and AT groups, but not in the more severe EP group. Unexpectedly, missense and frameshift mutations in the C-terminus of the protein, although theoretically predicted to give rise to less severe clinical presentations, were only present in the EP form of disease. Null mutations (those potentially leading to a total loss of function in the nucleus, or to degradation by the ubiquitin proteasome system) were absent from the MR forms and predominantly present in the EP group. Frameshift mutations affecting the NLS were also predominantly present in the EP group. In contrast, missense mutations at the MBD were more represented in the MR and AT groups (figure 2.15).

In summary, missense mutations in the MBD and missense and truncating mutations in the TRD (not affecting NLS) are predominantly found in the MR and AT groups. On the other hand, null alleles and mutations that impair transport of the protein to the nucleus are predominantly founding the more severe EP form. Surprisingly, mutations in the C-terminal region of the *MECP2* gene, thought to have a milder phenotype, are restricted to the EP group.

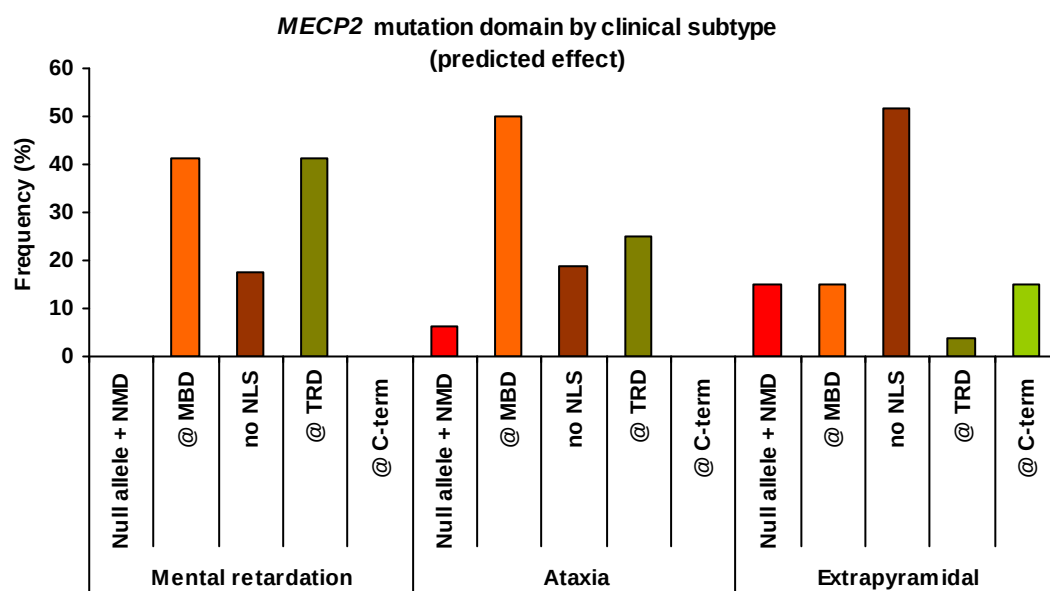


**Figure 2.15.** Frequency of predicted functional groups *MECP2* mutations in each domain by RTT clinical subtype. See also table 2.4, class I mutations. (MBD, methyl-CpG binding domain; NLS, nuclear localization signal; NMD, nonsense mediated decay; TRD, transcription repression domain; C-term, C-terminal region).



Considering the observed effects of mutations, we could see that mutations affecting binding to methylated DNA were rare in our series, and limited to the MR and EP groups. In contrast, mutations leading to a weaker (but not absent) binding to methylated DNA were present only in the MR and AT groups. Mutations leading to decreased stability of the mRNA/protein were also present predominantly in the MR and EP forms. Mutations that lead to a total loss of protein were present in the AT and EP groups, but their frequency increased with disease severity (AT<EP) (figure 2.16).

In summary, mutations that have an intermediate effect were predominant in the MR and AT groups. Responsible for the EP phenotype were predominantly mutations that impair repression and that lead to a total loss of the protein/function. Interestingly, mutations that lead to a decreased expression of the protein were present in both MR and EP groups, but not in AT; these phenotypic effects could be related to the sensitivity of different brain areas to changes of protein levels.

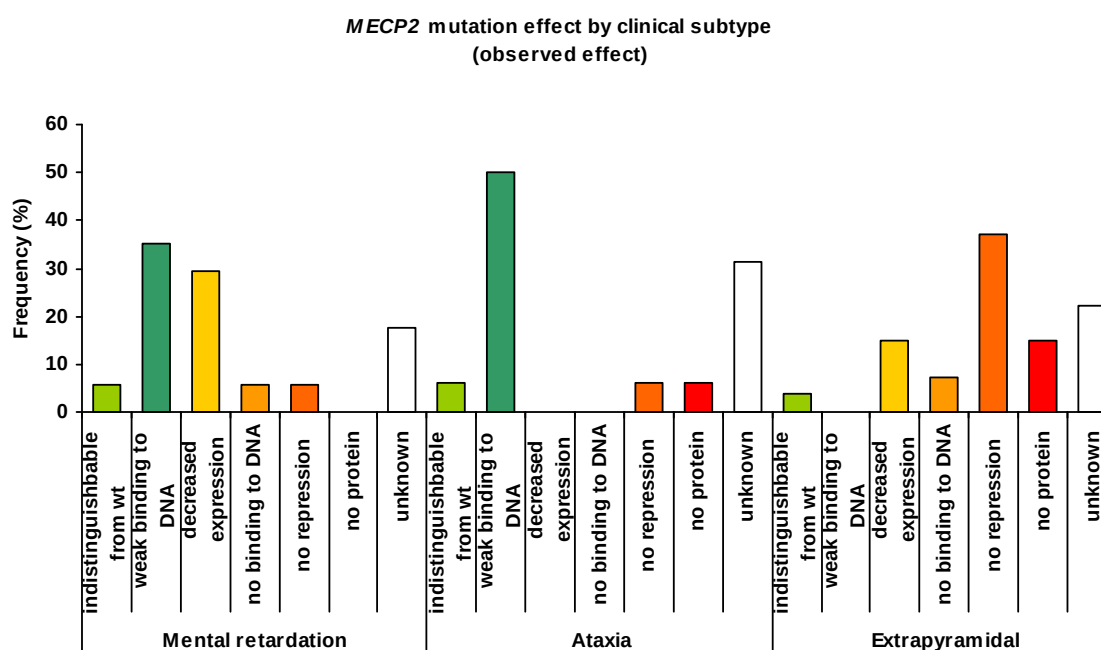


**Figure 2.16. Frequency of *MECP2* mutation effect by RTT clinical subtype.** See also table 2.4, class II mutations.

### Male patients with uncharacterized neurodevelopmental disorder

A total of 40 Portuguese male patients were sent to our laboratory to be tested for *MECP2* gene mutations (figure 2.2). A questionnaire asking for clinical, molecular and

familial information was filled in by the clinicians requesting the diagnosis. Sufficiently detailed clinical information was obtained only for 29 patients (figure 2.17).

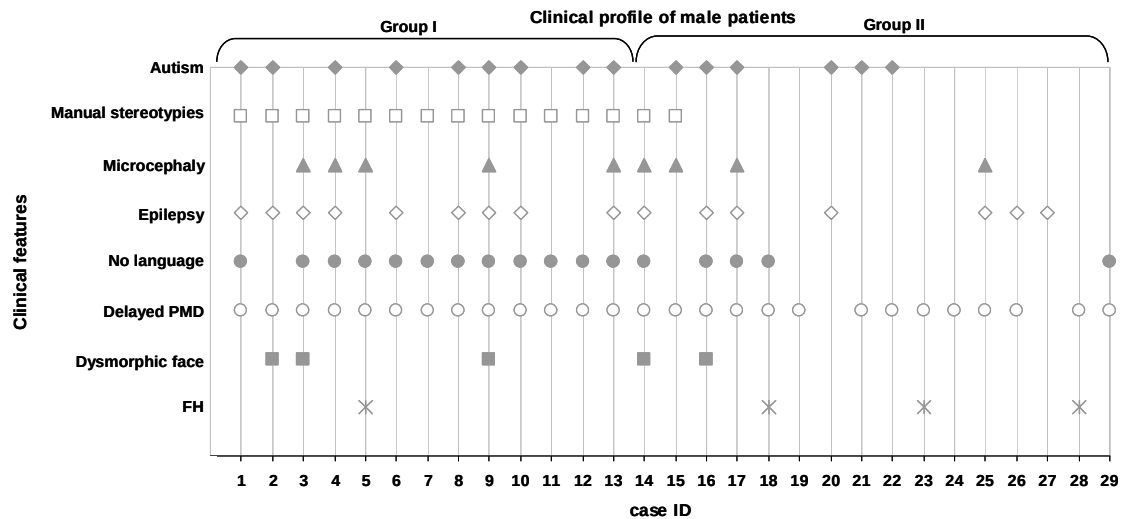


**Figure 2.17. Clinical manifestations presented by 29 male patients with a neurodevelopmental disorder.** Patients 1 to 13 had a clinical presentation compatible with a RTT-like disorder (group I) and patients 14 to 29, did not presented with clinical manifestations previously seen in *MECP2* mutation carrier males (group II). FH, family history of disease (mental retardation); PMD, psychomotor development..

**Table 2.5.** *MECP2* mutations identified in a Portuguese population with RTT or other neurodevelopmental disorder grouped by type of mutation, predicted (I) and observed (II) effect upon MeCP2 function and expression.

| Mutation        | Class (I) | Domains    | Predicted functional consequences             | Mutation | Class (II) | Observed functional effect     | Effect upon mRNA level | Reference                                                             |
|-----------------|-----------|------------|-----------------------------------------------|----------|------------|--------------------------------|------------------------|-----------------------------------------------------------------------|
| <b>Missense</b> | A         | MBD, ATR-X | Affect the binding of MeCP2 to methylated DNA | R106W    | b'         | Abolish binding to DNA         | Normal                 | (Yusufzai and Wolffe 2000; Kudo et al. 2001; Petel-Galil et al. 2006) |
|                 |           |            | Affect the binding of the ATRX protein        | S113P    | g'         | ?                              | ?                      |                                                                       |
|                 |           |            |                                               | R133C    | a'         | Wt/Intermediate binding + ATRX | ?                      | (Yusufzai and Wolffe 2000; Kudo et al. 2001)                          |
|                 |           |            |                                               | P152R    | f'         | Indistinguishable from wt      | ?                      |                                                                       |
|                 |           |            |                                               | T158M    | a'         | Intermediate binding           | Normal                 | (Yusufzai and Wolffe 2000; Kudo et al. 2001; Petel-Galil et al. 2006) |
|                 | B         | TRD        | Affect the repression potential of MeCP2      | P302L    | g'         | ?                              | ?                      |                                                                       |
|                 |           |            |                                               | K305R    | g'         | ?                              | ?                      |                                                                       |
|                 |           |            |                                               | R306C    | f'         | Indistinguishable from wt      | ?                      | (Yusufzai and Wolffe 2000)                                            |
|                 |           |            |                                               | R306H    | g'         | ?                              | ?                      |                                                                       |
|                 | C         | C-terminal | Affect the binding to nucleosomal DNA         | P322A    | g'         | ?                              | ?                      |                                                                       |

Legend: Class (I), classification of mutations according to the predicted functional consequence; Class (II), classification of mutations according to the observed functional effect; MBD, methyl-CpG binding domain; ATR-X, ATR-X-binding domain; TRD, transcription repression domain; NLS, nuclear localization signal; WW, group II WW domain; UPS, ubiquitin-proteasome system; wt, wild type, ?, not known.

Table 2.5 (continued)

**Truncating**

|                                       |            |             |                                                                       |                                                                       |            |                                              |           |                                                     |
|---------------------------------------|------------|-------------|-----------------------------------------------------------------------|-----------------------------------------------------------------------|------------|----------------------------------------------|-----------|-----------------------------------------------------|
| Nonsense and frameshift               | D          | all         | All functions impaired                                                | A7fsX37                                                               | e'         | ?                                            | ?         |                                                     |
|                                       |            |             | Nonsense-mediated mRNA decay                                          | K39fsX43                                                              | e'         | ?                                            | ?         |                                                     |
|                                       |            |             |                                                                       | Q110X                                                                 | e'         | ?                                            | ?         |                                                     |
| Nonsense                              | E          | TRD/NLS, WW | Affect the repression potential of MeCP2 and its nuclear localization | R168X                                                                 | c'         | Affects repression and binding to nucleosome | ?         | (Yusufzai and Wolffe 2000; Georgel et al. 2003)     |
|                                       |            |             |                                                                       | R255X                                                                 | c'         | Affects repression                           | Normal    | (Yusufzai and Wolffe 2000; Petel-Galil et al. 2006) |
|                                       |            |             |                                                                       | R270X                                                                 | d'         | Affects repression                           | Decreased | (Yusufzai and Wolffe 2000; Petel-Galil et al. 2006) |
|                                       | F          | TRD, WW     | Affect the repression potential of MeCP2                              | R294X                                                                 | d'         | Stability decreased and affects repression   | Decreased | (Yusufzai and Wolffe 2000; Petel-Galil et al. 2006) |
|                                       | Frameshift | G           | TRD/NLS, WW                                                           | Affect the repression potential of MeCP2 and its nuclear localization | T184fsX185 | g'                                           | ?         | ?                                                   |
| Affect the binding to nucleosomal DNA |            |             |                                                                       | R253fsX275                                                            | g'         | ?                                            | ?         |                                                     |
|                                       |            |             |                                                                       | G269fsX288                                                            | g'         | ?                                            | ?         |                                                     |
| <i>Degradation by ER-UPS</i>          |            |             |                                                                       | R270fsX288                                                            | g'         | ?                                            | ?         |                                                     |
| H                                     |            | TRD, WW     | Affect the repression potential of MeCP2                              | V300fsX318                                                            | g'         | ?                                            | ?         |                                                     |
|                                       |            |             | Affect the binding to nucleosomal DNA                                 | I303fsX477                                                            | g'         | ?                                            | ?         |                                                     |
| <i>Degradation by ER-UPS</i>          |            |             |                                                                       |                                                                       |            |                                              |           |                                                     |
| I                                     |            | C-terminal  | Affect the binding of MeCP2 to nucleosomal DNA                        | L386fsX389                                                            | d'         | ?                                            | Decreased | (Petel-Galil et al. 2006)                           |
|                                       |            |             |                                                                       | L386fsX390                                                            | d'         | ?                                            | Decreased | (Petel-Galil et al. 2006)                           |
|                                       |            |             |                                                                       | <i>Degradation by ER-UPS</i>                                          | L386fsX399 | d'                                           | ?         | Decreased                                           |
|                                       | P388fsX392 |             |                                                                       | g'                                                                    | ?          | ?                                            |           |                                                     |
| Large rearrangments                   | D          | all         | All functions impaired                                                | exon 3                                                                | e'         | ?                                            | ?         |                                                     |
|                                       |            |             | Nonsense-mediated mRNA decay                                          | exons 3&4                                                             | e'         | ?                                            | ?         |                                                     |
|                                       |            |             |                                                                       | all cds                                                               | e'         | ?                                            | NA        |                                                     |

Thirteen of the 29 patients presented features compatible with the male presentation of a Rett syndrome-like (RTT-like) phenotype (group I). Some of these 13 RTT-like male patients had previous molecular exclusion of other clinical conditions (table 2.6) and their karyotype had been assessed as normal (table 2.6). Their age ranged from 4 to 19 years (mean age  $10 \pm 5.4$  years).

**Table 2.6.** Date of birth, karyotype and molecular exclusions of 13 RTT-like boys.

| Case Id | Age | Karyotype | Angelman Syndrome | ATR-X    | Fragile X | Other                      |
|---------|-----|-----------|-------------------|----------|-----------|----------------------------|
| 1       | 8   | 46, XY    | negative          | negative | negative  | FISH (subtelomeric probes) |
| 2       | 6   |           | negative*         |          |           |                            |
| 3       | 19  |           | negative          |          |           |                            |
| 4       | 5   |           | negative          |          |           |                            |
| 5       | 5   |           |                   |          | negative  |                            |
| 6       | 11  |           | negative          |          |           |                            |
| 7       | 15  |           | negative          |          |           |                            |
| 8       | 5   |           |                   |          | negative  |                            |
| 9       | 17  |           | negative          |          | negative  |                            |
| 10      | 17  |           | negative          |          | negative  |                            |
| 11      | 12  |           | negative          |          |           |                            |
| 12      | 7   |           | negative          |          |           |                            |
| 13      | 4   |           |                   |          |           | FISH (subtelomeric probes) |

Legend: \* UBE3A

The coding region and exon/intron boundaries of the *MECP2* gene were analysed for point mutations and small rearrangements and no mutations were found, except for one patient (ID: 18, included in group II). We found a silent polymorphism in the *MECP2* gene in one patient (ID: 3; 897C>T; T299T), and an intronic deletion of one nucleotide (ID: 10; IVS3-17delT) in another patient, both already described as polymorphisms (<http://mecp2.chw.edu.au/mecp2/>). One patient (ID: 18) had a deletion of one nucleotide (c.808delC; R270fsX288) that created a frameshift and the premature truncation of the MeCP2 protein. We also searched for large duplications or deletions in the *MECP2* gene in this group of patients, but we did not find any alteration in gene dosage.

The boy with a mutation in the *MECP2* gene (ID: 18, included in group II) was the younger brother of a proband, a girl in which the same mutation had previously been identified. The molecular analysis of *MECP2* gene of their parents' peripheral blood revealed that neither of them was a carrier of that mutation (Venancio et al. 2007). The girl had a classical presentation of RTT and the boy a more severe and atypical presentation. He died at 21 months of age due to severe metabolic disequilibrium during a gastrointestinal infectious disease.

Analysis of DNA sequence of the exons where mutations had been previously described, in the *NLGN3* (c.1186insT; R451C) and *NLGN4* (c.1253delAG) genes, in all of the 40 male patients with a neurodevelopmental phenotype, also did not reveal any potentially pathogenic mutation. We did find one nucleotide substitution in the *NLGN4* gene that lead to a silent mutation in one patient (ID: 15): R417R, not identified until now, as far as we know.

## 2.5. Discussion

### Optimization of the molecular diagnostic method

Most *MECP2* gene mutations occur *de novo* and throughout the entire gene (Lee et al. 2001; Bienvenu et al. 2002; Miltenberger-Miltenyi and Laccone 2003). In this study, all types of mutations were found, from missense and nonsense to small and large rearrangements; to date, more than 200 different mutations have been described in *MECP2* (<http://mecp2.chw.edu.au/>). Based on this idea, the initial diagnostic strategy adopted by us, as others (Bienvenu et al. 2002), was to perform a first screen of the coding region (at that time exons 2, 3 and 4) and exon/intron boundaries of the gene by SSCP of the *MECP2* gene. The variants for each fragment displaying an abnormal migration were then identified by automated sequencing. The specificity of the SSCP technique established was in general reduced (30%); we had a large percentage of “false positives”, since we detected several alterations in the pattern of migration of fragments that did not reveal to be true upon sequencing.

The SSCP detection rate is described to range between 35 to 65% of all mutations. In our case, for the first segment of exon 4, the frequency of mutations identified by this method was higher (11/26; 78.6%), but for all the other segments the frequency was lower than that described, ranging between 16.7% to 55.6% (table 2.1). In order to improve the specificity of the technique, the PCR products should be analysed in different SSCP experimental conditions simultaneously (temperature, matrix of gel, additives, such as glycerol, etc), which is laborious and very time consuming.

For the group of patients analysed by SSCP, we have also performed direct sequencing of the coding and exon/intron boundaries and have identified several other mutations (n=12) that had been missed by the SSCP analysis (table 2.1 and table 2.2).

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This showed that we had a considerable number of “false negatives” in the SSCP (12/40; 35%).

We also optimized allele-specific PCR (AS-PCR) techniques to directly assess the recurrent mutations that we found in the *MECP2* gene, in the 84 patients that were analysed initially: R106W, R133C, T158M, R168X and R255X. In the total population, 3 additional recurrent mutations were identified (R270X, R294X, R306C; figure 2.11). Although these mutations were recurrent, the number of occurrences was reduced (the most frequent has nine occurrences in a population of 60 *MECP2*-positive patients); in our opinion, this gain does not compensate the effort invested in performing eight double PCRs (for the normal and mutant alleles) for each patient, particularly in patient series less enriched for *MECP2* mutation-positive cases.

In spite of the sporadic nature of *MECP2* mutations and their distribution throughout the entire gene, given (1) the unfavourable results obtained in the SSCP initial screen, (2) the low individual occurrence of the recurrent mutations, and (3) the relatively small coding region of this gene, we propose that the best approach for scanning mutations in the *MECP2* gene is by direct sequencing of the entire coding region.

Alternatively to the direct sequencing, if an initial screening approach is preferred, multiplex AS-PCR, denaturing high performance liquid chromatography (DHPLC) or DOVAM-S (followed by confirmation through direct sequencing), could constitute good alternatives for an initial screen of *MECP2* gene. Optimization of an AS-PCR multiplex reaction in which, in one PCR reaction, the presence of all recurrent mutations could be checked could also be of interest. The application of this technique to our population (n=250) would allow the identification of a recurrent mutation in 71.7% (43/60) of the cases positive cases.

Additionally, detection of large rearrangements should also be carried out in patients to whom no point mutations were found. Southern blotting is the classical method used for analysis of genomic rearrangements that normally are skipped by routine PCR based methods; however, it is very time consuming and difficult to optimize. Fluorescent in situ hybridization (FISH) is also a helpful tool, but it requires a deletion with a minimum size of 1000 bp. Quantitative-PCR (qPCR) methods, including real-time PCR, have recently been used for detection of large rearrangements in *MECP2* (Ariani et al. 2004; Laccone et al.

2004) following the use of Southern blotting (Bourdon et al. 2001; Yaron et al. 2002; Schollen et al. 2003). Here, the RD-PCR technique revealed to be a rapid and efficient assay, and one of easy optimization (Shi et al. 2005). The RD-PCR, a duplex PCR, amplifies an endogenous internal control and a target locus. The internal control has a known gene copy number per cell, while the target has an unknown number per cell. The ratio of yield (ROY) of the PCR reaction is directly proportional to the ratio of the two input templates, so that the copy number of the *MECP2* gene could be obtained according to the ROY and the known copy number of the internal control. Using this technique we identified four large deletions in the *MECP2* gene, in girls without a point mutation. We still have to optimize this method for exon 1.

Strategically, as most mutations are localized in exon 4 (84.4%), the molecular approach to RTT diagnosis should start by exon 4, followed by exons 3 and 1 (9.4%) and, lastly, large rearrangements should be searched (6.2%). No mutations have been detected until today in exon 2; this exon should be scanned lastly if no mutation was found.

Ideally, if mutations were still not found, these *MECP2*-negative patients should then enter a dynamic research program searching for mutations in other candidate genes. To enter this “program” the first step is to obtain from the physician detailed clinical information on each patient. This will help in the selection of the future studies in which the patient should be included or not (figure 2.18).

### **Prenatal diagnosis: yes or no?**

We received five requests for prenatal diagnosis (PND), including samples from the proband, parents and amniotic fluid and cultured amniocytes. The sporadic nature of the mutation was first confirmed in the parents.

The familiar occurrences of RTT are rare (described as 1% in the literature). Recurrence within RTT families can be due to asymptomatic nonpenetrant carrier mothers (due to somatic mosaicism or skewed X chromosome inactivation) or to parental germinal mosaicism for the *MECP2* mutation. Since germline mosaicism can neither be predicted, nor detected, families with one affected patient, whose RTT-causing mutation has been previously identified, may benefit from prenatal diagnosis, which would then contribute to a decrease in the risk for the new pregnancy, which becomes comparable to that of the

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normal population. Additionally, despite the accompanying risk of an amniocentesis, PND could prove to be beneficial by reducing the anxiety created in the parents, in particular the mother of a RTT child.

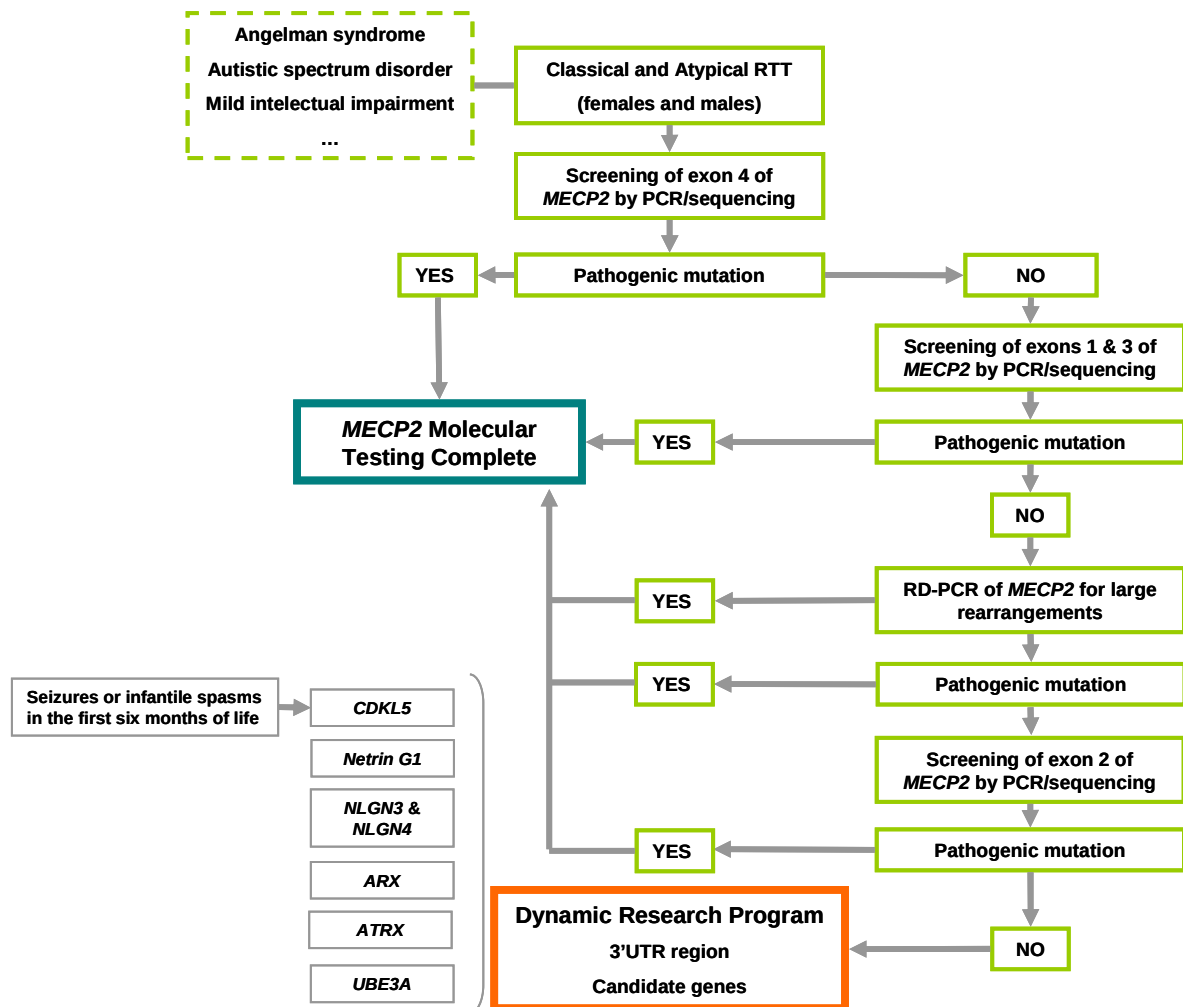


**Figure 2.18. Molecular diagnostic workflow for RTT.** Strategically, the scanning should start by exon 4, given the much higher mutation frequency (84.4%), followed by exons 1 and 3 and finally the search for large rearrangements. If no mutation was found, screening should be extended to non-coding regions and candidate genes.

### Boys with uncharacterized neurodevelopmental disorder

Male patients with neurodevelopmental disorders present a wide spectrum of phenotypes and share a combination of symptoms, which encompass mental retardation, autism and movement disorders. In most cases, the genetic basis of the pathology is unknown, and *MECP2* is an interesting candidate gene to be analyzed.

Mutations in *MECP2* are found in girl patients with heterogeneous clinical presentations; contributing to this fact are the effects of X-chromosome inactivation pattern, as well as a potentially significant influence of genetic, epigenetic or environmental modifiers. Furthermore, mutations known to be RTT-causing in females do not produce similar phenotypes in males, due to the X-linked dominance of the disorder (Ravn et al. 2003), and, possibly, to differences in the above-mentioned modifier effects.

We describe here one of the few familial cases of RTT, in which a maternal germline mosaicism is the most likely explanation. We detected a *MECP2* mutation (c.808delC; R270fsX288) in two children of a non-carrier couple: a girl with a classical form of RTT and a boy with a more severe and atypical presentation (Venancio et al. 2007).

In contrast, we were not able to identify any mutation in the *MECP2* gene in the remaining of our sample of boys with Rett syndrome-overlapping (RTT-like) phenotypes, including the large duplications of this gene, which have been described to be frequent in mentally retarded males with progressive neurological symptoms (Van Esch et al. 2005).

Our data suggests that, prior to the indication of systematic molecular testing of *MECP2* in all males with neurodevelopmental pathologies, the study of larger population series should be performed. In fact, the majority of male patients with RTT-like symptoms do not present mutations in the *MECP2* gene, which is in favour of the hypothesis that mutations in other gene(s) may be involved in this disorder (Schanen 2001). Even using a stricter phenotype definition, there are many males with a clinical diagnosis of RTT without an identified *MECP2* mutation (Leonard et al. 2001). Our *MECP2*-positive patient however, has a different phenotype than expected (Venancio et al. 2007), according to the first description of males with RTT (Jan et al. 1999). This should also be taken into account, regarding the indication for *MECP2* molecular studies in males.

There are a number of genes in which mutations have been found in patients with pathologies that partially overlap RTT, which would be interesting to test in patients with RTT or a RTT-like clinical presentation without a *MECP2* mutation: the neuroligin 3 (*NLGN3*) and neuroligin 4 (*NLGN4*) genes (mutated in patients with autism, mental retardation or Asperger syndrome) (Laumonnier et al. 2004); the study of the aristaless-related homeobox (*ARX*) (Stromme et al. 2002) and the serine/ threonine kinase 9 (*STK9*) genes, mutated in patients with West syndrome (Kalscheuer et al. 2003); and the study of

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the *UBE3A* gene (involved in Angelman syndrome) (Samaco et al. 2004). Brain derived neurotrophic factor (*BDNF*) and distal-less homeobox 5 (*DLX5*); two downstream target genes of MeCP2 regulation (Chen et al. 2003; Martinowich et al. 2003; Horike et al. 2005) would also be potentially important candidates for future analysis. Nevertheless, the possibility remains that additional novel genes may be identified as the molecular cause of disease in those patients.

### **Mutations versus polymorphisms in the *MECP2* gene**

We identified several polymorphisms (silent and synonymous changes), for which the pathogenic potential is minimal. Nevertheless, it is possible that these nucleotide changes, even if coding for the same amino acid (as is the case of silent changes) could affect other yet unknown mechanisms and, in this way, be responsible for the disease. For example, the DNA variants could affect the binding of “trans” elements, or affect an exonic splice enhancer (ESE) site and thus disturb the normal function of the protein (Cartegni et al. 2003; Smith et al. 2006).

The amino acid sequence of a protein specifies its secondary, and consequently, tertiary structure, which, in turn, affects the function of the protein. The two known and most studied domains of the MeCP2 protein are (1) a domain that binds methylated DNA (MBD), and (2) a domain that, through the interaction with other proteins, represses transcription (TRD). In this way, when a mutation occurs in MeCP2, at least one (or both) of these functions can be impaired; these features might be used in functional assays to assess the pathogenic nature of a mutation, especially of the missense type. Functional studies have been performed for certain MeCP2 mutations by others (Yusufzai and Wolffe 2000; Kudo et al. 2001; Georgel et al. 2003; Kudo et al. 2003; Petel-Galil et al. 2006) who showed that they are truly mutations that disturb the normal function of the MeCP2 protein.

Among the four variants of unknown significance identified in this study, S113P, K305R, P322A and V380M, only the last one seems to be a polymorphism. The alteration V380M was also present in the healthy mother of the patient (who had a random XCI). Evolutionarily, the substituted amino acid is not very conserved in paralogs and orthologs (figure 2.7 and figure 2.8) and the substitution of a valine for a methionine is conservative (both amino acids are hydrophobic). This data suggest that this alteration must not have consequences in the function of the protein. However, it may affect a potential group II

WW domain of MeCP2 (from amino acid 325 to C-terminal region), which is involved in splicing (Buschdorf and Stratling 2004). A functional assay addressing this feature should answer this question.

Data on the other 3 variants strongly suggest that they must play a role in the pathogenesis of RTT. The sporadic (i.e. not present in the parents) S113P change in amino acids was not conservative. The serine at position 113 is highly conserved, both between members of the same family, and across species, and it is localized in an important domain (MBD). This preliminary evidence suggests that it should have pathogenic consequences, but before any conclusion is taken, a control population should be tested for the presence of this alteration, and a proper functional assay designed to assess the binding capacity of the mutated protein to methylated DNA.

The K305R substitution appeared *de novo* in the patient, not being present in the parents. The K for R is a conservative substitution in terms of charge, but the lysine (K) amino acid at position 305 of MeCP2 is highly conserved across species (figure 2.8). This mutation has already been reported in three RTT cases (Buyse et al. 2000; Hoffbuhr et al. 2001; Monros et al. 2001), but it had never been tested before in a control population. This variant was not found in 226 X chromosomes of a Portuguese control population, suggesting it is in fact a causative mutation. Data from this preliminary evaluation indicates that it should constitute a good candidate to include in a functional assay, in this case to evaluate the transcriptional repression capacity of the normal and mutant alleles, as the alteration resides in the TRD of the protein.

The P322A alteration also appeared *de novo* and was not found in more than 100 control X-chromosomes in a population of European ancestry (Bienvenu et al. 2000). The change of a proline (P) by an alanine (A) is between amino acids of different groups, with implications for the folding of the protein. The P at position 322 is highly conserved across different species. The C-terminal region was also described to be involved in facilitating the binding of the protein to nucleosomal DNA (Chandler et al. 1999). The P322A substitution is also a good candidate to include in a functional assay, designed to assess the above referred functions.

We identified five different MeCP2 missense mutations in the MBD: class A - R106W, S113P, R133C, P152R and T158M (table 2.5).

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The MBD R106W, R133C and T158M mutations were previously found to completely abolish binding selectivity for methylated DNA (Yusufzai and Wolffe 2000). When the R106W missense mutation was assessed for its repression potential, in an experiment that did not involve methylation-dependent binding, it was shown that it was still able to repress transcription (Yusufzai and Wolffe 2000).

Another study showed that the nuclear localization of the protein and its binding to heterochromatin (in mouse L929 cells) was affected by the R106W mutation and, to a lower extent, by the T158M mutation. The R133C and the P152R mutations, however, did not affect nuclear localization of MeCP2, and these mutated forms were still able to bind to methylated DNA (Kudo et al. 2001; Kudo et al. 2003). Furthermore, using a *Drosophila* system (SL2 cells, expressing an exogenous Sp1 transcription factor that activates the methylated promoter of a reporter gene), the authors showed that, due to the impairment in the binding capacity of the R106W and T158M mutations, the transcriptional repressive potential of the resulting MeCP2 mutants was also affected (Kudo et al. 2001). In contrast, the R133C substitution exhibited a higher transcriptional repressive activity as compared to that of wild-type protein.

In the TRD we have identified four missense mutations: class B - P302L, K305R, R306H and R306C (table 2.5). Of these, the only functional assay reported was performed for the R306C; surprisingly, the results showed that MeCP2 with R306C mutation still had the ability to specifically bind to methylated DNA, and that its repression levels were comparable to those of the wild-type protein (Yusufzai and Wolffe 2000).

Therefore, not all mutations in the MBD disrupt the binding of the protein to the methylated DNA, and not all mutations in the TRD disrupt its repression potential; this is dependent on the aminoacid change and on particular position of a given mutation within each domain. Additionally, disrupting the binding will affect the repression potential.

Considering these functional studies, and particularly regarding the missense mutations in the MBD and in the TRD we found it possible to distinguish three main mutation groups according to the binding capacity and repression potential of the resulting proteins: (1) those that severely impair MeCP2 binding to methylated DNA (such as R106W), (2) those that present an intermediate pattern (such as T158M), and (3) those

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that are indistinguishable from the wild-type protein regarding these two functions (such as R133C, P152R, R306C). We thought this will be interesting to consider in a genotype-phenotype correlation (see below, genotype-phenotype correlation section).

Specifically in the case of R133C, P152R and R306C mutations, if they do not affect MeCP2 binding to DNA or its transcriptional repression capacity, how do they cause RTT or related phenotypes? Could they be important in other potential function/s of the MeCP2 protein, related to other less studied domains, such as the RG and ATRX-binding domains, or to other yet unknown domain/s?

Nan and colleagues (2007) showed that R133C mutation in fact did not affect binding to methylated DNA, but exerted its pathological effect by disrupting the interaction between MeCP2 and the protein mutated in ATR-X. *MECP2* mutations were also identified in patients with X-linked mental retardation (Meloni et al. 2000; Couvert et al. 2001; Gomot et al. 2003) and within this group it was described that subjects with the R133C mutation had a better overall function (Leonard et al. 2003).

Could it also be that in “live” neurons these changes in amino acid residues are more drastic than in the functional systems where they were studied?

Five different nonsense mutations were found (class D - Q110X; class E - R168X, R255X and R270X; and class F - R294X). Only mutation Q110X was located in exon 3, truncating the protein in the middle of its MBD. It would be interesting to see whether the Q110X mutant, as this mutation is localized in exon 3 (before the last exon) is able to produce a truncated protein, or whether (as would be predicted) it is directed to nonsense-mediated mRNA decay and hence no protein is produced at all. The other 4 nonsense mutations interrupted or excluded either the TRD and/or the NLS, leaving the MBD intact. Truncated R168X, R255X, R270X and R294X proteins must be produced, though they have decreased levels of stability *in vivo* (Yusufzai and Wolffe 2000).

Functional studies showed that R168X, R255X, R270X and R294X truncating mutations retain the ability to bind DNA (Yusufzai and Wolffe 2000; Nan et al. 2007), since their MBD is left intact. It was suggested that truncated MeCP2 proteins with an intact MBD might retain some degree of transcription silencing, either through a TRD-independent mechanism or by interfering with transcription-factor binding indirectly (Wan

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et al. 2001). In this way, it could be possible that these truncating mutations confer a milder phenotype than the missense mutations that disturb the MBD, not allowing the binding of MeCP2 to its targets. However, using the *Xenopus* oocyte system to evaluate the ability to repress transcription independently of DNA binding (GAL4), it was shown that MeCP2 with the R168X, R255X, R270X or R294X mutations was not able to repress the transcription of a reporter gene (Yusufzai and Wolffe 2000), as the wild-type protein did.

The CpG sites are one of the hotspots of MeCP2 mutation, as recurrent mutations often correspond to these sites. Most of the small rearrangements detected in our series (mainly deletions, but also insertions) occurred in the final portion of exon 4, suggesting that another hotspot for a different type of mutation might exist in the *MECP2* gene. The nucleotide sequence in exon 4 is very repetitive, which could lead to the creation of breakpoints.

The small rearrangements identified in our population created a frameshift in the sequence reading-frame and, after a few missense amino acids, truncated the protein at a premature position. The resulting mutated proteins, with altered folding, might be degraded by nonsense-mediated mRNA decay (A7fsX37 and K39fsX43) or the ubiquitin-proteasome system (T184fsX185, R253fsX275, G269fsX288, R270fsX288, V300fsX318, I303fsX477, L386fsX389, L386fsX390, L386fsX399 and P388fsX392).

A total of 60.9% (39/64) of all mutations found in our series predictably lead to the production of a truncated protein. MeCP2 truncating mutations, in terms of functional consequences in the protein, could be classified in four groups (table 2.5): those that (1) abolish MBD and TRD function, including the NLS and probably are null-alleles due to mRNA decay (class D mutations); (2) those that affect the TRD and NLS, disrupting the nuclear localization of MeCP2 and, hence, its function as a methyl-DNA binding protein (class E and class G); (3) those affecting the TRD function, but are still able to go to the nucleus and have their MBD intact (class F and H); and (4) the very late truncating mutations lying outside of the TRD, that might affect the binding of the protein to nucleosomal DNA and/or another function of a potential domain localized in this region (group II WW), but leave the MBD, TRD and NLS undisturbed (class I).

As discussed above these mutations could also affect other potential domains described in MeCP2, such as the RG domain or the ATRX-binding domain.

### Genotype-Phenotype correlation

Given the potentially very different effects of different mutations upon MeCP2 function, it could be expected that the clinical manifestations of RTT patients might be correlated with the mutation type. However, a correlation between *MECP2* mutation type, location and the clinical phenotype has been unclear.

The fact that the *MECP2* gene resides in the X-chromosome that undergoes XCI and, the type and localization of the several mutations often hampers a proper/powerful genotype-phenotype correlation. As discussed (chapter 1), the many attempts at studying this correlation did not contribute with consistent results across the different series of patients. The classical division of missense versus truncating mutations used in the correlation may not be the more useful approach. Nevertheless, the use of this classification in our series revealed that the median severity score was significantly higher in the group of patients with truncated mutations, than in the group with missense mutations (Temudo et al, in preparation), which is in accordance with other studies (Cheadle et al. 2000; Monros et al. 2001; Huppke et al. 2002; Schanen et al. 2004). Additionally, differences were found between patients with missense *versus* truncating mutations concerning acquisition of propositive words and independent gait before the beginning of the disease, and microcephaly, low weight and height and dystonia at the date of the patients' observation (Temudo T, in preparation). An association between the missense mutations and the ability to walk was also reported by (Monros et al. 2001; Huppke et al. 2002) and truncating mutations were reported to be associated with a worse language performance (Cheadle et al. 2000; Schanen et al. 2004) and a decelerated head growth (Huppke et al. 2002). Others however found different correlations or no correlation at all (Amir and Zoghbi 2000; Bienvenu et al. 2000; Auranen et al. 2001; Monros et al. 2001; Yamada et al. 2001; Weaving et al. 2003).

We attempted a different approach to genotype-phenotype correlations, more centered in the MeCP2 function or loss thereof: for this we established classes of mutations based on predicted or observed functional effects. We also adopted a classification of the *MECP2* mutation-positive RTT patient population into three different clinical groups, according to the major disease symptoms (mental retardation, ataxia and extrapyramidal), as proposed by Temudo et al. (in preparation). We found that missense mutations in the MBD and missense and truncating mutations in the TRD (not affecting NLS) were predominantly found in the MR and AT groups. On the other hand, null alleles

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and mutations that affect the NLS were predominantly found in the more severe EP form. Surprisingly, mutations in the C-terminal region of the *MECP2* gene, thought to have a milder phenotype, were restricted to the EP group. Considering the observed effect of mutations, we found that responsible for the EP phenotype were predominantly mutations that impair MeCP2 its major repression function and that lead to a total loss of the protein/function. Interestingly, mutations that lead to a decreased expression of the protein were present in both MR and EP groups, but not in AT, and it could be related to the sensitivity of different brain areas to changes of protein levels.

In spite of the low numbers used in the analysis, we showed that the results of this approach are quite interesting. This may be therefore a useful classification to use in a meta-analysis of a *MECP2* mutation- positive population, clinically well characterized. Analysis of animals or cellular models with particular mutations at these functional domains and comparison of their phenotypes would also be helpful in clarifying the role of specific mutations in pathogenesis as well for the development of directed drug therapies for RTT patients with different functional groups of mutations.

### **Analysis of the 3'UTR**

A restricted number of RTT cases remain without an identified genetic cause. Mutations in non-coding regions of the gene, untranslated regions (5' and 3'UTR) and introns (or in other genes not yet identified) may be the unidentified cause of the disorder in these cases.

The *MECP2* gene has one of the longest known 3'UTR tails, with 8.5-kb (Coy et al. 1999). Eight different transcripts result from alternative splicing and four different sites of polyadenylation, and the longest transcript is more than 10 kb and has several blocks of highly conserved residues between the human and mouse genomes (Coy et al. 1999). This argues in favour of a potential regulatory role of this 3'UTR in the expression pattern of the MeCP2 protein, in different cell types and at different developmental stages. The longest transcript is generally described as the predominant form in the brain (D'Esposito et al. 1996; Coy et al. 1999; Reichwald et al. 2000). The role of the 3'UTR of a gene might be in the regulation of its function at different levels, such as its "translatability", stability of the mRNA, nuclear export or the sub-cellular localization of the translated protein (Conne et al. 2000). As an example, the 3'UTR of CamKII gene was linked to the regulation of

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activity-dependent protein expression, via glutamate NMDAR activation, which is on the basis of synaptic plasticity, learning and memory formation (Wells et al. 2001), all disturbed in RTT.

The long and highly conserved 3'UTR of the gene *MECP2* suggested that mutations in this region could exist and explain a percentage of the RTT cases; from 20% to 70% in classical and atypical cases, respectively. Our data, however, indicated that mutations in this region must be rare and not account for a significant proportion of the RTT cases without genetic explanation.

A study based on data from the human gene mutation database (HGMD) estimated that around 0,2% of the disease-associated mutations reside in the regulatory regions of the 3'UTR (Chen et al. 2006). Mutations in the 3'UTR have been identified as the genetic cause of a number of diseases, all with a neurological component: IPEX syndrome (immune dysfunction, polyendocrinopathy, enteropathy, X-linked), caused by a mutation within the first polyadenylation signal of forkhead box P3 (*FOXP3*) gene (Bennett et al. 2001b); myotonic dystrophy, characterized by hypotonia, mental retardation and muscle development defects, due to a CTG repeat expansion in the *DMPK* (dystrophia myotonica protein kinase gene) (Fu et al. 1992); the Fukuyama-type congenital muscular dystrophy, presenting with mental retardation and brain defects, due to a defect in the Fukutin gene (Kondo-Iida et al. 1999); the familial Danish dementia caused by a decamer duplication in the integral membrane protein 2B gene (*BRI*) (Vidal et al. 2000); non-syndromic mental retardation suggested to be due to a nucleotide change found in the 3' regulatory region of cyclin-dependent kinase 5, regulatory subunit 1 gene (*CDK5R1*) (Venturin et al. 2006).

Mutations in 3'UTRs of other genes were found to be the cause of a number of neurological disorders, either by affecting the mRNA maturation, as is the case of the IPEX syndrome (Bennett et al. 2001a), the splicing of other genes as is the case with myotonic dystrophy (Ranum and Day 2004), expression levels as happens in familial Danish dementia (Vidal et al. 2000) and mRNA stability, in the case of the Fukuyama-type congenital dystrophy (Kondo-Iida et al. 1999).

However, although Shibayama and colleagues (2004) reported that 3'UTR variants in the *MECP2* gene seemed to be more frequent in autism patients than in the general population, we searched for mutations in the 3'UTR region of the *MECP2* gene in a group

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of Portuguese RTT females and did not find any pathogenic mutation, suggesting that this must be a rare cause of RTT. In agreement, in one study, the 3'UTR around the 10 kb polyadenylation signal of *MECP2* was scanned for mutations in RTT patients and no pathogenic variants were found (Bourdon et al. 2001). Additionally, the screening of the entire 3'UTR of *MECP2* in an autistic Portuguese population did not show any pathogenic variant or any increased representation of variants in this population, as compared to controls (Coutinho et al. 2007).

In order to clarify the contribution of the *MECP2* 3'UTR to RTT aetiology, a higher number of RTT patients of different RTT populations, without mutations in the coding region of the *MECP2* gene, should be screened, at least in these “blocks” of high conservation. Then again, this should only explain a small proportion of cases, and other candidate genes should be scanned, in particular those that are either being directly regulated by the MeCP2 protein, or interacting with one of the MeCP2 functional domains.

In summary, we established in the laboratory the molecular diagnostic method for detection of *MECP2* mutations. We found several different mutations in the coding region of *MECP2* in more than 90% of classical RTT cases and around 30% of atypical cases. We established interesting correlations between genotype and phenotype in mutation-positive patients taking into account the functional effects of mutations and the main subtypes of disease presentation. Our data also suggests that mutations in the 3'UTR of the *MECP2* gene are not responsible for the remaining cases of RTT (or related neurodevelopmental disorders) without a coding *MECP2* mutation and that *MECP2* mutations are not a major cause of the RTT-like phenotype in males.

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## CHAPTER 3

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### MeCP2 AND THE MOUSE NERVOUS SYSTEM: NEURODEVELOPMENT AND BEHAVIOUR OF *Mecp2*-NULL MICE



EVIDENCE FOR ABNORMAL EARLY DEVELOPMENT IN A MOUSE MODEL OF RETT SYNDROME

The results described in this chapter are included in the following peer-reviewed publication:

Mónica Santos, Anabela Silva-Fernandes, Pedro Oliveira, Nuno Sousa and Patrícia Maciel  
“Evidence for abnormal early development in a mouse model of Rett syndrome”. *Genes Brain & Behavior*, **2007** Apr 6(3): 277-86.





### 3-I.1 Abstract

Despite the classical description of RTT, researchers always questioned whether RTT patients did have subtle manifestations soon after birth. This issue was recently brought to light by several studies, using different approaches that revealed abnormalities in the early development of RTT patients.

Our hypothesis was that, in the mouse models of RTT as in patients, early neurodevelopment might be abnormal, but in a subtle manner, given the first descriptions of these models as initially normal. To address this issue, we performed a postnatal neurodevelopmental study in the *Mecp2*<sup>tm1.1Bird</sup> mouse. These animals are born healthy, and overt symptoms start to establish a few weeks later, including features of neurological disorder (tremors, hind limb claspings, weight loss). Different maturational parameters and neurological reflexes were analyzed in the pre-weaning period in the *Mecp2*-mutant mice and compared to wild type littermate controls. We found subtle but significant sex-dependent differences between mutant and wild type animals, namely a delay in the acquisition of the surface and postural reflexes, and impaired growth maturation. The mutant animals also show altered negative geotaxis and wire suspension behaviours, which may be early manifestations of later neurological symptoms. In the post-weaning period the juvenile mice presented hypoactivity that was probably due to motor impairments. The early anomalies identified in this model of RTT mimic the early motor abnormalities reported in the RTT patients, making this a good model for the study of the early disease process.

### 3-I.2 Introduction

The “classic” progression of RTT develops in four stages (Kerr & Engerstrom 2001). Stage I is characterized by an apparently normal development with uneventful pre and perinatal periods; in this stage (around 6 to 18 months) some of the patients learn some words and some are able to walk and feed themselves. In stage II (regression) a deceleration/arrest in the psychomotor development is noticed, with loss of stage I acquired skills, establishment of autistic behaviour and signs of intellectual dysfunction; hand skilful abilities are replaced by stereotypical hand movements, a hallmark of RTT. The pre-school/ school years correspond to stage III (pseudo-stationary) and here some

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improvement can be appreciated, with recovery of previously acquired skills. This is followed by the progressively incapacitating stage IV that can last for years (Hagberg et al. 2002); at this final stage patients develop trunk and gait ataxia, dystonia, autonomic dysfunction (breathing anomalies, sleep and gastrointestinal disturbances) and many of them have a sudden unexplained death in adulthood.

In spite of the classic RTT description, some researchers have questioned whether RTT patients displayed subtle signs of abnormal development soon after birth (Engerstrom 1992; Kerr 1995; Naidu 1997; Nomura & Segawa 1990). Huppke and colleagues (2003) described that their sample of RTT patients presented a significantly reduced occipito-frontal circumference, shorter length and lower weight at birth. This hypothesis has recently been confirmed by the work of Einspieler and colleagues (2005b), who analyzed video records of the first six months of life of 22 RTT patients and were able to notice abnormalities in several behaviours. All RTT patients presented an abnormal pattern of spontaneous movements within the first four weeks of life, with abnormal “fidgety” movements that were considered a sign of abnormal development (Einspieler et al. 2005a, b). Such abnormal movements were ascribed to problems in the central pattern generators in the brain (Einspieler et al. 2005a; Einspieler & Prechtel 2005). In a different study, midwives and health visitors blinded for the clinical status of the children, were able to identify in family videos potential anomalies in the early development of RTT patients, particularly anomalies in physical appearance and hand posture, as well as body movements and postures (Burford 2005). Segawa (2005), in a retrospective study of patients’ clinical files, also reported altered presentation of several motor milestones.

Animal models of RTT were created in mice, mimicking several motor and even the more emotional and social aspects of the syndrome (Chen et al. 2001; Guy et al. 2001; Shahbazian et al. 2002). The mutants are born normal and a few weeks later start to present a progressive motor deterioration, despite no gross abnormalities in the brain being noticed. Males carrying the mutation in hemizygoty display an earlier onset and are more severely affected than heterozygous females, probably due to X-chromosome inactivation that makes these females mosaics for the expression of the mutation, as is the case for the human condition.

The study presented here was performed using the *Mecp2*<sup>tm1.1Bird</sup> (Guy et al. 2001) mouse as a model. These mice were described as presenting no initial phenotype. Male

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*Mecp2*<sup>tm1.1Bird</sup> null animals begin to show symptoms at three-eight weeks whereas heterozygous female animals manifest the disease at three months of age. The phenotype of these animals mimics many of the motor symptoms of RTT: stiff and uncoordinated gait, reduced spontaneous movement, hind limb claspings, tremor and irregular breathing.

The goal of this study was to determine whether the early neurodevelopmental process was altered in the absence of MeCP2 in mice. We assessed achievement of milestones, considering different maturational and physical growth measures and neurological reflexes, two of the most well known and used neurobehavioral testing categories to address neurological disorder (Spear 1990), in the *Mecp2*<sup>tm1.1Bird</sup> mouse model of RTT (*Mecp2*-null males and *Mecp2*-heterozygous females). We identified an altered developmental progression of the mutant animals since the first postnatal week, in spite of their apparently normal phenotype. The differences seen suggest the presence of mild neurological deficits already at this age; the animals also presented significantly reduced activity, probably due to motor impairments soon in life. The abnormal achievement of the developmental hallmarks, although transient, could reflect abnormalities that are likely to impact the development of more mature behaviours.

### 3-1.3 Material and Methods

#### Animals

The strain used in this study was created by the Bird laboratory by transfecting the targeting vector in 129P2/OlaHsd E14TG2a embryonic stem cells and injecting these into C57Bl/6 blastocysts. According to information from the Jackson Laboratory, from whom we acquired the animals, the original strain was bred to C57Bl/6 mice and backcrossed to C57Bl/6 at least five times. Female *Mecp2*<sup>tm1.1Bird</sup> mice were bred with C57Bl/6 wild type (wt) male mice, in order to obtain wt and *Mecp2*-mutant animals. Mice were kept in an animal facility in a 12 hour light: 12 hour dark cycle, with food and water available *ad libitum*. A daily inspection for the presence of new litters in the cages was carried out twice a day and the day a litter was first observed was scored as day 0 for that litter.

After birth, animals were kept untouched in the home cage with their heterozygous mothers until postnatal day (PND) 3, and at PND4 animals were tagged in their feet or tip

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of the ears. Neurodevelopmental evaluation tests were started at PND4 and performed daily through PND21. Weaning was performed at 22/ 23 days of age. Males and females were separated and kept in independent cages, in groups of 3 to 7 animals per cage. At weaning the tip of the tail of the mice was cut for DNA extraction by Puregene DNA isolation kit (Gentra, Minneapolis, MN) and genotyping was performed according to the protocol supplied for this strain by the Jackson Laboratory.

At the fourth postnatal week animals were tested for spontaneous activity in the *Open field* apparatus (OF) and the day after animals were tested for anxiety-like behaviour in the *Elevated plus maze* apparatus (EPM). At the fifth postnatal week animals were tested in the *Rotarod* apparatus. After completing the experiment animals were rapidly decapitated, thus minimizing their suffering (in accordance to the European Communities Council Directive, 86/609/EEC).

All the tests described were evaluated by the same observer, who was blinded for the genotype of the animals and for the performance of the animals on the previous day. Tests were always performed in the same circadian period (between 11:00 and 18:00) and whenever possible at the same hour of the day. All the animals were separated from their parents at the beginning of each test session and kept with their littermates in a new cage, with towel paper and sawdust from their home cage. Once the test sessions finished for all the members of a litter, the animals were returned to their home cage. Table 3-I.1 shows attributable scores for each test. Throughout this chapter when we refer to “*Mecp2*-heterozygous animals” we always refer to females and “*Mecp2*-null animals” is always used to refer to male animals. All the controls used were littermates of the *Mecp2*-mutant (male and female) animals.

### **Pre-weaning behaviour**

#### **Maturation measures**

*Body Weight.* The body weight of mice was registered every day from PND4 through PND21 (weight  $\pm$  0,01g).

*Anogenital distance* (AGD). The distance between the opening of the anus and the opening of the genitalia was registered (distance  $\pm$  0.5mm).

*Ear opening.* The day when an opening in the ear was visualized was registered.

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*Eye opening.* We registered the state of the eyes from the day when animals start to open the eyes until the day when every animal in the litter has both eyes opened. An eye was considered open when any visible break in the membrane was noticed.

### Developmental measures

*Surface righting reflex (RR).* Mice were restrained in their back in a table and then released. The performance of the animal (to turn or not) was scored and the time taken to surface right, in a maximum of 30 seconds, in three consecutive trials, was registered. To determine the score for each day, the median value was calculated for the 3 trials.

*Postural reflex (PR).* Animals were put in a small box and shaken up and down and left and right. Existence of an appropriate response (animals splaying their four feet) was scored.

*Negative geotaxis (NG).* Animals were put in a horizontal grid and then the grid was turned 45°, so that the animal was facing down. The behavior of the animal was observed for 30 seconds and registered (as shown in table 3-I.1).

*Wire suspension (WS).* The animals were forced to grasp a 3 mm wire and hang from it on their forepaws. The ability of the animals to grasp the wire was scored and the time they held on the wire (maximum 30 seconds) was registered.

Table 3-I.1. Attributable scores for milestone performance of *Mecp2*-mutant and wild type animals

|                         | Score                     |                   |                         |              |
|-------------------------|---------------------------|-------------------|-------------------------|--------------|
|                         | 0                         | 1                 | 2                       | 3            |
| Ear opening             | close                     | open              |                         |              |
| Eye opening             | both closed               | one open          | both open               |              |
| Surface righting reflex | keeps in dorsal position  | fights to upright | rights itself up        |              |
| Postural reflex         | not present               | present           |                         |              |
| Negative geotaxis       | turns and climbs the grid | turns and freezes | moves but fails to turn | does not mov |
| Wire suspension         | not present               | present           |                         |              |

### Post-weaning behavioural tests

*Open field.* Animals were placed in the centre of an arena of 43.2 x 43.2 cm with transparent walls (MedAssociates Inc., St. Albans, Vermont) and their behaviour was

observed for 5 min. Activity parameters were collected (total distance travelled, speed, resting time and the distance travelled and time spent in the predefined centre of the arena versus the rest of the arena). The number of rears, the time that animals spent exploring vertically and the number of *bolus fecalis* was also registered by observation.

*Elevated Plus Maze.* Animals were placed in a EPM apparatus consisting of two opposite open arms (50.8 x 10.2 cm) and two opposite closed arms (50.8 x 10.2 x 40.6 cm) raised 72.4 cm above the floor (ENV-560, MedAssociates, Vermont, USA) and behaviour (number of entries in each arm and the time spent in each of the arms) was registered for 5 min.

*Rotarod.* Mice were tested in a rotarod (TSE systems, Germany) apparatus to evaluate their motor performance. The protocol consisted of 3 days of training at a constant speed (15 rpm) for a maximum of 60 seconds in four trials, with a 10 min interval between each trial. At the fourth day, animals were tested for each of 6 different velocities (5 rpm, 8 rpm, 15 rpm, 20 rpm, 24 rpm and 31 rpm) for a maximum of 60 seconds in two trials, with a 10 min interval between each trial. The latency to fall off the rod was registered.

### **Statistical analysis**

In the pre-weaning behaviour analysis, due to problems of the data in achieving the assumptions required for repeated measures testing, such as sphericity and homogeneity of variances, we used regression methods to compare the performance between *Mecp2*-mutant and wt littermate control mice. In order to do this, variables scored 0 or 1 were analyzed by logistic regression (Score= f (day, genotype, sex)). For continuous variables, a linear or a quadratic regression was applied. Interaction between the independent variables (day genotype and sex) was also studied and reported when it was observed. The surface righting reflex and wire suspension times were analyzed as survival times through the Kaplan-Meier test. The Negative Geotaxis was analyzed (classification in 3 classes) by a Chi-square test and the percentage of animals meeting criterion (score=0) by linear regression. In the post-weaning behaviour tests, data was analyzed with a Student t-test. A critical value for significance of  $p < 0.05$  was used throughout the study.

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### 3-I.4 Results

#### Pre-weaning behaviour analysis

In this and in all other variables under study we always analyzed male and female animals separately. The number of animals used in the analysis of maturation markers and neurological reflexes in the pre-weaning period was: *Mecp2*-null, n=13; wt littermate males, n=11; *Mecp2*-heterozygous, n=16; wt littermate females, n=9.

#### Physical growth and maturation

**Body weight.** We weighted *Mecp2*-mutant and wt littermate control mice everyday from PND4 to PND21 and analyzed the data with a quadratic regression. As expected, the body weight was statistically different between male and female animals, with female animals heavier than male mice ( $p=0.013$ ), and the day of analysis had a significant influence in the body weight ( $p<0.001$ ). When we analyzed the influence of the *Mecp2* genotype of mice in the body weight, we noticed that the body weight evolution of *Mecp2*-null mice was not different from that of the wt littermate controls, in the first 21 days of postnatal development ( $p=0.156$ ). Surprisingly, however, *Mecp2*-heterozygous mice presented a significantly reduced body weight when compared to their wt littermate controls ( $p<0.001$ ) (figure 3-I.1A-B). The effect of genotype was not seen from the beginning of the study, but from around PND10 onwards.

**Ear and Eye opening.** We observed mice daily from PND4 and registered the day when at least one eye was open and the day when both eyes were open. The day an aperture was seen in the ear was also registered. No differences existed between genotypes or gender regarding the mean day of aperture of eyes and ears (table 3-I.2).

Table 3-I.2. Maturation measures assessment in *Mecp2*-mutant and wild type animals

|                    | Female       |              |         | Male         |              |         |
|--------------------|--------------|--------------|---------|--------------|--------------|---------|
|                    | Wild-type    | Heterozygous | P-value | Wild type    | Knock out    | P-value |
| Day of ear opening | 12.89 ± 0.11 | 12.69 ± 0.12 | NS      | 12.81 ± 0.18 | 12.77 ± 0.17 | NS      |
| Day eye opening    | 13.56 ± 0.24 | 13.62 ± 0.16 | NS      | 13.82 ± 0.18 | 13.69 ± 0.13 | NS      |

Legend: NS, no statistical significant

**Anogenital distance.** We took this measure since PND4 through PND21 in all mice and analyzed data with a linear regression method. As body weight might influence the

anus-genitalia distance, previous studies (Degen et al. 2005) introduced a correction: The AGD value was divided by the weight of each animal at each postnatal day (AGD/weight). We calculated the coefficient of correlation between the AGD and the body weight of the mice ( $R=0.907$  for males and  $R=0.917$  for female mice), suggesting that these two variables were highly associated and so we decided not to use this correction.

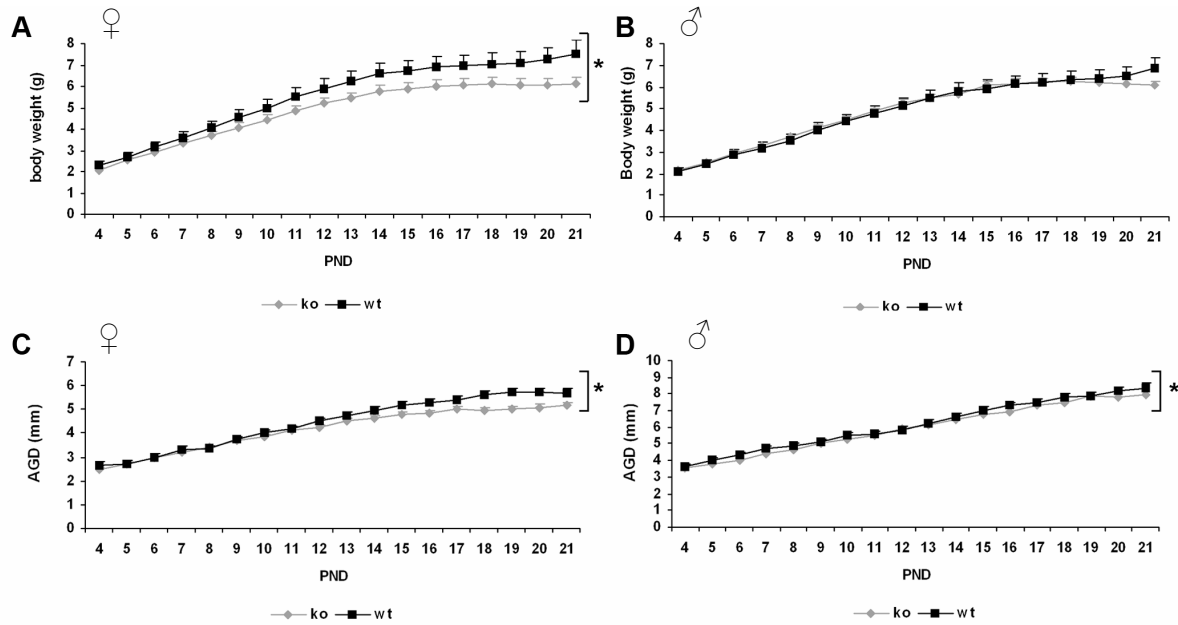


**Figure 3-I.1. Physical growth and maturation parameters of the *Mecp2*-heterozygous female and the *Mecp2*-null male mice during the pre-weaning period.** (A and B) Body weight evolution from PND4 to PND21 of *Mecp2*-mutant animals their wt littermate controls. *Mecp2*-heterozygous females presented a significant reduction in body weight that started to be notorious after PND10 ( $p < 0.001$ ). (C and D) Anogenital distance measurement from PND4 through PND21 of *Mecp2*-mutant animals their wt littermate controls. *Mecp2*-mutant mice presented a significant reduction in the AGD ( $p < 0.001$ ). (*Mecp2*-heterozygous females,  $n=16$ ; wt females,  $n=9$ ; *Mecp2*-null males,  $n=13$  and wt males,  $n=11$ . Values are mean  $\pm$  sem. AGD – anogenital distance, PND – postnatal day, ko – knock out, wt – wild type, \*  $p < 0.05$ , ♀ - female, ♂ - male).

The AGD of male mice was higher than that of female mice ( $p < 0.001$ ), as expected, and the day of testing affected this distance, which was higher the later the measure was taken ( $p < 0.001$ ). We found that male and female *Mecp2*-mutant animals presented a statistically significant reduction in the AGD along the pre-weaning period, when compared to their respective wt controls ( $p < 0.001$ ) (figure 3-I.1C-D).

### Neurological reflexes

**Surface righting reflex.** No differences between sexes were found in the acquisition of this reflex ( $p=0.668$ ), and the day improved the performance of the animals in the ability



to upright ( $p < 0.009$ ), as expected. *Mecp2*-mutant animals did not present differences in the age of acquisition of this reflex ( $p=0.534$  and  $p=0.161$  for *Mecp2*-null and *Mecp2*-heterozygous mice, respectively) (data not shown). When we considered the time these animals took to upright, *Mecp2*-heterozygous mice presented statistically significant differences, with mutant females taking longer time than wt littermates to upright ( $p=0.031$ ) (figure 3-I.2A-B). Nevertheless, when *Mecp2*-null mice and wt controls were compared no differences were found. There were no differences, in this last parameter, between sexes ( $p=0.216$ ).

*Postural reflex.* There were no differences between genders in the ontogeny of this reflex ( $p=0.118$ ) and, as expected, the day affected its establishment ( $p < 0.001$ ). The pattern of acquisition of the PR was statistically different between *Mecp2*-null ( $p < 0.001$ ) and *Mecp2*-heterozygous ( $p=0.006$ ) mice, when compared to their respective wt controls, with a worse outcome for mutant animals. Both *Mecp2*-null and *Mecp2*-heterozygous mice showed a delay in the acquisition of the PR reflex (figure 3-I.2C-D). The acquisition of the PR by wt animals started at PND9 for females and PND10 for males and at PND16 all wt animals presented the PR. In the mutant mice the first day of appearance of the reflex was PND11 for females and PND12 for males and only at PND17 did all mutant animals present the PR. In summary, *Mecp2*-mutant animals presented a delay of 2 days in relation to the day of first appearance of PR in the wt animals.

*Negative geotaxis.* In respect to mice behaviour, this reflex was scored from 0 to 3 (see table 3-I.1). Scores 2 and 3 were not frequent and so, in order to simplify the analysis of the data, we decided to recode the behaviours for the analysis. Score=0 and score=1 were maintained and score=2 was changed to include the previous scores 2 and 3. In this task, both male and female *Mecp2*-mutant mice had a worse performance than their respective wt littermate controls (figure 3-I.2E-F). The percentage of animals meeting the criterion for score=0 was dependent of the day ( $p < 0.01$ ) and genotype ( $p < 0.01$ ), whereas sex was not significant ( $p=0.07$ ). Moreover, differences were found in the acquisition of the NG reflex between genotypes in both sexes (in both cases  $p < 0.01$ ), resulting from a difference in the performance of the animals in classes 0 and 2. When we tested the animals in a weaker version of this test (at 30° inclination), *Mecp2*-null animals still presented a worse performance than wt controls in performing this task whereas heterozygous females did not differ significantly from wt animals (data not shown).

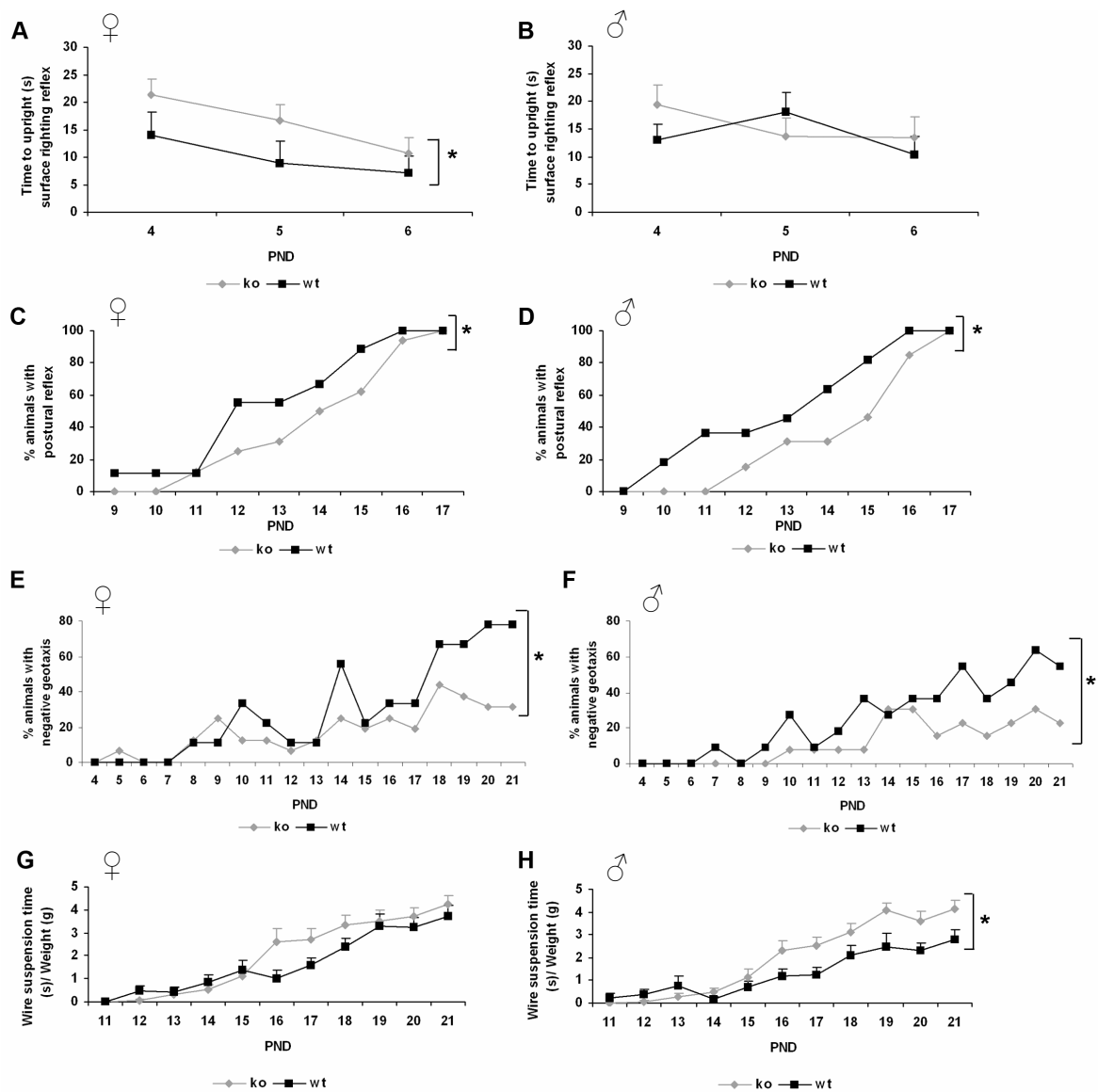


**Figure 3-I.2. Abnormalities in milestones achievement in the *Mecp2*-heterozygous and the *Mecp2*-null mice during the pre-weaning period.** (A and B) Time taken to upright in the surface righting reflex test. Female *Mecp2*-heterozygous mice take longer time to upright than their wt littermates ( $p < 0.05$ ). (C and D) Percentage of animals presenting the postural reflex between PND9 and PND17. A delay in the acquisition of this parameter was observed in both the *Mecp2*-null animals ( $p < 0.001$ ) and the *Mecp2*-heterozygous females ( $p = 0.006$ ). (E and F) Percentage of animals presenting the negative geotaxis reflex. Female *Mecp2*-heterozygous animals ( $p = 0.002$ ) and *Mecp2*-null males ( $p < 0.001$ ) presented a worse performance than wt littermates. (G and H) Time that animals hold in the wire suspension reflex (in a 30 seconds test). *Mecp2*-null male animals held longer in the wire ( $p = 0.010$ ), although differences in *Mecp2*-heterozygous females did not reach significance. (*Mecp2*-heterozygous females,  $n = 16$ ; wt females,  $n = 9$ ; *Mecp2*-null males,  $n = 13$  and wt males,  $n = 11$ ). Values are mean  $\pm$  sem. PND – postnatal day, ko – knock out, wt – wild type, \*  $p < 0.05$ , ♀ - female, ♂ - male).

*Wire suspension.* There were no differences in the establishment of this reflex between male and female mice ( $p=0.176$ ) and the day affected the establishment of the reflex ( $p<0.001$ ), as expected. The performance of *Mecp2*-null and *Mecp2*-heterozygous and their respective wt controls in the acquisition of the reflex (animals grasp the wire or do not grasp) was similar, with no statistical differences when compared among each other ( $p=0.605$  for males and  $p=0.214$  for females). This reflex was acquired between PND11 and PND18 for both *Mecp2*-mutant and wt mice of both genders. Another parameter that was taken from this analysis was the wire suspension time. As body weight might influence the time animals hold in the wire, the curves of the wire suspension holding time were corrected taking into account the curves of the body weight. We analyzed this parameter from PND15 onwards, since from this day more than 50% of the animals held in the wire more than 1 second. In the wt background females held a significantly longer time in the wire than male mice ( $p=0.046$ ), but there were no differences between mutant male and female mice ( $p=0.730$ ). Surprisingly, *Mecp2*-null and *Mecp2*-heterozygous mice stayed longer in the wire than their respective wt littermate controls and the differences were statistically significant between *Mecp2*-null and wt littermate controls ( $p<0.001$ ) (figure 3-I.2G-H). Even when we analyzed the data relative to all days (PND11-PND21), we achieved the same conclusions ( $p=0.010$ ).

### Post-weaning behaviour analysis

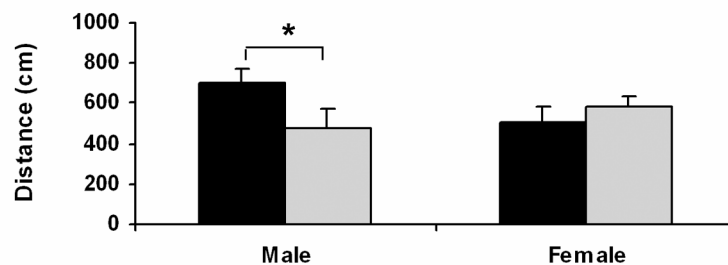
*Exploratory activity.* At the fourth week of age, animals were tested in the OF apparatus, to evaluate their spontaneous activity, for a period of 5 min (*Mecp2*-null,  $n=14$ ; wt littermate males,  $n=16$ ; *Mecp2*-heterozygous,  $n=12$ ; wt littermate females,  $n=10$ ). Globally, no differences were found between *Mecp2*-mutant and wt animals in the time they spent and distance they travelled in the centre of the arena in relation to the total area of the arena, in the time animals spent exploring vertically or in the number of rears (table 3-I.3). We found that *Mecp2*-null animals travelled a smaller total distance ( $p=0.049$ ) at a lower speed ( $p=0.000$ ) than wt controls (figure 3-I.3A-B). Null animals produced a significantly higher number of *bolus fecalis* ( $p=0.031$ ) (table 3-I.3), which could be a consequence of their neuroautonomic disorder.

Table 3-I.3. Performance of *Mecp2*-mutant and wild type animals in the Open field test

|                                | Female       |              |         | Male         |              |         |
|--------------------------------|--------------|--------------|---------|--------------|--------------|---------|
|                                | Wild-type    | Heterozygous | P-value | Wild type    | Knock out    | P-value |
| Ratio center:total - Distance  | 0.20 ± 0.04  | 0.16 ± 0.02  | NS      | 0.15 ± 0.01  | 0.14 ± 0.03  | NS      |
| Ratio center:total - Time      | 0.31 ± 0.05  | 0.38 ± 0.04  | NS      | 0.35 ± 0.03  | 0.33 ± 0.07  | NS      |
| Number of rears                | 23.50 ± 4.48 | 26.67 ± 4.22 | NS      | 33.25 ± 3.67 | 23 ± 4.71    | NS      |
| Time spent rearing             | 19.6 ± 4.66  | 25.92 ± 5.16 | NS      | 28.25 ± 3.73 | 20.43 ± 4.42 | NS      |
| Number of <i>bolus fecalis</i> | 1.60 ± 0.73  | 2.33 ± 0.68  | NS      | 1.19 ± 0.45  | 2.57 ± 0.40  | 0.031   |

legend: NS, no significant

### A Total distance travelled



### B Speed

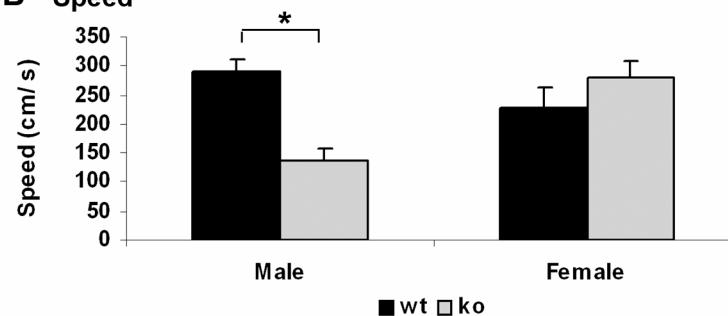


**Figure 3-I.3. *Mecp2*-mutant female and male mice present reduced spontaneous activity without altered exploratory capacity at 4 weeks of age, in the *open-field* paradigm.** (A) *Mecp2*-null male mice travelled a smaller total distance ( $p=0.022$ ), (B) at a lower speed ( $p=0.000$ ) and (C) spent more time resting ( $p=0.026$ ) than their respective wt littermate controls. Female heterozygous animals did not present differences in any of the parameters analysed. (*Mecp2*-heterozygous females,  $n=27$ ; wt females,  $n=19$ ; *Mecp2*-null males,  $n=21$  and wt males,  $n=22$ . Values are mean  $\pm$  sem. PND – postnatal day, ko – knock out, wt – wild type, \*  $p<0.05$ ).

**Anxiety-like behaviour.** The day after OF testing, animals were tested in the EPM apparatus, in a five minute session (*Mecp2*-null,  $n=13$ ; wt littermate males,  $n=13$ ; *Mecp2*-heterozygous,  $n=11$ ; wt littermate females,  $n=8$ ). There were no differences between *Mecp2*-mutant animals and wt controls in the percentage of time animals spent in the

open arms nor in the percentage of entries in the open arms in relation to total arms entries, but *Mecp2*-null animals presented a smaller number of closed arms entries ( $p=0.014$ ) (figure 3-I.4A-C).

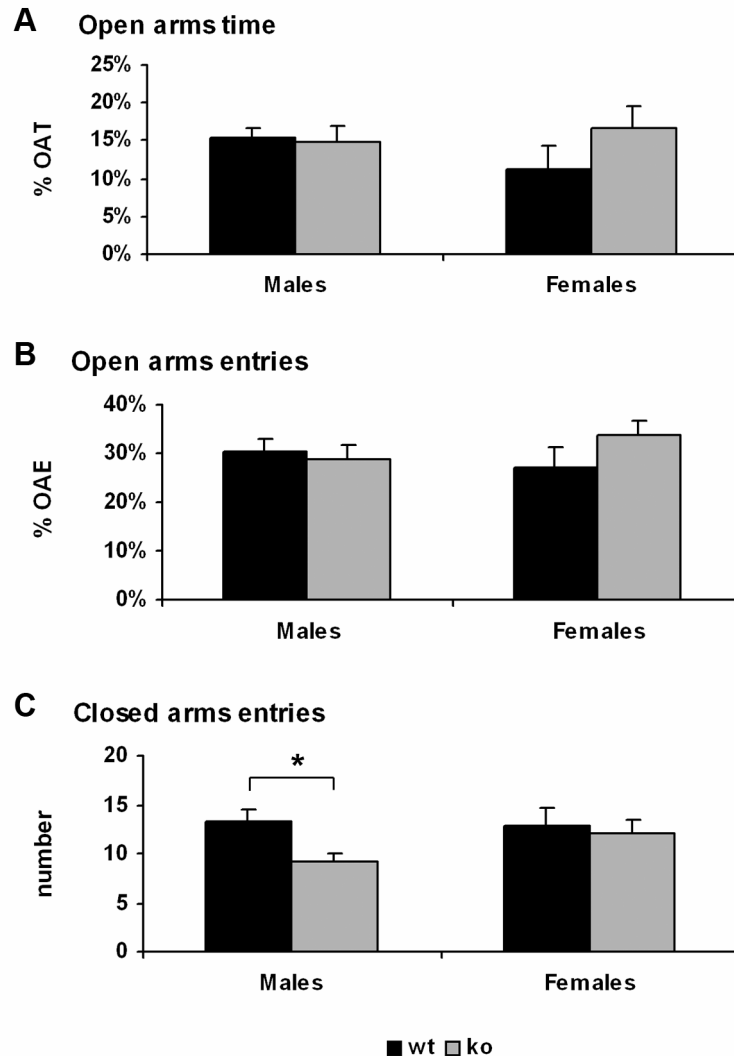


**Figure 3-I.4. *Mecp2*-mutant female and male mice do not present anxious-like behaviour at 4 weeks of age in the *elevated plus maze* paradigm.** Neither *Mecp2*-null male nor *Mecp2*-heterozygous female mice presented differences in (A) the percentage of open arms time and (B) the percentage of open arms entries, which are measures of the state of anxiety that the animals exhibit in a new environment. (C) *Mecp2*-null animals presented fewer number of entries in the closed arms than their wt littermate controls ( $p=0.000$ ) suggesting the existence of a locomotor impairment. (*Mecp2*-heterozygous females,  $n=26$ ; wt females,  $n=17$ ; *Mecp2*-null males,  $n=20$  and wt males,  $n=19$ . Values are mean  $\pm$  sem. PND – postnatal day, ko – knock out, wt – wild type, \*  $p<0.05$ ).

**Motor coordination.** At 5-weeks of age, *Mecp2*-mutant animals were tested in the rotarod in order to evaluate their motor coordination (*Mecp2*-null,  $n=11$ ; wt littermate males,  $n=11$ ; *Mecp2*-heterozygote,  $n=16$ ; wt littermate females,  $n=9$ ). After 3 days of

training, mice were tested at different speeds. *Mecp2*-null and *Mecp2*-heterozygous mice, when compared to wt control mice, presented a reduced latency to fall off the rod. This reduction was statistically significant at 15 rpm for male ( $p=0.046$ ) and at 20 rpm for female ( $p=0.023$ ) mice (figure 3-I.5A-B).

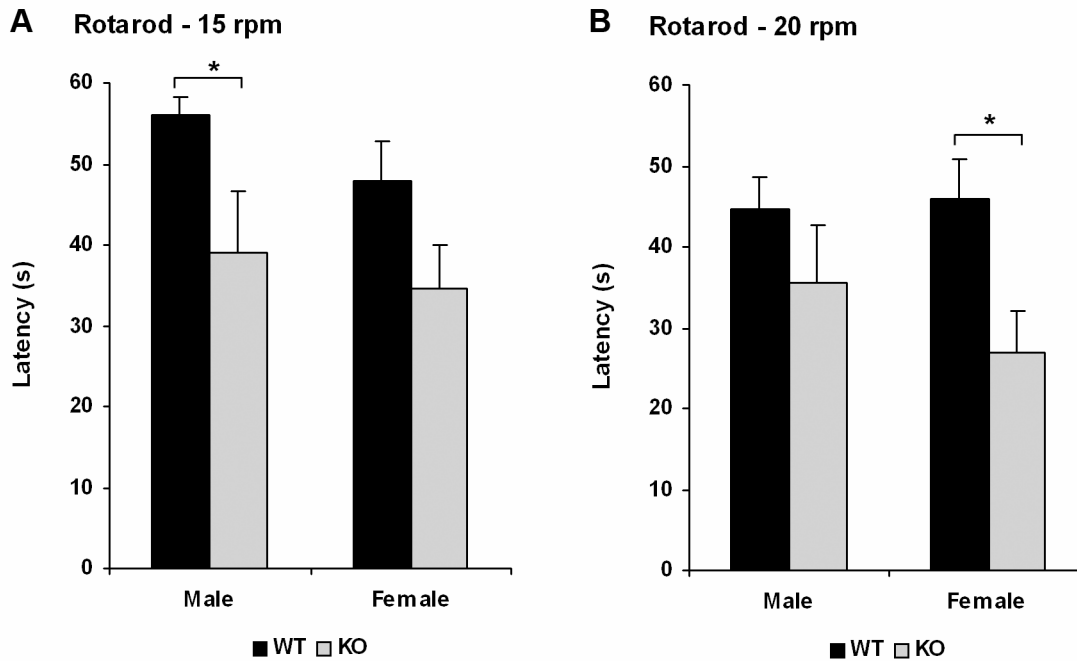


**Figure 3-I.5. *Mecp2*-mutant mice present motor problems at 5 weeks of age.** The latency to fall off the rod was lower for the *Mecp2*-null mice at 15 rpm (A) and for *Mecp2*-heterozygous females at 20 rpm (B) than the latency exhibited by their respective wt controls. (*Mecp2*-heterozygous females,  $n=16$ ; wt females,  $n=9$ ; *Mecp2*-null males,  $n=11$  and wt males,  $n=11$ . Values are mean  $\pm$  sem. PND – postnatal day, ko – knock out, wt – wild type, \*  $p<0.05$ ).

### 3-I.5 Discussion

#### Delayed somatic physical growth and maturation of *Mecp2*-mutant mice.

Among the physical growth and maturation parameters assessed in this study, differences were seen in body weight and in anogenital distance (AGD). The body weight was significantly reduced in the *Mecp2*-heterozygous, but, unexpectedly, this difference in body weight was not seen between *Mecp2*-null and wt control male mice in spite of their earlier disease onset. However, the curves of *Mecp2*-null and wt males start diverging at PND20 and would probably follow this trend at later ages. In fact, it is already known from

the original publication on this model that *Mecp2*-null mice present a smaller body weight than wt littermate controls at 4 weeks of age (Guy et al. 2001). The same authors suggested that, given the differences observed between mice of different genetic background, the effects of MeCP2 in body weight could be mediated by one or more modifier genes. One of these modifier genes could be sex-linked and thus provide a possible explanation for the results we obtained. Also, the AGD is reduced in both male and female *Mecp2*-mutant mice suggesting that these animals present a slower sexual maturation. In the case of *Mecp2*-null mice it has been reported that their testes are always internal and they do not mate since they are too debilitated or die before adulthood. However, adult *Mecp2*-heterozygous mice are fertile and, as far as we know, they do not present reduced fertility and raise normal litters (Guy et al. 2001). Taken together these results are also in support of the evidence that MeCP2 has an effect in somatic growth markers and not only in neuronal cells (Huppke et al. 2003; Nagai et al. 2005).

### **Pre-weaning behaviour in the *Mecp2*-mutant animals suggests early neurological dysfunction.**

In the present study, a delay in the achievement of the postural reflex and of the surface righting reflex (only in females) was evident between *Mecp2*-mutant and wt animals. Both reflexes depend on the development of dynamic postural adjustments and imply the integrity of muscular and motor function (Altman & Sudarshan 1975; Dierssen et al. 2002). Acquisition of the negative geotaxis reflex, a dynamic test which reflects sensorimotor function and depends on colliculi maturation (Dierssen et al. 2002) was also disturbed. Despite those impairments, in another neurological reflex, the static wire suspension test - that is highly compensated by information from the visual and proprioceptive systems, *Mecp2*-mutant animals did not perform worse than wt controls. *Mecp2*-null animals held in the wire for a longer time, even though there were no differences between *Mecp2*-null and wt controls in the moment when mice started to grasp the wire. Thus, the fine motricity of the forepaws does not appear to be affected in the mutant mice. The longer time in the wire could, however, reflect the incapacity of the mutant mice to initiate a voluntary movement, which could constitute a possible sign of dyspraxia, as observed in RTT patients (Kerr & Engerstrom 2001).

All the above-mentioned reflexes are sensitive to the function of the vestibular system, whose role is to provide information on the position and movement of body and

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head in space, and so they depend largely on brainstem (medullary) structures (Altman & Sudarshan 1975). The positional information is transmitted from the inner ear to the central vestibular system located in the hindbrain and integrated with information from other neural systems (for a review see Smith et al. 2005). The data we obtained on neurological reflexes is particularly interesting in the light of the studies in human RTT patients that suggest dysfunction of the brainstem, where the vestibular system is located, as responsible for the early pathogenesis in RTT (Einspieler et al. 2005b; Segawa 2005). Interestingly, MeCP2 binds directly to the brain derived neurotrophic factor (*Bdnf*) promoter region (Chen et al. 2003; Martinowich et al. 2003) and regulates its transcription in an activity-dependent manner. BDNF appears to have an important role in the maturation and maintenance of the vestibular system, as mice deficient for BDNF and its receptor TrkB present neuronal loss in the vestibular sensory ganglia (Huang & Reichardt 2001). It is, thus, possible to speculate that the levels of this neurotrophin in the vestibular pathways could be deregulated in the *Mecp2*-mutant mice and in this way also contribute to possible dysfunction in the vestibular system.

Abnormal acquisition of the NG reflex could reflect abnormalities in the maturation of the colliculi and the abnormal performance in the surface RR could reflect abnormalities in the labyrinthine function. Anomalies in the auditory canal must not be the source of this dysfunction, since mice with anomalies in this area present stereotypical behaviours (Khan et al. 2004) that are not exhibited by the *Mecp2*-mutants. Data on the pathology in this area of the mouse brain, as far as we know, is not yet available in the *Mecp2*<sup>tm1.1Bird</sup> mouse and future research is necessary to explore neuropathological correlates of the abnormal functional outcome in the first days of postnatal life of *Mecp2*-mutant mice.

The subtle but significant perturbations observed in the achievement of milestones are a first sign of early neurological pathology in the *Mecp2*<sup>tm1.1Bird</sup> mice. The motor problems that these mice experience later in life correlate with the developmental abnormalities and may even be a consequence of impaired neurodevelopment of pathways within the brainstem area.

### ***Mecp2*-mutant mice present reduced spontaneous activity due to motor impairments before the onset of overt symptoms.**

Adult *Mecp2*<sup>tm1.1Bird</sup> mice were initially described as presenting serious motor problems after a period of normal development (Guy et al. 2001). In fact, in their home

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cage at four weeks of age, juvenile *Mecp2*-mutant mice are, other than their reduced body weight, almost indistinguishable from their wt littermates. However, in the OF apparatus the *Mecp2*-null mice exhibit hypoactivity (Guy et al. 2001) despite a normal exploratory capacity. We were not able to notice any differences between *Mecp2*-heterozygous and wt control females in the OF, at four weeks of age, even though they were previously described to exhibit reduced spontaneous activity at later ages, when symptomatic (Guy et al. 2001). In the OF and EPM we did not identify an anxious-like behaviour, neither in male nor in female *Mecp2*<sup>tm1.1Bird</sup> animals, at four weeks of age. In accordance, performance of older symptomatic *Mecp2*-heterozygous animals in the OF also suggested that these mice do not present heightened anxiety (Guy et al. 2001). Anxiety was, however, described in other models of the RTT disorder (Gemelli et al. 2005; Moretti et al. 2005; Shahbazian et al. 2002).

At five weeks of age our data showed that *Mecp2*-null and *Mecp2*-heterozygous mice presented motor coordination impairment. This is, to the best of our knowledge, the first study to identify the effect of *Mecp2* mutation on the sensory-motor coordination in the rotarod test in five week old mice. Although differences in the locomotor profile of *Mecp2*-heterozygous mice when compared to wt controls were not identified in the OF and in the EPM apparatus, in the more sensitive and specific rotarod test, mutant females did present motor problems already at the age of five weeks. Motor coordination problems had already been previously reported in the other models of RTT, but not at such early age: the *Mecp2*<sup>308/y</sup> animals are not impaired up to 10 weeks of age (Moretti et al. 2005), but are impaired at later ages (Shahbazian et al. 2002). Our findings suggest that MeCP2 is important for the acquisition of motor coordination abilities and that deregulation of its levels causes slight motor problems that appear early in development and become increasingly evident as development proceeds. The deficits in the rotarod are not likely due to muscle weakness since the mutant animals held longer in the WS test than wt animals. Coordination is necessary for a good performance both in the dynamic reflexes and in the rotarod test. Hence, and regarding the data obtained in this study, a lack of limb coordination is apparently present in the *Mecp2*-mutant mice; given that both the NG reflex and the rotarod test are affected, we suggest that hind limbs are more severely involved. Rearing also presupposes hind limb strength (Altman & Sudarshan 1975) and as this parameter is not affected in these animals, the problem must reside in the coordination of hind limbs rather than in their strength.

The identification of early and subtle neurodevelopmental differences in the RTT mouse model provides an interesting analogy to the recent findings of minor neurological signs during the first months of life of RTT patients. Further analysis of neurodevelopment in these *Mecp2*-mutant mice, which mimic well the motor profile of RTT patients, should give insight into the underlying mechanisms of pathogenesis in this disease and contribute to a precocious RTT diagnosis that might be beneficial in terms of therapeutic approaches since the first months of life.

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EARLY DISTURBANCES OF MOTOR BEHAVIOUR IN *Mecp2*-NULL MICE



### 3-II.1. Abstract

Our data on the first part of this chapter suggested that the locomotor profile of the *Mecp2*<sup>tm1.1Bird</sup> mice is compromised at a precocious stage. Hence, in this second part of the chapter we explored further the onset and progression of the motor impairment in an attempt to understand its nature and map the affected brain structures.

In this way, we assessed the *Mecp2*-null mice (*Mecp2*<sup>tm1.1Bird</sup>) performance in three different motor paradigms and at two different timepoints: at three- and eight-weeks of age in the footprint pattern, gait onset and in the open field tests. We found that already at three weeks of age *Mecp2*-null mice exhibit motor impairments in gait onset and pattern, without hypoactivity; suggesting a potential involvement of the cortex, striatum and cerebellum brain structures.

### 3-II.2. Introduction

The motor impairment component of RTT pathology constitutes the most incapacitating aspect for the patients. Initially, RTT patients present hypotonia, failure in crawling and lack of skills in fine finger movements. After this initial presentation, the patients lose the purposeful use of hands, display dystonia and stereotyped hand movements, and become hypertonic. Gait is not acquired by all patients, but those that have it present an ataxic, not goal-directed gait.

Knowledge on the first motor impairments and the precise moment they are manifested contribute to the identification of the first neural substrates to be affected by the absence of MeCP2 protein, which ultimately may be helpful in developing therapeutic approaches.

In the different mouse models of RTT, hypoactivity has been consistently described for both male and female mice (Chen et al. 2001; Guy et al. 2001; Shahbazian et al. 2002; Pelka et al. 2006). Additionally, a motor coordination impairment was also shown (Shahbazian et al. 2002; Gemelli et al. 2005; Pelka et al. 2006). From our own data in the study of the *Mecp2*<sup>tm1.1Bird</sup> mouse model of RTT (chapter 3, part I) we observed that the first subtle (some of them transient) motor problems became evident already during the

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early postnatal weeks, and potentially suggested an impairment of neural pathways in the brainstem, as well as the involvement of the cerebellum.

In order to further define the early motor impairment of the *Mecp2*<sup>tm1.1Bird</sup> mouse model we evaluated their performance in three different paradigms used to assess motor behaviour. The performance of *Mecp2*-null males and their respective wt littermate controls was compared in the footprint pattern, gait onset test and in the open field (OF) at two different timepoints: before the onset of overt symptoms (three weeks of age) and when symptomatic (eight weeks of age).

### 3-II.3. Material and Methods

#### Animals

We used young three-week-old, immediately after weaning (*Mecp2*-null, n=10 and wt, n=15), and eight-week old (*Mecp2*-null, n=5 and wt, n=13) *Mecp2*-null mice (*Mecp2*<sup>tm1.1Bird</sup> mouse model) and their respective wt littermate controls in this study. At weaning (PND21-23) mice were group housed in standard laboratory cages, filled with sawdust and cardboard rolls, in an animal facility with controlled temperature and kept in a 12 hour light: 12 hour dark cycle, with food and water *ad libitum*. All experiments were performed in accordance with the European Communities Council Directive, 86/609/EEC.

#### Behavioural testing

*Open field.* Animals were placed in the centre of an arena of 43.2x43.2 cm with transparent walls (MedAssociates Inc., St. Albans, Vermont) and their behaviour was observed for 5 min. The following activity parameters were collected: total distance travelled, resting time, the distance travelled and time spent in the predefined centre of the arena *versus* the rest of the arena. The number of rears and time spent exploring vertically was registered by observation. The time taken by each animal (1) to start walking and (2) to reach the wall of the arena was also registered, as a measure of latency to movement onset.

*Gait onset.* Animals were placed in the centre of a white circle (ø 13 cm), and the time, in seconds, taken to move out of the circle with the four paws was recorded (30 sec was the maximum duration of the test).

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*Footprinting pattern.* Fore- and hindpaws of *Mecp2*-null and wt littermate mice were painted with two different colours and the animals were guided to walk in a tunnel on the top of a white paper sheet. The pattern of three consecutive steps (the first four steps were excluded from the analysis) was analyzed and the following parameters assessed: stride length, uniformity of step alternation, forepaw and hindpaw base width (figure 3-II.3C).

### Statistical analysis

The behavioural tests data were analysed using Student's t-test (SPSS version 15.0). A critical value for significance of  $P < 0.05$  was used throughout the study.

## 3-II.4. Results

The behaviour of *Mecp2*-null and their wt littermate control males was studied at two timepoints: (1) after weaning, at three weeks of age, and (2) at eight weeks of age. Animals were first weighted and then their behaviour was evaluated in the OF apparatus, followed by assessment of gait onset and, finally, footprinting pattern.

The body weight of both the three- and eight-week-old *Mecp2*-null male mice was significantly lower than their littermate wt animals, as expected ( $p < 0.01$ ; table 3-II.1).

### Exploratory activity

At three weeks of age, *Mecp2*-null and wt mice were tested in an OF apparatus to evaluate their motor activity. No differences were found between *Mecp2*-null and wt mice in all the parameters assessed: the total distance travelled and the time they spent resting, the time spent exploring vertically or the number of rears (see figure 3-II.1 and table 3-II.1). Also, no differences were found between the two groups in the time spent and distance travelled in the centre of the arena relatively to the total area of the arena, parameters indicative of heightened anxiety (table 3-II.1). Given that some animals died before eight weeks of age the number of animals studied at this age was lower. Unexpectedly, at this age, differences in the motor activity were not observed. *Mecp2*-null and wt animals were also tested in the OF but showed no differences in the above referred parameters.

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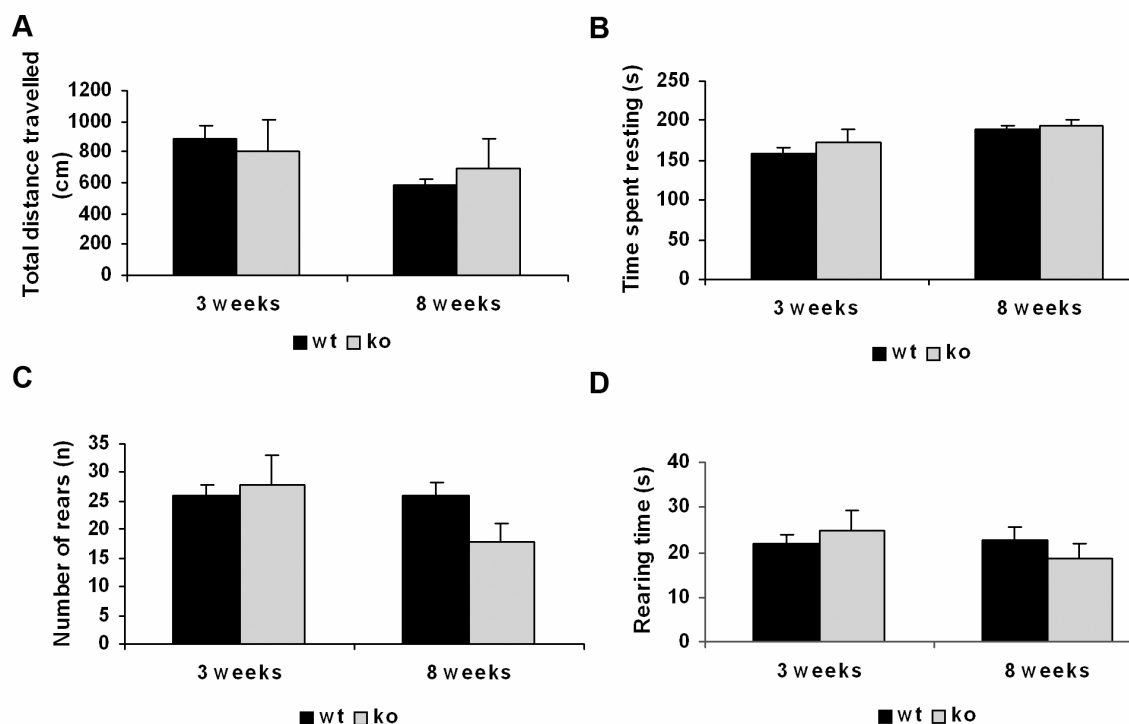


**Figure 3-II.1. *Mecp2*-null mice do not present exploratory or spontaneous motor activity deficits in the open field apparatus.** *Mecp2*-null mice did not present differences, neither at three nor at eight weeks of age, in the (a) total distance travelled, (b) time spent resting, (c) number of rears and (d) time spent rearing. (ko, *Mecp2*-null; wt, wild-type. Three-weeks-old group: ko, n=10; wt, n=15; eight-weeks-old group: ko, n=5; wt, n=13. Values represent mean + sem).

### Gait onset

In order to test whether *Mecp2*-null animals exhibited dyspraxia, we evaluated the time that animals took to initiate an action. For this purpose we analysed gait onset for both *Mecp2*-null and wt mice at three- and eight-weeks of age. Also, we used the OF apparatus to registered the time that mice took to (1) start walking after being placed in the centre of the arena, and (2) the time taken to get to the walls of the apparatus.

No statistical significant differences were observed between *Mecp2*-null and wt animals in the time they took to get off a circle (gait onset), the time they took to start moving and to get to the wall in the OF apparatus. However, a tendency for *Mecp2*-null animals to take more time to initiate these actions was observed at three weeks of age, which was even more notorious at eight weeks of age (table 3-II.1 and figure 3-II.2). The lack of statistical power is probably due to the great variability between the animals regarding this measure.



| Table 3-II.1.            |                |                 |              |                |                 |              |
|--------------------------|----------------|-----------------|--------------|----------------|-----------------|--------------|
| Measure                  | Three weeks    |                 |              | Eight weeks    |                 |              |
|                          | wt (n=15)      | ko (n=10)       | P-value      | wt (n=13)      | ko (n=5)        | P-value      |
| Body weight              | 6.64 ± 0.36    | 5.20 ± 0.30     | <b>0.009</b> | 22.24 ± 0.65   | 15.94 ± 1.69    | <b>0.001</b> |
| Total distance travelled | 887.32 ± 79.56 | 800.89 ± 211.76 | NS           | 592.76 ± 31.11 | 694.13 ± 196.77 | NS           |
| Time spent resting       | 159.25 ± 5.99  | 172.35 ± 16.46  | NS           | 189.48 ± 4.37  | 193.47 ± 7.30   | NS           |
| Ratio centre/total area  |                |                 |              |                |                 |              |
| time                     | 0.36 ± 0.02    | 0.28 ± 0.03     | NS           | 0.18 ± 0.02    | 0.21 ± 0.09     | NS           |
| distance                 | 1.29 ± 0.19    | 1.00 ± 0.42     | NS           | 0.19 ± 0.02    | 0.16 ± 0.04     | NS           |
| Time spent rearing       | 22.00 ± 1.72   | 24.70 ± 4.53    | NS           | 22.85 ± 2.66   | 18.40 ± 3.43    | NS           |
| Number rears             | 25.93 ± 1.97   | 28.00 ± 5.12    | NS           | 25.77 ± 2.28   | 17.80 ± 3.22    | NS           |
| <i>Bolus fecalis</i> (n) | 0.47 ± 0.16    | 0.40 ± 0.16     | NS           | 1.85 ± 0.32    | 3.80 ± 0.58     | <b>0.006</b> |
| Latency to:              |                |                 |              |                |                 |              |
| start movement           | 1.73 ± 0.38    | 1.00 ± 0.00     | NS           | 1.77 ± 0.53    | 12.60 ± 8.30    | NS           |
| get to the wall          | 4.13 ± 0.58    | 4.60 ± 1.12     | NS           | 3.46 ± 0.76    | 33.60 ± 26.68   | NS           |
| Gait onset               | 1.73 ± 0.34    | 4.50 ± 2.38     | NS           | 1.54 ± 0.54    | 9.0 ± 5.58      | NS           |

Legend: wt, wild type; ko, knock out; NS, no significant

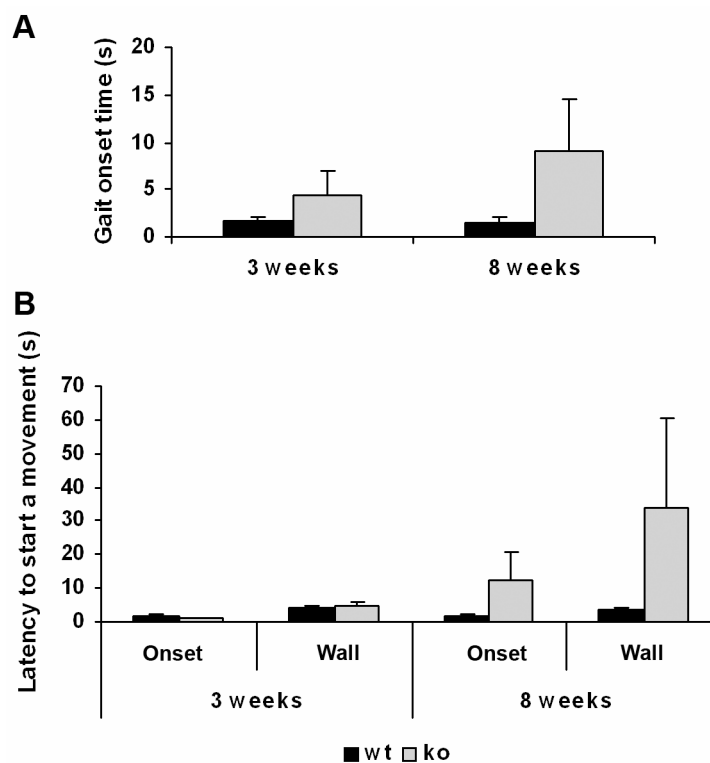


**Figure 3-II.2. Increased latency to start a movement exhibited by the *Mecp2*-null mice at three and eight weeks of age.** Although not reaching statistical significance, *Mecp2*-null mice, both at three and eight weeks of age, took more time (A) to move out of a circle in the gait onset test and (B) to start walking from the centre of the OF arena (onset) and then reach the walls of the apparatus (Wall). (wt, wild-type; ko, *Mecp2*-null. Three-week-old group: ko, n=10; wt, n=15; eight-week-old group: ko, n=5; wt, n=13. Values represent mean+sem).

### Gait pattern

The footprinting pattern of *Mecp2*-null and wt male mice was assessed at three and eight weeks of age. The discrepancy between the number of mice in the three-week-old group that we used to analyze footprint patterns and those used in the previous tests was due to the impossibility to obtain a valid footprint pattern for some animals (number of invalid assays not different between groups; wt, n=6 and *Mecp2*-null, n=5). At three weeks of age, *Mecp2*-null mice exhibited a larger front-base and a larger hind-base width than their wt littermate controls ( $p < 0.05$ ). At eight weeks of age, the difference in the front-base and hind-base width was maintained ( $p = 0.079$  and  $p < 0.05$ , respectively) (figure 3-II.3 and 3-II.4). Additionally, the stride length was significantly smaller in the *Mecp2*-null mice than in the wt animals ( $p < 0.05$ ), which could be due to the fact that *Mecp2*-null animals are smaller. In order to control for the differences in body size and because we did not measure the length of each animal, we introduced a correction in the analysis dividing the

stride length by the animal body weight. With this correction we no longer detected the difference first observed in the eight-week-old group, but we did detect a significant difference in this parameter at three weeks of age ( $p=0.028$ ) in the opposite sense (wt displaying a lower ratio), the meaning of which is unclear to us (figure 3-II.4). One possible explanation is that at three weeks of age the mutant animals do not differ from wt in length, but more so in weight, whereas at eight weeks both length and weight seem reduced. Thus, we may have introduced an overcorrection at three weeks. In summary, our data do not support a significant difference in stride length, but more so in the base width as usually present in ataxia.

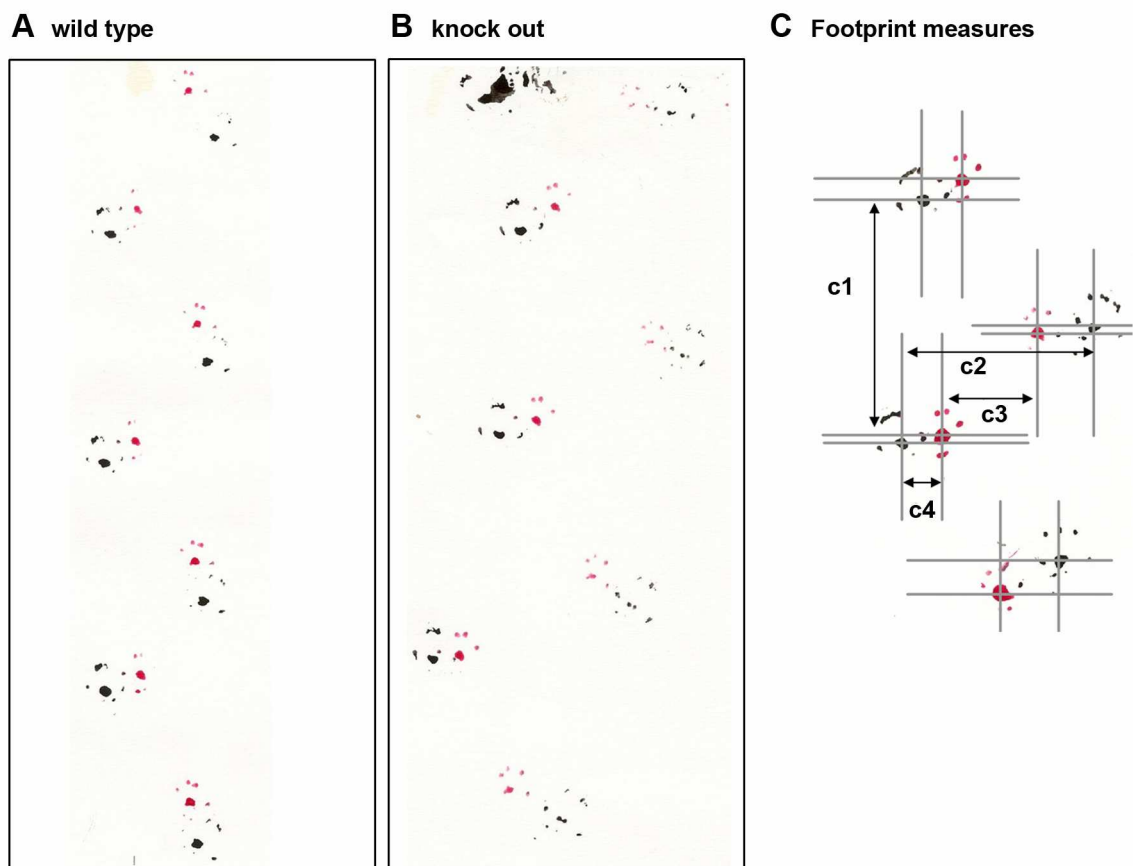


**Figure 3-II.3. Representative walking footprint patterns of three-week-old mice.** (A) wt and (B) *Mecp2*-null (ko) male mice. (C) Schematic representation of the footprint measures taken: (c1) stride length; (c2) hind-base width; (c3) front-base width and (c4) uniformity of step alternation.

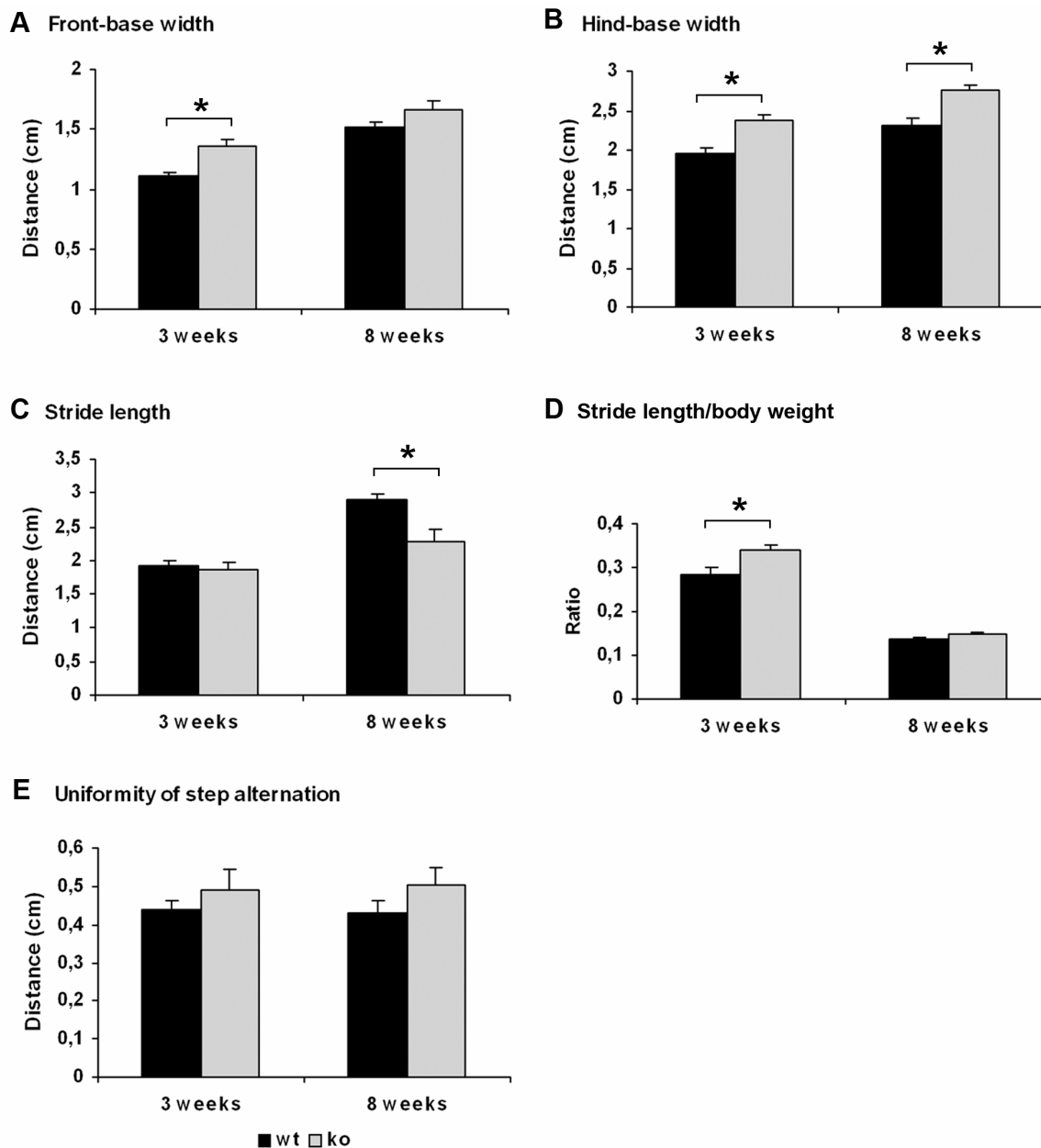


**Figure 3-II.4. Abnormal gait pattern exhibited by *Mecp2*-null and wt mice at three and eight weeks of age.** Quantitative analysis of the walking footprint patterns produced by wt and *Mecp2*-null mice at three and eight weeks of age showed a significant statistical difference between genotypes. Both groups of *Mecp2*-null mice presented a broader (A) front-base width and a broader (B) hind-base width as compared with controls; ko mice presented a significantly smaller relative to controls (C) stride length at eight weeks of age. At three weeks of age (D) the ratio stride length/body weight higher in the *Mecp2*-null mice than in wt controls. No differences were found for the (E) uniformity of step alternation. (ko, knock-out; wt, wild-type. Three-week-old group: ko, n=6; wt, n=8; eight-week-old: ko, n=5; wt, n=14. Values represent mean+sem; \* p<0.05).

### 3-II.5. Discussion

#### ***Mecp2*-null mice do not exhibit spontaneous motor and exploratory activity impairments at an early age.**

In this study, the assessment of *Mecp2*-null motor behaviour shows that (1) *Mecp2*-null mice presented an abnormal gait already at three weeks of age, with a higher front- and hind-base width and (2) there was a clear tendency of the *Mecp2*-null mice to exhibit a higher latency to start a purposeful movement.

Nevertheless, no differences were found in any of the parameters assessed in the OF apparatus at three weeks of age, which in accordance with the given “apparently” normal period of development of this model (Guy et al. 2001). Surprisingly no differences were also observed at eight weeks of age, when overt symptoms are already established and male *Mecp2*-null mice start to die (Guy et al. 2001). In fact, we and others (Guy et al. 2001; Santos et al. 2006) have already shown that at four weeks of age *Mecp2*-null mice do exhibit hypoactivity. Because the genetic background used in this study is the same as that of the original colony it is difficult to explain these results. Since a considerable number of *Mecp2*-null mice in our sample died before the age of eight weeks (likely the most affected ones) it is plausible that, in this study, the surviving male *Mecp2*-null mice that we analysed were a “selected” sample of those with better motor outcome.

#### ***Mecp2*-null mice exhibit a higher latency to start a movement**

Subtle deficits in the motor performance of *Mecp2*-null and wt mice were evident in other paradigms already at three weeks of age. The higher latency for gait onset (both in the gait onset test and in the OF paradigm, figure 3-II.3) exhibited by the *Mecp2*-null mice may reflect an incapacity of mutant animals to initiate a purposeful movement. In fact, this has already been observed by us when *Mecp2*-null male mice were tested in the wire suspension test and held in the wire for a significantly longer time than wt mice (Santos et al. 2006 and chapter 3, part I), a possible sign of dyspraxia, as observed in RTT patients (Kerr and Engerstrom 2001). The inability to initiate a voluntary motor response (akinesia) is also known to be one of the outcomes of basal ganglia dysfunction, particularly involving the caudate-putamen (Hauber 1998).

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***Mecp2*-null mice exhibit abnormal gait already at three weeks of age**

The results of the footprinting pattern analysis confirmed the gait disturbance observed in *Mecp2*-null mice. Already at three weeks of age, instead of walking in a straight line with evenly spaced and accurately positioned footprints, *Mecp2*-null mice presented a wider base (figure 3-II.4A-B). Again, the *Mecp2*-null mice replicated the gait disturbance features seen in RTT patients: rocking of the trunk side by side with wide based posture due to a failure of the interlimb coordination between the upper and the lower extremities (Segawa 2001). These results emphasize our previous observations of motor uncoordination of the *Mecp2*-null mice, at five weeks of age, which exhibited a worse performance in the rotarod apparatus (Santos et al. 2006) and chapter 3, part I). These motor deficits could be attributable to both striatal and/or cerebellar dysfunction.

Although, at three weeks of age, the *Mecp2*-null mice did not present deficits in motor execution (OF results) they already exhibited a delayed motor initiation and coordination. Several processes precede the movement onset and our data seems to indicate that the initial problem may not be in the execution of movement itself but instead on its planning. If this is the case, forebrain structures could potentially be as important as the brainstem, which is generally considered as the origin of the problem, in the initial establishment of the motor profile of RTT (Einspieler et al. 2005; Segawa 2005).

This possibility has been strengthened by the generation of a conditional *Mecp2*-null mouse model restricted to forebrain structures (prefrontal cortex, striatum, nucleus accumbens, hippocampus and amygdala) (Gemelli et al. 2005). These mice resemble in many aspects the RTT phenotype and, although they show, at around four months of age, a normal locomotor activity, they display impaired motor coordination, among other deficits.

In summary, in this work we characterized in more detail the motor behaviour of the *Mecp2*-null mice at an early age and we showed that this model replicates RTT motor components. The relevance of this study is even higher in terms of using this model to test the efficiency of therapies for RTT, the effect of which can be evaluated from very early stages.

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## CHAPTER 4

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### AGE- AND REGION-SPECIFIC DISTURBANCES OF MONOAMINERGIC SYSTEMS IN THE BRAIN OF *Mecp2*-NULL MICE

The results described in this chapter are included in the following manuscript (in preparation):

Mónica Santos, Teresa Summavielle, Sérgio Teixeira, Anabela Silva-Fernandes, Andreia Teixeira-Castro, Pedro Oliveira, Nuno Sousa and Patrícia Maciel. "Age- and region-specific disturbances of monoaminergic systems in the brain of *Mecp2*-null mice."





#### 4.1. Abstract

RTT is a pervasive disorder that affects a multitude of brain neural systems, resulting in breathing and sleep dysfunction, an autonomic dysfunction, a characteristic loss of locomotor abilities and a movement disorder including dystonia and stereotypies, as well as profound cognitive impairments. This wide involvement may suggest a dysfunction of the modulatory monoaminergic brain systems of the brain in RTT pathophysiology.

In fact, neurotransmitters such as norepinephrine, dopamine and serotonin have repeatedly, although not always consistently, been shown to be altered in the brain and cerebrospinal fluid of RTT patients. Furthermore, the *Mecp2*-null mice, an animal model of RTT, showed reduced levels of these neurotransmitters and its metabolites both in total brain extracts and in the medulla oblongata as compared to wt mice.

In order to clarify the contribution of monoamines to the different clinical components of the RTT phenotype, we performed a neurochemical study of different brain regions of the *Mecp2*-null<sup>tm1.1Bird</sup> mouse potentially playing a role in RTT-like pathophysiology, at two different timepoints: before and after the establishment of overt symptoms.

We found that the serotonergic and noradrenergic systems are affected in this model, with a reduction in the levels of the neurotransmitters and their metabolites, as well as a dysregulation of their degradation, already at three weeks of age. Additionally, we verified that the prefrontal and motor cortices were the primarily affected regions, whereas the hippocampus and cerebellum may play a role in later stages of the disorder.

#### 4.2. Introduction

Several lines of evidence indicate that a dysfunction of the monoaminergic systems may contribute to the neuropathology of RTT. This idea was first brought to light by Drs Nomura and Segawa who suggested, based on clinical and polysomnographic studies, that the primary lesion of RTT involved the raphe nuclei and the locus coeruleus (Nomura et al. 1987).

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RTT patients present an overall reduction in the size of the brain, but no other gross neuropathological abnormalities are seen, such as hypoplasia or ectopias (Armstrong 2005). This suggests that MeCP2 dysfunction does not disrupt the initial events of CNS development, neurogenesis and neuronal migration. The reduction in brain size appears to result mainly from a reduction of cortical thickness, which in turn corresponds to a markedly reduced neuronal size and increased cell packing density (Armstrong 2001). Post-mortem studies of RTT brains showed that in layers III and V of frontal, motor and inferior temporal cortices, the dendritic arborisation pattern of pyramidal neurons was simplified (Armstrong 1995). Additionally, the number of dendritic spines and the synaptic density were also decreased in the frontal lobe (reviewed in (Armstrong 2002; Armstrong 2005). These results point to a role of MeCP2 in the maturation of the neuronal circuitry. Consistently, the pattern of expression of MeCP2 in the CNS is coincident with the beginning of maturation and differentiation of cortical cells in the embryo (Meehan et al. 1992; LaSalle et al. 2001; Shahbazian et al. 2002; Balmer et al. 2003; Cohen et al. 2003; Jung et al. 2003; Cassel et al. 2004; Kishi and Macklis 2004; Samaco et al. 2004).

Neuromorphological studies in *Mecp2*-null mice have also shown that the projection layers of the neocortex are thinner and that pyramidal neurons are smaller and less complex than those in wt mice, although with no differences in the density of spines (Kishi and Macklis 2004). Electrophysiology studies in *Mecp2*-null mice revealed synaptic deficits (altered LTP and LTD) in the hippocampus of symptomatic, but not asymptomatic, mutants (Asaka et al. 2006). Moretti and colleagues (2006), using as a RTT model a transgenic mouse carrying an hypomorphic allele of the human *MECP2* gene, also reported, by electrophysiology studies, synaptic deficits in the *Mecp2*<sup>308X/Y</sup> mutant, both in the hippocampus and the neocortex, but failed to detect any corresponding abnormalities in the neuronal morphology or in the dendritic arborisation of pyramidal neurons in the frontal cortex.

Previous studies in RTT post mortem brains and CSF have revealed alterations in neurotransmitter levels of biogenic amines and of amino acids. Researchers have claimed reduced levels of the metabolites of norepinephrine (NE), dopamine (DA) and serotonin (5-HT) in the CSF of RTT patients as compared to controls (Zoghbi et al. 1985; Percy et al. 1987; Zoghbi et al. 1989; Ramaekers et al. 2003; Ormazabal et al. 2005), but others did not achieve the same results (Perry et al. 1988; Lekman et al. 1990). In post mortem RTT brain studies, some researchers reported reduced levels of NE, DA, 5-HT and their

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metabolites (Lekman et al. 1989), but again other researchers did not reproduce these results (Wenk and Mobley 1996) (summary in table 1.4, chapter 1). The excitatory glutamatergic and the inhibitory GABAergic transmissions appeared elevated in the brains of deceased RTT patients during the first decade of life and then reduced, when compared to controls (Lappalainen and Riikonen 1996; Blue et al. 1999a; Blue et al. 1999b). However, Perry and colleagues (1988) did not find any differences in the levels of amino acids in RTT patients' CSF.

Overall, the neurochemistry results are conflicting and it is difficult to draw a clear conclusion from the human data due to the fact that only a small number of cases were studied, at different ages, and thus different stages of the disease. However, the limitations of the post-mortem studies, as well as the limitation of extrapolating the CSF data to the brain, cannot be ignored when interpreting the results of these studies.

With the appearance of animal models of the RTT, new possibilities of study arose. A neurochemical study has also been performed in the total brain of *Mecp2*<sup>tm1.1Bird</sup> hemizygous males and their wt littermates. Data revealed that the concentration of the biogenic amines NE, DA and 5-HT in *Mecp2* hemizygous males was lower than in wt animals and the differences were stronger with increasing age (Ide et al. 2005). Reduced NE levels in *Mecp2*-null in comparison to wt animals reached statistical significance at PND 28, which was achieved for DA and 5-HT only at PND 42. In another study using this RTT mouse model, Viemari and colleagues (2005) mapped the breathing disturbances presented by the *Mecp2*-null animals to a deficiency in the noradrenergic and serotonergic modulation of the medullary respiratory circuitry. At 2 months of age *Mecp2*-null mice presented deficits in NE and 5-HT levels in the medulla, but not in the pons or forebrain. NE levels were already significantly reduced at one month of age, before the establishment of the breathing dysfunction.

Given the wide clinical presentation of RTT, with dysfunction of multiple body systems, in addition to the neuropathological data and the, although inconclusive, neurochemical data, it appears wise to think of a general dysregulation of the modulatory monoaminergic systems of the brain.

In order to clarify the role of neuromodulator monoaminergic systems in the establishment of the RTT pathology, we measured, by high performance liquid

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chromatography with electrochemical detection (HPLC/EC), the levels of NE, DA, 5-HT and their metabolites homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA) in several brain areas (prefrontal cortex, motor cortex, caudate-putamen, hippocampus, dorsal/medial raphe nuclei, ventral mesencephalon (substantia nigra + ventral tegmental area), vestibular area and cerebellum) of the *Mecp2*-null (male) mice and their respective wt littermate controls at two different timepoints: before the onset of overt symptoms (three weeks of age) and when symptomatic (eight weeks of age). Our results revealed significant age- and region-dependent impairments in the neuromodulatory neurotransmitters systems that correlate with the phenotype displayed by these mice.

### 4.3. Material and Methods

#### Animals

*Mecp2*<sup>tm1.1Bird</sup> mice were purchased from Jackson Laboratory. The colony was maintained by crossing heterozygous *Mecp2*<sup>tm1.1Bird</sup> females with wt C57Bl/6 males. Around PND21-23 pups were weaned and group housed (three to five animals) by sex. At weaning animals were individually tagged and the tip of their tails cut for posterior DNA extraction and genotyping. Animals were maintained in an animal facility with controlled temperature, in a 12 hour light: 12 hour dark cycle and with food and water *ad libitum*. DNA was extracted from tail tips using the Puregene DNA isolation kit (Gentra, Minneapolis, MN). Genotype was determined by polymerase chain reaction according to protocol provided by the Jackson Laboratory for this strain.

#### Neurochemical determinations by HPLC-EC system

Male *Mecp2*-null and their wt littermate controls were sacrificed by decapitation at three and eight weeks of age and their brains were rapidly removed and snap frozen in isopentane cooled in liquid nitrogen. Brains were kept at -80 °C until neurochemical determinations.

Brains were dissected on ice with the help of a 2X magnifying lens, following orientation marks provided by stereotaxic brain atlas (Paxinos and Franklin 2001). Eight brain areas were dissected (figure 4.1): prefrontal cortex (PFCx), motor cortex (MCx), caudate-putamen (CPu), hippocampus, ventral mesencephalon (SN-VTA, substantia

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nigra + ventral tegmental area), dorsal and medial raphe nuclei (D/MRN), vestibular area, and cerebellum. Once dissected, the tissue was kept in 150  $\mu$ l 0.2N perchloric acid and stored at -80°C. On the day before the neurochemical determination, samples were passed to -20°C. On the day of the analysis samples were defrosted on ice and sonicated for 2 min. After, samples were centrifuged at 5000 rpm for 3 min at 4°C, the supernatant was filtered through a 0.22  $\mu$ m SpinX HPLC columns (Costar) and centrifuged at 10000 rpm for 5 min at 4°C. 50  $\mu$ l of the filtrated solution were analyzed by the HPLC-EC system, using a mobile phase of 0.7 M aqueous potassium phosphate (monobasic) (pH 3.0) in 10% methanol, 1-heptanesulfonic acid (222 mg/L) and Na-EDTA (40 mg/L), (Gilson Inc., Middleton, WI, USA), fitted with an analytical column (Supelco Supelcosil LC-18 3  $\mu$ m, 7.5 cm x 4.6mm, Supelco, Bellefonte, PA, USA) (flow rate: 1.0-1.5 ml/min) for NE, DA, DOPAC, HVA, 5-HT and 5-HIAA. Pellets were stored at -20°C for posterior total protein quantification.

Known amounts of standard: NE, DA, 5-HT, DOPAC, HVA and 5-HIAA (purchased from Sigma, St.Louis, MO) were used to generate calibration curves to determine the concentration of each neurotransmitter and metabolite in our sample. Data were normalized to total protein concentration.

### **Total protein determination**

Fifty microliters of phosphate buffer 0.2 M were added to the pellet of tissue (100  $\mu$ l in the case of the pellet of cerebellum) and the samples were sonicated for 3 min and centrifuged at 3000 rpm for 5 min, at room temperature. BSA was used as a standard protein (0.01 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL, 0.4 mg/mL, 0.5 mg/mL).

Ten microliters of sample or BSA standard in duplicate were loaded into a microplate and added 200  $\mu$ L of 1:5 diluted Protein assay dye reagent (BioRad) to each well. After 5 min incubation at room temperature, absorbance was read at 595 nm (SUNRISE, TECAN).

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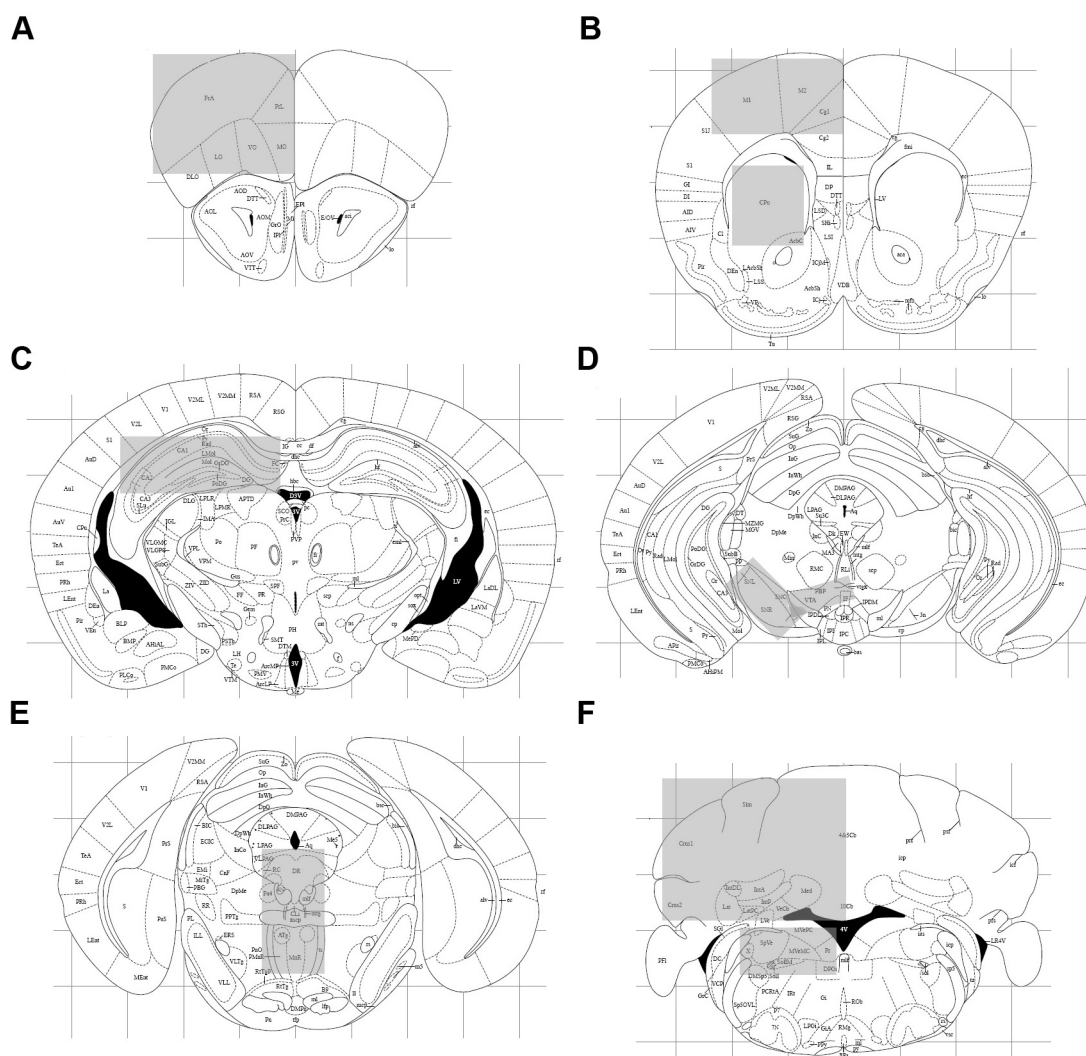


**Figure 4.1. Schematic representation of the brain areas dissected for further HPLC analysis.**  
(adapted from (Paxinos and Franklin 2001)).

## Immunohistochemistry

In order to assess the serotonergic innervation in the PFCx and MCx of three-week-old *Mecp2*-null and their wt littermate controls, mice (n=5 for each genotype) were anesthetized (ketamine/medetomidine) and intracardially perfused with phosphate buffered saline (PBS, pH 7.6) and 4% paraformaldehyde (PFA, pH 7.6). Brains were removed and kept in 4% PFA overnight, at 4°C, and then placed in a 30% saccharose solution for at least 24 hours, at 4°C. Brains were coronally sliced at 40 µm using a vibratome (Leica VT 1000S) and free-floating sections were collected in PBS.

Forty µm free-floating coronal brain sections were treated with 0.3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in PBS to eliminate endogenous peroxidase activity and blocked with 0.4% BSA in PBS/0.3% Triton X-100 (PBS/T) for 1 hour. Sections were then incubated in rabbit 5-HT primary antibody (1:5000) (kindly provided by Professor John Parnavelas from the University College of London, United Kingdom) for 48 hours at 4°C. Antigen visualization was performed using a universal detection system (BioGenex, San Ramon, CA, USA) and diaminobenzidine (DAB: 0.025% and 0.5% H<sub>2</sub>O<sub>2</sub> in Tris-HCl 0.05M, pH 7.2). Sections were mounted on Superfrost slides and lightly counterstained with hematoxylin.

## Stereological analysis

Every 6<sup>th</sup> section was used in the analysis of 5-HT innervation density. Following orientation marks provided by (Paxinos and Franklin 2001) PFCx and MCx areas were drawn using the StereoInvestigator software (Microbrightfield, VT) and a camera (DXC-390, Sony, Japan) attached to a motorized microscope (Axioplan 2, Carl Zeiss, Germany). The density of 5-HT fibres in these two areas was estimated using the L-cycloid optical fractionator software. The total number of intersections of the cycloid arcs with the stained fibers was obtained on randomly selected probes (parameters: grid size: 300 x 300 µm, cycloid width: 10 µm).

## mRNA expression levels

*Mecp2*-null (3 weeks: n=7, 8 weeks: n=5) and their wt littermate control (3 weeks: n=6, 8 weeks: n=7) mice were sacrificed by decapitation and their brains removed. Dissection of PFCx and MCx brain areas was performed and the tissue was stored at -80°C. Total RNA was extracted from the PFCx and MCx using the TRIzol reagent

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(Invitrogen, Carlsbad, CA, USA) and quantified in a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Two  $\mu\text{g}$  of RNA were reverse transcribed, using the Superscript<sup>TM</sup> First-Strand Synthesis System for RT-PCR (Invitrogen, CA, USA).

The expression levels of mRNA transcripts for noradrenergic receptors (*Adra2a* and *Adrb2*), serotonergic receptors (*Htr1a*, *Htr2a*, *Htr2b* and *Htr3a*) and the NE and 5-HT transporters (NET (*Slc6a2*) and *SERT* (*Slc6a4*), respectively) were measured in the above referred brain areas by qRT-PCR. The reference gene, hypoxanthine guanine phosphoribosyl transferase (*Hprt*), was used as internal standard for normalization. The real-time PCR reactions, using equal amounts of total RNA from each sample, were performed on a LightCycler instrument (Roche Diagnostics, Basel, Switzerland) using QuantiTect SYBR Green RT-PCR reagent kit (Qiagen, Hamburg, Germany). Product fluorescence was detected at the end of the elongation cycle. All melting curves exhibited a single sharp peak at a temperature characteristic of the primer used (see supplementary table S4.1, in appendix I, for primer sequences and annealing temperatures for each gene).

### Statistical analysis

The levels of monoamine neurotransmitters (NE, 5-HT and DA) and of its metabolites (DOPAC, HVA and 5-HIAA) were compared between *Mecp2*-null and wt mice in eight different brain areas (PFCx, MCx, CPu, Hippocampus, SN-VTA, D/MRN, vestibular area and cerebellum), at two different timepoints, by 2-way ANOVA (age x genotype). Due to problems of the data in achieving the assumptions required for ANOVA, such as homogeneity of variances, the data was transformed in its Ln values and homogeneity of variances was achieved in most of the data. Interaction between the independent variables (age and genotype) was also studied and reported when it was observed.

Serotonergic innervation data was compared between both genotypes by a Student's t-test.

Expression levels of the different NE and 5-HT receptors and its transporters, obtained by qRT-PCR, between groups was performed using the 2-way ANOVA, as described above.

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The results are represented graphically as mean + standard error mean (sem). Differences were considered to be statistically significant when P-value <0.05.

## 4.4 Results

### Neurotransmitter and metabolite analyses by HPLC-EC

We used HPLC/EC to measure the levels of monoamine neurotransmitters and their metabolites (figure 4.2) in the PFCx, MCx, CPu, Hippocampus, SN-VTA, D/MRN, vestibular area and cerebellum of male *Mecp2*-null and wt littermate controls, at three and eight weeks of age. These different brain areas were selected based on a possible involvement in the pathology of RTT; particularly, we have focused on areas mediating learning, exploratory behaviour and motor activity.

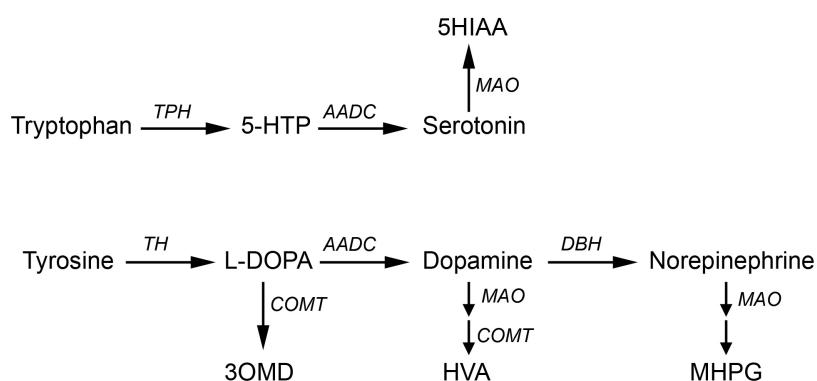


**Figure 4.2. Metabolic pathway of monoaminergic and serotonergic neurotransmitters.** (adapted from Neuroscience: Exploring the Brain)

We found significant age- and region-specific differences in the levels of the monoamine neurotransmitters and their metabolites between *Mecp2*-null mice and their wt littermates. Overall, the ontogenic profile of neurotransmitter and metabolites levels as well as their metabolism were parallel between the two genotypes, unless otherwise stated in the text.

In the PFCx, differences in the levels of NE, 5-HT and its metabolite 5-HIAA, but not of DA or its metabolite HVA, between *Mecp2*-null and wt males were found, both at three and eight weeks of age (figure 4.3A; NE:  $F_{3,29}=18.54$ ,  $p=0.000$ ; 5-HT:  $F_{3,29}=21.66$ ,  $p=0.000$ ; 5-HIAA:  $F_{3,29}=5.95$ ,  $p=0.021$ ). *Mecp2*-null mice presented a decrease of NE (31%), 5-HT (36%) and 5-HIAA (17%) levels at three weeks of age, which was even more

marked at eight weeks of age; NE (51%), 5-HT (47%) and 5-HIAA (34%). The ontogenic profile of DOPAC levels differed between *Mecp2*-null and wt mice through age (as given by an interaction age x genotype, DOPAC:  $F_{3,27}=7.67$ ;  $p=0.010$ ); we observed an increase in the DOPAC levels from three to eight weeks of age in the wt group that was not accompanied by the *Mecp2*-null mice group, that showed a decrease of DOPAC levels (figure 4.3A).

The 5-HT turnover was assessed by the ratio of 5-HIAA to 5-HT, which was significantly increased in this region in the *Mecp2*-null as compared to wt mice, both at three and eight weeks of age (figure 4.3B;  $F_{3,29}=5.80$ ,  $p=0.023$ ). The DA turnover was also analyzed, as given by the ratios of DOPAC+HVA to DA and of each one of its metabolites to DA (DOPAC/DA and HVA/DA) between the *Mecp2*-null and wt controls and no differences were found (figure 4.3B).

In the MCx, comparisons revealed that the levels of NE and 5-HT were decreased, in the *Mecp2*-null mice, at both three (26% and 32%, respectively) and eight (33% and 28%, respectively) weeks of age, as compared to wt mice (figure 4.4A; NE:  $F_{3,27}=7.26$ ,  $p=0.012$ ; 5-HT:  $F_{3,27}=6.45$ ,  $p=0.017$ ). No differences were detected in DA or its metabolites, and no differences were detected in the 5-HIAA levels between genotypes.

The turnover of 5-HT showed an increase in the MCx in the *Mecp2*-null as compared to wt mice (figure 4.4B;  $F_{3,27}=18.73$ ,  $p=0.000$ ). No differences were detected between genotypes in the metabolism of DA in this brain region.

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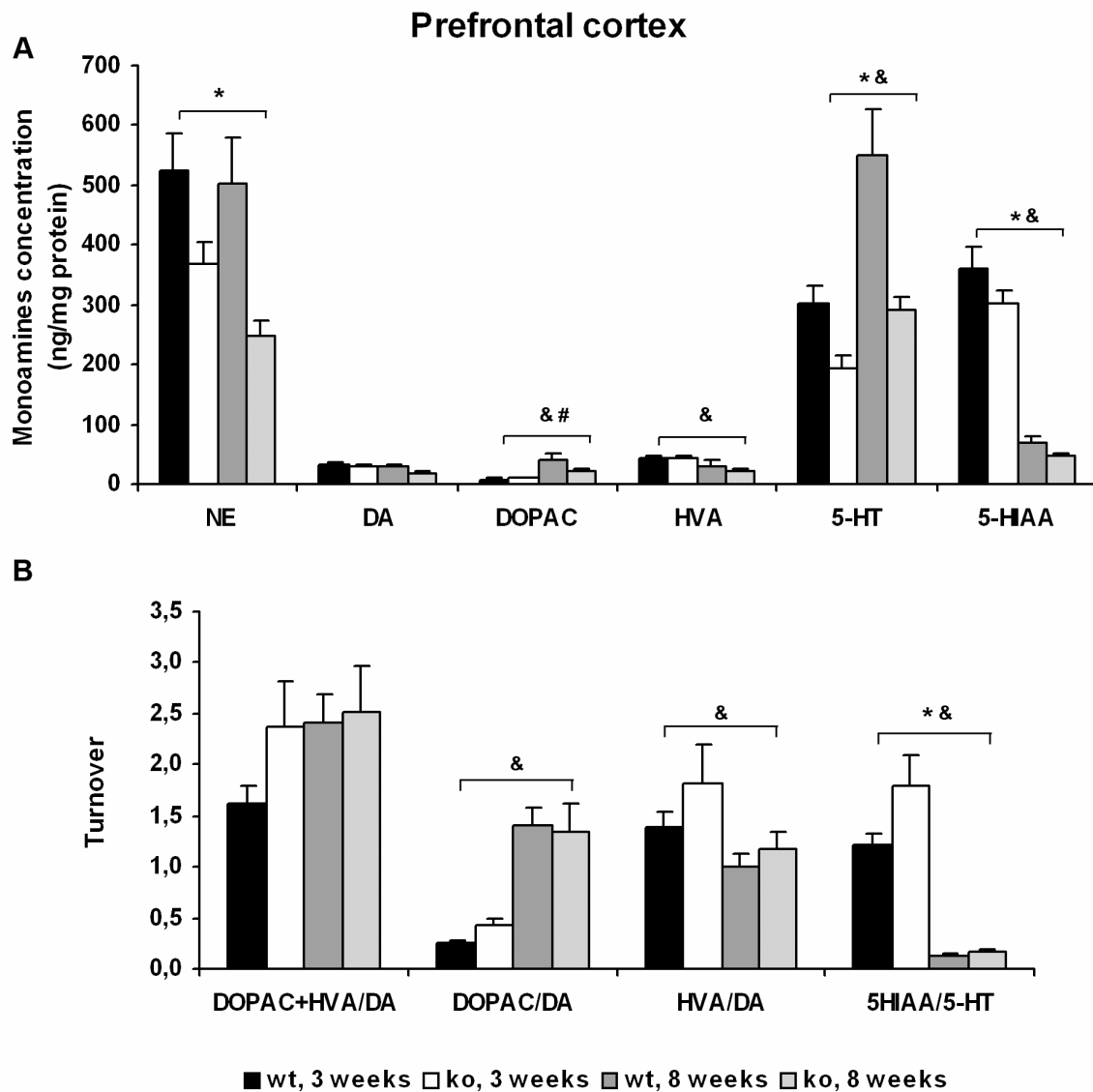


**Figure 4.3. Neurochemical analysis of the prefrontal cortex region of wt and *Mecp2*-null mice at three and eight weeks of age.** (A) Concentration of each neurotransmitter and metabolite and (B) neurotransmitter (DA and 5-HT) turnover. (wt, wild type; ko, *Mecp2*-null. All values are mean + sem. \*, genotype effect; &, age effect and #, age x genotype interaction; all  $P < 0.05$ ).

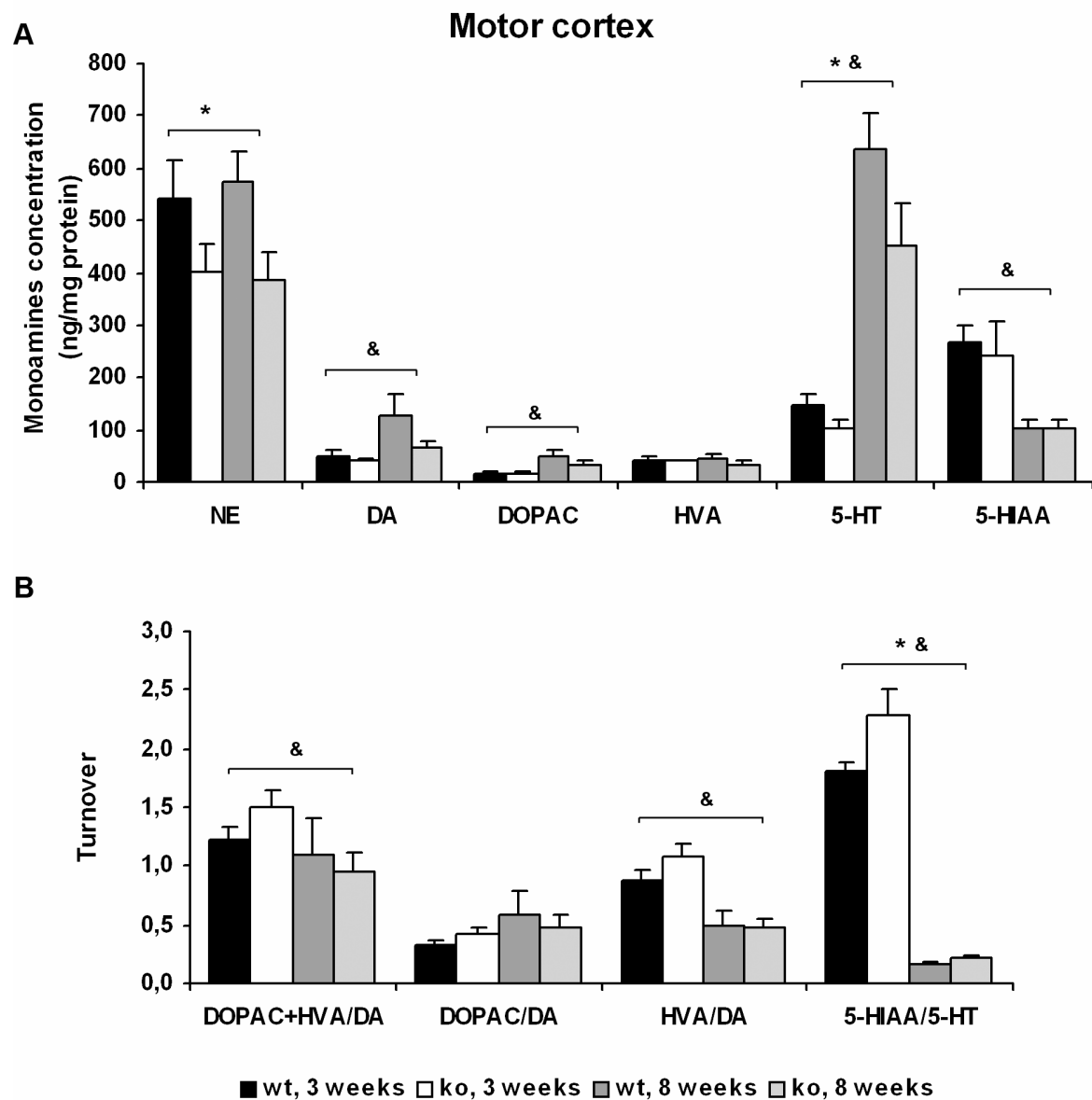


**Figure 4.4. Neurochemical analysis of the motor cortex region of wt and *Mecp2*-null mice at three and eight weeks of age.** (A) Concentration of each neurotransmitter and metabolites and (B) neurotransmitter (DA and 5-HT) turnover. (wt, wild type; ko, *Mecp2*-null. All values are mean+sem. \* genotype effect, & age effect; all  $P < 0.05$ ).

In the CPu, *Mecp2*-null mice showed levels of HVA and 5-HIAA lower than the wt levels, both at three (34% and 27%, respectively) and at eight weeks of age, when it was even more notorious (45% and 37%, respectively) (figure 4.5A; HVA:  $F_{3,29}=8.99$ ,  $p=0.006$ ; 5-HIAA:  $F_{3,29}=7.09$ ,  $p=0.013$ ). No other differences were noticed in the levels neurotransmitters or its metabolites.

The decrease observed in the DOPAC+HVA/DA, HVA/DA and 5-HIAA/5-HT ratios in the *Mecp2*-null as compared to wt mice showed that the metabolism of both DA and 5-HT was altered (figure 4.5B; DOPAC+HVA/DA:  $F_{3,29}=7.54$ ,  $p=0.010$ ; HVA/DA:  $F_{3,29}=16.66$ ,  $p=0.000$ ; 5-HIAA/5-HT:  $F_{3,29}=4.41$ ,  $p=0.044$ ) in the CPu.

In the hippocampus, no significantly different levels of the neurotransmitters or their metabolites were observed between *Mecp2*-null and wt mice (figure 4.6A). However, at eight weeks of age *Mecp2*-null mice showed a decrease, although not statistically significant, in the levels of NE and 5-HT (42% and 41%, respectively) as compared to wt. Interestingly, the ontogenic profile of HVA and 5-HT in this region was significantly different between wt and *Mecp2*-null mice, which evolved differently from three to eight weeks of age (figure 4.6A; HVA:  $F_{3,23}=7.989$ ,  $p=0.010$ ; 5-HT:  $F_{3,26}=7.74$ ,  $p=0.031$ ).

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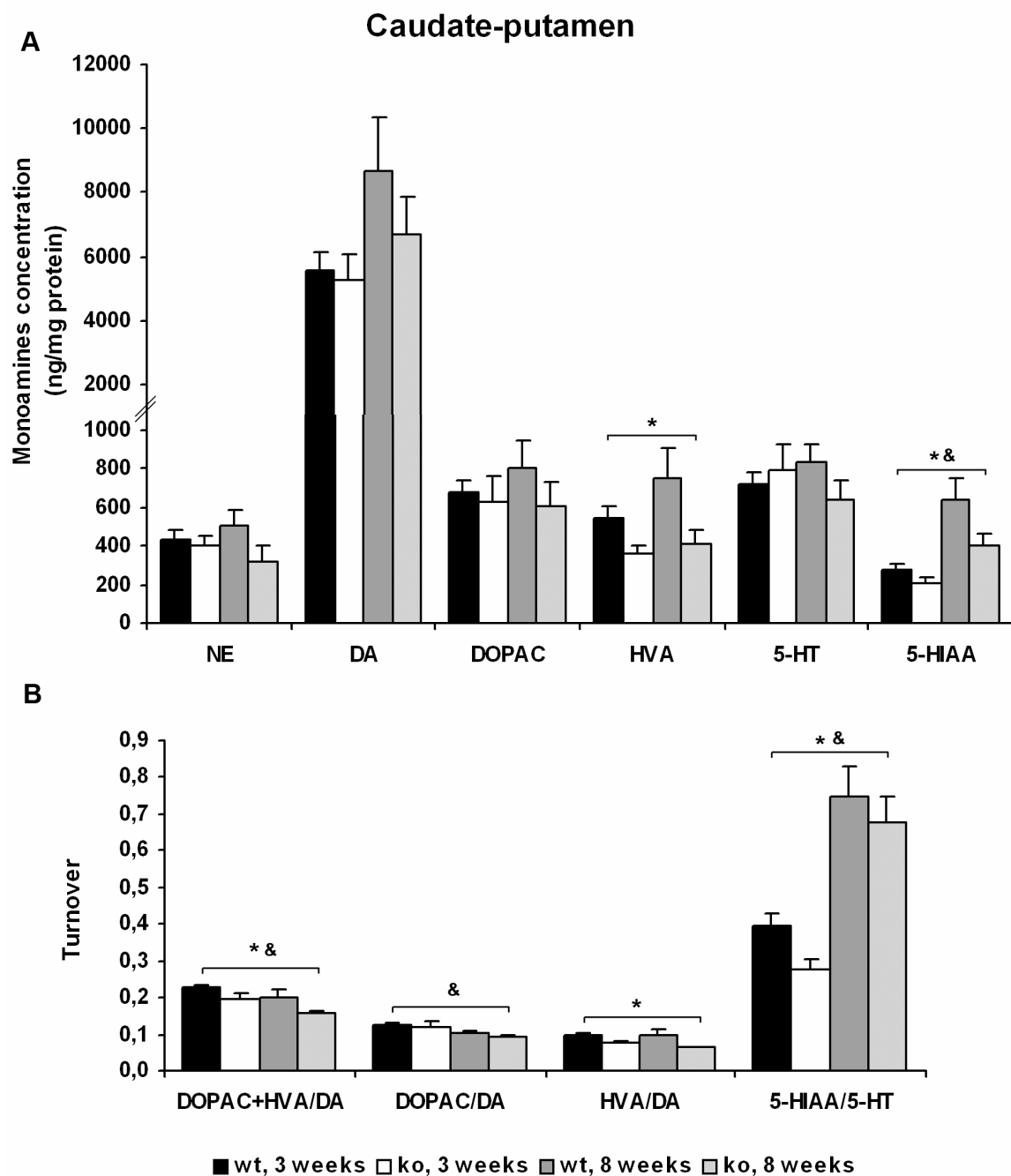


**Figure 4.5. Neurochemical analysis of the caudate-putamen region of wt and *Mecp2*-null mice at three and eight weeks of age.** (A) Concentration of each neurotransmitter and metabolites and (B) neurotransmitter (DA and 5-HT) turnover. (wt, wild type; ko, *Mecp2*-null. All values are mean+sem. \* genotype effect, & age effect; all  $P < 0.05$ ).

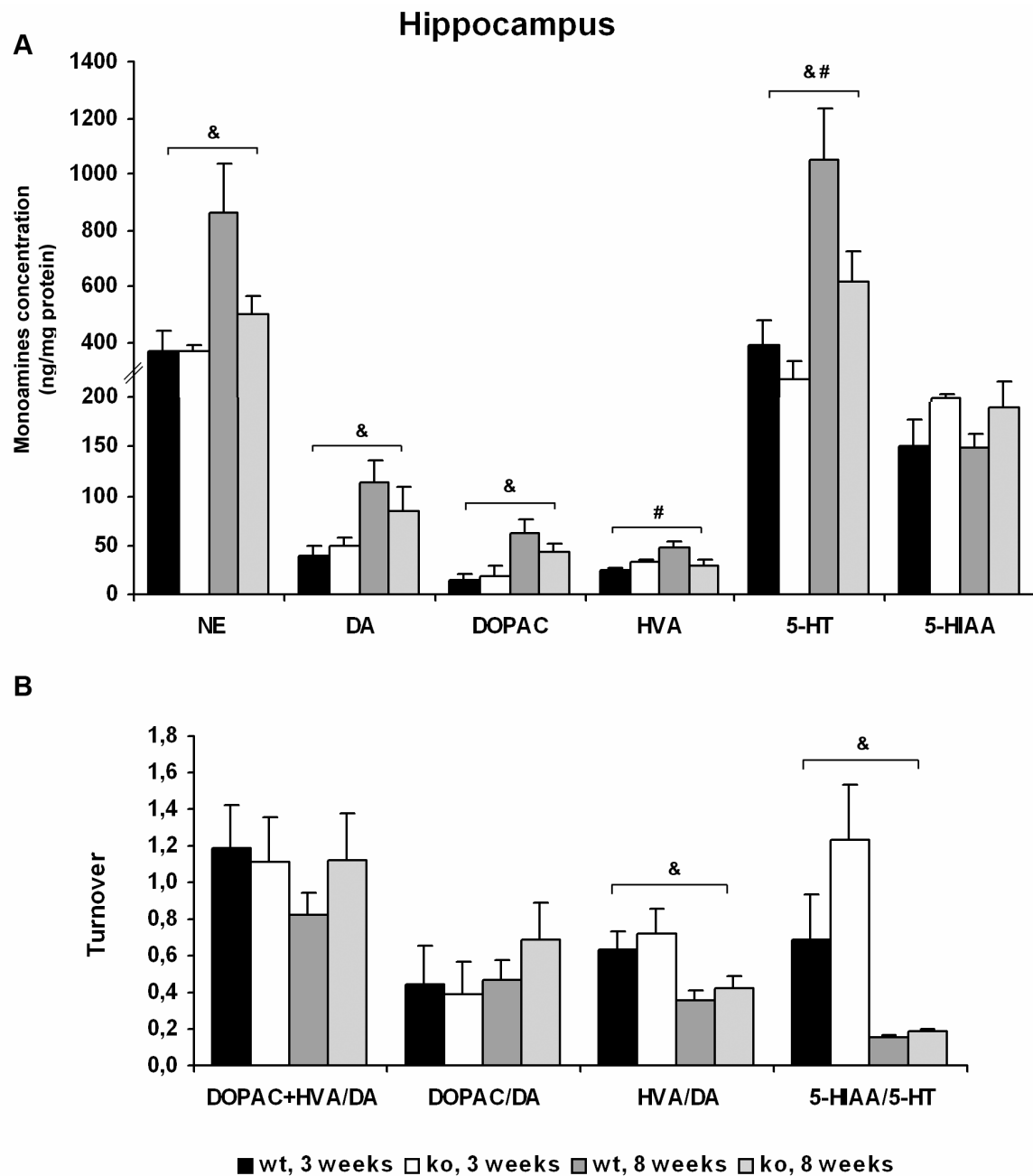


**Figure 4.6. Neurochemical analysis of the hippocampus region of wt and *Mecp2*-null mice at three and eight weeks of age.** (A) Concentration of each neurotransmitter and metabolites and (B) neurotransmitter (DA and 5-HT) turnover. (wt, wild type; ko, *Mecp2*-null. All values are mean+sem. \* genotype effect and & age effect; all  $P < 0.05$ .)

The nuclei of dopaminergic neurons are localized in the midbrain, arranged in the substantia nigra and ventral tegmental areas, here dissected as ventral mesencephalon. Interestingly, the levels of DA, its metabolites (DOPAC and HVA) and the DA turnover in the area of origin (SN-VTA) did not differ between *Mecp2*-null and wt male mice. Also, no other differences were found in the levels of the other neurotransmitters or metabolites between genotypes (figure 4.7A).

The only significant difference that we found between *Mecp2*-null and wt mice in this brain region was in the 5-HIAA/5-HT ratio, that was decreased in the *Mecp2*-null relative to wt mice (figure 4.7B;  $F_{3,22}=4.83$ ,  $p=0.039$ ).

The D/MRN is the region of origin of the serotonin-producing cell bodies. These neurons project their axons to virtually all brain region, including the frontal regions of the brain. No differences at all were found in this brain region in the levels of monoamines and their metabolites or their turnover (figure 4.8A-B).

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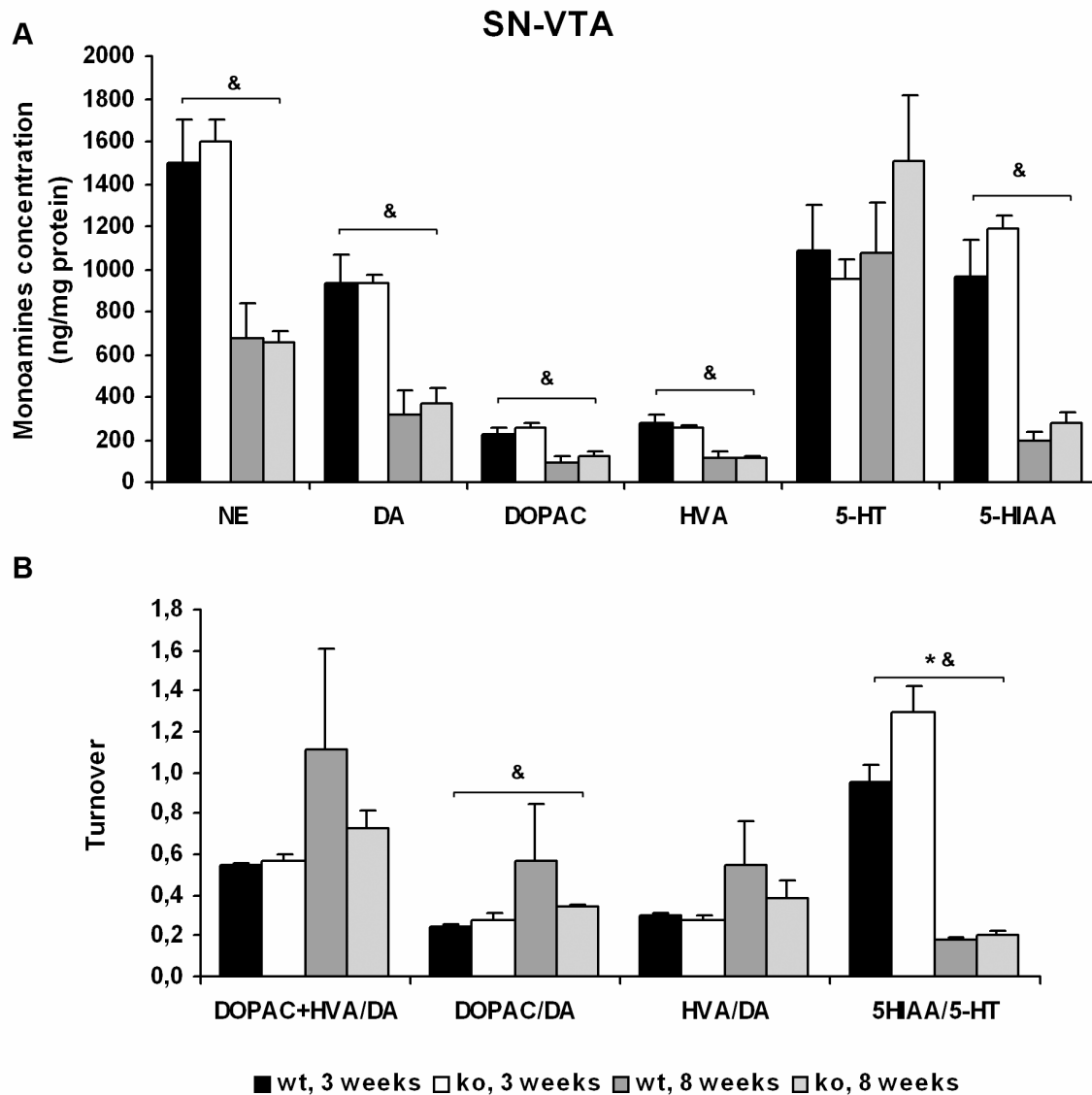


**Figure 4.7. Neurochemical analysis of the ventral mesencephalon region of wt and *Mecp2*-null mice at three and eight weeks of age.** (A) Concentration of each neurotransmitter and metabolites and (B) neurotransmitter (DA and 5-HT) turnover. (wt, wild type; ko, *Mecp2*-null. All values are mean+sem. \* genotypic effect and & age effect; all  $P < 0.05$ ).

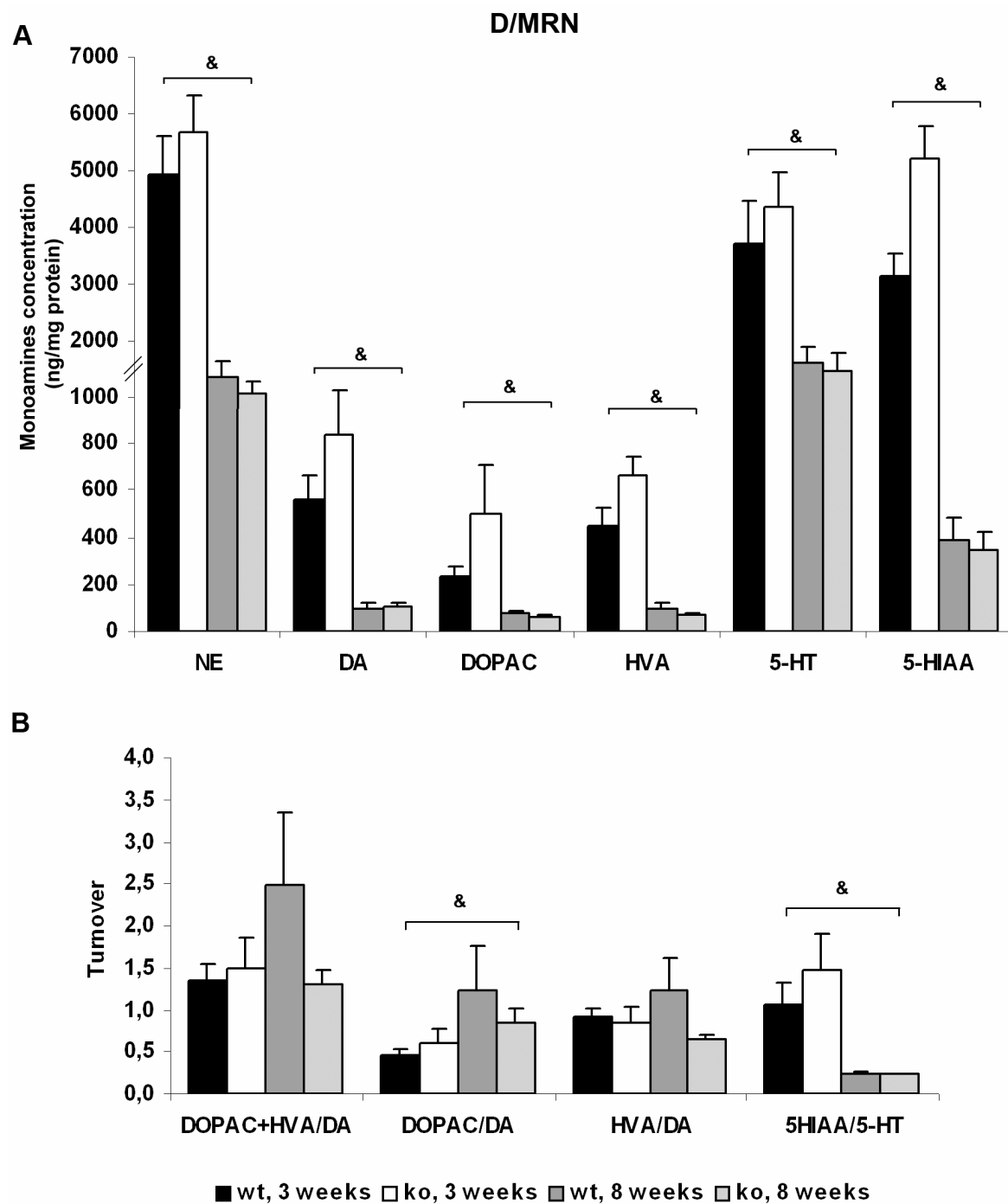


**Figure 4.8 Neurochemical analysis of the D/MRN region of wt and *Mecp2*-null mice at three and eight weeks of age.** (A) Concentration of each neurotransmitter and metabolites and (B) neurotransmitter (DA and 5-HT) turnover. (wt, wild type; ko, *Mecp2*-null. All values are mean+sem. \* genotype effect and & age effect; all  $P < 0.05$ ).

In the vestibular area, the only difference found between *Mecp2*-null and wt mice was a reduction of 36% in the levels of NE in the *Mecp2*-null as compared to wt mice, already from three and which was maintained at eight weeks of age (figure 4.9A;  $F_{3,23}=7.41$ ,  $p=0.012$ ).

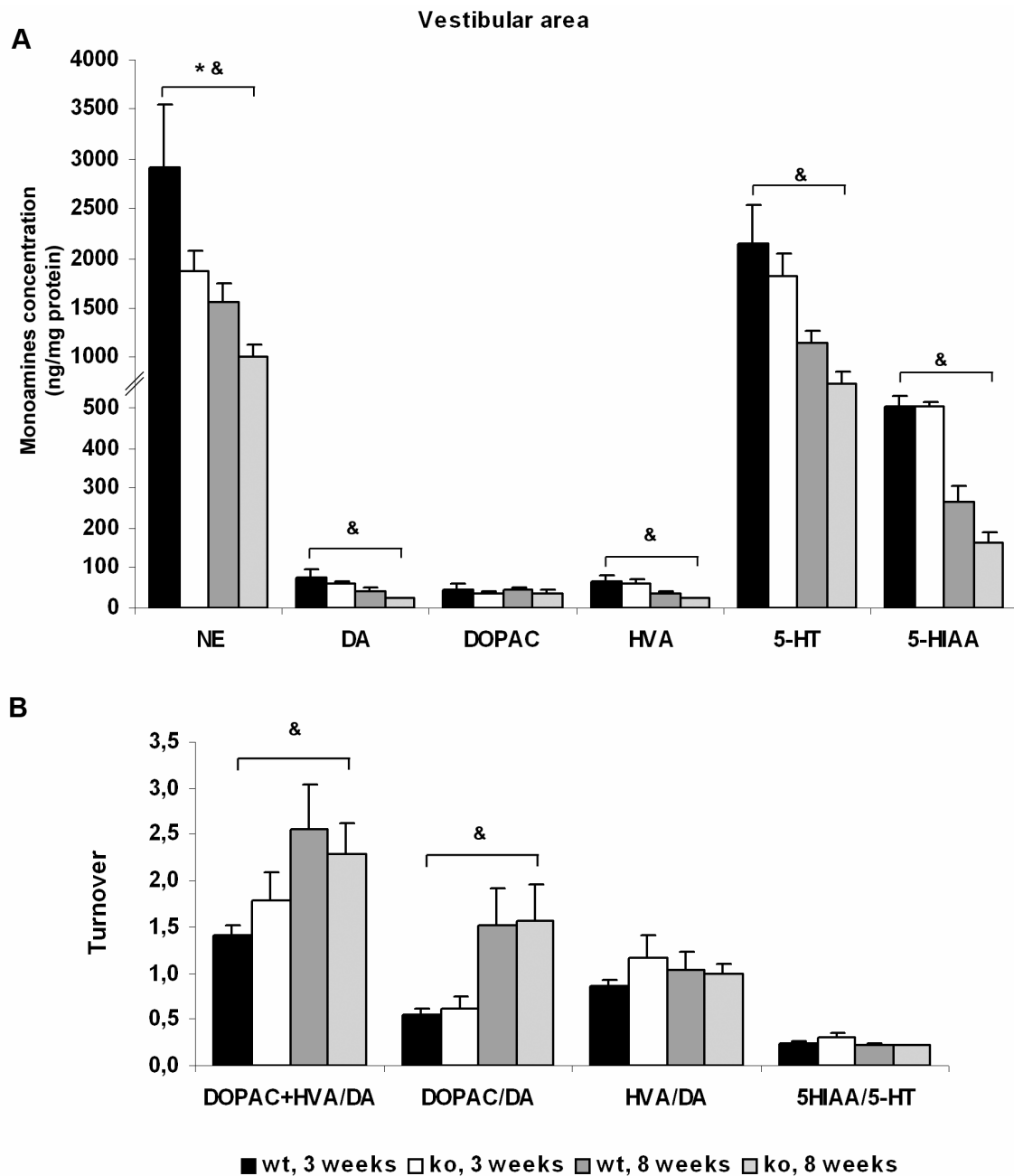


**Figure 4.9. Neurochemical analysis of the vestibular region of wt and *Mecp2*-null mice at three and eight weeks of age.** (A) Concentration of each neurotransmitter and metabolites and (B) neurotransmitter (DA and 5-HT) turnover. (wt, wild type; ko, *Mecp2*-null. All values are mean+sem. \* genotype effect and & age effect; all  $P<0.05$ ).

In the cerebellum, an interaction between age and genotype was observed for most neurotransmitters, which means that the response variable (neurotransmitter/metabolite) did not respond linearly to the variation of the factors (age and genotype). This means, for example, that for a given genotype, the change from one age to another generates a response whereas for the other genotype the response is much different (increasing or decreasing).

This age x genotype interaction was noticed for NE, 5-HT and 5-HIAA in the cerebellum. At three-weeks of age *Mecp2*-null NE, 5-HT and 5-HIAA levels all showed an increase of approximately 30% of the wt levels. However, at eight weeks of age the NE, 5-HT and 5-HIAA levels were 61%, 55% and 35% decreased, respectively, as compared to the wt levels (figure 4.10A; NE:  $F_{3,26}=13.682$ ,  $p=0.001$ ; 5-HT:  $F_{3,26}=7.830$ ,  $p=0.010$ ; 5-HIAA:  $F_{3,26}=8.821$ ,  $p=0.006$ ).

Regarding all the other amines, the ontogenic profiles of *Mecp2*-null and wt mice were similar. In the cerebellum, *Mecp2*-null mice showed levels of DA lower than the wt levels, both at three (39%) and at eight weeks of age, which was even more notorious (60%) (figure 4.10A; DA:  $F_{3,26}=4.957$ ,  $p=0.003$ ). No other differences were noticed in the levels of neurotransmitters or its metabolites.

The turnover ratio DOPAC/DA was increased in the *Mecp2*-null as compared to wt (figure 4.10B; DOPAC/DA:  $F_{3,24}=4.640$ ,  $p=0.041$ ).

At three weeks of age, cerebellar levels of HVA were not detectable by HPLC/EC in a considerable number of samples. Therefore, in this brain region, only the levels of DA and DOPAC were considered in the analysis.

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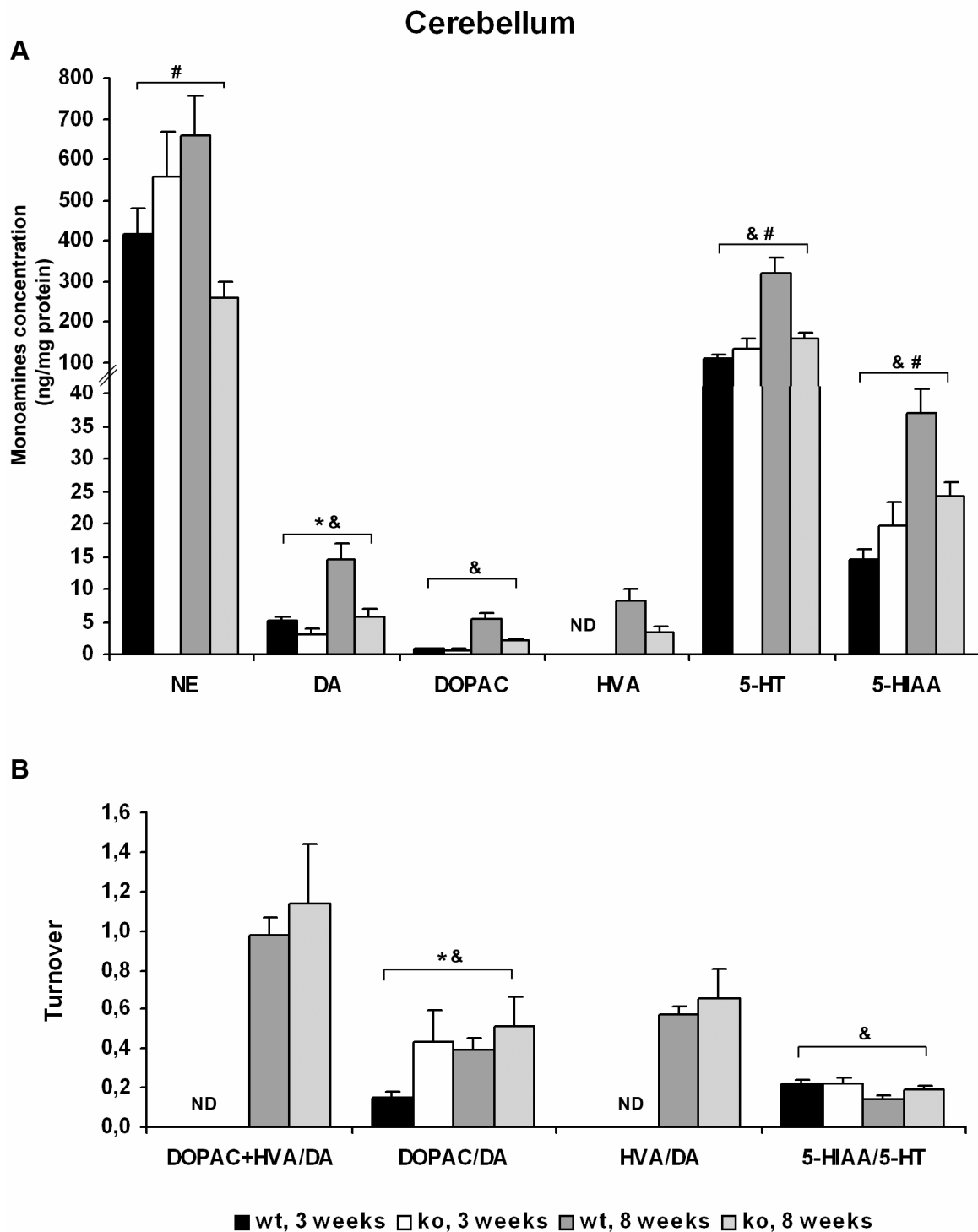


**Figure 4.10. Neurochemical analysis of the cerebellum region of wt and *Mecp2*-null mice at three and eight weeks of age.** (A) Concentration of each neurotransmitter and metabolites and (B) neurotransmitter (DA and 5-HT) turnover. (wt, wild type; ko, *Mecp2*-null. All values are mean+sem. \* genotype effect, & age effect and # age x genotype interaction; all  $P < 0.05$ ).

### Serotonergic innervation

Given that in *Mecp2*-null mice at three weeks of age the levels of 5-HT were reduced in both the PFCx and the MCx, two brain regions highly innervated by serotonergic fibres, we explored whether the observed reduction in the levels of the neurotransmitter was associated with a reduction in the number of serotonergic fibres in these regions.

The serotonergic innervation was evaluated in the PFCx (ko, n=4; wt, n=3) and MCx (n=4 for each genotype) areas of three-week-old *Mecp2*-null and wt mice by counting the number of serotonergic fibre intersections with L-cycloids. Overall, the analysis of the 5-HT immunohistochemical sections failed to show any significant difference in the density of 5-HT fibres between *Mecp2*-null and wt groups (figure 4.11; t-test; PFCx, p=0.275 and MCx, p=0.488).

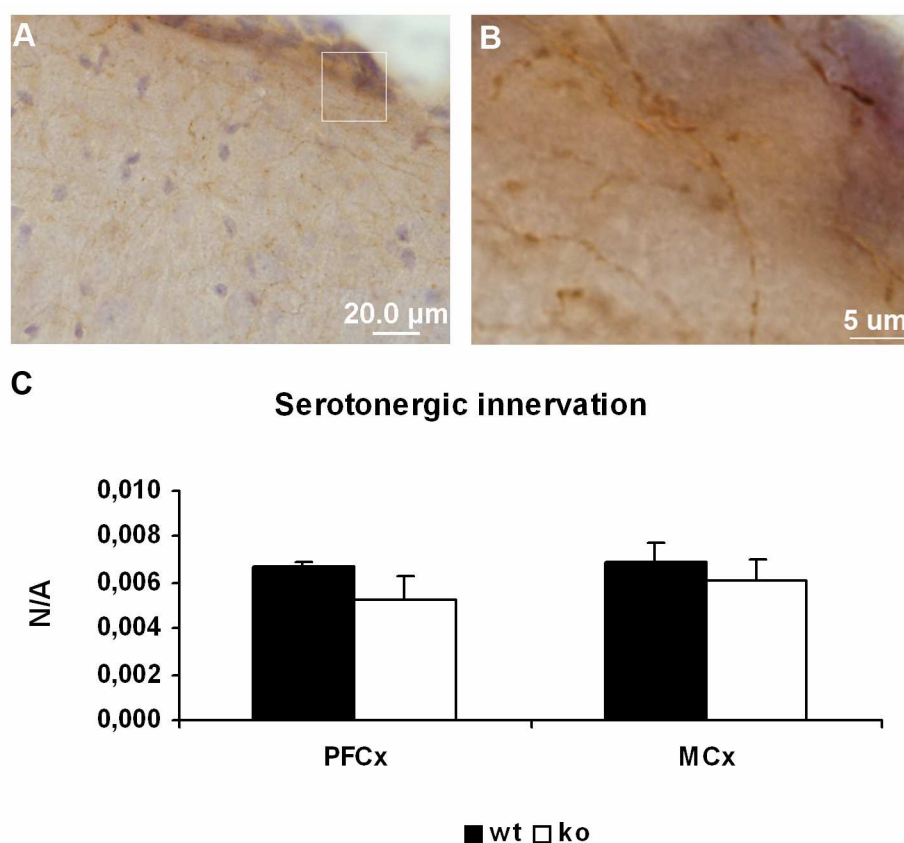


**Figure 4.11. Serotonergic innervation in the motor cortices of *Mecp2*-null mice and their wt littermate controls at 3 weeks of age.** (A-B) Representative photomicrograph of 5-HT immunohistochemistry reactivity in the MCx region. (C) Quantification of the serotonergic innervation in the PFCx and MCx brain areas. Values represent mean+sem, wt, wild-type; ko, *Mecp2*-null; N/A, number of fibre intersections per area; PFCx, prefrontal cortex; MCx, motor cortex.

### mRNA expression levels of NE and 5-HT receptors and transporters

We then assessed, by qRT-PCR, the levels of mRNA expression of several noradrenergic and serotonergic receptors and transporters in the PFCx and MCx of three- and eight-week-old *Mecp2*-null and wt animals. The receptors in study were selected based on their expression in these brain areas and on the way they mediate the effects of the neurotransmitter (role in behaviour).

We found that in the PFCx the mRNA expression levels of NET and of the adrenergic receptor *Adra2a*, both at three and eight weeks of age, were reduced in the *Mecp2*-null as compared to wt mice (figure 4.12; NET:  $F_{3,18}=9.76$ ,  $p=0.006$ ; *Adra2a*:  $F_{3,20}=8.392$ ,  $p=0.009$ ). A trend was observed as the levels of *Adra2a* in both wt and *Mecp2*-null mice behave differently at the two ages, as given by the almost significant age and genotype interaction (figure 4.12; *Adra2a*:  $F_{3,20}=4.237$ ,  $p=0.053$ ). The serotonergic receptors *Htr2a* and *Htr3a* were also altered in this brain region of *Mecp2*-null mice. Interestingly, the ontogenic profile of *Htr2a* expression was significantly different between wt and *Mecp2*-null mice, which evolved differently from three to eight weeks of age (figure 4.12; *Htr2a*:  $F_{3,23}=4.32$ ,  $p=0.05$ ). In the wt animals there was an increase of the *Htr2a* mRNA levels, which did not happen in the *Mecp2*-null mice. Additionally, the levels of *Htr3a* mRNA, were reduced in the *Mecp2*-null mice, at both timepoints, as compared to wt levels (*Htr3a*:  $F_{3,20}=4.91$ ,  $p=0.038$ ). No differences were detected in the levels of expression of the other genes studied.

In the MCx differences were found in the serotonergic receptors *Htr2a* and *Htr3a*. Relative to wt values, the levels of expression of *Htr2a* receptor in the *Mecp2*-null mice were reduced (figure 4.12; *Htr2a*:  $F_{3,18}=7.690$ ,  $p=0.013$ ). The ontogenic profile of *Htr3a* receptor expression was significantly different between wt and *Mecp2*-null mice, which evolved differently from three to eight weeks of age, as given by an interaction between age and genotype for the expression levels of this receptor (figure 4.12; *Htr3a*:  $F_{3,20}=6.86$ ,  $p=0.016$ ).

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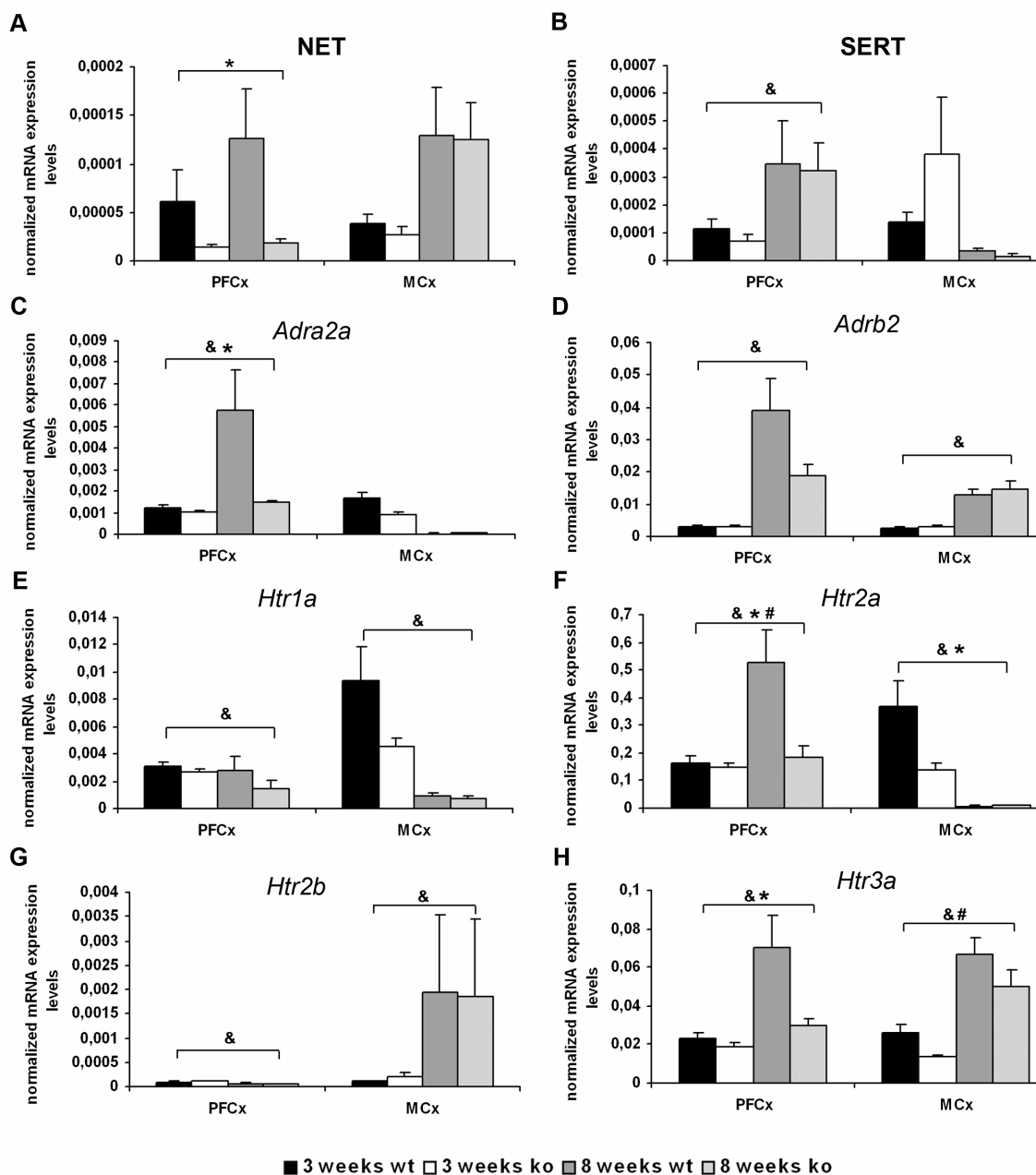


**Figure 4.12. Expression levels of NE and 5-HT receptors and transporters in the *Mecp2*-null mice at 8 weeks of age.** ( $n > 5$ ; wt, wild type; ko, *Mecp2*-null. Values represent mean+sem. &, age effect; \*, genotype effect; #, age x genotype interaction; all  $P < 0.05$ . Data analysed by 2-way ANOVA).



## 4.5. Discussion

### ***Mecp2*-null mice display monoaminergic disturbances in brain regions involved in higher level motor control.**

In this work we examined the impact of the absence of the MeCP2 protein upon the brain modulatory monoaminergic systems and evaluated its consequences in the pathogenesis of the *Mecp2*-null male mice, as a model of human RTT pathology. We found important differences between *Mecp2*-null and wt mice in the bioaminergic systems which were (1) brain region-dependent and (2) age-dependent; we observed that (3) the most affected monoamines were 5-HT and NE, both showing decreased levels in specific brain regions of the *Mecp2* ko mice; our results also showed that (4) the metabolism of 5-HT was altered in these mice, the PFCx, MCx and SN-VTA showing an increase, while in the CPu there was a decreased turnover ratio. Additionally, dopaminergic system imbalances were present in the CPu and cerebellum regions.

As others, we also observed that overall the levels of biogenic amines were decreased in the brain of *Mecp2*-null mice as compared to wt controls (Ide et al. 2005; Viemari et al. 2005). In our study, however, we were able to further map the differences found in the biogenic amines levels in *Mecp2*-null mice, as we studied eight different brain regions that play a role in motor function and in learning processes, which are dysfunctional in RTT. Additionally, we were also able to narrow the time-window of onset of this monoaminergic dysregulation to the first three weeks of age, as we observed differences in the levels of biogenic amines of *Mecp2*-null mice never reported at such an early age.

Data from other studies also confirms our observation that one of the first bioaminergic systems to be affected by the absence of MeCP2 protein was the noradrenergic one. The levels of NE, but not of 5-HT and DA, were shown to be decreased in the *Mecp2*-null mice as compared to wt controls, at four weeks of age, both in whole brain (Ide et al. 2005) and in medulla oblongata extracts (Viemari et al. 2005). Viemari and colleagues (2005) also studied the levels of these neurotransmitters in the pons and forebrain of *Mecp2*-null and wt mice and they did not find any differences between genotypes. In their studies, differences in the serotonergic system became evident later, between five and eight weeks of age (Ide et al. 2005; Viemari et al. 2005). Our results, however, show that already at three weeks of age the levels of both NE and

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5-HT were decreased in specific brain regions of *Mecp2*-null mice when compared to wt controls; namely in the PFCx and in the MCx. The fact that differences in the serotonergic system were not noticed before at earlier ages in the *Mecp2*-null mice could be attributed to the lack of sensitivity given the gross dissection of brain regions (whole brain or forebrain) used in the analysis. Additionally, no differences of the biogenic amine levels were found between *Mecp2*-null and wt neonates (Ide et al. 2005; Viemari et al. 2005) or at postnatal day 14 (Ide et al. 2005); we have not analysed brains at this age given the technical difficulty to appropriately dissect the regions of interest. So, at this time, we can only conclude that onset of the serotonergic imbalance occurs before the age of three weeks.

The diffuse monoaminergic modulatory systems of the brain originate in a core of subcortical nuclei and send extensive projections to several brain areas (figure 4.13) (Herlenius and Lagercrantz 2001). No differences in the levels of biogenic amines were found between *Mecp2*-null and wt mice in the D/MRN and in the SN-VTA brain regions where 5-HT and DA, respectively, are produced, both at three and at eight weeks of age (figure 4.7 and 4.8). The most obvious differences that we found were a reduction in the levels of NE and 5-HT in the PFCx, MCx and cerebellum of *Mecp2*-null mice as compared to wt controls (figures 4.3, 4.4 and 4.10), which are known for their involvement in higher and mid- level motor control, in the planning of movement. This aspect of RTT phenotype is well modelled in the *Mecp2*-null mouse we used for the current study already at an early age (see chapter 3).

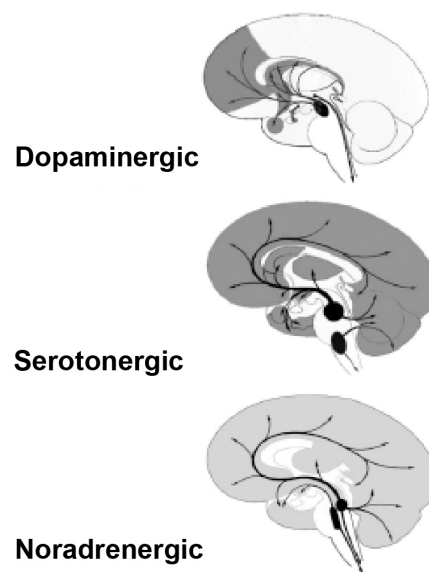


**Figure 4.13. Brain modulatory monoaminergic systems** (adapted from (Herlenius and Lagercrantz 2001)).

The PFCx, together with the CPu, plays a role in motor control, in the integration of the new presented situation with former memories, in order to envisage possible outcomes of an action. On the other hand, the MCx sets a plan in order to achieve the aimed outcome. The cerebellar input is related to the coordination of the movement and, finally, execution of the movement is determined by brainstem nuclei and the spinal cord. A disturbance in the crosstalk between all these areas, even subtle, may result in a serious motor deficit. The data we obtained in this study in the *Mecp2*-null mice showed that the above-referred regions presented an impaired bioaminergic modulation. This could explain some of the motor behavioural problems exhibited by this model and also the phenotypic manifestations of the human RTT patients: the non-directed and wide-base walking gait (when acquired), the hand stereotypies and the dyspraxia (figure 4.14).

## Motor Control

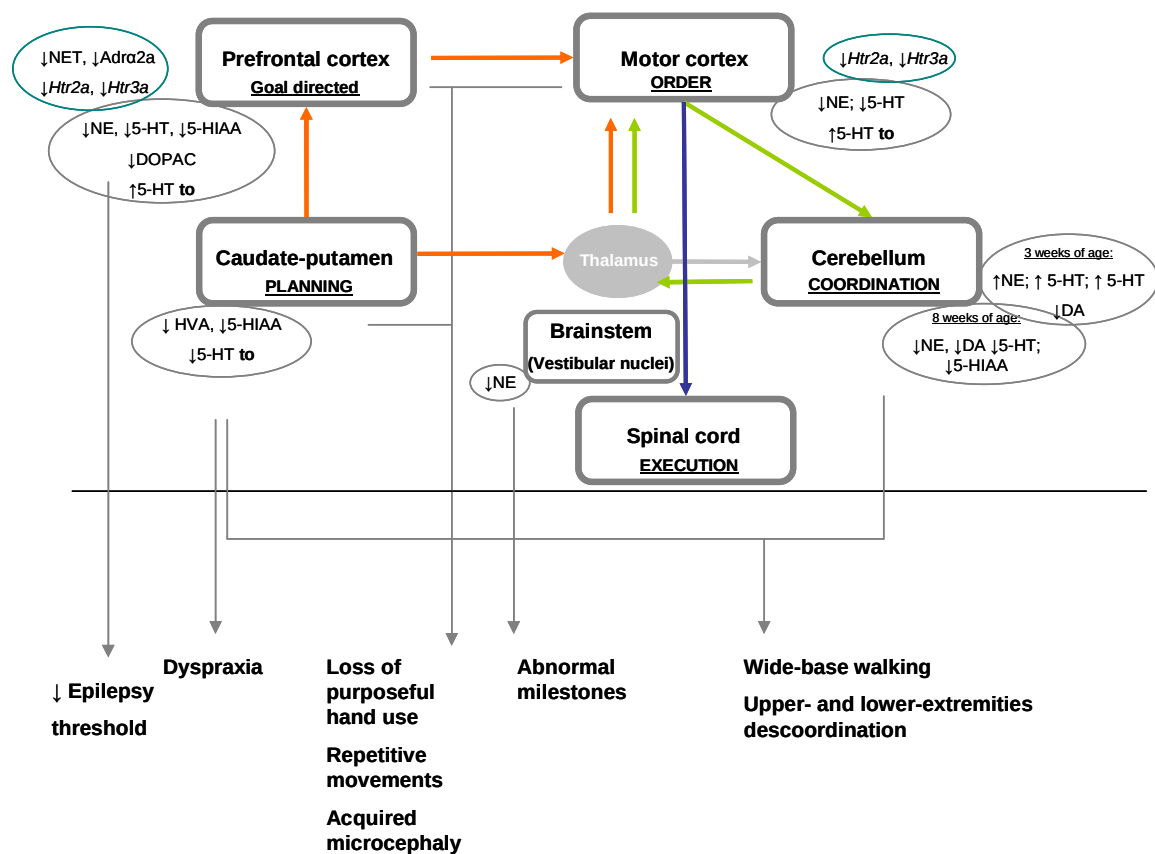


**Figure 4.14. Brain structures involved in the motor control.** Represented are the differences found at each brain region of the *Mecp2*-null mouse model. (Arrows: orange, regions involved in the “high level” control of movement, green, regions involved in the “mid level” control of movement and blue, regions involved in the “low- level” control of movement).

What could be the physiological significance of the observed alterations of neurotransmitters and their metabolites, and what could be their impact on the developing brain?

The noradrenergic, dopaminergic and serotonergic systems achieve their modulatory role partly through an influence on neuronal maturation; in the regulation of physiological processes such as synaptic transmission, synaptic modification (dendritic length and arborisation, spine density and morphology) and neuronal adaptation (influencing LTP) (Hasselmo 1995; Berger-Sweeney and Hohmann 1997). For example, the apical dendritic branches of the infralimbic pyramidal neurons in SERT ko mice, which are characterized by high levels of extracellular 5-HT, were significantly increased in length relative to wt mice (Wellman et al. 2007). Interestingly, alterations in all these processes have already been reported in the brains of human RTT patients (Armstrong et al. 1995; Armstrong 2001; Armstrong 2002; Armstrong 2005) and in its different mouse models (Kishi and Macklis 2004; Asaka et al. 2006; Moretti et al. 2006)

The performance on behavioural tasks is also affected by monoamine dysregulation. A depletion of NE is related to an impaired performance in attention paradigms, whereas 5-HT is more related to the postural control and locomotor function; its influence, through the descending pathways, in the central pattern generators of locomotion has been described (Pflieger et al. 2002; Vinay et al. 2002). DA is more closely linked to motor response initiation (Hauber 1998), which we have seen to be impaired in this mouse model.

### **Primarily affected brain regions in RTT**

Our results clearly implicate a dysfunction of the noradrenergic and serotonergic pathways in the neuropathology of *Mecp2*-null mice since the earlier stages. The main affected brain areas are those involved in the higher and mid-level motor control, such as the prefrontal cortex and the motor cortex. The hippocampus and cerebellum seem to play a role only in the later stages of the disease. Altered bioaminergic modulation in these brain regions could be responsible for important components of the phenotype present in human RTT patients, partially modelled in these *Mecp2*-null mice.

The neurochemical changes detected in the vestibular area also support our previous results on the abnormal development of neurological reflexes of the *Mecp2*-null

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mice (chapter 3-I), which suggested an impaired neurodevelopment of pathways within the brainstem, particularly vestibular nuclei (Santos et al. 2006b). In the present study, we could confirm that this abnormal early motor development may be in part due to a dysfunction of the noradrenergic system, as *Mecp2*-null mice presented reduced levels of NE as compared to wt controls, already at 3-weeks of age, in the vestibular nuclei.

In the CPu of *Mecp2*-null mice we found that the levels of HVA (a metabolite of DA) and the turnover rate of 5-HIAA/5-HT were decreased. Impaired DA transmission within CPu delays motor initiation whereas enhanced serotonergic activity promotes akinesia (for a review see Hauber 1998), which is in agreement with our behavioural data (see chapter 3-II).

In summary, our data on neurochemical measurements suggests that the effect of *Mecp2* mutation upon the brain modulatory monoaminergic systems is reflected in several of their projection target regions and not in the regions of their origin. In this way, MeCP2 may affect not the synthesis of monoamines but instead affect their release, their degradation or the pathways that are activated by monoamine receptor stimulation. We can say that the disease has a progressive course at the neurochemical level given that, overall, the mean differences detected between *Mecp2*-null and wt mice were higher at eight weeks of age than at three weeks of age.

### **Cerebellar involvement and RTT progression**

Little attention has been given to the cerebellum, in respect to RTT pathology. However, our data showed that neurochemically the cerebellum, although not affected from the beginning, becomes progressively involved, being severely altered at later stages, as has been described for RTT patients (Gotoh et al. 2001). At eight weeks of age the noradrenergic, dopaminergic and serotonergic pathways were significantly impaired (figure 4.10A,B), highlighting the importance of the cerebellum in the later phases and in the progression of the disorder. The cerebellum is the area of the brain responsible for coordinating muscular activity and complex movement. The serotonergic innervation to the cerebellum affects all parts of the cerebellar circuitry (for a review see Schweighofer et al. 2004) and disturbances of the cerebellar input have been related to cerebellar ataxia (Trouillas 1993) and to changes in spontaneous behavioural activity (Mendlin et al. 1996). The cerebellar noradrenergic modulation is also very important, and noradrenergic terminals make close contacts with granule cell and Purkinje cell dendrites; NE levels

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have also been related to cerebellar learning (for a review see Schweighofer et al. 2004). The cerebellum of RTT patients exhibited a progressive atrophy with loss of specific neurons, such as Purkinje neurons (Oldfors et al. 1990; Armstrong 2002).

### **The hippocampus and cognitive defects in RTT**

In the hippocampus, no major neurochemical differences were found in the *Mecp2*-null as compared to wt mice, although at eight weeks of age there was a clear tendency for decreased levels of NE, 5-HT and HVA (figure 4.6A). Additionally, the interaction age x genotype may suggest an involvement of the hippocampus in the later stages of the disease. At three weeks of age, we observed that *Mecp2*-null mice performed as well as wt controls in the homing test, which assesses spatial learning in young juveniles (data not shown). Our data on neurotransmitter levels was in agreement with this unimpaired learning at three weeks of age. At eight weeks of age, given their severe motor impairment, it is impossible to perform any kind of learning task in this mouse model. However, it has been reported that when symptomatic (mean eight weeks of age), but not at asymptomatic ages, *Mecp2*-null mice exhibited an impaired hippocampal LTP (Asaka et al. 2006), which underlies some forms of learning and memory, further supporting our neurochemical data. In another model of RTT, with an hypomorphic *MECP2* allele (*Mecp2*<sup>308/Y</sup>), the performance of the *Mecp2*<sup>308/Y</sup> males in hippocampal-dependent learning and memory tasks was also significantly impaired and synaptic deficits at the hippocampus (LTP and LTD) were reported (Moretti et al. 2006).

### **Possible causes**

The cause for the altered biogenic amine levels in these brain regions of the *Mecp2*-null mice remains elusive. A defect in the synthesis of monoamines does not appear to be the cause of the deregulated neuromodulation found in the *Mecp2*-null mice, as no differences in the levels of these amines were found in the regions of production of 5-HT and DA (D/MRN and SN-VTA, respectively).

In order to determine the mechanism by which the *Mecp2*-null mice exhibit decreased levels of NE and 5-HT we have explored some possible causes of such a difference. For example, a reduction in the levels of 5-HT could result from a reduction in the number of 5-HT fibres that innervate a given region. In the PFCx and MCx of *Mecp2*-null the levels of 5-HT were reduced as compared to wt mice at three weeks of age;

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however, this was not accompanied by a statistically significant reduction in the number of 5-HT positive fibres that innervate these regions.

As a transcriptional repressor, the absence of MeCP2 could also, directly or indirectly, affect the levels of expression of NE and 5-HT receptors and/or transporters. In fact, differences in the expression levels of other downstream target genes involved in neurotransmission had already been reported in *Mecp2*-null mice, such as the *Dlx5* (Horike et al. 2005) and the human *GABRB3* genes (Samaco et al. 2005) (for a review see Santos et al. 2006a).

NE and 5-HT release is modulated by the  $\alpha 2$  adrenergic receptors (Baraban and Aghajanian 1980; Baraban and Aghajanian 1981). The action of NE is terminated, in part, by its uptake into presynaptic noradrenergic neurons by the plasma-membrane NET, and serotonergic neurotransmission is regulated by clearance of 5-HT from the extracellular space by SERT. The serotonergic *Htr2a* is highly expressed in the frontal cortex and *Htr3a*, also expressed in the cortex, is the only 5-HT receptor that it is not G-protein-coupled but ligand-gated Na/K channel. Both the *Htr2a* and *Htr3a* act as heteroreceptors by regulating the synthesis and/or the release of other neurotransmitters, such as GABA and glutamate, which are involved in learning and memory.

Interestingly, we found that in the PFCx of *Mecp2*-null mice, the mRNA levels of the NE transporter, of the adrenergic receptor *Adra2a* and the mRNA levels of the serotonin receptors *Htr2a* and *Htr3a* were reduced as compared to wt levels. Additionally, in the MCx of the *Mecp2*-null mice also the levels of *Htr2a* and *Htr3a* receptors were reduced.

We have observed a decreased expression levels of three receptors (*Adra2a*, *Htr2a* and *Htr3a*) from 3 weeks of age, which were maintained at low levels at eight weeks of age. If the cause of the reduced levels of both 5-HT and NE was in the low levels of the neurotransmitter itself, then through time the receptors would have adapted to the condition, by increasing their expression levels in order to compensate that dysregulation, which does not happen. However, our data appear to indicate that the problem must be at the transcription level. MeCP2 is a repressor and must be regulating the repression of another receptor that in turn modulates the expression of *Adra2a*, *Htr2a* and *Htr3a* receptors, as their transcription was reduced in the *Mecp2*-null mice. Since 5-HT receptor levels may be crucial for strengthening of the synapses during development, this reduction

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may have as a consequence the loss of serotonergic synapses and a posterior decrease of serotonin. A further decrease of this neurotransmitter may result from the decrease of the  $\text{Adra}2\text{a}$  receptor, which regulates the release of 5-HT and NE.

The lower levels of NET in the *Mecp2*-null mice are more likely to be a consequence of a chronic depletion of NE. It was shown that NET ko mice have increased levels of  $\text{Adra}2\text{a}$  (Gilsbach et al. 2006). This again suggests that, in a normal situation, the receptor adapts to compensate the levels of its neurotransmitter,.

The PFCx has an important role in both cognitive and executive functions and is one of the brain structures involved in “higher level” control of movement, in the planning of an action (for a review see Berger-Sweeney and Hohmann 1997; Dalley et al. 2004; Arnsten and Li 2005). The action of NE is particularly relevant in the PFCx (reviewed in Dalley et al. 2004; Arnsten and Li 2005) and mediated by the adrenergic receptor  $\alpha 2\text{A}$  (Franowicz et al. 2002). We showed that in the PFCx of *Mecp2*-null mice the levels of *Adra2A* receptor were reduced and this fact may affect the performance of the *Mecp2*-null mouse in the planning of a motor action. Moreover, the increased levels of extracellular NE of the NET ko mice is related to a decreased vulnerability to seizures (Kaminski et al. 2005). The lower levels of NE in the *Mecp2*-null mice may thus contribute to the seizures presented by most of the RTT patients.

Both 5-HT and NE were shown to induce an increase in the frequency and amplitude of excitatory postsynaptic potentials (EPSPs) in apical dendrites of neocortex and medial prefrontal cortex layer V pyramidal cells (Aghajanian and Marek 1997) and these effects are mediated by the serotonergic receptor *Htr2a* but not the adrenergic receptor *Adra2A* (Marek and Aghajanian 1999). Interestingly, the *Mecp2*-null mouse we studied (1) has, as we showed here, decreased levels of 5-HT, NE neurotransmitters as well as of *Htr2a* receptor in PFCx and MCx, and (2) has reduced amplitude and frequency of mEPSPs in cortical pyramidal cells (Dani et al. 2005; Nelson et al. 2006). In this way, this data seems to suggest a role for NE and 5-HT, through *Htr2a*, in the neuronal activity levels of *Mecp2*-null mice.

Beyond the altered expression levels of the transcripts analyzed it would also be useful to analyze their binding activity/functional binding. It would be interesting to evaluate the binding activity of these monoaminergic receptors, through pharmacological

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studies, in the *Mecp2*-null mice, in order to clarify their involvement in the decreased availability of NE and 5-HT in these brain regions.

In the PFCx and MCx the reduction observed in the levels of 5-HT are accompanied by an increase in the turnover of this neurotransmitter. This evidence may suggest that the regulation of 5-HT turnover is compromised. Assessment of the enzymatic activity of monoamine oxidases may provide clues as to the biochemical basis of this increased turnover rate. Additionally, it would also be important to evaluate the expression levels of vesicular monoamine transporter, which could be further contributing to the decrease of 5-HT and NE in the *Mecp2*-null mice.

Our future studies will also address whether manipulation of the noradrenergic and serotonergic systems with agonists and antagonists influence the phenotype, in particular the motor performance, of *Mecp2*-null mice, and should provide further evidence as to the mechanism of neurotransmitter imbalances in this model. This knowledge should be helpful in defining future therapeutic approaches to RTT.

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INCREASED NEUROGENESIS IN THE HIPPOCAMPUS OF *Mecp2*-NULL MICE

The results described in this chapter are included in the following manuscript (in preparation):

Mónica Santos, Andreia Teixeira-Castro, Anabela Silva-Fernandes, Hugo Tavares, Nuno Sousa and Patrícia Maciel. "Increased neurogenesis in the hippocampal subgranular zone of *Mecp2*-null mice."



## 5.1. Abstract

Adult hippocampal neurogenesis has been described in several species and, although its functional significance is still controversial, evidence points to a major role in cognition. It has been proposed that dysregulation of adult neurogenesis may also play a role in brain pathophysiology and/or capacity of brain repair. Postnatal hippocampal neurogenesis can be dynamically regulated through external as well as internal stimuli, such as neurotransmitters, neurotrophins and hormones, but also by physical exercise and rearing in an enriched environment.

Mental retardation is one of the most important features in RTT, with most of the affected patients presenting moderate to profound cognitive impairments. Additionally, factors known to regulate hippocampal neurogenesis, such as 5-HT, NE, BDNF, steroid hormones and neuronal activity, were found to be impaired both in RTT patients and in mouse models of the disorder.

Our goal in this chapter was to explore the role of adult hippocampal neurogenesis in RTT. For this, four-week-old *Mecp2*-null mice were injected with 5-bromodeoxyuridine, for three consecutive days, and the number of proliferating cells, TUNEL-positive cells and the phenotype of the newly generated cells was analysed. We found an increased hippocampal neurogenesis in the *Mecp2*-null mice as compared to wt littermates. Further studies are needed in order to elucidate the clinical relevance of this finding.

## 5.2. Introduction

The generation of new neurons within the postnatal and the adult brain (neurogenesis) has been described from invertebrate to vertebrate species (Altman and Das 1965; Eriksson et al. 1998; Gould et al. 1999a; Gould et al. 1999b; van Praag et al. 1999b; Sullivan et al. 2007). Both embryonic and adult neurogenesis involves cellular proliferation, migration and differentiation of the new neurons, which then integrate the neural network. There are essentially two neurogenic areas in the adult mammalian brain, the sub-ventricular zone, where cells migrate through the rostral migratory stream to the olfactory bulb, and the sub-granular zone (SGZ) of the hippocampal formation, where neural stem/progenitor cells can generate neuronal lineage (reviewed in Mackowiak et al.

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2004). In the hippocampus, new neurons extend their dendrites through the granular cell layer into the molecular layer and project axons, through mossy fibres, to CA3 region where they synapse (Markakis and Gage 1999) (figure 5.1). Functional integration of these newly generated cells into the hippocampal circuitry has been shown, as evidenced by their responsiveness to stimulation of the perforant path and their ability to extend axonal projections to appropriate target areas (van Praag et al. 2002).

Neurogenesis can be positively or negatively regulated by several factors. For example, the oestrogen hormones, neuromodulators (NE, 5-HT), growth factors (brain derived neurotrophic factor – BDNF and vascular endothelial growth factor - VEGF), as well as environmental enrichment or physical activity (van Praag et al. 1999b), all have been shown to stimulate the production of new granule cells in the adult hippocampus. On the other hand, adrenal steroids, glutamate neurotransmission (via NMDA receptors), stimulus deprivation and stressful experience reduce the number of granule cells in the hippocampus (reviewed in Gould et al. 2000; Mackowiak et al. 2004; Lehmann et al. 2005). Pathological conditions might also alter the number of granule cells as shown for epileptic seizures, in which the levels of adult generated granule cells are increased, whereas they are decreased in stroke/ischemia and Parkinson's disease (reviewed in Mackowiak et al. 2004).

The functional significance of adult neurogenesis is still controversial, however studies point to a major role in cognition. For example rearing in an enriched environment or voluntary exercise, as well as training on a hippocampal-dependent task, increase neurogenesis and concomitantly improve the performance in learning and memory tasks and, enhanced long-term potentiation (van Praag et al. 1999a; Cao et al. 2004).

Mental retardation is one of the most important features in RTT, with most of the affected patients presenting moderate to profound cognitive impairments. Remarkably, *Mecp2* mutant mice exhibit impaired spatial and emotional cognition tasks (Gemelli et al. 2005; Moretti et al. 2006; Pelka et al. 2006). Impairments of both LTP and LTD were described in the CA1 region of hippocampus of *Mecp2*-null (Asaka et al. 2006), and in the neocortex and hippocampus of the transgenic *Mecp2*<sup>308/Y</sup> (Moretti et al. 2006) mouse models, which is consistent with the clinical finding of mental retardation in RTT patients.

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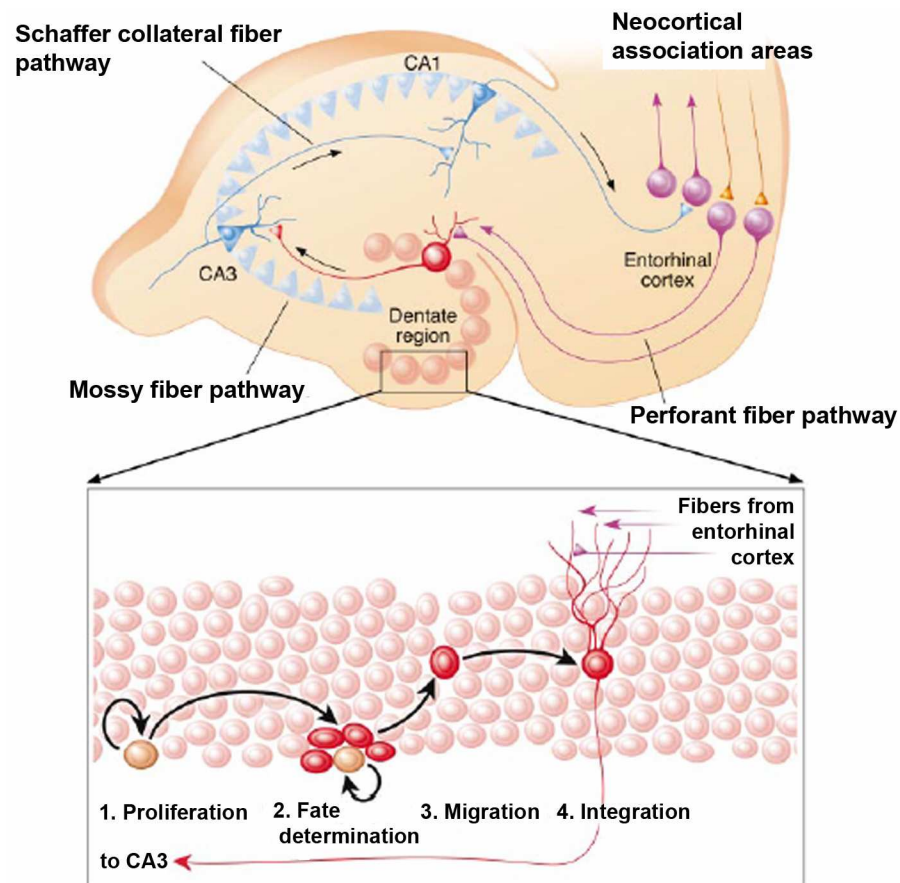


**Figure 5.1. Schematic representation of adult hippocampal neurogenesis.** Hippocampal neurogenesis comprises at least four distinct stages: stem cells in the subgranular zone of the hippocampus proliferate and give rise to immature neurons (1 and 2). (3) These immature neurons migrate into the granular layer and (4) mature into new granule cells that will receive inputs from entorhinal cortex, project axons into CA3 thus integrating into the hippocampal network. (Adapted from Lie et al. 2004).

Interestingly, one of the targets of MeCP2 identified so far is the gene encoding Brain derived neurotrophic factor (BDNF), a gene for which transcription is regulated in a neuronal activity-dependent manner (Lu 2003). MeCP2 binds methylated rat *Bdnf* promoter III (equivalent to *Bdnf* promoter IV in the mouse) and is responsible for its silencing; upon membrane depolarization of cultured cortical neurons, MeCP2 dissociates from the promoter allowing a higher transcription of the *Bdnf* gene (Chen et al. 2003; Martinowich et al. 2003). Moreover, *in vitro*, the absence or dysfunction of MeCP2 led to increased levels of *Bdnf* transcript in the absence of neuronal activation. In accordance, *in vivo*, in basal conditions, the levels of *Bdnf* transcript were significantly higher in cultured cortical neurons of *Mecp2*-null than of wt mice (Chen et al. 2003). This loss of regulation

of BDNF expression may be responsible for several neuronal effects such as altered synaptic plasticity and unbalanced neurogenesis.

The MeCP2 homologue of *Xenopus* targets the *Hairy2a* gene during development, and the absence or presence of a mutant MeCP2 misregulated the expression of the *xHairy2a* gene, affecting embryonic neurogenesis (Stancheva et al. 2003).

Given (i) the mental retardation of RTT patients and the corresponding alterations in the *Mecp2*-null mouse, (ii) the fact that the levels of neuromodulators (such as NE and 5-HT) have been reported to be altered in the brain of both RTT patients and in animal models of the disorder and (iii) BDNF and xHairy2a have been reported to be targets of MeCP2, we decided to investigate whether lack of MeCP2 could affect the formation of new neurons in the postnatal mouse brain. For this, we assessed the postnatal neurogenesis in the subgranular zone (SGZ) of the hippocampus *Mecp2*-null mice.

### 5.3. Material and Methods

#### Animals

We used young four-week-old male *Mecp2*-null (*Mecp2*<sup>tm1.1Bird</sup> mouse model) and their wt littermate controls in this study. Mice were group housed in standard laboratory cages, filled with sawdust and cardboard rolls, in an animal facility with 55% humidity and 22°C and kept in a 12 hour light: 12 hour dark cycle, with food and water *ad libitum*. The manipulation of animals was always performed by the same researcher. All experiments were performed in accordance with the European Communities Council Directive, 86/609/EEC.

#### 5-Bromodeoxyuridine (BrdU) injections

BrdU (Sigma, St Louis, MO) in 0,9% sodium chloride was administered intraperitoneally, once a day for three consecutive days, at 50 mg/kg of body weight. BrdU is a thymidine analog that will incorporate and label dividing cells which are replicating their DNA.

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### **Imunohistochemistry and TUNEL assay**

For imunohistochemistry and TUNEL assay, mice were sacrificed at four days after the first BrdU injection. Mice were decapitated and, after dissection, the brains (n=5 for each genotype) were involved in OCT (Tissue-Tek, Torrance, Japan) and rapidly immersed in isopentane cooled in liquid nitrogen. The entire length of the hippocampal dentate gyrus was sliced in serial coronal sections in a cryostat (Leica CM1900) at 20  $\mu$ m and collected in slides.

Every 8<sup>th</sup> section was stained for BrdU using a rat anti-BrdU antibody (1:50, Abcam, Cambridge, UK) after fixation in 4% paraformaldehyde (PFA), for 30 min; permeabilization with 0.2% Triton X-100 in Tris buffered saline (TBS), for 10 min; for antigen retrieval in 0.1M citrate buffer, for 20 min; acidification with 2M HCl, for 30 min and peroxidase and nonspecific blocking with 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and 4% bovine serum albumin (BSA), respectively. Visualization of the antigen was carried out using a universal detection system (BioGenex, San Ramon, USA) and diaminobenzidine (DAB) as a chromogen. Specimens were lightly counterstained with hematoxylin.

In order to detect apoptotic cells 20  $\mu$ m sections, contiguous to BrdU labelled sections, were stained using the TUNEL assay. After 4% PFA fixation for 30 min, sections were permeabilized in a two-step procedure with 0.1% trypsin in PBS (pH 7.2) for 15 min at 37°C followed by 0.1% Triton X-100 in PBS, for 5 min, at room temperature, and then treated with 3% H<sub>2</sub>O<sub>2</sub> in PBS, for 3 min, to block endogenous peroxidases. Sections were pre-incubated with terminal deoxynucleotidyl transferase (TdT) buffer and incubated with the reaction mixture containing TdT enzyme (MBI Fermentas, Burlington, Canada), dUTP-Biotin (Roche diagnostics, Basel, Switzerland), TdT buffer and TdT enzyme buffer (MBI Fermentas), for one hour at 37°C. Antigen visualization was performed with a commercial avidin-biotin/DAB system (Vector Labs, CA) and lightly counterstained with hematoxylin.

### **Stereology**

The area of interest (dentate gyrus (DG) of hippocampus) was drawn, subdivided in the SGZ, subgranular infrapyramidal cell layer (SGI) and subgranular suprapyramidal cell layer (SGS). The number of BrdU-positive cells was counted in one of every eight series of sections (160  $\mu$ m apart) throughout the entire DG of the hippocampus. The number of TUNEL-positive cells was counted in sections contiguous to the BrdU sections.

Stereoinvestigator software (Microbrightfield, VT) and a camera (DXC-390, Sony, Japan) attached to a motorized microscope (Axioplan 2, Carl Zeiss, Germany) and a 40X objective was used. The total number of BrdU-positive cells and TUNEL-positive cells per total area (N/A) was calculated.

### **Imunofluorescence**

For imunofluorescence, another set of mice (n=5 per genotype) were anesthetized (ketamine/medetomidine) and intracardially perfused with PBS and 4% PFA (pH 7.6). Brains were removed, kept in 4% PFA over night, at 4°C, and then passed to a 30% saccharose solution for 72 hours, at 4°C. Brains were coronally sliced at 40 µm using a vibratome (Leica VT 1000S, Nussloch, Germany) and free-floating sections were collected in PBS.

Double labeling for BrdU (1:50, Abcam), and either the neuronal specific marker (NeuN; 1:100, Chemicon) or the glia fibrillary acidic protein marker (GFAP; 1:500, DAKO), were performed. Brain sections were pre-treated with 50% formamide/50% 2xSSC at 65°C, for 2 hours; acidified in 2M HCl at 37°C, for 30 min, and washed in 0.1M borate buffer. Unspecific binding was blocked in 0.1% Triton X-100/3% goat serum in TBS for one hour. Incubation in primary antibodies was carried at room temperature for 24 hours. The secondary antibodies used to visualize antigens were Alexa-Fluor goat anti-rat IgG 647 (1:1000) or Alexa-Fluor goat anti-rat IgG 594 (1:1000) with either Alexa-Fluor goat anti-mouse IgG 568 (1:1000) or Alexa-Fluor goat anti-rabbit IgG 488 (1:1000) (all from Molecular Probes, Eugene, OR, USA).

### **Confocal microscopy**

Imunofluorescence images were obtained on an Olympus FV1000 confocal laser scanning biological microscope (Japan) under a 60x objective using a 559 nm laser line excitation for Alexa Fluor 594 or 568; a 488 nm laser line for Alexa Fluor 488 nm and 635 nm for Alexa Fluor 647. The pinhole was adjusted to 1.0 µm of optical slice and a scan was taken every ~0.5 µm along the Z-axis.

To determine the phenotype of the new generated cells in the hippocampus, more than 50 BrdU positive cells, randomly selected through the entire dentate gyrus, were analyzed for co-localization with either the expression of the neuronal or the glial marker (NeuN or GFAP, respectively), by a person who was blind to the genotypes of the mice. In

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the case of the GFAP marker, we considered that the two markers were in the same cell when they were both present close to each other in at least six of ten (3  $\mu$ m) z-axis planes.

### **mRNA expression levels**

Four-week-old male *Mecp2*-null and their wt littermate control mice (n=5 and n=7 for each genotype, respectively) were sacrificed by decapitation and their brains removed. Dissection of left and right hippocampus was performed and the tissue was stored at -80°C. Total RNA was extracted from the hippocampus using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and quantified in a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Two micrograms of RNA were reverse transcribed, using the Superscript™ First-Strand Synthesis System for RT-PCR (Invitrogen, CA, USA).

The expression levels of mRNA transcript IV of mouse *Bdnf* were measured in the hippocampus by qRT-PCR. The reference gene *Hprt*, was used as internal standard for normalization. The real-time PCR reactions were performed on a LightCycler instrument (Roche Diagnostics, Basel, Switzerland) using the QuantiTect SYBR Green RT-PCR reagent kit (Qiagen, Hamburg, Germany). Product fluorescence was detected at the end of the elongation cycle. All melting curves exhibited a single sharp peak at a temperature characteristic of the primer used (see supplementary table S5.1 in appendix I for primer sequences and annealing temperatures for each gene).

### **Statistical analysis**

Student's t-test was used to compare variables between *Mecp2*-null and wt mice groups. A value of  $P < 0.05$  was considered as statistically significant.

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## 5.4. Results

### Cellular proliferation

To evaluate the effect of the absence of MeCP2 protein upon adult hippocampal DG neurogenesis, we administered BrdU in four-week-old *Mecp2*-null male mice and their wt littermates, in order to label cells in proliferation. We counted the number of BrdU-positive cells in the DG-SGZ, further divided in the SGS and in the SGI of the DG. Our results showed that there was no difference in the number of proliferating cells between the right and left hemispheres in both genotypes in the SGZ or in the SGI and SGS cell layers, when considered individually (data not shown). In this way, and in order to gain statistical power, we pooled data from both right and left hemispheres and performed the analysis. The number of BrdU-positive cells in the SGZ of *Mecp2*-null mice was significantly higher than in the wt littermates ( $p=0.023$ ; figure 5.2A-B, E).

### Apoptosis

In order to evaluate whether the number of dying cells was affected by the absence of MeCP2, we counted the number of TUNEL-positive cells in the SGZ (SGI and SGS) of the hippocampal DG of male *Mecp2*-null and their wt littermate controls. The number of TUNEL-positive cells did not differ significantly between the left and right hemispheres in both genotypes in all the layers analyzed (data not shown). Thus, we pooled the data from both hemispheres and we analyzed the number of TUNEL-positive cells per hippocampus. No differences were found between *Mecp2*-null and wt littermates in the number of cells in apoptosis in the SGZ, as labelled by the TUNEL assay ( $p=0.288$ ; figure 5.2C-D, F). Thus, the increased proliferation in the SGZ of *Mecp2*-null mice did not correspond to an increased cell demise.

### Phenotype of proliferating cells

Given the higher cellular proliferation in the *Mecp2*-null as compared to wt animals we analysed the phenotype of these newly formed cells. We used confocal microscopy to assess the phenotype of BrdU-positive cells as either neuronal or glial, i.e. expressing the NeuN or GFAP markers, respectively. We found that both in the *Mecp2*-null and in the wt mice a large percentage of the newly formed cells, 91.1% and 86.4%, respectively, expressed the NeuN marker. The percentage of the new mature neurons was not significantly different between the two genotypes ( $p=0.67$ ; figure 5.3A-F, M). Additionally,

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the percentage of the newly generated cells that express the GFAP marker was low in both genotypes, and also did not differ significantly between *Mecp2*-null and wt mice ( $p=0.121$ ; figure 5.3G-L, N).

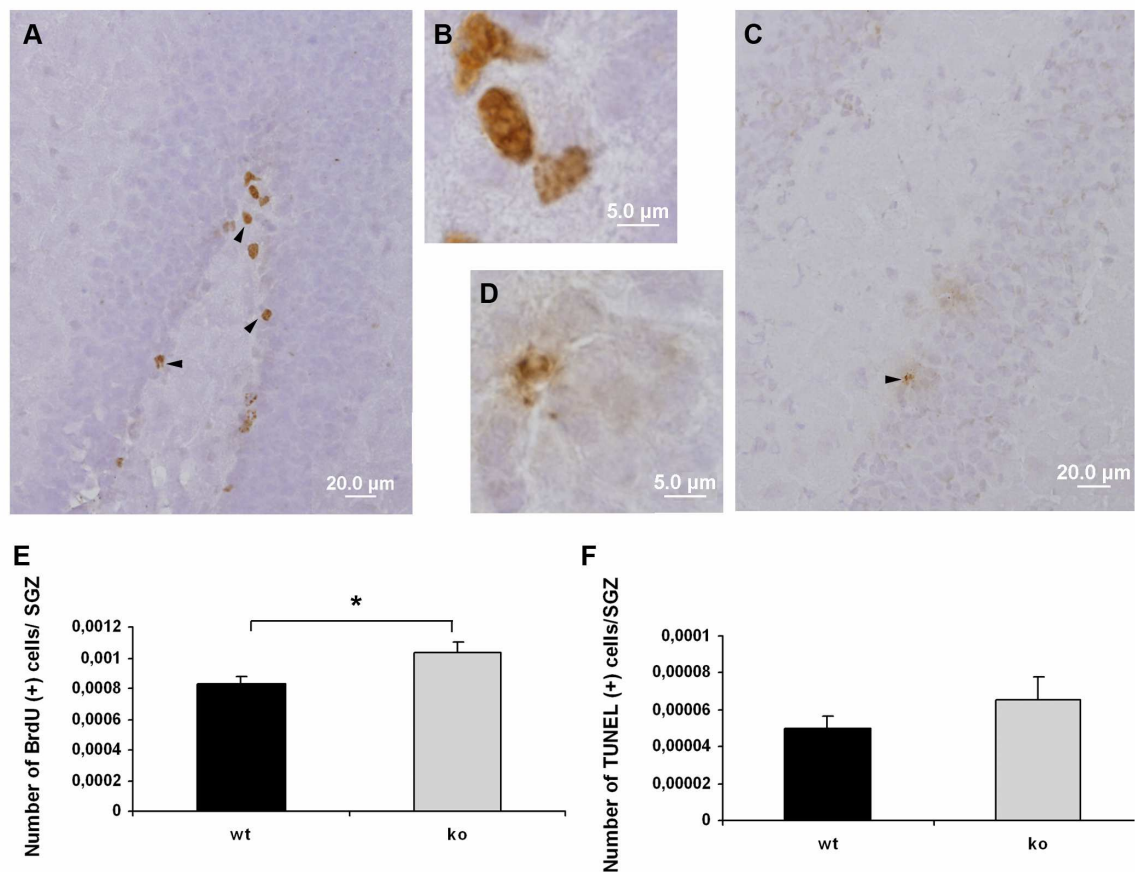


**Figure 5.2. *Mecp2*-null mice exhibit a higher number of proliferating cells without increased apoptosis.**

Photomicrographs of hippocampal dentate gyrus showing (A, B) the BrdU and (C, D) TUNEL staining used in the analysis of cellular proliferation and apoptosis, respectively. (E) Quantitative analysis of BrdU-positive cells indicated a higher cellular proliferation of in the SGZ of *Mecp2*-null mice as compared to wt controls ( $p=0.023$ ;  $n=5$  per genotype). (F) Quantitative analysis of TUNEL-positive cells indicated no significant difference in apoptosis between *Mecp2*-null and wt animals ( $n=4$  per genotype). (Values are mean+sem; SGZ, subgranular zone; wt, wild-type; ko, *Mecp2*-null; \*  $p<0.05$ ).

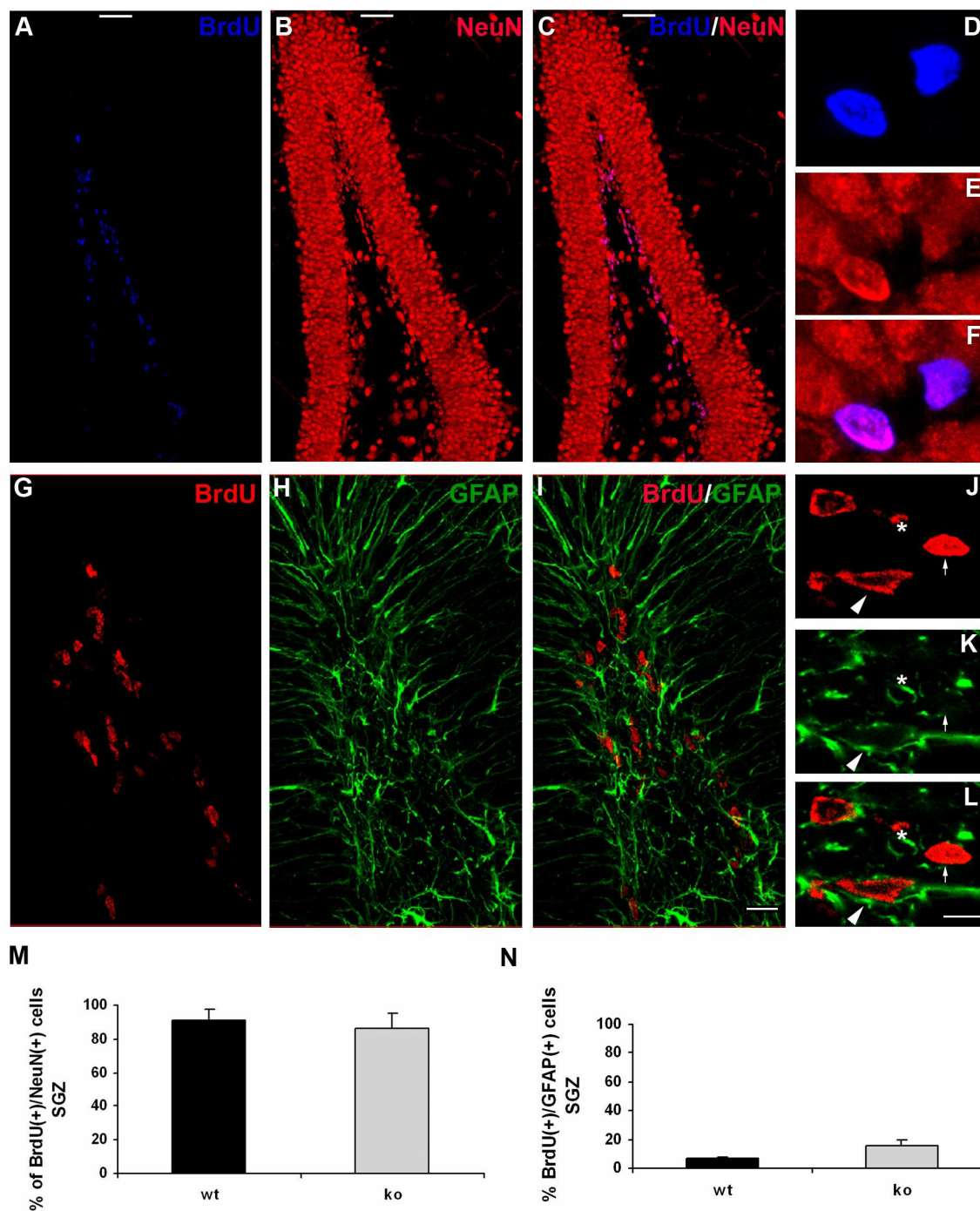


**Figure 5.3. *Mecp2*-null newly generated cells differentiate into mature neurons similarly to wt.** Confocal images showing DG of the hippocampus labelled for both (A) BrdU and (B) NeuN, which were used for analysis of neurogenesis. (C) An overlay of A-B. Scale bar=40  $\mu$ m. (D-F) Higher magnification images of SGZ cells. Scale bar=10  $\mu$ m. Confocal photomicrograph of hippocampal DG labelled for both (G) BrdU and (H) GFAP markers; used to quantify the percentage of new astrocytes in both wt and *Mecp2*-null mice. (I) Merged image of G-H. Scale bar=40  $\mu$ m. (J-L) higher magnification images of SGZ cells. Scale bar=10  $\mu$ m. (n=5 animals per genotype and approximately 100 cells per animal were analyzed; values are mean+sem; wt, wild type; ko, *Mecp2*-null).

### mRNA expression levels of *Bdnf* transcript

*Bdnf* is thought to be directly regulated by the MeCP2 protein, which represses its transcription. It was reported that the loss of MeCP2 function causes the overexpression of *Bdnf* (Chen et al. 2003; Martinowich et al. 2003). We assessed by qRT-PCR the mRNA expression levels of the transcript IV of *Bdnf* in the hippocampus of four-week-old *Mecp2* ko and wt mice. *Bdnf* was differentially expressed in the right and left hemispheres in both genotypes (wt,  $p=0.078$ ; ko,  $p=0.0029$ ). In this way, we analysed the *Bdnf* expression levels in both hemispheres separately and we found that both *Mecp2*-null and wt mice exhibited similar levels of *Bdnf* transcript IV in the hippocampus, in both hemispheres ( $p>0.05$ ; figure 5.4).

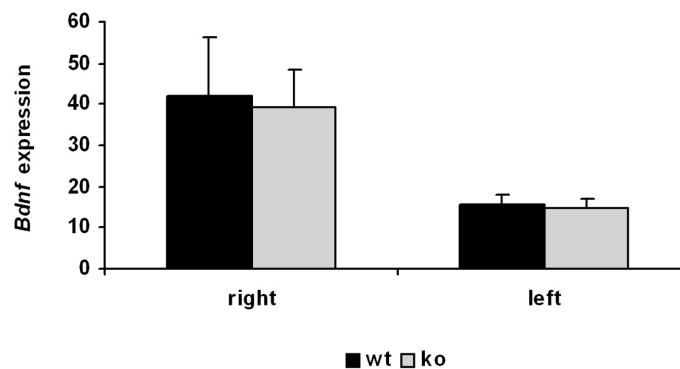


**Figure 5.4. *Bdnf* gene expression in the hippocampus of *Mecp2*-null mice.** Real time PCR analysis showed that the levels of *Bdnf* transcript IV were not significantly different between *Mecp2*-null and wt control mice hippocampi. (values are mean+sem; wt, wild-type; ko, *Mecp2*-null).

## 5.5. Discussion

Several lines of evidence support a role of MeCP2 in the mature brain, rather than during early CNS development. The first evidence comes from the timing of manifestation of the disease itself; RTT symptoms start to manifest between 6 and 18 months of age, long after primary neurogenesis has occurred. The generation of several mouse models of RTT has also shown that MeCP2 is not essential during embryonic development. Moreover, the proliferation or differentiation of embryonic neural precursors was not affected by absence of MeCP2 expression in a “neurosphere generation assay” (Kishi and Macklis 2004). Additionally, the highest levels of MeCP2 expression were found in the postnatal brain in regions such as the olfactory bulb, the cerebral cortex, the caudate-

putamen, the dentate gyrus, the brainstem and the cerebellum (see chapter 1, section 1.2.3).

The hippocampus is one of the regions that have persistent structural plasticity in the adult brain. Classically, it is known to have a role in cognitive processes like learning and memory formation, which are compromised in RTT. Changes in learning have been associated with changes in the levels of neurogenesis in the adult DG (Lemaire et al. 2000; Shors et al. 2002; Prickaerts et al. 2004).

In this study, we explored the possibility that dysfunction or loss of function of the transcriptional repressor MeCP2 is affecting adult neurogenesis in the DG. Our results show that four-week-old *Mecp2*-null mice presented a significantly higher cellular proliferation in the SGZ of the hippocampus. We observed no differences in the apoptosis levels, as compared to wt controls. Additionally, the percentage of the newly generated cells that differentiate into mature neurons (BrdU<sup>+</sup>/NeuN<sup>+</sup> cells) was high in both wt and *Mecp2*-null mice and similar between both genotypes, suggesting that neuronal survival is maintained. Moreover, the percentage of the new cells that express the glial marker GFAP (BrdU<sup>+</sup>/GFAP<sup>+</sup> cells) is also similar between both wt and *Mecp2*-null.

Our results are in agreement with other studies that have previously referred to the role of MeCP2 in adult cell proliferation and neurogenesis. Using a different mouse model and by analyzing neurogenesis in the olfactory epithelium, Matarazzo and colleagues (2004) found that cellular proliferation was transiently increased, without differences in apoptosis, in *Mecp2*-null mice as compared to wt controls, at four weeks of age.

In contrast to our data, in another study performed in the hippocampus of *Mecp2*-null mice no differences were observed in the cellular proliferation of four- and eight-week-old dentate gyrus granule cells (Smrt et al. 2007). The reason(s) for this discrepancy is currently unknown, but the fact that (1) different *Mecp2*-null mouse models were used (we used the *Mecp2*<sup>tm1.1Bird</sup> mouse, while the other study was performed with the *Mecp2*<sup>tm1.1Jae</sup>); (2) the protocols of BrdU administration are different (we performed a daily injection for three consecutive days *versus* daily injection for seven consecutive days in the other study) or (3) the methods used to quantify BrdU-labeling are different (we counted all BrdU-positive cells in one in every eighth section through entire hippocampus *versus* a random sampling stereology method) may underlie the differences observed.

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### **Increased proliferative activity observed in the dentate gyrus of *Mecp2*-null mice: possible mechanisms**

The increased cell proliferation we observed in the *Mecp2*-null mice could be attributed either to features of the surrounding environment of the hippocampus and adjacent brain regions, or to intrinsic properties of the neural precursors. Data from a “neurosphere generation assay” has shown a normal proliferative and differentiation capacity of neural stem cells derived from embryonic cortical cells of *Mecp2* mutant as compared to wt cells (Kishi and Macklis 2004), suggesting the later is not the most likely explanation for the higher proliferative capacity exhibited by *Mecp2*-null mice.

Several studies performed both in RTT patients and in the different mouse models of the disease reported a dysregulation of factors known to be involved in the regulation of adult cellular proliferation and neurogenesis.

#### **1. Neuromodulatory systems of the brain**

The neuromodulatory systems (5-HT, NE and DA) have been described to increase basal levels of adult hippocampal neurogenesis (reviewed in Mackowiak et al. 2004). The inhibition of 5-HT synthesis leads to a reduction in the proliferation of granule neurons in the rat DG hippocampus (Brezun and Daszuta 2000). The 5-HT<sub>1A</sub> receptor might be involved in the control of adult neurogenesis and of dendritic maturation in the hippocampus (Banasr et al. 2004). In fact, ko animals for the 5-HT<sub>1A</sub> receptor gene showed increased anxiety and altered dendritic maturation and neurogenesis (Santarelli et al. 2003); the 5-HT<sub>2A</sub> receptor has also been implicated in the extent of cell proliferation in the SGZ of the DG (Banasr et al. 2004). Administration of a selective NE neurotoxin (*N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine hydrochloride) was shown to reduce the proliferation of cells in the adult DG, but not the survival or differentiation of the granule cell progenitors (Kulkarni et al. 2002). Depletion of DA in rodents was also reported to decrease precursor cell proliferation in the SGZ (Hoglinger et al. 2004). The role of 5-HT and NE in neurogenesis has also been described by showing that 5-HT- or NE-selective reuptake inhibitor antidepressants can stimulate adult hippocampal neurogenesis (Duman et al. 2001).

The neuromodulatory monoaminergic systems such as the noradrenergic, dopaminergic and serotonergic have been suggested to be dysregulated in RTT patients, despite some data inconsistency (see chapter 4). More recently, studies performed with

mouse models of RTT, including our own (chapter 4) showed that the levels of NE and 5-HT, but also DA, were decreased in some regions of the brain of the *Mecp2*-null mice as compared to wt animals (Ide et al. 2005; Viemari et al. 2005). However, our neurochemical study showed that the levels of these neurotransmitters and their metabolites were not significantly different in the hippocampus region of *Mecp2*-null mice, at three weeks of age (see chapter 4). This suggests that the higher cellular proliferation observed in the DG-SGZ of *Mecp2*-null mice cannot be explained by altered local levels of monoamines.

## 2. Up-regulation of stress-responsive genes

Several findings indicate that adrenal steroids suppress the production of new neurons (Cameron et al. 1998; Gould and Tanapat 1999). Different glucocorticoid regulated genes, such as serum glucocorticoid-inducible kinase 1(*Sgk*) and FK506-binding protein 51 (*Fkbp5*), were found to be up-regulated in *Mecp2* mutant mice by transcriptional profiling (Nuber et al. 2005). Also, the corticotrophin-releasing hormone (*Crh*) was shown to be a direct target of MeCP2, and up-regulation of its levels might be responsible for the enhanced corticosterone response to stress exhibited by *Mecp2*<sup>308/Y</sup> mice (McGill et al. 2006). Hence, we would expect a decrease in cellular proliferation, which was not what we observed. Therefore, this not contributes to the higher cellular proliferation we observed in the *Mecp2*-null mice.

## 3. BDNF protein levels

Neurotrophins, like BDNF, play an important role during the CNS formation, in the regulation of neural survival and development but also in the mature brain, assuring the maintenance of its function and its plasticity (reviewed in Huang and Reichardt 2001). Several studies have established a positive correlation between BDNF levels and the extent of adult neurogenesis (Lee et al. 2002; Scharfman et al. 2005; Rossi et al. 2006).

We did not observe a difference in the basal levels of *Bdnf* transcript between four-week-old *Mecp2*-null and wt mouse hippocampi. In agreement, Moretti and colleagues (2006) observed no differences in the levels of *Bdnf* transcript in the hippocampus of the *Mecp2*<sup>308/Y</sup> transgenic mice as compared to wt levels.

*Bdnf* expression is directly regulated by MeCP2 (Chen et al. 2003; Martinowich et al. 2003). It was shown that, in the absence of neuronal activity, the levels of *Bdnf* transcript

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were higher in cultured *Mecp2*-null than in cultured wt cortical cells (Chen et al. 2003). However, MeCP2 does not affect the activity-dependent up-regulation of *Bdnf* (Chen et al. 2003). Hence, it would be plausible to expect that the levels of BDNF in *Mecp2*-null mice would be higher or equal to that of wt levels. This would be the case if the neuronal activity in both genotypes was similar, which in fact is not, as recently reported (Dani et al. 2005; Nelson et al. 2006). In fact, in the whole brain extract (and in dissected cortex and cerebellum brain region) of *Mecp2* mutant mice, the levels of BDNF protein were found to be decreased in relation to the wt levels, in symptomatic but not in asymptomatic ages (Chang et al. 2006). In summary, the global levels of BDNF also do not account for the differences we found in the adult SGZ-DG cellular proliferation.

#### 4. Neuronal activity

Dani and colleagues (2005) showed that in the *Mecp2*-null mice, already at two weeks of age, spontaneous activity of cortical pyramidal neurons is reduced due to a higher inhibition over excitation ratio. The miniature excitatory postsynaptic currents (mEPSCs), a synaptic transmission property, were also shown to be altered in *Mecp2*-null mice: *Mecp2*-null mice exhibited in pyramidal cortical cells a reduced mEPSCs amplitude (Dani et al. 2005), and, in hippocampal neurons, a decrease in mEPSCs frequency as compared to wt (Nelson et al. 2006).

The higher cellular proliferation found in the SGZ-DG of *Mecp2*-null mice may be due to an electrophysiological dysfunction of the hippocampus, namely due to a reduced neuronal activity. In fact, it has been shown that proliferation of cells in the adult hippocampus is decreased by administration of NMDA, and increased by administration of NMDA receptor antagonist. Moreover, lesions of the entorhinal cortex, which provides a major glutamatergic input to the hippocampus, increased hippocampal cell proliferation (Cameron et al. 1995; Gould et al. 2000; Nacher et al. 2001; Meltzer et al. 2005). This is very interesting, considering that the expression of NMDA receptors subtypes NR2A and NR2B was described to be altered in the hippocampus of symptomatic *Mecp2*-null mice: while the levels of NR2A were significantly reduced, the levels of NR2B were significantly increased as compared to wt controls (Asaka et al. 2006). Developmentally, the replacement of NR2B by NR2A occurs, which is linked to the ability of neural circuits to undergo experience- or activity-dependent synaptic plasticity (van Zundert et al. 2004). This does not seem to be happening properly in the *Mecp2*-null mice and so might be on the basis of altered neuronal activity.

In summary, our interpretation is that the higher cellular proliferation that we observed in the DG-SGZ could be due to a decreased neuronal activity of the *Mecp2*-null hippocampus.

### **Increased cellular proliferation in the adult hippocampus: the consequences**

Several experiments established a relationship between hippocampus-dependent learning and adult granule cell proliferation and neurogenesis in the adult DG, but some controversy persists, in particular to what refers to proliferation (Gould et al. 1999a; van Praag et al. 1999b; Lemaire et al. 2000; Prickaerts et al. 2004; Rola et al. 2004).

Additionally, an increase in the cellular proliferation may not necessarily promote normal function and be beneficial. For example, inappropriate migration and maturation of the new cells may impair hippocampal function. It has been shown that infusion of BDNF in the hippocampus of rat increased neurogenesis but also increased the number of new neurons in the hilar region of these animals (ectopia) (Scharfman et al. 2005). The negative effects of an increased neurogenesis have also been evidenced in the human temporal lobe epilepsy and in an animal model of epilepsy: the seizure-induced neurogenesis contributes to aberrant axonal reorganization, disrupted migration and hilar-ectopic localization of granule cells (Pierce et al. 2007; Scharfman and Gray 2007).

The increase in the cellular proliferation observed in the DG of *Mecp2*-null mice may be an adjustment of the system in order to maintain the homeostasis of the network, which may be altered due to a decrease of the neuronal activity, and ultimately to prevent more dramatic consequences of a deregulated hippocampal system.

Smrt and colleagues (2007), using a different mouse model of RTT (*Mecp2*<sup>tm1.1Jae</sup>), assessed long-term survival of the newly born DG neurons and found that the number of new cells that survived was similar between *Mecp2*-null and wt mice, and that they differentiate into the same type of granule cells (NeuN and GFAP) at equal ratios.

However, the same *Mecp2*-null mice (*Mecp2*<sup>tm1.1Jae</sup>) were described to exhibit a transient delay in the terminal differentiation of olfactory receptor neurons, at two-weeks of age (Matarazzo et al. 2004). At this age, the animals presented a higher number of immature neurons, a similar number of mature neurons but with a reduced cell in death of

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mature neurons and a delay in terminal differentiation. Evidence for altered neuronal maturation was also reported by studies in the hippocampus of *Mecp2*-null mice (*Mecp2*<sup>tm1.1Jae</sup>), which presented a failure in the maturation of the new neurons, at eight weeks of age, as exhibited by a higher percentage of “transitioning” neurons (DCX<sup>+</sup>/NeuN<sup>+</sup>) (Smrt et al. 2007). Thus, it will be also important to assess whether both differentiation and long-term survival would also be affected by MeCP2 dysfunction in our model.

In summary, the involvement of the hippocampus in RTT pathology is supported by the mental retardation phenotype presented by RTT patients and the behavioural learning and memory impairments seen in the different mouse models of the disorder. Additionally, neuroanatomical correlates of this cognitive dysfunction were found both in human RTT and *Mecp2* mutant mice brains, such as morphological (simplified dendritic arborizations) and electrophysiological (impaired LTP and LTD, decreased neuronal activity) impairments. So, it would be of great importance to understand the mechanisms by which impaired MeCP2 activity produces this component of the phenotype of RTT patients. The cognitive deficits and the anxiety of RTT patients could be due to alteration of the hippocampus function, through altered adult granule cell proliferation and/or neurogenesis.

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## CHAPTER 6

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### GENERAL DISCUSSION AND FUTURE PERSPECTIVES





## 6.1. General discussion

The first description of RTT was published in 1966 by Andreas Rett, but it took almost twenty years for the disorder to be internationally recognized (Hagberg et al. 1983), and more than fifteen years until, in 1999, causative mutations in the *MECP2* gene were reported by the group of Huda Zoghbi (Amir et al. 1999). Ever since, research in RTT has advanced vertiginously and this has become one of the most exciting and promising areas, and an example of research in Molecular Medicine. In less than ten years, several mouse models of the disorder were created (Chen et al. 2001; Guy et al. 2001; Shahbazian et al. 2002; Pelka et al. 2006) and made available for research. In the beginning of this year, researchers showed that it was possible to reverse the RTT-like symptoms in mice (Giacometti et al. 2007; Guy et al. 2007) and put RTT research one step forward and closer to its ultimate goal. In this context, ours is a small but hopefully significant contribution.

### How do mutations in MeCP2 affect the CNS in humans?

The determination of the spectrum of *MECP2* mutations and their associated phenotypes is important in clinical terms for a molecular diagnosis strategy, in the field of child neurology and psychiatry; it is also interesting from the functional genomics perspective, since the correlation between the loss of MeCP2 function(s) and the resulting phenotype(s) in humans may help to elucidate the function of this protein and of the pathways that it integrates in the normal development, maturation and function of the nervous system.

The MeCP2 protein seems not to be essential to the embryonic development of the nervous system, in general, not affecting embryonic neurogenesis or neuronal migration, but seems to be important to the maintenance of the nervous system, particularly to the appropriate formation of the synapse and its plasticity, essential to the functioning of the system. Its absence simultaneously affects cognition and motor control at the CNS level. It is also associated to the occurrence of epilepsy.

Interestingly, and in contrast to what was being proposed at the time of onset of this study, our genetic analysis of Portuguese population (Temudo et al, in preparation) revealed that the clinical picture associated with *MECP2* mutations really overlaps very strictly with the initial description of RTT, and very few cases do display the variant presentations that have been proposed. Those that constitute “variants”, can often be interpreted as milder or more

severe forms within a continuum of RTT presentations. Temudo et al (in preparation) proposed that the phenotype of MeCP2-positive patients can be classified in three major subtypes, within this continuum: the MR, AT and EP forms. This classification may be useful to define alternative pathways within the disorders.

### **Does disruption of different functional domains of the protein originate different phenotypes?**

Mutations in the *MECP2* gene are responsible for most cases of RTT, but also, at a much lower proportion, for a wide range of related neurodevelopmental disorders, including autism and mental retardation. RTT presents with a wide range of clinical signs that affect females at varying degrees of severity and that are not necessarily all of them present in every patient. The explanation for such a wide phenotypic heterogeneity must reside in the pleiotropic function(s) of the MeCP2 protein.

In the first place, the nature of *MECP2* mutations contributes to the variability seen in RTT clinical outcome: several different types of mutations (missense, nonsense, small and large rearrangements and splice mutations), spread throughout the entire gene, have been identified. The type and position of the mutation will affect the function of the protein differently by disruption of certain functional domains, hence originating specific phenotypes.

So the next question arises, why is the same mutation present in patients with different clinical phenotypes? For this also contributes the fact that *MECP2* gene is localized in the X-chromosome, which in females is subjected to the XCI phenomenon (lyonisation). Although in most of the patients we studied the XCI pattern at peripheral lymphocytes did not exhibit a general skewing, we do not know the XCI pattern in their brain.

Evidence points to a major role of MeCP2 as transcriptional repressor, through deacetylation of histones (Jones et al. 1998). In this way, the functional effect of mutations has been assessed as to whether they disrupt the binding of MeCP2 to methylated DNA, its nuclear transport and repression capacity, and/or its expression levels (Yusufzai and Wolffe 2000; Kudo et al. 2001; Georgel et al. 2003; Kudo et al. 2003; Petel-Galil et al. 2006). However, for some *MECP2* mutations associated with classical RTT, these functions do not appear to be impaired and the mechanism through which they cause RTT is not known yet. These mutations could for example affect the interaction of MeCP2 with other proteins, such

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as ATRX (Nan et al. 2007), YB-1 (Young et al. 2005) and DNMT (Kimura and Shiota 2003), described as MeCP2 partners. The potential effect of mutations in these MeCP2 interactions and their functional consequences has not been extensively explored.

We made an original attempt of a genotype-phenotype correlation in a RTT *MECP2* mutation-positive population. Our goal was to “map” a specific phenotype to a disruption of a certain functional domain. According to our data, mutations that originated a null allele and those that affected the NLS function (such as R168X), hence aborting completely its repression capacity, were predominantly associated with the more severe forms of the disease (EP), with a high motor incapacity. Additionally, the T158M mutation, described to confer an intermediate impairment to MeCP2 function is predominantly abundant in the AT group of patients, who present a form of the disease that can be considered of intermediate severity. Interestingly, the R133C mutation, described to disrupt the binding of MeCP2 to ATRX (Nan et al. 2007), but not to affect its binding to methylated DNA (Kudo et al. 2001) was present predominantly in the MR group.

Our results show that if *MECP2* mutations are classified based on what is known about their functional effect(s) a tighter correlation may be found with their phenotypic manifestation. This needs to be explored in larger patient series.

### **Do mutations in non-coding regions of *MECP2* cause neurodevelopmental disorders?**

In a significant proportion of RTT cases without a genetic cause, mutations in the non-coding regions of *MECP2* and the involvement of another gene in the RTT aetiology have been considered. However, and in spite of its striking inter-species conservation, our data suggested that the 3'UTR may not be an important source of *MECP2* mutations. The resemblance between the phenotypes of *MECP2* mutation-positive and *MECP2* mutation-negative clinically diagnosed RTT patients may suggest that common MeCP2-related biochemical pathways might be affected; this may provide additional candidate genes to search for potential mutations.

### **How does absence of MeCP2 affect CNS function in the mouse?**

The genotype-phenotype correlation that we described in the human RTT patients also seems to apply to the mouse, i.e., mouse models that had a more severe mutation, such as

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the *Mecp3*-null models (Chen et al. 2001; Guy et al. 2001) are more severely impaired and die within the first 6 to 10 weeks of age (in the case of males). We identified four mutations that we predict to be null alleles (due to NMD) and all these four mutations were present in patients in the EP (more severe) form of the disease. Conversely, mice that have a hypomorphic *Mecp2* allele (*Mecp2*<sup>308</sup>; an allele that truncates the protein at position 308), known to produce a milder phenotype in RTT patients, predominantly display a behavioural/cognitive phenotype. For example, the two mutations that we detected around MeCP2 aminoacid position 308 (I303fs and V300fs), giving rise to similarly truncated versions of the protein, were both present in patients with the MR form.

In this way, the analysis of allelic series of mice, with *Mecp2* alleles mutated at functionally interesting sites, will be crucial to study the functional effect of different mutations and unveil their phenotype; and hence (1) better understand the role of different functional domains of MeCP2, (2) characterize the pathways involved and (3) develop differential therapeutic approaches directed for each specific phenotype (pharmacogenomics).

The RTT mouse model that we used in this study (*Mecp2*<sup>tm1.1Bird</sup>; (Guy et al. 2001) parallels a specific group of mutations present in RTT-total loss of function. The phenotype of this model has been partially described in respect to behavioural, neuroanatomical and electrophysiological aspects (see chapter 1); we, in particular, focused on the very early stages of the disease, in an attempt to identify the primary lesion(s) associated with their RTT-like phenotype.

It is now known that in the apparently normal initial period of development of RTT patients subtle, but significant, abnormalities may be recognized (Huppke et al. 2003; Burford 2005; Einspieler et al. 2005a; Einspieler et al. 2005b; Segawa 2005; Temudo et al. 2007). Careful and systematic observation of RTT patients allows the identification of motor development anomalies from the first days after birth, which are thought to originate in brainstem structures (Einspieler et al. 2005a; Segawa 2005). Of course this new evidence has (1) direct implications in the early diagnosis of RTT, when intervention is likely to be most effective and future management of the disorder and (2) provides clues on the primary lesion of RTT pathology.

We analysed the postnatal neurodevelopmental period of *Mecp2*-mutant mice (null males and heterozygous females) and showed that several abnormalities in the achievement

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and establishment of neurological reflexes were present (chapter 3, part I). Neurological reflexes are useful in assessing the degree of neural maturation and reliable indicators of normal development (Fox 1965). In this way, these abnormalities constitute the first sign of early neurological pathology in the *Mecp2*-mutant mice. Additionally, the altered neurological reflexes had in common the fact of being sensitive to the function of the vestibular system, which is involved in motor development and activity, and so they depend largely on brainstem (medullary) structures (Altman and Sudarshan 1975). Thus, our data is particularly interesting in the light of the studies in human RTT patients that suggest dysfunction of the brainstem, where the vestibular system is located, as responsible for the early stages of the natural history of disease (Einspieler et al. 2005b; Segawa 2005).

We further characterized the early motor impairments of this RTT mutant mouse model, but also their motor performance at later ages, when overt symptoms are established. *Mecp2*-null mice exhibit motor impairments already at three weeks of age, such as a higher latency to initiate a voluntary movement and a gait pattern characterized by a higher front- and hind-base width, with normal activity levels. Reduced exploratory activity however has been noticed at four weeks of age in this model, and at five weeks of age co-ordination impairments were also present (Guy et al. 2001; Santos et al. 2006). These motor impairments resemble the gait apraxia and ataxia that characterizes RTT pathology (Kerr and Engerstrom 2001; Segawa 2001).

Thus, the *Mecp2*-null mouse model in particular appears to mimic very well all the motor profile of RTT (in our opinion not so much the emotional aspects of it) and with this work, we defined the first behavioural alterations to be noticed. This will be of most relevance when the time arrives to test the efficiency of potential therapies to RTT, since critical time-windows and “clinical” markers are now defined.

As discussed, our data from the neurodevelopmental study and the motor behaviour characterization of the *Mecp2*-null mice suggested the possible involvement of different brain areas in the RTT-like phenotype in mice. Undoubtedly, the vestibular system was one of these areas, but others also could be involved such as the caudate-putamen, the cerebellum and eventually the cortex, given their role in the motor control. The inability to initiate a voluntary motor response (akinesia) is one of the outcomes of basal ganglia dysfunction, particularly involving the caudate-putamen and cortical dysfunction (Hauber 1998) and the ataxia may involve the cerebellum, but also caudate-putamen. We then proceeded to clarify

the basis of the observed neurological dysfunction. Our first approach was to perform a volumetric analysis of the PFCx, MCx and CPu regions, which did not reveal any significant differences between wt and *Mecp2*-null mice, when data was corrected for total brain size (data not shown). Next, a neurochemical study of different brain areas of the *Mecp2*-null mice was performed, in order to (1) characterize the neural substrates underneath this motor impairment and (2) clarify the controversial role of the neuromodulatory systems of the brain in RTT pathology.

The results from the neurochemical study were quite surprising and exciting. The global involvement of 5-HT and NE in human RTT and RTT-like phenotype in mice had already been reported (Ide et al. 2005; Viemari et al. 2005). Our data showed that both 5-HT and NE are the main monoamines involved in the pathology of RTT, and added the concept that they are both reduced already at three weeks of age. Moreover, we showed the reduction of 5-HT and NE specifically in the PFCx and MCx brain regions from three weeks of age. NE was also reduced in the vestibular nuclei, thus confirming the neural basis of the altered neurological reflexes.

Extensive monoamine disturbances were also found at eight weeks of age in the cerebellum and hippocampus, suggesting an involvement of these brain areas at later stages, most likely in the progression of the RTT-like disease.

In summary, at three weeks of age *Mecp2*-null mice (1) had normal spontaneous locomotion, (2) had impairments in the ability to initiate a voluntary movement and a gait pattern with a wide base suggestive of ataxia, (3) had decreased levels of NE and 5-HT specifically in the PFCx and MCx brain regions. Several processes precede the movement onset and it appears that the initial problem in the motor component of the phenotype of *Mecp2*-null mice is related not to the execution of the movement but more so to a problem at the mid- and higher levels of motor control, which are involved in the planning and coordination of movement. In this way, forebrain structures could potentially be as important as the brainstem, which is widely considered in the literature as the origin of the problem, in the initial establishment of the motor profile of RTT (Einspieler et al. 2005b; Segawa 2005). These new data may lead us to re-direct our focus and re-think about the aetiology of this disorder, bringing the discussion of the origin of RTT pathology to more frontal/superior regions of the brain. In accordance with our results is the RTT-like phenotype exhibited by the conditional *Mecp2*-null mouse restricted to forebrain structures (Gemelli et al. 2005).

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Nevertheless, the decreased NE levels we found in the vestibular system suggest that the brainstem is equally involved from the early beginning, which is also in agreement with our results on the abnormal development of neurological reflexes of the *Mecp2*-null mice (chapter 3-I) that suggested impaired neurodevelopment of pathways within the brainstem (vestibular nuclei) (Santos et al. 2006).

In terms of the molecular mechanism underlying the observed monoaminergic imbalances, one could speculate that the reduced levels of 5-HT and of NE in the PFCx and MCx brain regions may potentially result from the low expression of the serotonergic *Htr2a*, *Htr3a* in both areas, which may result, during the developmental stage, in the impaired reinforcement of the serotonergic synapses, thus leading to their loss, and consequent reduction of 5-HT in the projections regions. In parallel, the reduction of the expression levels of the noradrenergic *Adra2a* receptor may be a cause of noradrenergic synapse loss during development and consequent NE reduction. Furthermore, the *Adra2a* receptor also plays a role in the release of both NE and 5-HT.

Moreover the role of NE and of 5-HT, this last one mediated through the *Htr2a* receptor, in inducing cortical excitatory postsynaptic potentials (EPSP) has been described (Aghajanian and Marek 1997; Aghajanian and Marek 1999). Interestingly, reduced cortical and hippocampal EPSPs were also reported in the *Mecp2*-null mouse (Dani et al. 2005; Nelson et al. 2006).

As mentioned, the mRNA expression levels of NE transporter (NET) were reduced in the *Mecp2*-null mouse. This reduction is probably a consequence of the long-term reduced NE levels. Intriguingly, the levels of *Adra2a* receptor were not increased, although it is known that the NET ko mouse model has increased levels of *Adra2a* (Gilsbach et al. 2006). Additionally, it is now known that the antidepressant effect of desipramine, a NET antagonist, is mediated through the *Adra2a* receptors. This is interesting since very recently it was found that the treatment of *Mecp2*-null mice with desipramine alleviates their breathing impairment (Roux et al. 2007; Zanella et al. 2007). Since our data suggest an inability of *Mecp2*-null mice to appropriately increase transcription of several monoamine related genes, it will be interesting to evaluate in the long-term the effect of these drugs upon the brain circuits of *Mecp2*-null mice; particularly to evaluate whether the changes detected in the

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levels of noradrenergic receptors are a cause or a consequence of the neurotransmitter depletion.

One more interesting note is that the *Bdnf* conditional ko mice had normal presynaptic serotonergic function, but an abnormal 5-HT<sub>2A</sub>-mediated glutamate and GABA postsynaptic potential, reduced prefrontal cortex levels of 5-HT<sub>2A</sub> and reduced EPSP (Rios et al. 2006). This is interesting given that the cortical levels of BDNF are also reduced in *Mecp2*-null mice. Thus, it may be that part of the neurochemical imbalance is a downstream effect of loss in RTT of appropriate BDNF expression (Chen et al. 2003; Martinowich et al. 2003; Chang et al. 2006)

Although more work needs to be performed to clarify the source of the observed early monoaminergic imbalance, we have further characterized it, and we may have identified potential new targets for drugs to use in RTT pathology.

Where to go from here? Future studies should focus on understanding of what is on the basis of the dysfunction of the monoaminergic systems. Challenging *Mecp2*-null mice and wt controls with NE and 5-HT modulating agents (particularly directed to *Htr2a*, *Htr3a* and *Adra2a* receptors and NET mediated actions) to: (1) evaluate their behavioural attention and motor performance, (2) evaluate the release of the neurotransmitters by microdialysis experiments and (3) by micro positron emission tomography scan assess PFCx and MCx activation.

This knowledge is essential for clarify the role of MeCP2 in the proper function of noradrenergic and serotonergic neurons and for the development of proper therapies for RTT.

### **Increased postnatal neurogenesis**

The involvement of the hippocampus in RTT pathology is supported by the cognitive deficits phenotype presented by RTT patients and the behavioural learning and memory impairments and its neuroanatomical correlates seen in the different mouse models of the disorder (Kishi and Macklis 2004; Moretti et al. 2006; Pelka et al. 2006).

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One question that arises is whether the mental retardation component of RTT is a neurodevelopmental problem or a consequence of the lack of plasticity in the hippocampus due to later impairments?

Synaptic impairments, such as LTP and LTD, which are the basis of learning and memory, were not detected at four, but only at eight weeks of age in the *Mecp2*-null mouse (Asaka et al. 2006). Additionally, we did not detect, at three weeks of age, impaired performance of *Mecp2*-null mice in the homing test, which assesses juvenile motor behaviour (data not shown). This could be due to specific features of our model of study, but it also could suggest that the cognitive dysfunction arises as a consequence of other neurological impairments.

The recent and extremely surprising finding that it is possible to reverse the phenotype of *Mecp2*-mutant mice, once the symptoms were established suggests that the problem must not be a developmental one, since this would cause a permanent damage to the brain (Giacometti et al. 2007; Guy et al. 2007). This raises another question emerges that may direct the research in RTT in the next years: is RTT a neurodevelopmental or a neurodegenerative disorder? Degeneration is now known to occur mostly at synaptic level in some situations, without affecting the neuronal cell body, and this could be the case in RTT.

Finally, although we had not characterized ourselves the cognitive impairment in this model, given that we did not find neurochemical imbalances in the hippocampus at an early age and given the preliminary results of the homing test, we explored the idea that the cognitive defects in this region could correlate with impaired neurogenesis. Neurogenesis is a BDNF-dependent process, known to be affected by neurotrophins and corticosteroid signalling, both of which found to be affected in RTT (Chen et al. 2003; Martinowich et al. 2003; Nuber et al. 2005; Chang et al. 2006; McGill et al. 2006), and by neuromodulatory systems (which we have shown are affected). Furthermore, neuronal activity also modulates the hippocampal neurogenesis, and *Mecp2*-null mice were described to have an imbalance in the excitatory versus inhibitory cortical and hippocampal activity (Dani et al. 2005; Nelson et al. 2006).

We found an altered neurogenesis in the SGZ-DG of the hippocampus of *Mecp2*-null mice. Although another study did not corroborate our data (Smrt et al. 2007), Matarazzo and

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colleagues (2004) have also showed a transient increase of the cellular proliferation in the olfactory epithelium, a region which also displays adult neurogenesis.

Our results are consistent with a loss of balance in which a decreased neuronal activity leads to increased neurogenesis, as discussed by (Gould et al. 2000; Mackowiak et al. 2004). An increase in neurogenesis may not necessarily promote normal function and be beneficial. Whether the increase we showed in the neurogenesis of the SGZ is a disruptive effect or an effort of the system to cope with injury (or both simultaneously) remains to be determined.

Although according to our data the local levels of monoamines are not the main contributors to the altered hippocampal neurogenesis at four weeks of age, an indirect effect of the decrease of monoamines, through connections of other brain areas within the hippocampus (and hence, activity), such as the frontal cortices, where noradrenergic and serotonergic systems are de-regulated, may be of relevance.

In this context, it is interesting to notice that serotonin was found to induce a marked increase in glutamatergic spontaneous excitatory postsynaptic currents EPSCs in apical dendrites of layer V pyramidal cells of prefrontal cortex; this effect was mediated by 5-HT receptors *Htr2a*, which we showed were reduced in the *Mecp2*-null (Aghajanian and Marek 1997; Aghajanian and Marek 1999). Also, NE enhances neurotransmitter release from glutamate terminals that innervate apical dendrites of layer V pyramidal cells. (Marek and Aghajanian 1999). In this way, the decrease of 5-HT and NE levels we observed in the cortex, may also contribute to the reduced neuronal activity and, indirectly, to the increased neurogenesis in the hippocampus, through the hippocampus-cortex projections.

## 6.2. Future perspectives

In order to answer some of the questions raised in this work, it would be interesting to:

1. Implement a “dynamic research program” in order to identify within the *MECP2* mutation-negative population specific phenotypes that could share the same affected pathways and search for mutations in genes acting downstream of MeCP2.
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2. Generate an allelic series of transgenic mice with mutations affecting the function of specific MeCP2 domains and characterize their phenotypes.
  3. Confirm the involvement of the *Htr2a*, *Htr3a*, *Adra2a* and NET in the pathology of RTT, and clarify the mechanism of causality of the monoaminergic imbalance.
  4. Assess the functional ability or potentially disruptive role of the newly generated neurons in the hippocampus of *Mecp2*-null mice.
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APPENDIX I

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SUPPLEMENTARY TABLES



**Table S2.1.** Primer pairs and their sequences, PCR segments size and Ta in the scanning of *MECP2* by SSCP/sequencing.

| Gene         | Exon | Fragment | Primer   | Sequence (5'>3')       | Size (bp) | Tm (°C) | Ta (°C) |
|--------------|------|----------|----------|------------------------|-----------|---------|---------|
| <i>MECP2</i> | 2    |          | RTT2F(2) | TGTGTTTATCTTCAAAATGT   | 273       | 50      | 56,5    |
|              |      |          | RTT2R    | AGATGGCCAAACCAGGACAT   |           | 60      |         |
|              |      | 3.1      | RTT31F   | CCTGCCTCTGCTCACTTGTT   | 196       | 62      | 59      |
|              |      |          | RTT31R   | GGCTGATGGCTGCACGGGCT   |           | 68      |         |
|              | 3    | 3.2      | RTT32F   | AGCCCGTGCAGCCATCAGCC   | 165       | 68      | 50      |
|              |      |          | RTT32R   | GGGGTCATCATACATGGGTC   |           | 62      |         |
|              |      | 3.3      | RTT33F   | GACCCATGTATGATGACCCC   | 206       | 62      | 57      |
|              |      |          | RTT33R   | GTTCCCCCGACCCACCCT     |           | 70      |         |
|              |      | 4.1      | RTT41F   | TTTGTCTAGAGCGTTGTCACC  | 380       | 60      | 60      |
|              |      |          | RTT41R   | CTTCCCAGGACTTTTCTCCA   |           | 60      |         |
|              |      | 4.2      | RTT42F   | AACCACCTAAGAAGCCCCAAA  | 380       | 58      | 54      |
|              |      |          | RTT42R   | CTGCACAGATCGGATAGAAGAC |           | 66      |         |
|              | 4    | 4.3      | RTT43F   | GGCAGGAAGCGAAAAGCTGAG  | 366       | 66      | 60      |
|              |      |          | RTT43R   | TGAGTGGTGGTGATGGTGG    |           | 60      |         |
|              |      | 4.4      | RTT44F   | TGGTGAAGCCCCTGCTGGT    | 414       | 62      | 60      |
|              |      |          | RTT44R   | CTCCCTCCCCTCGGTGTTTG   |           | 66      |         |
|              |      | 4.5      | RTT45F   | GGAGAAGATGCCAGAGGAG    | 411       | 64      | 60      |
|              |      |          | RTT45R   | CGGTAAGAAAAACATCCCCAA  |           | 60      |         |

**Table S2.2.** Primer pairs and their sequences, PCR fragments size and Ta of the AS-PCR.

| Gene             | Exon                | Primer                | Sequence (5'>3')       | Size (bp) | Tm (°C) | Ta (°C) |
|------------------|---------------------|-----------------------|------------------------|-----------|---------|---------|
| MECP2            | Recurrent mutations |                       |                        |           |         |         |
|                  | 3                   | RTTnR106W_F           | CTGCCTGAAGGCTGGACAC    | 133       | 62      | 59,2    |
|                  |                     | RTTmR106W_F           | CTGCCTGAAGGCTGGACAT    |           | 60      | 64,5    |
|                  |                     | RTTR106W_R            | GATCCTTGTCCTGCCCTCC    |           | 66      |         |
|                  | 4                   | RTTnR133C_F           | TCCCCAGGGAAAAGCCTTTC   | 296       | 62      | 57,8    |
|                  |                     | RTTmR133C_F           | TCCCCAGGGAAAAGCCTTTT   |           | 60      | 52,9    |
|                  |                     | RTTR133C_R            | CTTGACAAGGAGCTTCCCAG   |           | 62      |         |
|                  | 4                   | RTTnT158M_F           | GCTCTAAAGTGGAGTTGATTGC | 174       | 64      | 56,8    |
|                  |                     | RTTmT158M_F           | GCTCTAAAGTGGAGTTGATTGT |           | 58      | -       |
|                  |                     | RTTT158M_R            | CCCCGGCCTCTGCCAGTTCC   |           | 70      |         |
|                  | 4                   | RTTnR168X_R           | TTAGGTGGTTTCTGCTGTCTG  | 238       | 60      | 54,9    |
|                  |                     | RTTmR168X_R           | TTAGGTGGTTTCTGCTGTCA   |           | 58      | 52,3    |
|                  |                     | RTTR168X_F            | TTGTCACCACCATCCGCTCTG  |           | 66      |         |
|                  | 4                   | RTTnR255X_F           | AAACGCCCCGGCAGGAAGC    | 100       | 64      | 64,6    |
|                  |                     | RTTmR255X_F           | AAACGCCCCGGCAGGAAGT    |           | 62      | 64,6    |
| RTTR255X_R       |                     | CGGCGGCAGCGGCTGCCACC  | 74                     |           |         |         |
| Unknown function |                     |                       |                        |           |         |         |
| 4                | RTTnK305R_F         | GACCGTACTCCCCATCAAGAA | 207                    | 64        | 66      |         |
|                  | RTTmK305R_F         | GACCGTACTCCCCATCAAGAG |                        | 66        |         |         |
|                  | RTT43R              | TGAGTGGTGGTGATGGTGG   |                        |           |         |         |

**Table S2.3.** Primer pairs and their sequences, PCR segments size and Ta used in the direct sequencing of *MECP2*.

| Gene         | Exon | Primer    | Sequence (5'>3')       | Size (bp) | Tm (°C) | Ta (°C) |
|--------------|------|-----------|------------------------|-----------|---------|---------|
| <i>MECP2</i> | 1    | RTT1F(3)  | CCAAAGGGCGGGGCGCGAC    | 256       | 68      | 63      |
|              |      | RTT1R(3)  | TCCGCCAGCCGTGTCGTCCG   |           | 70      |         |
|              | 2    | RTT2F(3)  | AAATGTCCCAAATAGCCCTGG  | 273       | 62      | 56      |
|              |      | RTT2R     | AGATGGCCAAACCAGGACAT   |           | 60      |         |
|              | 3    | RTT31F    | CCTGCCTCTGCTCACTTGTT   | 526       | 62      | 64      |
|              |      | RTT33R    | GTTCCCCCGACCCACCCCT    |           | 70      |         |
|              |      | RTT41F(2) | GTCACCACCATCCGCTCTGC   | 616       | 66      | 64      |
|              |      | RTT42R    | CTGCACAGATCGGATAGAAGAC |           | 66      |         |
|              | 4    | RTT43F    | GGCAGGAAGCGAAAAGCTGAG  | 614       | 66      | 64      |
|              |      | RTT44R    | CTCCCTCCCCTCGGTGTTTG   |           | 66      |         |
|              |      | RTT45F    | GGAGAAGATGCCAGAGGAG    | 423       | 64      | 63      |
|              |      | RTT45R    | CGGTAAGAAAAACATCCCCAA  |           | 60      |         |

**Table S2.4.** Primer pairs and their sequences, PCR segments size and Ta used in the PCR and sequencing of the 3'UTR of *MECP2*.

| Gene         | Region | NM_004992  | Primer | Sequence (5'>3')         | Size (bp) | Tm (°C) | Ta (°C) |
|--------------|--------|------------|--------|--------------------------|-----------|---------|---------|
| <i>MECP2</i> | 3'UTR  | 1607-1956  | MD14   | CGTGACCGAGAGAGTTAGC      | 350       | 60      | 55      |
|              |        |            | MU14   | CGGGAGGGGAGGTGCC         |           | 58      |         |
|              |        | 2561-2891  | MD15   | CCAGTTACTTTCCAATTCTCC    | 331       | 58      | 55      |
|              |        |            | MU15   | AGAAGTGAAAGGATGAAATGAA   |           | 58      |         |
|              |        | 3551-3805  | MD16   | GCTTAGAGGCATGGGCTTG      | 255       | 60      | 55      |
|              |        |            | MU16   | CAGCAGCTCACATGGGACA      |           | 60      |         |
|              |        | 3768-4128  | MD17   | CCAGAAACACCCACAGGCA      | 361       | 60      | 55      |
|              |        |            | MU17   | TTGGGCTGATGGGAGTTTG      |           | 58      |         |
|              |        | 6851-7029  | MD18   | GCAGATGAGGTGAAAAGGC      | 179       | 58      | 55      |
|              |        |            | MU18   | GCAAAACAAAAGCCCAGGAT     |           | 58      |         |
|              |        | 7116-7436  | MD19   | CTGTATATTGCACAATTATAAAC  | 321       | 58      | 55      |
|              |        |            | MU19   | ACCCAGAACCTTGGGACC       |           | 58      |         |
|              |        | 8372-8645  | MD20   | GGAGTGCAAAGGCTTGCA       | 274       | 58      | 55      |
|              |        |            | MU20   | GAAAAACCCAGAAAGACAAG     |           | 60      |         |
|              |        | 8607-8872  | MD21   | TTCCTTCTTTGCCCTTTACTTGTC | 266       | 58      | 55      |
|              |        |            | MU21   | GTAAAGAAAAAGTGTCTAGAAAT  |           | 58      |         |
|              |        | 9844-10182 | MD22   | GGCCGGGACACACTTAGC       | 339       | 60      | 55      |
|              |        |            | MU22   | AAATTTATAAGGCAAACTCTTTAT |           | 58      |         |

**Table S2.5.** Primer pairs and their sequences, PCR segments size and Ta used in the RD-PCR of *MECP2* gene

| Gene         | Group | Exon | Primer      | Sequence (5'>3')                  | Size (bp) | Tm (°C) | Ta (°C) |
|--------------|-------|------|-------------|-----------------------------------|-----------|---------|---------|
| <i>MECP2</i> | I     | 2    | MECP2-2-D   | GGCCAAGTGT TTTAGTCTTTGGGTACTTTTA  | 492       | 58      | 55      |
|              |       |      | MECP2-2-U   | GGCCAAGTGT GGCTTGTGATAGTGTGATTCT  |           | 62      |         |
|              |       | 3    | MECP2-3-D   | GGCCAAGTGT ACCTGGTCTCAGGTTTATTGT  | 486       | 62      | 55      |
|              |       |      | MECP2-3-U   | GGCCAAGTGT CTTCAAGGAAGAAAAGTCAGAA |           | 62      |         |
| <i>ATM</i>   |       | 12   | ATM-D       | GGCCAAGTGT ATCCTGCAAGTTTACCTAAC   | 418       | 56      |         |
|              |       |      | ATM-U       | GGCCAAGTGT GATCAGGGATATGTGAGTGT   |           | 58      |         |
| <i>MECP2</i> | II    | 4I   | MECP2-4I-D  | GGCCAAGTGT CTTTGTGAGAGCCCTACCCATA | 447       | 66      | 60      |
|              |       |      | MECP2-4I-U  | GGCCAAGTGT CCACCATCACCACCACTCAGAG |           | 70      |         |
|              |       | 4II  | MECP2-4II-D | GGCCAAGTGT CCCCCTGGCGAAGTTTGAAAAG | 400       | 68      | 60      |
|              |       |      | MECP2-4II-U | GGCCAGTGT CCACCATCCGCTCTGCCCTATC  |           | 72      |         |
| <i>FUT</i>   |       | FUT2 | FUT-D       | GGCCAAGTGT TTCACCGGCTACCCCTGCTC   | 504       | 66      |         |
|              |       |      | FUT-U       | GGCCAAGTGT GGAGTCGGGGAGGGTGTAAT   |           | 64      |         |

**Table S2.6.** Primer pairs and their sequences, probe size and Ta of the amplification of probes used in the Southern blot.

| Gene         | Probe         | Primer   | Sequence (5'>3')      | Size (bp) | Tm (°C) | Ta (°C) |
|--------------|---------------|----------|-----------------------|-----------|---------|---------|
| <i>MECP2</i> | <b>RTT2</b>   | RTT2F(2) | TGTGTTTATCTTCAAAATGT  | 273       | 50      | 56,5    |
|              |               | RTT2R    | AGATGGCCAAACCAGGACAT  |           | 60      |         |
|              | <b>RTT3</b>   | RTT31F   | CCTGCCTCTGCTCACTTGTT  | 526       | 62      | 64      |
|              |               | RTT33R   | GTTCCCCCGACCCACCCT    |           | 70      |         |
|              | <b>p(A)10</b> | p(a)10F  | GGCCGGGACACACTTAGC    | 424       | 60      | 55      |
|              |               | p(a)10R  | CTGCCCATCTTTTCCAATAGT |           | 60      |         |

**Table S2.7.** Primer pair and their sequences, PCR segment size and Ta used in the XCI assay

| Gene      | Exon | Primer | Sequence (5'>3')         | Size (bp) | Tm (°C) | Ta (°C) |
|-----------|------|--------|--------------------------|-----------|---------|---------|
| <i>AR</i> | 1    | RTTX_F | TCCAGAATCTGTTCCAGAGCGTGC | 279       | 74      | 60      |
|           |      | RTTX_R | GCTGTGAAGGTGCTGTTCTCAT   |           | 72      |         |

**Table S2.8.** Primer pairs and their sequences, PCR segments size and Ta used in the PCR and sequencing of *NLGN3* and *NLGN4* genes.

| Gene         | Exon | Primer | Sequence (5'>3')      | Size (bp) | Tm (°C) | Ta (°C) |
|--------------|------|--------|-----------------------|-----------|---------|---------|
| <i>NLGN3</i> | 6    | NLGN3F | GGTGTCTCTGGCACTGACTT  | 511       | 62      | 60      |
|              |      | NLGN3R | AGGTTTAGCTAGAGGAGCAGA |           | 62      |         |
| <i>NLGN4</i> | 5    | NLGN4F | ATCCTGATGGAGCAAGGCGA  | 565       | 62      | 60      |
|              |      | NLGN4R | ATACCCCAACACGAAGATGAA |           | 60      |         |

**Table S4.1.** Primer pairs and their sequences, PCR segment size and Ta used in the qRT-PCR of 5-HT and NE receptors and transporters.

| Gene          | Primer    | Sequence (5'>3')      | Size (bp) | Tm (°C) | Ta (°C) |
|---------------|-----------|-----------------------|-----------|---------|---------|
| <i>Htr1a</i>  | mHtr1a_F  | CTGTTTATCGCCCTGGATGT  | 168       | 60      | 61      |
|               | mHtr1a_R  | GAGAAAGCCAATGAGCCAAG  |           | 60      |         |
| <i>Htr2a</i>  | mHtr2a_F  | AGCGGTCCATCCACAGAG    | 108       | 58      | 59      |
|               | mHtr2a_R  | AACAGAAAGAACACGATGC   |           | 54      |         |
| <i>Htr2b</i>  | mHtr2b_F  | CCCTTGGAGTCGTGTTTTTC  | 148       | 60      | 61      |
|               | mHtr2b_R  | CCCGAGGAAACGTAGCCTAT  |           | 62      |         |
| <i>Htr3a</i>  | mHtr3a_F  | CGGCAGTACTGGACTGATGA  | 127       | 62      | 59      |
|               | mHtr3a_R  | CCACGTCCACAACTCATTG   |           | 60      |         |
| <i>Adra2a</i> | mAdra2a_F | CAGGCCATCGAGTACAACCT  | 192       | 62      | 63      |
|               | mAdra2a_R | TCTGGTCGTTGATCTTGACAG |           | 60      |         |
| <i>Adrb2</i>  | mAdrb2_F  | TTCGAAAACCTATGGGAACG  | 121       | 58      | 58      |
|               | mAdrb2_R  | GGGATCCTCACACAGCAGTT  |           | 62      |         |
| <i>Slc6a4</i> | mSert_F   | ACTCCGCAGTTCCCAGTACA  | 181       | 62      | 63      |
|               | mSert_R   | GTAGGGAAAACGCCAGATGT  |           | 60      |         |
| <i>Slc6a2</i> | mNet_F    | GCAAAACCGCCGATCTACTA  | 192       | 60      | 63      |
|               | mNet_R    | CCACCACCATTCTTGTAGCA  |           | 60      |         |
| <i>Hprt</i>   | Hprt_F    | GCTGGTGAAAAGGACCTCT   | 249       | 58      | 59      |
|               | Hprt_R    | CACAGGACTAGAACACCTGC  |           | 62      |         |

**Table S5.1.** Primer pairs and their sequences, PCR segments size and Ta used in qRT-PCR

| Gene        | Primer     | Sequence (5'>3')        | Size (bp) | Tm (°C) | Ta (°C) |
|-------------|------------|-------------------------|-----------|---------|---------|
| <i>Bdnf</i> | mBdnf_IV_F | CAGGAGTACATATCGGCCACCA  | 300       | 68      | 63      |
|             | mBdnf_R    | GTAGGCCAAGTTGCCTTGTCCGT |           | 72      |         |
| <i>Hprt</i> | Hprt_F     | GCTGGTGAAAAGGACCTCT     | 249       | 58      | 59      |
|             | Hprt_R     | CACAGGACTAGAACACCTGC    |           | 62      |         |

## APPENDIX II

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### PUBLISHED ARTICLES





**ARTICLE 1**

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“Chromatin remodelling and neuronal function: exciting links”

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## Review

# Chromatin remodeling and neuronal function: exciting links

M. Santos<sup>†,‡</sup>, P. A. Coelho<sup>§</sup> and P. Maciel<sup>\*,†,‡</sup>

<sup>†</sup>Life and Health Sciences Research Institute, Health Sciences School, University of Minho, Braga, <sup>‡</sup>Institute for Biomedical Sciences Abel Salazar, University of Porto, and <sup>§</sup>Laboratório de Genética Molecular da Mitose, Instituto de Biologia Molecular e Celular, Porto, Portugal

\*Corresponding author: P. Maciel, PhD, Life and Health Sciences Research Institute, Health Sciences School, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal. E-mail: pmaciel@eicsaude.uminho.pt

**Regulation of gene expression occurs at different levels, from DNA to protein, and through various mechanisms. One of them is modification of the chromatin structure, which is involved in the definition of transcriptional active and inactive regions of the chromosomes. These phenomena are associated with reversible chemical modifications of the genetic material rather than with variability within the DNA sequences inherited by the individual and are therefore called 'epigenetic' modifications. Ablation of the molecular players responsible for epigenetic modifications often gives rise to neurological and behavioral phenotypes in humans and in mouse models, suggesting a relevant function for chromatin remodeling in central nervous system function, particularly in the adaptive response of the brain to stimuli. We will discuss several human disorders that are due to altered epigenetic mechanisms, with special focus on Rett syndrome.**

Keywords: DNA methyltransferase, epigenetics, imprinting, methyl-binding protein, neuronal plasticity, Rett syndrome

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Several articles in this issue address the role of a timely and appropriate regulation of gene expression in the function(s) of the nervous system. Gene expression is particularly varied in neurons, with a large proportion of the coding genome being expressed at any time point (Geschwind 2000; Sandberg *et al.* 2000) often with increased variability of gene products due to alternative splicing of mRNA. Additionally, in the central nervous system (CNS), given the high level of structural and functional specialization,

transcripts that are expressed at very low levels may have crucial roles for specific groups of cells and therefore be very important for the function of the whole system.

Regulation of gene expression occurs at different levels, from DNA to protein, and through various mechanisms, some of which are rigidly pre-established and genetically defined, whereas others are necessarily more flexible, as they are required for an adequate response to environmental stimuli. From the point of view of energy cost, it is more useful for the cells to regulate expression at the earliest possible level, i.e. gene transcription, so that mRNAs for unnecessary products are not synthesized. This can be controlled (i) by the availability, in each cell, of transcription factors (in the appropriate activation state and in the presence of the necessary molecular partners) that bind to specific regulating sequences upstream of the genes, allowing their transcription through positioning of the RNA synthesis machinery, but also (ii) by mechanisms of long-range action of other DNA sequences, involving different regulating proteins, with a transcription-enhancing or repressing effect, and/or (iii) through the modification of the chromatin structure, which will define the accessibility of the DNA to these transcription regulators and to the RNA polymerases. The latter mechanisms are involved in the definition of active and inactive regions of the chromosomes, in the dosage-compensation processes such as the inactivation of one of the X-chromosomes in mammalian females, and in the parental imprinting of genes, which makes the expression of a given gene allele dependent on its parental origin. These phenomena are associated with reversible chemical modifications of the genetic material rather than with variability within the DNA sequences inherited by the individual and are therefore called 'epigenetic' modifications.

The link between epigenetic modifications and neuronal function is an exciting new field of investigation in the neurosciences emerging in the post-Human Genome era (Shahbazian & Zoghbi 2002; Tucker 2001). In this review, we will discuss the recent developments in this area of research.

## Mechanisms of epigenetic modification

The study of epigenetic instability began more than 75 years ago, when Muller (1940) recovered several examples of flies displaying variegating eye phenotypes after X-irradiation. In

several human diseases, phenotypic variation has normally been attributed to differences in genetic background and the influences of environment on that genetic background. In disagreement with this idea, experiments in isogenic populations of model organisms such as *Drosophila melanogaster* showed that phenotypic variation does persist and can be transmitted through mitosis and meiosis. These data clearly support the existence of mechanisms providing stable or semi-stable regulation of gene expression apart from nucleotide sequence. This regulation is achieved through the action of epigenetic factors, chromatin-modifying enzymes that can be divided into three distinct categories: (i) histone-modifying enzymes which covalently acetylate, phosphorylate, ubiquitinate, or methylate histones; (ii) DNA-modifying enzymes which methylate CpG-rich sequences; and (iii) ATP-dependent chromatin-remodeling complexes which can disrupt nucleosome structure and increase accessibility to DNA and histones, using the energy from ATP hydrolysis to move histone octamers along DNA molecules (Becker & Horz 2002; Gregory *et al.* 2001; Narlikar *et al.* 2002).

Eukaryotic genome assembles into chromatin; the basic building block of chromatin is the nucleosome, which contains 147 base pairs (bp) of DNA wrapped in a left-handed superhelix 1.7 times around a core histone octamer (two copies each of histones H2A, H2B, H3, and H4). Each core histone contains two separated functional domains: a signature 'histone-fold' motif sufficient for both histone–histone and histone–DNA contacts within the nucleosome, and NH<sub>2</sub>-terminal and COOH-terminal 'tail' domains that contain sites for post-translational modifications referred above. Histone covalent modifications can work as recognition signals, directing to chromatin the binding of non-histone proteins that determine its function and subsequently the transcriptional state of the genes. Nearly 40 years of research has resulted in the documentation of a variety of post-translation modification of the histones. The covalent modifications that take place on histones include the acetylation of lysines, the methylation of lysines and arginines, the phosphorylation of serines and threonines, the ubiquitination of lysines, the sumoylation of lysines, and the ADP-ribosylation of glutamic acid residues. All these modifications, except methylation, appear to be reversible. These are the histone modifications that allow the transition between open and condensed states and regulate the accessibility of DNA to several biological processes such as transcription, recombination, replication, and DNA repair. Covalent histone modifications and the histone positioning constitute a potential histone code defining actual or potential transcription sites (Jenuwein & Allis 2001; Richards & Elgin 2002).

The acetylation of lysine residues in histone tails has several roles in the regulation of the nucleosome, such as decreasing the histone–DNA interactions and increasing the accessibility of the DNA for transcription activation. Acetylation can also regulate DNA replication, histone deposition, and DNA repair, by recruiting proteins that have

an acetyl-lysine binding module – the bromodomain. The histone acetyltransferase (HAT) is a multisubunit protein responsible for the acetylation of lysines. This acetylation, which promotes transcription, is reversed by histone deacetylases (HDACs) (Marmorstein & Roth 2001). Another histone modification, lysine methylation, has been directly implicated in epigenetic inheritance. Two distinct epigenetic silencing mechanisms are linked to methylation of lysines 9 and 27 on histone H3. Heterochromatic proteins, such as HP1, bind histone H3-containing methyl-lysine 9 and promote gene silencing. The Polycomb protein also binds to histone H3, specifically at methyl-lysine 27, thus promoting gene silencing during development (Sims *et al.* 2003). The histone ubiquitination or sumoylation plays an important role in the regulation of transcription either through proteasome-dependent degradation of transcription factors or through other mechanisms related to the recruitment of modification complexes. Histone ubiquitination is usually involved in positive regulation of transcription, unlike sumoylation of histone H4, which is important for transcriptional repression (Berger 2002; Iizuka & Smith 2003; Zhang 2003). Finally, serine phosphorylation of histone H3 at Ser 10 and Ser 28 has been correlated with mitotic chromosome condensation (Nowak & Corces 2004). Other serine phosphorylation sites have been identified on histone H2A, H2B, and H4. For instance, phosphorylation of histone H2A at Ser 1 is reported to be a hallmark for mitotic chromosome condensation (Barber *et al.* 2004).

In addition to the histone modifications, DNA is also subjected to covalent modifications that are important for gene repression. So far, DNA methylation has been identified in several eukaryotes except in yeast, *Caenorhabditis elegans*, and *D. melanogaster*. In mammals, DNA methylation occurs exclusively at CpG dinucleotides, and different patterns of DNA methylation have been correlated with genome imprinting, inactivation of the X chromosome, and embryonic development. There are essentially two classes of DNA methyltransferases, the *de novo* DNA methyltransferases (DNMT3A and DNMT3B) which define new methylation patterns, and the maintenance DNA methyltransferases. The first identified member of DNA methyltransferases, DNA methyltransferase 1 (DNMT1), is a maintenance DNA methyltransferase. This enzyme uses as substrate hemi-methylated DNA and copies the pattern already established during DNA replication. As a maintenance DNA methyltransferase, one could expect DNMT1 levels in adult brain to be low, as neurons do not undergo mitosis. Instead, not only the level of this protein is quite high but also the level of DNA methylation is higher in adult brain than in other tissues (Brooks *et al.* 1996; Goto *et al.* 1994; Inano *et al.* 2000; Tawa *et al.* 1990). DNA methylation must have a role in the maturation process of the brain as the ablation of DNA methylation maintenance pathway, through a targeted disruption of the *Dnmt1* gene, in mouse CNS precursor cells (but not in postnatal neurons) causes global DNA hypomethylation and neonatal death, due

to defects in neuronal respiratory control of the mutant animals (Fan *et al.* 2001).

DNMT3A and DNMT3B, *de novo* methyltransferases, are essential for mammalian development. Both proteins might be partially redundant, but the critical timing and mutant outcomes of both proteins are different, as shown by the studies of Okano and collaborators (1999) (mutant phenotypes summarized in Table 1). DNMT3A and DNMT3B expression studies performed by Feng and collaborators (2005) also suggest that these proteins have a different functional significance: while DNMT3B is predominant at the beginning of embryonic neurogenesis, DNMT3A appears to play a role at this developmental stage but also later, at postnatal stages, in CNS function. Mutations in the catalytic domain of *DNMT3B* gene have been recognized in a subset of patients with the autosomal recessive human disorder Immunodeficiency, Centromeric instability, Facial anomalies syndrome (ICF, OMIM #242860) characterized by variable immunological defects, centromeric heterochromatin instability, facial anomalies, and mental retardation (Okano *et al.* 1999; Xu *et al.* 1999).

In *Neurospora*, cytosine methylation depends on a conserved DNA methyltransferase, which is directed to chromatin by the histone H3 lysine methyltransferase DIM-5, linking these two types of epigenetic modification.

### Imprinting as modification of genetic information affecting behavior

The general idea that genetic information inherited from both parents is equivalent, except for the sex chromosomes, was questioned 20 years ago with experiments that showed that proper development of mice embryos required information from both maternal and paternal genomes (McGrath & Solter 1984; Surani *et al.* 1984). This idea has been consolidated with the identification of several imprinted genes, i.e. genes that display a pattern of expression that is dependent on their parental origin (Smith *et al.* 2004).

The mechanisms underlying the establishment and maintenance of imprinting are not clearly understood, but it is known that the epigenetic mark of the imprinted genes occurs early in the gametogenesis (gonocyte and oocyte development). After the erasing of the inherited methylation pattern, a new one is defined according to the origin of the genetic material (the sex of the parent) (Kafri *et al.* 1992; Monk *et al.* 1987; Sanford *et al.* 1987). For numerous genes, imprinting may not be ubiquitous, but rather tissue-specific, specific to developmental stage or species-specific (Yamasaki *et al.* 2003). Interestingly, there seems to be a differential distribution of the expression of imprinted genes within the brain. This was elegantly demonstrated with studies in mouse chimeras, in which cells that were disomic for maternal genome survived especially in the neocortex, striatum, and hippocampus, while cells disomic for paternal genome were virtually absent in telencephalic structures but

present in the hypothalamus, preoptic area, structures important for primary motivated behavior (Allen *et al.* 1995; Keverne *et al.* 1996).

Many explanations for the evolution and origin of genomic imprinting have been proposed, including regulation of gene dosage (Solter 1988) and the conflict over parental investment (Moore 2001). Parental imprinting can be seen as a form of selection of the regions of maternal/paternal genomes contributing for the behavior of the offspring. Maternal investment over its offspring is influenced by the paternal contribution to the offspring genome, and the conflict created might be solved through gene imprinting, each player's (mother, father, and offspring) involvement defending each one's best interest. An example is the involvement of the father in determining the size of the litter and of the mother in provisioning it (Hager & Johnstone 2003).

Clearly, a disturbance in the balance of the two imprinted genomes can result in brain dysfunction, and imprinted genes are recognized to play important roles in a number of different human conditions and in altered social behavior in mammals. Angelman's syndrome (AS, OMIM #105830) is a human disorder presenting severe speech delay, happy affect, epilepsy, and movement disorders (Williams *et al.* 2001). Prader-Willi syndrome (PWS, OMIM #176270) is characterized by diminished fetal activity, obesity, muscular hypotonia, mental retardation, short stature, and small hands and feet. The most common mutations in these two syndromes are deletions of chromosome 15q, and depending on whether the affected allele is the maternal or the paternal one, PWS or AS will develop. Mutations in one particular gene located on chromosome 15q, *UBE3A*, have also been identified in patients with AS without deletions in 15q (Kishino *et al.* 1997; Matsuura *et al.* 1997). *UBE3A* shows an imprinted mode of inheritance, consistent with a gene exclusively or preferentially active on the maternal chromosome. The absence of a functional maternal allele causes AS. The restricted neurobehavioral phenotype of this syndrome might suggest a brain-specific imprinting of *UBE3A*. In fact, Yamasaki and collaborators (2003) showed that *Ube3a*-deficient mice exhibit a neurological phenotype that resembles AS in humans and that *Ube3a* in mice is imprinted specifically in neurons but not in glial cells.

Other very interesting examples of imprinted genes with a role in behavior are the paternally expressed genes (Peg). The *Peg1* gene (also known as *Mest*) is highly expressed in various brain regions of mice and presents an imprinted pattern, with expression of the paternal allele. *Peg1*-deficient mice are viable and fertile; however, the paternal transmission of a mutant allele causes a growth retardation, increased perinatal and postnatal lethality, and abnormal maternal behavior, without placentophagy (Lefebvre *et al.* 1998). *Peg3* also has an imprinted monoallelic paternal expression. *Peg3*-mutant mice have a complete deficit in all aspects of maternal behavior (retrieving, nest building, and crouching). The hypothalamic medial preoptic area (MPOA) is

**Table 1:** Behavioral phenotypes of mouse mutants for methyl-binding proteins and DNA methyltransferases

| Gene Function          | <i>MBD1</i>                                                                                                                                                                                                                                                                                                                                     | <i>MBD2</i>                                                                                                                                                                                                                                                                                                                |
|------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Animal model phenotype | Methyl-CpG binding<br>Transcriptional repression<br><b>Mbd1<sup>-/-</sup> (Zhao et al. 2003)</b><br>Normal development<br>Apparently healthy as adults<br>Reduced neurogenesis<br>Impaired spatial learning ability<br>Marked reduction in DG-specific LTP<br>Histological analysis: no detectable developmental defects                        | Methyl-CpG binding<br>Transcriptional repression<br><b>Mbd2<sup>-/-</sup> (Hendrich et al. 2001)</b><br>Possess N-terminal 183 aa of the protein<br>Viable, fertile, and with normal appearance<br>Maternal nurturing defect<br>Reduced litter size and pup weight<br>Failure of the mothers to adequately feed their pups |
| Observation            | Adult neural stem cells (ANCs) from Mbd1 null:<br>Reduced neurogenesis<br>Increased genomic instability                                                                                                                                                                                                                                         | Genomic DNA methylation is not affected<br>Lack intact MeCP1 complex<br>Normal imprinting pattern of various analyzed genes                                                                                                                                                                                                |
| Gene Function          | <b><i>DNMT1</i></b><br>Methylation maintenance                                                                                                                                                                                                                                                                                                  | <b><i>DNMT3A and DNMT3B</i></b><br><i>de novo</i> methylation                                                                                                                                                                                                                                                              |
| Animal model phenotype | <b>Dnmt1<sup>-/-</sup> (Fan et al. 2001)</b><br>Dnmt1 deficiency in embryonic postmitotic neurons:<br>Viability is not affected<br>No obvious demethylation in mutant neurons                                                                                                                                                                   | <b>Dnmt3a<sup>+/-</sup> and Dnmt3b<sup>+/-</sup> mice (Okano et al. 1999)</b><br>Grossly normal and fertile<br>Dnmt3a <sup>-/-</sup> :<br>Normal at birth<br>Died at about 4 weeks of age                                                                                                                                  |
| Observation            | Dnmt1 deficiency in embryonic CNS precursor cells:<br>Neonatal death (respiratory failure due to abnormal neural control)<br>Substantial demethylation in the brain of mutant embryos<br>No obvious defect in brain structure<br>No increase in embryonic cell death<br>Dnmt1 is crucial for the function and survival of postnatal CNS neurons | Dnmt3b <sup>-/-</sup> :<br>Not viable at birth<br>Growth impairment<br>Rostral neural tube defects<br>Dnmt3a <sup>-/-</sup> ; Dnmt3b <sup>-/-</sup> :<br>Blocks <i>de novo</i> methylation                                                                                                                                 |

Table 1: Continued

| Gene Function          | MECP2                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
|------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                        | Methyl-CpG-binding domain<br>Transcriptional repression                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| Animal model phenotype | <b>MeCP2 null-mice (Guy et al. 2001)</b><br>Exons 3 and 4 deleted<br>Born normal<br>Develop neurological symptoms from 3 to 8 weeks of age<br>No signs of cortical lamination or ectopias in the brain<br>Specific loss of MeCP2 in the brain: indistinguishable phenotype<br>Death at around 54 days<br>MeCP2; Mbd2 double mutant:<br>Phenotype was not different from the single mutant<br><b>MeCP2-deficient mice (Chen et al. 2001)</b><br>Exon 3 deleted<br>Born healthy<br>Showed abnormal behaviour at around 5 weeks of age<br>Reduced brain size and weight<br>Cell bodies and nuclei of neurons: smaller and more densely packed<br>Mice with specific mutant MeCP2 in postmitotic neurons:<br>Similar but delayed and less severe phenotype<br><b>MeCP2-deficient mice (Shahbazian et al. 2002a)</b><br>Born healthy<br>From 6 weeks of age start to develop neurological symptoms<br>Abnormal motor function and activity<br>Heightened anxiety<br>Abnormal social interactions<br>No abnormalities in the CNS, normal brain weight<br>H3 hyperacetylation in the brain | <b>Mice expressing exogenous MeCP2</b><br><b>Tau-MeCP2 expression in neurons (Luikenhuis et al. 2004)</b><br>WT heterozygous for the transgene<br>Healthy and fertile<br><br>Mutant MeCP2 308 heterozygous for the transgene<br>Healthy and fertile<br>Body and brain weight indistinguishable from wt littermates<br><br>WT and MeCP2 308 mutants homozygous for the transgene<br>Motor dysfunction<br>Mutant pups smaller than wt littermates at weaning<br>No reduction in brain weight<br>Disheveled look<br>Excessive stereotypic scratching<br><br><b>MeCP2 transgenic mice (Collins et al. 2004)</b><br>Mice expressing MeCP2 approximately 2 times endogenous levels<br>Normal at birth<br>Developed severe neurological phenotype at around 10–12 weeks of age<br><br>Less anxiety-like behavior<br>Enhanced cerebellar motor learning and hippocampal learning<br>Enhanced synaptic plasticity<br>Older animals develop:<br>Clonic and akinetic seizures with abnormal EEG recordings<br>Double mutant MeCP2Tg1; MeCP2 null<br>at 33 weeks remain indistinguishable from wt littermates |

known as a regulatory center for maternal behavior, and oxytocin released from the hypothalamic paraventricular and supraoptic nuclei neurons controls milk ejection. The suggestion that *Peg3* could be involved in the modulation of the 'maternal response' is supported by the neural expression pattern of *Peg3* in hypothalamic nuclei, including MPOA, medial amygdala, and hippocampus, and the reduced number of oxytocin-positive neurons in mutant *Peg3* females (Li *et al.* 1999). Interestingly, however, Szeto and collaborators (2004) created a transgenic mouse in a mutant *Peg3* background (Li *et al.* 1999) in which they were not able to see the recovery of the wild-type phenotype; they propose that this result could be due to the low expression level of the transgene during early embryonic development, probably due to the absence of important regulatory elements in the transgene.

### Chromatin remodeling and behavior

Chromatin-remodeling complexes were first identified by genetic screens in yeast as targets of mutations that alter the transcription of genes induced in response to extracellular signals (Winston & Carlson 1992). The identified mutant strains were named SWI/SNF (mating type SWItching/Sucrose Non-Fermenting). All different chromatin-remodeling multisubunit complexes contain a core SNF2-related ATPase region. SNF2 family members can be subdivided into several subfamilies according to the presence of protein motifs outside the ATPase region. The SNF2 subfamily includes the human BRG-1, and hBRM subunits of SWI/SNF-related complexes in *Drosophila* and humans. The BRG1- and BRM-associated chromatin-remodeling complexes have been implicated indirectly in the pathology of Williams-Beuren syndrome (WBS, OMIM #194050), an autosomal dominant disorder caused by heterozygosity of a microdeletion at 7q11.2. WBS is characterized by congenital heart disease, infantile hypercalcemia, a characteristic facies (described as elfin facies), and mental retardation. Socially, WBS children present a unique social behavior. Often they take the initiative to approach others, are overly friendly, and are always noted in a group. However, they also present behavioral problems such as attention deficits and anxiety (Morris & Mervis 2000). Interestingly, the Williams syndrome transcription factor (WSTF) encoded by the *WBSCR9/BAZ1B* gene, one of the genes deleted in WBS, is needed to recruit BRG1 and BRM and their associated chromatin-remodeling factors to vitamin D-regulated promoters (Kitagawa *et al.* 2003). Haploinsufficiency of this gene has been implicated as a possible cause of hypocalcemia in WBS patients. WSTF also interacts with ISWI, a SWI/SNF-related ATPase, to form a chromatin-remodeling complex, WHICH, that participates in DNA replication through interaction with PCNA (Bozhenok *et al.* 2002; Poot *et al.* 2004). On the basis of these findings, aberrant chromatin remodeling might play a

key role in the pathophysiology of WBS. Another disorder in which chromatin remodeling seems to be affected is Rett syndrome (RTT), which we will explore in greater detail in the next sections, given the abundance of recent data regarding its pathophysiology.

### MECP2 and Rett syndrome

The relevance of chromatin modification and remodeling for the function of the mammalian nervous system was first brought to attention when the genetic basis of the pervasive neurodevelopmental disorder known as Rett syndrome was clarified, in 1999 (Amir *et al.* 1999). This syndrome is a major cause of mental retardation in females, affecting 1/10 000–1/22 000 born females; it is characterized by an apparently normal pre- and perinatal development (6–18 months of age), followed by a growth deceleration/arrest and a loss of motor, language, and social acquisitions, leading to lifetime mental retardation, autistic behavior, and motor deterioration (clinical diagnosis criteria reviewed and recently updated by Hagberg and colleagues) (Hagberg *et al.* 1983; Hagberg *et al.* 2002). Stereotypical hand movements (hand washing/wringing, hand clapping/patting or hand mouthing) are often present and constitute a hallmark of the syndrome. Pathologically, a reduction of cortical thickness is observed, in spite of relative preservation of neuronal number, corresponding to a markedly reduced neuronal size and increased cell packing density, with loss of neuronal arborization and decreased synaptic density (Armstrong 2001). The majority of patients with classic RTT are heterozygous for mutations in the *MECP2* gene (Amir *et al.* 1999), which encodes a methyl-CpG-binding protein, MeCP2, known to bind symmetrically methylated CpG dinucleotides and recruit Sin3A and HDACs to repress transcription (Jones *et al.* 1998).

Several animal models for the study of the MeCP2 function *in vivo* have been created in mice (mutants summarized in Table 1), which mimic in many aspects Rett syndrome: a knock-out (*ko*) mouse for the *Mecp2* gene (Guy *et al.* 2001), a mutant that possesses only the C-terminal region of the gene (Chen *et al.* 2001), and a transgenic mouse, MeCP2<sup>308</sup>, with a hypomorphic allele that truncates the protein at the position 308 (Shahbazian *et al.* 2002a). All these mutants are born normal and symptoms start to develop a few weeks later with progressive motor deterioration, males displaying an earlier onset and being more severely affected than females. As in RTT patients, no gross abnormalities in the brain were detected. The MeCP2<sup>308</sup> mutant also presented emotional and social behavior abnormalities along with the motor dysfunction.

Expression of MeCP2 in mutant mice that are deficient for the *Mecp2* gene (models by Guy *et al.* 2001 and Chen *et al.* 2001) was shown to rescue the neurological RTT-like phenotype of mutants, the mutant mice expressing the transgene becoming indistinguishable from wild-type (*wt*) littermates.



In the study by Luikenhuis *et al.* (2004), the expression of a mutant *Mecp2* transgene in the postmitotic neurons of mutant mice was sufficient to recover the RTT-like phenotype in these animals. This suggests that the function of MeCP2 must be not in the embryonic development, but at later stages. However, overexpression of MeCP2 had a deleterious effect both on *wt* and on *ko* mice and induced a neurological phenotype that varied in severity according to the protein level (Collins *et al.* 2004; Luikenhuis *et al.* 2004). The MeCP2 protein appears to be highly regulated and its deregulation seems to have severe consequences specifically in the brain. In mice overexpressing MeCP2 (Collins *et al.* 2004), its upregulation affects pathways leading to cerebellar and hippocampal learning and increases synaptic plasticity, in an antagonistic way to the mental retardation presented by RTT patients.

In the embryonic development of humans and mice, MeCP2 expression starts to be detected very early and in the ontogenetically older brain areas (Shahbazian *et al.* 2002b). However, it is only in the mature brain that MeCP2 is expressed at the strongest levels. LaSalle and collaborators (2001) showed that in brain, one can find subpopulations of cells that are MeCP2 'high expression' and MeCP2 'low expression' cells. In RTT pathogenesis, the MeCP2 'high expression' cells seem to be selectively affected. The subpopulation of MeCP2 'high expression' cells was more represented in developed cerebrum than in immature brain (Balmer *et al.* 2002). The results by Mullaney and collaborators (2004) in the rat brain further narrowed the window of MeCP2 critical role to synaptogenesis. The authors showed a higher expression of MeCP2 and higher number of synapses in layer V than in layer VI of the cerebral cortex (first generated), as well as a concordant timing between the expression of MeCP2 and a higher number of synapses in the granule cells of the cerebellum and in the hippocampus, suggesting that MeCP2 might be regulating genes that are important for synapse formation, function, or maintenance rather than previous stages of nervous system development (such as neuronal differentiation or migration).

## Neuronal targets of MeCP2

Mutations in the *MECP2* gene are responsible for hyperacetylation of histone H4 in cultured cells from patients with RTT, through impaired formation of the co-repressor complex Sin3A/HDAC, which in turn can affect chromatin architecture (Wan *et al.* 2001). Also, mutant MeCP2<sup>308</sup> mice display hyperacetylation of H3 in cerebral cortex and cerebellum (Shahbazian *et al.* 2002a). Additionally, MeCP2 has been shown to facilitate lysine 9 methylation in H3 and may serve as a bridge between DNA methylation and histone methylation (Fuks *et al.* 2003; Horike *et al.* 2005). Finally, during postnatal brain development, pairing of homologous 15q11–13 alleles occurs (Thatcher *et al.* 2005) and MeCP2 is involved in

this specific pairing that is disrupted in several neurodevelopmental disorders such as RTT. How disruption of these functions leads to the specific developmental dysfunctions that occur in RTT remains unknown. The identification of neuronal targets of MeCP2 is one avenue of research that may provide a clue to RTT pathogenesis, and possibly to an increased understanding of other pervasive developmental disorders such as autism and AS, in which MeCP2 levels appear to be low (Samaco *et al.* 2004).

Most microarray studies have failed to identify any substantial and consistent changes in transcription levels in *Mecp2*-null mice (Tudor *et al.* 2002), clonal cell cultures from individuals with RTT (Traynor *et al.* 2002), or in post-mortem RTT brains (Colantuoni *et al.* 2001). These results suggest functional redundancy between the different methyl-binding proteins or a more focused action of MeCP2 as a selective regulator – be it region-specific actions of the protein in the brain, action at a specific developmental stage, involvement of MeCP2 in specific epigenetic events (such as imprinting of certain genes), or in activity-dependent transcription. In any of these scenarios, important differences in the transcription levels of certain genes may exist in the absence of MeCP2, but their detection will only be possible if suitable experimental designs are used.

A recent study by Ballestar and collaborators (2005) combining microarray studies, chromatin immunoprecipitation analysis, bisulfite genomic sequencing, and treatment with demethylating agents, in lymphoblastoid cell lines derived from RTT patients, revealed the deregulated expression of a number of genes, which were shown to have methylated promoters, directly bound by MeCP2. Approximately half of these target genes presented high expression levels in RTT cells when compared with *wt* cells, whereas the remaining half were downregulated, most likely because of an indirect effect of MeCP2 on genes that are in turn regulating these ones. The role of these target genes in the pathogenesis of RTT remains to be clarified.

MeCP2 was shown to be involved in the imprinting control region of the *H19* gene (Drewell *et al.* 2002). *H19* is an example of a gene for which imprinting occurs for the paternal allele. The promoter region of the paternal allele is highly methylated and its silencing was shown to be methylation-dependent and mediated by MeCP2 (Drewell *et al.* 2002). However, the analysis of different imprinted genes, including the *H19* gene, in cultured T-cell clones from blood and in brains from patients with mutations in the *MECP2* gene revealed normal monoallelic expression in all clones and brain samples (Balmer *et al.* 2002), which might suggest an *in vivo* redundancy amongst the methyl-binding domain-containing (MBD) family of proteins.

Horike and collaborators (2005) recently found that *DLX5*, a gene whose product is involved in the synthesis of gamma aminobutyric acid (GABA), is upregulated in RTT. In humans, *DLX5* has an imprinted pattern with expression of the maternal allele, while in mice *Dlx5* is biallelically transcribed, but

preferentially from the maternal allele. The authors found that in the cortex of *Mecp2*-null mice and in human lymphoblastoid cells from individuals with RTT (i) transcription levels were higher than normal and (ii) there was an altered parental imprinting of the gene that was dependent on the type of mutation. Although the target region through which MeCP2 regulates *Dlx5* expression is not known yet, this strengthens the possible link between MeCP2 and imprinting and, for the first time, connects RTT to this epigenetic mechanism. It also provides useful clues to RTT pathogenesis, as affected GABA neurotransmission could explain some of the cognitive symptoms of RTT.

Two other candidate targets of MeCP2 are the *UBE3A* and *GABRB3* genes. These are particularly interesting, as *UBE3A* is linked to AS and *GABRB3* (which encodes the protein GABA receptor  $\beta 3$  subunit), have been consistently implicated in autism, in association studies, and both disorders present some phenotypic overlap with RTT. *UBE3A* and *GABRB3* levels were found to be decreased in RTT, AS, and autism brains. *Mecp2*-deficient mice also display decreased levels of *Ube3a* and *Gabrb3*, in spite of the lack of alterations in the imprinting pattern of the *Ube3a* gene (Samaco *et al.* 2005). A possible mechanism through which MeCP2 regulates the expression of *UBE3A* has recently been proposed: MeCP2 binding to the methylated PWS-imprinting center at the maternal allele where the antisense *UBE3A* gene resides. Mutant MeCP2 would cause an epimutation at this center, affecting the expression of *UBE3A* (Makedonski *et al.* 2005).

Experiments performed in *Xenopus* embryos showed that MeCP2 targets the gene *xHairy2a* during development. In the absence or presence of a mutant form of MeCP2, the expression of the *xHairy2a* gene was misregulated, with consequences in neuronal differentiation. This study showed that MeCP2 interacts with SMRT complex via Sin3A and that mutant MeCP2 had defective binding to SMRT co-repressor complex. It is possible that DNA methylation and MeCP2 binding can modulate the levels of *xHairy2a* expression and have an essential role in early neurogenesis (Stancheva *et al.* 2003).

The most interesting target of MeCP2 identified so far is doubtlessly the gene encoding the brain-derived neurotrophic factor (*BDNF*), one of the genes for which transcription is regulated in a neuronal activity-dependent manner. Data from two different studies showed that MeCP2 is involved in the *Bdnf* gene silencing in the absence of neuronal activation. MeCP2 was shown to bind to the methylated rat *Bdnf* promoter III (equivalent to promoter IV in the mouse) and, upon membrane depolarization of cultured cortical neurons, to dissociate from the promoter and lead to a higher transcription level of the *Bdnf* gene (Chen *et al.* 2003; Martinowich *et al.* 2003). Chen and collaborators (2003) also showed that the release of MeCP2 protein was due to calcium influx that caused a phosphorylation of MeCP2. Given the role of *BDNF* in development and neuronal plasticity

(McAllister *et al.* 1999; Binder & Scharfman 2004) and the timing when MeCP2 demand becomes crucial, that coincides with moments of synapse development and maturation, the aforementioned evidence easily fits a model in which MeCP2-regulated chromatin remodeling would underlie neuronal plasticity, which could explain some symptoms of the RTT phenotype, such as reduced dendritic arborization and complexity in some areas of the brain (Armstrong 2001) as well as the clinical finding of mental retardation.

## Methyl-DNA-binding proteins and DNA methyltransferases

In addition to MeCP2, four other MBD-containing proteins (MBD1, MBD2, MBD3, and MBD4) exist (Ballestar & Wolffe 2001). Interestingly, null mutations in several of these proteins lead to behavioral phenotypes, as do some mutations in DNA methyltransferases (summarized in Table 1).

MBD1 is expressed in neurons throughout the brain, with highest concentration in the hippocampus (CA1 and DG), and is not expressed in glia. Mice *ko* for the *Mbd1* gene display reduced neurogenesis in the hippocampus, perform worse than *wt* animals when tested in the Morris water maze, and have a reduction in dentate gyrus long-term potentiation (LTP) (Zhao *et al.* 2003).

*Mbd2*<sup>-/-</sup>-mutant mothers do not present a proper nurturing behavior of their offspring (Hendrich *et al.* 2001). This phenotype resembles the *Peg3*-mutant mothers (discussed above), highlighting a potential connection between *Mbd2* and imprinting. However, altered expression of *Peg3* or other imprinted genes was not detected in *Mbd2*<sup>-/-</sup> animals. It is possible that if differences exist, the deregulation occurs in a localized and functionally related area of the brain, such as MPOA of the hypothalamus. *Mbd3*<sup>-/-</sup> animals die before birth, suggesting an essential role of this protein during development (Hendrich *et al.* 2001). The different phenotypes of these two mutants might be explained, in part, by the expression pattern of the corresponding proteins. Expression profiles of MBD2 and MBD3 in the developing brain are not parallel: during development and in adulthood, MBD3 is expressed in ontogenetically younger brain regions, in contrast with MBD2 expression, that is weak in embryonic brain, but pronounced in the adult brain (Jung *et al.* 2003).

In addition to RTT and WBS, there are other human disorders in which mutations affecting chromatin remodeling lead to behavioral phenotypes where, for most of the cases, MR is a cardinal feature. Mutations in the *JARID1C* gene have been recently identified in patients with X-linked mental retardation (XLMR). The protein encoded by this gene belongs to the ARID protein family, which contains several DNA-binding motifs, and is involved in transcriptional regulation and chromatin remodeling (Jensen *et al.* 2005).

All this evidence suggests a role for 'brain chromatin' and its epigenetic modifications in mental retardation. This link

seems to be established early in development and when perturbed has consequences for life.

### Chromatin remodeling and interaction with the environment

Some chromatin modification patterns need to be rigidly pre-established and even irreversible, such as the ones involved in developmental determination and differentiation, relevant to the appropriate formation of clearly defined circuits in the nervous system. However, there are many recent pieces of evidence suggesting that in many other cases, a process of dynamic chromatin remodeling is connected to phenomena of cellular and/or system response to extracellular and environmental stimuli.

The first example of this is the response to ischemia. After cerebral ischemia, DNA methylation is known to augment in *wt* mice, rendering the brain more susceptible to damage (Endres *et al.* 2000). The mechanisms through which this happens are not clear, but they might involve altered gene expression, DNA repair mechanisms or changes in mitotic activity. *Ko* animals for the *Dnmt1* gene do not present, after mild brain ischemia, this elevation in the level of DNA methylation, and have a better stroke outcome than *wt* mice, with reduced lesion size and higher number of neurons in the striatum (Endres *et al.* 2000).

Another example is the role of chromatin modifications in rhythmicity of expression of the Clock genes. Organisms learn how to properly respond to the environmental changes that occur through the 24-h day or through the different seasons of the year such as temperature and light intensity. The mammalian core timekeeping has been identified as the suprachiasmatic nucleus (SCN) in the hypothalamus (Hastings & Maywood 2000) and allows mammals to adapt behavior and physiological responses to the day: night 24-h cycle (see Oster 2006). The entrainment of the SCN is done by a light pulse which induces a burst of expression of the clock genes (*Per1* and *Per2*) and immediate early genes (*c-Fos*, *Fos-B*, and *Jun-B*) (Albrecht *et al.* 1997; Kornhauser *et al.* 1990; Morris *et al.* 1998). One of the mechanisms involved in transcriptional regulation is chromatin remodeling through histone modification. The data obtained by Crosio *et al.* (2000) support the idea that circadian gene expression might be controlled at the histone level. When a pulse of light was given to mice kept in a 12-h light/12-h dark cycle for 2 weeks, and then for 4 days in constant dark, an increase of the H3 phosphorylation was detected and closely accompanied by the expression of the early gene *c-Fos*. In another study, Etchegaray and collaborators (2003) were able to identify rhythmicity in RNA polymerase II binding and acetylation of H3 in the *Per1* and *Per2* genes and showed that these rhythms were synchronous in the peripheral liver oscillator. It has also been demonstrated that p300, which has intrinsic HAT activity, is part of the CLOCK/BMAL1 complex and that the negative loop of CRY protein in the transcription

regulation of *Per* genes is through the p300 protein (Etchegaray *et al.* 2003). Thus, in addition to mutations in circadian genes, loss of function of genes involved in epigenetic modification, namely acetylation and phosphorylation, might be responsible for impairment of rhythmicity.

### Activity-dependent gene transcription and chromatin modification: role in synaptic plasticity

Synaptic plasticity underlies the brain's adaptive response to the environment. The mechanisms involved operate through post-translational modifications of proteins at the level of the dendrites (short-term responses) but may also involve the synthesis of new proteins through regulation of gene expression in the nucleus, when long-term responses/long-term memories are concerned (Levenson & Sweatt 2005; West *et al.* 2001). Synaptic activity induced either by external or by endogenous stimuli leads to a calcium influx and depolarization of the membrane. This  $\text{Ca}^{2+}$  rise is an important element in the activity-dependent gene transcription in the nucleus of neurons.  $\text{Ca}^{2+}$  influx can be perceived by the cell in different ways (temporal pattern of electrical activity or spatial pattern of  $\text{Ca}^{2+}$  influx) and by different molecules (second messengers) and the manner in which the signal gets to the nucleus ( $\text{Ca}^{2+}$  channels, Calmodulin, CREB, and MAP kinase) has a consequence in the interpretation of the different stimuli. This leads to different pathways being activated and consequently different genes activated and proteins expressed (Bradley & Finkbeiner 2002).

In the nucleus, CREB-dependent gene expression plays a crucial role in associating synaptic activity with long-term changes in synaptic circuitry in many kinds of neuronal systems. The phosphorylation of CREB by PKA increases the stability of the complex formed by CREB and CBP, a histone acetyltransferase, and thus regulates CREB-dependent gene expression through chromatin modification (Bito & Takemoto-Kimura 2003). The work by Guan and collaborators (2002) with the early response gene *C/EBP* also showed that the integration of stimuli that were repeatedly presented at independent synapses occurs at the nucleus by changes in chromatin structure that regulate gene expression/protein synthesis.

The data available for MeCP2 (Chen *et al.* 2003; Martinowich *et al.* 2003) provide the first evidence strongly supporting a link between chromatin remodeling and the synaptic or dendritic modifications that underlie the learning process, impaired in RTT and in many other related developmental disorders associated with cognitive deficits which share the clinical outcome of mental retardation.

It can be concluded that epigenetic modifications are essential for proper neuronal development, survival, and function and they may play a role in this system's adaptive response to the environment. We begin to have some evidence for an involvement of chromatin remodeling in plastic CNS

processes, such as the synaptic or dendritic modifications underlying learning. Transcriptional changes and modification of protein expression are known to be crucial for the establishment of many types of long-term memory. Thus, it is conceivable that modification of chromatin could affect these processes, either through an effect on global repression of gene activity or through specific modification of the expression of genes involved in such processes. An increased understanding of the mechanisms of epigenetic modifications and their role in neuronal function should shed light on the basis of many human cognitive and behavioral disorders.

### Note added in proof

After this article as been accepted for publication, two independent studies revealed the impairment of synaptic plasticity, LTP and LTD in mouse models of RTT (Asaka Y, 2005; Moretti P, 2006).

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**ARTICLE 2**

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“Detection of heterozygous deletions and duplications in the *MECP2* gene in Rett syndrome by  
Robust Dosage PCR (RD-PCR)”

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**MUTATION IN BRIEF**

# **Detection of Heterozygous Deletions and Duplications in the MECP2 Gene in Rett Syndrome by Robust Dosage PCR (RD-PCR)**

Jinxiu Shi<sup>1</sup>, Akane Shibayama<sup>1</sup>, Qiang Liu<sup>1</sup>, Vu Q. Nguyen<sup>1</sup>, Jinong Feng<sup>1</sup>, Mónica Santos<sup>2</sup>, Teresa Temudo<sup>3</sup>, Patricia Maciel<sup>2</sup>, Steve S. Sommer<sup>1\*</sup>

<sup>1</sup>Department of Molecular Genetics and Molecular Diagnosis, City of Hope National Medical Center, Duarte, California; <sup>2</sup>Hospital de Sto. António, Porto, Portugal; <sup>3</sup>Health Sciences School, University of Minho, Braga, Portugal

\*Correspondence to: Steve S. Sommer M.D., Ph.D. City of Hope National Medical Center, 1500 East Duarte Road, Duarte, California 91010-3000; Phone: (626) 359-8111 x64333; Fax: (626) 301-8142; E-mail: sommerlab@coh.org

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**Fifty to eighty percent of Rett syndrome (RTT) cases have point mutations in the gene encoding methyl-CpG-binding protein-2 (MECP2). A fraction of MECP2 negative classical RTT patients has large heterozygous deletions. Robust Dosage PCR (RD-PCR) assays were developed as a rapid, convenient and accurate method to detect large heterozygous deletions and duplications. A blinded analysis was performed for 65 RTT cases from Portugal by RD-PCR in the coding exons 2-4 of the MECP2 gene. Neither the patients with point mutations nor the non-classical RTT patients without point mutation had a deletion or duplication. One of remaining eight female patients with classical RTT without point mutation had a heterozygous deletion. This is the first report of a deletion spanning the entire MECP2 gene. The deletion was confirmed by southern blotting analysis and the deletion junction was localized 37kb upstream from exon 1 and 18kb downstream from exon 4. No duplications were detected. Our results suggest that RD-PCR is an accurate and convenient molecular diagnostic method. © 2005 Wiley-Liss, Inc.**

KEY WORDS: Rett Syndrome; MECP2; RD-PCR; heterozygous deletion

## **INTRODUCTION**

Fifty to eighty percent of Rett syndrome (RTT; MIM# 312750) cases have point mutations in the MECP2 gene (Methyl-CpG-binding protein 2; MIM# 300005)(Amir et al., 1999; Miltenberger-Miltenyi., 2003). Southern blotting analysis, quantitative PCR and MLPA (Multiplex Ligation-Dependent probe amplification) have been used to detect large deletions and duplications in the MECP2 gene of RTT patients without point mutation (Schollen et al., 2003; Erlandson et al., 2003; Ariani et al., 2004; Laccone et al., 2004). In those reports, 12 out of 59 (20.3%) classical RTT patients without point mutation have large deletions in MECP2 gene.

Robust dosage PCR (RD-PCR) has been developed as a rapid, convenient and accurate method to detect heterozygous deletions and duplications (Liu et al., 2003). The accuracy and consistency of RD-PCR has previously been validated in multiple blinded analyzes with 100% accuracy (Liu et al., 2003; Nguyen et al., 2004). RD-PCR has the advantages of rapid optimization and validation of new assays, and inclusion of positive controls without the requirement of the heterozygous deletion. The enhanced RD-PCR protocol has the additional advantages of tolerance toward genomic DNA of variable quality and uniform and unbiased performance across

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regions of variable sequence context and GC content (Nguyen et al., 2004; Shi. et al., 2004).

In our study, we used, for the first time, RD-PCR to detect heterozygous deletions in the *MECP2* gene. A blinded analysis was performed for 65 RTT cases from Portugal. One of eight patients with classical RTT without point mutation had a heterozygous deletion spanning the entire coding sequence. The deletion was confirmed by southern blotting. This is the first biological analysis report on RD-PCR.

## MATERIALS AND METHODS

### Samples

The DNA was prepared from peripheral blood by Puregene DNA Isolation Kit (Gentra, Minneapolis, MN). The concentrations were measured by UV spectrophotometer at 260 nm and adjusted to a working concentration of 30 ng/μl in TE buffer. Forty-eight control samples were analyzed in a blinded validation analysis, in which gender was used as a surrogate for heterozygous deletions.

Sixty-five RTT patient samples from Portugal and two blinded male controls were screened in the study. Patients were diagnosed according to the RTT diagnostic criteria defined by Hagberg et al (Hagberg et al., 1985 and 2002), which includes psychomotor regression after a period of normal development, severe mental retardation, deceleration of head growth and loss of purposeful hand skills with appearance of stereotypical hand movements. 30 of the RTT samples were classical RTT patients, and 8 of these were negative *MECP2* mutation according to our previous work.

### RD-PCR assay

Genomic DNA samples were incubated at 90°C in TE buffer for 10 minutes to minimize RD-PCR bias (Shi *et al.*, 2004). Four RD-PCR assays for the three coding exons of *MECP2* gene were designed (Table 1) according to Liu et al (Liu et al., 2003), except for the 5' universal tail of 5' ggccaagtgt- 3'. These assays were divided into two groups depending on whether the *ATM* or *FUT* gene was used as the autosomal control segment. Group I had two assays in exon2 and exon3 of the *MECP2* gene, exon 12 of the ataxia telangiectasia mutated (*ATM*) gene was the internal control. Group II had the other two assays in the coding part of exon 4 of the *MECP2* gene; the fucosyltransferase 2 (*FUT*) gene was the internal control.

Ten more RD-PCR assays were developed in the 3' and 5' flanking regions of the *MECP2* gene to localize the deletion junction. The primers were designed according to the genomic sequence from NT\_025965 (GenBank accession number) (data not shown).

The PCR mixtures contained a total volume of 25μl: 1xExpand™ High Fidelity buffer#3 (Roche), 4.5 mM MgCl<sub>2</sub>, 200 μM of each dNTP for Group I or 3.0 mM MgCl<sub>2</sub> and 150 μM dGTP/50 μM deaza-dGTP, 200 μM of each other dNTPs and 10% DMSO for Group II, 0.1-0.2 μM of each pair of primers, 1U Platinum *Taq* DNA polymerase (Invitrogen) and 1U platinum *Taq* DNA polymerase High Fidelity (Invitrogen), 0.5 μg of BSA, and 60 ng of genomic DNA. The cycling entailed denaturation at 94°C for 15 sec, annealing at 55°C for Group I or 65°C for Group II for 30 sec, and elongation at 72°C for 1 min for 23 cycles.

### Quantitation

Twelve μl of PCR product was electrophoresed through a standard 2% agarose gel. Gels were stained in 0.2μg/ml Ethidium Bromide for 1 hour and scanned by Typhoon 9410 Imager (Amersham) with the following parameters: focal plane =+3 mm, laser wavelength= 532 nm, Green, emission filter =610 BP 30, photomultiplier voltage =600 V, pixel size =100μm and sensitivity =normal.

ImageQuant™ software was used to quantitate the PCR yield. Net signal of a product band was obtained by subtracting local background signal from total signal in arbitrary unit. The ratio of yields (ROY) is calculated by dividing the target net signal by the internal control net signal. For normalization, the ROY of the patient sample was divided by the average ROY of the normal females.

Table 1. List of Primer Pairs and PCR Segments

|          | 3' sequence-specific region |                                 | T <sub>m</sub><br>(°C) | GC% | Core PCR segment <sup>c</sup> |      |                        |     |  |
|----------|-----------------------------|---------------------------------|------------------------|-----|-------------------------------|------|------------------------|-----|--|
|          | Name <sup>a</sup>           | Sequence <sup>b</sup> (5' - 3') |                        |     | Region                        | Size | T <sub>m</sub><br>(°C) | GC% |  |
| Group I  | Assay 1                     |                                 |                        |     |                               |      |                        |     |  |
|          | Target                      |                                 |                        |     |                               |      |                        |     |  |
|          | MECP2-2(709463)D            | 5' TTTAGTCTTTGGGGTACTTTTA3'     | 45.4                   | 32  | Exon 2 of MECP2               | 492  | 74.1                   | 38  |  |
|          | MECP2-2(709954)U            | 5' GGCTTGTGATAGTGTTGATTCT3'     | 47.8                   | 41  |                               |      |                        |     |  |
|          | Control <sup>d</sup>        |                                 |                        |     |                               |      |                        |     |  |
|          | ATM(38415)D                 | 5' ATCCTGCAAGTTTACCTAAC3'       | 44.9                   | 41  | Exon 12 of ATM                | 418  | 75.2                   | 41  |  |
|          | ATM(38829)U                 | 5' GATCAGGGATATGTGAGTGT3'       | 46.4                   | 43  |                               |      |                        |     |  |
| Group II | Assay 2                     |                                 |                        |     |                               |      |                        |     |  |
|          | Target                      |                                 |                        |     |                               |      |                        |     |  |
|          | MECP2-3(649492)D            | 5' ACCTGGTCTCAGTGTTTCATTGT3'    | 50.0                   | 46  | Exon 3 of MECP2               | 486  | 81.2                   | 55  |  |
|          | MECP2-3(649977)U            | 5' CTTTCAGGGAAGAAAAGTCAGAA3'    | 49.8                   | 41  |                               |      |                        |     |  |
|          | Assay 3                     |                                 |                        |     |                               |      |                        |     |  |
|          | Target                      |                                 |                        |     |                               |      |                        |     |  |
|          | MECP2-4-1(647695)D          | 5' CTTTGTGTCAGAGCCCTACCCATA3'   | 52.7                   | 50  | Exon 4-1 of MECP2             | 447  | 83.5                   | 61  |  |
|          | MECP2-4-1(648141)U          | 5' CCACCATCACCACCACTCAGAG3'     | 56.5                   | 59  |                               |      |                        |     |  |
|          | Control                     |                                 |                        |     |                               |      |                        |     |  |
|          | FUT(502)D                   | 5' TTCACCGGCTACCCCTGCTC3'       | 58.6                   | 65  | FUT2                          | 504  | 83.5                   | 61  |  |
|          | FUT(1006)U                  | 5' GGAGTCGGGGAGGGTGTAAAT3'      | 54.1                   | 60  |                               |      |                        |     |  |
|          | Assay 4                     |                                 |                        |     |                               |      |                        |     |  |
|          | Target                      |                                 |                        |     |                               |      |                        |     |  |
|          | MECP2-4-2(648547)D          | 5' CCCCCTGGCGAAGTTTGAAAAG3'     | 60.5                   | 55  | Exon 4-2 of MECP2             | 400  | 81.2                   | 56  |  |
|          | MECP2-4-2(648946)U          | 5' CCACCATCCGCTCTGCCCTATC3'     | 61.4                   | 64  |                               |      |                        |     |  |

a. For example, MECP2-2(709463)D: MECP2=methyl CpG binding protein 2, Xq28, its sequence is from NT\_025965.13 (GenBank accession number); 5' end of the 3' sequence-specific region of the primer begins at 709463; and D, downstream. The precise sizes and locations of the PCR fragment can be obtained from the information names. ATM=ataxia telangiectasia mutated, 11q22-q23, its sequence is from U82828; FUT=fucosyltransferase 2,19q13, its sequence is from D82933.

b. The sequence of the 3' sequence-specific region is shown. A 10-nucleotide universal tail (5' ggccaagtgt 3') is attached to the 5' end of each primer. Note that the control primers have been redesigned relative to previous report (Liu et al., 2003) to incorporate the 10-nucleotide universal tail.

c. The core PCR segment does not include the tails.

d. Exon 12 of ATM gene and FUT gene were internal controls of the Group I and Group II. They are listed in one assay in each group and left out in others.

#### Southern blotting analysis

Southern blot was performed using probes RTT2 (709610-709766, sequence is from NT\_025965.13), RTT3 (649518-650043) and p(A)10 (639141-639564) that hybridized with exon 2, exon 3 and the end of the 3'UTR. Probes were generated by PCR from genomic DNA, purified from 1% agarose gel by QIAEX II (QIAGEN, Valencia, CA) and labeled with <sup>32</sup>P dCTP by Prime-It II Random primer (Stratagene, Cedar Creek, TX). The genomic DNA (8μg) of female control, male control and patient P3 was digested with *Hind III* and *Pst I* for probe RTT2, *Sac I* for probe RTT3 and *Hind III* and *Sac I* for probe p(A)10. Digested DNA fragments were separated in

a 1.5% agarose gel and blotted into a nylon membrane (Hybond H-N+; Amersham Pharmacia Biotech, Buckinghamshire, England). Hybridization was performed overnight at 65°C and washings were carried out in a series of SSC/SDS solutions (0.1%SDS, 2%-0.1% SSC). Membranes were exposed to storage phosphor screen, scanned by Typhoon 9410 Imager (Amersham, Molecular Dynamics, Sunnyvale, CA). ImageQuant™ software was used to quantitate the signals.

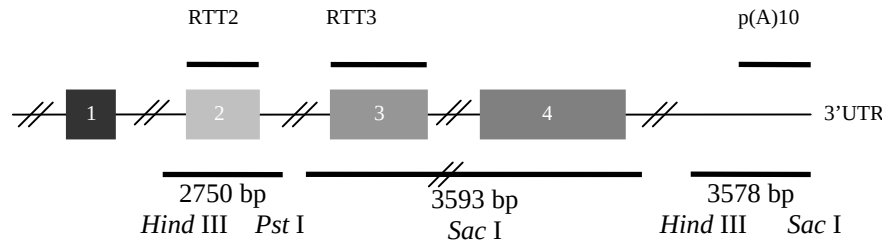


**Figure 1.** Schematic representation of the *MECP2* gene regions analysed by Southern blotting and localization of the probes used in the assay (figure is not to scale).

## RESULTS AND DISCUSSION

### Validation by blinded analysis with 100% accuracy

RD-PCR, a duplex PCR, amplifies an endogenous internal control and a target locus. The internal control has a known gene copy number per cell while the target has an unknown number per cell. The ROY was directly proportional to the ratio of the two input templates, so the copy number of the *MECP2* gene could be obtained according to the ROY and the known copy number of the internal control.

For validation of the four assays in the *MECP2* gene, a blinded analysis was performed with 48 blinded genomic DNA samples where either the sex status or the number of each status were unknown (Fig. 2A). The male sample was functionally equivalent to a RTT patient with large heterozygous deletion. All the males and females were determined with 100% accuracy. The standard deviations of ROY were around 0.04 in both male and female samples in each of the assay.

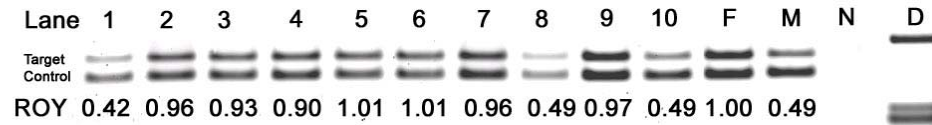
### Large heterozygous deletion found in one patient

Exons 2, 3 and 4 of the *MECP2* gene were analyzed for deletions by four RD-PCR assays. ROYs of each assay were obtained and the copy numbers of the three coding exons 2-4 of the *MECP2* gene were determined in the 65 patient samples and two blinded male controls (Fig. 2B). All the 65 patient samples were previously scanned for point mutation in coding exons and immediate flanking intronic regions of *MECP2* gene by DOVAM-S (Detection of virtually all mutations-SSCP). The RD-PCR analysis was performed blinded to previous point mutation scanning. Only one of eight female patients with classical RTT without point mutation (P3) and two blinded male controls, P1 and P2 showed much lower ROY values than all the other female patients in all the four assays. ROYs of the female patient P3 were 0.44, 0.49, 0.51, and 0.52, indicating that the patient carried a large heterozygous deletion spanning the completely coding region of the *MECP2* gene. None of 22 patients with non-classical RTT with point mutations had a heterozygous deletion. No patient with duplication was observed.

Except for the three samples, P1, P2 and P3, the means and standard deviations of the ROYs were  $1.00 \pm 0.09$ ,  $1.02 \pm 0.08$ ,  $1.00 \pm 0.09$  and  $1.00 \pm 0.10$  respectively; the ranges of ROYs were 0.83-1.22 for Assay 1, 0.83-1.21 for Assay 2, 0.83-1.21 for Assay 3 and 0.79-1.22 for Assay 4. The two blinded male controls were both detected as heterozygous deletions in every assay. All female patients without deletions or duplications had ROYs clearly distinguishable from the male controls and the patient with deletion. These strongly supported the accuracy of the RD-PCR assays.

Southern blotting analysis was used to confirm the deletion identified by RD-PCR method. Signal intensity of patient P3 was similar to that of the male control with probes RTT2, RTT3 and p(A)10 (Fig. 3), indicating only one copy of the *MECP2* gene in patient P3.

A



B

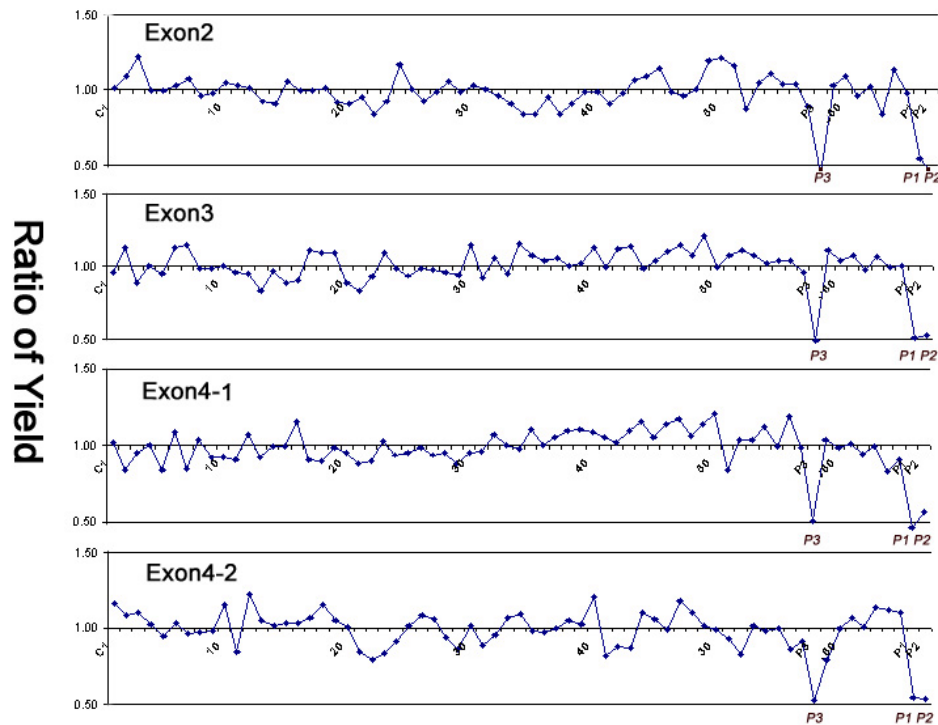


**Figure 2.** Analysis of copy number of the coding region of the *MECP2* gene. **A:** Blinded RD-PCR analysis for exon2. Lanes 1 to 10 are blinded normal control samples, where the gender is unknown. F is a normal female control, M is a normal male control, N is a negative control without DNA added. D is PhiX174 DNA/Hae III Markers, in which three bands of 603bp, 310bp, 281bp+217bp were shown. The ROY of each sample is indicated. **B:** ROY for four RD-PCR assays. Sixty-seven samples were tested for each assay. Y-axis is ROY, crossed with X-axis at 1.0. P1, P2, and P3 have much lower ROYs, indicating only one copy of the *MECP2* gene. P3 is the RTT patient with the deletion; P1 and P2 are male controls.

### Characterization of the large deletion in the female patient P3

To localize the deletion junction, ten more RD-PCR assays were developed flanking the *MECP2* gene. The deletion junction was located within a region of 37.2kb upstream from 5' end of exon 1 and 18.1kb downstream from 3' end of exon 4 (Fig. 4). Long-distance PCR approaches were designed, but unfortunately failed, probably because the DNA was partially degraded.

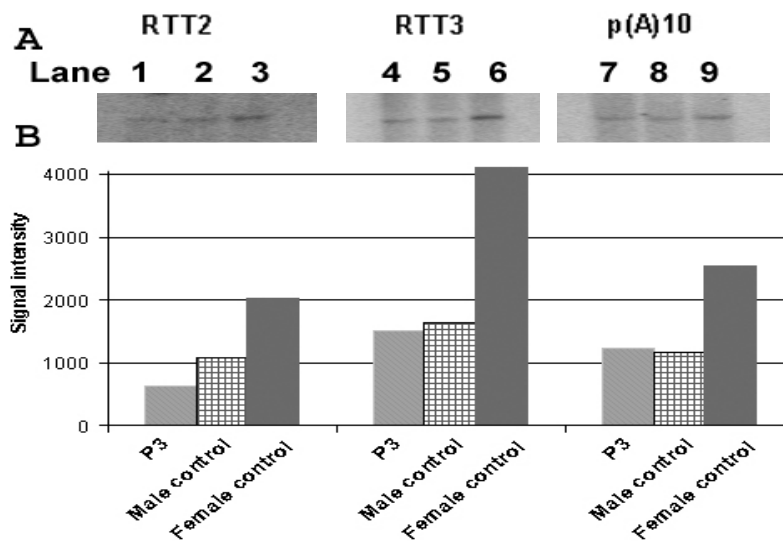


**Figure 3.** Southern blotting analysis. **A:** Images of southern blotting with probes RTT2, RTT3, and p(A)10. Lanes 1, 4, and 7 are patient P3; Lanes 2, 5, and 8 are male control; and lanes 3, 6, and 9 are female control. **B:** Quantitation of each individual. Signal intensity of patient P3 was similar to that of the male control, indicating only one copy of the *MECP2* gene in patient P3.

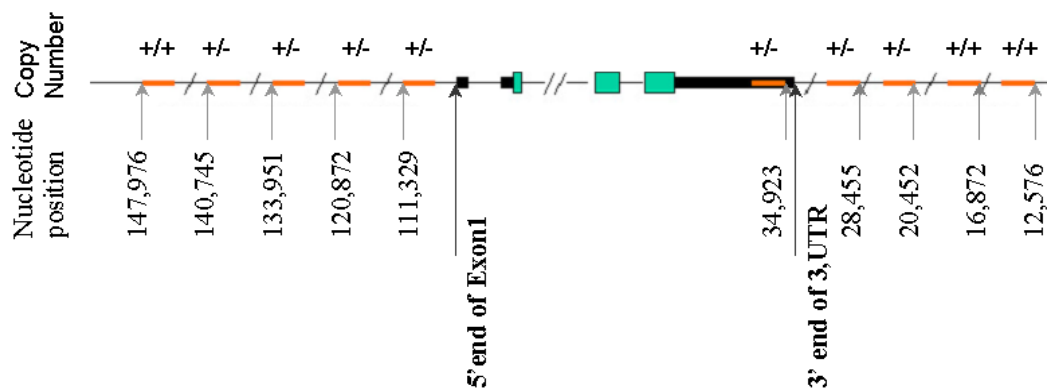


**Figure 4.** Localization of the deletion junction in the female patient P3. Ten RD-PCR assays were developed in the flanking region of the *MECP2* gene on X-chromosome. Primers were designed according to the genomic sequence from NT\_025965 and the nucleotide positions are shown. +/+ indicates two gene copies at the test locus, while +/- indicates only one copy. The deletion junction was located within a region of 37kb upstream from 5' end of exon 1 and 18kb downstream from 3' end of exon 4 (3'UTR).

RD-PCR was used for detection of heterozygous deletions and duplications in the *MECP2* gene in RTT patients. One large deletion was identified in one of eight classical RTT patients without point mutations. The prevalence of *MECP2* gene heterozygous deletions detected by RD-PCR in our patients is 12.5% (1 out of 8), not significantly lower than the aggregate of the previous reports (12 out of 59) (Schollen et al., 2003; Erlandson et al., 2003; Ariani et al., 2004; Laccone et al., 2004). As illustrated by the ten additional dosage assays developed to characterize the deletion, rapid assay development and optimization are two important advantages of RD-PCR.

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### ARTICLE 3

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“An explanation for another familial case of Rett syndrome: maternal germline mosaicism”

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## SHORT REPORT

# An explanation for another familial case of Rett syndrome: maternal germline mosaicism

Margarida Venâncio<sup>1</sup>, Mónica Santos<sup>2</sup>, Susana Aires Pereira<sup>3</sup>, Patrícia Maciel<sup>2</sup> and Jorge M Saraiva<sup>\*,1</sup>

<sup>1</sup>Serviço de Genética Médica, Hospital Pediátrico de Coimbra, Coimbra, Portugal; <sup>2</sup>Instituto de Ciências da Vida e da Saúde (ICVS), Escola das Ciências da Saúde, Universidade do Minho, Braga, Portugal; <sup>3</sup>Serviço de Pediatria, Centro Hospitalar de Vila Nova de Gaia, Portugal

Rett syndrome (RTT; OMIM#312750) is a severe neurodevelopmental disorder that affects mainly girls. It has an estimated incidence of 1:10 000–15 000 females. Mutations in the X-linked gene methyl CpG-binding protein 2 (*MECP2*) have been found in most patients. The most accepted explanation for the sex bias is that the Rett mutation in sporadic cases has its origin in the paternal germline X chromosome and can thus only be transmitted to females. The majority of cases are sporadic (99.5%) but some familial cases have been described. These cases can either be explained by germline mosaicism or by asymptomatic carrier mothers with skewing of X-inactivation towards the wild-type *MECP2* allele. We describe one of the few familial cases of RTT in which a maternal germline mosaicism is the most likely explanation. The mutation p.Arg270fs (c.808delC) was identified in both a girl with classical RTT and her brother who had the severe neurological phenotype usually described in males. The mutation was absent in DNA extracted from blood of both parents. These type of events must be taken into consideration in the genetic counselling of families after the diagnosis of a first case of RTT in a female or a *MECP2* mutation in a male. *European Journal of Human Genetics* advance online publication, 18 April 2007; doi:10.1038/sj.ejhg.5201835

**Keywords:** Rett syndrome; maternal germline mosaicism; *MECP2*

## Introduction

Rett syndrome (RTT; OMIM#312750) is a severe neurodevelopmental disorder that affects mainly girls.<sup>1</sup> It has an estimated incidence of 1:10 000–15 000 females<sup>2</sup> and is one of the leading causes of mental retardation in this sex.<sup>3</sup> The diagnosis is based on the established criteria defined by Hagberg.<sup>4</sup> Mutations in the X-linked gene methyl CpG-binding protein 2 (*MECP2*) have been found in most patients.<sup>5</sup> Recently other genes (*CDKL5*<sup>6</sup> and *Netrin G1*<sup>7</sup>) have been linked to this disease. Male lethality and uniparental disomy have been proposed as possible

explanations for the sex bias. The most interesting suggestion, proposed by several authors<sup>8,9</sup> is that the Rett mutation in sporadic cases has its origin in the paternal germline X chromosome and can thus only be transmitted to females. The majority of cases are sporadic (99.5%).<sup>10</sup> Only a few familial cases with documented *MECP2* mutation have been reported. Some were explained by skewing of X-inactivation towards the wild type allele of *MECP2* in an asymptomatic carrier. In others, five, germline mosaicism (four maternal<sup>2,8,11,12</sup> and one paternal<sup>13</sup>) was a possible explanation.

\*Correspondence: Professor JM Saraiva, Serviço de Genética Médica, Hospital Pediátrico de Coimbra, Av Bissaya Barreto, 3000-075 Coimbra, Portugal. Tel: +351 239 480 638; Fax: +351 239 717 216;

E-mail: j.saraiva@hpc.chc.min-saude.pt

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## Clinical description

### Case 1

The index case is a 3-year-old girl who was referred to us due to moderate developmental delay, severe growth

retardation (weight, 11.010 kg (<P5), standing height, 85 cm (<P5), head circumference, 46 cm (<P5)) since birth, and desacceleration of the head growth, ataxic gait, and hand stereotypies since 24 months of age. The prenatal and perinatal history was considered normal. A thorough investigation (for chromosomal, neurological and metabolic disorders) had already been performed without any diagnostic results. In our observation, non-relevant dysmorphisms were noted, and the diagnoses of RTT and Angelman syndrome (AS) were considered. The molecular study for the AS revealed a biparental pattern of methylation. The molecular analysis of *MECP2* gene detected the mutation p.Arg270fs (c.808delC) (described in the RettBASE: IRSA *MECP2* Variation Database, <http://mecp2.chw.edu.au/mecp2/>), thus confirming the diagnosis of RTT.

### Case 2

Her younger brother was 1-year-old when the diagnosis of RTT was carried out after being confirmed on his sister. The same mutation was found on the *MECP2* gene. His prenatal history was considered normal, but since early age severe growth retardation and development delay was noted. At birth, his somatometric parameters were within the normal range for a 38 weeks' gestation (weight, 2330 g (P3), recumbent height, 44 cm (P3–10), head circumference, 32.3 cm (P10–25)) as well as his Apgar score (7 at the first minute and 9 at the fifth minute). However, due to food refusal, he was admitted in a neonatal intensive care unit for a week. When he was 8 months old, his somatometric evaluation was consistent with severe growth retardation (weight, 5150 g (<P5), recumbent height, 60.1 cm (<P5), head circumference, 38.3 cm (<P5)). He was hypotonic and had very poor visual contact and facial mimic, weak crying and repetitive oral facial and lingual movements. He showed synophris, upslanting palpebral fissures and micrognathia. His cytogenetic study was normal (46,XY). An electroencephalogram was also performed; it revealed low paroxysmal activity at the right temporal region.

The developmental assessment, at 17 months of age, using the Ruth Griffiths Mental Development Scales confirmed the severe developmental delay. The overall developmental quotient was extremely difficult to evaluate (5%). He had a mental age corresponding to 1 month and his functional level was below 3 months. The speech and hearing and eye-hand tests coordination presented the worse scores (3%). The performance, locomotor and personal-social subscales showed a value of 6%. The locomotor evaluation showed that when on ventral position he could only push our hands with his feet but not raise his head and upper body. On ventral prone, he merely tried to rotate the head and was not able to raise it or to pull himself into a crawl position. He could not sit without support but held the head erect and the back straightened for only very short periods of time. The personal-social development revealed that he sporadically

smiled responsively and liked having bath. He felt secure when he was held but did not try to reach the person. Regarding the hearing and language area, his skills were very poor (he reacted badly to loud sounds; did not turn his head towards a sound source; and could simply make some vocalizations). On the hand and eye coordination testing, he sometimes followed a light horizontally, but did not stare at or follow an object. He could not change his look between two objects or tried to grab it in the midline. The performance analysis showed that he reacted to paper when it was in front of him but did not try to reach it. His hands were more or less open and he put them sometimes on the mouth but he was not able to hold a cube on his hand.

He died at 21 months of age due to severe metabolic disequilibrium during a gastrointestinal infectious disease.

The molecular analysis of *MECP2* gene of their parents peripheral blood revealed that neither of them was a carrier for that mutation.

### Conclusion

We describe one of the few familial cases of RTT, in which a maternal germline mosaicism is the most likely explanation. We have described two children of a non-carrier couple, a girl with a classical form of RTT and a boy with a more severe and atypical presentation.

As reviewed previously in a paper by Williamson *et al*,<sup>5</sup> most RTT patients are sporadic and the recurrence risk is low. However, maternal heterozygosity with X-inactivation is a possibility that can be excluded by molecular studies. Even then maternal gonadal mosaicism may exist as once again described in this family.

The presence of the same mutation in a male sib has a recognized phenotype difference. However, our patient has a milder disease than expected. This should be taken into account regarding the indication for *MECP2* molecular studies in males.

Being the proband male, the probability of a maternal heterozygosity or germline mosaicism is greater and the parents should be made aware of this fact.

The knowledge of the rare events described here will be useful regarding the genetic counselling of families where a first diagnosis of RTT in a female or *MECP2* mutation in a male is carried out.<sup>10,12</sup>

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#### ARTICLE 4

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“Stereotypies in Rett Syndrome: analysis of 83 patients with and without detected *MECP2* mutations”

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# NEUROLOGY

## **Stereotypies in Rett syndrome: Analysis of 83 patients with and without detected MECP2 mutations**

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# Stereotypies in Rett syndrome

## Analysis of 83 patients with and without detected MECP2 mutations

T. Temudo, MD; P. Oliveira, PhD; M. Santos, BSc; K. Dias, MD; J. Vieira, MD; A. Moreira, MD; E. Calado, MD; I. Carrilho, MD; G. Oliveira, MD, PhD; A. Levy, MD; C. Barbot, MD; M. Fonseca, MD; A. Cabral, MD; A. Dias, MD; P. Cabral, MD; J. Monteiro, MD; L. Borges, MD; R. Gomes, MD; C. Barbosa, MD; G. Mira, MD; F. Eusébio, MD; M. Santos, MD; J. Sequeiros, PhD, MD; and P. Maciel, PhD

**Abstract—Background:** Hand stereotypies are considered a hallmark of Rett syndrome (RTT) and are usually described as symmetric movements at the midline. However, related pathologies may show the same type of involuntary movement. Furthermore, patients with RTT also have stereotypies with other localizations that are less well characterized. **Methods:** We analyzed stereotypies in 83 patients with RTT, 53 with and 30 without a mutation detected in the *MECP2* gene. Patients were observed and videotaped always by the same pediatric neurologist. Stereotypies were classified, and data were submitted to statistical analysis for comparison of mutation-positive and -negative patients and analysis of their evolution with the disease. **Results:** All the patients showed hand stereotypies that coincided with or preceded the loss of purposeful hand movements in 62% of the patients with *MECP2* mutations. The hair pulling stereotypy was more frequent in the group with detected mutations, whereas hand washing was not. Hand gaze was absent in all RTT patients with *MECP2* mutations. Patients with *MECP2* mutations also had more varied stereotypies, and the number of stereotypies displayed by each patient decreased significantly with age in this group. In all patients, stereotypies other than manual tended to disappear with the evolution of the disease. **Conclusions:** Although symmetric midline hand stereotypies were not specific to patients with an *MECP2* mutation, some of the other stereotypies seemed to be more characteristic of this group. In patients younger than 10 years and meeting the necessary diagnostic criteria of Rett syndrome, the association of hand stereotypies without hand gaze, bruxism, and two or more of the other stereotypies seemed to be highly indicative of the presence of an *MECP2* mutation.

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Rett syndrome (RTT) was discovered by Andreas Rett who noted that two girls waiting for his consultation presented the same movement disorder: hand stereotypies.

In 1966, Rett<sup>1</sup> published data on 22 girls with progressive cerebral atrophy, stereotyped hand

movements, dementia, alalia, gait apraxia, and a tendency toward epileptic attacks. As the disease is primarily sporadic in nature and familial cases are rare,<sup>2</sup> it took more than 30 years to determine its genetic basis: mutations in the methyl-CpG-binding protein 2 (*MECP2*) gene.

Stereotypies may be defined as involuntary, rhythmic, patterned, coordinated, repetitive, and seemingly purposeless movements or utterances that are usually continuous, in contrast with mannerisms or tics.<sup>3</sup> They can be transient (physiologic)<sup>4,5</sup> or persistent (pathologic).<sup>6,7</sup>

Additional material related to this article can be found on the *Neurology* Web site. Go to [www.neurology.org](http://www.neurology.org) and scroll down the Table of Contents for the April 10 issue to find the title link for this article.

From the Unidade de Neuropediatria (T.T.), Serviço de Pediatria, Hospital Geral de Santo António, Porto, Portugal; Departamento de Produção e Sistemas (P.O.), Escola de Engenharia, Universidade do Minho, Guimarães, Portugal; Instituto de Investigação em Ciências da Vida e da Saúde (M.S., P.M.), Escola de Ciências da Saúde, Universidade Minho, Braga, Portugal; Departamento de Estudos de Populações (M.S., J.S.), ICBAS, Universidade do Porto, Portugal; Serviço de Neuropediatria (K.D., J.V., A.M., E.C., A.D.), Hospital D<sup>a</sup> Estefânia, Lisboa, Portugal; Serviço de Neuropediatria (I.C., C. Barbot, M.S.), Hospital de Crianças Maria Pia, Porto, Portugal; Centro de Neuropediatria (G.O., A.C., L.B.), Hospital Pediátrico, Coimbra, Portugal; Serviço de Pediatria (A.L., F.E.), Hospital Santa Maria, Lisboa, Portugal; Serviço de Pediatria (M.F., J.M.), Hospital Garcia da Horta, Almada, Portugal; Serviço de Neurologia (P.C.), Hospital Egas Moniz, Lisboa, Portugal; Serviço de Pediatria (R.G., C. Barbosa), Hospital Pedro Hispano, Matosinhos, Portugal; and Serviço de Pediatria (G.M.), Hospital Espírito Santo, Évora, Portugal; UNIGENE (J.S.), IBMC, Porto, Portugal.

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Address correspondence and reprint requests to Dr. Teresa Temudo, Unidade de Neuropediatria, Serviço de Pediatria, Hospital de Santo António, SA, Largo Abel Salazar, 4099/001 Porto, Portugal; e-mail: [teresatemudo@netcabo.pt](mailto:teresatemudo@netcabo.pt)

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In addition to motor and phonic, stereotypies can be classified as either simple (e.g., tapping, mouthing, clapping) or complex (e.g., a sequence of different movements always performed in the same way and sometimes seeming to have a purpose); they can also be described according to the predominant site involved (e.g., head, trunk, hand, inferior limbs).<sup>3,6</sup>

Stereotyped hand movements are a hallmark of RTT, and one of its necessary diagnostic criteria.<sup>8</sup> Usually they are associated with or follow the disappearance of purposeful hand movements, but can also be present before developmental regression begins.<sup>9</sup> These almost continuous, repetitive, compulsive automatisms disappear during sleep. Environmental manipulation was shown to have a limited effect on their frequency, suggesting that these movements are reinforced through neurochemical processes.<sup>10</sup>

Other stereotyped movements and behaviors can also be present in RTT, but are much less well described.<sup>10-13</sup>

We describe the stereotypies that can be present in RTT and their lifelong evolution, based on an observational study of 83 patients with a clinical diagnosis of RTT, all studied for the presence or absence of *MECP2* mutations. We also compare the stereotypies present in patients with and without a positive molecular diagnosis.

**Methods.** All Portuguese pediatric neurologists were asked to indicate their patients with possible RTT. Patients were always observed and videotaped by the same pediatric neurologist, and a clinical checklist for RTT was completed. A classification of stereotypies was used that was modified from Jankovick<sup>3</sup> and Fernandez-Alvarez and Aicardi.<sup>6</sup> Informed consent was obtained from all parents to collect blood, and to take and use video and photographs.

Blood samples from patients and their parents were received at our laboratory, and genomic DNA was extracted using the Puregene DNA isolation kit (Gentra, Minneapolis, MN). The coding region and exon-intron boundaries of the *MECP2* gene were amplified by PCR and sequenced. The robust dosage PCR method was used, as described,<sup>14</sup> for the detection of large rearrangements in the *MECP2* gene. Primers and PCR conditions are available upon request.

Contingency tables were obtained, and  $\chi^2$  and Fisher exact tests were used. Groups were compared with Student's *t* test. The SPSS (v.14) statistical package was used to analyze the data.

**Results.** We observed 117 cases with possible RTT; 33 were excluded because they did not fulfill the revised diagnostic criteria and one because the patient was a man with a severe encephalopathy who never presented stereotypies.<sup>8</sup> The mean age at the first observation of the 83 patients who fulfilled the diagnostic criteria was 10.0 years (range, 1 to 31, median, 7 years). Twenty patients were observed and videotaped two or more times at 6-month intervals.

Cases were classified as classic (60.2%) and variant (39.8%) forms of RTT. Mutations were found in 63.9% of all patients (*n* = 53), corresponding to 84.0% of the classic forms and 33.3% of the variants; all were *de novo*. Patients were thus divided into two groups: those with a positive molecular diagnosis (Group I) and those without (Group II).

All cases presented hand stereotypies that appeared

at a mean age of 22.3 months in Group I and 25.4 months in Group II, after decrease or loss of purposeful hand movements (22.2 months in Group I and 17.4 months in Group II), and several months after losing social contact (17.0 months in Group I and 11.1 month in Group II). In 25 cases (23 in Group I and two in Group II), loss of purposeful hand movements and appearance of stereotypies coincided; in 11 cases (10 cases in Group I and one in Group II), stereotypies preceded the loss of purposeful hand movements (mean time, 9.5 months; range, 5.0 to 25).

The most frequent hand movement observed was the compulsive wringing, washing-like movement of both hands, usually at the midline, most often in front of the body (73.26% in Group I and 80.0% in Group II). Other symmetric movements of both hands were also present (table; video E-1 on the *Neurology* Web site at [www.neurology.org](http://www.neurology.org); figure 1). Stereotypies with separated hands, more often each hand performing a different movement and coexisting or not with midline hand stereotypies, were present in 60.2% of all the patients (60.4% in Group I and, 60.0% in Group II) (table; video E-2; figure 1). Of the Group I patients, 20.4% showed only hands-apart stereotypies. None of the patients in Group I looked at their hands while performing hand stereotypies, whereas 40.0% of those in Group II did (*p* < 0.001).

The second most frequent stereotypy in RTT was bruxism. We found bruxism in 90.4% of 83.0 RTT patients (94.3% in Group I and 83.3% in Group II); in all patients, it occurred while awake and disappeared during sleep.

Stereotypies with other topographies are described in the table (see also videos E-3 and E-4; figure 1). They could be very complex at the beginning of the disease; two girls had a stereotyped dancelike behavior involving many sites of the body, always performed in the same sequence, and seemingly with a purpose (table; video E-5).

Most patients (97.6%) had more than one stereotypy, and 31.7% (*n* = 26) had five or more different ones (38.5% in Group I and 20.0% in Group II). The number of stereotypies per patient was larger in Group I (a mean of 4.8 stereotypies in Group I and 3.7 in Group II), and the patients in this group exhibited a significantly greater number of topographies of stereotypies: 28 different stereotypies were found in Group I, whereas only 19 stereotypies were seen in Group II. Although 54.6% of the patients in Group I performed five stereotypies, only three were performed by 52.7% of the patients in Group II. Patients who acquired independent gait had a significantly larger number of stereotypies, in both groups. Group I (but not Group II) patients with rigidity showed a significantly smaller number of stereotypies.

The most frequent association was that of the hand-washing stereotypy with bruxism (69.1%; 70.6% in Group I, 66.7% in Group II). Among patients with more than one manual stereotypy, mouthing and hand washing were the most frequently associated (43.4%; 39.6% in Group I, 50.0% in Group II).

Significant differences between the mutation-positive (Group I) and mutation-negative groups (Group II) regarding the frequency of specific stereotypies were found (table).

We also found that the number of stereotypies decreased with age (particularly after the age of 10), mainly

**Table** Stereotypies found in a series of 83 patients with Rett syndrome

|                                                     | Total, %<br>(n = 83) | Group I, %<br>(n = 53) | Group II, %<br>(n = 30) | $\chi^2$ |
|-----------------------------------------------------|----------------------|------------------------|-------------------------|----------|
| Motor stereotypy                                    |                      |                        |                         |          |
| Simple                                              |                      |                        |                         |          |
| Head                                                |                      |                        |                         |          |
| Rolling                                             | 1.2                  | 1.9                    | 0.0                     | 0.573    |
| Retropulsion                                        | 7.2                  | 11.2                   | 0.0                     | 3.661*   |
| Grimacing                                           | 8.4                  | 11.2                   | 3.3                     | 1.583    |
| Bruxism                                             | 90.4                 | 94.3                   | 83.3                    | 5.956†   |
| Protrusion of the lips                              | 6.0                  | 9.4                    | 0.0                     | 3.012    |
| Repetitive closure of the eyes                      | 7.2                  | 9.4                    | 3.3                     | 1.063    |
| Eye rolling                                         | 12.0                 | 15.1                   | 6.7                     | 1.284    |
| Joined hands                                        | 80.7                 | 81.1                   | 80.0                    | 0.016    |
| Washing                                             | 75.9                 | 73.6                   | 80.0                    | 0.431    |
| Clapping                                            | 14.5                 | 15.1                   | 13.3                    | 0.048    |
| Mouthing                                            | 21.7                 | 22.6                   | 20.0                    | 0.079    |
| Separated hands                                     | 60.2                 | 60.4                   | 60.0                    | 0.001    |
| Mouthing                                            | 36.1                 | 37.7                   | 33.3                    | 0.161    |
| Hair pulling                                        | 10.8                 | 17.0                   | 0.0                     | 5.714†   |
| Pill rolling                                        | 8.4                  | 3.8                    | 16.7                    | 4.124*   |
| One hand behind the neck                            | 4.8                  | 5.7                    | 3.3                     | 0.226    |
| Castanets                                           | 1.2                  | 1.9                    | 0.0                     | 0.573    |
| Twisting two or three fingers                       | 3.6                  | 3.8                    | 3.3                     | 0.011    |
| Flapping                                            | 2.4                  | 1.9                    | 3.3                     | 0.170    |
| Tapping                                             | 30.1                 | 28.3                   | 33.3                    | 0.230    |
| "Sevillana"                                         | 2.4                  | 3.8                    | 0.0                     | 1.160    |
| Hand twirling                                       | 8.4                  | 9.4                    | 6.7                     | 0.190    |
| Hand gaze                                           | 14.5                 | 0.0                    | 40.0                    | 24.783‡  |
| Arms                                                |                      |                        |                         |          |
| Repetitive and rhythmic flexion of the arms         | 4.8                  | 5.7                    | 3.3                     | 0.226    |
| Legs                                                |                      |                        |                         |          |
| Intermittent leg elevation and tapping of the floor | 10.8                 | 11.3                   | 10.0                    | 0.035    |
| Toe walking                                         | 15.7                 | 17.0                   | 13.3                    | 0.193    |
| Jumping                                             | 1.2                  | 1.9                    | 0.0                     | 0.573    |
| Feet                                                |                      |                        |                         |          |
| Feet twirling                                       | 3.6                  | 5.7                    | 0.0                     | 1.762    |
| Whole body                                          |                      |                        |                         |          |
| Trunk rocking                                       | 21.7                 | 22.6                   | 20.0                    | 0.079    |
| Shifting weight from one leg to the other           | 20.5                 | 24.5                   | 13.3                    | 1.474    |
| Complex                                             | 2.4                  | 3.8                    | 0.0                     | 1.160    |
| Phonic stereotypy                                   |                      |                        |                         |          |
| Repetitive sounds                                   | 4.8                  | 5.7                    | 3.3                     | 0.226    |
| Repetitive words or phrases                         | 1.2                  | 1.9                    | 0.0                     | 0.573    |

\*  $p < 0.1$ .†  $p < 0.05$ .‡  $p < 0.010$ .

in Group I (figure 2): a difference was observed between patients younger and older than 10 years of age ( $t = 3.749$ ,  $p = 0.001$ ) in Group I.

In the 20 patients observed various times (15 in Group I and five in Group II), the mean length of follow-up was 3.75 years (range, 1 to 6 years; median, 4

years). All maintained the pattern of manual stereotypies, but there was a change in the pattern of stereotypies with other localizations: new stereotypies might be added to or replace one of previous ones. Stereotypies other than manual ones tended to disappear with evolution of the disease.





**Figure 1.** Different topographies of stereotypies in Group I Rett syndrome patients. Typical hand washing stereotypies (A to C); mouthing with hands apart (D to G); mouthing with joined hands (H); hair pulling (I, J); cervical retropulsion (K to M) (K and L with simultaneous closure of the eyes); twisting of the fingers with hands apart (N); shifting weight from one leg to the other (O, P).

**Discussion.** Stereotyped hand movements are considered a hallmark of RTT, and it has been assumed that they are symmetric and at midline.<sup>10-13,15-19</sup> The aim of this study was to analyze the specificity of the stereotypies in patients with a positive molecular diagnosis of RTT. There are a number of limitations to this study, including the fact that it was a single-rater, cross-sectional, and observational, and, therefore, state-dependent study; however, 24% of the patients were observed several times, and complete video records allowed reanalysis and increased objectivity.

Although washing-like movements of both hands are considered an indicator of RTT, this finding is not specific of the syndrome; remarkably, in this study, this stereotypy was more frequent in the group with no mutation in the *MECP2* gene. In addition,

a significant proportion of the mutation-positive patients showed only hands-apart stereotypies. One of the singularities of *MECP2* mutation-positive RTT patients was that they tended not to look at their hands when performing hand stereotypies, possibly because they have very poor ocular-manual coordination.

Differences were found in the frequency of four stereotypies, and of these, hair pulling, bruxism, and cervical retropulsion were more frequent in the mutation-positive group.

In general, mutation-positive patients had more diverse stereotypies that diminished after the age of 10. These patients also had a larger number of stereotypies per patient. In patients younger than 10 years and with the necessary diagnostic criteria of RTT, the association of hand stereotypies without

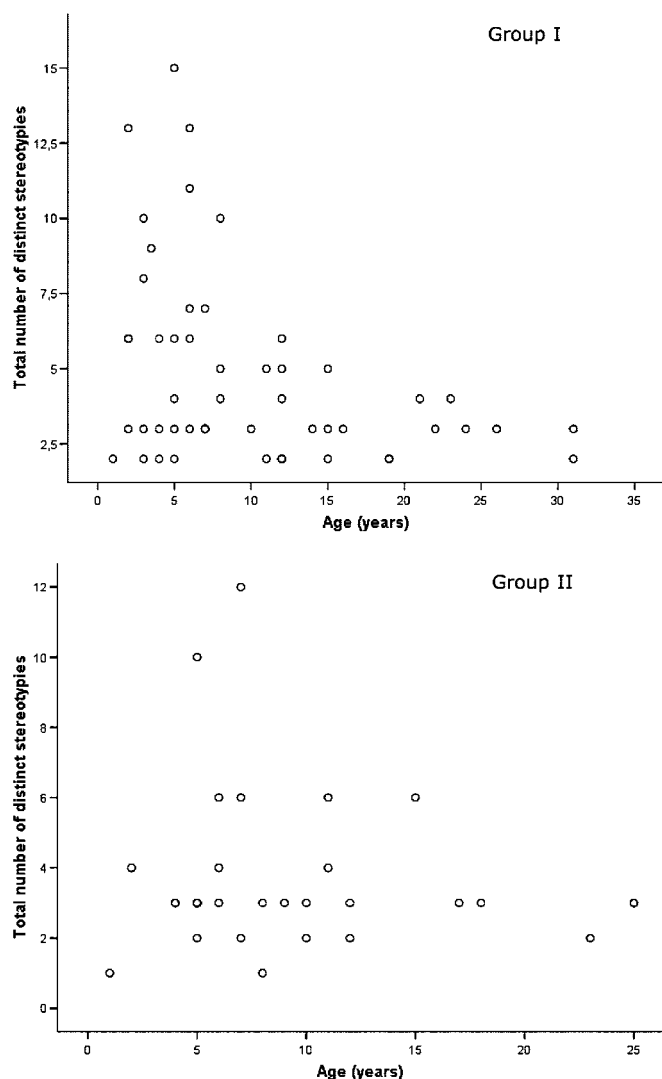


Figure 2. Total number of distinct stereotypies by age (some points in the graph may correspond to several patients). The number of stereotypies decreased with age (particularly after age 10 years), especially in Group I, in which a significant difference was observed between patients younger and older than 10 years ( $t = 3.749$ ,  $p = 0.001$ ).

hand gaze, bruxism, and two or more of the other stereotypies seemed to be highly indicative of the presence of an *MECP2* mutation.

The pathophysiologic basis of stereotypies in RTT remains elusive, and we do not know whether these involuntary movements interfere with motor learning. Most studies stress that hand stereotyped movements coincide with or follow the disappearance of purposeful prehension in RTT; however, a video analysis of 22 patients in the first 6 months of life, before the beginning of regression, showed stereotyped hand movements in 42% of cases.<sup>9</sup> In our series, in the group with *MECP2* mutations, stereotypies were also described by the parents as coinciding with the loss of purposeful hand movements in 43.3% and preceding it in 18.8%. We thus

believe that the particularly compulsive behavior of stereotypies in patients with RTT may have a role in the complex process of loss or reduction of hand use.

Like other authors,<sup>12,13</sup> we found that manual stereotypies became simpler and slower with the progress of the disease, as patients become hypokinetic and rigid; however, each patient maintained the same type of hand movements throughout the period of observation. Other stereotypies tended to disappear and behaved like tics, with one stereotyped movement replacing another. This allows us to speculate that the physiopathology of hand stereotypies may be different from that of other topographies.

We conclude that stereotypies in RTT can be pleomorphic, mainly in the first decade of life. Nevertheless, the pattern of some repetitive movements associated with this disorder suggests that they have underlying monotonous motor programming, the basis of which should be investigated.

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**Stereotypies in Rett syndrome: Analysis of 83 patients with and without detected MECP2 mutations**

T. Temudo, P. Oliveira, M. Santos, K. Dias, J. Vieira, A. Moreira, E. Calado, I. Carrilho, G. Oliveira, A. Levy, C. Barbot, M. Fonseca, A. Cabral, A. Dias, P. Cabral, J. Monteiro, L. Borges, R. Gomes, C. Barbosa, G. Mira, F. Eusébio, M. Santos, J. Sequeiros and P. Maciel

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**ARTICLE 5**

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*“MECP2 coding sequence and 3'UTR variation in 172 unrelated autistic patients”*

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## MECP2 Coding Sequence and 3'UTR Variation in 172 Unrelated Autistic Patients

Ana M. Coutinho,<sup>1</sup> Guiomar Oliveira,<sup>2</sup> Cécile Katz,<sup>3</sup> Jinong Feng,<sup>3</sup> Jin Yan,<sup>3</sup> Chunmei Yang,<sup>3</sup> Carla Marques,<sup>2</sup> Assunção Ataíde,<sup>4</sup> Teresa S. Miguel,<sup>4</sup> Luís Borges,<sup>2</sup> Joana Almeida,<sup>2</sup> Catarina Correia,<sup>1,9</sup> António Currais,<sup>1</sup> Celeste Bento,<sup>2</sup> Luísa Mota-Vieira,<sup>5</sup> Teresa Temudo,<sup>6</sup> Mónica Santos,<sup>7,8</sup> Patrícia Maciel,<sup>7</sup> Steve S. Sommer,<sup>3</sup> and Astrid M. Vicente<sup>1,9\*</sup>

<sup>1</sup>Instituto Gulbenkian de Ciência, Oeiras, Portugal

<sup>2</sup>Hospital Pediátrico de Coimbra, Coimbra, Portugal

<sup>3</sup>Department of Molecular Genetics, City of Hope National Medical Centre and Beckman Research Institute, Duarte, California

<sup>4</sup>Direcção Regional de Educação da Região Centro, Coimbra, Portugal

<sup>5</sup>Unidade de Genética e Patologia Moleculares, Hospital do Divino Espírito Santo, Ponta Delgada, Açores, Portugal

<sup>6</sup>Hospital de Sto. António, Porto, Portugal

<sup>7</sup>ICVS/Escola de Ciências da Saúde, Universidade do Minho, Braga, Portugal

<sup>8</sup>ICBAS, Universidade do Porto, Porto, Portugal

<sup>9</sup>Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisboa, Portugal

Mutations in the coding sequence of the methyl-CpG-binding protein 2 gene (*MECP2*), which cause Rett syndrome (RTT), have been found in male and female autistic subjects without, however, a causal relation having unequivocally been established. In this study, the *MECP2* gene was scanned in a Portuguese autistic population, hypothesizing that the phenotypic spectrum of mutations extends beyond the traditional diagnosis of RTT and X-linked mental retardation, leading to a non-lethal phenotype in male autistic patients. The coding region, exon–intron boundaries, and the whole 3'UTR were scanned in 172 patients and 143 controls, by *Detection of Virtually All Mutations-SSCP* (DOVAM-S). Exon 1 was sequenced in 103 patients. We report 15 novel variants, not found in controls: one missense, two intronic, and 12 in the 3'UTR (seven in conserved nucleotides). The novel missense change, c.617G > C (p.G206A), was present in one autistic male with severe mental retardation and absence of language, and segregates in his maternal family. This change is located in a highly conserved residue within a region involved in an alternative transcriptional repression pathway, and likely alters the secondary structure of the MeCP2 protein. It is therefore plausible that it leads to a functional modification of MeCP2. *MECP2* mRNA levels measured in four patients with 3'UTR conserved changes were below the control range, suggesting

an alteration in the stability of the transcripts. Our results suggest that *MECP2* can play a role in autism etiology, although very rarely, supporting the notion that *MECP2* mutations underlie several neurodevelopmental disorders.

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**KEY WORDS:** autism; *MECP2*; 3'UTR; exon 1; *Detection of Virtually All Mutations-SSCP*

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### INTRODUCTION

Mutations in the coding region of the methyl-CpG-binding protein 2 gene (*MECP2*) are responsible for about 80% of Rett Syndrome (RTT, OMIM #312750) cases [Amir et al., 1999]. Most RTT patients develop microcephaly, seizures, and autism. Autism (OMIM #209850) is a neurodevelopmental disorder characterized by deficits in social interaction and communication, and by restricted and stereotyped patterns of behavior. It affects more males than females, in a ratio of 3–4:1, which led to the hypothesis of the involvement of an X-linked gene. As autism and RTT share a range of symptoms, it was speculated that specific mutations in the *MECP2* coding region could also be involved in autism etiology. Evidence for linkage to autism was recently found at chromosome Xq27–q28, in the region where *MECP2* maps, supporting it as a candidate gene for this disorder [Vincent et al., 2005]. So far, a number of mutations previously found in RTT have been reported in autism studies [van Karnebeek et al., 2002; Carney et al., 2003; Lobo-Menendez et al., 2003; Zappella et al., 2003]. In two studies novel alterations were reported, one missense change in one autistic male [Beyer et al., 2002] and a de novo intronic variation in an autistic female with mental retardation [Lam et al., 2000], although their functional significance was not demonstrated. Mutations in *MECP2* have also been found in other syndromes, including non-specific X-linked mental retardation in males (reviewed in Bienvenu and Chelly [2006]). Although severe *MECP2* mutations leading to RTT are thought to be lethal in hemizygous males, these studies

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\*Correspondence to: Astrid M. Vicente, Ph.D., Instituto Nacional de Saúde Dr. Ricardo Jorge, Av. Padre Cruz, 1649-016 Lisboa, Portugal, and Instituto Gulbenkian de Ciência, Ap. 14, 2781-901, Oeiras, Portugal. E-mail: avicente@igc.gulbenkian.pt

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show the existence of sequence changes not found in RTT that segregate in the families of males with autism, mental retardation, and occasionally language problems. Hemizyosity for some *MECP2* mutations, leading to a less severe functional alteration of the protein, may therefore be compatible with life, with heterozygosity for these same mutations insufficient to cause disease in the female carriers.

The *MECP2* gene originates two protein isoforms: MeCP2\_e2, encoded by exons 2–4, and MeCP2\_e1, encoded by exons 1, 3, and 4, which is more abundantly expressed in the brain [Kriaucionis and Bird, 2004; Mnatzakanian et al., 2004]. MeCP2 acts as a transcriptional repressor and is mainly expressed in the central nervous system (CNS), indicating a role in the regulation of brain gene expression. Although its target genes are not fully known, microarray studies have found several genes with altered expression in RTT, some showing a direct regulation by MeCP2 (reviewed in Bienvenu and Chelly [2006]). In addition, it has been demonstrated that it regulates the expression of the brain-derived neurotrophic factor gene (*BDNF*), which encodes a molecule essential in neurodevelopment and neuronal plasticity, learning, and memory [Chen et al., 2003; Martinowich et al., 2003]. The 3'UTR in exon 4 is unusually long (8.5 kb) and well conserved between human and mouse, with at least eight blocks of strong sequence similarity that can represent important functional domains [Coy et al., 1999]. The size and conservation of this region, as well as expression studies in *post-mortem* brain, indicate an important role in the transcriptional regulation of *MECP2*. Samaco et al. [2004] found altered levels of *MECP2* expression in the brain of four out of five autistic individuals, none of which had mutations in the coding region of *MECP2*, suggesting that variations in the 5'UTR or 3'UTR could be responsible for these changes. These findings support the hypothesis that variants in the 3'UTR, and not only in the coding region of *MECP2*, may be involved in some clinical manifestations.

A previous report has shown a higher frequency of missense and 3'UTR variants in a sample of 24 autistic patients, as compared to other psychiatric diseases [Shibayama et al., 2004]. In the present study, we extend the search for *MECP2* mutations in the coding region, exon–intron boundaries, and in the whole 3'UTR in 172 patients with autism. Our goal was to determine if the phenotypic spectrum of mutations extends beyond the traditional diagnosis in RTT and mental retardation, leading to a less severe phenotype in autistic male and female patients.

## MATERIALS AND METHODS

### Subjects and Clinical Assessments

One hundred seventy two caucasian unrelated autistic children (141 males and 31 females; age range of 2–14 years old), originating from mainland Portugal and the Azorean islands, were recruited at the Autism Clinic from Hospital Pediátrico de Coimbra (HP). Diagnosis and assessment of the children followed a comprehensive evaluation protocol by a clinical team including a developmental pediatrician, two psychologists, one special education teacher, and a social worker. Observation of the children entailed extensive interaction and semi-structured activities in a clinical setting. ASD was diagnosed using DSM-IV (American Psychiatric Association 1994) criteria, the Autism Diagnostic Interview-Revised (ADI-R) [Lord et al., 1994], and the Childhood Autism Rating Scale (CARS) [Schopler et al., 1988]. Diagnosis required fulfillment of DSM-IV criteria and meeting the ADI-R algorithm cutoff for autistic disorder, and a functional level of 12 months or above. Consensus diagnosis among the clinical team was obtained for all patients. About 90% of these patients

are routinely followed by the same clinical team at HP and therefore are monitored over several years until age 18.

Developmental or intellectual quotients were determined using the Ruth Griffiths Mental Development Scale II [Griffiths, 1984] or the Wechsler Intelligence Scale for Children (WISC 1974). Functional level was assessed using the Vineland Scales for Adaptive Behavior [Carter et al., 1998]. Idiopathic subjects were included after clinical assessment and screening for known medical and genetic conditions associated with autism, including testing for Fragile X mutations (FRAXA and FRAXE), chromosomal abnormalities (karyotype study), neurocutaneous syndromes, endocrine, and metabolic disorders. Control Portuguese unrelated adult individuals consisted of 143 caucasian healthy blood donors (113 males and 30 females), with no family history of neuropsychiatric diseases. Control Portuguese unrelated children consisted of 36 healthy individuals (21 males and 15 females; age range of 2–15 years old), recruited at the surgery service at the HP where they were undergoing minor surgery procedures, requiring blood sample collection for pre-surgery baseline evaluation. The study was approved by the HP ethical committee, and all participants or legal representatives signed an informed consent.

### *MECP2* Mutation Detection

The coding region of *MECP2* comprising exons 2–4, exon–intron boundaries, and its whole 3'UTR, were scanned for mutations with *Detection Of Virtually All Mutations*-SSCP (DOVAM-S) [Liu et al., 1999]. DOVAM-S is a robotically enhanced and highly redundant form of *Single Strand Conformational Polymorphism* (SSCP) with virtually 100% sensitivity of mutation detection. The gene was first amplified robotically in 42 segments ranging in size from 150 to 476 bp, pooled, denatured, and electrophoresed under five non-denaturing conditions varying in gel matrix, buffer, temperature, and additive. PCR products with mobility shifts were sequenced with the ABI model 377 (Perkin-Elmer Model 377, Norwalk, CT) and nucleotide alterations were analyzed with Sequencher 4.1 software (Gene Codes, Ann Arbor, MI). Sequence changes were confirmed by reamplification with genomic DNA and sequencing in the opposite direction. Exon 1 was analyzed by direct sequencing of the corresponding region. Information on the primers used for amplification of the coding region and the 3'UTR of *MECP2*, as well as the PCR conditions used, is provided on the Supplementary Information section.

### Bioinformatic Analysis of the *MECP2* Sequence

In order to assess if the novel intronic changes were affecting normal splicing by altering a consensus sequence, the program GenScan version 1.0 was used, which performs predictions of exon/intron splice sites based on local nucleotide sequence properties of the genomic DNA. Protein secondary structure predictions were performed for the novel missense changes found in *MECP2* using the programs PeptideStructure (GCG package, version 10.0), Garnier (Emboss, version 2.8.0), and SSpro8, which use several algorithms for prediction based on the primary structure and properties of the amino acid residues. Searching for sequence patterns in the 3'UTR of *MECP2* was performed using UTRscan database, which looks for known UTR functional elements, and the program FindPatterns (GCG package, version 10.0), which locates short sequence patterns specified by the user.

### RNA Isolation and Quantification of the *MECP2* mRNA

Total RNA was extracted from  $5 \times 10^6$  peripheral blood mononuclear cells (PBMCs) using the RNeasy Mini Kit (Qiagen, Valencia, CA), and used for amplification of the first

strand of cDNA by reverse transcription with oligo-dT primer (Invitrogen, Carlsbad, CA). Total *MECP2* mRNA (*MECP2\_e1* and *MECP2\_e2* transcripts) levels were quantified by quantitative PCR with LightCycler Fast Start SYBR Green I (Roche Molecular Biochemicals, Mannheim, Germany). For each sample, 50 ng of first-stranded cDNA were amplified in duplicate by PCR and real-time fluorimetric intensity of SYBR green I was monitored. The levels of *MECP2* mRNA for each sample were normalized by the amount of mRNA of the housekeeping gene *HPRT1*. Details for the quantitative PCR reaction, including primers used for *MECP2* and *HPRT1* are provided in the Supplementary Information section.

### Quantitative X-chromosome Inactivation Assay

X-chromosome inactivation (XCI) assays were performed in DNA isolated from peripheral blood leukocytes, to assess the pattern of XCI from the carrier mother and maternal grandmother of the autistic male who presented the novel p.G206A missense change. It was also performed in the proband, in order to determine which allele was inherited from his mother. The assay was based on a previously described method [Allen et al., 1992], which allows the determination of the X-inactivation status using a trinucleotide repeat polymorphism in the androgen receptor gene (*AR*) flanked by two methylation-sensitive restriction enzyme sites. These sites are methylated on the inactive X chromosome, and are unmethylated on the active X chromosome. This allowed the development of an assay that distinguishes between the maternal and paternal alleles (through the repeat number) and identifies their methylation status (through enzymatic restriction). Details for the assay are provided in the Supplementary Information section.

### Quantification of BDNF Levels in Plasma

Levels of BDNF were quantified in plasma using BDNF Emax Immunoassay System kit (Promega Corp., Madison, WI), according to the manufacturer's instructions. The assays were performed in duplicate for each sample.

## RESULTS

### Variation of the *MECP2* Coding Region and Exon-Intron Boundaries in Autistic Patients and Controls

Scanning of *MECP2* sequence changes in exons 2–4 and exon–intron boundaries was performed in 172 patients (141 males and 31 females; 203 X-chromosomes total), revealing that 12 patients (7.0%), 10 males and 2 females, have sequence changes corresponding to 11 different variants (Table I). Of these, four are missense changes observed in males, of which one is novel. In addition, we found four silent and three intronic changes (two novel); of these, two silent and one intronic change were also present in the controls, and have been previously reported [Trappe et al., 2001; Kleefstra et al., 2004]. The novel missense change is a c.617G > C transition found in one autistic male, resulting in a p.G206A amino acid replacement in the inter-domain region of MeCP2, and was not found in 143 controls. Protein secondary structure predictions revealed that this alteration can disturb a  $\alpha$ -helix in the inter-domain region of the protein, due to the alteration in amino acid properties from polar to hydrophobic. This amino acid position is included in a region involved in a histone deacetylase-independent pathway of transcriptional repression by MeCP2 [Yu et al., 2000], and thus this sequence change is likely to lead to an alteration in protein function. Predictions of exon/intron splice sites were performed for the novel intronic changes found, and do not alter any putative consensus

sequence in the genomic DNA, therefore it is not likely that they affect normal splicing of *MECP2*. None of the sequence variants found in our autistic children sample have been reported as pathogenic mutations in Rett syndrome studies. Sequencing of exon 1 was performed in 103 autistic patients (88 males and 15 females; 118 X-chromosomes total), and no sequence changes were found, indicating that alterations in this region in autistic patients are likely very rare.

In 143 Portuguese healthy controls (113 males and 30 females; 173 X-chromosomes total), we found 11 individuals (7.7%), 5 males and 6 females, with sequence changes in exons 2–4 and exon–intron boundaries, corresponding to six distinct variants (Table II). Of these, one is a novel missense change (p.K82R) occurring in one male, one intronic change, and four silent changes. The p.K82R missense change does not lead to a change in amino acid properties and does not alter the secondary structure of the protein, so it likely represents a rare polymorphism. None of the sequence variants found in the control samples have been reported as pathogenic mutations in previous studies. Of the sequence changes observed in this study, three were common to both patients and controls (c.378-19delT, p.A131A, and p.T445T), suggesting that they are polymorphisms with no pathogenic effect, as reported before [Trappe et al., 2001; Kleefstra et al., 2004].

### Variation of the *MECP2* 3'UTR in Autistic Patients and Controls

Scanning of mutations was performed in the whole 3'UTR of *MECP2*. We found 46 patients out of 172 (26.7%) with 3'UTR variations, some having more than one change. In total, 24 unique sequence changes were found (13 in conserved nucleotides), of which 21 are novel (Table III). In 96 Portuguese controls (76 males and 20 females; 116 X-chromosomes total) we observed 26 individuals (27.1%) with 3'UTR variations, in a total of 20 unique sequence changes (12 in conserved nucleotides) (Table IV). Again, some of the individuals had more than one sequence change, indicating that there is a high variability in the 3'UTR. All of the variations encountered were novel, except the c.9964insC [Bourdon et al., 2001]. Ten of the changes were common to both patients and controls, suggesting that they do not have any pathogenic effect. A high degree of sequence variability in the 3'UTR was found, comparable in patients and controls, even in the most conserved regions between human and mouse. However, of the 21 novel changes found in patients, 12 were not present in the controls. Of these, seven were located in conserved nucleotides in seven autistic males, and only one was found in one autistic female: c.4167G > A (heterozygous). One of these novel changes, c.1655G > A, was localized in a region of strong sequence identity with mouse, suggesting that it may alter the regulation of *MECP2* expression.

Little is known about the functionality of the long 3'UTR of *MECP2*. In order to understand the meaning of the alterations found in this study we performed scans for known sequence patterns in 3'UTR regions. We found eight matches for 15-lipoxygenase differentiation control elements (15-LOX-DICE), which are CU-rich sequences involved in mRNA stabilization and translation inhibition, and at least two matches for AU-rich elements (AREs) and several C-rich regions, involved in the regulation of mRNA stability. However, none of the 3'UTR alterations found exclusively in the patients were localized within any of these regions. *MECP2* mRNA levels were measured in PBMCs from four autistic males presenting changes in conserved nucleotides: c.1832G > C, c.2015G > A, c.4017T > A, and c.4417G > A (Fig. 1). *MECP2* mRNA levels measured in four autistic males in the same age range, but without any detected *MECP2* alteration, were used as controls for specificity of these *MECP2* changes within the autistic

TABLE I. *MECP2* Sequence Changes Identified in the Coding Region and Exon–Intron Boundaries in 172 Unrelated Autistic Patients

| Nucleotide change <sup>a</sup> | Amino acid change <sup>b</sup> | Domain              | Type of sequence change | Amino acid conservation <sup>b</sup>                                                                                                            | Number of samples | Mut/PM <sup>c</sup> | References                                  |
|--------------------------------|--------------------------------|---------------------|-------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------|-------------------|---------------------|---------------------------------------------|
| c.27-55G > A                   |                                | Intron              | Intronic variation      |                                                                                                                                                 | 1                 |                     | This report                                 |
| c.377 + 18C > G                |                                | Intron              | Intronic variation      |                                                                                                                                                 | 1                 |                     | This report                                 |
| c.378-19delT                   |                                | Intron              | Intronic variation      |                                                                                                                                                 | 1                 | PM                  | Trappe et al. [2001]                        |
| c.393C > G                     | p.A131A                        | MBD                 | Silent                  | <i>Macaca fascicularis</i> , <i>Mus musculus</i> , <i>Rattus norvegicus</i> , <i>Danio rerio</i> , <i>Gallus gallus</i> , <i>Xenopus laevis</i> | 1                 | PM                  | Kleefstra et al. [2004]                     |
| c.602C > T                     | p.A201V                        | Inter-domain region | Missense                | <i>Macaca fascicularis</i> , <i>Mus musculus</i> , <i>Rattus norvegicus</i>                                                                     | 1                 | PM                  | Amano et al. [2000], Lam et al. [2000]      |
| c.617G > C                     | p.G206A                        | Inter-domain region | Missense                | <i>Macaca fascicularis</i> , <i>Mus musculus</i> , <i>Rattus norvegicus</i> , <i>Danio rerio</i> , <i>Gallus gallus</i> , <i>Xenopus laevis</i> | 1                 |                     | This report                                 |
| c.1189G > A                    | p.E397K                        | C-term              | Missense                | <i>Mus musculus</i> , <i>Rattus norvegicus</i>                                                                                                  | 1                 | PM                  | Wan et al. [1999]                           |
| c.1197C > T                    | p.P399P                        | C-term              | Silent                  | <i>Mus musculus</i> , <i>Rattus norvegicus</i>                                                                                                  | 2                 | PM                  | Cheadle et al. [2000]                       |
| c.1233C > T                    | p.S411S                        | C-term              | Silent                  | <i>Macaca fascicularis</i> , <i>Mus musculus</i> , <i>Rattus norvegicus</i>                                                                     | 1                 | PM                  | Amir et al. [1999]                          |
| c.1330G > A                    | p.A444T                        | C-term              | Missense                |                                                                                                                                                 | 1                 | PM                  | Buyse et al. [2000]                         |
| c.1335G > A                    | p.T445T                        | C-term              | Silent                  | <i>Mus musculus</i> , <i>Rattus norvegicus</i>                                                                                                  | 1                 | PM                  | Directly submitted to RettBASE <sup>d</sup> |

<sup>a</sup>RefSeq ID: NM\_004992.2 (mRNA).<sup>b</sup>Protein ID: NP\_004983.1 (human), AAK97131.1 (*M. fascicularis*), NP\_034918.1 (*M. musculus*), NP\_073164.1 (*R. norvegicus*), NP\_997901.1 (*D. rerio*), CAA74577.1 (*G. gallus*), AAD03736.1 (*X. laevis*).<sup>c</sup>Mut, mutation; PM, polymorphism.<sup>d</sup>TRSA (International Rett Syndrome Association) *MECP2* Gene Variation Database (RettBASE), <http://mecp2.chw.edu.au/>.TABLE II. *MECP2* Sequence Changes Identified in the Coding Region and Exon–Intron Boundaries in 143 Healthy Control Individuals

| Nucleotide change <sup>a</sup> | Amino acid change <sup>b</sup> | Domain | Type of sequence change | Amino acid conservation <sup>b</sup>                                                                                                            | Number of samples | Mut/PM <sup>c</sup> | References                                  |
|--------------------------------|--------------------------------|--------|-------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------|-------------------|---------------------|---------------------------------------------|
| c.245A > G                     | p.K82R                         | MBD    | Missense                | <i>Macaca fascicularis</i> , <i>Mus musculus</i> , <i>Rattus norvegicus</i> , <i>Danio rerio</i> , <i>Gallus gallus</i> , <i>Xenopus laevis</i> | 1                 |                     | This report                                 |
| c.378-19delT                   |                                | Intron | Intronic variation      |                                                                                                                                                 | 5                 |                     | Trappe et al. [2001]                        |
| c.393C > G                     | p.A131A                        | MBD    | Silent                  | <i>Macaca fascicularis</i> , <i>Mus musculus</i> , <i>Rattus norvegicus</i> , <i>Danio rerio</i> , <i>Gallus gallus</i> , <i>Xenopus laevis</i> | 1                 | PM                  | Kleefstra et al. [2004]                     |
| c.834C > T                     | p.A278A                        | TRD    | Silent                  | <i>Mus musculus</i> , <i>Rattus norvegicus</i> , <i>Xenopus laevis</i>                                                                          | 1                 | PM                  | Hoffbuhr et al. [2001]                      |
| c.948C > G                     | p.V316V                        | C-term | Silent                  | <i>Macaca fascicularis</i> , <i>Mus musculus</i> , <i>Rattus norvegicus</i> , <i>Xenopus laevis</i>                                             | 1                 | PM                  | Directly submitted to RettBASE <sup>d</sup> |
| c.1335G > A                    | p.T445T                        | C-term | Silent                  | <i>Mus musculus</i> , <i>Rattus norvegicus</i>                                                                                                  | 2                 | PM                  | Directly submitted to RettBASE <sup>d</sup> |

<sup>a</sup>RefSeq ID: NM\_004992.2 (mRNA).<sup>b</sup>Protein ID: NP\_004983.1 (human), AAK97131.1 (*M. fascicularis*), NP\_034918.1 (*M. musculus*), NP\_073164.1 (*R. norvegicus*), NP\_997901.1 (*D. rerio*), CAA74577.1 (*G. gallus*), AAD03736.1 (*X. laevis*).<sup>c</sup>Mut, mutation; PM, polymorphism.<sup>d</sup>TRSA (International Rett Syndrome Association) *MECP2* Gene Variation Database (RettBASE), <http://mecp2.chw.edu.au/>.

TABLE III. MECP2 3'UTR Variants Identified in 172 Unrelated Autistic Patients

| Nucleotide change <sup>a</sup> | Nucleotide conservation <sup>a</sup> | Number of samples | Mut/PM <sup>b</sup> | References                  |
|--------------------------------|--------------------------------------|-------------------|---------------------|-----------------------------|
| c.1470G > A                    |                                      | 1                 | PM                  | Lam et al. [2000]           |
| c.1554G > A                    | <i>Mus musculus</i>                  | 1                 | PM                  | Ylisaukko-Oja et al. [2005] |
| c.1655G > A                    | <i>Mus musculus</i>                  | 1                 |                     | This report                 |
| c.1832G > C                    | <i>Mus musculus</i>                  | 1                 |                     | This report                 |
| c.2005G > A                    |                                      | 1                 |                     | This report                 |
| c.2015G > A                    | <i>Mus musculus</i>                  | 1                 |                     | This report                 |
| c.2228G > T                    |                                      | 1                 |                     | This report                 |
| c.2322T > G                    | <i>Mus musculus</i>                  | 1                 |                     | This report                 |
| c.2339C > G                    |                                      | 15                |                     | This report                 |
| c.2829C > A                    | <i>Mus musculus</i>                  | 1                 |                     | This report                 |
| c.3198G > A                    | <i>Mus musculus</i>                  | 8                 |                     | This report                 |
| c.4017T > A                    | <i>Mus musculus</i>                  | 1                 |                     | This report                 |
| c.4118G > A                    |                                      | 1                 |                     | This report                 |
| c.4167G > A                    |                                      | 1                 |                     | This report                 |
| c.4417G > A                    | <i>Mus musculus</i>                  | 1                 |                     | This report                 |
| c.4938G > A                    |                                      | 2                 |                     | This report                 |
| c.5119C > T                    |                                      | 1                 |                     | This report                 |
| c.5339G > C                    |                                      | 1                 |                     | This report                 |
| c.6037A > C                    | <i>Mus musculus</i>                  | 1                 |                     | This report                 |
| c.6948ins(AT)                  | <i>Mus musculus</i>                  | 1                 |                     | This report                 |
| c.9209C > T                    | <i>Mus musculus</i>                  | 1                 |                     | This report                 |
| c.9317A > C                    | <i>Mus musculus</i>                  | 13                |                     | This report                 |
| c.9964delC                     |                                      | 2                 |                     | This report                 |
| c.9964insC                     |                                      | 3                 | PM                  | Bourdon et al. [2001]       |

<sup>a</sup>RefSeq ID: NM\_004992.2 (human), AF158181.1 (*M. musculus*).<sup>b</sup>Mut, mutation; PM, polymorphism.

phenotype. *MECP2* mRNA levels of the patients with 3'UTR changes were significantly lower when compared with these controls (Kruskal–Wallis test,  $P = 0.021$ ). Although mRNA levels were measured in very few individuals, all four patients with 3'UTR alterations had lower levels than any of the patients with no *MECP2* sequence changes (Fig. 1), suggesting that at least these alterations in 3'UTR conserved nucleotides may render the *MECP2* transcripts more unstable and subject to degradation.

#### Analysis of the Novel p.G206A Missense Change

Segregation analysis was carried out in the family of the autistic male in which the novel p.G206A missense change was found (Fig. 2A). Clinical, neuropsychological, and behavioral data on this patient is shown in Table V. This patient was 10 years old at the time of collection, and had severe autism (positive ADI-R and DSM-IV with a CARS score of 50.5), severe mental retardation (Global Developmental Quotient of

TABLE IV. MECP2 3'UTR Variants Identified in 96 Healthy Control Individuals

| Nucleotide change <sup>a</sup> | Nucleotide conservation <sup>a</sup> | Number of samples | Mut/PM <sup>b</sup> | Reference             |
|--------------------------------|--------------------------------------|-------------------|---------------------|-----------------------|
| c.1854G > A                    |                                      | 1                 |                     | This report           |
| c.1950G > C                    | <i>Mus musculus</i>                  | 1                 |                     | This report           |
| c.1990G > T                    | <i>Mus musculus</i>                  | 1                 |                     | This report           |
| c.2267G > A                    | <i>Mus musculus</i>                  | 1                 |                     | This report           |
| c.2292G > C                    | <i>Mus musculus</i>                  | 1                 |                     | This report           |
| c.2336insA                     |                                      | 1                 |                     | This report           |
| c.2339C > G                    |                                      | 8                 |                     | This report           |
| c.2698T > C                    | <i>Mus musculus</i>                  | 1                 |                     | This report           |
| c.3198G > A                    | <i>Mus musculus</i>                  | 5                 |                     | This report           |
| c.4938G > A                    |                                      | 3                 |                     | This report           |
| c.5123A > G                    | <i>Mus musculus</i>                  | 1                 |                     | This report           |
| c.5339G > C                    |                                      | 1                 |                     | This report           |
| c.5547del(GT)                  |                                      | 1                 |                     | This report           |
| c.6037A > C                    | <i>Mus musculus</i>                  | 2                 |                     | This report           |
| c.6948ins(AT)                  | <i>Mus musculus</i>                  | 1                 |                     | This report           |
| c.7300C > T                    | <i>Mus musculus</i>                  | 1                 |                     | This report           |
| c.9209C > T                    | <i>Mus musculus</i>                  | 1                 |                     | This report           |
| c.9317A > C                    | <i>Mus musculus</i>                  | 5                 |                     | This report           |
| c.9964delC                     |                                      | 5                 |                     | This report           |
| c.9964insC                     |                                      | 2                 | PM                  | Bourdon et al. [2001] |

<sup>a</sup>RefSeq ID: NM\_004992.2 (human), AF158181.1 (*M. musculus*).<sup>b</sup>Mut, mutation; PM, polymorphism.

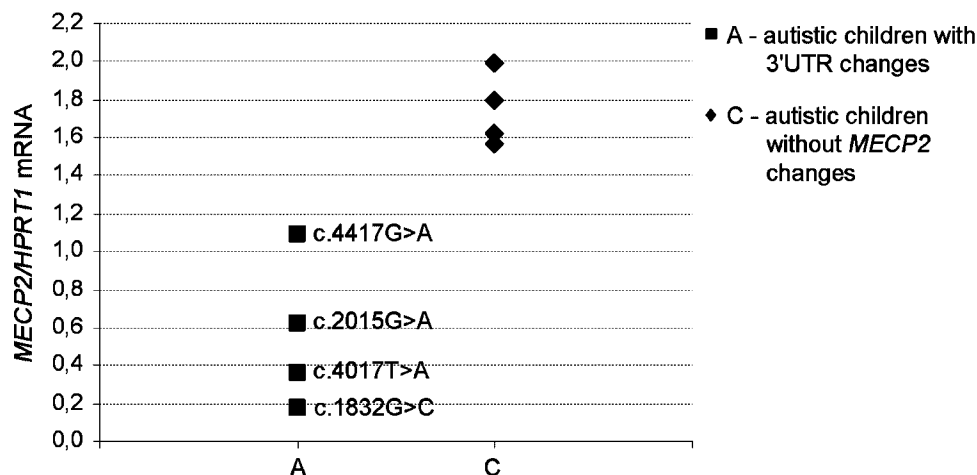


Fig. 1. *MECP2* mRNA quantification in PBMCs from four autistic males with different 3'UTR alterations in conserved nucleotides: c.1832G>C, c.2015G>A, c.4017T>A, and c.4417G>A, compared with four autistic males without *MECP2* changes. Results were normalized for *HPRT1* mRNA levels.

25), and absence of language. He had purposeful hand manipulation of objects and hand stereotypes not characteristic of Rett syndrome. Breathing irregularities were never noticed by the family and he never had epilepsy. In the first year of life he had developmental delay without history of

regression, the weight and height were in the percentile 5, and the cephalic perimeter was in the percentile 50 (without posterior deceleration of head growth). Presently 15 years old, he shows the same symptomatology and his neurologic examination does not show any abnormality besides mental retardation and autistic behavior. This patient has a younger male sibling who is 3 years of age and has a normal development to this day. A maternal aunt died at age 7 and was reported to have mental retardation, abnormal motor development, and uncontrolled seizures of unknown etiology. The available relatives from the proband were sequenced for the p.G206A alteration: his parents, the maternal grandmother, and a maternal uncle (see Fig. 2A). The mother and maternal grandmother are heterozygous asymptomatic carriers of this sequence change. Quantitative XCI assays were then performed in the female relatives; the proband was also tested, to determine which of the alleles was inherited from his mother. A pattern of moderately skewed XCI ratio of  $\pm 30\%$ :70% was found in the mother, and a normal random pattern of  $\pm 40\%$ :60% was found in the grandmother (Fig. 2B). Although the three individuals (proband, mother, and maternal grandmother) share the same alteration, they do not share one common androgen receptor gene (*AR*) allele. Because *AR* (Xq12) and *MECP2* (Xq28) are located far apart in the X chromosome, this suggests that a crossing-over event has occurred between generations. If this is correct, the mutation must be segregating with the low molecular weight allele from the grandmother to the mother (allele c in Fig. 2B), and then passed to the proband together with the intermediate molecular weight allele (allele b in Fig. 2B), due to recombination in the mother's germline. Densitometry analysis shows that in the mother, who inherited the mutated *MECP2* allele from the grandmother (associated with allele c in Fig. 2B), the X chromosome carrying the wild type *MECP2* allele (inherited

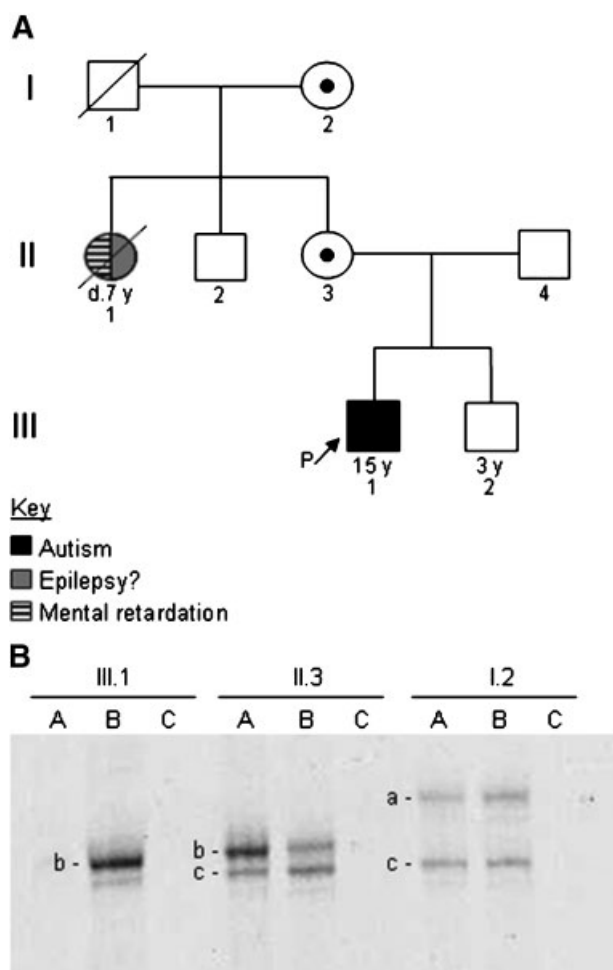


Fig. 2. Pedigree and X-chromosome inactivation (XCI) assay results in the family of the autistic proband who presents the novel p.G206A missense change in *MECP2*. **Panel A:** pedigree showing the structure of the family of the autistic patient in whom the novel p.G206A missense change was found. **Panel B:** XCI assay results, performed in leukocytes of the autistic proband with the p.G206A alteration and the family female carriers; the autoradiography shows the *AR* allelic band pattern after PCR amplification of the DNA samples. III.1—proband; II.3—proband's mother; I.2—proband's maternal grandmother; A—PCR amplification after restriction of genomic DNA with *HhaI* (only methylated, inactivated alleles are amplified); B—PCR amplification of intact genomic DNA; C—PCR amplification with no DNA (negative control); a, b, c—*AR* trinucleotide repeat alleles.



TABLE V. Clinical, Neuropsychological, and Behavioral Data of the Patient Presenting the p.G206A Missense Change in *MECP2*

| Variable                                                                                                                          | Clinical data   |
|-----------------------------------------------------------------------------------------------------------------------------------|-----------------|
| Age at examination (years)                                                                                                        | 10              |
| Physical measurements                                                                                                             |                 |
| Height                                                                                                                            | 5th percentile  |
| Occipital-frontal circumference (OFC)                                                                                             | 50th percentile |
| Weight                                                                                                                            | 5th percentile  |
| Neurological symptoms                                                                                                             |                 |
| Seizures                                                                                                                          | No              |
| Brisk tendon reflexes                                                                                                             | No              |
| Tremor                                                                                                                            | No              |
| Developmental history                                                                                                             |                 |
| First remarkable signs of autism (months)                                                                                         | <12             |
| Walked independently (months)                                                                                                     | 12              |
| Motor skills                                                                                                                      |                 |
| Poor-motor coordination                                                                                                           | No              |
| Slow                                                                                                                              | No              |
| Clumsy                                                                                                                            | No              |
| Regression                                                                                                                        | No              |
| Sleep problems                                                                                                                    | No              |
| Developmental quotient (Ruth Griffiths Mental Development Scale II)                                                               |                 |
| Global developmental quotient (GDQ)                                                                                               | 25              |
| Motor developmental quotient (MDQ)                                                                                                | 30              |
| Performance developmental quotient (PDQ)                                                                                          | 23              |
| Language developmental quotient (LDQ)                                                                                             | 13              |
| Vineland adaptive behavior scales domain scores                                                                                   |                 |
| Communication (percentile rank <sup>a</sup> )                                                                                     | 40              |
| Daily living (percentile rank <sup>a</sup> )                                                                                      | 50              |
| Socialization (percentile rank <sup>a</sup> )                                                                                     | 40              |
| Adaptive behavior composite (percentile rank <sup>a</sup> )                                                                       | 50              |
| DSM-IV positive criteria                                                                                                          |                 |
| Qualitative impairment in social interaction                                                                                      | 3 out of 4      |
| Qualitative impairments in communication                                                                                          | 2 out of 4      |
| Restricted repetitive and stereotyped patterns of behavior, interests, and activities                                             | 4 out of 4      |
| Delays or abnormal functioning, with onset prior to age 3 years, in social interaction, language and symbolic or imaginative play | Yes             |
| Disturbance not better accounted for by Rett's disorder or CDD                                                                    | No              |
| ADI-R domain scores at final diagnosis                                                                                            |                 |
| Social interaction (cutoff = 10; max. = 30)                                                                                       | 29 out of 30    |
| Communication: nonverbal (cutoff = 7; max. = 14)                                                                                  | 13 out of 14    |
| Repetitive behavior (cutoff = 3; max. = 12)                                                                                       | 4 out of 12     |

<sup>a</sup>Supplemental Norm Group percentiles ranks—Autism Special Population [Carter et al., 1998].

from her father, associated with allele b in Fig. 2B), is preferentially inactivated. Because both the mother and maternal grandmother show a normal phenotype, any pathogenic effect of the p.G206A missense change can likely be compensated by the expression of the normal allele in the carrier females.

In view of these results, we evaluated the role of the p.G206A missense change in the functionality of MeCP2. Because *BDNF* is one of the few genes known to be directly regulated by MeCP2 [Chen et al., 2003; Martinowich et al., 2003], we quantified plasma BDNF levels in this autistic patient (19.5 ng/ml), which were found to be within normal levels when compared to 36 age-matched control children (range: 8.9–69.8 ng/ml; mean: 28.6 ng/ml  $\pm$  13.9 SD). This result suggests that the potential pathogenic mechanism of this missense change does not involve BDNF level changes in this patient, or that they are too mild in plasma to be detected at this level.

## DISCUSSION

In the present study we hypothesized that sequence changes in specific locations within the *MECP2* gene that are less deleterious for MeCP2 function might be involved in a pathway

leading to a milder, non-lethal phenotype in autistic male and female patients, extending beyond the traditional RTT diagnosis.

We found low sequence variability in the coding region and exon–intron boundaries of *MECP2*, comparable in autistic patients (7.0%) and controls (7.7%), in agreement with previous studies in which *MECP2* sequence changes were found only rarely in subjects with autism [Lam et al., 2000; Beyer et al., 2002; van Karnebeek et al., 2002; Carney et al., 2003; Lobo-Menendez et al., 2003; Zappella et al., 2003; Shibayama et al., 2004]. We report two intronic variations and one missense change that were not present in the control sample. The novel missense change (p.G206A) was found in one autistic male, and segregates in his family. It is localized in a highly conserved amino acid, within a region implicated in an alternative transcriptional repression pathway independent of histone-deacetylation [Yu et al., 2000], and likely leads to a change in protein structure, implying that it may alter the function of MeCP2. These findings are compatible with an association of this change with autism in the proband. In the proband's mother and grandmother, who have a normal phenotype, either the *MECP2* mutated allele has a low penetrance or its deleterious effect is compensated by the

expression of the normal allele, which may be sufficient to avoid the appearance of symptoms. It is possible that the maternal aunt, affected with mental retardation, and who died early in life, had the p.G206A alteration with a skewed XCI favoring the expression of the mutated allele. Plasma BDNF levels of the autistic proband were found to be within normality, suggesting that the pathogenic mechanism does not involve BDNF level changes in this patient. However, we cannot exclude that an alteration in BDNF levels in the CNS mediated by the altered MeCP2 is not accompanied by a change in plasma levels. The expression of other target genes which may be involved in the clinical phenotype of this patient was not investigated.

We sequenced exon 1 of *MECP2* in 103 autistic patients, and found no sequence changes in this region. Mutations in exon 1, and thus in MeCP2\_e1, either do not play a role in autism etiology or occur very rarely associated with this disorder, possibly with a very severe effect.

To our knowledge, this is the first report in which the whole 3'UTR of *MECP2* was scanned for sequence variations. We found a high degree of sequence variability in the 3'UTR, comparable between patients (26.7%) and controls (27.1%). In 172 patients, 12 novel changes were found, of which 7 were located in conserved nucleotides. In the patients carrying four of these conserved 3'UTR alterations *MECP2* mRNA levels were always lower than in autistic patients without any *MECP2* sequence changes. While the use of autistic individuals with no *MECP2* alterations as controls does not provide us with the normal range of mRNA levels, it suggests that lower expression, and a consequent abnormal overexpression of target genes, may be a specific cause of autism for the individuals bearing these *MECP2* sequence alterations, among autistic individuals. Given the etiological heterogeneity of autism, with multiple genetic alterations known to lead to an autistic phenotype, this hypothesis is plausible. These results are however to be considered preliminary, as the sample size is very small. If confirmed in a larger sample, these observations would indicate the existence of important regulatory regions within the 3'UTR of *MECP2*.

Taken together, our results suggest that mutations in the *MECP2* coding region and 3'UTR alterations in conserved regions may play only a minor role in autism etiology. We report a novel missense change, which may have a non-lethal but severe pathogenic effect in males. Functional studies in order to demonstrate its pathogenicity are under way. Mutations in the coding region, however, are probably restricted to a very small subgroup of subjects with autism, as we found only one patient out of 172 (0.58%) with a potential pathogenic mutation; if we consider exclusively the autistic male sample ( $n = 141$ ), this frequency increases to 0.71%. Additionally, we report seven novel 3'UTR alterations in conserved nucleotides, present in seven autistic males out of 172 patients (4.07%; 4.96% in the male sample), of which at least four possibly alter the stability of the *MECP2* transcripts. The frequency of *MECP2* alterations in autism might be higher, as we did not scan the promoter region and the method used cannot detect large deletions or duplications that could be missed in heterozygous females (and duplications in males). Our patient sample included only idiopathic cases, which were screened for common medical conditions associated with autism including tuberous sclerosis, Fragile X syndrome, and chromosomal abnormalities, as a standard procedure. The prevalence of these conditions in autism spectrum disorders is estimated to be 1–3%, 2–3%, and 5%, respectively [Rutter, 2005], with full mutations of the gene causing the Fragile X syndrome (*FMR1*) observed in less than 1% of children with autism [Lord et al., 2000]. Similarly, rare mutations in *MECP2* may increase the susceptibility to develop autism in a minority of cases. Screening for *MECP2* mutations in cases of autism

associated with mental retardation, particularly in males who may have a variable phenotype, may be useful for research purposes, to decrease genetic heterogeneity in the study samples and thus facilitate the identification of other genes predisposing to autism.

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**ARTICLE 6**

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“Evidence for abnormal early development in a mouse model of Rett syndrome”

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# Evidence for abnormal early development in a mouse model of Rett syndrome

M. Santos<sup>†,‡</sup>, A. Silva-Fernandes<sup>†</sup>, P. Oliveira<sup>§</sup>,  
Nuno Sousa<sup>†</sup> and Patrícia Maciel<sup>\*,†</sup>

<sup>†</sup>Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, <sup>‡</sup>Institute for Biomedical Sciences Abel Salazar, University of Porto, Porto, and <sup>§</sup>Department of Production and Systems Engineering, School of Engineering, University of Minho, Braga, Portugal

\*Corresponding author: P. Maciel, Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal. E-mail: pmaciel@ecsaude.uminho.pt

**Rett syndrome (RTT) is a neurodevelopmental disorder that affects mainly females, associated in most cases to mutations in the *MECP2* gene. After an apparently normal prenatal and perinatal period, patients display an arrest in growth and in psychomotor development, with autistic behaviour, hand stereotypies and mental retardation. Despite this classical description, researchers always questioned whether RTT patients did have subtle manifestations soon after birth. This issue was recently brought to light by several studies using different approaches that revealed abnormalities in the early development of RTT patients. Our hypothesis was that, in the mouse models of RTT as in patients, early neurodevelopment might be abnormal, but in a subtle manner, given the first descriptions of these models as initially normal. To address this issue, we performed a postnatal neurodevelopmental study in the *Mecp2*<sup>tm1.1Brd</sup> mouse. These animals are born healthy, and overt symptoms start to establish a few weeks later, including features of neurological disorder (tremors, hind limb claspings, weight loss). Different maturational parameters and neurological reflexes were analysed in the pre-weaning period in the *Mecp2*-mutant mice and compared to wild-type littermate controls. We found subtle but significant sex-dependent differences between mutant and wild-type animals, namely a delay in the acquisition of the surface and postural reflexes, and impaired growth maturation. The mutant animals also show altered negative geotaxis and wire suspension behaviours, which may be early manifestations of later neurological symptoms. In the post-weaning period the juvenile mice presented hypoactivity that was probably the result of motor impairments. The early anomalies identified in this model of RTT mimic the early motor abnormalities**

**reported in the RTT patients, making this a good model for the study of the early disease process.**

Keywords: autism, MeCP2, neurodevelopment, postnatal, reflexes

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Rett syndrome (RTT) is a major cause of mental retardation in females, affecting 1 per 10 000 to 1 per 22 000 females born (Percy 2002). The 'classic' progression of RTT has four stages (Kerr & Engerstrom 2001). Stage I is characterized by an apparently normal development with uneventful prenatal and perinatal periods; in this stage (around 6–18 months) some of the patients learn some words and some are able to walk and feed themselves. In stage II (regression) a deceleration/arrest in the psychomotor development is noticed, with loss of stage I acquired skills, establishment of autistic behaviour and signs of intellectual dysfunction; the hands' skilful abilities are replaced by stereotypical hand movements, a hallmark of RTT. The pre-school/school years correspond to stage III (pseudo-stationary) and here some improvement can be appreciated, with recovery of previously acquired skills. This is followed by the progressively incapacitating stage IV that can last for years (Hagberg *et al.* 2002); at this final stage patients develop trunk and gait ataxia, dystonia, autonomic dysfunction (breathing anomalies, sleep and gastrointestinal disturbances) and many of them have a sudden unexplained death in adulthood.

In spite of the classic RTT description, some researchers have questioned whether RTT patients display subtle signs of abnormal development soon after birth (Engerstrom 1992; Kerr 1995; Naidu 1997; Nomura & Segawa 1990). Huppke and colleagues reported on a sample of RTT patients who presented a significantly reduced occipito-frontal circumference, shorter length and lower weight at birth (Huppke *et al.* 2003). This hypothesis has recently been confirmed by the work of Einspieler and colleagues (Einspieler *et al.* 2005b), who analysed video records of the first 6 months of life of 22 RTT patients and were able to notice abnormalities in several behaviours. All RTT patients presented an abnormal pattern of spontaneous movements within the first 4 weeks of life, with abnormal 'fidgety' movements that were considered a sign of abnormal development (Einspieler *et al.* 2005a,b). Such abnormal movements were ascribed to problems in the central pattern generators in the brain (Einspieler *et al.*

2005a; Einspieler & Prechtel 2005). In a different study, midwives and health visitors blinded for the clinical status of the children were able to identify in family videos potential anomalies in the early development of RTT patients, particularly anomalies in physical appearance and hand posture, as well as body movements and postures (Burford 2005). Segawa, in a retrospective study of patients' clinical files (Segawa 2005), also reported altered presentation of several motor milestones.

Most patients with classic RTT are heterozygous for mutations in the X-linked methyl-CpG binding protein gene (*MECP2*) (Amir *et al.* 1999), which encodes the methyl-CpG binding protein, MeCP2; this is known to bind symmetrically methylated CpG dinucleotides, and to recruit the co-repressors Sin3 yeast homologue A and histone deacetylase 1 and histone deacetylase 2 to repress transcription (Jones *et al.* 1998). When mutated, MeCP2 does not bind or binds ineffectively to its targets and, as a consequence, deregulation of transcription is thought to occur. Animal models of RTT were created in mice, mimicking several motor aspects of RTT and even the more emotional and social aspects of the syndrome (Chen *et al.* 2001; Guy *et al.* 2001; Shahbazian *et al.* 2002). The mutants are born normal and a few weeks later start to present a progressive motor deterioration, despite no gross abnormalities in the brain being noticed. Males carrying the mutation in hemizyosity display an earlier onset and are more severely affected than heterozygous females, probably as the result of X-chromosome inactivation that makes these females mosaics for the expression of the mutation, as is the case for the human condition.

The study presented here was performed using the *Mecp2*<sup>tm1.1Bird</sup> (Guy *et al.* 2001) mouse as a model. These mice were described as presenting no initial phenotype. Male *Mecp2*<sup>tm1.1Bird</sup> null animals begin to show symptoms at 3–8 weeks whereas heterozygous female animals manifest the disease at 3 months of age. The phenotype of these animals mimics many of the motor symptoms of RTT: stiff and uncoordinated gait, reduced spontaneous movement, hind limb claspings, tremor and irregular breathing. Pathologically, no obvious histological abnormalities were detected in peripheral organs or in the brain. However, more recently, Kishi and Macklis reported that in the *Mecp2*-null mice the neocortical projection layers were thinner and the pyramidal neurons in layer II/III had smaller somas and less complex dendritic trees in symptomatic animals than in wild-type mice (Kishi & Macklis 2004). Another study in this animal model suggested an essential role of MeCP2 in the mechanisms of synaptic plasticity (LTP and LTD) in the mature hippocampal neurons (Asaka *et al.* 2005).

The goal of this study was to determine whether the early neurodevelopmental process was altered in the absence of MeCP2 in mice. We assessed the achievement of milestones, considering different maturational and physical growth measures and neurological reflexes, two of the most well-known and most used neurobehavioural testing

categories to address neurological disorder (Spear 1990), in the *Mecp2*<sup>tm1.1Bird</sup> mouse model of RTT (*Mecp2*-null males and *Mecp2*-heterozygous females). We identified an altered developmental progression of the mutant animals since the first postnatal week, in spite of their apparently normal phenotype. The differences seen suggest the presence of mild neurological deficits already at this age; the animals also presented significantly reduced activity, probably as a result of motor impairments early in life. The abnormal achievement of the developmental hallmarks, although transient, could reflect abnormalities that are likely to impact the development of more mature behaviours.

## Materials and Methods

### Animals

The strain used in this study was created by the Bird laboratory by transfecting the targeting vector in 129P2/OlaHsd E14TG2a embryonic stem cells and injecting these into C57BL/6 blastocysts (Guy *et al.* 2001). According to information from the Jackson Laboratory, from whom we acquired the animals, the original strain was bred to C57BL/6 mice and backcrossed to C57BL/6 at least five times. Female *Mecp2*<sup>tm1.1Bird</sup> mice were bred with C57BL/6 wild-type (wt) male mice, to obtain wt and *Mecp2*-mutant animals. Mice were kept in an animal facility in a 12-hour light: 12-hour dark cycle, with food and water available *ad libitum*. A daily inspection for the presence of new litters in the cages was carried out twice a day and the day a litter was first observed was scored as day 0 for that litter. After birth, animals were kept untouched in the home cage with their heterozygous mothers until postnatal day (PND) 3, and at PND4 animals were tagged in their feet or the tip of the ears. Neurodevelopmental evaluation tests were started at PND4 and performed daily through to PND21. Weaning was performed at 22/23 days of age. Males and females were separated and kept in independent cages, in groups of three to seven animals per cage. At weaning the tip of the tail of the mice was cut for DNA extraction by Puregene DNA isolation kit (Gentra, Minneapolis, MN) and genotyping was performed according to the protocol supplied for this strain by the Jackson Laboratory. At the fourth postnatal week animals were tested for spontaneous activity in the Open-field (OF) apparatus and the day after this animals were tested for anxiety-like behaviour in the Elevated plus-maze (EPM) apparatus. At the fifth postnatal week animals were tested in the rotarod apparatus. After completing the experiment animals were rapidly killed by decapitation, thus minimizing their suffering (in accordance with the European Communities Council Directive, 86/609/EEC).

The same observer, who was blinded for the genotype of the animals and for the performance of the animals on the previous day, evaluated all the described tests. Tests were always performed in the same circadian period (between



1100 and 1800 h) and whenever possible at the same hour of the day. All the animals were separated from their parents at the beginning of each test session and kept with their littermates in a new cage, with towel paper and sawdust from their home cage. Once the test sessions finished for all the members of a litter, the animals were returned to their home cage. Table 1 shows attributable scores for each test. Throughout the text when *Mecp2*-heterozygous animals are referred to they are always females and *Mecp2*-null animals is always used to refer to male animals. All the controls used were littermates of the *Mecp2*-mutant (male and female) animals.

### Pre-weaning behaviour

#### Maturation measures

**Body weight.** The body weight of mice was registered every day from PND4 through to PND21 (weight  $\pm$  0.01 g).

**Anogenital distance (AGD).** The distance between the opening of the anus and the opening of the genitalia was registered (distance  $\pm$  0.5 mm).

**Ear opening.** The day when an opening in the ear was visualized was registered.

**Eye opening.** We registered the state of the eyes from the day when animals started to open the eyes until the day when every animal in the litter had both eyes opened. An eye was considered open when any visible break in the membrane was noticed.

#### Developmental measures

**Surface righting reflex (RR).** Mice were restrained on their back on a table and then released. The performance of the animal (to turn or not) was scored and the time taken to surface-right, in a maximum of 30 seconds, in three consecutive trials, was registered. To determine the score for each day, the median value was calculated for the three trials.

**Postural reflex (PR).** Animals were put in a small box and shaken up and down and left and right. Existence of an appropriate response (animals splaying their four feet) was scored.

**Negative geotaxis (NG).** Animals were put in a horizontal grid and then the grid was turned through 45° so that the animal was facing down. The behaviour of the animal was observed for 30 seconds and registered as shown in Table 1.

**Wire suspension (WS).** The animals were forced to grasp a 3-mm wire and hang from it on their forepaws. The ability of the animals to grasp the wire was scored and the time for which they held the wire (maximum 30 seconds) was registered.

### Post-weaning behavioural tests

#### Open field

Animals were placed in the centre of a 43.2  $\times$  43.2-cm arena with transparent walls (MedAssociates Inc., St Albans, VT) and their behaviour was observed for 5 min. Activity parameters were collected (total distance travelled, speed, resting time and the distance travelled and time spent in the predefined centre of the arena versus the rest of the arena). The number of rears, the time that animals spent exploring vertically and the number of *bolus faecalis* were also registered by observation.

#### Elevated plus maze

Animals were placed in an EPM apparatus consisting of two opposite open arms (50.8  $\times$  10.2 cm) and two opposite closed arms (50.8  $\times$  10.2  $\times$  40.6 cm) raised 72.4 cm above the floor (ENV-560, MedAssociates Inc.) and behaviour (number of entries in each arm and the time spent in each of the arms) was registered for 5 minutes.

#### Rotarod

Mice were tested in a rotarod (TSE systems, Bad Hamburg, Germany) apparatus to evaluate their motor performance. The protocol consisted of 3 days of training at a constant speed (15 r.p.m.) for a maximum of 60 seconds in four trials, with a 10-min interval between each trial. At the fourth day, animals were tested for each of six different velocities (5 r.p.m., 8 r.p.m., 15 r.p.m., 20 r.p.m., 24 r.p.m. and 31 r.p.m.) for a maximum of 60 seconds in two trials, with a 10-min interval between each trial. The latency to fall off the rod was registered.

**Table 1:** Attributable scores in milestones performance of *Mecp2*-mutant and wild-type animals

|                         | Score                    |                   |                         |               |
|-------------------------|--------------------------|-------------------|-------------------------|---------------|
|                         | 0                        | 1                 | 2                       | 3             |
| Ear opening             | closed                   | open              |                         |               |
| Eye opening             | both closed              | one open          | both open               |               |
| Surface righting reflex | stays in dorsal position | fights to upright | rights itself           |               |
| Postural reflex         | not present              | present           |                         |               |
| Negative geotaxis       | turns and climbs grid    | turns and freezes | moves but fails to turn | does not move |
| Wire suspension         | not present              | present           |                         |               |

### Statistical analysis

In the pre-weaning behaviour analysis, because there were problems with achieving the assumptions required for repeated measures testing, such as sphericity and homogeneity of variances, using the data obtained, we used regression methods to compare the performance between *Mecp2*-mutant and wt littermate control mice. To do this, variables scored 0 or 1 were analysed by logistic regression [Score = f(day, genotype sex)]. For continuous variables, a linear or a quadratic regression was applied. Interaction between the independent variables (day, genotype and sex) was also studied and reported when it was observed. The surface righting reflex and wire suspension times were analysed as survival times through the Kaplan–Meier test. The Negative Geotaxis was analysed (classification in three classes) by a  $\chi^2$  test and the percentage of animals meeting the criterion (score = 0) by linear regression was found. In the post-weaning behaviour tests, data were analysed using Student's *t*-test. A critical value for significance of  $P < 0.05$  was used throughout the study.

## Results

### Pre-weaning behaviour analysis

In this and in all other variables under study we always analysed male and female animals separately. The number of animals used in the analysis of maturation markers and neurological reflexes in the pre-weaning period was: *Mecp2*-null  $n = 13$ , wt littermate males  $n = 11$ , *Mecp2*-heterozygotes  $n = 16$ , wt littermate females  $n = 9$ .

### Physical growth and maturation

#### Body weight

We weighed *Mecp2*-mutant and wt littermate control mice everyday from PND4 to PND21 and analysed the data with a quadratic regression. As expected, the body weight was statistically different between male and female animals, with female mice being heavier than male mice ( $P = 0.013$ ), and the day of analysis had a significant influence on the body weight ( $P < 0.001$ ). When we analysed the influence of the *Mecp2* genotype of mice in the body weight, we noticed that the body weight evolution of *Mecp2*-null mice was not different from that of the wt littermate controls, in the first 21 days of postnatal development ( $P = 0.156$ ). Surprisingly, however, *Mecp2*-heterozygous mice presented a significantly reduced body weight when compared to their wt littermate controls ( $P < 0.001$ ) (Fig. 1a,b). The effect of genotype was not seen from the beginning of the study, but from around PND10 onwards.

#### Ear and eye opening

We observed mice daily from PND4 and registered the day when at least one eye was open and the day when both eyes

were open. The day an aperture was seen in the ear was also registered. No differences existed between genotypes or gender regarding the mean day of aperture of eyes and ears (supplemental table 1).

### Anogenital distance

We took this measure from PND4 to PND21 in all mice and analysed the data using a linear regression method. As body weight might influence the anus–genitalia distance, previous studies (Degen *et al.* 2005) introduced a correction: the AGD value was divided by the weight of each animal at each postnatal day (AGD/weight). We calculated the coefficient of correlation between the AGD and the body weight of the mice ( $R = 0.907$  for male mice and  $R = 0.917$  for female mice) and because our findings suggested that these two variables were highly associated we decided not to use this correction.

The AGD of male mice was higher than that of female mice ( $P < 0.001$ ), as expected, and the day of testing affected this distance, which was higher the later the measure was taken ( $P < 0.001$ ). We found that male and female *Mecp2*-mutant animals presented a statistically significant reduction in the AGD throughout the pre-weaning period, when compared to their respective wt controls ( $P < 0.001$ ) (Fig. 1c,d).

### Neurological reflexes

#### Surface righting reflex

No differences between sexes were found in the acquisition of this reflex ( $P = 0.668$ ), and the animals' ability to regain an upright position improved with age ( $P < 0.009$ ), as expected. *Mecp2*-mutant animals did not present differences in the age of acquisition of this reflex ( $P = 0.534$  and  $P = 0.161$  for *Mecp2*-null and *Mecp2*-heterozygous mice, respectively) (supplemental Fig. 1a,b). When we considered the time these animals took to surface-right, *Mecp2*-heterozygous mice presented statistically significant differences, with mutant females taking longer than wt littermates to regain an upright position ( $P = 0.031$ ) (Fig. 2a,b). Nevertheless, when *Mecp2*-null mice and wt controls were compared no differences were found. There were no differences, in this last parameter, between sexes ( $P = 0.216$ ).

#### Postural reflex

There were no differences between genders in the ontogeny of this reflex ( $P = 0.118$ ) and, as expected, the day affected its establishment ( $P < 0.001$ ). The pattern of acquisition of the PR was statistically different between *Mecp2*-null ( $P < 0.001$ ) and *Mecp2*-heterozygous ( $P = 0.006$ ) mice, when compared to their respective wt controls, with a worse outcome for mutant animals. Both *Mecp2*-null and *Mecp2*-heterozygous mice showed a delay in the acquisition of the PR reflex (Fig. 2 c,d). The acquisition of the PR by wt animals started at PND9 for females and PND10 for males and at PND16 all wt animals presented the PR. In the mutant mice the reflex first appeared on PND11 for females and PND12 for males and only at PND17

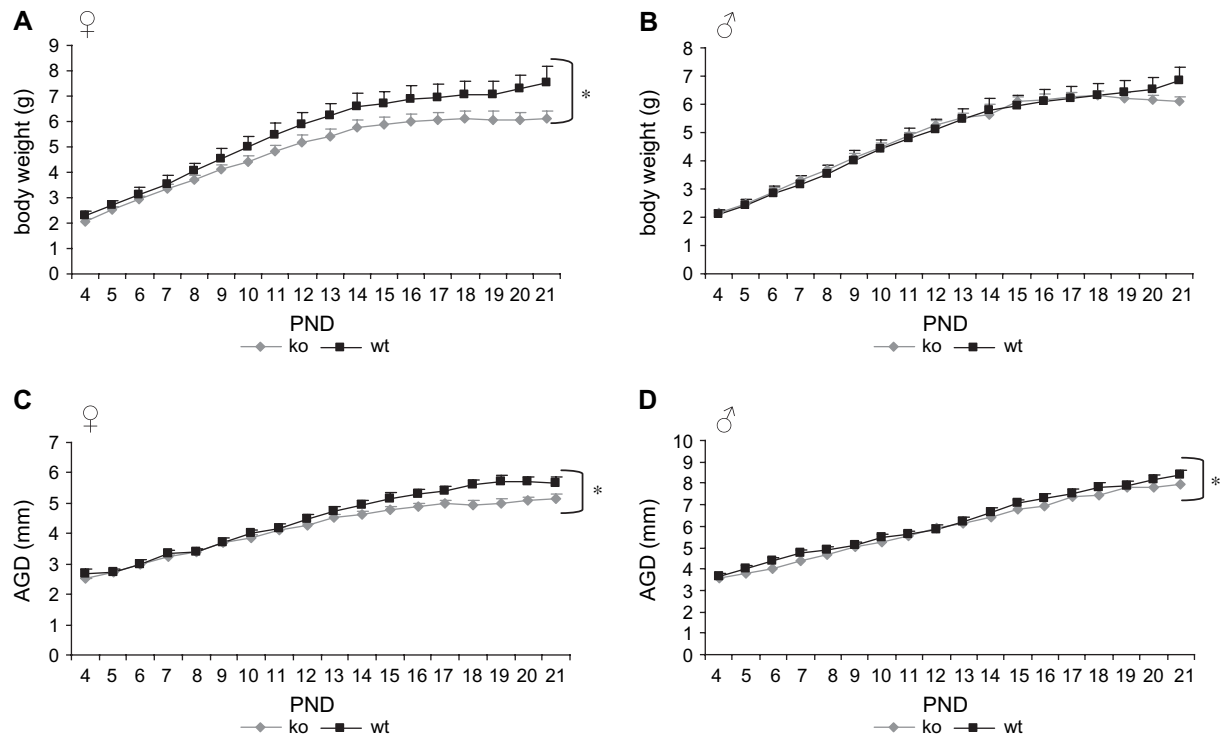


**Figure 1: Physical growth and maturation parameters of the *Mecp2*-heterozygous female mice and the *Mecp2*-null male mice during the pre-weaning period.** (a,b) Body weight evolution from PND4 to PND21 of *Mecp2*-mutant animals and their wt littermate controls. *Mecp2*-heterozygous females had a significant reduction in body weight that started to be noticeable after PND10 ( $P < 0.001$ ). (c,d) Anogenital distance measurement from PND4 through PND21 of *Mecp2*-mutant animals and their wt littermate controls. *Mecp2*-mutant mice presented a significant reduction in the AGD ( $P < 0.001$ ). (*Mecp2*-heterozygous females,  $n = 16$ ; wt females,  $n = 9$ ; *Mecp2*-null males,  $n = 13$  and wt males,  $n = 11$ . Values are mean  $\pm$  SEM. AGD, anogenital distance; PND, postnatal day; ko, knock-out; wt, wild-type; \* $P < 0.05$ ).

did all mutant animals present the PR. The *Mecp2*-mutant animals showed a delay of 2 days in relation to the day of first appearance of PR in the wt animals.

#### Negative geotaxis

In respect to mouse behaviour, this reflex was scored from 0 to 3 (see Table 1). Scores 2 and 3 were not frequent and so, to simplify the analysis of the data, we decided to recode the behaviours for the analysis. Score 0 and score 1 were maintained and score 2 was changed to include the previous scores 2 and 3. In this task, both male and female *Mecp2*-mutant mice had a worse performance than their respective wt littermate controls (Fig. 2e,f). The percentage of animals meeting the criterion for a score of 0 was dependent on the day ( $P < 0.01$ ) and genotype ( $P < 0.01$ ), whereas sex was not significant ( $P = 0.07$ ). Moreover, differences were found in the acquisition of the NG reflex between genotypes in both sexes (in both cases  $P < 0.01$ ), resulting from a difference in the performance of the animals in classes 0 and 2. When we tested the animals in a weaker version of this test (at  $30^\circ$  inclination), *Mecp2*-null animals still performed worse than wt controls in this task whereas heterozygous females did not differ significantly from wt animals (data not shown).

#### Wire suspension

There were no differences in the establishment of this reflex between male and female mice ( $P = 0.176$ ) and the day affected the establishment of the reflex ( $P < 0.001$ ), as expected. The performance of *Mecp2*-null and *Mecp2*-heterozygous mice and their respective wt controls in the acquisition of the reflex (animals grasp the wire or do not grasp) was similar, with no statistical differences when compared among each other ( $P = 0.605$  for males and  $P = 0.214$  for females). This reflex was acquired between PND11 and PND18 for both *Mecp2*-mutant and wt mice of both genders. Another parameter that was taken from this analysis was the wire suspension time. As body weight might influence the time animals hold on to the wire, the curves of the wire suspension holding time were corrected taking into account the body weight. We analysed this parameter from PND15 onwards because from this day more than 50% of the animals held on to the wire for more than 1 second. The wt females held the wire for a significantly longer time than wt male mice ( $P = 0.046$ ), but there were no differences between mutant male and female mice ( $P = 0.730$ ). Surprisingly, *Mecp2*-null and *Mecp2*-heterozygous mice stayed on the wire longer than their respective wt littermate controls

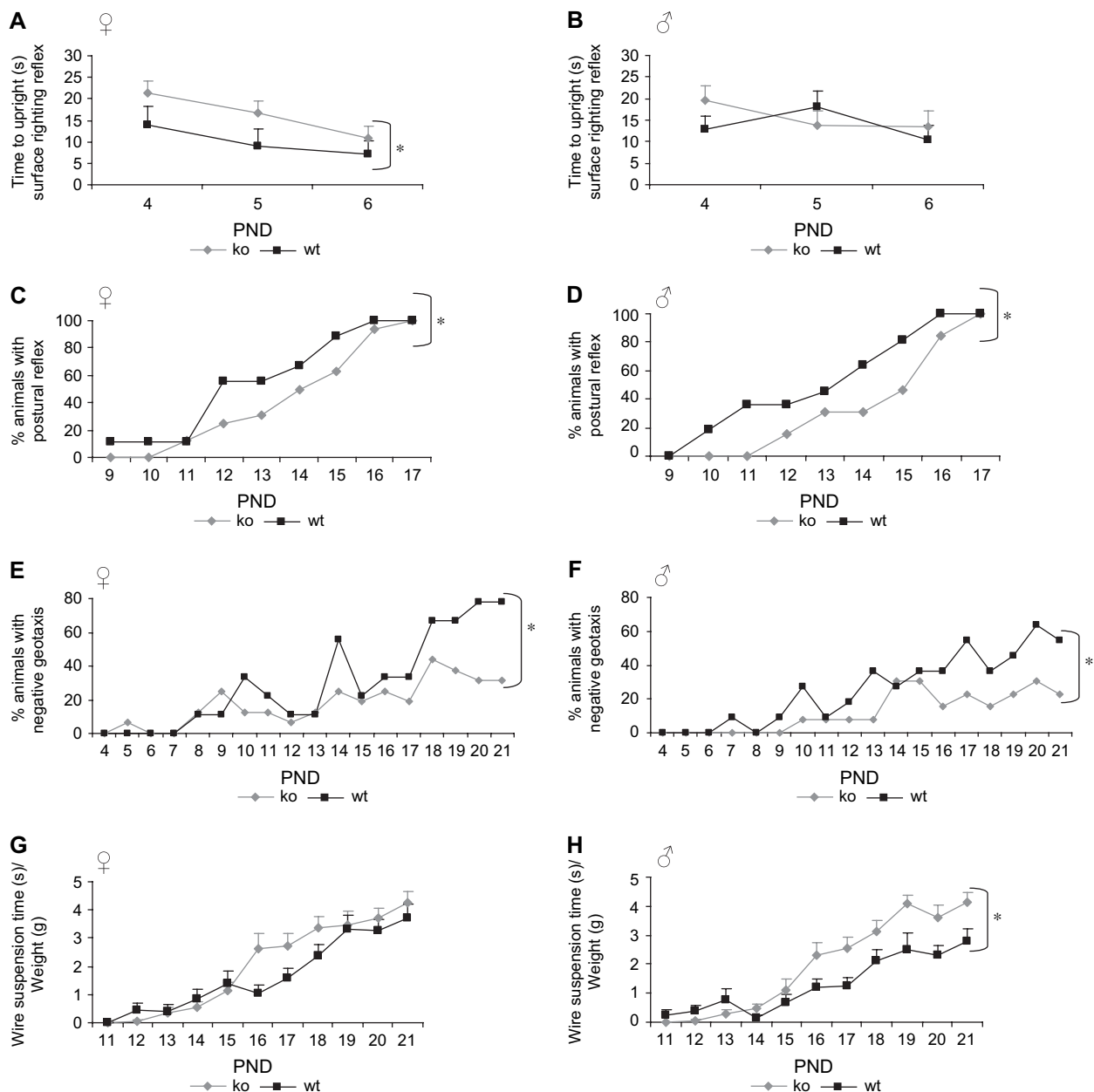


**Figure 2: Abnormalities in milestone achievement in the *Mecp2*-heterozygous and the *Mecp2*-null mice during the pre-weaning period.** (a,b) Time taken to surface-right in the surface righting reflex test. Female *Mecp2*-heterozygous mice took longer to regain an upright position than their wt littermates ( $P < 0.05$ ). (c,d) Percentage of animals presenting the postural reflex between PND9 and PND17. A delay in the acquisition of this parameter was observed in both the *Mecp2*-null animals ( $P < 0.001$ ) and the *Mecp2*-heterozygous females ( $P = 0.006$ ). (e,f) Percentage of animals presenting the negative geotaxis reflex. Female *Mecp2*-heterozygous animals ( $P = 0.002$ ) and *Mecp2*-null males ( $P < 0.001$ ) showed a worse performance than wt littermates. (g,h) Time that animals held the wire in the wire suspension reflex (in a 30-second test). *Mecp2*-null male animals held the wire for longer ( $P = 0.010$ ), although differences in *Mecp2*-heterozygous females did not reach significance. (*Mecp2*-heterozygous females,  $n = 16$ ; wt females,  $n = 9$ ; *Mecp2*-null males,  $n = 13$  and wt males,  $n = 11$ . Values are mean  $\pm$  SEM. PND, postnatal day; ko, knock-out; wt, wild-type,  $*P < 0.05$ ).

and the differences were statistically significant between *Mecp2*-null and wt littermate controls ( $P < 0.001$ ) (Fig. 2g,h). Even when we analysed the data relative to all days (PND11–PND21), the same conclusions were reached ( $P = 0.010$ ).

### Post-weaning behaviour analysis

#### Exploratory activity

At the fourth week of age, animals were tested in the OF apparatus, to evaluate their spontaneous activity, for a period

of 5 min (*Mecp2*-null  $n = 14$ , wt littermate males  $n = 16$ , *Mecp2*-heterozygous  $n = 12$ , wt littermate females  $n = 10$ ). Globally, no differences were found between *Mecp2*-mutant and wt animals in the time they spent and distance they travelled in the centre of the arena in relation to the total area of the arena, in the time animals spent exploring vertically or in the number of rears (Supplemental table 2). We found that *Mecp2*-null animals travelled a smaller total distance ( $P = 0.049$ ) at a lower speed ( $P = 0.000$ ) than wt controls (Fig. 3a–c). Null animals produced a significantly higher number of *bolus faecalis* ( $P = 0.031$ ) (Supplemental table 2), which could be a consequence of their neuroautonomic disorder.

#### Anxiety-like behaviour

The day after OF testing, animals were tested in the EPM apparatus, in a 5-min session (*Mecp2*-null  $n = 13$ , wt littermate males  $n = 13$ , *Mecp2*-heterozygous  $n = 11$ , wt littermate females  $n = 8$ ). There were no differences between *Mecp2*-mutant animals and wt controls in the percentage of time animals spent in the open arms nor in the percentage of entries in the open arms in relation to total arms entries, but *Mecp2*-null animals presented a smaller number of closed arms entries ( $P = 0.014$ ) (Fig. 4a–c).

#### Motor co-ordination

At 5 weeks of age, *Mecp2*-mutant animals were tested in the rotarod to evaluate their motor co-ordination (*Mecp2*-null

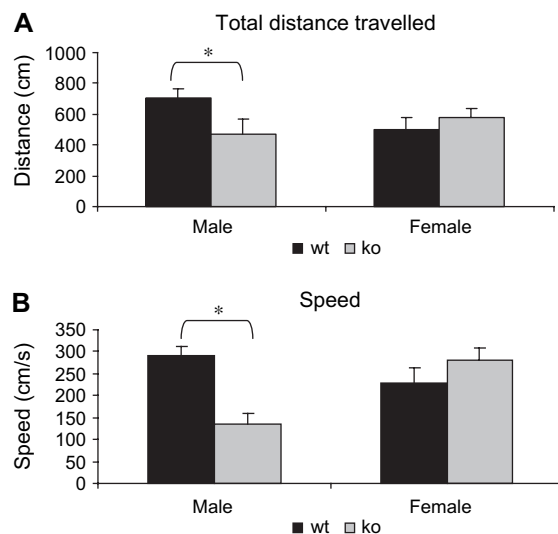


**Figure 3: *Mecp2*-mutant female and male mice present reduced spontaneous activity without altered exploratory capacity at 4 weeks of age, in the open-field paradigm.** (a) *Mecp2*-null male mice travelled a smaller total distance ( $P = 0.049$ ), (b) at a lower speed ( $P = 0.000$ ) than their respective wt littermate controls. Female heterozygous animals did not present differences in any of the parameters analysed. (*Mecp2*-heterozygous females,  $n = 12$ ; wt females,  $n = 10$ ; *Mecp2*-null males,  $n = 14$  and wt males,  $n = 16$ . Values are mean  $\pm$  SEM. PND, postnatal day; ko, knock-out; wt, wild-type, \* $P < 0.05$ ).

$n = 11$ , wt littermate males  $n = 11$ , *Mecp2*-heterozygote  $n = 16$ , wt littermate females  $n = 9$ ). After 3 days of training, mice were tested at different speeds. *Mecp2*-null and *Mecp2*-heterozygous mice, when compared to wt control mice, presented a reduced latency to fall off the rod. This reduction was statistically significant at 15 r.p.m. for male ( $P = 0.046$ ) and at 20 r.p.m. for female ( $P = 0.023$ ) mice (Fig. 5a,b).

## Discussion

### Delayed somatic physical growth and maturation of *Mecp2*-mutant mice

Among the physical growth and maturation parameters assessed in this study, differences were seen in body weight and in AGD. The body weight was significantly

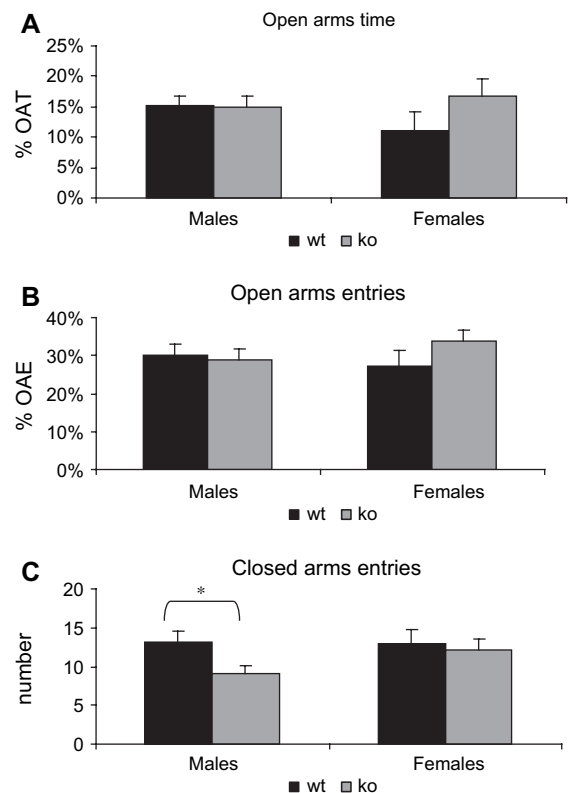


**Figure 4: *Mecp2*-mutant female and male mice do not present anxiety-like behaviour at 4 weeks of age in the elevated plus-maze paradigm.** Neither *Mecp2*-null male nor *Mecp2*-heterozygous female mice presented differences in (a) the percentage of open arms time and (b) the percentage of open arms entries, which are measures of the state of anxiety that the animals exhibit in a new environment. (c) *Mecp2*-null animals had fewer entries into the closed arms than their wt littermate controls ( $P = 0.014$ ) suggesting the existence of a locomotor impairment. (*Mecp2*-heterozygous females,  $n = 11$ ; wt females,  $n = 8$ ; *Mecp2*-null males,  $n = 13$  and wt males,  $n = 13$ . Values are mean  $\pm$  SEM. PND, postnatal day; ko, knock-out; wt, wild-type, \* $P < 0.05$ ).

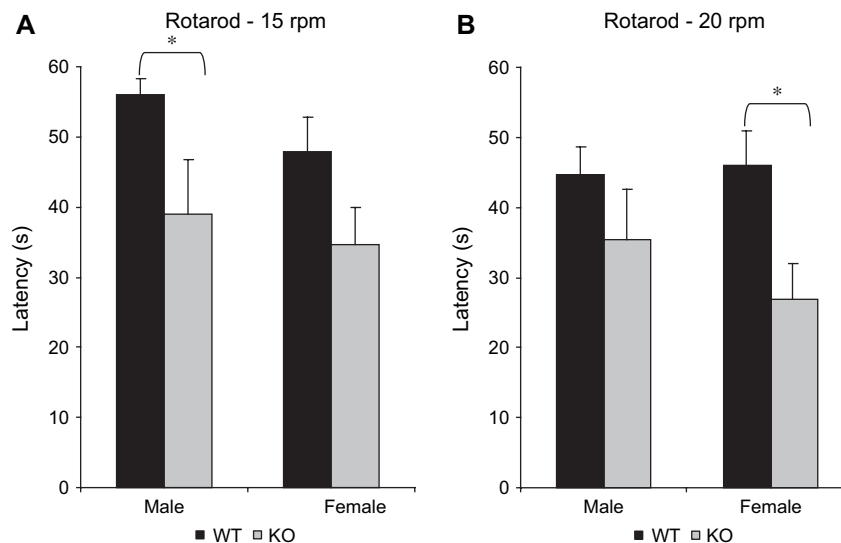


**Figure 5: *Mecp2*-mutant mice present motor problems at 5 weeks of age.** The latency to fall off the rod was lower for the *Mecp2*-null mice at 15 r.p.m. (a) and for *Mecp2*-heterozygous females at 20 r.p.m. (b) than the latency exhibited by their respective wt controls. (*Mecp2*-heterozygous females,  $n = 16$ ; wt females,  $n = 9$ ; *Mecp2*-null males,  $n = 11$  and wt males,  $n = 11$ . Values are mean  $\pm$  SEM. PND, postnatal day; ko, knock-out; wt, wild-type,  $*P < 0.05$ ).

reduced in the *Mecp2*-heterozygotes, but, unexpectedly, this difference in body weight was not seen between *Mecp2*-null and wt control male mice in spite of their earlier disease onset. However, the curves of *Mecp2*-null and wt males start diverging at PND20 and would probably follow this trend at later ages. In fact, it is already known from the original publication on this model that *Mecp2*-null mice present a smaller body weight than wt littermate controls at 4 weeks of age (Guy *et al.* 2001). The same authors suggested that, given the differences observed between mice with different genetic backgrounds, the effects of MeCP2 in body weight could be mediated by one or more modifier genes. One of these modifier genes could be sex-linked and thus provide a possible explanation for the results we obtained. Also, the AGD is reduced in both male and female *Mecp2*-mutant mice suggesting that these animals present a slower sexual maturation. In the case of *Mecp2*-null mice it has been reported that their testes are always internal and they do not mate because they are too debilitated or die before adulthood. However, adult *Mecp2*-heterozygous mice are fertile and, as far as we know, they do not present reduced fertility and they raise normal litters (Guy *et al.* 2001). Taken together these results also support the evidence that MeCP2 has an effect in somatic growth markers and not only in neuronal cells (Huppke *et al.* 2003; Nagai *et al.* 2005).

#### **Pre-weaning behaviour in the *Mecp2*-mutant animals suggests early neurological dysfunction**

In the present study, a delay in the achievement of the postural reflex and of the surface righting reflex (only in

females) was evident between *Mecp2*-mutant and wt animals. Both reflexes depend on the development of dynamic postural adjustments and imply the integrity of muscular and motor function (Altman & Sudarshan 1975; Dierssen *et al.* 2002). Acquisition of the negative geotaxis reflex, a dynamic test that reflects sensorimotor function and depends on colliculus maturation (Dierssen *et al.* 2002) was also disturbed. Despite those impairments, in another neurological reflex – the static wire suspension test, which is highly compensated by information from the visual and proprioceptive systems – *Mecp2*-mutant animals did not perform worse than wt controls. *Mecp2*-null animals held the wire for a longer time, even though there were no differences between *Mecp2*-null and wt controls as to when the mice started to grasp the wire. Thus, the fine motor skills of the forepaws did not appear to be affected in the mutant mice. The longer time holding the wire could, however, reflect the incapacity of the mutant mice to initiate a voluntary movement, which could constitute a possible sign of dyspraxia, as observed in RTT patients (Kerr & Engerstrom 2001).

All the above-mentioned reflexes are sensitive to the function of the vestibular system, of which the role is to provide information on the position and movement of body and head in space, and so they depend largely on brainstem (medullary) structures (Altman & Sudarshan 1975). The positional information is transmitted from the inner ear to the central vestibular system located in the hindbrain and integrated with information from other neural systems [for a review see (Smith *et al.* 2005)]. The data we obtained on neurological reflexes is particularly interesting in the light of

the studies in human RTT patients that suggest dysfunction of the brainstem, where the vestibular system is located, as responsible for the early pathogenesis in RTT (Einspieler *et al.* 2005b; Segawa 2005). Interestingly, MeCP2 binds directly to the brain-derived neurotrophic factor (BDNF) promoter region (Chen *et al.* 2003; Martinowich *et al.* 2003) and regulates its transcription in an activity-dependent manner. BDNF appears to have an important role in the maturation and maintenance of the vestibular system, as mice deficient for BDNF and its receptor TrkB demonstrate neuronal loss in the vestibular sensory ganglia (Huang & Reichardt 2001). It is, thus, possible to speculate that the levels of this neurotrophin in the vestibular pathways could be deregulated in the *Mecp2*-mutant mice and in this way could also contribute to possible dysfunction in the vestibular system.

Abnormal acquisition of the NG reflex could reflect abnormalities in the maturation of the colliculi and the abnormal performance in the surface RR could reflect abnormalities in the labyrinthine function. Anomalies in the auditory canal cannot be the source of this dysfunction because mice with anomalies in this area present stereotypical behaviours (Khan *et al.* 2004) that are not exhibited by the *Mecp2*-mutants. Data on the pathology in this area of the mouse brain, as far as we know, is not yet available in the *Mecp2*<sup>tm1.1Bird</sup> mouse and future research is necessary to explore neuropathological correlates of the abnormal functional outcome in the first days of postnatal life of *Mecp2*-mutant mice.

The subtle but significant perturbations observed in the achievement of milestones are a first sign of early neurological pathology in the *Mecp2*<sup>tm1.1Bird</sup> mice. The motor problems that these mice experience later in life correlate with the developmental abnormalities and may even be a consequence of impaired neurodevelopment of pathways within the brainstem area.

### ***Mecp2*-mutant mice present reduced spontaneous activity as a results of motor impairments before the onset of overt symptoms**

Adult *Mecp2*<sup>tm1.1Bird</sup> mice were initially described as presenting serious motor problems after a period of normal development (Guy *et al.* 2001). In fact, in their home cage at 4 weeks of age, juvenile *Mecp2*-mutant mice are, other than their reduced body weight, almost indistinguishable from their wt littermates. However, in the OF apparatus the *Mecp2*-null mice exhibit hypoactivity (Guy *et al.* 2001) despite a normal exploratory capacity. We were not able to notice any differences between *Mecp2*-heterozygous and wt control females in the OF, at 4 weeks of age, even though they were previously described to exhibit reduced spontaneous activity at later ages, when symptomatic (Guy *et al.* 2001). In the OF and EPM we did not identify an anxiety-like behaviour in either male or female *Mecp2*<sup>tm1.1Bird</sup> animals at 4 weeks of age. In accordance, performance of older symptomatic *Mecp2*-heterozygous animals in the OF also suggested that these mice do not present heightened anxiety (Guy *et al.* 2001).

Anxiety is, however, described in other models of the RTT disorder (Gemelli *et al.* 2005; Moretti *et al.* 2005; Shahbazian *et al.* 2002).

In this study, at 5 weeks of age the *Mecp2*-null and *Mecp2*-heterozygous mice demonstrated motor co-ordination impairment. This is, to the best of our knowledge, the first study to identify the effect of *Mecp2* mutation on sensorimotor co-ordination in the rotarod test in 5-week-old mice. Although differences in the locomotor profile of *Mecp2*-heterozygous mice when compared to wt controls were not identified in the OF and in the EPM apparatus, in the more sensitive and specific rotarod test, mutant females already showed motor problems at the age of 5 weeks. Motor co-ordination problems had already been previously reported in the other models of RTT, but not at such an early age: the *Mecp2*<sup>308/y</sup> animals are not impaired up to 10 weeks of age (Moretti *et al.* 2005), but are impaired at later ages (Shahbazian *et al.* 2002). Our findings suggest that MeCP2 is important for the acquisition of motor co-ordination abilities and that deregulation of its levels causes slight motor problems that appear early in development and become increasingly evident as development proceeds. The deficits in the rotarod are not likely to be the result of muscle weakness because the mutant animals held on longer in the WS test than the wt animals. Co-ordination is necessary for a good performance both in the dynamic reflexes and in the rotarod test. Hence, and regarding the data obtained in this study, a lack of limb co-ordination is apparently present in the *Mecp2*-mutant mice; given that both the NG reflex and the rotarod test are affected, we suggest that hind limbs are more severely involved. Rearing also presupposes hind limb strength (Altman & Sudarshan 1975) and as this parameter is not affected in these animals, the problem must reside in co-ordination of the hind limbs rather than in their strength.

The identification of early and subtle neurodevelopmental differences in the RTT mouse model provides an interesting analogy to the recent findings of minor neurological signs during the first months of life of RTT patients. Further analysis of neurodevelopment in these *Mecp2*-mutant mice, which mimic well the motor profile of RTT patients, should provide an insight into the underlying mechanisms of pathogenesis in this disease and contribute to a precocious RTT diagnosis that might be beneficial in terms of therapeutic approaches since the first months of life.

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## Supplementary Material

The following material is available for this article online:

**Table S1:** Maturation measures assessment in *Mecp2*-mutant and wild type animals

**Table S2:** Performance of *Mecp2*-mutant and wild type animals in the Open field test

**Figure S1:** Milestones achievement in the *Mecp2*-heterozygous and the *Mecp2*-null mice during the pre-weaning period

This material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/abs/10.1111/j.1601-183x.2006.00258.x>

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