

Modulation of neuropathic pain:
a role for imprinted genes

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Pain is temporary
(if a chronification does not happen,
or a disease in the somatosensory system,
or a dysregulation in facilitatory/inhibitory mechanisms...).

Pain of quitting lasts forever.

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Abstract

Chronic pain is a serious debilitating condition affecting one-third of the population of developed countries and up to one-tenth of the adult population worldwide, thus representing a major public health problem. Poor diagnostic accuracy of different chronic pain types coupled with the lack of effective treatments make this pathology particularly impactful to patients, but also burdensome to families, caregivers and state economies. Therefore, relevant studies of the molecular mechanisms underlying the pathological processes of both neuropathic and nociceptive pain - the two types of chronic pain - are warranted.

In this work, we have studied a mutant mouse line subjected to the SNI model of neuropathic pain to explore the role of imprinted genes in this pathology. *Begain* is a gene of the *Dlk1-Dio3* imprinted domain that has been recently implicated in pathological pain transmission following peripheral nerve injury in mice. *Begain* is widely expressed in the central nervous system where it is thought to sustain the structure of the postsynaptic density in neurons, by binding to scaffold proteins and interacting with N-methyl-D-aspartate (NMDA) receptors at synapses. Interestingly, it encodes a biallelically-expressed protein and an imprinted (paternally-expressed) variant from alternative exon usage. The individual role of these isoforms, namely that of the imprinted variant, has not been explored to date. The mutant model used herein, termed Rep^{L1del}, is characterized by a severe downregulation of the imprinted *Begain* (1B) isoform in embryonic brain while retaining normal expression levels of the biallelically expressed (1A) isoform. In spite of displaying a moderate effect in the expression of the neighboring *Dlk1* gene in embryonic and adult brain, the Rep^{L1del} mutant constitutes the closest model to an isoform-specific knockdown of imprinted *Begain* 1B.

Our results show the same patterns of dysregulation in mutant embryos in two essential elements of the pain circuitry, the dorsal root ganglia (DRG) and spinal cord (SC): downregulation of imprinted *Begain* 1B in DRG and SC and of *Dlk1* in embryonic SC only, along with no detectable changes in the expression of *Begain* 1A in either tissue. Notably, in the SNI model of neuropathic pain, the Rep^{L1del} mutant mice exhibited exacerbated mechanical allodynia as assessed by the von Frey test. Mutant animals subjected to

peripheral nerve injury consistently showed substantially more sensitivity to monofilament stimuli than the Wt mice subjected to the same condition, as well as than both mutant and Wt sham controls. Our results unequivocally show a pronounced phenotype of pain sensitization in Rep^{L1del} mutant mice, which implicate imprinted *Begain* (and/or *Dlk1*) in the modulation of neuropathic pain. This is the first description of a physiological phenotype in the Rep^{L1del} mouse line.

To investigate the dynamics of *Begain* and *Dlk1* expression in the SC and DRG of adult Rep^{L1del} mutants and wildtype littermates subjected to the SNI model of neuropathic pain, gene and protein expression studies were conducted at a single timepoint, 28 days following SNI. In DRG, imprinted *Begain 1B* expression was unaffected; conversely, *Begain 1A* was significantly upregulated in mice subjected to SNI comparing to animals subjected to sham surgery (18%), particularly within the mutants group, where SNI animals showed a 38% upregulation. These changes were also seen at the protein level, with total *Begain* increasing by 33% (SNI vs. sham) and 42% (mutant SNI vs. mutant sham). Similarly, *Dlk1* displayed a response to SNI in adult DRG, seen only at the protein level. The most common isoform detected by Western blot (60 kDa) was tentatively increased in SNI relatively to sham animals, irrespective of genotype. This peptide, together with the less abundant variant of 55 kDa, showed a tentative strong increase in SNI Wt animals. Both proteins were increased following SNI in the Wt group relative to the Rep^{L1del} mutant group, particularly the 55 kDa isoform, which showed a massive 4.5-fold increase. Overall, the analyses conducted in adult DRG at this timepoint appear to implicate *Begain 1A* and *Dlk1* in a response to nerve injury. This response was mutant-specific in the case of *Begain 1A*, suggesting that it may be pathological. Conversely, for *Dlk1* the response to SNI seemed to be physiological, as it was seen in Wt animals, but dysregulated in Rep^{L1del} mice.

In adult spinal cord, imprinted *Begain 1B* expression was significantly downregulated by 16% in mutants relative to Wt littermates, a behaviour tentatively followed by *Begain 1A*. Protein analyses corroborated these findings, showing similar levels of downregulation of total *Begain* (16 to 20%). However, at this timepoint, there was no response to SNI in either case. *Dlk1* expression revealed much the same behaviour as that of *Begain*, although with a more pronounced downregulation in Rep^{L1del} mutants,

both at the gene and protein level (up to 53% and 37%, respectively). At the surveyed timepoint, no significant differences were noted when comparing SNI- and sham surgery-subjected animals of either genotype.

Imprinting studies in the DRG and SC of mutant and wildtype embryos and adult mice revealed the characteristic parent-of-origin specific expression of the three transcripts, indicating that Rep^{L1del} has no effect on the regulation of imprinting in these tissues.

In all, we show that in adult mice *Begain 1B* is not overtly responding to SNI at the timepoint tested; thus, any effects on pain transmission would have to stem from the downregulation of the isoform elicited by the Rep^{L1del} mutation as seen in the spinal cord. Conversely, *Begain 1A* and *Dlk1* were found to respond to nerve injury, but only in the DRG. Therefore, they may be involved in the modulation of neuropathic pain in this tissue. Any effects in the spinal cord would have to stem from their downregulation caused by the mutation. Importantly, gene and protein expression of *Begain* or *Dlk1* could be compromised earlier in time, following the insult, which would not be picked up at the timepoint surveyed.

In the course of this work, we have also produced a suitable *Begain 1B*-specific knockout mouse model using CRISPR/Cas9-based genome editing, for future studies. Altogether, the study with the Rep^{L1del} mouse line has enable us to identify a clear pain sensitization phenotype, which has been associated to imprinted genes, for the first time, and is thus suggestive of a putative role for genomic imprinting in modulating neuropathic and, perhaps more generally, chronic pain. Further studies will follow to deepen our understating of relevant molecular events in the nociceptive circuit upon nerve lesion, namely the analysis of additional timepoints closer to the time of injury, as well as histological studies to explore potential structural and molecular phenotypes *in situ*. The recently produced *Begain 1B* knockout mouse will prove an invaluable tool to probe the specific role of imprinted *Begain* in pathological pain.

Resumo

A dor crónica é uma condição debilitante grave que afeta um terço da população em países desenvolvidos e cerca de um décimo da população adulta em todo o mundo, constituindo-se por isso como um sério problema de saúde pública. A falta de métodos diagnósticos precisos que distingam os diferentes tipos de dor crónica, associada à inexistência de terapêuticas eficazes, têm um forte impacto na vida dos doentes e são particularmente onerosos para familiares, cuidadores e para a economia em geral. Consequentemente, a realização de estudos relevantes que clarifiquem os mecanismos moleculares subjacentes aos processos patológicos da dor neuropática e nociceptiva - os dois tipos de dor crónica - é de grande importância.

Neste trabalho foi estudada uma linha mutante de murganho (rato-doméstico) sujeito a lesão de dois dos três ramos do nervo ciático (modelo de dor neuropática SNI) com o objetivo de explorar o envolvimento de genes “imprinted” (de expressão monoalélica dependente da origem da transmissão parental) nesta patologia de dor. *Begain* é um gene pertencente ao domínio “imprinted” Dlk1-Dio3 e que foi recentemente implicado na transmissão de dor patológica após lesão de nervos periféricos em murganho. O *Begain* é amplamente expresso no sistema nervoso central onde se crê que sustém a estrutura da densidade pós-sináptica através da sua ligação a proteínas “scaffold” e da sua interação com recetores NMDA nas sinapses. Curiosamente, o gene *Begain* codifica duas variantes proteicas, uma de expressão bialélica e a outra “imprinted” (de expressão paterna), resultantes do uso alternativo do primeiro exão. Contudo, o papel de cada uma das isoformas, nomeadamente a da variante “imprinted”, não foi explorado até à data. O modelo mutante utilizado neste trabalho, designado Rep^{L1del}, é caracterizado por apresentar uma forte subexpressão da variante “imprinted” do gene *Begain* (variante 1B) em cérebro embrionário, enquanto que os níveis de expressão da isoforma bialélica (1A) se mantêm inalterados. Apesar de apresentar um efeito moderado na expressão do gene vizinho *Dlk1* em cérebro embrionário e adulto, o mutante Rep^{L1Del} constitui o modelo mais próximo ao de um “knockdown” específico do *Begain* 1B “imprinted”.

Os nossos resultados mostram, em embriões mutantes, os mesmos padrões de desregulação, em dois elementos essenciais dos circuitos da dor, os gânglios das raízes dorsais (DRG) e a medula espinal (SC): subexpressão do *Begain* “imprinted” em DRG e SC, e subexpressão do *Dlk1* apenas em SC, assim como a ausência de alterações detetáveis na expressão do *Begain* bialélico em ambos os tecidos. Crucialmente, verificou-se que perante a indução do modelo de dor neuropática os mutantes Rep^{L1Del} apresentaram alodinia mecânica exacerbada, tal como avaliado através do uso do teste de von Frey. Quando submetidos a lesão do nervo periférico, os animais mutantes exibiram consistentemente um fenótipo de maior sensibilidade ao estímulo provocado pelos monofilamentos de von Frey do que os animais Wt submetidos à mesma lesão, bem como relativamente aos controlos (mutantes e Wt) sujeitos a uma cirurgia “sham” (sem lesão do nervo). Os nossos resultados mostram inequivocamente um pronunciado fenótipo de sensibilização à dor em murganhos mutantes Rep^{L1Del}, que implicam o *Begain* “imprinted” (e/ou o *Dlk1*) na modulação da dor neuropática. Esta é a primeira descrição de um fenótipo fisiológico na linha de murganhos Rep^{L1Del}.

Com o intuito de investigar a dinâmica da expressão de *Begain* e *Dlk1* em SC e DRG adulto de animais mutantes Rep^{L1del} e Wt sujeitos ao modelo de dor neuropática SNI, foram realizados estudos de expressão génica e proteica num ponto de tempo único: a 28 dias após o SNI. Em DRG, a expressão de *Begain 1B* (variante “imprinted”) não estava alterada; por outro lado, a de *Begain 1A* aumentou significativamente em murganhos submetidos a SNI em comparação com animais submetidos a cirurgia “sham” (18%), particularmente no grupo dos mutantes, no qual os animais sujeitos a SNI apresentaram uma sobreexpressão de 38%. Essas alterações de expressão foram também observadas ao nível proteico, com um aumento de proteína total de *Begain* em 33% (SNI vs. “sham”) e 42% (mutante SNI vs. mutante “sham”). Da mesma forma, o *Dlk1* revelou uma resposta ao SNI em DRG adulto, detetado apenas a nível proteico. A isoforma de *Dlk1* mais comum detetada por Western blot (com 60 kDa) apresentou um aumento tendencial em animais SNI relativamente à dos animais “sham”, independentemente do genótipo. Este péptido, juntamente com a variante menos abundante de 55 kDa, mostrou tendência para um forte aumento em animais SNI Wt. Ambas as proteínas aumentaram após SNI no grupo Wt em relação ao grupo mutante Rep^{L1del}, particularmente a isoforma de 55 kDa, que

apresentou um aumento muito expressivo de 4,5 vezes. No geral, as análises realizadas em DRG adulto neste ponto de tempo parecem implicar o *Begain 1A* e *Dlk1* na resposta à lesão do nervo. No caso do *Begain 1A* esta resposta foi específica dos animais mutantes, sugerindo que pode ser patológica. Por outro lado, a resposta do *Dlk1* ao SNI parece ser fisiológica, uma vez que se verificou também em animais Wt, mas desregulada em mutantes *Rep^{L1del}*.

Na medula espinal de adulto, a expressão da variante “imprinted” *Begain 1B* revelou uma redução significativa de 16% em animais mutantes quando comparada com os Wt, um comportamento tendencialmente seguido pelo *Begain 1A*. A análise proteica corroborou estas observações, mostrando níveis de redução semelhantes da proteína total do *Begain* (16 a 20%). Contudo, neste ponto de tempo analisado, não se verificou qualquer resposta ao SNI em nenhum dos casos. A expressão de *Dlk1* revelou o mesmo comportamento que a de *Begain*, embora com redução mais acentuadas nos mutantes *Rep^{L1del}*, tanto ao nível génico como ao nível proteico (até 53% e 37%, respetivamente). No ponto de tempo analisado não foram registadas diferenças significativas ao comparar animais submetidos a SNI com animais sujeitos à cirurgia “sham”, em ambos os genótipos.

Estudos de “imprinting” em DRG e SC de embriões e animais adultos mutantes e Wt, mostraram a expressão específica de origem parental típica de cada um dos três transcritos, indicando que a mutação *Rep^{L1del}* não tem influência na regulação do estado de “imprinting” nesses tecidos.

Em suma, no ponto de tempo examinado, mostramos que em murganhos adultos o *Begain 1B* não responde de forma evidente ao SNI; assim, quaisquer efeitos na transmissão da dor teriam que resultar da subexpressão desta isoforma provocada pela mutação *Rep^{L1del}*, como verificado na medula espinal. Contrariamente, verificou-se que *Begain 1A* e *Dlk1* respondem à lesão do nervo, mas apenas em DRG. Podem, portanto, estar envolvidos na modulação da dor neuropática neste tecido. Quaisquer efeitos na medula espinal teriam por isso que resultar da sua subexpressão causada pela mutação. É importante salientar que a expressão génica e proteica do *Begain* ou do *Dlk1* podem estar comprometidas mais cedo, após a lesão, o que não seria necessariamente detetável no ponto de tempo analisado neste estudo.

No decurso deste trabalho, produzimos ainda um modelo adequado de ablação (“knockout”) específica da isoforma *Begain 1B* em murganho, usando a ferramenta de edição do genoma CRISPR/Cas9, para estudos futuros. No geral, o estudo com a linha mutante de murganho *Rep^{L1del}* permitiu identificar um fenótipo claro de hipersensibilidade à dor que é associado, pela primeira vez, a genes “imprinted” e, como tal, sugestivo de um possível papel para o “imprinting” genómico na modulação da dor neuropática ou, possivelmente de forma mais genérica, da dor crónica. Seguir-se-ão estudos adicionais para aprofundar a compreensão de eventos moleculares relevantes no circuito nociceptivo desencadeados pela lesão do nervo, nomeadamente a análise de pontos temporais adicionais mais próximos do momento da lesão, bem como estudos histológicos para explorar possíveis fenótipos estruturais e moleculares *in situ*. O recém-produzido “knockout” do *Begain 1B* será uma ferramenta de valor inestimável para investigar o papel específico do *Begain* “imprinted” na dor patológica.

Table of contents

Acknowledgements	1
Abstract	4
Resumo	7
Table of contents	11
List of figures and tables	13
List of abbreviations	15
Introduction	
Part I	18
1. Pain	18
1.1 Chronic pain	18
1.2 Neuropathic pain	19
1.3 Peripheral neuropathic pain models	20
1.4 Peripheral neuropathic pain mechanisms	21
1.5 NMDA receptors and scaffold proteins	24
2. Epigenetics	25
2.1 Genomic imprinting	26
2.2 Mouse Chr. 12 (Dlk1-Dio3) imprinted domain	28
2.3 Del ^{L1rep} mouse mutant model	30
2.4 Begain	31
2.5 Dlk1	33
Part II	35
1. Genome editing	35
1.1 CRISPR-Cas 9	35
AIMS	40
Material and Methods	
Part I	43
1. Animals	43
2. Spared Nerve Injury Surgery	43
3. Nociceptive Behaviour - von Frey test	45
4. Tissue collection	47
5. RNA extraction	47
5.1 cDNA synthesis	48
5.2 Real-Time qPCR	48
6. Protein extraction	49
6.1 Western Blotting (WB)	50
7. Statistical Analysis	50
8. Imprinting	51

Part II – Production of a Begain 1B knockout mouse	53
1. Begain 1B Isoform-specific targeting using CRISPR/Cas9	53
2. Assembly of the CRISPR-Cas9 complex and in vitro testing of the crRNAs	54
3. Microinjection of the CRISPR-Cas9 complex	55
4. Genotyping of pups	55
Results	
Part I	58
Pain behaviour studies	58
Rep ^{L1del} mutants exhibit mechanical allodynia	58
Begain and Dlk1 gene and protein expression studies	61
1. E16.5 embryos	61
1.1 Embryonic spinal cord	61
Begain 1B and Dlk1 are downregulated in the SC of Rep ^{L1del} embryos ..	61
1.2 Embryonic DRG	62
Begain 1B alone is downregulated in the DRG of Rep ^{L1del} embryos	62
2. Adult animals	64
2.1 Adult DRG	65
Begain 1A is upregulated in the DRG of Rep ^{L1del} mice subjected to SNI	65
Begain 1B expression is unaffected in the DRG of Rep ^{L1del} mutants and following SNI	67
Dlk1 expression is unaffected in the DRG of Rep ^{L1del} mutants and following SNI	68
The Dlk1 protein is dysregulated in DRG following SNI	69
2.2 Adult spinal cord.....	71
Begain expression is seemingly downregulated in SC of Rep ^{L1del} mutant mice but not responding to SNI	71
Dlk1 is downregulated in SC of Rep ^{L1del} mutant mice but not responding to nerve injury.....	75
Imprinting studies	79
Part II	81
Production of a Begain 1B knockout mouse	81
In vitro testing of the selected crRNAs	81
In vivo targeting of mouse zygotes for genome editing	82
Discussion	85
Conclusion and future perspectives	95
References	96

List of figures and tables

Figure 1. Types and characteristics of sensitive fibres from peripheral nerves	24
Figure 2. Schematic representation of imprinted and biallelic gene expression	27
Figure 3. Schematic representation of the <i>Dlk1-Dio3</i> imprinted domain	29
Figure 4. Schematic diagram of <i>Begain</i> transcript variants in the mouse	32
Figure 5. Schematic representation of <i>Dlk1</i> gene variants	33
Figure 6. Graphical representation of <i>Dlk1</i> expression during development	34
Figure 7. Mechanism of CRISPR-Cas9 action	37
Figure 8. CRISPR-Cas9 cleavage of target sequences	38
Figure 9. Spared Nerve Injury (SNI) surgical procedure	45
Figure 10. Schematic representation of the experiment timeline	47
Figure 11. Schematic representation of the approach employed for specific PCR amplification of <i>Begain</i> SNP rs252334243	52
Figure 12. Relative frequency of paw withdrawal (FPW) of Rep ^{L1del} mutant and Wt littermate mice following SNI and sham surgery.	60
Figure 13. Relative gene expression of <i>Begain 1A</i> isoform in spinal cord of Rep ^{L1del} mutant and wildtype E16.5 embryos	61
Figure 14. Relative gene expression of <i>Begain 1B</i> isoform and <i>Dlk1</i> in spinal cord of Rep ^{L1del} mutant and wildtype E16.5 embryos	62
Figure 15. Relative gene expression of <i>Begain 1A</i> , <i>Begain 1B</i> and <i>Dlk1</i> in dorsal root ganglia of Rep ^{L1del} mutant and wildtype E16.5 embryos	63
Figure 16. Representative images of Western blot analyses of <i>Begain</i> and <i>Dlk1</i> in dorsal root ganglia (A) and spinal cord (B) of adult Rep ^{L1del} mutants and wildtype littermates	64
Figure 17. Relative gene expression of <i>Begain 1A</i> and <i>1B</i> in dorsal root ganglia of Rep ^{L1del} mutants and wildtype littermates	66
Figure 18. Relative protein expression of total <i>Begain</i> in dorsal root ganglia of Rep ^{L1del} mutants and wildtype littermates	67
Figure 19. Relative gene expression of <i>Dlk1</i> in dorsal root ganglia of Rep ^{L1del} mutants and wildtype littermates	68
Figure 20. Relative protein expression of <i>DLK1</i> in dorsal root ganglia of Rep ^{L1del} mutants and wildtype littermates	70
Figure 21. Relative gene expression of <i>Begain 1A</i> and <i>Begain 1B</i> in spinal cord of Rep ^{L1del} mutants and wildtype littermates	72

Figure 22. Relative protein expression of total Begain in spinal cord of Rep ^{L1del} mutants and wildtype littermates	74
Figure 23. Relative gene expression of <i>Dlk1</i> in spinal cord of Rep ^{L1del} mutants and wildtype littermates	76
Figure 24. Relative protein expression of <i>Dlk1</i> in spinal cord of Rep ^{L1del} mutants and wildtype littermates	78, 79
Figure 25. Allele-specific expression of <i>Begain 1A</i> , <i>Begain 1B</i> and <i>Dlk1</i> in spinal cord of Rep ^{L1del} mutant and wildtype mice	80
Figure 26. <i>In vitro</i> testing of the selected crRNAs for <i>Begain</i> exon 1B targeting	81
Figure 27. Detection of genomic alterations at the <i>Begain 1B</i> locus in born pups targeted with CRISPR-Cas9 at the zygote stage	82
Figure 28. Multiplex PCR genotyping of the first litter (5 males and 3 females) of the <i>Begain 1B</i> KO mutant founder	83
Figure 29. Gel electrophoresis of PCR amplification products from the <i>Begain 1B</i> KO and wildtype alleles	83
Figure 30. Gel electrophoresis of qPCR amplification products of <i>Begain 1A</i> and <i>1B</i> brain and spinal cord cDNA	84

Table 1. Primer sequences and annealing conditions used in RT-qPCR	49
Table 2. Primer sequences and annealing conditions used for transcript-specific PCR amplification	52
Table 3. Primer sequences and annealing conditions for PCR genotyping of <i>Begain</i> KO mice	56

List of abbreviations

AMPA - α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BDNF - brain-derived neurotrophic factor

Begain - brain-enriched guanylate kinase associated protein

bp – base pairs

CAMKII - Ca^{2+} calmodulin-dependent protein kinase II

CK2 - casein kinase 2

CNS - central nervous system

CRISPR - clustered regularly interspaced short palindromic repeats

crRNA - CRISPR RNA

Cas – CRISPR-associated

Dio3 - type III iodothyronine deiodinase

Dlk1 - delta-like homolog 1

DMR - differentially methylated region

DRG - dorsal root ganglion

DSB - double-strand breaks

GABA - γ -aminobutyric acid

gDMR - germline differentially methylated region

gRNA - guide RNA

hCG - human chorionic gonadotropin

HDR - homology directed repair

IASP - International Association for the Study of Pain

ICR - imprinting control region

lncRNA - long non-coding RNA

Meg3 or Gtl2- maternally expressed 3

mRNA - messenger RNA

ncRNA - non-coding RNA

NHEJ - non-homologous end joining

NMDA - N-methyl-D-aspartate

NMDAR - N-methyl-D-aspartate receptors

PAM - protospacer adjacent motif
PCR - polymerase chain reaction
PKA - protein kinase A
PMSG - pregnant mare's serum gonadotropin
PSD - post synaptic density
PWF - paw withdrawal frequency
PWT - paw withdrawal threshold
Rtl1 - retrotransposon-like 1
Rtl1as - retrotransposon-like 1 anti-sense
SAP - synapse-associated protein
SNI - spared nerve injury
ssDNA - single-strand DNA
tracrRNA - trans-activating RNA
TRPV1 - transient receptor potential vanilloid 1
Y1472F - tyrosine residue 1472 to phenylalanine

Introduction

Introduction

- Part I -

1. Pain

According to the International Association for the Study of Pain (IASP), pain is defined as *“an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”* (Merskey & Bogduk, 1994). By definition, pain could be assumed as a biological mechanism to maintain the body integrity acting as a warning signal to potential threat. The sensory component of pain describes the noxious stimuli in terms of time duration, location, intensity and modality while the emotional component is responsible for the behavioural response and, at the same time, the pain subjectivity (Walk & Poliak-Tunis, 2016). Pain might be classified as acute or chronic according to its time duration. In an acute condition, nociceptive primary sensory neurons that innervate the skin, deep tissues and internal organs are turned on by a wide variety of noxious mechanical, thermal and chemical stimuli, and a complex biological process is initiated through activation of modality-specific sensory transduction molecules located in specialized primary afferent neurons, called nociceptors, that are capable of transducing noxious stimuli into action potentials (Peirs & Seal, 2016). In this condition, the process is intense, phasic and characterized by a short-time period. When pain persists for a period after the primary noxious stimulus is over and/or the wound has healed (when tissue injury occurs) there is a chronification of pain which is characterized by a long-lasting, tonic and persistent pathologic event with a spontaneous nature and a lack of biological evident reason (Tracey & Bushnell, 2009).

1.1 Chronic pain

Chronic pain is a frequent condition seen in clinical practice: it is estimated that 1 in 10 adults worldwide are diagnosed with this pathology making pain a serious medical problem (Golberg & McGee, 2011). In fact, in Portugal, it is estimated that about 37% of the population suffers from chronic pain (Azevedo et al., 2012) which is in line with the high prevalence found in other developed countries (Merskey & Bogduk, 1994). This pathology is not only a major burden for patients, but also for the family due to the

incapability to perform normal daily tasks (Reid et al., 2011, Gorczyca et al., 2013) as it is normally accompanied by sleep disturbances, anxiety and depression (Attal et al., 2011; Finnerup et al., 2016). The absence of effective treatment for chronic pain is also a problem to governments due to the financial burden caused by direct and indirect costs, namely with health-related services and lower productivity of these patients and their families, respectively (Reid et al., 2011; Breivik et al., 2013).

Two types of chronic pain can be distinguished according to the pain origin: nociceptive and neuropathic pain. Nociceptive pain results from an activation of the nociceptors, in a normal functioning of the somatosensory nervous system, in response to an actual or a perceived damage in non-neuronal tissue. Neuropathic pain is assumed as caused by a disorder or lesion in the somatosensory nervous system, including central neurons and peripheral fibres (Merskey & Bogduk, 1994; Baron et al., 2010).

1.2 Neuropathic pain

It is estimated that neuropathic pain affects 7% to 10% of the general population (van Hecke et al., 2014; Bouhassira et al., 2008) being more frequent in aged people (8.9% vs 5.6% in under 49 years of age) and women (8% vs 5.7% in men) (Bouhassira et al., 2008). Diagnosing neuropathic pain and distinguishing it from nociceptive pain can be challenging due to the lack of efficient tests to discriminate between the two types. Therefore, an accurate diagnosis based in a detailed medical history, detailed examination and an appropriate investigation of what kind of pain the patient suffers from, as well as its etiology, should be performed (Gierthmühlen & Baron, 2016). Contrarily to nociceptive pain, neuropathic pain is characterized by positive and/or negative symptoms arising from gain or loss of somatosensory function, respectively. Evoked pain, burning pain and an abnormal temporal summation are some of those symptoms. In addition, allodynia, a condition of pain triggered by stimuli that usually do not provoke pain (Merskey & Bogduk, 1994), and hyperalgesia, an abnormally increased sensitivity to pain, are also commonly observed.

Neuropathic pain can arise from disturbances at the level of the central or peripheral nervous systems. At the central level, cerebrovascular disease and neurodegenerative disorders, such as Parkinson, are brain conditions that could be in the

origin of central neuropathic pain. Spinal cord injury, syringomyelia and other demyelinating diseases, such as multiple sclerosis, are conditions that affect the spinal cord and are associated with the same type of pain. Peripheral neuropathic pain on the other hand, is caused by peripheral lesions or diseases affecting the unmyelinated C fibres and the myelinated A fibres, namely, the A β and A δ fibres, of the peripheral nervous system (Fig. 1). A tendency to an increase of peripheral neuropathic pathologies is expected due to the aging of the world population, to the increasing incidence of diabetes mellitus, and to other putative as well as proven underlying conditions, such as cancer surviving following chemotherapy, among other causes.

1.3 Peripheral neuropathic pain models

From a clinical point of view, the management of neuropathic pain is generally focused on the reduction and attenuation of symptoms as the underlying causes are seldom known and/or can rarely be treated. Consequently, the lack of effective treatments for this pathology underscores the importance of this field of study, not only for the understanding of pain-related mechanisms but also for the development of new and effective targeted therapies (Wolff & Decosterd, 1999; Dworkin et al., 2003). In order to mimic the pathological conditions observed in humans, such as hyperalgesia and allodynia, rat and mouse experimental models of neuropathic pain have been developed. Among these, the models of toxic and inflammatory neurites (Courteix et al., 1993; Eliav et al., 1999; Polomano et al., 2001), spinal cord injuries (Hao et al., 1992), and partial or complete nerve transections (Bennett & Xie, 1988; Seltzer et al., 1990; Kim & Chung, 1992; Decosterd & Woolf, 2000), stand out. These studies highlight the contribution of non-injured neurons to neuropathic pain development and maintenance, where increased expression of BDNF, TRPV1, and activation of p38 intracellular signalling pathway are observed, along with activation of Remak Schwann cells, differential expression of sensitive-specific voltage-gated channels (sodium channels) and ectopic activity in C-fibres (Fukuoka et al., 2001; Hudson et al., 2001; Wu et al., 2001; Decosterd et al., 2002; Gold et al., 2003; Obata et al., 2004; Murinson et al., 2005).

Of particular interest to this work is the spared nerve injury (SNI) model. This well-established procedure consists on the lesion (sectioning) of two branches of the sciatic

nerve (commonly the peroneal and tibial nerves) while leaving the other branch intact, usually the sural nerve. The SNI induces a long-lasting mechanical allodynia-like behaviour with high reproducibility. This mechanical allodynia can be measured by assessing the sural non-injured skin area in the foot pad of animal's hind limb (Decosterd & Woolf, 2000; El Khoury et al., 2002; Broom et al., 2004; Decosterd et al., 2004; Zhao et al., 2004; Howard et al., 2005). The SNI model was first developed in rats and later adapted to mice to be used in combination with genetic modifying technologies available in this model organism. By employing robust mechanical allodynia-like behavioural tests, such as the stimulus-evoked Von Frey test, it is possible to correlate key biochemical factors with measurable functional changes (Woolf et al., 1998).

1.4 Peripheral neuropathic pain mechanisms

Pain transmission is initiated in the peripheral nervous system by an activation of nociceptors, which have the capability of encoding and transducing the noxious stimuli. The transduction process is characterized by a membrane depolarization of the primary afferent neuron, whose cell body is located in specialized sensory ganglia, the trigeminal ganglia (for stimuli from the face and head) or the dorsal root ganglia (DRG; for stimuli arising from the remaining body), and the stimuli encoded information is transmitted (via the primary afferent neuron central axonal branch) to the spinal cord. The action potential generated in primary afferent neurons induces neurotransmitter release in axon terminals at the dorsal horn of the spinal cord. These neurons not only codify the stimuli type but also the intensity and location. The intensity is given by the number and frequency of action potentials generated while the location depends on the distribution of the nociceptor terminals in the spinal dorsal horn. Subsequently, the nociceptive signals reach the brain via two major ascending pathways: the lateral- and medial-spinothalamic tracts, which convey the nociceptive information from the spinal dorsal horn to the thalamus and from these to the first and second somatosensory cortices. The first somatosensory cortex will discriminate the location and intensity, while aspects such as the type of stimuli and cognitive pain perception are processed by the second somatosensory cortex. Pain can be controlled and/or modulated by descending pathways

that will inhibit and/or excite neurons at the spinal cord that, ultimately, control the signals reaching the supraspinal centres of pain processing.

In peripheral neuropathic pain the electrical properties of sensory nerves are impaired, leading to imbalances between excitatory and inhibitory signalling, such as the inhibition of interneurons and descending inhibitory control systems. The facilitation or inhibition mechanisms may be altered at the level of the spinal cord dorsal horn neurons with increased transmission of sensory signals. In fact, these alterations lead to an excitation or facilitation gain that may also be associated with loss of inhibitory mechanisms producing a state of hyperexcitability and, consequently, altering the central integration of these signals, which might contribute to pain chronification. Neuropathy impacts on ion channels functioning as well: increased expression and improved function of sodium channels at the nociceptors peripheral terminals lead to an enhanced excitability with an increased neurotransmitter release, augmenting the synaptic transmission in this circuit. Furthermore, enhanced excitability of the spinal dorsal neurons gives rise to an increased sensitivity to many sensory modalities, such as mechanosensitivity, enabling low-threshold afferent fibres, namely the A β and A δ fibres, to activate second order nociceptive neurons that have expansive receptive fields, generating central sensitization and thus mechanosensitivity (Woolf, 2011; Baron, 2013). Central sensitization events also include post-synaptic changes such as the phosphorylation of N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, explaining the physical allodynia reflected by enhanced sensory thalamic neuronal activity (Patel & Dickenson, 2016; Peyron, 2016). Hyperexcitability can also be caused by loss of γ -aminobutyric acid (GABA) release by interneurons, which can switch the normal inhibition function to an excitation state at the spinal cord (Gagnon et al., 2013) together with the functional changes occurring in non-neuronal cells such as microglia and astrocytes (Tsuda, 2013).

In early 1949, the dorsal root ganglion (DRG) was recognized as playing a role in chronic pain and was established as a target for anaesthetic infiltration to reduce pain (Soresi et al., 1949). Today, as explained above, it is known that the DRG is a critical structure in sensory transduction and modulation, and therefore in pain transmission (Hogan, 2010). As such, the DRG is implicated in the development and maintenance of

neuropathic pain (Krames, 2014). The DRG is a bilateral structure containing the cell bodies of primary sensory neurons whose extended axons make the connection between the receptor endings at the periphery and the central nervous system (CNS). The axons of these neurons are packed into nerve fibres that contain a mix of types with variable excitability modalities and conduction velocities, including low-threshold mechanical fibres (named A β), high-threshold C nociceptor fibres and A δ nociceptor fibres (Fig. 1). The activation signals of the fibres' peripheral receptors will be carried through the axon's peripheral branch to their respective soma in the DRG and from there to the spinal cord dorsal horn in the CNS through the axon's central branch. A β , A δ and C fibres can be classified regarding their degree of myelination, diameter and the type of "information" they convey. The A δ are medium diameter, lightly myelinated fibres, carrying thermal, mechanical and chemical nociceptive information with a relatively high velocity. The C fibres are small diameter unmyelinated fibres with slower conduction velocity, which also carry nociceptive information, although contributing to a more diffuse and deeper secondary pain. The A β are the largest, myelinated, and faster carrying proprioception and light touch information fibres.

DRG are also composed by a large population of glial cells, the satellite glial cells, which involve each primary sensory neuron and separate neurons' cells bodies from each other. After a nerve injury, DRG undergo several changes including the infiltration of immune cells and the action on glial cells which, together, release inflammatory mediators and excitatory cytokines that contribute to pain development and maintenance (Hu & McLachlan, 2003; Znaor et al., 2007). Moreover, increased expression of neurotrophic factors in satellite glial cells within DRG trigger a persistent mechanical allodynia (Sapunar et al., 2012; Krames, 2014). There are also multiple changes in the expression of genes that encode receptors, ion channels, synaptic vesicle proteins, neuropeptides, and signal transduction molecules, among others (Xiao et al., 2002), resulting in the sensitization and hyperexcitability of DRG neurons, or peripheral sensitization. Thus, DRG function may be characterized by the impediment, filtration or enhancement of signals from the periphery towards the CNS.

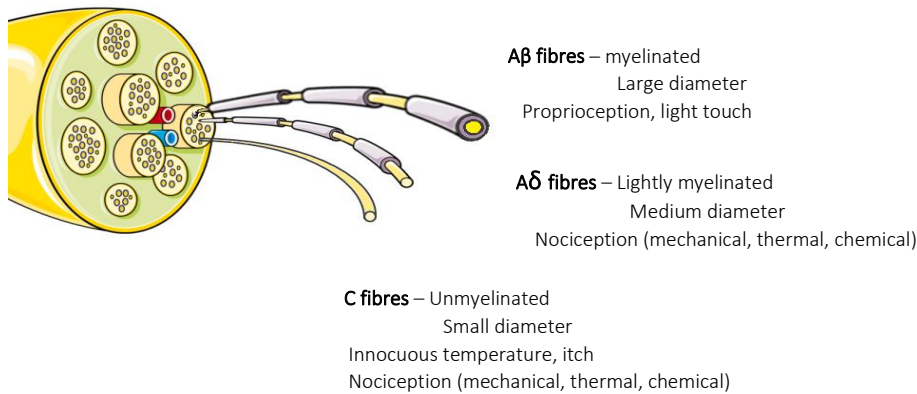


Figure 1. Types and characteristics of sensitive fibres from peripheral nerves.

(Adapted from Julius & Basbaum, 2001)

1.5 NMDA receptors and scaffold proteins in neuropathic pain and pain transmission

N-methyl-D-aspartate (NMDA) receptors (NMDAR) are glutamate receptors and ion channel proteins with an essential role in the synaptic transmission between nerve cells. These receptors are important players in the transmission of nociceptive inputs in the spinal cord dorsal horn and are, therefore, also implicated in pain transmission. They are heterotetramer protein complexes made up of variants of three major subunits: GluN1, GluN2 and GluN3. (Kutsuwada et al., 1992; Monyer et al., 1992; Sun et al., 1998; Nishi et al., 2001). The subunit composition of the NMDA receptor determines their physiological and pharmacological properties such as the time for activation/deactivation (Monyer et al., 1992; Vicini et al., 1998).

NMDAR activity is intrinsically correlated with phosphorylation of its subunits promoted by kinase proteins, such as protein kinase A (PKA), casein kinase 2 (CK2) or Ca^{2+} calmodulin-dependent protein kinase II (CAMKII). For example, GluN2B phosphorylation at Tyr1472 is highly associated with neuropathic pain maintenance in the spinal cord after nerve injury. Indeed, in knock-in mice with a substitution of the GluN2B tyrosine residue

1472 to phenylalanine (Y1472F-KI), which inhibits GluN2B phosphorylation, neuropathic pain is attenuated (Abe et al., 2005; Matsumura et al., 2010; Katano et al., 2011). Moreover, there is a strong suggestion that increased NMDAR function is potentiated by interaction with other components of the postsynaptic density (PSD), namely the PSD-95/SAP90 (also known as synapse-associated protein 90). The interaction between PSD-95 and GluN2B is accelerated after nerve injury (Peng et al., 2013) and PSD95/GluN2B complex disruption, on the other hand, contributes to neuropathic and inflammatory pain attenuation (Tao et al., 2001, 2003; D'Mello et al., 2011). Indeed, PSD proteins (including NMDARs) and scaffold proteins participate in physiological pain as well as abnormal pain transmission through the activation of intracellular signalling cascades in the spinal cord dorsal horn and brain (Craven & Brecht, 1998; Garner et al., 2000; Luo et al., 2014). *Shank* is one such component that interacts directly with PSD-95 to stabilise the PSD complex, and is thought to have a regulatory relationship with the NMDAR (Katano et al., 2016).

2. Epigenetics

Epigenetics was coined for the first time in the 1940's as "the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being." (Waddington, 1942). The rapid development of the genetics field prompted the narrowing of the first definition to "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence" (Wu & Morris, 2001). Indeed, epigenetic modifications modulate gene expression without altering the actual DNA sequence. DNA methylation, histone modifications and the expression of non-coding RNA (ncRNA) are examples of epigenetic processes that can be acutely elicited as consequence of stress, tissue damage or diseases affecting physiological and pathological processes (Smith & Meissner, 2013; Bai et al., 2015; Descalzi et al., 2015). DNA methylation and histone tail post-translation modifications impact on DNA access by changing the chromatin state between euchromatin and heterochromatin, thus allowing or preventing the physical binding of transcriptional factor proteins, and regulating gene transcription (Jenuwein & Allis, 2001;

Ruthenburg et al., 2007; Birney et al., 2007). On the other hand, ncRNAs act as translation inhibitors and/or promote mRNA degradation (Grishok, 2001; Bartel, 2004; He & Hannon 2004).

DNA methylation is one of the most common epigenetic processes that have considerable influence on gene expression. During gestational development different tissues acquire different patterns of methylation that confer cell-type differentiation by regulating gene function (Razin & Szyf, 1984). Studies conducted with monozygotic twins revealed that even sharing the same genetic material, the methylation profiles were slightly different (Ziller, 2013). Although initially it was thought that the DNA could not lose the attached methyl groups by an active process of demethylation, it was latter established that DNA methylation is a reversible process, and that methylation profiles vary under different conditions and through time (Guo et al., 2011).

There is now strong evidence about the influence of epigenetic mechanisms controlling the expression of certain genes following lesions or diseases in the somatosensory system. Those mechanisms are inherited and capable of maintaining long-lasting effects in gene expression (Penas & Navarro, 2018). For example, it was demonstrated that the DNA methylation patterns following spinal cord injury in rats were altered (Wang et al., 2011). Furthermore, Qi and collaborators showed that intraplantar CFA-induced peripheral insults also triggered DNA demethylation resulting in an increase in cystathionine-beta-synthetase, a hydrogen sulphide enzyme in DRG neurons (Qi et al., 2012). Thus, it is conceivable that chronic pain pathologies could be modulated (enhanced or attenuated) by epigenetic mechanisms which could represent potential new therapeutic targets.

2.1 Genomic imprinting

Mammalians are diploid organisms containing two matched sets of chromosomes, one inherited from the father and the other inherited from the mother. Accordantly, each gene is composed by two alleles (one on each parents' corresponding chromosome) potentially having the same pattern of expression within a given cell, also termed as biallelic expression. Genomic imprinting is an epigenetic process in which a gene is preferentially (or exclusively) expressed from only one of the inherited

chromosomes, in a parental-specific manner, thus exhibiting monoallelic expression (Barlow & Bartolomei, 2014). This mechanism affects both male and female offspring, and therefore is a consequence of inherited parental chromosomes and not sex. For example, if a gene is paternally imprinted the allele on the maternally-inherited chromosome is active, while the paternally-inherited allele will be silenced in all male and female offspring (Fig. 2).

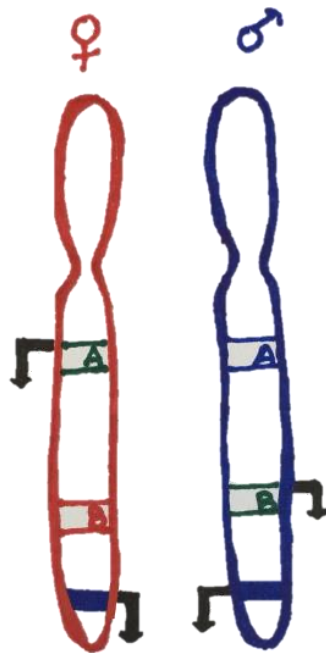


Figure 2. Schematic representation of imprinted and biallelic gene expression. A: gene expressed from the maternally-inherited chromosome only (repressed on the paternally-inherited chromosome); B: gene expressed from the paternally-inherited chromosome only (repressed on the maternally-inherited chromosome). A biallelically expressed gene is represented at the bottom.

In humans, over 50 imprinted genes have been identified to date (Baran et al., 2015; Court et al., 2014; Smith et al., 2014). Many imprinted genes have an important role in human development, neonatal growth and neuronal plasticity, and disruption of their expression could lead to an augmented risk of cancer, transient neonatal diabetes mellitus, Prader-Willi syndrome, among others (Peters, 2014). The majority of imprinted genes are found in clusters, called imprinted domains, allowing coordinated regulation

by differentially methylated regions (DMRs), which are regions with different patterns of methylation between maternally- and paternally-inherited chromosomes, and by long non-coding RNAs (lncRNAs). Each cluster of imprinted genes is controlled by an independent imprinting centre, or imprinting control region (ICR). Normally, these regions are differentially methylated in the germline, and thus are known as germline differentially methylated regions (gDMRs). In the human genome, 35 gDMRs associated with imprinted domains were identified (Monk et al., 2018). The chromatin configuration of the gDMRs differs between the parental chromosomes. Open chromatin histone marks, such as histone 3 lysine 4 dimethylation (H3K4me2) and trimethylation (H3K4me3) are found on the unmethylated allele; whereas closed chromatin marks (H3K9me2, H3K9me3 and H4K20me3) are found on the methylated allele (Court et al., 2014; Sanli & Feil, 2015; Singh et al., 2011). Different transcription factors recognize different DMR methylation patterns, bringing about differential epigenetic modifications and imprinted gene expression (Riso et al., 2016).

Imprinted genes frequently display monoallelic expression in most or all cell types. However, monoallelic expression of some genes is restricted to a specific tissue or developmental window and, in some cases, this monoallelic expression and/or methylation can differ between individuals (Rougeulle, Glatt & Lalande, 1997; Vu & Hoffman, 1997; Travers et al., 2013; Hanna et al., 2016; Zink et al., 2018; Sanchez-Delgado et al., 2016).

2.2 Mouse Chr. 12 (Dlk1-Dio3) imprinted domain

The Dlk1-Dio3 imprinted domain, located in chromosome 12 in the mouse (chromosome 14 in human), is an imprinted gene cluster with four paternally expressed protein-coding genes [brain-enriched guanylate kinase associated protein (BEGAIN) isoform 1B, delta-like homolog 1 (*Dlk1*), retrotransposon-like 1 (*Rtl1*), and type III iodothyronine deiodinase (*Dio3*)] and four maternally expressed non-coding RNAs, including microRNAs [maternally expressed 3 (*Gtl2* or *Meg3*), retrotransposon-like 1 anti-sense (*Rtl1as*), RNA imprinted and accumulated in nucleus (*Rian*), and miRNA containing gene (*Mirg*)] (Fig. 3) (Takada et al., 2000; Miyoshi et al., 2000; Schmidt et al., 2000; Hernandez et al., 2002; Seitz et al., 2003; Hatada, 2001).

The imprinted genes from this region are essential in the late stages of embryonic development as shown by studies with maternal and paternal uniparental Chr. 12 disomy (mUPD12 and pUPD12, respectively) and in knockout (KO) mice (Georgiades, 2000; Moon et al., 2002; Hernandez et al., 2006; Takahashi et al., 2009; Sekita et al., 2008). Imprinted genes at the *Dlk1–Dio3* domain are cis-regulated by a germline-derived intergenic DMR (IG-DMR) located between *Dlk1* and *Gtl2*, which is the ICR of this domain with sperm-derived methylation (Takada et al., 2002). Removal of the IG-DMR in mice by targeted deletion on the maternal chromosome results in bidirectional loss of imprinting of all genes in the cluster, whereas if the deletion is borne by the paternal chromosome imprinting is unaltered, indicating that the allele-specific gene expression within the domain is controlled differently on the two parental chromosomes (Lin et al., 2003).

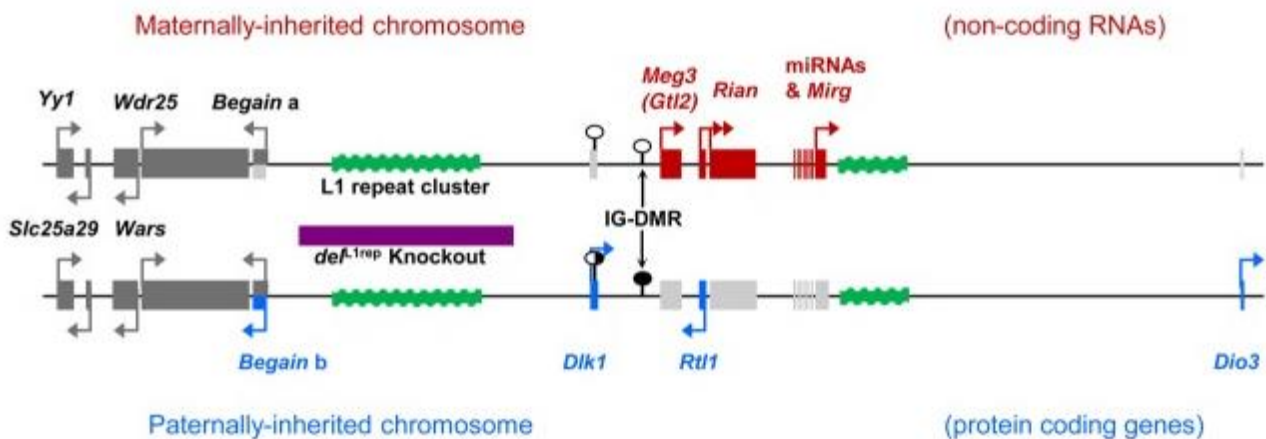


Figure 3. Schematic representation of the *Dlk1–Dio3* imprinted domain. Maternally expressed noncoding transcripts are shown in red, and paternally expressed protein coding genes are shown in blue. On the maternally inherited chromosome, noncoding RNAs *Meg3* (*Gtl2*), *Rtl1* anti-sense (AS), *Rian*, and microRNA-containing *Mirg* (red) are transcribed, whereas the protein-coding genes (light gray) are repressed. The paternally inherited chromosome expresses protein-coding *Begain* variant 1b, *Dlk1*, *Rtl1*, and *Dio3* (blue), while the transcription of the noncoding RNAs is silenced (light gray). The imprinting control region (IG-DMR) and the *Dlk1* DMR are unmethylated on the maternally inherited chromosome (white circles), and hypermethylated/partially methylated, respectively, on the paternally inherited chromosome (black and shady gray circles). Biallelic genes are shown in dark gray, and arrows denote the direction of transcription.

(From Soares et al., 2018)

2.3 Del^{L1rep} mouse mutant model

In the context of a study evaluating the influence of a 170-kb cluster of LINE-1 repeats in regional control within the imprinted *Dlk1-Dio3* domain, Soares et al. (2018) targeted 260 kb of a highly repetitive genomic sequence located between *Begain* and *Dlk1* encompassing the LINE-1 array. Tandem repeats are an exceptional feature of genome imprinting centres (Hutter et al., 2006) functioning as high concentrated binding sites for transcription factors that regulate imprinted genes. Disruption or alterations of the tandem repeats can result in genomic imprinting defects (De Crescenzo et al., 2011). LINE-1 recombination is one of the major sources of population variation and also plays a role in gene expression modulation by alternative splicing, disruption of transcription or by generating alternative promoters (Daria et al., 2007).

The mutant line, generated by homologous recombination in embryonic stem cells (termed Rep^{L1del}), was established on a C57BL/6J background and showed no obvious phenotype in terms of viability, reproductive fitness and postnatal growth. Molecular analysis did not reveal any influence of the deletion on the imprinting status of the imprinted genes within the domain, nor a spread of imprinting to the upstream biallelically-expressed genes. Indeed, analysis of allele-specific expression of *Begain 1B*, *Dlk1*, *Gtl2*, *Rtl1as* and *Dio3* revealed that all five transcripts retained their imprinted status and thus the original parent-of-origin specific expression, whereas five genes outside the imprinted domain upstream the deletion (*Yy1*, *Slc25a29*, *Wars* and *Wdr25*), as well as isoform 1A of *Begain*, retained their biallelic status. Thus, the LINE-1-rich repetitive DNA array is not involved in or required for imprinting establishment or maintenance within the domain.

Gene expression of twelve genes flanking the deletion were analysed by RT-qPCR in foetal brain, placenta and liver, which are tissues with abundant embryonic expression of most of these genes. Maternal transmission of Rep^{L1del} did not show differences in gene expression in all tissues analysed. Similarly, no differences were observed in liver and placenta following paternal transmission. However, in foetal brain, Rep^{L1del} mutants showed a significant downregulation of the protein-coding paternally expressed transcripts upon paternal transmission of the deletion. *Begain 1B* was most affected with a decrease of 63%, followed by *Dlk1* and *Dio3*, both downregulated by ~30%. These

effects were observed both in heterozygotes following paternal transmission (Pats) and in homozygotes. Gene expression was also studied in adult mice, but in brain only. In adult brain, only the expression of *Dlk1* was downregulated: ~33% at the mRNA level, as assessed by RT-qPCR and ~50% at the protein level (total Dlk1) as determined by ELISA. *Begain1B* and *Dio3* levels were unaltered in mutants (Pats and homozygotes) when compared to wildtype littermates.

Soares and collaborators hypothesised that the effect of the deletion on the expression of the paternal chromosome-specific transcripts could be due to the removal of a putative local (for *Begain* and *Dlk1*) and long-range (for *Dio3*) enhancer contained within the targeted region.

2.4 Begain

Begain (brain-enriched guanylate kinase-associated protein) is a PSD-95/SAP90 binding protein highly expressed in the brain and thought to sustain the structure of the postsynaptic density PSD (Deguchi et al., 1998; Naisbitt et al., 1997). The *Begain* gene encodes two isoforms termed *Begain 1A* and *Begain 1B* (Soares et al., 2018), which result from the usage of alternative first exons, thus having different N-termini. Specifically, the N-terminus amino acid composition of the isoforms is MWTGGRRPGRLRRA and MGS HQSSQ for *Begain 1A* and *Begain 1B* respectively; whereas the remainder 601 amino acids that make up the proteins are fully shared among the two (Fig. 4). The *Begain 1A* N-terminus contains two nuclear localization signals, while that of the *Begain 1B* isoform contains a single one. These are recognized in both neuronal and non-neuronal cells. In non-neuronal cells, namely epithelial cells, Begain is restricted to the nucleus, whereas in neurons the protein is also localized at synapses (Yao et al., 2002). Indeed, Yao and collaborators suggest that Begain recruitment to synapses may occur in two steps: first, there may be neuron-specific components that bind the N-terminal of Begain and either recruit it from the nucleus to the dendrites or sequester it at the dendrites; second, Begain is recruited from dendrites to synapses by PSD-95/SAP90 binding in a NMDAR activity-dependent manner. In fact, Begain has a leucine zipper and it has been shown to form dimers or tetramers by the N-terminal region, suggesting it may be involved in NMDAR/PSD-95 clustering (Yao et al., 2002). The lack of specific antibodies

recognizing each Begain isoform precludes the assessment of where each protein is recruited to, as well as their relative amounts at each location. Be that as it may, the dual localization of Begain suggests that it may transfer signals directly between nuclei and synapses.

Strikingly, whereas the 1A isoform is biallelically expressed, the *Begain 1B* isoform is imprinted (paternally expressed), as previously demonstrated in sheep (Smit et al., 2005) and in the mouse (Tierling et al., 2009; Soares et al., 2018).



Figure 4. Schematic diagram of Begain transcript variants in the mouse. The genomic distance between the 5' end of exon 1A and the 3' end of exon 7 comprises 36 070 bp (GenBank accession no. NC_000078.6). The genomic distance between the alternative first exons and exon 2 are indicated. Untranslated regions (UTR) are shown in white.

(From Tierling et al., 2009)

Recently, Begain - which is also expressed in spinal cord, specifically in the lamina II of the dorsal horn - has been implicated in the maintenance of neuropathic pain following peripheral nerve injury (Katano et al., 2016). In wild type mice, Begain was upregulated in the spinal dorsal horn upon spared nerve injury (SNI). Furthermore, in Begain knockout mice, neuropathic pain was significantly attenuated after SNI suggesting that Begain was involved in pathological pain transmission through activation of NMDA receptors in the spinal dorsal horn (Katano et al., 2016). However, these functional studies were conducted following the simultaneous knockout of *Begain 1A* and *1B* since the gene was targeted through the removal of exons 4 and 5, which are common to both isoforms. Thus, the specific involvement of each isoform in pathological pain remains elusive. Importantly, the role and/or relative contribution of imprinted *Begain 1B* in neuropathic pain is yet to be demonstrated, raising the question of whether imprinting (a crucial epigenetic regulatory process) is in any way associated with abnormal neural plasticity in the context of pathological pain.

2.5 Dlk1

The Delta like non-canonical notch ligand 1, also known as Delta-like homolog (*Dlk1*), encodes a transmembrane glycoprotein with an epidermal growth factor (EGF)-like motif in its extramembrane domain (Smas et al., 1993; Takada et al., 2000). In the mouse, *Dlk1* is alternatively spliced and processed to six splice isoforms, resulting in two different transmembrane proteins in which the unique difference is the presence or absence of a protease recognition site. Two isoforms with protease recognition site (A and B isoforms) and four isoforms lacking recognition site (C, C2, D and D2 isoforms) have been identified (Fig. 5) (Smas et al., 1997; Mei et al., 2002). Lack of protease recognition site results in a membrane-tethered protein whereas the presence of this cleavage site originates a large soluble isoform released into circulation (Jensen et al., 1994; Wang & Sul, 2006).

Structurally, the Dlk1 protein is similar to that of the canonical delta-like ligands of the Notch signalling molecules, although the DSL (Delta/Serrate/LAG-2) domain is absent. Dlk1 contains the conserved DOS (Delta and OMS-11) domain, thought to be involved in NOTCH/DSL ligand activation (Laborda, 2000; Luca et al., 2015). Therefore, it is commonly accepted that Dlk1 acts as an inhibitory modulator of NOTCH signalling by cis-inhibition, competing with canonical ligands. Thus, Dlk1 is considered a member of a diverse group of molecules that play a modulatory role on NOTCH signalling by exerting modulatory changes, even without being canonical ligands.

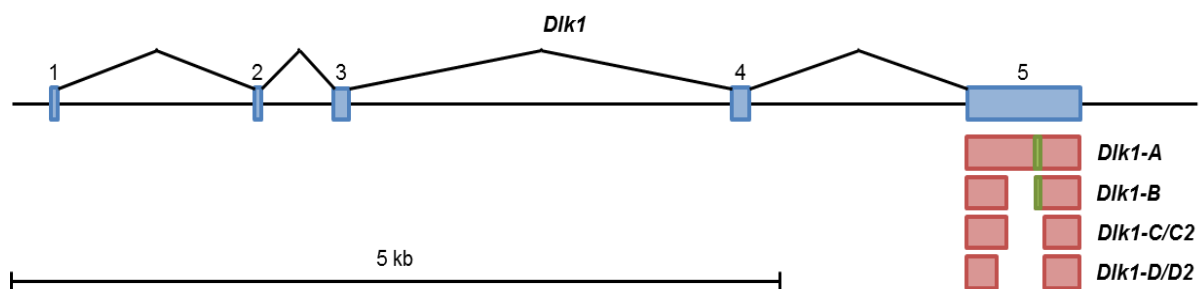


Figure 5. Schematic representation of Dlk1 isoforms. The *Dlk1* gene has 5 exons represented by blue squares; black lines represent intronic regions. Six Dlk1 isoforms resulting of alternative splicing are represented in red.

(Kindly provided by Dr. Mitsu Ito)

The main function of Dlk1 is thought to be as a pre-adipocyte factor (Wang & Sul, 2006) with impact in adipogenesis and metabolism (Smas & Sul, 1993; Garces et al., 1999; Moon et al., 2002; Lee et al., 2003; Charalambous et al., 2014; Abdallah et al., 2015). Indeed, Dlk1 plays an important role in cell differentiation by keeping cell progenitors undifferentiated until the appropriate time has come for differentiation. Thus, Dlk1 is involved in multiple differentiation processes in mice, including hematopoiesis (Moore et al., 1997), pancreatic islet cell differentiation (Carlsson et al., 1997), Schwann cell differentiation (Costaglioli et al., 2001), hepatic cell differentiation (Tanimizu et al., 2004), and differentiation of muscle satellite cells. Additionally, Dlk1 plays a role following injury/disease affecting progenitor cells by inducing tissue regeneration (J. Laborda, 2000; Li & Clevers, 2010). *Dlk1* is widely expressed in tissues derived from the three germ layers throughout development but its expression is downregulated either just before or right after birth, and then restricted to a few tissues (namely brain, pituitary, pancreas, adrenals, testes and lung endothelium) or stem cells (Fig. 6) (Smas & Sul, 1993; Bachmann et al., 1996; Rodriguez et al., 2012; Cheung et al., 2013; Christophersen et al., 2007).

Dlk1 mutant mouse models show poor placental and foetal growth, and accelerated adiposity (Moon et al., 2002), and compromised neurogenesis in adult brain (Ferron SR, 2011). Overexpression of Dlk1 causes lethality associated with major organ abnormalities (da Rocha et al., 2009), whereas overexpression in adipose tissue exhibits decreased fat mass (Villena et al., 2008).

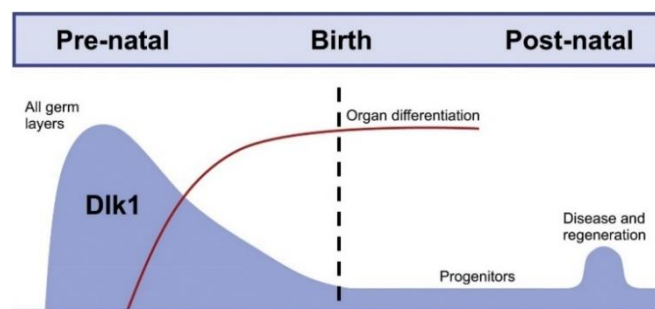


Figure 6. Graphical representation of Dlk1 expression during development. In embryonic development, Dlk1 expression is very high due to tissue differentiation, which is maintained until birth. After birth, Dlk1 levels decrease to a basal state and become restricted to some tissues or progenitor cells. After a disease or lesion, Dlk1 levels increase until the lesion is healed.

(From Traustadóttir, et al. 2019)

- Part II -

1. Genome editing

Genetic engineering is widely used in laboratory research due to the numerous and powerful applications in multiple scientific domains. The first attempts to achieve genetically modified organisms started in the late 60's, and consisted mainly on the random (non-targeted) delivery of foreign DNA into cells or organisms. The first precise gene-editing techniques targeting specific loci through the use of genomic recombination were developed in the 1980's (Szostak, 1983). Homologous recombination allows the recognition of a genomic sequence at a specific genome location and subsequent integration of a DNA molecule with sequence homology to the target site, thus providing low risk of random integration. In spite of being widely used and very popular among the scientific community until the early 2000's, as the best available gene/locus targeting tool, this method has major limitations, namely low efficiency.

The discovery that targeted DNA double-strand breaks (DSBs) could be used to stimulate the endogenous cellular repair machinery (Rouet, Smih & Jasin, 1994), and that the efficiency of gene targeting through homologous recombination in mammalian cells could be increased by several orders of magnitude by introducing a DSB at the target site (Smih et al., 1995), prompted the introduction of programmable nucleases for high-efficiency genome editing through precise targeting. Zinc-finger nucleases were first used with this purpose (Klug, 2010), along with the homing nucleases (Jasin, 1996), followed by the transcription activator-like effector nucleases (TALENs) (Joung & Sander, 2013). In the last decade, seminal studies in bacterial biology have uncovered a naturally occurring genome editing system in archeabacteria (most notably *Streptococcus pyogenes*), termed CRISPR-Cas9, which was quickly adapted as a revolutionary tool for inexpensive, fast, high-efficiency, high-specificity genome editing (Barrangou et al., 2007; Jinek et al, 2012; Gasiunas et al., 2012; Wang et al., 2013).

1.1 CRISPR-Cas 9

In bacteria, the clustered regularly interspaced short palindromic repeats (CRISPR) locus is composed by short repeat sequences, usually ranging from 28-37 base pairs (bp)

(Barrangou & Marraffini, 2014), separated by spacers bearing a unique sequence of similar length. The repeats are arranged in a palindromic fashion, which is characterized by an identical sequence when read from 5' to 3' on one strand and 5' to 3' on the other, complementary, strand. Upstream the CRISPR loci there is a leader sequence (adenine- and thymine-rich) of 500 bp in length carrying promoter elements and signals that are crucial to the transcription of the CRISPR RNA (crRNA) and for the successful integration of exogenous DNA into CRISPR sequences (Pul et al., 2010; Yosef et al., 2012). Adjacent to the CRISPR loci and the leader sequence are the CRISPR-associated (Cas) genes, which encode the Cas endonuclease proteins. Cas proteins “coupled” with crRNA constitute the CRISPR-Cas effector complex, which mediates the cleavage and “destruction” of foreign “invading” bacteriophage nucleic acids. One of such Cas endonucleases, Cas9, is the signature nuclease for the type II CRISPR-Cas system, which relies on host RNases, Cas9 proteins and small trans-activating RNA molecules (tracrRNA). TracrRNAs are complementary to the repeated sequence of CRISPRs and contain 3 hairpin structures (Chylinski et al., 2013; Nishimasu et al., 2014) that bind the crRNA molecules and Cas9, and act as a stabilizer of the CRISPR-Cas9 complex (Deltcheva et al., 2011). In June of 2012, Jinek et al. proposed that the tracrRNA and crRNA molecules could be fused in a single chimeric RNA molecule later designed as guide RNA (gRNA) (Jinek et al., 2012), ready to be assembled in a complex with the Cas9 endonuclease (Fig. 7).

The CRISPR-Cas 9 system (originally described in and “adapted” from *Streptococcus pyogenes*) is the most widely used of all CRISPR-Cas systems. It requires that the target DNA contains a short 3-nucleotide sequence that follows the DNA region targeted for cleavage by the CRISPR-Cas9 complex, called the protospacer adjacent motif (PAM). The PAM is required for Cas nuclease to cut and is generally found 3-4 nucleotides downstream from the cut site. The canonical PAM recognized by Cas9 is 5'-NGG-3'. In bacteria, this ensures that Cas9 does not target its own DNA, since the spacer in the bacterial CRISPR loci will not contain a PAM sequence, whereas the protospacer in the invading virus or plasmid will have the sequence and will, therefore, be cleaved by Cas9 (Jinek et al., 2012). The CRISPR-Cas9 complex thus binds the first 8 to 12 nucleotides near a PAM sequence and will produce a double-strand break (Fig. 8). This prompts the cell machinery to undergo DSB repair.

Cellular DSB repair can be achieved by non-homologous end joining (NHEJ) or by homology directed repair (HDR). NHEJ is an active endogenous repair mechanism, in which the DNA is randomly repaired by DNA sequence alignment, resulting in small insertions or deletions, called indels (as small as two nucleotides), at the cut site that may lead to frameshift mutations in coding sequence (Bennardo et al., 2009; Waters et al., 2014). In contrast to NHEJ, homology-directed repair acts as a more specific and precise method of DSB repair by the incorporation of a specific sequence with homology to the repair site through homologous recombination. Thus, genome editing using HDR requires

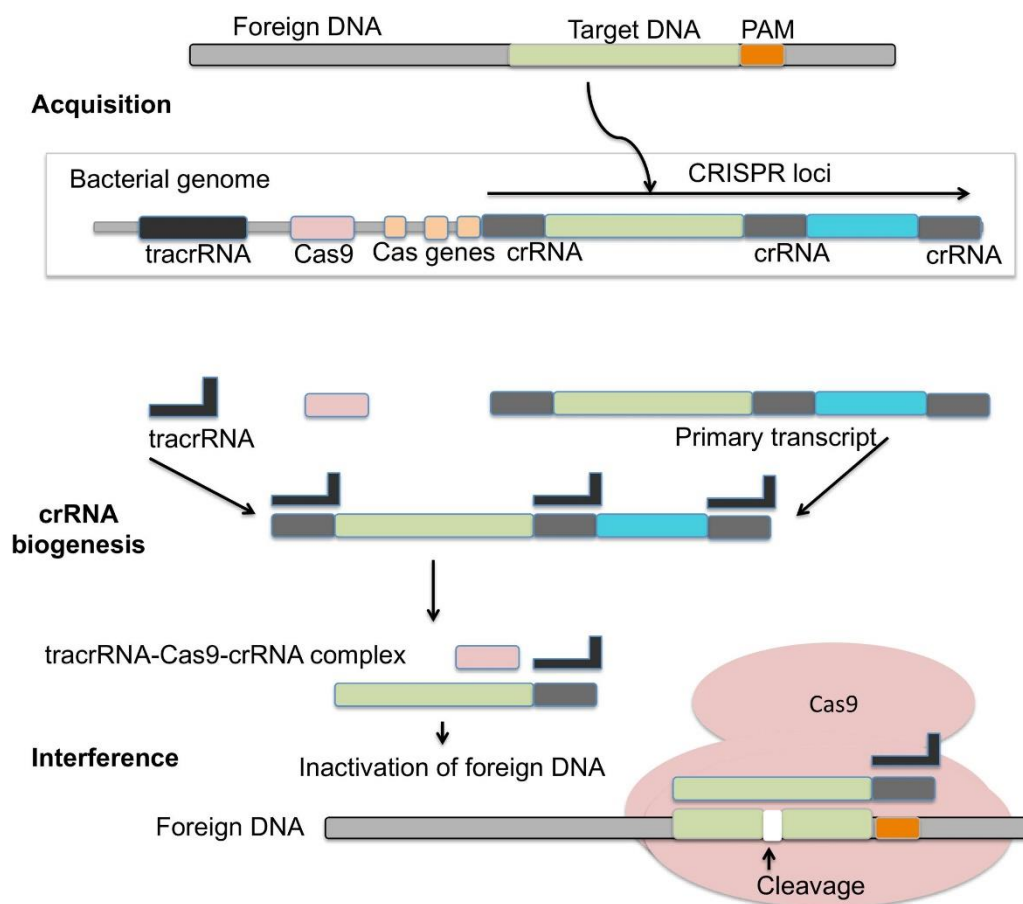


Figure 7. Mechanism of CRISPR-Cas9 action: in the acquisition phase fragments of viral foreign DNA are incorporated into the CRISPR loci of the bacterial genome. After transcription of CRISPR, the resulting primary transcript is processed into mature CRISPR RNAs. For interference, Cas9 endonuclease is complexed with a crRNA and tracrRNA, and cleaves foreign DNA near the PAM region.

(From Arora and Narula, 2017)

a DNA template with a sequence with homology arms to be delivered into the cell along with Cas9 and the gRNA (Shrivastav et al., 2008; Pardo et al., 2009).

Different strategies and techniques have been developed for the successful delivery of all of these components into the cells. Microinjection and electroporation are the traditional physical methods mostly used to deliver CRISPR-Cas9 components into zygotes and embryonic stem cells, respectively, that subsequently will give rise to a genetically modified organism (Horii et al., 2014; Crispo et al., 2015; Kaneko et al., 2015).

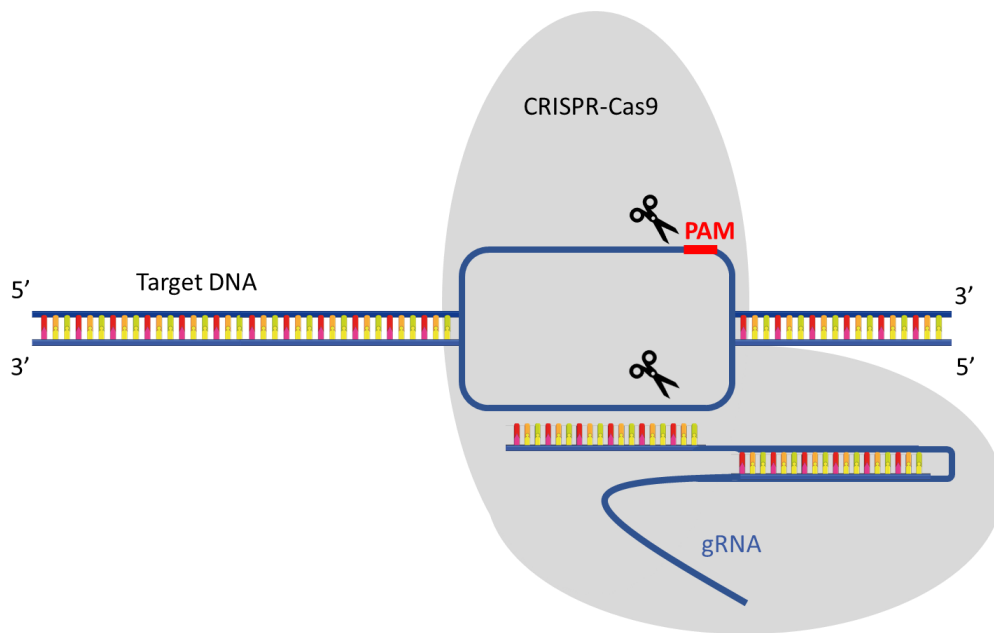


Figure 8. CRISPR-Cas9 cleavage of target sequences within genes results in gene-specific disruption in host cells. The CRISPR-Cas9 complex aligns with the DNA target region through complementary base pairing of the crRNA to the target strand, and the Cas9 endonuclease produces a site-specific DNA double strand break adjacent to a PAM motif within the gene of interest.

An increasing number of protocols for the generation of mutant mice using the CRISPR-Cas system coupled with microinjection of zygotes has been published, whether to create simple frameshifts for gene knockout or to generate conditional and insertion alleles (knock-in) using ssDNA donor sequences (Ran et al., 2013; Harms et al., 2014; Yang, Wang and Jaenisch, 2014; Quadros et al. 2017; Miura et al., 2018). Therefore, the technology is well established and available for seamless genome editing in purpose-equipped laboratories.

AIMS

AIMS

With this work, we aim to contribute to the understanding of whether and how genes from the Dlk1-Dio3 imprinted domain - namely the imprinted *Begain 1B* isoform - may be involved in the modulation of neuropathic pain. To that effect, we tested the suitability of the Rep^{L1del} mutant model, which is characterized by a strong downregulation of *Begain 1B* (and a more moderate downregulation of *Dlk1*) in embryonic brain upon paternal transmission of the mutant allele, by conducting suitable, hypothesis-driven behavioural and molecular studies. Thus, nociceptive behaviour studies and molecular analyses were carried out in mutant mice (paternal transmission crosses) and wild type littermates, in adulthood and at embryonic day (E)16.5, as follows:

Nociceptive behaviour analyses following SNI of Rep^{L1del} mutants and wildtype littermates

To evaluate the nociceptive response following SNI-induced neuropathic pain, the animals were subjected to a peripheral nerve lesion by transecting the peroneal and tibial nerves, and tested at four different timepoints to assess the pain model progression and maintenance. The nociceptive response to mechanical stimuli was measured using the von Frey test of monofilaments applied vertically on the injured hind paw.

Gene expression studies of *Begain* (1A and 1B isoforms) and *Dlk1* in spinal cord and dorsal root ganglia of Rep^{L1del} mutants and wildtype littermates

To assess the expression levels of the three transcripts in spinal cord and DRG, essential components of the pain circuitry, tissue samples of E16.5 embryos and adult mice (subjected to the SNI model of neuropathic pain) were dissected and snap frozen. Prior to freezing, the spinal cord was divided in ipsilateral and contralateral halves, which were treated as different samples. The levels of gene expression were determined by reverse transcription-quantitative PCR (RT-qPCR).

Protein expression studies of *Begain* and *Dlk1* in spinal cord and DRG of Rep^{L1del} mutants and wildtype littermates

Proteins were extracted from the dissected tissues mentioned above and subjected to Western blot analysis for quantification. Since antibodies capable of distinguishing the two *Begain* isoforms are not commercially available, the quantification at the protein level was performed as total *Begain*.

Analysis of imprinting of *Begain* and *Dlk1* in spinal cord and DRG of Rep^{L1del} mutants and wildtype littermates

To determine the imprinting status of *Begain* (isoforms 1A and 1B) and *Dlk1* in spinal cord and DRG, wildtype and heterozygous animals with expressed polymorphisms in these transcripts were obtained from reciprocal crosses between the Rep^{L1del} mutant (C57BL/6J background) and mice from the C3H/HeJ strain. Following reverse transcription of extracted RNA at E16.5 and adulthood, the polymorphic alleles were ascertained by Sanger sequencing. This analysis aimed not only to establish the normal imprinting status of these genes in the spinal cord and DRG of wildtype animals, but also to probe for any effects of the Rep^{L1del} mutation on imprinting in these tissues.

Additionally, with the objective of obtaining the most suitable model to investigate the specific involvement of the imprinted *Begain 1B* isoform in the modulation of chronic pain, as well as for wider studies of the biological function of this epigenetically-regulated transcript, we aimed at producing an isoform-specific knockout mouse. To that effect, we have resorted to CRISPR-Cas9 technology to specifically target exon 1B of *Begain*, while leaving isoform 1A intact. In order to disrupt exon 1B with minimal impact on the genomic locus, which contains a CpG island that extends up to the exon's location, an inversion strategy was pursued.

Material and Methods

MATERIAL AND METHODS

- Part I -

1. Animals

Experiments were performed in mice housed at the Animal Facility of the Centre of Medical Research of the Faculty of Medicine of Porto. All procedures were conducted in accordance to Portuguese government's Direção Geral de Alimentação e Veterinária licensing and regulations, as well as the guidelines set forth by the institution's Animal Welfare and Ethics Body (Orgão Responsável pelo Bem-estar e Ética Animal, ORBEA) on the use of animals for research purposes based on the 2010/63/EU directive. Additionally, all experimental procedures were performed in accordance to the ethical guidelines for the study of experimental pain in conscious animals from the International Association for Study of Pain (IASP) (Zimmermann, 1983).

Animals were housed with water and food ad libitum under controlled conditions of temperature and humidity, $22\pm 2^{\circ}\text{C}$ and $50\pm 5\%$ respectively, in a 12-hour light/12-hour dark cycle.

All experimental animals resulted from crosses between C57BL/6J females and heterozygote $\text{Rep}^{\text{L1del}}$ mutant males (C57BL/6J background), ensuring paternal transmission of the deletion bordering the *Dlk1-Dio3* imprinted domain (Soares et al., 2018). The experimental groups were composed by $\text{Rep}^{\text{L1del}}$ mutants and wild type littermates, genotyped by multiple polymerase chain reaction (PCR) analyses.

2. Spared Nerve Injury Surgery

To induce the spared nerve injury model of neuropathic pain a protocol previously described in the rat and later improved for mice was followed (Decosterd & Wolf, 2000; Suter et al., 2003), as described below. Surgery was performed under 1.5-2.5% isoflurane-induced deep general anaesthesia (Decosterd & Wolf, 2000; Suter et al., 2003). For this, the mice were placed in a container to induce anaesthesia with 2.5% of isoflurane, which was then maintained under 1.5% isoflurane with a head mask during the whole protocol. The left hindlimb was immobilized and extended in a lateral position and slightly elevated. The femur and knee joint were used as reference marks to perform the incision at the

mid-thigh level (Fig. 9A). The *biceps femoris* muscle was exposed and the artery *genus descendens* was used to direct the incision following the orientation of the muscle fibres to avoid major lesion of the muscle. After muscle incision, the three branches of the sciatic nerve (sural, common peroneal and tibial nerves) were exposed without stretching the nerve structures and the peroneal and tibial nerves were both ligated and transected together, while the sural nerve was left intact (Fig. 9B). For ligation and transection, a silk 5.0 suture was tightly wrapped around the peroneal and tibial nerves, and then a 1-2mm wide section was removed (Fig. 9C). During the procedure the integrity of the sural nerve was closely monitored as it was essential to avoid contact with the surgical tools to preclude the stretching of the nerve. After nerve transection, the muscle tissue and the skin were sutured in two distinct layers, and the head mask was removed to revert the anaesthesia effect. The sham group was submitted to the same surgical protocol without performing the nerve lesion.

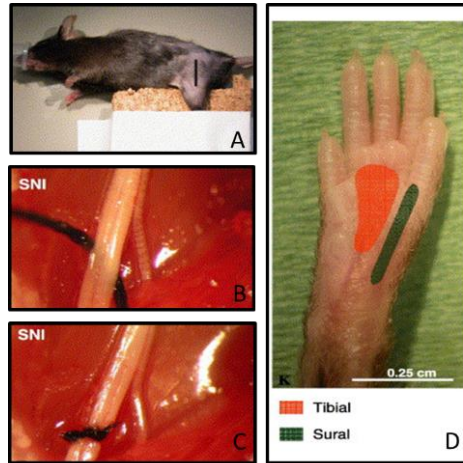


Figure 9. Spared Nerve Injury (SNI) surgical procedure. A) Mouse hindlimb immobilization and orientation in an elevated platform. The black line represents the incision mark. B) Surgical thread under the peroneal and tibial nerves. The nerve dissection is reduced to a minimum, avoiding the contact with the sural nerve branch. C) The surgical suture is tied around the common branches of the peroneal and tibial nerves to be transected, and a 2mm section of these nerves is removed. D) Representation of the areas enervated by the tibial and sural nerves in the plantar region of the mouse hind paw, which are used for the mechanical allodynia tests.

(From Bourquin et al., 2006)

3. Nociceptive Behaviour - von Frey test

For nociceptive behavioural analyses, the animal groups were randomly divided in 3 different sets. Each set was composed by Rep^{L1del} mutant mice (sham and SNI) and wild type (Wt) littermates (sham and SNI) to ensure that all the genotypes and SNI/sham surgeries were represented in each set. A total of 35 animals were used, divided in four groups: Mut SNI (n=9); Mut sham (n=9); Wt SNI (n=8); and Wt sham (n=9).

All animals were habituated to the experimenter and testing conditions for a period of one week before behavioural evaluation, each day for 30 minutes per mouse, to reduce the stress during the experiments. For this, mice were allowed to recognize the testing room and the testing material, such as the von Frey filaments and the elevated platform. In addition, handling familiarizes the mice with the experimenter, as well as

with the smells and sounds of the behaviour room. Throughout the experimental period, the health state of the animals was ascertained by monitoring the weight, healing from surgical procedures, opening of sutures, self-mutilation and locomotion, as well as any signs of stress and abnormal aggressiveness.

Mechanical sensitivity was recorded before and after surgery at different timepoints: at 2 days before surgery, to use as a baseline of the response to specific von Frey filaments, and at 7, 14, 21 and 28 days after surgery to analyse the potential mechanical sensitivity changes within groups associated with the development and maintenance of neuropathic pain induced by the SNI surgery (Fig. 10). All measurements were performed blindly with respect to mice genotype and SNI or sham surgeries.

On the days of behavioural evaluation, mice were placed in the test room for a period of 15 to 30 minutes before the test to become habituated to the characteristics of the testing room. Subsequently, each mouse was placed on an elevated platform with a 20 mm soft wire mesh floor surrounded by a transparent acrylic box (10x10x13 cm) and was then familiarized with the application of the von Frey filaments in the paw pad. To perform the test, the plantar side of the paw was stimulated by an ascendant perpendicular movement of the von Frey filaments (Stoelting Co., Chicago, USA) through the wire mesh floor where the animals were placed, in order to apply a specific force on the plantar territory of the sural (spared) nerve. The applied force was considered sufficient when it was enough to cause filament bending. To measure mechanical sensitivity, ten stimuli were applied with each of the eleven monofilaments by ascending order of force (0.02, 0.04, 0.07, 0.16, 0.40, 0.60, 1.0, 1.4, 2.0, 4.0 and 6.0 g). The relative frequency of paw withdrawal (FPW) was assessed by a method used for rats and mice (Song et al., 1999; Mansikka et al., 2000), which consists on measuring the total number of positive responses per filament in the total of ten stimuli applied. A response was considered positive when the animal abruptly removed, shaken or licked the hindpaw. The paw withdrawal threshold (PWT) corresponds to the force of the filament to which each animal displays five positive responses (out of ten), and this is considered the lowest force required to trigger a pain reaction.

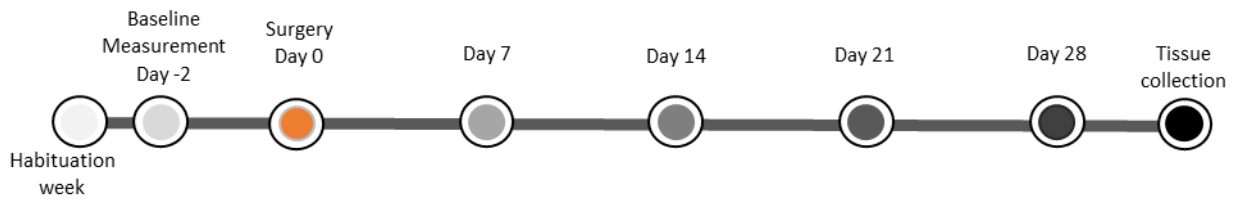


Figure 10. Schematic representation of the experiment timeline. Habituation, surgery, behavioural analyses before surgery (baseline) and on days 7, 14, 21 and 28 days after surgery, and tissue collection.

4. Tissue collection

To assess gene expression levels in Rep^{L1del} mutant mice and wildtype littermates, the brain, spinal cord and dorsal root ganglia were collected from embryo at E16.5 and adult animals. For this, following euthanasia of adult mice by cervical dislocation or retrieval of embryos by C-section, the tissues were collected into Eppendorf tubes and snap-frozen in liquid nitrogen. All samples were stored at -80 °C until use.

Adult spinal cord samples were collected from the region corresponding to the L3 to L6 lumbar segments, which is the area that receives all the inputs from sensory fibres in the hindpaw, namely the sural, tibial and peroneal nerve fibres. Before freezing, the spinal cord sample was further divided into the ipsilateral and contralateral halves. DRG were bilaterally collected from the same L3 to L6 region and pooled together.

In embryos, the entire spinal cord and all DRG were collected since they were not submitted to any experimental pain protocol.

5. RNA extraction

Total RNA was extracted from embryonic and adult mice with the NZYTech Total RNA Isolation Kit according to the manufacturer's instructions, with some modifications, and quantified using spectrophotometry by the Nanodrop 2000 (Thermofisher). Briefly, 0.35 to 1 mL of QIAzol Lysis reagent (Qiagen) was added to the tissue samples according to size and weight in RNase-free tubes with magnetic beads for homogenizing in a MagNA Lyser instrument (Roche). Following the addition of 200 µl of chloroform per 1 mL of

QIAzol, and phase separation by centrifugation (15 min at 12000 g), the upper aqueous phase was transferred into a 1.5 mL Eppendorf tube, to which 1 volume of 70% ethanol was added and mixed by up and down pipetting. The mixture was then pipetted into an NZYSpin Binding column for RNA extraction according to the manufacturer's protocol (NZYtech, Portugal). Following RNA binding, DNase treatment and several washing steps, the RNA was eluted with 40-60 µL RNase-free water and quantified. RNA integrity was ascertained using the 2100 Bioanalyzer Instrument (Agilent, USA). RNA samples were all stored at -80°C until subsequent analysis.

5.1 cDNA synthesis

Reverse transcription was carried out with 0.5 to 1 µg of total RNA using the NZY First-Strand cDNA Synthesis Kit (NZYtech, Portugal). The reaction mix included a combination of random hexamers and oligo(dT)₁₈ primers in order to increase sensitivity. All cDNA samples were RNase-treated following synthesis, according to the protocol. The cDNA was diluted 1:20, aliquoted, and stored at 4 °C for subsequent use. Negative control reactions - without reverse transcriptase - were also set up for a number of samples to ascertain the absence of genomic DNA contamination in the subsequent qPCR analysis.

5.2 Real-Time qPCR

Quantitative real time PCR (qPCR) was performed in a StepOne Plus qPCR system (Applied Biosystems) using the SensiFAST SYBR Hi-Rox Kit (Bioline, UK) in 12.5 µL reactions with 200 nM (*Begain 1A* and *1B*) or 400 nM of primers (*Dlk1* and *Gap3dh*). The reactions were assayed in triplicate, containing 3 µL of cDNA synthesized and diluted as described. A standard curve made up of doubling dilutions of pooled cDNA from the samples being assayed was run on each plate, and quantification was performed relative to the standard curve. Target gene expression was normalized to the expression of *Gap3dh*. qPCR reaction efficiencies for all sets of primers ranged from 90% to 100%. Primer sequences and annealing temperatures are shown in Table 1.

TRANSCRIPT PRIMER	PRIMER SEQUENCE	ANNEALING
<i>Begain 1A_ Fw</i>	GTAGTGGCGCGTGGAGTC	68 °C
<i>Begain 1A_ Rv</i>	GCAGAGCCGGGGCCATGT	
<i>Begain1B_ Fw</i>	GTAGTGGCGCGTGGAGTC	65 °C
<i>Begain 1B_ Rv</i>	ATGGGCAGCCATCAGTCTT	
<i>Dlk1_ Fw</i>	GAAAGGACTGCCAGCACAAG	60 °C
<i>Dlk1_ Rv</i>	CACAGAAGTTGCCTGAGAAGC	
<i>GAPDH_ Fw</i>	CCATCACCATCTTCCAGGAG	60 °C
<i>GAPDH_ Rv</i>	GCATGGACTGTGGTCATGAG	

Table 1. Primer sequences and annealing conditions used in RT-qPCR.

6. Protein extraction

Protein extraction was performed from the interphase and phenol phases obtained in the RNA extraction protocol, after Trizol/chloroform centrifugation and subsequent aqueous phase removal. To separate DNA, 100% ethanol was added (0.3 mL ethanol per 1 mL of Trizol used), and samples were mixed and incubated at room temperature for 3 min. Following centrifugation at 2,000 g for 5 min at 4 °C, the supernatant was collected and used for protein extraction. Protein precipitation was produced by the addition of isopropanol to the supernatant (1.5 mL isopropanol per 1 mL of Trizol), mixing, incubation at room temperature for 10 min, and centrifugation at 12,000 g for 5 min at 4 °C. Protein precipitates were washed with 2 mL of 0.1 M ammonium acetate (in 80% methanol), through mixing and incubation at room temperature for 20 min. To remove washing solution, samples were centrifuged at 7,500 g for 5 min at 4 °C, and the washing step was repeated two times. Afterwards, 2 mL of 100% ethanol were added, and samples were vortexed, incubated for 20 min at room temperature and then centrifuged at the same conditions used in previous washing steps. Finally, ethanol was removed, and protein pellets were dissolved in 1% SDS (sodium dodecyl sulphate) by up and down pipetting, and incubating at 50 °C for 15 min. Protein samples were then stored at 4 °C.

6.1 Western Blotting (WB)

Proteins were quantified by the bicinchoninic acid (BCA) protein assay. After denaturation at 65 °C for 15 min, 20 µg of protein extracts were loaded onto 10% SDS-polyacrylamide (SDS/PAGE) gels. Gel electrophoresis was performed for 1h at constant electric current (30 mA per gel, and maximum 200 V) to separate proteins according to their molecular weight. Proteins were then transferred into nitrocellulose membranes by blotting for 1h30 at 30 V. Following Ponceau staining, and using the protein ladder (Broad Range Protein Ladder, ThermoFisher) as reference, membranes were cut between 35 kDa and 42 kDa bands for Gapdh detection, between 42 kDa and 70 kDa for Dlk1 detection, and between 70 and 95 kDa for Begain detection.

Prior to incubation with primary antibodies, all membrane fragments were blocked for 1h at room temperature with Bovine Serum Albumin (BSA) (4% BSA powder diluted in Tris buffer saline, with 0.1% Tween 20 [TBST buffer]). Incubation with primary antibodies was performed at 4 °C with mouse anti-Gapdh antibody (Abcam ab8245) diluted 1:10000 in BSA 4%; rabbit anti-Dlk1 antibody (Abcam ab210471) diluted 1:500 in BSA 4% or rabbit anti-Begain antibody (ThermoFisher PA5-31568) diluted 1:3000 in BSA 4%. Finally, blots were incubated in HRP-conjugated anti-mouse secondary antibody (GE Healthcare NA931V) diluted 1:10000 in TBST with 4% BSA or HRP-conjugated anti-rabbit secondary antibody (GE Healthcare NA934V) 1:10000 in TBST with 4% BSA for 1h at room temperature. The chemiluminescent signal was detected using Clarity Western ECL Substrate (BioRad) in the ChemiDoc™ system (BioRad).

7. Statistical Analyses

Statistical analyses were performed using the GraphPad Prism software (version 8.0) for Windows. Data is presented as the Mean ± SEM (standard error of the mean). To assess the significance of the differences between groups, two-way ANOVA with repeated measures followed by the Tukey's Multiple Comparisons post-hoc test was used for the nociceptive behaviour, while the remaining data were analysed by two-way ANOVA (using genotype and SNI/sham surgeries as variables) followed by the Tukey's Multiple Comparisons post-hoc test and the unpaired Student's t test. For analyses of the

ipsilateral versus contralateral samples, the paired Student's t test and the Wilcoxon matched-pairs signed rank test (for normal and non-normal distributions, respectively) were used. Continuous variables distributions were tested for normality using the Shapiro-Wilk and the Kolmogorov-Smirnov tests. The statistical tests applied and respective significance values are indicated in each figure legend. $P < 0.05$ was deemed as statistically significant.

8. Imprinting

To assess the imprinting status of *Begain* and *Dlk1* in the spinal cord and DRG of *Rep^{L1del}* mutants and Wt littermates, expressed single-nucleotide polymorphisms (SNPs) were identified between the C57BL/6J and C3H/HeJ strains *in silico* (Wellcome Sanger Institute Mouse SnpViewer). A SNP was identified in exon 7 of *Begain* (rs252334243, alleles G and C, respectively); and another in exon 5 of *Dlk1* (rs253217941, alleles A and T, respectively). PCR amplification of genomic DNA of both strains with primers flanking the SNPs (Table 2) followed by sequencing, confirmed the variants.

Allele-specific expression was assessed in embryos and adult mice obtained from reciprocal crosses between *del^{L1rep}* heterozygous mutants (C57BL/6J background) and C3H/HeJ. DRG and SC from eleven embryos from a *Rep^{L1del}* x C3H cross (2 litters, 4 mutants and 4 Wt) and a C3H x *Rep^{L1del}* cross (1 litter, 2 mutants and 1 Wt); and four adults from a C3H x *Rep^{L1del}* cross (1 litter, 2 mutant and 2 Wt) were examined. *Begain* and *Dlk1* cDNA was produced by reverse transcription using the NZY First-Strand cDNA Synthesis Kit according to the manufacturer's protocol (NZYtech). For *Begain*, a gene-specific primer was used in order to generate *Begain* cDNA only, whereas for *Dlk1* a combination of random hexamers and oligo(dT)₁₈ was employed. To ascertain the transcript-specific alleles in *Begain*, the cDNA was first amplified with transcript specific-primers. The PCR conditions were as follows: 95 °C initial denaturing for 5 min; 40 cycles (*Begain 1A*) or 42 cycles (*Begain 1B*) of 95 °C denaturing for 30 sec; 60 °C annealing for 40 sec; 72 °C extension for 1 min and 30 sec; and final extension at 72 °C for 10 min. The amplicons were run on a 1% agarose gel, gel extracted and purified, and subjected to a second PCR amplification with primers flanking the SNP as follows: 95 °C initial denaturing for 5 min; 36 cycles of 95 °C denaturing for 30 sec; 60 °C annealing for 30 sec; 72 °C

extension for 45 sec; and final extension at 72 °C for 10 min. For *Dlk1*, a single amplification was carried out (same conditions as those for *Begain* second PCR amplification but with 40 cycles), directly in the SNP region, since exon 5 is common to all isoforms. All resulting amplicons were sequenced by Sanger sequencing.

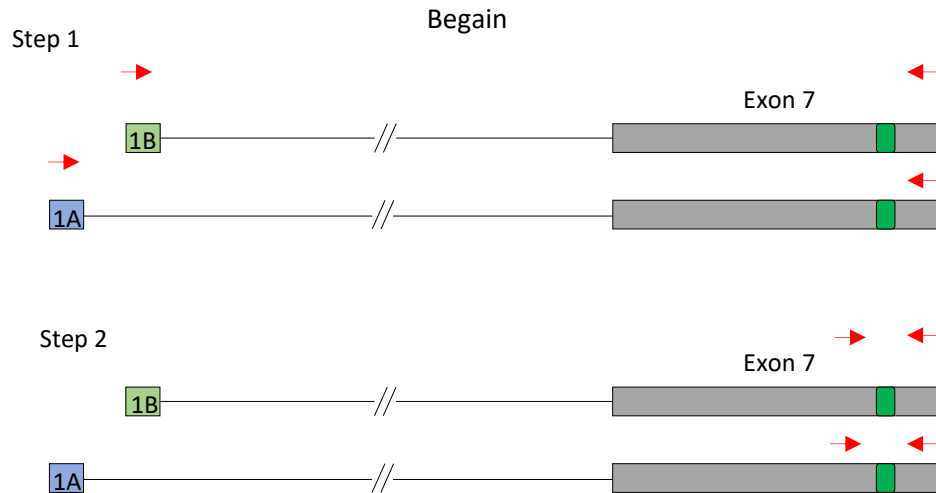


Figure 11. Schematic representation of the approach employed for specific PCR amplification of *Begain* SNP rs252334243 in *Begain 1A* and *Begain 1B* transcripts.

PRIMER	SEQUENCE	ANNEALING
<i>Begain</i> RT GSP	AGCTTCCGAGTAGTTGCAGG	n/a
<i>Begain 1A</i> cDNA_Fw	GCTCCACAGGTTTGGAGGCA	60 °C
<i>Begain 1A</i> _Rv	GCAGAGCCGGGGCCATGT	
<i>Begain1B</i> _Fw (<i>Begain</i> _rs252334243_Fw)	ACAGGTTTGGAGGCATGAGA	60 °C
<i>Begain 1B</i> _Rv	ATGGGCAGCCATCAGTCTT	
<i>Begain</i> _rs252334243_Fw	ACAGGTTTGGAGGCATGAGA	60 °C
<i>Begain</i> _rs252334243_Rv	CCGCGACGAGTTCTTCAAC	
<i>Dlk1</i> _rs253217941_Fw	CAAACCAGCCCCATCACAAT	60 °C
<i>Dlk1</i> _rs253217941_Rv	GCGTGGAGAAATAGGCTGTT	

Table 2. Primer sequences and annealing conditions used for transcript-specific PCR amplification.

- Part II -

1. *Begain 1B* Isoform-specific targeting using CRISPR/Cas9

To target the *Begain 1B* isoform, a strategy was devised that entailed the inversion of the genomic area containing exon 1B. This would result in the disruption of the transcript, while maintaining the genomic region mainly intact. Adequate target CRISPR sites surrounding exon 1B were selected resorting to CRISPR guide design software tools available online: CRISPOR (<http://crispor.tefor.net>); CHOPCHOP (<https://chopchop.cbu.uib.no>) and IDT (https://eu.idtdna.com/site/order/designtool/index/CRISPR_SEQUENCE). The sequences of the CRISPR sites - 5'-ACTCCAGCACTGCCTTTCGC **GGG**-3', and 5'-CCTGAAGGATGATTCGTAGA **AGG** -3' (PAM sequences in bold) - were characterized by high specificity (lowest number of off-targets) and predicted efficiencies. Since we aimed at the replacement of the sequence delimited by the CRISPR-Cas target sites and not just the removal, a long single-strand DNA (ssDNA) fragment with arms of homology to the endogenous locus, flanking the inverted target sequence, were designed, to be introduced by homology directed repair following Cas9-mediated cleavage and removal of the endogenous target sequence.

Endogenous sequence:

```
CCCGCGAAAGGCAGTGCTGGAGTCTGAACCCAGGGACGACGCCACAGACCTGGGAAGACTGATG  
GCTGCCCCATCCCGGACCCTCACGCCGGGCGCCGCCCGCGTCCGCCGGGAGCGCGCTCCGCTCGGTCCG  
GGCCCCACGCCGCTCCCCACCGCCCCGCCGAGCGAGGGCGCAGCCCGGCCTCGGAGCCGCGGCTAGGC  
TCGGCCAGCAACTTCCCCCGGCGCCCGAGCAGGCCTCCGGGCTCTCCCCGAGCCTCAGTTTCTCTCCTG  
AAATCTGGAGGAGGGAAGGCGGGAGGAGCTCGGACAGGCGCTCTGCTGCCCTGCAGCTCTGGCGGGCTGC  
AAAGGGGCTGGGTTAGGAAAAGGACCCAGGGGCGCCGGTGGGCTTCCCTCCCCGCACACTTTAATAAA  
AGTTTACAAAAAGCACGAGTCCAGACCCCCTTCTACGAATCATCCTTCAGG
```

Inverted sequence:

ATCGCACTTCCTGCGCGCAACGGGCTCTCCTGCGCCCGGCCGAAGCCGCCCCGGGGCTGCCCCC
GCCCTGCGGAGAAATTGAAGTGTGGAGTATAACTTCGTATAGCATACATTATACGAAGTTATGTCTGGAC
TCGTGCTTTTTGTAAACTTTTATTAAAGTGTGCGGGGAGGGAAGCCCACCGGGCGCCCTGGGGTCCTTT
TCCTAACCAGCCCCCTTTCAGAGCCCGCCAGAGCTGCAGGGCAGCAGAGCGCCTGTCCGAGCTCCTCCCGC
CTTCCCTCCTCCAGATTTTCAGGAGAGGAAACTGAGGCTCGGGGAGATGCTCGAAGTTGCTGGCCGAGCCT
ATCGATGCGCTTTACCGAGCGGAGCGCGCTCCCGGCGGACGCGGCGGCGGCGCCCGGCGTGAGGGTCCGG
GATGGGCAGCCATCAGTCTTCCCAGGTCTGTGGCGTGTCCCTGGGTTTCAGATAACTTCGTATAATGTAT
GCTATACGAAGTTATCCCGTTGTACTTATCATCCTTCAGGACTAGAGTTCTCTAGTTAGAGTTTGGAGCA
TATCAGCACGATCCCCAGAGCCCCTCTGCCCCAGGCCACCTTTTCGAGTCCCCATCACAGT

Green highlight: CRISPR target sequences; Turquoise highlight: Exon 1B; Yellow highlight: mismatches introduced in CRISPR target sequences; Italic: homology arms.

2. Assembly of the CRISPR-Cas9 complex and *in vitro* testing of the crRNAs

The CRISPR RNA (crRNA) molecules and ssDNA donor fragment were synthesized *in vitro* (Integrated DNA Technologies, IDT, U.S.). Prior to being used *in vivo*, crRNAs were tested *in vitro* by incubating with tracrRNA and Cas9 (IDT) and a PCR amplicon of genomic DNA containing the region with the target sites. TracrRNA and crRNA were suspended in microinjection buffer (10mM Tris-HCl pH 7.5 and 0.1 mM EDTA) to a stock concentration of 10 μ M. One μ g (1 μ L) of tracrRNA and 250 ng crRNA (each) were mixed with 5.9 μ L of H₂O, incubated at 95 °C for 5 min and then left cooling down at room temperature to promote crRNA:tracrRNA complex formation (also known as guide RNA). One hundred and fifty ng of target sequence DNA amplicon, 2 μ L of NEB 3.1 buffer and 1 μ g of Cas9 nuclease were added and the mixture was incubated at 37 °C for 1h30min. After incubation, 1 μ L of RNase A (4 μ g/ μ L) was added for gRNA digestion at 37 °C for 15 min. The reaction was terminated with stop solution (30% glycerol, 1.2% SDS and 250mM EDTA) in a further incubation for 15 min at 37 °C. The product was run on a 2% agarose gel.

3. Microinjection of the CRISPR-Cas9 complex

The CRISPR-Cas9 complex targeting *Begain* exon 1B was delivered into mice zygotes by pronuclear injection. To the effect, C57BL/6J females were superovulated by intraperitoneal injection of 5 units of pregnant mare's serum gonadotropin (PMSG) (Intervet) and, after 46h, with 5 units of human chorionic gonadotropin (hCG) (Intervet). Superovulated females were crossed with C57BL/6J males for fertilization. Early next morning, the presence of vaginal plugs was checked and plugged females were sacrificed. The zygotes were collected from the ampulla, incubated briefly in hyaluronidase (Sigma 9001-54-1) to remove cumulus cells, washed in M2 medium (Sigma M7167), transferred to M16 medium (Sigma M7292) covered with mineral oil (Sigma 8042-47-5), and incubated at 37 °C under a 5% CO₂ atmosphere until injection.

The CRISPR-Cas9 complex was assembled as mentioned above. The mixture containing 125 ng of the ssDNA donor fragment and the CRISPR-Cas9 complex was injected into the zygotes' male pronuclei using a Femtojet microinjector (Eppendorf) with continuous flux (50 hPa). The injection was performed in a Leica AM6000 Micromanipulator System (Leica, Eppendorf). After injection, the zygotes were washed and incubated in M16 at 37 °C (5% CO₂) until implantation. The embryos were transferred into the oviducts of pseudo-pregnant females 0.5 days post coitum, which had been superovulated as above and mated with vasectomized males.

After birth, pups were genotyped by PCR to confirm the ssDNA donor genomic integration.

4. Genotyping of pups

Ear punch samples of over 12 days-old mice were collected and boiled in 600 µl of 100 mM NaOH followed by the addition of 100 µl of 1M Tris-HCl (pH 8.0). One µL of gDNA template was PCR amplified in a 12.5 µL reaction with Kapa Ready Mix (Sigma), 3% DMSO and primer set A (Table 3). The PCR cycling conditions were as follows: 95 °C initial denaturing for 5 min; 35 cycles of 95 °C denaturing for 30 sec; 58 °C annealing for 40 sec; 72 °C extension for 1 min; and final extension at 72 °C for 10 min. The amplicons were run on a native 5% polyacrylamide gel, which separates sequence hybrids (variants of the

original sequence) by conformation. The genomic DNA of putative mutants was reamplified by PCR and sequenced in both orientations.

For genotyping of the founder progeny once the *Begain 1B* mutant line was established, gDNA was PCR amplified using primer sets B and C (Table 3), each distinguishing the mutant and the wildtype allele, respectively. The PCR cycling conditions were as follows: 95 °C initial denaturing for 5 min; 36 cycles of 95 °C denaturing for 30 sec; 60 °C annealing for 30 sec; 72 °C extension for 30 sec; and final extension at 72 °C for 5 min.

PRIMER	SEQUENCE	ANNEALING, AMPLICON
SET A		58 °C, 778 b
Bg1b_CRISPR_5Pgenot_Fw	ACCCACTAGCCCCTGTTAAC	
Bg1b_CRISPR_3Pgenot_Rv	ACTTCTGTGCCATAGGACCC	
SET B		60 °C, 242 bp
Bg1b_CRISPR_5Pgenot_Fw	CCCTCCCGCACACTTTAAT	
Bg1b_CRISPR_5Pgenot_Inv_Rv		
SET C		60 °C, 400 bp
Bg1b_CRISPR_5Pgenot_Fw	AACTGAGGCTCGGGGAGA	
Bg1b_CRISPR_5Pgenot_Rv		

Table 3. Primer sequences and annealing conditions for PCR genotyping of *Begain* KO mice.

Results

Results

- Part I -

Pain behaviour studies

Rep^{L1del} mutants exhibit mechanical allodynia

All animals, whether subjected to SNI or sham surgery, were assayed in three independent sets and tested using the von Frey test. Each set was composed by a number of animals belonging to four groups defined according to the genotype and treatment: SNI Mut (n=9); SNI Wt (n=8); sham Mut (n=9) and sham Wt (n=9). Von Frey baseline values showed no differences among groups. Indeed, the frequency of response to the application of each monofilament was similar in all experimental groups, before SNI induction. As expected, the FPW increased proportionally to the increase of the force of the von Frey filaments in all experimental groups, so that the higher force (0.8 of force (log)) exerted a frequency of responses close to 100% (Fig. 12 A). However, with the development of neuropathic pain following the SNI surgery, the FPW to the monofilaments of lower force progressively increased in the SNI animals of both genotypes, compared to sham animals (Fig. 12). Thus, as expected, from 7 days after SNI onwards, the SNI Wt mice group showed increased sensitivity to von Frey monofilaments when compared to Sham Wt animals, denoting a well-induced SNI pain model and validating the testing conditions. This effect was particular evident (and significant) for the mid-range filaments of -0.4, -0.2 and 0.0 log g of force, and increased significance with the evolution of the neuropathy, at day 28 (Fig 12 B-E).

At day 7, the SNI Mut group exhibited increased sensitivity to lower von Frey monofilaments, with a higher FPW, which was more evident than that of SNI Wt mice and was not accompanied by the sham groups. The sensitivity to the von Frey filaments was significantly higher ($p<0.01$) when compared with sham groups (regardless of genotype) from the second monofilament onwards (-1.4 of force (log)) and most significantly increased ($p<0.0001$) from the third monofilament onwards (-1.2 of force (log)), notably when compared to the SNI Wt group as well (Fig. 12 B).

At day 14, a slight decrease of PWF was observed in SNI mutants when compared with day 7 values and, at the same time, a slightly increase in the SNI Wt group was registered. Indeed, at this time point the differences observed between these groups were no longer statistically significant, apart from those measurements made with the fourth filament (-0.8 log g force). Nonetheless, the differences relative to the sham groups, which did not differ among themselves, were still highly statistically significant from the second to the ninth filaments (Fig. 12 C).

Interestingly, at day 21, similar results to those from day 7 were observed: in the SNI Mut group the mechanical sensitivity to von Frey filaments was significantly higher than that registered in SNI Wt animals, as indicated by the higher frequency of response. Indeed, from this day onwards, SNI Mut animals showed significantly increased responses to a lower force monofilament (-1.4 log g force) that had not been detected in the previous timepoints. The SNI Wt group also exhibited significantly higher sensitivity when compared with Wt sham animals, which was significant for the mid-range filaments of -0.4, -0.2 and 0.0 log g of force. Regarding the sham groups, there were no significant differences among them and, as before, they were significantly much less responsive to the filaments than the SNI-subjected animals (Fig. 12 D).

Finally, at 28 days after surgery, the tendency verified at 7 and 21 days was confirmed (Fig. 12 E), with SNI mutant animals showing significantly increased frequency of response to lower force von Frey monofilaments comparing to other groups. The differences were not only statistically significant but also the statistical significance grew stronger when comparing the SNI Mut with sham groups (not shown). At this last time point of disease progression, SNI Wt animals also displayed higher sensitivity comparing with sham groups, while maintaining a statistically significant lower sensitivity when compared to SNI mutants.

In all, this experiment clearly shows that following SNI-induced neuropathic pain the Rep^{L1del} mutants exhibit mechanical allodynia and increased sensitivity compared to wild type littermates subjected to the same condition and to animals subjected to sham surgery regardless of the genotype.

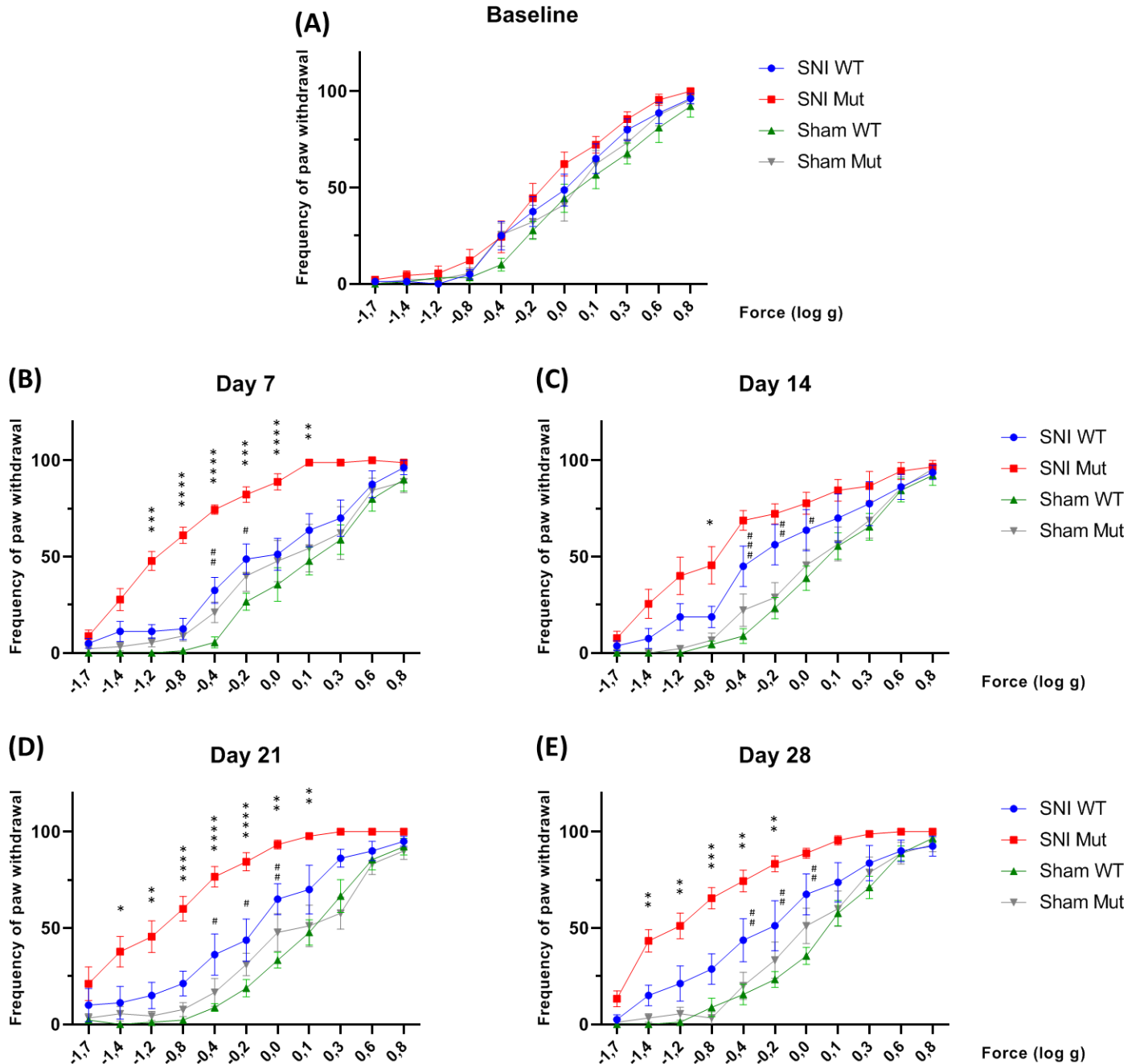


Figure 12. Relative frequency of paw withdrawal (FPW) of Rep^{L1del} mutant and Wt littermate mice following SNI and sham surgery. The relative frequency of paw withdrawal after 10 stimulations of the sural nerve skin territory was determined for each of the 11 von Frey monofilaments (0.02, 0.04, 0.070, 0.16, 0.40, 0.60, 1.0, 1.4, 2.0, 4.0 and 6g) on the ipsilateral paw. Mean $\pm$ SEM are shown. (*) Statistically significant difference between SNI Rep^{L1del} mutants and SNI Wt mice. (#) Statistically significant difference between SNI and sham Wt animals. *, # $p < 0.05$; **, ## $p < 0.01$; ***, ### $p < 0.001$; ****, #### $p < 0.0001$, Two-way ANOVA with repeated measures followed by Tukey's Multiple Comparisons post-hoc test. SNI Wt: n=8; Sham Wt: n=9; SNI Mut: n=9; Sham Mut: n=9.

Begain and Dlk1 gene and protein expression studies

1. Embryo

Following the molecular characterisation of the Rep^{L1del} mutant carried out by Soares et al. (2018), which showed a downregulation of *Begain 1B* and *Dlk1* in embryonic nervous tissue (more specifically in brain), the levels of expression of these two genes were studied in embryonic spinal cord and dorsal root ganglia, essential components of the pain circuitry, at the same embryonic timepoint. To that effect, RT-qPCR analyses were conducted in these tissues at E16.5.

1.1 Embryonic Spinal Cord

Begain 1B and *Dlk1* are downregulated in the SC of Rep^{L1del} embryos

In spinal cord, the expression levels of the biallelic *Begain* isoform 1A did not show differences between mutant and wildtype embryos (Fig. 13), whereas the imprinted *Begain* isoform 1B was ~53% downregulated in mutants relative to wildtype levels ($p < 0.0001$) (Fig. 14 A). The expression of *Dlk1* in spinal cord was also downregulated by 40% in Rep^{L1del} mutant embryos when compared to Wt animals ($p < 0.0001$) (Fig. 14 B). These results are in accordance with the findings of Soares et al. (2018) in embryonic brain: the expression of imprinted transcripts *Begain 1B* and *Dlk1* is affected by the Rep^{L1del} mutation, whereas that of biallelic *Begain 1A* is not.

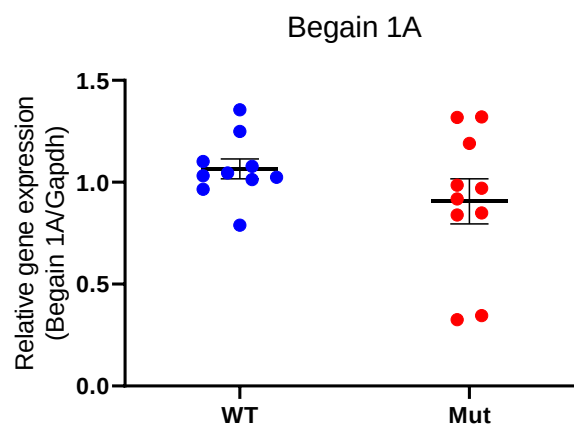


Figure 13. Relative gene expression of *Begain 1A* isoform in spinal cord of Rep^{L1del} mutant and wildtype E16.5 embryos. Target gene expression was normalized to *Gapdh* expression. Bars represent the mean $\pm$ SEM. $P > 0.05$, unpaired two-tailed Student's t-test. Wt: $n = 10$; Mut: $n = 10$.

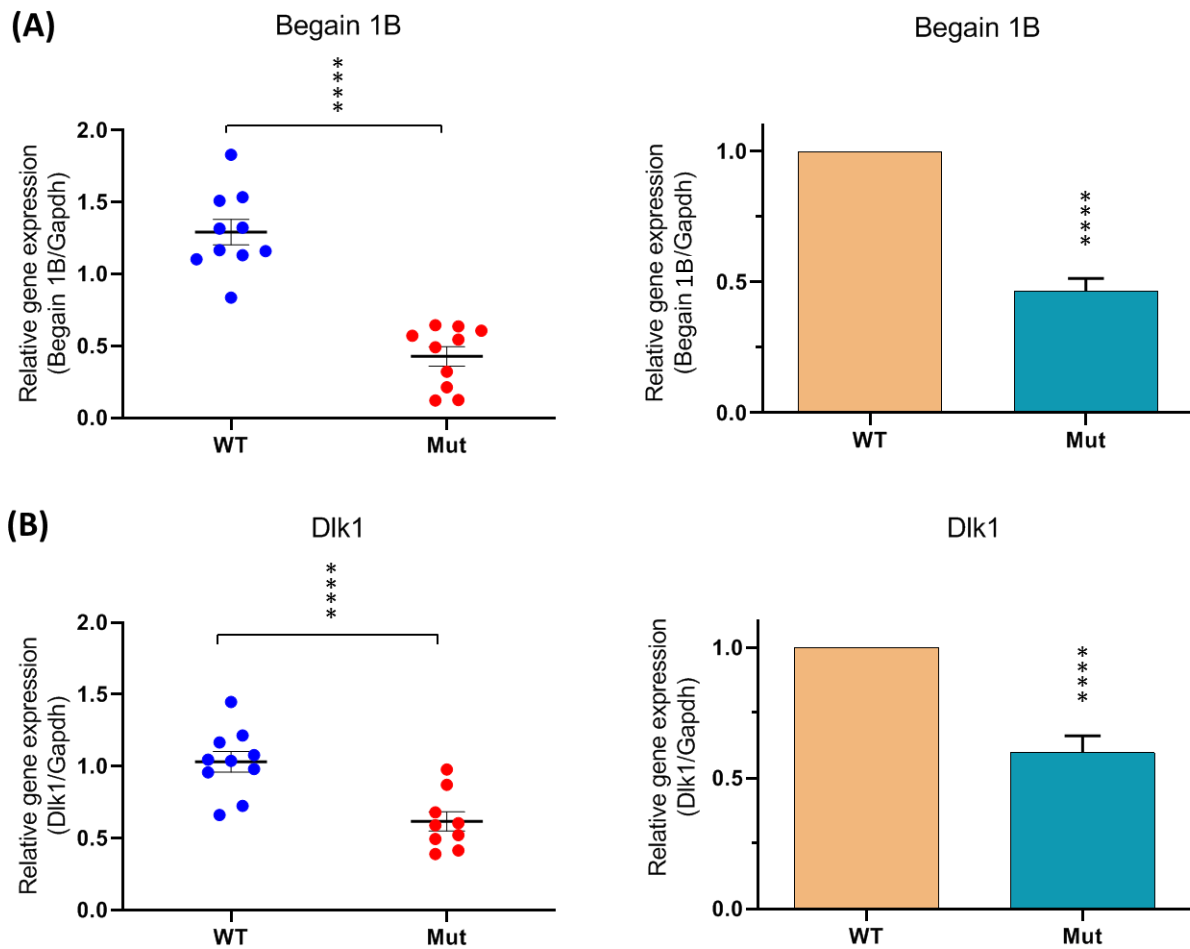


Figure 14. Relative gene expression of *Begain 1B* isoform and *Dlk1* in spinal cord of Rep^{L1del} mutant and wildtype E16.5 embryos. Target gene expression was normalized to *Gapdh* expression. Scatter plot charts: bars represent the mean $\pm$ SEM. Column bar charts: data are shown relative to WT controls (=1). **** $p < 0.0001$, unpaired two-tailed Student's t-test. Wt: n=10; Mut: n=10 (in B, n=9).

1.2 Embryonic DRG

Begain 1B alone is downregulated in the DRG of Rep^{L1del} embryos

In dorsal root ganglia, the levels of expression of both *Begain* transcripts followed the same pattern as that shown in the spinal cord. RT-qPCR revealed a ~46% downregulation of *Begain 1B* isoform in Rep^{L1del} mutants relative to Wt embryos ($p = 0.0006$) (Fig. 15 B and C), and no differences in the expression of *Begain 1A* (Fig. 15 A). Interestingly, *Dlk1* expression was not affected by the mutation in embryonic DRG (Fig. 15 D and E). Indeed,

although showing a tendency for a slight decrease in Rep^{L1del} mutants (~9%), it was not statistically significant ($p=0.068$). These results are somewhat surprising given the effect of the Rep^{L1del} mutation in *Dlk1* expression in all embryonic tissues probed thus far.

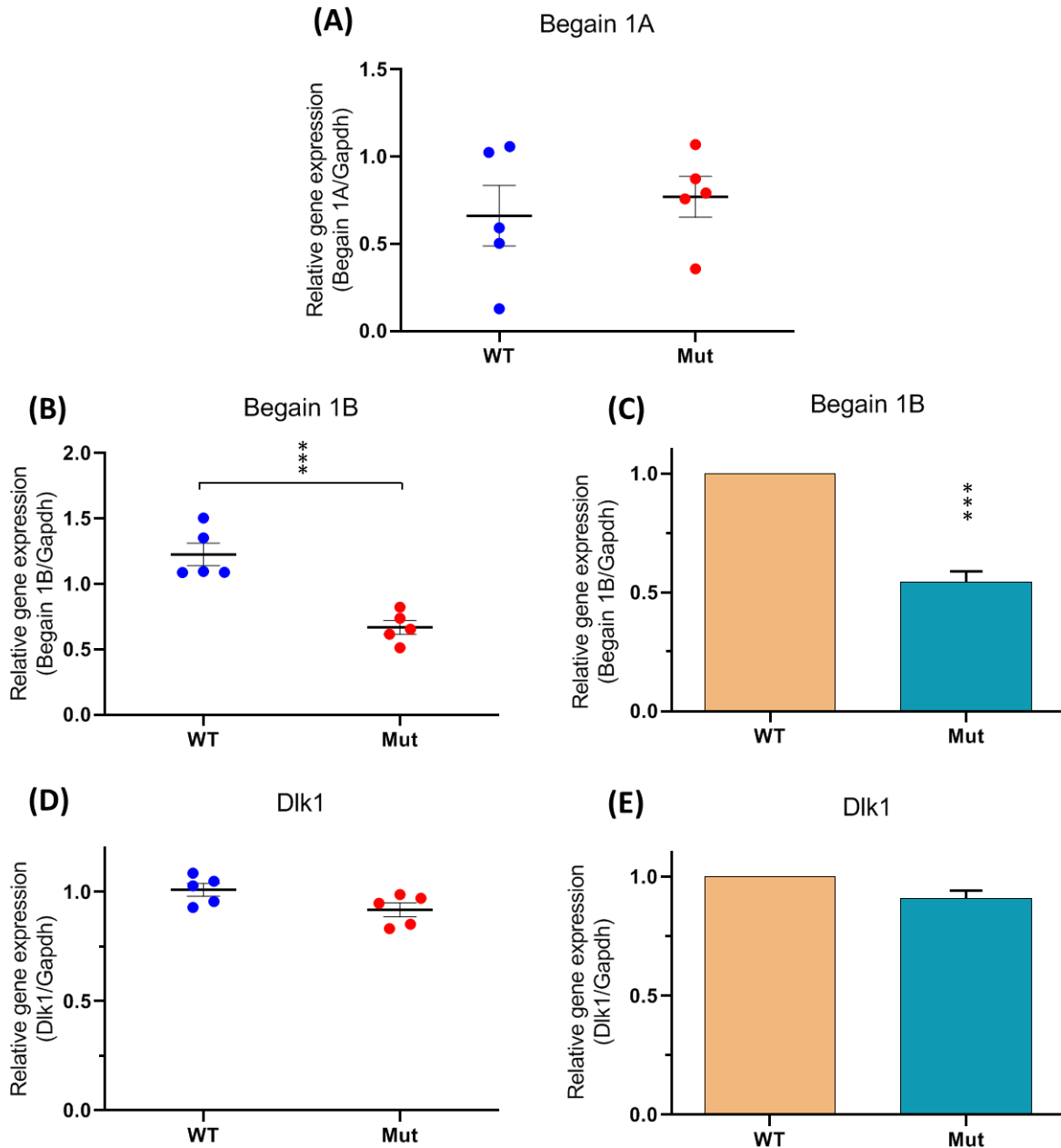


Figure 15. Relative gene expression of *Begain 1A*, *Begain 1B* and *Dlk1* in dorsal root ganglia of Rep^{L1del} mutant and wildtype E16.5 embryos. Each sample represents a pool of 3 embryos. Target gene expression was normalized to *Gapdh* expression. Scatter plot charts: bars represent the mean $\pm$ SEM. Column bar charts: data are shown relative to WT controls (=1). *** $p<0.001$, unpaired two-tailed Student's t-test. Wt: $n=5$; Mut: $n=5$.

2. Adult

To study the dynamics of *Begain* and *Dlk1* expression and respective protein products in neuropathic pain, gene and protein expression studies were conducted in adult mice following the SNI pain model. The levels of gene expression were determined by RT-qPCR, as carried out for embryonic tissues, and the protein expression studies were conducted by Western blot analysis. All studies were performed in L3-L6 sectioned spinal cord and respective DRG in Rep^{L1del} and Wt littermate animals subjected to SNI and sham surgery. Since the tissues were collected following the mechanical allodynia measurements, only a single time point was analysed: at 28 days after injury. The spinal cord was further divided in ipsilateral and contralateral halves by longitudinal sectioning through the ventral median sulcus.

Under our experimental conditions, the Begain-encoded protein showed a molecular weight of ~72 kDa (slightly heavier than the predicted 65 kDa) in both tissues. Importantly, the antibody used in our study precluded the distinction of the Begain's isoforms since it was raised against an antigen shared by both protein variants. Indeed, most of the sequence is shared among the two isoforms - with the exception of the first exon – which is why no isoform-specific Begain antibodies are commercially available, to our knowledge. Thus, all protein quantifications shown herein pertain to total Begain (1A- and 1B-encoded products).

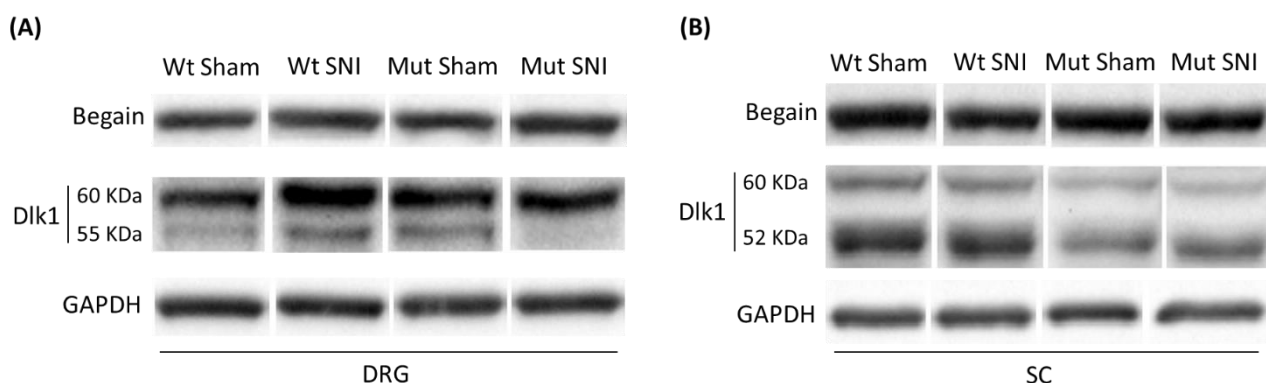


Figure 16. Representative images of Western blot analyses of Begain and Dlk1 in dorsal root ganglia (A) and spinal cord (B) of adult Rep^{L1del} mutants and wildtype littermates subjected to SNI or sham surgery.

Dlk1 showed two bands with molecular weights of approximately 52 kDa and 60 kDa in the spinal cord (Fig. 16 B) and 55 kDa and 60 kDa in DRG (Fig. 16 A). As for *Begain*, the Dlk1 antibody used is not isoform-specific, recognizing an epitope common to all the transmembrane and soluble isoforms. In this regard, it is important to point out that the Dlk1 soluble forms, with a molecular weight of ~25 kDa, were not quantified.

2.1 Adult DRG

***Begain 1A* is upregulated in the DRG of Rep^{L1del} mice subjected to SNI**

The analyses of *Begain 1A* gene expression showed a statistically significant effect of the SNI versus *sham* surgeries among groups: *Begain 1A* was upregulated by 18% in SNI versus *sham* animals, regardless of genotype ($p=0.041$) (Fig. 17 A, B). Multiple comparisons *post hoc* analyses showed that this effect was brought by a substantial (38%) increase in expression of *Begain 1A* seen in Rep^{L1del} mutant mice subjected to nerve injury comparing with mutant *sham* animals ($p=0.0188$) (Fig. 17 A, C). Additionally, a 14% upregulation in SNI-subjected Rep^{L1del} mutants relative to SNI Wt mice was observed but no statistical significance was reached. These results were corroborated by the proteomic studies, which showed a 33% increase of total *Begain* in nerve injured mice compared to the *sham* group (irrespective of genotype) ($p=0.023$) (Fig 18 A, B). In accordance, multiple comparisons *post hoc* analyses showed that after nerve injury, mutant mice had higher protein levels of total *Begain* (42%) relatively to those of the mutant *sham* animals, although the difference is only close to statistical significance ($p=0.0790$) (Fig. 18 A, C). Furthermore, in animals subjected to SNI, mutant mice displayed a tentative increase of total *Begain* (31%) when compared to Wt mice, although it did not reach statistical significance likely due to the small sample size ($p=0.2767$) (Fig. 18 A, D).

Among *sham* animals (mutant and wildtype) there were no significant differences in gene expression, which was expected due to the lack of nerve injury and to the absence of a genotype effect. Indeed, *Begain 1A* was unaffected by the Rep^{L1del} mutation as seen in the embryo DRG (Fig. 17 A). These results were also observed at the protein level as *sham* animals did not show any differences in total *Begain* protein (Fig. 18 A). In wild type littermates, nerve injury seems not to affect *Begain 1A* gene expression or total *Begain* protein levels in the DRG.

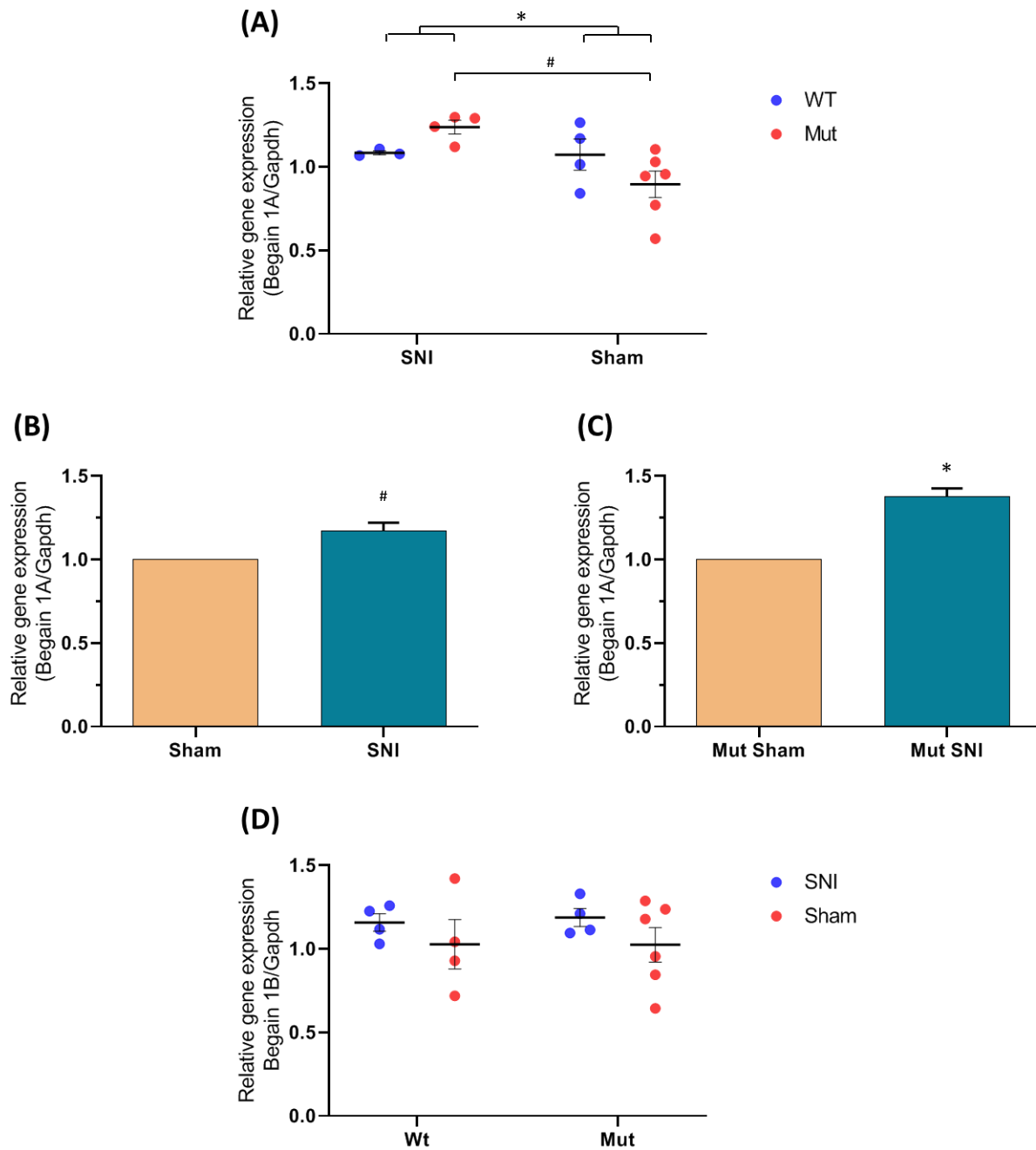


Figure 17. Relative gene expression of *Begain 1A* and *1B* in dorsal root ganglia of *Rep^{L1del}* mutants and wildtype littermates. Target gene expression was normalized to *Gapdh* expression. Scatter plot charts: bars represent the mean $\pm$ SEM. Column bar charts: data are shown relative to Wt controls (=1). Two-way ANOVA followed by Tukey's Multiple Comparisons post-hoc test. * $p < 0.05$, for the effect of the SNI versus sham surgeries in two-way ANOVA; # $p < 0.05$, for the multiple comparisons Tukey's *post hoc* test; Wt SNI: $n=3$ (in D, $n=4$); Wt Sham: $n=4$; Mut SNI: $n=4$; Mut Sham: $n=6$.

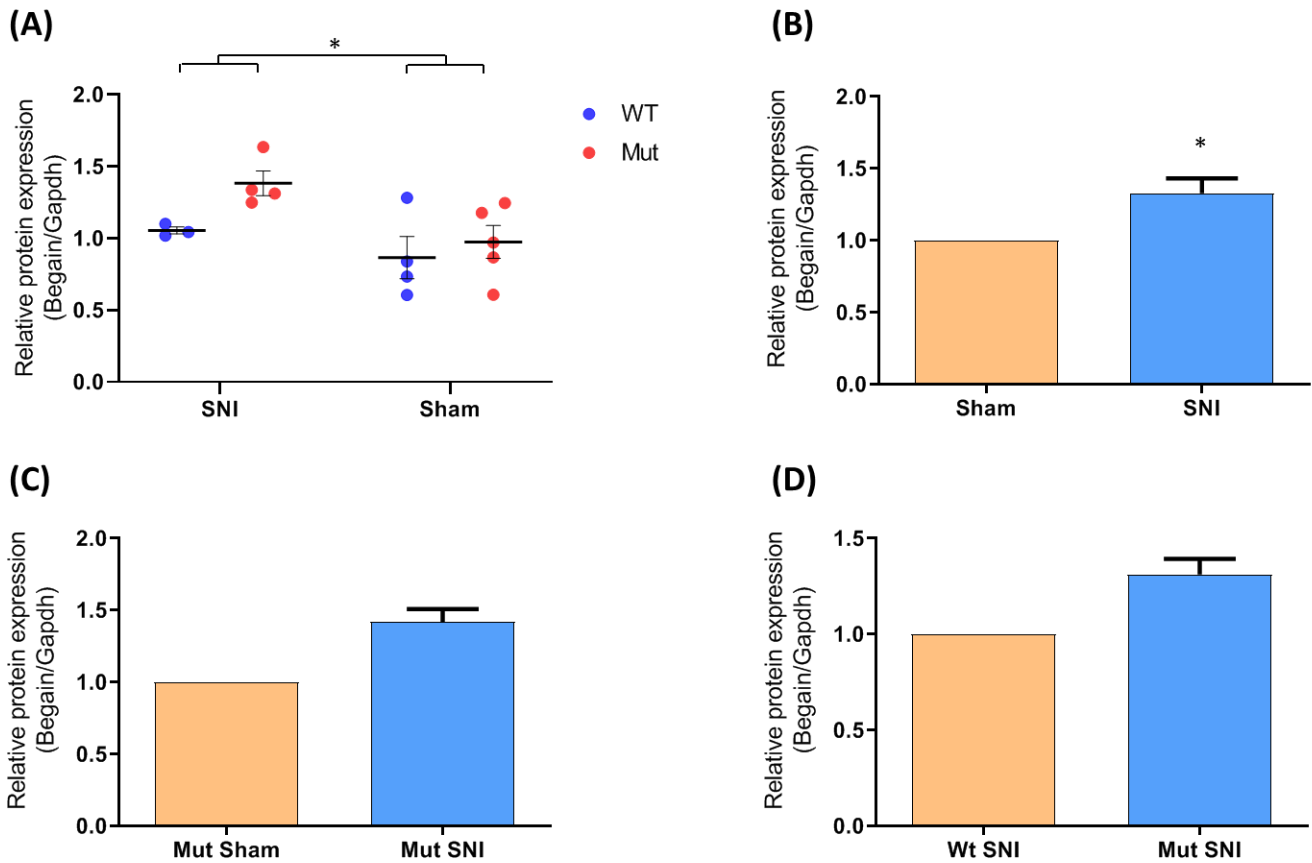


Figure 18. Relative protein expression of total Begain in dorsal root ganglia of Rep^{L1del} mutants and wildtype littermates. Protein expression was normalized to *Gapdh*. Scatter plot charts: bars represent the mean $\pm$ SEM. Column bar charts: data are shown relative to Wt controls (=1). Two-way ANOVA followed by Tukey's Multiple Comparisons post-hoc test. * $p < 0.05$ for the effect of the SNI versus sham surgeries in two-way ANOVA; # $p < 0.05$, for the multiple comparisons Tukey's *post hoc* test; Wt SNI: $n = 3$; Wt Sham: $n = 4$; Mut SNI: $n = 4$; Mut Sham: $n = 5$.

***Begain 1B* expression is unaffected in the DRG of Rep^{L1del} mutants and following SNI**

Contrary to the findings for *Begain 1A*, two-way ANOVA showed no significant effect of either the surgery or of the genotype, although a large dispersion of gene expression levels was observed in the sham groups of both genotypes (Fig. 17 D). Thus, the expression of *Begain 1B* isoform in DRG seems not to be affected by the nerve lesion neither in Rep^{L1del} mutant mice nor in Wt littermates. Thus, the effects of SNI on total Begain protein observed in Rep^{L1del} mutants seem to pertain, mostly, to the biallelic 1A isoform. Also, regardless of experimental treatment, no significant differences were seen

between mutants and wildtype animals, indicating that the Rep^{L1del} mutation has no effect on imprinted *Begain 1B* in this adult tissue at this timepoint. Again, this observation is in accordance with the findings of Soares et al. in adult brain.

***Dlk1* expression is unaffected in the DRG of Rep^{L1del} mutants and following SNI**

The analyses of *Dlk1* expression showed no dysregulation in adult DRG at the timepoint surveyed, as no genotype or treatment effects were observed by two-way ANOVA. Rep^{L1del} mutant mice subjected to SNI did not show differences in expression comparing to the sham group, a feature shared by Wt mouse littermates (Fig. 19). Furthermore, the expression levels did not vary between mutant and wildtype animals, whether after nerve transection or in sham animals. These results are in line with those obtained for embryonic DRG, where the levels of the *Dlk1* transcript were unaffected by the Rep^{L1del} mutation.

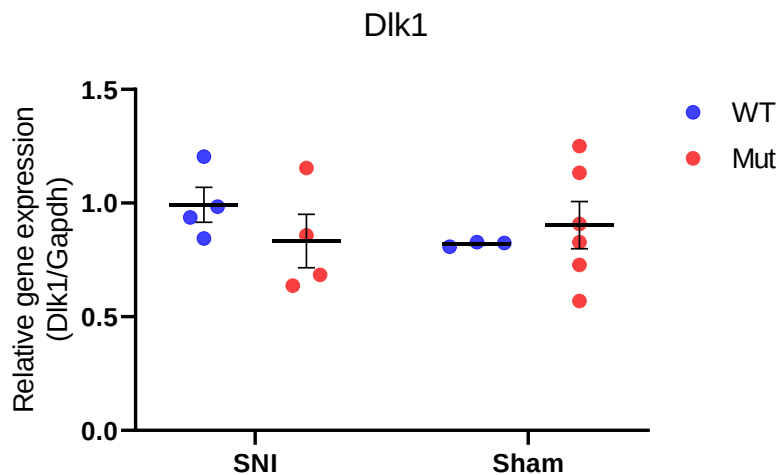


Figure 19. Relative gene expression of *Dlk1* in dorsal root ganglia of Rep^{L1del} mutants and wildtype littermates. Target gene expression was normalized to *Gapdh* expression. Bars represent the mean $\pm$ SEM. $P > 0.05$, two-way ANOVA followed by Tukey's Multiple Comparisons post-hoc test. Wt SNI: n=4; Wt Sham: n=3; Mut SNI: n=4; Mut Sham: n=6.

The Dlk1 protein is dysregulated in DRG following SNI

Following spared nerve injury, the levels of Dlk1 protein in adult DRG of Rep^{L1del} mutant mice and Wt littermates seem to show different behaviours. As mentioned above, Dlk1 has two distinct bands in this tissue with molecular weights of 55 kDa and 60 kDa; the former showing an intensity of approximately 35% of the latter. Thus, these bands were analysed independently as well as jointly (referred to as total Dlk1).

Analysis of total Dlk1 showed a treatment effect, with a significant 39% increase in SNI-subjected mice relatively to sham animals (regardless of genotype) ($p=0.0358$). Such effect was seen specifically for the more abundant 60 kDa protein, which displayed an almost significant 35% increase in the SNI group ($p=0.0599$), but not for the less abundant 55 kDa peptide (Fig. 20 A-E). The upregulation seen in SNI animals was not observed in Rep^{L1del} mutants; rather, it stemmed from the Wt animals where a pronounced increase of 59% was seen for the 60 kDa peptide (and total Dlk1) in mice subjected to SNI compared to sham animals, although this tendency did not reach statistical significance likely due to the small sample size ($p=0.1380$ and $p=0.1351$, respectively) (Fig. 20 A and B). The 55 kDa protein also showed a trend for an even stronger increase (103%) in Wt animals following SNI ($p=0.1380$), but this increase was offset by a putative decrease in Rep^{L1del} mutants subjected to SNI (relatively to sham mutants), although not at all statistically significant ($p=0.4408$) (Fig. 20 C, G and H). On the other hand, sham animals did not show any difference in all Dlk1 bands analysed (Fig. 20 A-C).

Interestingly, a genotype effect was also seen for the less abundant 55 kDa protein, with a pronounced increase of almost twofold (90%) in wildtype mice relatively to Rep^{L1del} mutants (regardless of treatment) ($p=0.0457$). Multiple comparisons post hoc tests showed that this result undoubtedly reflects the massive 350% increase of the 55 kDa protein levels seen in Wt animals comparing to Rep^{L1del} mutants following SNI ($p=0.0237$), given that no differences were observed between mutant and Wt mice in the sham group (Fig. 20 C, F and I).

Taken together, these results seem to indicate that nerve injury elicits a physiological upregulation of Dlk1 in Wt animals, which is impaired in Rep^{L1del} mutants, as shown by the behaviour of both the 60 kDa and 55 kDa peptides.

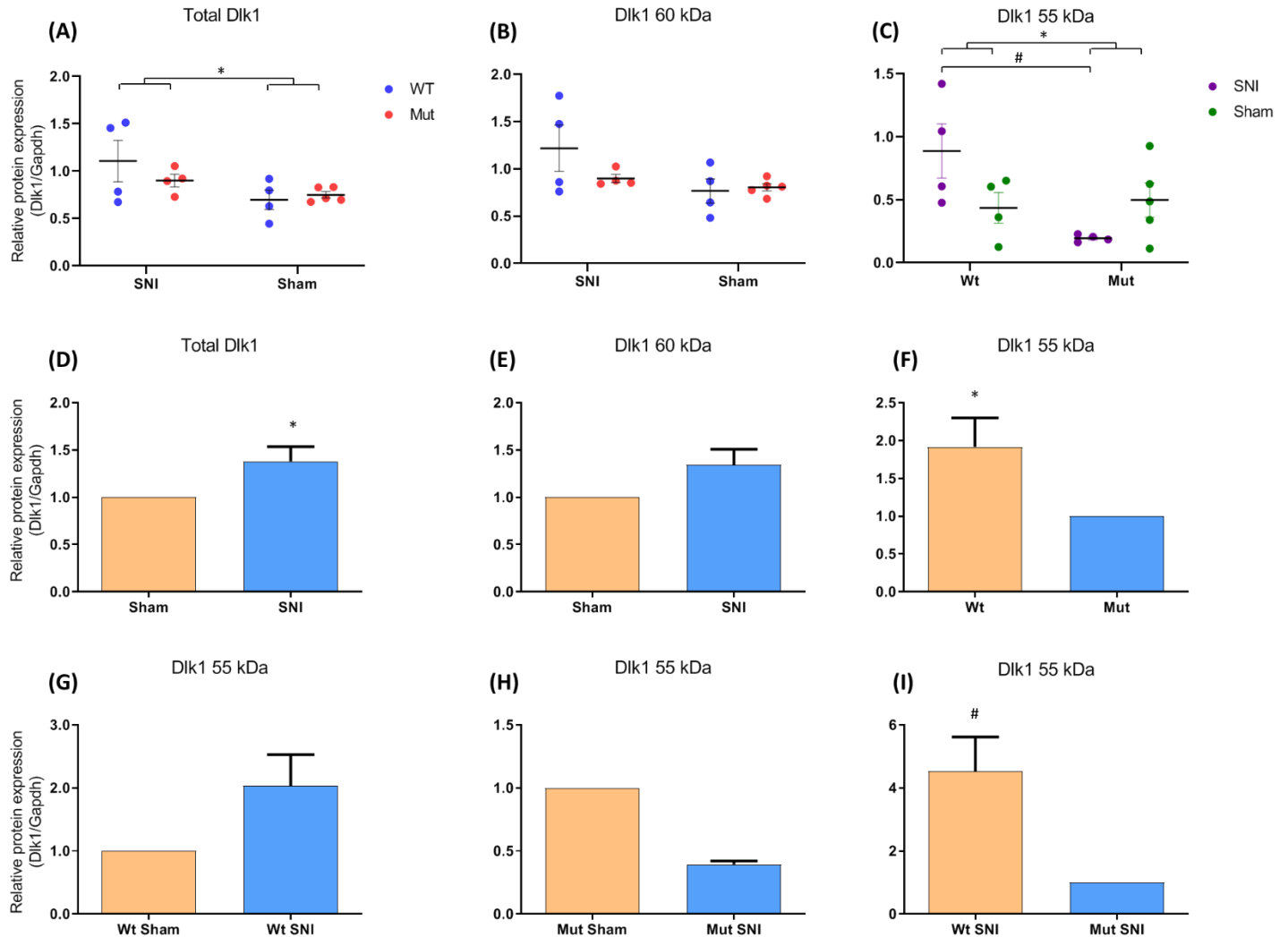


Figure 20. Relative protein expression of DIK1 in dorsal root ganglia of Rep^{L1del} mutants and wildtype littermates. Expression was normalized to Gapdh. Scatter plot charts: bars represent the mean $\pm$ SEM. Column bar charts: data are shown relative to Sham (D and E), to Mut (F), to Wt Sham (G), to Mut Sham (H) and to Mut SNI (I) (=1). Two-way ANOVA followed by Tukey's Multiple Comparisons post-hoc test. * $p < 0.05$, for the effect of the SNI *versus* sham surgeries or the effect of genotype in two-way ANOVA; # $p < 0.05$, for the results of the multiple comparisons Tukey's *post hoc* test; Wt SNI: $n = 4$; Wt Sham: $n = 4$; Mut SNI: $n = 4$; Mut Sham: $n = 5$.

2.2 Adult spinal cord

As mentioned above, the spinal cord was divided in ipsilateral and contralateral halves by longitudinal sectioning, meaning that two additional groups needed to be considered in the analyses. Globally, no statistically significant differences were seen in the expression of *Begain* and *Dlk1* between the ipsilateral and contralateral halves, whether genotype and surgical treatment were considered or not, except where noted. Therefore, having “two samples” per animal, we have elected to use the ipsilateral as the sample representative of the individual in all comparisons. In addition, to confirm the results obtained at the individual level, the analyses were also carried out by pooling the samples from both sides (as biological replicates) in order to increase the statistical power.

***Begain* expression is seemingly downregulated in the SC of Rep^{L1del} mutant mice but not responding to SNI**

In adult spinal cord, the expression levels of *Begain 1A* were unchanged in Rep^{L1del} mutant mice relatively to those of the Wt control group. Indeed, at the timepoint surveyed two-way ANOVA did not show a treatment effect as the transcript levels were similar among SNI-subjected mice and sham animals of either genotype. Additionally, no genotype effect was identified, and thus no significant differences were observed between genotypes in the groups subjected to SNI or sham surgery. (Fig. 21 A and B). However, there was a tendency for a 16% downregulation in mutant animals compared to Wt littermates, irrespective of treatment, but this was not statistically significant ($p=0.1214$).

The expression of *Begain 1B* showed a behaviour similar to that of *Begain 1A*. However, the two-way ANOVA analyses revealed a significant genotype effect corresponding to a 16% downregulation of the imprinted transcript in Rep^{L1del} mutants relatively to Wt littermates, irrespective of treatment ($p=0.0384$) (Fig. 21 C). Upon examining the levels of expression among genotypes in the SNI and sham groups separately, a downregulation of the same magnitude (16%) was observed, but it did not reach statistical significance in the *post hoc* test ($p=0.4449$ and $p=0.3186$, respectively).

At the timepoint analysed, 28 days after SNI, there was no detectable effect of the injury on the expression of *Begain 1B*.

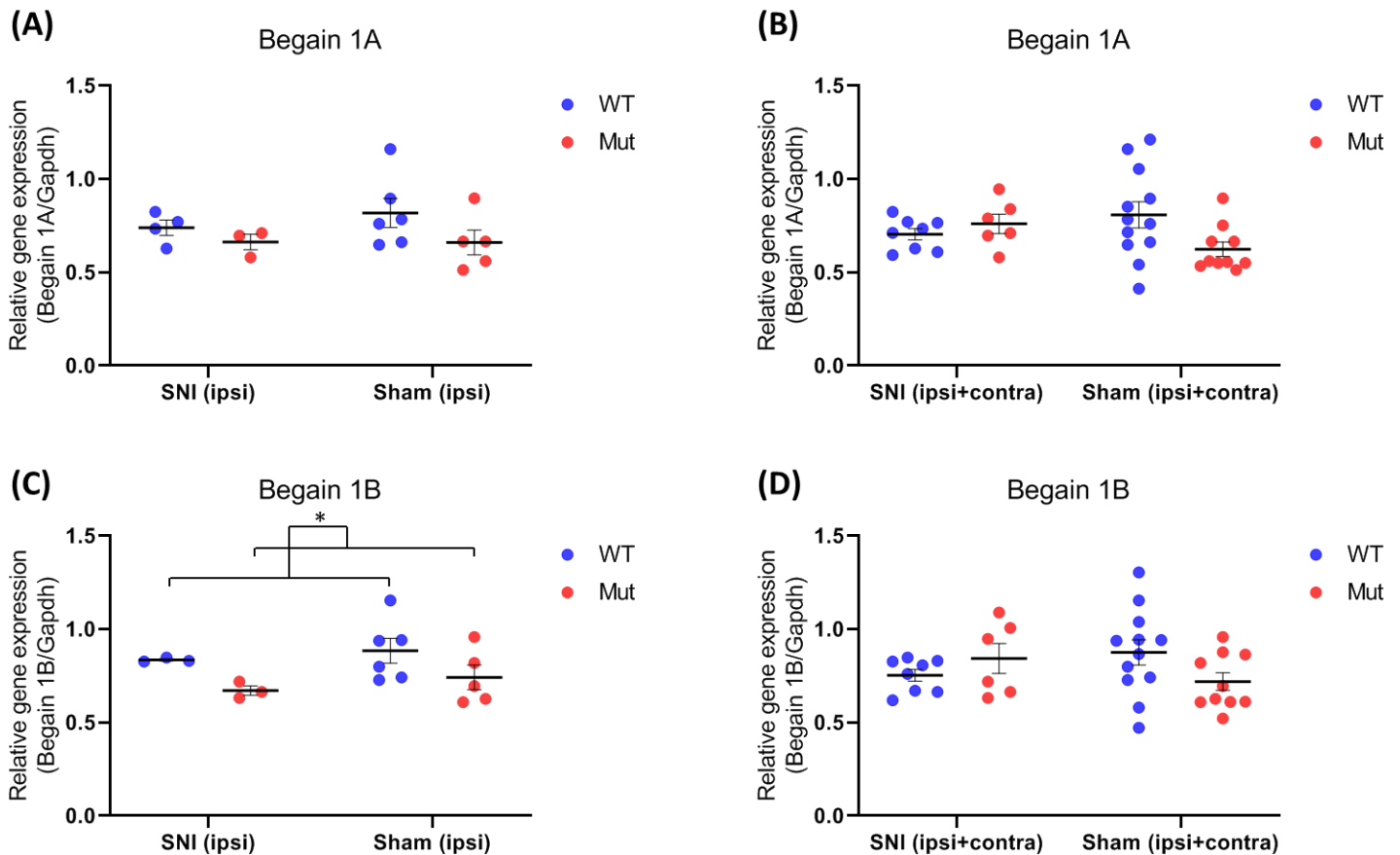


Figure 21. Relative gene expression of *Begain 1A* and *Begain 1B* in spinal cord of *Rep^{L1del}* mutants and wildtype littermates. Target gene expression was normalized to *Gapdh* expression. A) and C) Ipsilateral samples only (representing individuals). Wt SNI: n = 4 (in C, n=3); Wt Sham: n=6; Mut SNI: n = 3; Mut Sham: n = 5. B) and D) Ipsilateral and contralateral pooled samples. Wt SNI: n = 8; Wt Sham: n=12; Mut SNI: n = 6; Mut Sham: n = 10. Bars represent the mean $\pm$ SEM. Two-way ANOVA followed by Tukey's Multiple Comparisons post-hoc test. * $p < 0.05$, for the genotype effect in two-way ANOVA.

The behaviour of the *Begain* transcripts was recapitulated at the protein level (Fig. 22). Although two-way ANOVA showed no significant effects of treatment or genotype, protein analyses showed a 16% decrease of total Begain in Rep^{L1del} mutants relative to wildtype littermates, regardless of treatment, very close to statistical significance ($p=0.0798$) (Fig. 21 A and C). This putative decrease was mostly seen in the sham group, where the amount of total protein was tentatively reduced by 20% in mutants relatively to the wildtype animals, but not significantly so ($p=0.1456$) (Fig. 22 A and B). These results are in accordance with the putative downregulation seen at the transcript level of both 1A and 1B isoforms in the sham group, which, once again, were not statistically significant in either case ($p=0.3206$ and $p=0.3186$, respectively). Interestingly, when pooling the ipsilateral and contralateral samples together, thus increasing the sample size, the downregulation of both variants seen in the sham mutants was closer to statistical significance, particularly for Begain 1A ($p=0.0713$), suggesting that the observed trend may be real (Fig. 21 B and D). These results seem to indicate that *Begain 1A* and *1B* are affected by the Rep^{L1del} mutation in adult spinal cord, constituting the first instance in which either isoform is seen downregulated in an adult tissue in the context of the Rep^{L1del} model.

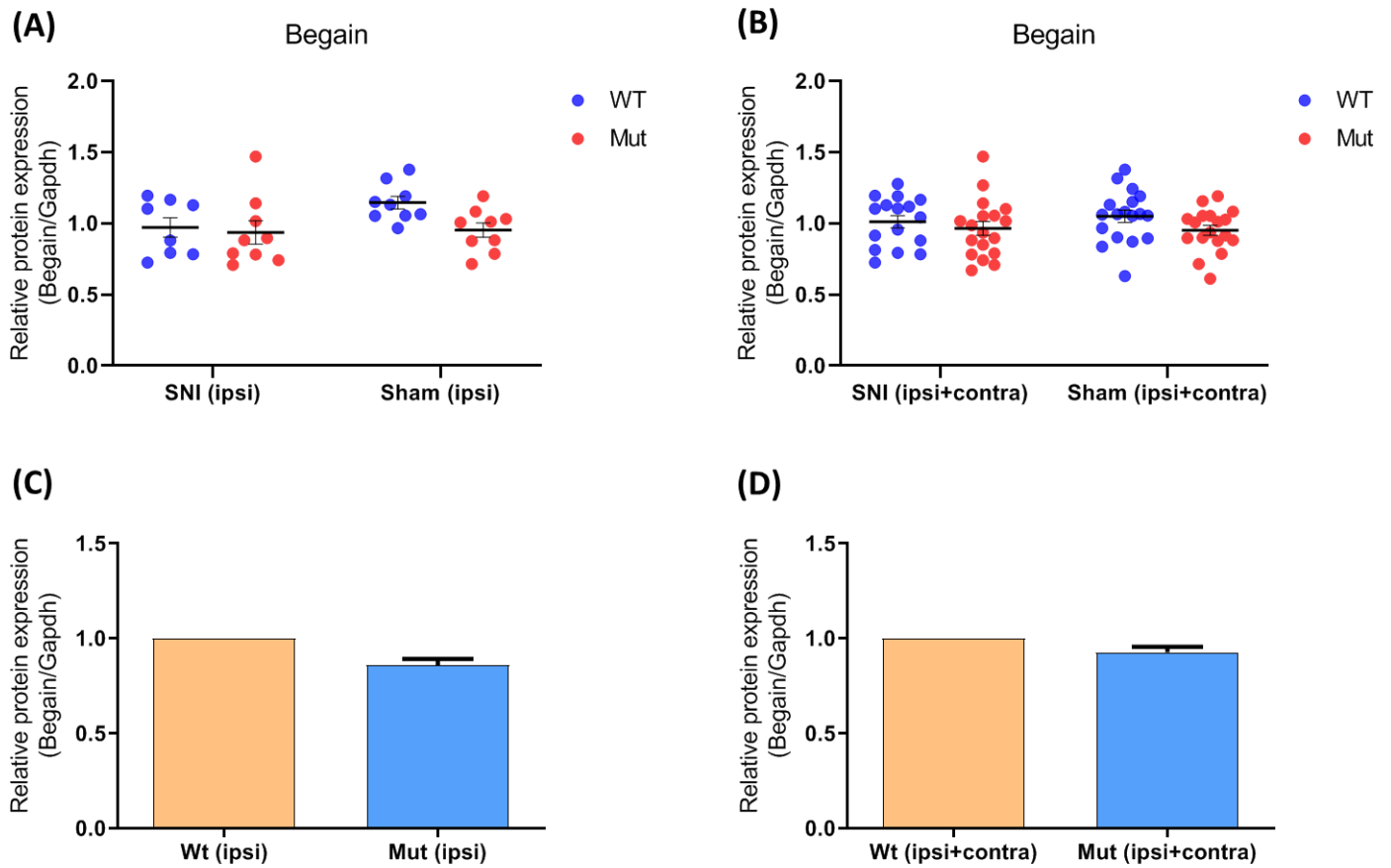


Figure 22. Relative protein expression of total Begain in spinal cord of Rep^{L1del} mutants and wildtype littermates. Expression was normalized to Gapdh. A) and C) Ipsilateral samples only (representing individuals) Wt SNI: n = 8; Wt Sham: n=9; Mut SNI: n = 9; Mut Sham: n = 9; B) and D) Ipsilateral and contralateral pooled samples. Wt SNI: n = 16; Wt Sham: n=18; Mut SNI: n = 18; Mut Sham: n = 18. Scatter plot charts: bars represent the mean ± SEM. Column bar charts: data are shown relative to WT controls (=1). p>0.05, two-way ANOVA followed by Tukey's Multiple Comparisons post-hoc test.

***Dlk1* is downregulated in SC of Rep^{L1del} mutant mice but not responding to nerve injury**

Two-way ANOVA analyses showed a strong significant effect of the genotype for the expression of the *Dlk1* gene in the spinal cord, either when considering only the ipsilateral samples or when pooling the ipsilateral and contralateral halves (Fig. 23 A, B). Thus, Rep^{L1del} mutant mice showed a significant downregulation of *Dlk1* gene expression relative to wildtype littermates, irrespective of surgical treatment. When all samples (ipsi and contralateral combined) were considered, *Dlk1* was downregulated by 52% ($p < 0.0001$); the analysis of the ipsilateral samples revealed a similar downregulation of 55% ($p < 0.0001$) (Fig. 23). These results were corroborated by the protein studies, which showed a significant reduction of the *Dlk1* variants identified by Western blot analyses (Fig. 24). Two *Dlk1* peptides with approximately 52 kDa and 60 kDa were identified in the spinal cord, the latter presenting approximately 30% of band intensity of the former. Indeed, when comparing all samples, *Dlk1* protein was reduced in mutants by 23% ($p = 0.0129$) and 24% ($p = 0.0111$), respectively for the 52 kDa and 60 kDa bands; and by 24% when both bands were quantified together ($p = 0.0075$) (Fig. 24 A-C, G-I). Accordingly, when considering the ipsilateral samples only, the observed reduction was of 30% ($p = 0.0198$) and 37% ($p = 0.0104$) for the 52 kDa and 60 kDa bands, respectively, and of 31% for total *DLK1* ($p = 0.0149$) (Fig. 24 D-F, J-L).

In accordance, the *post hoc* test showed statistically significant differences when comparing the levels of the transcript in mutant and wildtype mice subjected to SNI or sham surgery: considering the ipsilateral samples only, transcript levels were downregulated in Rep^{L1del} mutants relative to control Wt by 52% ($p = 0.0036$) and 53% ($p = 0.0012$), in SNI and sham groups, respectively (Fig. 23 A, E, and F); while in pooled ipsi and contra halves a 49% ($p < 0.0001$) and 54% ($p < 0.0001$) downregulation, respectively, was seen (Fig. 23 B).

At the protein level, the *post-hoc* analyses showed no significant differences among genotypes in the SNI group, perhaps due to the high variation of the Wt values. Nonetheless, in the sham group, the differences were more pronounced and close to statistical significance: *Dlk1* was decreased by 36% ($p = 0.1205$), 42% ($p = 0.0852$) and 32% ($p = 0.0895$) in mutants, as shown by quantification of the 52 kDa band, the 60 kDa band and total protein, respectively, in ipsilateral samples (Fig. 24 D-F). Concordant protein

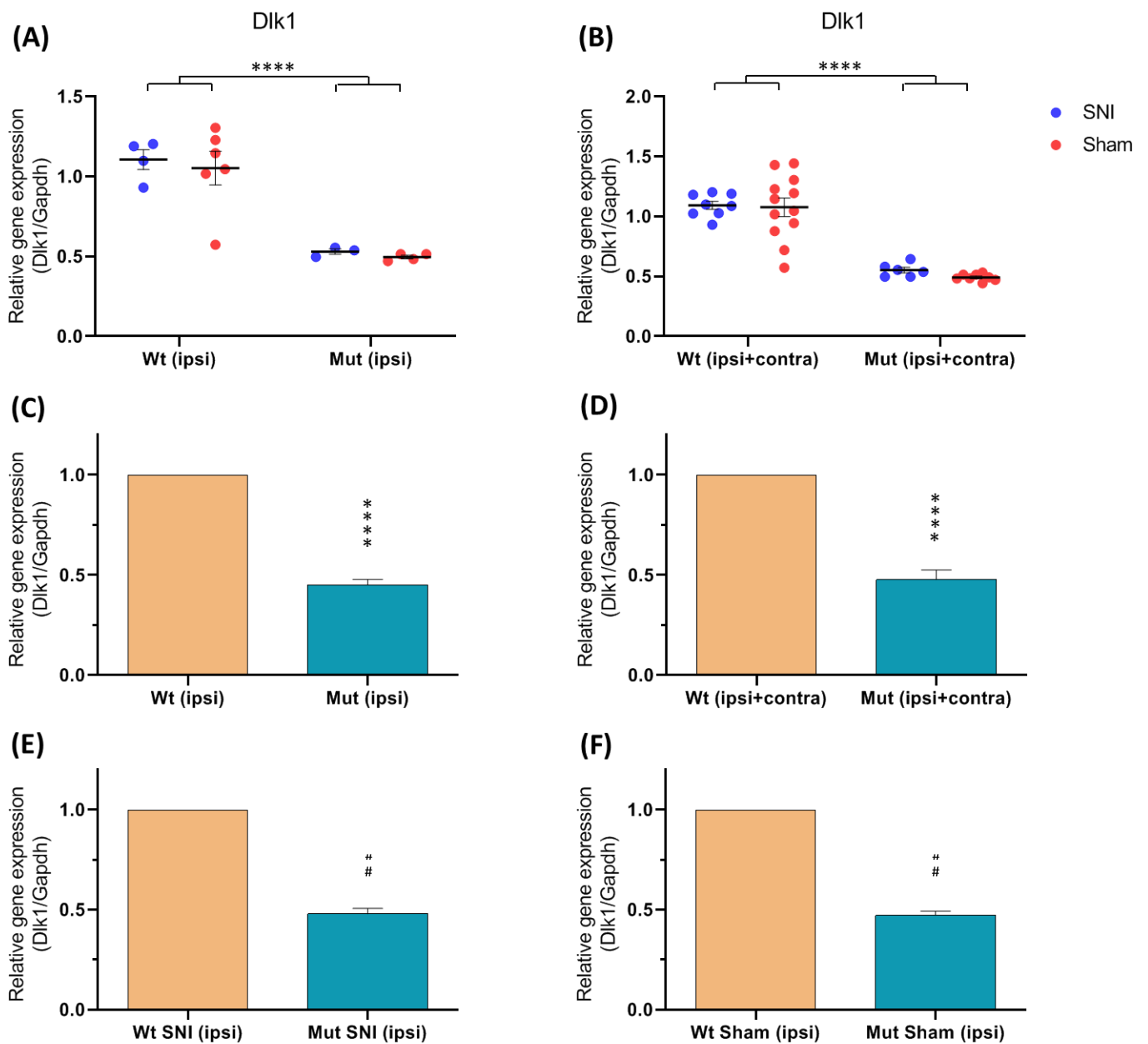


Figure 23. Relative gene expression of *Dlk1* in spinal cord of Rep^{L1del} mutants and wildtype littermates. Target gene expression was normalized to *Gapdh* expression. A), C) and E) Ipsilateral samples only (representing individuals) Wt SNI: n=4; Wt Sham: n=6; Mut SNI: n=3; Mut Sham: n=4; B), D) and F) Ipsilateral and contralateral pooled samples. Wt SNI: n=8; Wt Sham: n=12; Mut SNI: n=6; Mut Sham: n=8. Scatter plot charts: bars represent the mean $\pm$ SEM. Column bar charts: data are shown relative to WT controls (=1). Two-way ANOVA followed by Tukey's Multiple Comparisons *post-hoc* test. ****p<0.0001, for the effect of the genotype in two-way ANOVA; ##p<0.01, for the results of the *post hoc* test.

expression results were obtained when analysing the levels of expression in ipsi and contralateral pooled samples of the sham group: 31% ($p=0.0677$) and 32% ($p=0.0715$) for the 52 kDa and 60 kDa bands, respectively. When analysing the total protein, the 32% decrease in Sham mutant mice relatively to Wt reached statistical significance ($p=0.0381$) (Fig. 24 A-C and M-O).

Despite the unequivocal genotype effect found in adult spinal cord, no treatment effect was apparent for Dlk1 at the timepoint surveyed. Peripheral nerve transection did not show any influence on Dlk1 gene or protein expression levels, either in mutant or wildtype mice, indicating that spinal cord Dlk1 seems not to be functionally involved in the modulation of pain 28 days after the lesion (Fig. 23 A and B; Fig. 24 A-F).

As mentioned earlier, in general, no statistically significant differences were found in the expression of Dlk1 between the ipsilateral and contralateral samples. This was certainly the case for the gene expression studies, where in no instance differences were observed, not even close to statistical significance. Nonetheless, in protein expression analyses, a significant lower amount of Dlk1 was found in the ipsi- versus the contralateral samples following SNI (irrespective of genotype): 18% ($p=0.0301$), 15% ($p=0.0797$, close to significance) and 17% ($p=0.0192$) less protein for the 52 kDa peptide, the 60 kDa peptide and total Dlk1, respectively (not shown). By breaking down the SNI group into SNI mutants and SNI Wt littermates, these results were roughly recapitulated but significantly so in mutants only. Mutant mice subjected to SNI showed 23% less protein for the 52 kDa more abundant peptide ($p=0.0365$) and for total Dlk1 ($p=0.0451$) in the ipsilateral versus the contralateral half. The 60 kDa protein also showed a tentative lower amount (21% less) but this was far from statistical significance ($p=0.2157$) (not shown). Conversely, in mice subjected to sham surgery (irrespective of genotype), a higher amount of protein was seen in the ipsilateral sample relatively to the contralateral one, with 19% more protein for total Dlk1 ($p=0.0464$) and for the 60 kDa peptide ($p=0.0693$, close to statistical significance) and 20% more for the 52 kDa peptide ($p=0.0576$, close to statistical significance). However, although displaying a similar trend, the differences in protein expression between the ipsilateral and contralateral samples in animals of both the mutant sham and wildtype sham groups were not statistically significant (not shown).

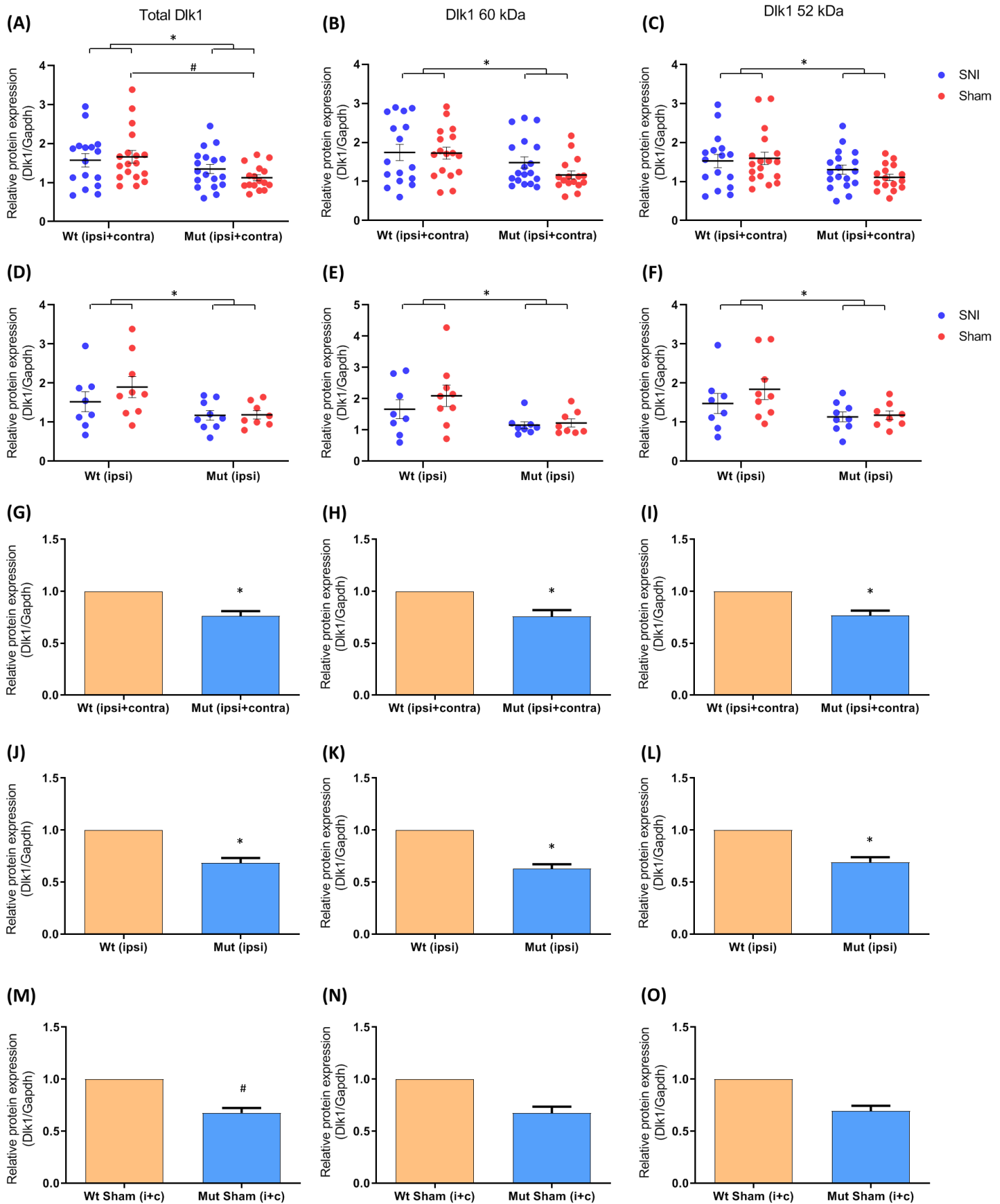


Figure 24. Relative protein expression of *Dlk1* in spinal cord of Rep^{L1del} mutants and wildtype littermates. Expression was normalized to *Gapdh*. A) to C) Ipsilateral and contralateral samples Wt SNI: n = 16; Wt Sham: n=18; Mut SNI: n = 18; Mut Sham: n = 16; D) to F) Ipsilateral samples only (representing individuals). Wt SNI: n = 8; Wt Sham: n=9; Mut SNI: n = 9; Mut Sham: n = 8. Scatter plot charts: bars represent the mean $\pm$ SEM. Column bar charts: data are shown relative to WT controls (=1). Two-way ANOVA followed by Tukey's Multiple Comparisons *post-hoc* test. *p<0.05, for the effect of the genotype in two-way ANOVA; #p<0.05, for the results of the *post hoc* test.

Imprinting studies

Allele-specific expression of *Begain* 1A, 1B and *Dlk1* was assessed in embryonic and adult DRG and spinal cord, in order to a) ascertain the normal imprinting status of the transcripts, and b) to determine if the Rep^{L1del} mutation had an impact on the regulation of imprinting in these two tissues. Wildtype and heterozygous mice (embryos and adults) were obtained from reciprocal crosses between del^{L1rep} heterozygous mutants (C57BL/6J background) and C3H/HeJ, and allele-specific expression was ascertained by RT-PCR followed by Sanger sequencing.

The three transcripts were confirmed to be imprinted in embryos and in adults, displaying their characteristic parent-of-origin specific expression as described in other tissues (Fig. 25). This was seen both in wildtype and in Rep^{L1del} mutants, indicating that the mutation has no effect on imprinting of *Begain* and *Dlk1* in dorsal root ganglia and spinal cord.

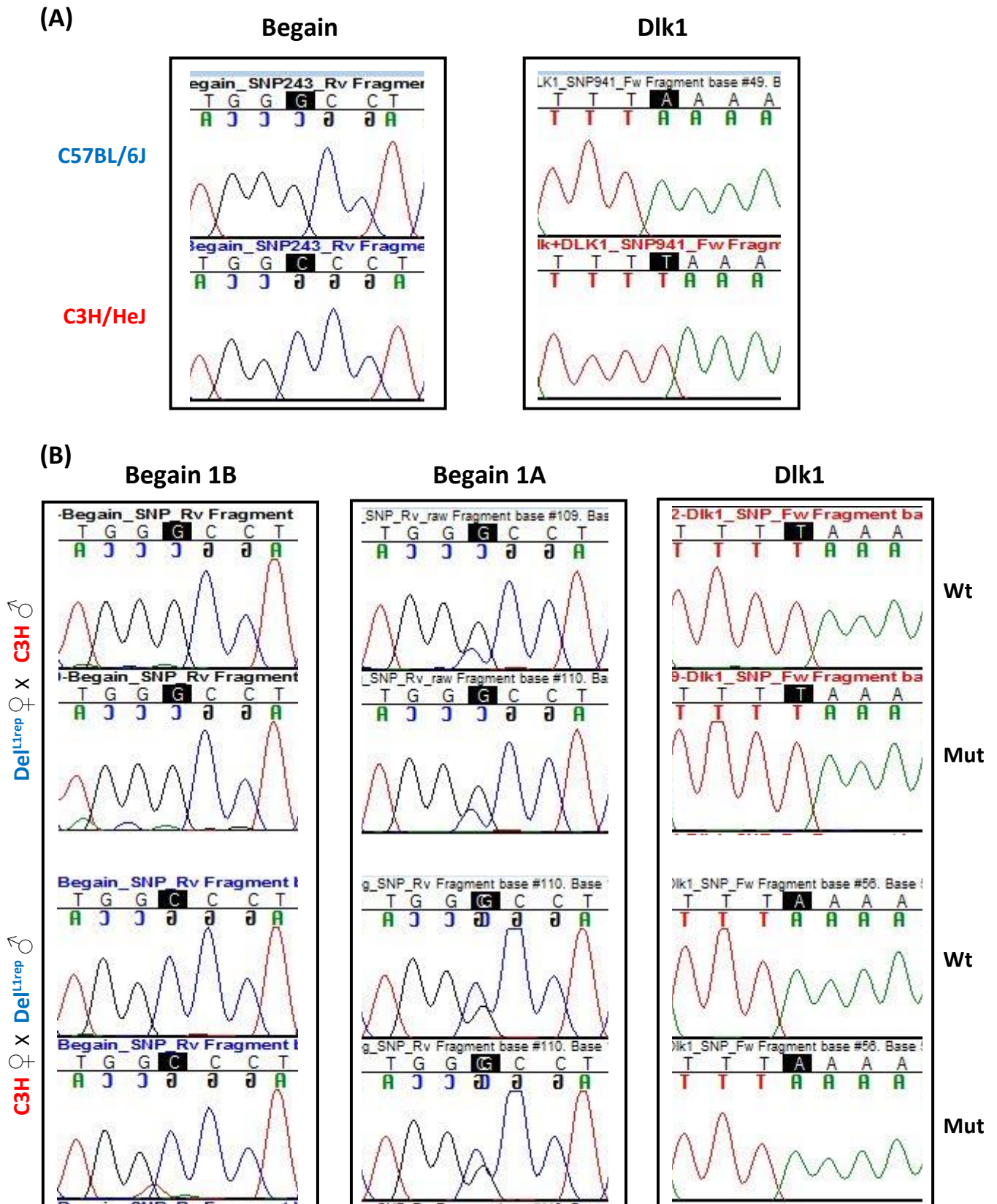


Figure 25. Allele-specific expression of *Begain 1A*, *Begain 1B* and *Dlk1* in embryonic spinal cord of Rep^{L1del} mutant and wildtype mice. Sequencing electropherograms of genomic DNA (A) and spinal cord cDNA (B). *Begain* SNP rs252334243 and *Dlk1* SNP rs253217941.

- Part II -

Production of a *Begain 1B* knockout mouse

In vitro testing of the selected crRNAs

Following the incubation of the CRISPR-Cas9 complex with a 778 bp PCR amplicon containing the crRNA target sequences, the reaction product was run on a 1% agarose gel. In a fully efficient “digestion”, the fragment was expected to be cut twice, yielding 3 bands of 142 bp, 442 bp and 194 bp. The bands observed on the gel corresponded to the intact amplicon (uncut) and to two bands pertaining the 142 bp fragment and a 636 bp fragment, which amounts to the sum of the remaining two expected fragments together (Fig. 26). These results indicate partial “digestion” of the target sequence, with the 5-prime crRNA showing robust efficiency, contrarily to that of the 3-prime crRNA. Nonetheless, the PCR product - run on its own in a separate lane of the gel in an amount equivalent to that subjected to CRISPR digestion - showed a very strong intensity denoting an excessive amount of template in the reaction, which could explain the presence of uncut amplicon (Fig. 26). Notwithstanding, and considering that in the zygote there are only 2 molecules to target (as opposed to the million generated by PCR), we decided to carry on to the *in vivo* targeting.

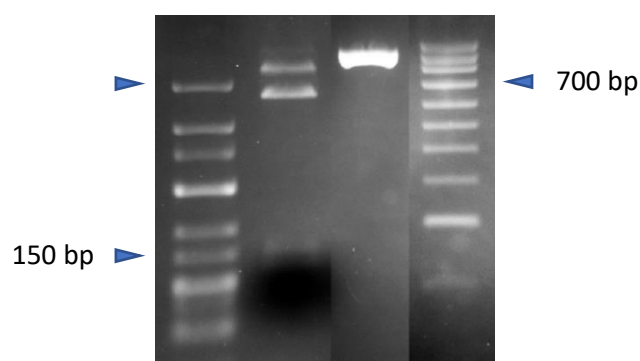


Figure 26. *In vitro* testing of the selected crRNAs for *Begain* exon 1B targeting. The crRNAs were complexed with tracrRNA and Cas9, and incubated with a PCR amplicon containing the respective target sequences (left lane). Uncut amplicon is shown on the right. Molecular weight markers: GRS Low Range Ladder (GRISP) and NZYDNA Ladder V (NZYtech).

***In vivo* targeting of mouse zygotes for genome editing**

The CRISPR-Cas9 complex targeting *Begain*'s exon 1B along with the ssDNA donor fragment were delivered into zygotes by pronuclear injection. From a total of 523 injected zygotes, 476 were transferred to pseudopregnant females (~15 zygotes per oviduct, totalling 16 females). Of an unknown number of born animals (there was evidence of some peri- and early post-natal mortality, including from cannibalism), 38 live pups were obtained and genotyped prior to weaning. Candidate samples were identified by running the PCR amplicons in 5% native PAGE (Fig. 27). These were then analysed by Sanger sequencing revealing several instances of genome alterations, ranging from cuts at a single or both CRISPR target sites, repaired by NHEJ and resulting in indels, to the removal (deletion) of the target sequence without integration of the ssDNA donor. Importantly, 1 of the 38 pups showed successful ssDNA integration. Further analysis of the region by Sanger sequencing confirmed the correct editing of the *Begain 1B* locus.

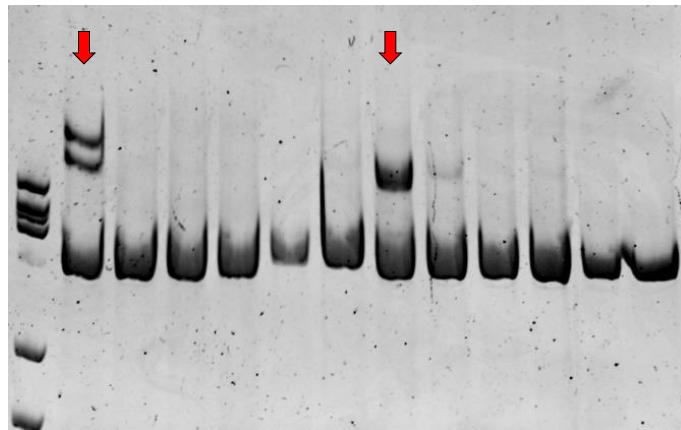


Figure 27. Detection of genomic alterations at the *Begain 1B* locus in born pups targeted with CRISPR-Cas9 at the zygote stage. PCR amplicons from the targeted region were run on a 5% native polyacrylamide gel for detection of heteroduplexes (red arrows), which indicate genomic editing. Candidate samples were then subjected to sequence analysis for characterization. Molecular weight marker: NZYDNA Ladder V (NZYtech).

The founder, a male, was crossed with C57BL/6J females and the progeny was genotyped by PCR, showing transmission of the mutant allele with an expected Mendelian ratio (Fig. 28).

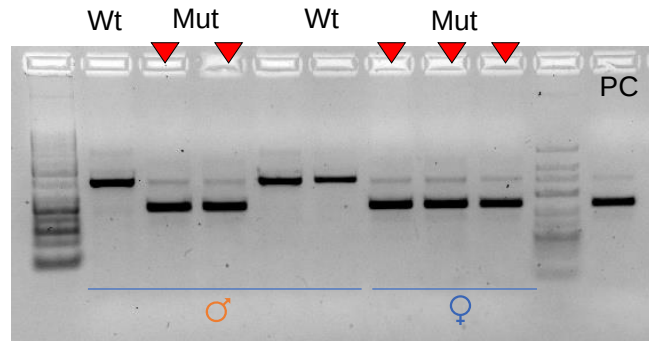


Figure 28. Multiplex PCR genotyping of the first litter (5 males and 3 females) of the *Begain 1B* KO mutant founder. The upper band corresponds to the Wt allele (400 bp fragment) and the lower band to the KO allele (242 bp fragment). The genotyping reactions are flanked by molecular weight markers (GRS Ladder 50bp, on the left, and GRS Low Range Ladder (GRISP), on the right (NZYtech)). On the far right, the genotyping PCR of the founder male was used as positive control. Mutant animals are marked with red arrowheads.

Two of the daughter mutant females were backcrossed with the founder male to generate homozygous null *Begain 1B* mice. The first 12 pups (from two litters born together in the same cage) displayed the expected Mendelian ratio of allelic inheritance: 4 wildtype, 6 heterozygotes and 2 homozygous KO animals (Fig. 29).

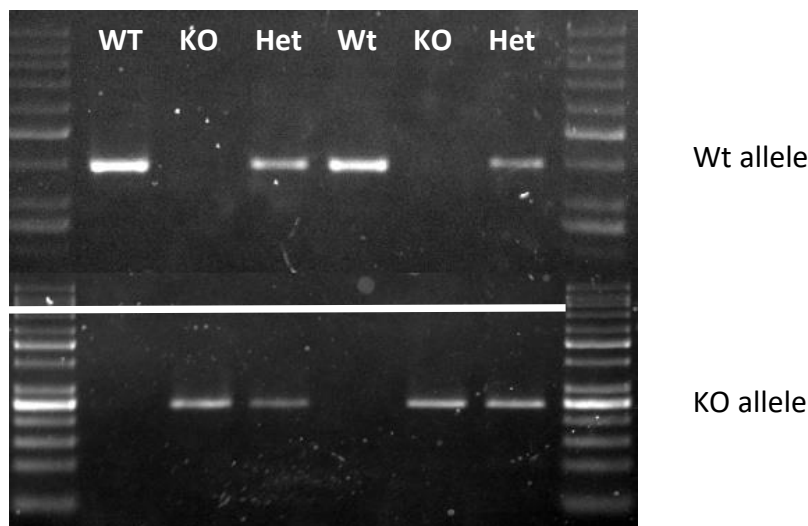


Figure 29. Gel electrophoresis of PCR amplification products from the *Begain 1B* KO and wildtype alleles. Amplicon sizes are 242 bp (upper panel) and 400 bp (lower panel), respectively. Molecular weight markers: GRS Low Range Ladder (left) (GRISP) and NZYDNA Ladder V (right) (NZYtech).

To ascertain the effect of the genomic mutation at the transcript level, total RNA from brain and spinal cord of 5 adult animals (2 Wt, 1 heterozygote and 2 KO) was extracted and reverse transcribed. *Begain 1A* and *1B* transcripts were quantified by qPCR. *Begain 1A* was expressed normally and at equivalent levels among all genotypes, indicating that the editing of exon 1B sequence at the *Begain* locus did not affect the expression of the 1A isoform, neither qualitatively nor quantitatively. Conversely, no expression of imprinted *Begain 1B* was observed in KO mutants, whereas the levels of transcription were similar between wildtype and heterozygote littermates (Fig. 30). These results indicate that the heterozygote had inherited the mutant allele from the mother (hence showing a normal expression of *Begain 1B* from the paternal allele) and, importantly, that the CRISPR-Cas9 mediated genetic manipulation and the production of a *Begain 1B* isoform-specific KO model were successful.

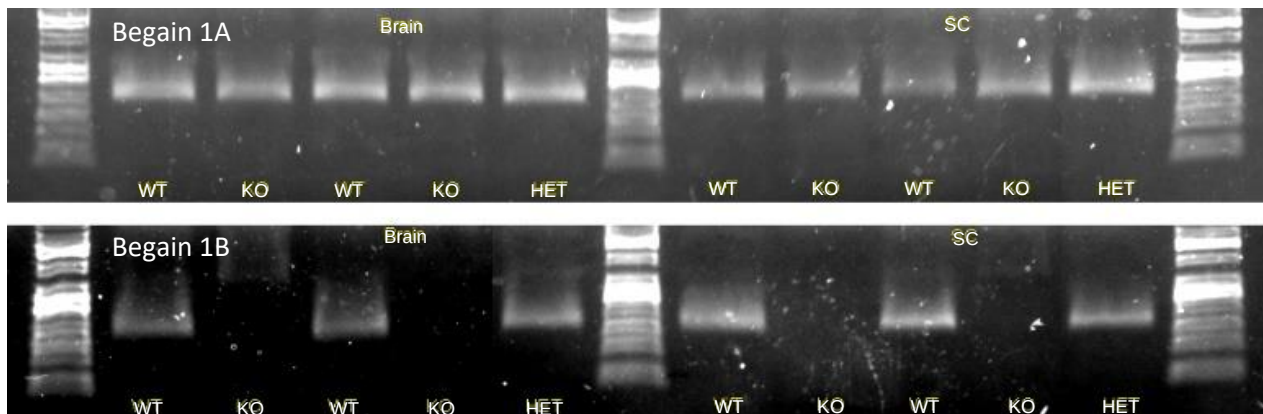


Figure 30. Gel electrophoresis of qPCR amplification products of *Begain 1A* and *1B* brain and spinal cord cDNA. Amplicon sizes are 185 bp and 153 bp, respectively. Molecular weight markers: GRS Low Range Ladder (GRISP).

Discussion

Discussion

Begain, a poorly studied gene from the *Dlk1-Dio3* imprinted domain, has been recently implicated in pathological pain transmission following peripheral nerve injury in mice (Katano et al., 2016). Interestingly, *Begain* encodes distinct paternally and biallelically expressed proteins, whose individual roles have not been explored to date. With this work, we aimed to contribute to the understanding of the role of the imprinted isoform of *Begain* (isoform 1B) in the modulation of neuropathic pain. To that effect, we have used an existing mutant mouse line closest to an isoform-specific knock-down of *Begain* 1B for pain behaviour and molecular analyses, and have ourselves produced a suitable *Begain* 1B-specific knockout mouse model for future studies. The previously generated mutant line, termed Rep^{L1del}, shows a severe downregulation of imprinted *Begain* 1B in embryonic brain while retaining normal expression levels of the biallelically expressed *Begain* 1A isoform (Soares et al., 2018). The mutation - a deletion of a 260 kb L1-rich array of repetitive sequence located between *Begain* and *Dlk1* - also affects the expression of the latter in the brain, both in embryos and in adulthood, where it was shown to be downregulated by 31-34%. In the original study, no expression analyses were conducted in other nervous tissues, namely in the dorsal root ganglia and spinal cord, both involved in pain processing.

Our own gene expression studies, performed at embryonic day 16.5 in dorsal root ganglia and spinal cord - essential components of the pain circuitry - showed a marked downregulation of imprinted *Begain* 1B in both tissues (43% and 53%, respectively) in Rep^{L1del} mutants, whereas no effect on the expression of *Begain* 1A was observed. These results are consistent with the findings of Soares and collaborators (2018) in embryonic brain, where the effect of the Rep^{L1del} mutation was seen solely on the imprinted isoform of *Begain*. The level of downregulation found here in these two nervous tissues was, however, less pronounced than that observed in the whole brain (63%). Similarly, *Dlk1* showed a 40% downregulation in embryonic spinal cord - to an equivalent level as that seen in brain - but this was not recapitulated in the DRG, where only a tendency for a (very small) downregulation was found. The results in SC are in line with previous observations in embryonic brain (Soares et al., 2018), while those in DRG represent the

first instance in which the Rep^{L1del} mutation seems not to have any effect on *Dlk1* expression in the embryo. Taken together, our results in embryonic SC and DGR proved to be in line with previous data from embryonic brain, and confirmed the putative suitability of the Rep^{L1del} mutant line as the best available model to conduct neuropathic pain behavioural studies and respective molecular analyses, in the context of the hypotheses to be tested.

Notably, Rep^{L1del} mutant mice exhibited exacerbated mechanical allodynia, a condition of abnormal pain induced by innocuous stimuli. Indeed, following SNI, von Frey test analyses showed that as soon as day 7, mutant animals displayed substantially more sensitivity to filament stimuli than the Wt animals. The results obtained were highly reproducible among 3 independent sets of animals examined, and the differences were consistently maintained at day 21 and 28. On day 14, however, even though the SNI mutants were still displaying higher sensitivity, the difference towards that of the SNI Wt animals became less pronounced and not statistically significant, due to both a decrease observed in the mutant group and an increase displayed by the Wt group. This fluctuation in the otherwise unequivocal phenotype seen at the other timepoints is difficult to explain, and may reflect inherent oscillations at the molecular level throughout the evolution of the disease. Importantly, in the wildtype group the SNI-subjected animals showed significantly increased sensitivity compared to the mice subjected to sham surgery, indicating that the pain model had been well implemented and that the von Frey assessment had been successful, as thoroughly documented in the literature (Bourquin et al., 2006). Furthermore, the differences between SNI mutants and sham animals (whether mutant or wildtype) were even more pronounced, with compelling statistical significance in all timepoints analysed. These results unequivocally show a pronounced phenotype of pain sensitization in Rep^{L1del} mutant mice relatively to wildtype littermates, and thus implicate either imprinted *Begain* or *Dlk1* (or both) in the modulation of neuropathic pain.

Begain is undoubtedly a natural candidate, not least because of its binding to NMDA receptor-associated scaffold proteins (namely PSD-95) (Deguchi et al., 1998, Naisbitt et al., 1997), as well as to its proposed recruitment to synapses in a NMDA receptor activity-dependent manner (Yao I, 2002), indicating a role in synaptic transmission. Moreover, following SNI, Begain was shown to be upregulated in lamina II

of the spinal cord dorsal horn by immunohistochemistry, and a full *Begain* knockout showed attenuated mechanical allodynia in a SNI model (Katano et al. 2016). Thus, while there is compelling evidence implicating *Begain* in neuronal activity from a functional standpoint, little is known about a putative structural role in the development of the nervous system, namely in the formation of the nociceptive circuitry during embryogenesis. Conversely, there are no data supporting a functional role for *Dlk1* in neurotransmission; rather, *Dlk1* is a well-documented regulator of differentiation in many tissues (Moore et al., 1997; Carlsson et al., 1997; Costaglioli et al., 2001; Tanimizu et al., 2004), and highly expressed through embryonic development in many cell progenitors (Smas & Sul, 1993; Bachmann et al., 1996; Christophersen, et al., 2007; Rodriguez et al., 2012; Cheung et al., 2013). Notably, *Dlk1* was shown to be highly expressed in the developing peripheral nerve, where it may regulate the myelination program during Schwann cell differentiation (Costaglioli et al., 2001). Furthermore, *Dlk1* knockout mice show compromised neurogenesis in adult brain (Ferron, 2011). These data would be more consistent with a hypothetical requirement of *Dlk1* for the proper development of the nociceptive circuitry of the embryo.

Gene and protein expression studies were conducted to investigate the dynamics of *Begain* and *Dlk1* expression in the DRG and SC of adult *Rep^{L1del}* and wildtype mice subjected to the SNI model of neuropathic pain and behaviourally assessed by the stimulus-evoked Von Frey test. Thus, these analyses were performed at a single timepoint and after a period of 28 days following SNI (or sham surgery). In adult DRG, imprinted *Begain 1B* expression was unaffected: contrarily to the downregulation observed in embryonic DRG, no genotype effect was seen in this adult tissue. Furthermore, gene levels were also unresponsive to SNI in both genotypes, suggesting that if imprinted *Begain 1B* in DRG is functionally involved in pain modulation, this is not detectable at the surveyed timepoint. Conversely, there was an upregulation of *Begain 1A* expression in DRG of mutant animals subjected to nerve injury (18%). Interestingly, this upregulation, which was corroborated by protein analyses (33%), was not seen in wildtype animals (Wt SNI vs. Wt sham), indicating that it is mutant-specific. As suggested by our results in embryonic tissue, it would not be elicited by the *Rep^{L1del}* mutation alone, as no differences in expression were found between mutants and Wt animals in sham groups. Given that

the antibody used for protein detection by Western blot analysis does not discriminate between Begain isoforms, it can be concluded that the protein increase seen in SNI mutants is likely to be that of Begain 1A. It could be speculated that this upregulation could stem from a relaxation of transcriptional control of the Begain 1A isoform caused by the Rep^{L1del} mutation, which would preclude the maintenance of the isoform steady-state levels under a situation of nerve injury. Nonetheless, we currently have no data to support this hypothesis.

Similarly, Dlk1 displayed a response to SNI in adult DRG but, in this case, it was seen only at the protein level. Indeed, *Dlk1* transcript levels were unaffected in mutants and Wt littermates following SNI or sham surgery. On the contrary, protein levels were dysregulated with the two peptides detected by Western blot analysis showing an abnormal behaviour in mutant mice. The most common isoform (60 kDa) was strongly increased in SNI relative to sham animals, irrespective of genotype. This treatment effect was clearly seen in Wt mice, where both peptides showed a tendency for a strong upregulation in SNI Wt animals (relatively to Wt sham). However, this was not seen in Rep^{L1del} mutants. Conversely, when looking to SNI-subjected animals only, the 55 KDa Dlk1 protein was significantly upregulated in Wt animals comparing to the mutant group.

Taken together, these results suggest that Dlk1 responds to nerve injury in DRG, irrespective of genotype, at this timepoint. This response appears not to involve any changes in expression at the transcript level; rather, it seems to entail a regulation at the protein level. However, whereas in wildtype mice the response is a tentatively strong increment of both Dlk1 proteins, in mutants the response is impaired and bivalent, with no changes in the 60 kDa protein levels and a tentative reduction of the 55 kDa peptide. These observations may reflect significant changes in gene expression earlier in time, in response to SNI, which could be disrupted in the mutant.

Overall, the analyses conducted in adult DRG at this timepoint appear to implicate Begain 1A and Dlk1 in a response to nerve injury. This response is mutant-specific in case of Begain 1A, suggesting that it may be pathological. Conversely, for Dlk1 the response to SNI in adult DRG seems to be physiological (since it is displayed by Wt animals as well) but dysregulated in Rep^{L1del} mutants.

In adult spinal cord, imprinted *Begain 1B* expression was downregulated by 16% in mutants relative to Wt littermates, irrespective of surgical treatment. Separating the group by treatment, *Begain 1B* was tentatively downregulated by the same amount in mutant animals of SNI and sham groups. As observed in adult DRG, no differences in transcript levels were detected in SNI-subjected animals when compared to sham controls, suggesting that if imprinted *Begain 1B* is functionally involved in pain modulation in the SC, such instance is not detectable at the level of gene expression, at the timepoint surveyed. The genotype-elicited dysregulation of *Begain 1B* seen in adult SC is found for the first time in an adult tissue of the Rep^{L1del} mutant, and is in line with the downregulation observed in the same tissue in the embryo.

The expression of *Begain 1A* showed a trend that paralleled the behaviour of the 1B isoform, as far as the genotype effect is concerned. In addition, the transcript levels of the 1A variant did not reveal a response to SNI in adult SC, either.

The gene expression results were mostly corroborated by the protein analyses, which showed the same level of downregulation of total Begain, both in a general comparison between mutant and Wt mice, irrespective of treatment, and also in the sham group, but without statistical significance. Our results are partially in disagreement with those obtained by Katano et al. (2016), who showed by immunohistochemistry that in wildtype mice subjected to SNI there was an increase of total Begain (~32%) in the spinal dorsal horn. However, it should be noted that the comparison was made between SNI-subjected mice and naïve mice (rather than animals subjected to sham surgery). Thus, the comparisons in both studies are of a different nature. Regardless, the discrepancy, if existent, may be easily explained by either the timepoint of analysis (day 7 vs. day 28 (our work)) or, conceivably, by a higher sensitivity of the immunohistochemistry method, particularly for a spatially restricted pattern of expression such as that of Begain in the spinal dorsal horn. The latter hypothesis may also explain the absence of differences in expression between the ipsilateral and contralateral samples. Indeed, the unsegmented (joint) analysis of all spinal cord laminae could have resulted in a dilution of regional or localized small differences, which ended up by not being detected. Alternatively, the contralateral side may mirror the ipsilateral stimuli-

evoked responses given the considerable time elapsed between the nerve injury and the molecular analyses (discussed ahead).

Dlk1 expression in adult SC revealed much the same trend as that of Begain, although in a much clearer fashion, displaying a substantial downregulation at both the gene and protein level in Rep^{L1del} mutants (up to approximately 54% and 31%, respectively). This behaviour was registered irrespective of treatment. Our results are in agreement with the findings of Soares and collaborators (2018) in adult brain, although in this tissue the reduction of Dlk1 protein was of ~50%, thus more consistent with the levels of downregulation seen by RT-qPCR. At the surveyed timepoint, no significant differences were noted when comparing SNI- and sham- subjected animals of either genotype. These results do not support a functional role for spinal cord Dlk1 in the modulation of neuropathic pain, at least not at 28 days after surgery, but are consistent with the Rep^{L1del}-elicited downregulation of *Dlk1* seen in embryonic spinal cord.

All of the analyses conducted in adult spinal cord were performed on the ipsilateral and contralateral halves obtained by longitudinal sectioning through the ventral median sulcus. This is common procedure when animals are subjected to a unilateral intervention with respect to the symmetry axis of the body, under the expectation that one side will serve as an internal control for the other. Notably, we found no significant differences between ipsi- and contralateral samples in most of the comparisons performed among groups for either gene. The only exceptions were those noted for Dlk1 protein expression following SNI, more specifically in mutant animals, where a smaller amount of Dlk1 was found in the ipsilateral side relatively to the contralateral half; and in sham animals, with a higher amount of protein in the ipsilateral side but that was not significant within either genotypic group. These observations are difficult to interpret, particularly considering that for the ipsilateral half there were no differences between SNI mutants and sham mutants, or between SNI Wt and sham Wt animals. It seems as though we are not seeing either an effective reduction of Dlk1 protein in the ipsilateral side of mutant mice in response to SNI, or a general increase in animals subjected to sham surgery, but instead a higher and a lower amount of protein in the contralateral side, respectively, which we cannot explain. In spite of these exceptions, our results are consistent with a wide range of examples in the literature in

which unilateral interventions produce bilateral effects (reviewed by Koltzenburg, Wall and McMahon, 1999). Indeed, contralateral reactions paralleling the ipsilateral stimuli-evoked responses have been documented, namely congruent contralateral reactions have already been described in experimental animal studies of unilateral nerve lesion.

Overall, our expression studies show a downregulation of imprinted *Begain 1B* and *Dlk1* in embryonic SC and *Begain 1B* only in embryonic DRG in Rep^{L1del} mutants, along with no detectable changes in the expression of *Begain 1A*. Together with the findings of Soares et. al (2018) in embryonic brain, the transcript levels of the imprinted *Begain* isoform 1B are thus markedly reduced in three essential components of the nervous system in the embryo, contrarily to those of the biallelically expressed isoform 1A, which is unaffected. In the adult, at the timepoint tested in our study, *Begain 1B* is not overtly responding to SNI; any effects on pain transmission would have to stem from its downregulation elicited by the Rep^{L1del} mutation in the spinal cord. On the other hand, the expression of *Begain 1A* was found to respond to nerve injury, but only in the DRG. As such, it is conceivable that it may be involved in neuropathic pain modulation in this tissue. As with *Begain 1B*, any effects on pain transmission in the spinal cord would have to stem from its downregulation caused by the mutation.

The Rep^{L1del} mutant phenotype described in this work is the opposite of that described for a *Begain* null mouse, which showed attenuated mechanical allodynia following SNI (Katano et al., 2016). In this null model, both *Begain* isoforms were knocked-out through targeting of exon 4, common to both transcripts, whereas our model is a *Begain 1B* isoform-specific knockdown, at best. Assuming that the downregulation of *Begain 1B* seen in the Rep^{L1del} mutant is sufficient to disrupt the integrity of key synaptic complexes, thus negatively impacting on pain response mechanisms, one would not necessarily expect to see a phenotype recapitulating that of the *Begain* null mouse. It is noteworthy that *Begain* has been shown to form dimers or tetramers by the N-terminal region (Yao et al, 2002), suggesting that multimerization plays a role on *Begain*'s function, conceivably in the clustering of receptors. One could speculate that the interplay between the *Begain* isoforms, the balance between the nuclear and synaptic forms, as well as the level and/or type of multimerization, may regulate a dynamic behaviour in

response to specific sensory stimuli. Conceivably, the main driver of this “plasticity” could be the imprinted isoform of *Begain*, which by definition is subjected to a tighter regulation and that may dynamically integrate environmental stimuli through epigenetic processes. Whereas a dysregulation of *Begain 1B* was not unequivocally observed in the DRG or SC 28 days after surgery in response to nerve injury, we cannot exclude the possibility that the expression of the imprinted isoform could have been severely affected earlier in time. Indeed, this is also true for *Begain 1A* although perhaps less expected considering that the *Rep^{L1del}* mutation has no effect on this isoform in the embryo. This is one of the limitations of our work, whose molecular analyses were confined to a single timepoint and perhaps far too distant from the initial insult. Additionally, a structural analysis of the peripheral nociceptive circuitry would have proven useful to investigate any malformations or structural changes that could be attributed to the *Rep^{L1del}* mutation and, in turn, to the downregulation of *Begain 1B* and/or *Dlk1*. However, having done immunohistochemical analyses, Katano et al. (2016) did not report any obvious structural changes in the nociceptive system of the *Begain* null mice, although the data focused mainly on the spinal cord (the DRG were not investigated).

The downregulation of *Dlk1* in the spinal cord - both pre-natal and post-natal - may also underlie the phenotype seen in *Rep^{L1del}* mutant mice. Although gene and protein expression were unaffected in mutant animals in response to nerve injury, at the surveyed timepoint, they may be compromised earlier in time, following the insult. Indeed, we saw such an effect in adult DRG, where *Dlk1* protein levels (the more abundant 55 kDa peptide) respond to SNI differently depending on the genotype, with a significant strong SNI-elicited increase in Wt animals and a tendential decrease in mutants. To our knowledge *Dlk1* has never been implicated in synaptic transmission or neuronal plasticity; however, it is highly expressed in the developing peripheral nerve (Costaglioli et al., 2001). Conceivably, a full dosage of *Dlk1* may be required for normal development of the embryonic peripheral nerve by regulating neuronal cell differentiation, and/or its maintenance for proper operation in the adult. We are not aware of peripheral nervous system abnormalities having been described in *Dlk1* null mutants, but this may be because these animals display neonatal lethality and a whole range of malformations (mainly arising from compromised homeostasis/metabolism)

(Moon et al., 2002), which mask less obvious phenotypes or preclude their assessment. Consequently, we cannot resort to the analysis of the *Dlk1* KO mouse model to investigate the involvement of *Dlk1* in the modulation of chronic pain. Indeed, since *Dlk1* is paternally expressed, a heterozygous *Dlk1* mutant with the mutation on the maternal allele (*Dlk1*^{-/-}) would be phenotypically equivalent to a wildtype, whereas a heterozygote bearing the mutation on the paternally-inherited allele (*Dlk1*^{+/-}) would be equivalent to the KO null homozygote. The use of a tissue-specific inducible conditional knockout of *Dlk1* in DRG and spinal cord could possibly circumvent these limitations and constitute a way forward.

However and whenever any changes of gene expression may occur in DRG and spinal cord of Rep^{L1del} mutant mice, they are not caused by a relaxation (loss) of imprinting of *Begain 1B* and *Dlk1*, nor by acquisition of imprinting of *Begain 1A*. Our imprinting studies show that Rep^{L1del} has no effect in the imprinting status of any of the genes in these two tissues, where the characteristic patterns of allele-specific expression were observed. These results are in agreement with the work by Soares et al. (2018), which showed that, at least in brain, the repeat array has gained no role in the control of monoallelic gene expression within the Chr.12 imprinted cluster under normal developmental or physiological conditions. However, it should be noted that our imprinting analyses were carried out on naïve animals. Whether nerve injury or similar insults underlying pathological pain may influence imprinting of *Begain* and *Dlk1* in dorsal root ganglia and/or spinal cord remains to be determined.

Conclusion and future perspectives

Given the above constraints and, until recently, the lack of a KO model of imprinted *Begain 1B*, the Rep^{L1del} line was a good compromise model to probe for a putative involvement of imprinted genes (particularly *Begain*) in the modulation of neuropathic pain. The study of this model has enabled us to identify a clear pain sensitization phenotype, which, for the first time, has been associated to imprinted genes, and is thus suggestive of a putative role for genomic imprinting in modulating neuropathic and, perhaps more generally, chronic pain. Further studies are warranted to produce a clearer picture of what is unfolding at the molecular level in the nociceptive circuit upon nerve lesion, namely the analysis of additional timepoints closer to the time of injury, as well as histological studies to ascertain structural and molecular phenotypes *in situ*. Importantly, with the production of the isoform-specific *Begain 1B* KO model, we can now probe the effect of imprinted *Begain*, specifically, in chronic pain processing in the context of a nearly intact genomic locus and in the absence of confounding players.

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