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ARMANDO CARDOSO

O SISTEMA HIPOCAMPO - CÓRTEX RETROSPLÉNICO NA EPILEPSIA EXPERIMENTAL:

ESTUDOS MORFOLÓGICO E COMPORTAMENTAL

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Dissertação de candidatura ao grau de Doutor em Neurociências apresentada à

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Esta investigação foi realizada no Centro de Morfologia Experimental – Unidade 121/94 da Fundação para a Ciência e a Tecnologia (FCT), sediado no Instituto de Anatomia da Faculdade de Medicina da Universidade do Porto. O candidato realizou o trabalho experimental com o apoio de uma bolsa de Doutoramento (SFRH/BD/21596/2005) atribuída pela FCT.

Orientador: Professor Doutor Nikolay Lukoyanov (Professor Associado da Faculdade de Medicina da Universidade do Porto).

Co-orientador: Professora Doutora Maria Dulce Madeira (Professora Catedrática da Faculdade de Medicina da Universidade do Porto).

O candidato declara que teve contribuição determinante na realização do trabalho experimental (execução de protocolos e elaboração técnica), interpretação dos resultados e discussão dos mesmos. Além disso, contribuiu activamente para a redacção dos trabalhos apresentados.

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Doutor João José Oliveira Malva, investigador do Centro de Neurociências e Biologia Celular da Universidade de Coimbra.

Divisão Académica, 24 de Fevereiro de 2011

O Presidente do Conselho Científico

(Doutor J. Agostinho Marques)

À minha mãe e ao meu pai.

Ao Alexandre e ao Carlos.

À Carla.

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LISTA DE ABREVIATURAS

BDNF	*Brain-derived neurotrophic factor*
CA3	Região 3 do Corno de Ámon
CA1	Região 1 do Corno de Ámon
ECS	Choques electroconvulsivos
ELT	Epilepsia do lobo temporal
FH	Formação do hipocampo
GABA	Ácido gama-aminobutírico
ILAE	Liga internacional contra a epilepsia
NGF	*Nerve growth factor*
NMDA	N-metil-D-aspartato
NPY	Neuropeptídeo Y
TrkB	Tirosina cínase B

ÍNDICE

INTRODUÇÃO

A epilepsia é uma manifestação clínica que surge num vasto grupo de patologias neurológicas e se caracteriza por convulsões espontâneas e recorrentes (Fisher et al., 2005). É consequente à hiperactividade anómala e sincronizada de populações neuronais em áreas específicas do sistema nervoso central (Fisher et al., 2005). A epilepsia do lobo temporal (ELT) é a mais frequente nos adultos (Engel, 1996) e foi descrita pela primeira vez por Hughlings Jackson que associou diversos sintomas clínicos, actualmente classificados como crises parciais complexas, com lesões estruturais no lobo temporal (Jackson e Stewart, 1899; Taylor e Marsh, 1980). As crises com origem no lobo temporal caracterizam-se, geralmente, por um baixo limiar epileptogénico, sensações de *déjà vu* e *jamais vu*, e automatismos oro-alimentares, como por exemplo *chewing*, *lip smacking*, *tooth grinding* (Fakhoury et al., 1994; French et al., 1993). As crises mais comuns na ELT são as parciais (focais) complexas com origem numa região do lobo temporal bem definida, nomeadamente no hipocampo, córtex entorrinal ou amígdala, que se acompanham de alteração temporária do estado de consciência (ILAE, 1989). Registe-se, todavia, que a actividade paroxística mencionada pode propagar-se a partir do seu local de origem para outras áreas corticais e subcorticais, incluindo o hemisfério cerebral contra-lateral e, assim, originar crises generalizadas secundárias (Engel, 1989; Fakhoury et al., 1994; French et al., 1993).

A ELT está frequentemente associada a morte neuronal e gliose na fáscia denteada e no hipocampo próprio (Babb e Brown, 1987; Blümcke et al., 2000; Bruton, 1988; Margerison e Corsellis, 1966). Idênticas alterações degenerativas, embora menos acentuadas, foram também descritas noutras áreas do lobo temporal, nomeadamente na amígdala e nos córtices entorrinal, perirrinal e piriforme (Du et al., 1993; Fujikawa et al., 2000; Margerison e Corsellis, 1966; Pitkänen et al., 1998). A ELT pode ainda ser acompanhada de neurogénese (Scharfman e McCloskey, 2009; Siebzehnrubl e Blumcke, 2008), *regrowing* axonal e dendrítico (Isokawa, 2000; Mathern et al., 1995a; Sutula et al., 1989; von Campe et al., 1997) e alterações das características neuroquímicas de diversas populações neuronais (Bernard et al., 2004; de Lanerolle et al., 1995; During e Spencer, 1993; Mathern et al., 1997).

A ELT estabelece-se, geralmente, de modo faseado. O processo inicia-se com a formação da lesão inicial, de origem multifactorial, como por exemplo convulsões febris,

traumatismos cranianos ou acidentes vasculares cerebrais (French et al., 1993; Mathern et al., 1996). Instalada a lesão, segue-se a segunda fase onde avulta o processo epileptogénico propriamente dito, que envolve uma cascata de alterações moleculares e estruturais que modificam, progressivamente, o padrão de conectividade neuronal na zona da lesão (Jutila et al., 2002; Pitkänen e Lukasiuk, 2009). Durante este período, que é de duração variável, o doente não apresenta ainda convulsões epilépticas espontâneas (French et al., 1993; Mathern et al., 1995b; Spencer e Spencer, 1994). Por fim, desencadeia-se a fase crónica da ELT que se caracteriza pela formação de uma rede neuronal epileptogénica que tem um padrão anómalo de sincronização e hiper-excitabilidade. É a actividade desta rede que está na génese das crises epilépticas espontâneas (Ben-Ari et al., 2008; Jutila et al., 2002). Uma vez estabelecida a fase crónica, a rede neuronal reveste-se de características dinâmicas, podendo as convulsões epilépticas recorrentes contribuir, por si só, para a progressão da patologia e agravamento dos demais sintomas, como por exemplo, os défices cognitivos (Bernhardt et al., 2009; Nearing et al., 2007; Pitkänen e Sutula, 2002; Sutula et al., 2003; Williams et al., 2009).

É importante realçar que, em muitos pacientes com ELT, podem existir modificações estruturais na região hipocampal, nomeadamente esclerose hipocampal, que estão na base de graves problemas neurológicos. Destes, salientam-se a resistência das crises convulsivas aos tratamentos farmacológicos, que pode ocorrer em cerca de um terço dos pacientes com ELT (Regesta e Tanganelli, 1999; Sander, 2003). A nível clínico salientam-se graves défices cognitivos e várias alterações de foro afectivo (Helmstaedter e Elger, 2009; Marques et al., 2007; Walpole et al., 2008). Estas alterações não surpreendem, atendendo ao facto dos circuitos neuronais do lobo temporal estarem envolvidos na memória para factos e eventos recentes (*recent memory*), na orientação espacial, no reconhecimento de objectos e na regulação emocional (Buffalo et al., 2006; Olson et al., 2007; Phelps, 2004; Ryan et al., 2010; Shrager et al., 2007). Trata-se, pois, de uma temática ainda pouco conhecida mas muito atractiva, o que explica serem em número elevado os investigadores que se dedicam a tentar esclarecer os mecanismos moleculares e celulares da epileptogénese com objectivo de melhor compreenderem as causas e as consequências das crises epilépticas.

Um dos aspectos chave da ELT é que incide sobre um conjunto de estruturas corticais que estão interligadas, entre as quais se destacam a formação do hipocampo (FH), o córtex entorrinal e o córtex retrosplénico (Du et al., 1993; Düzel et al., 2006; Fujikawa et al., 2000; Margerison e Corsellis, 1966). A FH, que engloba a fáscia denteada, o hipocampo próprio e o complexo subicular, é a região mais afectada na ELT. A esclerose hipocampal é o tipo de neurodegenerescência mais comum nestes pacientes (Margerison e Corsellis, 1966), tendo sido observada em cerca de 65% dos pacientes sujeitos a cirurgia de ressecção hipocampal para tratamento da ELT refractária (Bruton, 1988; Margerison e Corsellis, 1966; Xu et al., 2007). Caracteriza-se pela morte dos interneurónios GABAérgicos e das células musgosas do hilo da fáscia denteada (Blümcke et al., 1999; de Lanerolle et al., 1989), bem como das células piramidais das regiões CA3 e CA1 do hipocampo (Babb e Brown, 1987; Bruton, 1988; Margerison e Corsellis, 1966). Além disso, a ELT pode acompanhar-se de diversas alterações das células granulares da fáscia denteada, nomeadamente dispersão e diminuição da sua densidade (El Bahh et al., 1999; Houser, 1990), neurogénese (Blümcke et al., 2001; Siebzehnrubl e Blümcke, 2008), *sprouting* axonal (Sutula et al., 1989), modificação do padrão de arborização dendrítica (von Campe et al., 1997), alteração do número e do tamanho das espinhas dendríticas (Isokawa, 2000) e sinaptogénese (Mathern et al., 1995a; Wittner et al., 2001). Pode ainda ser acompanhada de alteração dos níveis de síntese e expressão de neurotransmissores (During e Spencer, 1993), neuropeptídeos (de Lanerolle et al., 1995; Sundstrom et al., 2001), neurotrofinas (Mathern et al., 1997; Takahashi et al., 1999), receptores (de Lanerolle et al., 1995; Ozbas-Gerçeker et al., 2004) e canais iónicos (Bernard et al., 2004; Huang et al., 2009; Pitkänen e Lukasiuk, 2009) nas regiões envolvidas na génese e propagação das convulsões epilépticas.

A FH é fundamental para a aquisição e consolidação da memória explícita (Eichenbaum, 1999; Squire, 1992; Squire e Zola-Morgan, 1991). O seu envolvimento na memória foi descoberto no paciente H.M. que sofria de ELT com esclerose hipocampal unilateral e que após hipocampectomia bilateral começou a evidenciar amnésia anterógrada (Scoville e Milner, 1957). A integridade estrutural e funcional da FH é particularmente importante na memória espacial. A este respeito refira-se que muitos dos neurónios hipocampais e subiculares actuam como *place cells* e *head direction cells*, estabelecendo assim uma representação neuronal de determinado ambiente espacial. A

FH exerce, também, papel importante na memória de trabalho, visto estabelecer conexões recíprocas com o córtex prefrontal dorsolateral. Mais ainda, a FH, embora de modo não primordial, participa na aquisição da memória emocional (LeDoux, 2000) e na regulação da resposta hipotalámica ao stresse (Sapolsky et al., 1984).

O complexo subicular é constituído pelo subículo, pré-subículo e para-subículo (Witter et al., 1989) embora haja autores que consideram uma 4ª região denominada pós-subículo (van Groen e Wyss, 1990b). O complexo subicular é a principal área de projecção da FH e representa um centro de interligação do hipocampo próprio e dos córtices entorrinal e retrosplénico (Amaral e Witter, 1995; Lopes da Silva et al., 1990). Estudos realizados em secções hipocampais de pacientes com ELT sugerem que o subículo é local de origem de actividade convulsiva espontânea e sincronizada (Cohen et al., 2002). Por sua vez, o pré-subículo e o para-subículo parecem, também, estar implicados na ELT devido às conexões que estabelecem com as camadas III e II do córtex entorrinal, respectivamente (Eid et al., 1996). Refira-se, aliás, que a actividade anómala das fibras pré-subiculares parece estar na base da morte neuronal da camada III do córtex entorrinal observada nos modelos experimentais de ELT (Eid et al., 2001).

O córtex entorrinal, por alguns autores considerado como parte integrante da FH (Amaral e Witter, 1995), dá origem à principal aferência da fáscia denteada, a via perfurante, e recebe grande parte das eferências subiculares (Amaral e Witter, 1995; Lopes da Silva et al., 1990; Witter et al., 2000). É consensualmente reconhecido estar implicado na ELT, uma vez que nesta situação se observa morte neuronal na camada III, acompanhada de perda, embora menos acentuada, de células estreladas da camada II (Du et al., 1993; Schwarcz et al., 2000). É de salientar que os axónios destas células participam na formação da maioria das sinapses excitatórias da camada molecular da fáscia denteada, e a sua hiperexcitabilidade exerce um papel importante no processo epileptogénico (Bear et al., 1996; Scharfman et al., 1998). Por este motivo, alguns autores defendem que a via entorrino-denteada tem um papel pivô no estabelecimento de redes epileptogénicas na ELT (Bartolomei et al., 2005; Roch et al., 2002). Funcionalmente, o córtex entorrinal exerce papel preponderante na formação da memória a longo prazo (Squire e Zola-Morgan, 1991) e na representação de mapas neuronais de ambientes espaciais, através de múltiplos *place fields* organizados em

padrão hexagonal e que correspondem à estrutura geométrica do espaço (*grid cells*) (Fyhn et al., 2004; Hafting et al., 2005).

O córtex retrosplénico estabelece marcadas conexões com o córtex entorrinal e com o complexo subicular (van Groen e Wyss, 1990a, 2003; Wyss e van Groen, 1992). Tal como o córtex entorrinal, funciona como ponte entre as estruturas arquicorticais da FH e o neocórtex. Apesar desta estreita relação com estruturas implicadas na epilepsia, nomeadamente a FH (Wyss e van Groen, 1992), tem sido objecto de poucos estudos. Trabalhos recentes indicam que o córtex retrosplénico está implicado na ELT. Düzel e colaboradores (2006) demonstraram haver correlação, em pacientes com ELT, entre atrofia hipocampal e perda de substância cinzenta no córtex retrosplénico. Noutro estudo, verificou-se que convulsões generalizadas secundárias tónico-clónicas estão associadas à redução da sua espessura (Bernhardt et al., 2008). Além destes, um estudo experimental realizado em ratos que sofreram *status epilepticus* parece também apoiar o envolvimento do córtex retrosplénico na epilepsia, uma vez que demonstrou haver alterações das arborizações dendríticas nos neurónios piramidais da camada V deste córtex (Ampuero et al., 2007). O córtex retrosplénico é constituído por duas áreas, granular e desgranular, sendo a primeira subdividida em córtices granular *a* e granular *b*. Refira-se que destas subdivisões, o córtex retrosplénico granular *b* parece estar especialmente implicado na epilepsia, uma vez que, contrariamente às outras subdivisões, recebe fortes projecções do núcleo talâmico anteroventral (van Groen e Wyss, 2003; Wyss e van Groen, 1992), que é fundamental para a propagação das convulsões (Dubé et al., 1998; Mraovitch e Calando, 1999). Estudos recentes demonstraram que o córtex retrosplénico está envolvido em funções cognitivas, dado que lesões específicas deste córtex provocam alterações significativas da aprendizagem e memória espacial (Lukoyanov et al., 2005; van Groen et al., 2004; Vann et al., 2003, 2009). Além disso, está implicado nos comportamentos designados por *discriminative avoidance* e *active avoidance* (Freeman et al., 1996; Gabriel e Sparenborg, 1987; Lukoyanov e Lukoyanova, 2006), aprendizagem visual discriminativa (Bussey et al., 1996, 1997), armazenamento das associações acção-recompensa, e medo condicionado contextual (Keene e Bucci, 2008). Estudos imagiológicos realizados no Homem sugerem ainda que este córtex tem papel na interface entre as emoções e a memória episódica (Maddock, 1999).

Apesar dos numerosos estudos realizados nos domínios da morfologia, electrofisiologia e genética, os mecanismos que levam determinadas populações neuronais a tornarem-se hiperactivas e sincronizadas ainda estão longe de estarem esclarecidos. Uma das hipóteses avançadas para explicar a epileptogénese defende a necessidade de uma lesão inicial, indutora de *sprouting* das fibras excitatórias que converterá as respectivas células alvo em populações neuronais hiperexcitáveis (Tauck e Nadler, 1985). É a designada hipótese de reorganização axonal e sináptica da epileptogénese que, a nível experimental, tem sido testada no circuito neuronal da fáscia denteada de roedores. Tal deve-se ao facto da excitabilidade das células granulares ser modulada por interneurónios GABAérgicos, muitos dos quais capazes de expressar neuropeptídeo Y (NPY) e/ou somatostatina, e pelas células musgosas, as principais células glutamatérgicas do hilo da fáscia denteada. As células musgosas recebem aferências excitatórias das células granulares e projectam para a camada molecular interna da fáscia denteada. A morte das células musgosas, uma das populações neuronais mais vulneráveis às convulsões epilépticas, conduz à perda de sinapses na camada molecular interna, o que despoleta o *sprouting* dos axónios das células granulares, as fibras musgosas, para esta área. O *sprouting* destas fibras para os dendritos dos mesmos neurónios torna as células granulares auto-excitáveis, promovendo assim a formação de um circuito recorrente excitatório (Babb et al., 1991; Tauck e Nadler, 1985; Wuarin e Dudek, 1996).

Uma hipótese alternativa, denominada *dormant basket cell hypothesis*, defende que a diminuição da actividade das células em cesto da fáscia dentada provocaria um desequilíbrio no circuito hipocampal com diminuição da inibição (Sloviter, 1987, 1991). As células em cesto são GABAérgicas, e portanto inibitórias e resistentes às convulsões epilépticas. Recebem aferências excitatórias das células musgosas e formam sinapses perissomáticas com as células granulares, exercendo acção inibitória nesta população. Assim, a morte das células musgosas levaria à deaferenciação das células em cesto que, desprovidas do tono excitatório, se tornariam inactivas e incapazes de promover a inibição das células granulares (Sloviter, 1991). Esta hipótese sugere que a hiperexcitabilidade do circuito hipocampal resulta da perda de neurónios vulneráveis que normalmente são responsáveis pela excitação das células em cesto inibitórias.

Outro possível mecanismo do processo epileptogénico fundamenta-se na perda de interneurónios GABAérgicos responsáveis pelo controlo inibitório dos neurónios. Destes interneurónios salientam-se os produtores de NPY. Este neuropeptídeo exerce efeitos anticonvulsivantes e neuroprotectores através da inibição da transmissão glutamatérgica na fáscia denteada e nas regiões CA3 e CA1 do hipocampo (Bacci et al., 2002; Baraban, 2004; Qian et al., 1997; Silva et al., 2001; Sperk et al., 2007). A depleção de NPY levaria à desinibição dos neurónios hipocampais, tornando-os mais susceptíveis às convulsões. Esta hipótese é sustentada por trabalhos experimentais onde se demonstrou que ratinhos *knockout* do gene NPY desenvolvem convulsões mais acentuadas (Baraban et al., 1997), enquanto ratos transgénicos com expressão aumentada de NPY (Vezzani et al., 2002b) ou ratos sujeitos a injecção de vector viral adeno-associado recombinante contendo o gene humano NPY recombinante (Richichi et al., 2004) são mais resistentes às mesmas. Nesta linha estão também os estudos onde se detectou aumento da expressão de NPY nos interneurónios GABAérgicos durante e logo após as convulsões (Mikkelsen e Woldbye, 2006; Vezzani et al., 1996; Vezzani e Sperk, 2004). Além disso, tem sido demonstrado que as convulsões, induzidas por *status epilepticus* ou *kindling*, podem aumentar a síntese de NPY nas células granulares glutamatérgicas, efeito que pode persistir por longos períodos de tempo (Marksteiner et al., 1990; Nadler et al., 2007; Sperk et al., 2007; Vezzani e Sperk, 2004). A expressão aumentada de NPY nessas regiões pode representar um mecanismo adaptativo de compensação da hiperexcitabilidade associada às convulsões (Vezzani et al., 1999).

Existem ainda outros mecanismos moleculares e celulares que podem potencialmente contribuir para o processo epileptogénico. Tal é o caso do aumento da neurogénese pós-natal na fáscia denteada que ocorre em modelos experimentais de epilepsia (Bengzon et al., 1997; Parent et al., 1997) e em crianças com ELT (Blümcke et al., 2001), das alterações nos sistemas de neurotransmissão, como por exemplo a insuficiente libertação de GABA (During et al., 1995) ou as alterações dos receptores $GABA_A$ (Brooks-Kayal et al., 1998; Kamphuis et al., 1995), das modificações dos canais iónicos (Aronica et al., 2001), das alterações nos níveis de expressão de neurotrofinas (Zhu e Roper, 2001), da gliose (Wetherington et al., 2008), da angiogénese (Rigau et al., 2007) ou, ainda, de mecanismos epigenéticos (Huang et al., 2002; Sng et al., 2006; Tsankova et al., 2004).

Os modelos experimentais de ELT permitem analisar as diferentes variáveis que alegadamente estão relacionadas com a doença, tais como a frequência e a duração das crises e características genéticas. São vários os modelos experimentais animais de ELT, embora englobáveis em dois grupos: os que se centram nas modificações morfofuncionais características da fase crónica da ELT e os que incidem nas alterações cerebrais que ocorrem imediatamente após a lesão inicial.

Os modelos crónicos de ELT são geralmente induzidos por *kindling* ou *status epilepticus*. O *kindling* consiste na administração de estimulações eléctricas sub-convulsivas que provocam uma progressiva redução do limiar convulsivo (Goddard et al., 1969). O *status epilepticus* é caracterizado por crises epilépticas contínuas que se prolongam por vários minutos (DeLorenzo et al., 1995; Knake et al., 2009). Pode ser induzido por estimulação eléctrica de diferentes estruturas límbicas (Bertram e Cornett, 1994; Brandt et al., 2004; Gorter et al., 2001; Mazarati et al., 1998; Sloviter, 1987) ou administração sistémica de agentes químicos convulsivantes, como a pilocarpina ou o ácido caínico (Covolan e Mello, 2000; Leite et al., 2002). Destes, destaca-se o recurso à pilocarpina, que é, provavelmente, o modelo de *status epilepticus* mais utilizado. A pilocarpina é um agonista colinérgico muscarínico que, após injecção sistémica, induz crises epilépticas, *status epilepticus* e danos cerebrais (Covolan e Mello, 2000; Leite et al., 2002; Turski et al., 1983). A capacidade da pilocarpina induzir *status epilepticus* depende dos receptores muscarínicos M1 (Hamilton et al., 1997), cuja activação provoca alteração no equilíbrio entre a transmissão excitatória e a inibitória e, consequentemente, *status epilepticus* (Priel e Albuquerque, 2002). Após activação dos receptores M1, as crises são mantidas pelos receptores NMDA (Smolders et al., 1997). Este modelo apresenta numerosas vantagens, apesar de possuir também alguns inconvenientes. Das primeiras salientam-se: 1) a indução de *status epilepticus*, que ocorre mais rapidamente que nos outros modelos, 2) o período de latência entre a fase aguda (*status epilepticus*) e a fase crónica, onde ocorrem crises epilépticas espontâneas e recorrentes, é mais curto que nos demais modelos de *status epilepticus* (Cavalheiro et al., 1991), 3) as alterações estruturais das regiões hipocampal e para-hipocampal são semelhantes às observadas na ELT (Wieser, 2004) e 4) as crises epilépticas são pouco sensíveis a drogas antiepilépticas (Chakir et al., 2006). No que aos inconvenientes diz respeito, salienta-se o facto das lesões provocadas por este modelo serem pouco

específicas, dado afectarem também regiões não envolvidas na ELT (Harvey e Sloviter, 2005).

Os modelos experimentais que utilizam pentilenotetrazole ou choques electroconvulsivos (ECS) são os mais utilizados em estudos da fase aguda das alterações cerebrais associadas à actividade convulsiva (Fisher, 1989; Löscher, 2002). O pentilenotetrazole é um antagonista dos receptores $GABA_A$ e produz convulsões epilépticas tónico-clónicas generalizadas que podem durar vários minutos, culminando tipicamente na morte do animal. Os electrochoques são utilizados como modelo de convulsões epilépticas generalizadas breves, com duração de 10-15 segundos, principalmente em estudos destinados a avaliar o potencial antiepiléptico de novos fármacos. Existem diversos protocolos de ECS que podem ser administrados com eléctrodos via transauricular ou através da córnea, utilizando diferentes voltagens e intensidades eléctricas. A administração repetida de ECS em animais de experiência acarreta alterações no cérebro que se assemelham às observadas em pacientes com ELT, tais como alterações na síntese e expressão de neuropeptídeos, neurogénese de células granulares da fáscia denteada e *sprouting* das fibras musgosas (Gombos et al., 1999; Scott et al., 2000; Vaidya et al., 1999; Zachrisson et al., 1995). Apesar dessas alterações, os ECS não produzem, aparentemente, focos epileptogénicos.

Estudos recentes têm demonstrado que a actividade convulsiva pode, por si só, induzir lesões cerebrais potencialmente epileptogénicas, e que as convulsões frequentes não controladas podem levar ao agravamento da lesão inicial, dando assim origem a um ciclo progressivo que pode culminar com o aparecimento ou agravamento de alterações comportamentais (Bernhardt et al., 2009; Nearing et al., 2007; Pitkänen e Sutula, 2002; Sutula et al., 2003; Williams et al., 2009). Todavia, os efeitos das convulsões epilépticas na função e estrutura cerebral são ainda pouco conhecidos. Uma das razões deve-se ao facto de grande parte dos estudos empregarem modelos experimentais de convulsões prolongadas que provocam lesões extensas (Buckmaster e Dudek, 1997; Mello et al., 1993; Turski et al., 1983). Com efeito, poucos são os trabalhos centrados nos efeitos neuronais das convulsões de curta duração (Cavazos et al., 1994; Cavazos e Sutula, 1990; Kotloski et al., 2002). Por esta razão, a caracterização detalhada das alterações morfológicas, neuroquímicas e comportamentais induzidas por convulsões de curta

duração, bem como a sua potencial implicação no evoluir dos processos epileptogénicos foi um dos principais objectivos do presente estudo. Para tal, desenvolveu-se, numa série de estudos piloto, um novo modelo experimental, assente na indução repetida de convulsões de curta duração desencadeadas por ECS e caracterizado por produzir apenas moderada morte celular em populações vulneráveis. Além disso, comparam-se as alterações morfofuncionais induzidas por este modelo com as observadas nas convulsões prolongadas.

Estes objectivos foram conseguidos através da realização de uma série de estudos cujos resultados foram relatados nos seguintes trabalhos:

1) "*Retrosplenial granular b cortex in normal and epileptic rats: a stereological study*" (Cardoso et al., 2008b). Pretendeu-se avaliar se a actividade convulsiva induzida por ECS ou pilocarpina é capaz de provocar alterações estruturais no córtex retrosplénico granular *b*. Aplicando metodologias estereológicas, determinaram-se os volumes e o número total de neurónios das diferentes camadas deste córtex em ratos controlo, ratos tratados com ECS e ratos submetidos a *status epilepticus* induzido por pilocarpina.

2) "*Seizure-induced structural and functional changes in the rat hippocampal formation: comparison between brief seizures and status epilepticus*" (submetido). Quis-se determinar se as convulsões epilépticas induzidas por electrochoques provocam alterações estruturais e funcionais na FH, e compará-las com as observadas em animais com *status epilepticus* induzido por pilocarpina. O estado cognitivo dos animais foi avaliado recorrendo aos testes comportamentais *Morris water maze*, *active* e *passive avoidance*. O número total de neurónios da FH, pré-subículo, para-subículo e córtex entorrinal foi determinado utilizando metodologias esterológicas.

3) "*Loss of synapses in the entorhinal-dentate gyrus pathway following repeated induction of electroshock seizures in the rat*" (Cardoso et al., 2008a). Pretendeu-se aquilatar se as convulsões epilépticas induzidas por electrochoques provocam alterações estruturais no circuito córtex entorrinal – fáscia denteada. Para tal, determinaram-se os

números totais de neurónios e sinapses deste circuito em ratos controlo e noutros submetidos a ECS. Analisaram-se as arborizações dendríticas das células granulares da fáscia denteada em material impregnado pela prata segundo o método de Golgi.

4) "*Effects of repeated electroconvulsive shock seizures and pilocarpine-induced status epilepticus on emotional behavior in the rat*" (Cardoso et al., 2009). Pretendeu-se escrutinar as alterações comportamentais afectivas consequência das convulsões epilépticas induzidas por electrochoques e compararam-se com as observadas em animais submetidos a *status epilepticus* induzido por pilocarpina. Para tal, utilizaram-se os testes comportamentais *open-field*, *elevated plus-maze* e *fear conditioning*.

5) "*Seizure-induced changes in neuropeptide Y-containing cortical neurons: potential role for seizure threshold and epileptogenesis*" (Cardoso et al., 2010). Tentou-se clarificar se as convulsões epilépticas induzem alterações nos níveis de expressão de NPY, e se essas alterações são específicas de regiões cerebrais implicadas na epilepsia. Para isso, analisaram-se os níveis de expressão dos interneurónios NPYérgicos no hilo da fáscia denteada e nos córtices retrosplénico e somatossensorial em animais tratados com ECS e noutros tratados com pilocarpina. Analisou-se ainda a susceptibilidade às convulsões epilépticas através do modelo de pentilenotetrazole.

6) "*Reduced density of neuropeptide Y neurons in the somatosensory cortex of old male and female rats: relation to cholinergic depletion and recovery after nerve growth factor treatment*" (Cardoso et al., 2006). Pretendeu-se avaliar os efeitos do envelhecimento e do dimorfismo sexual sobre os sistemas NPYérgico e colinérgico no córtex somatossensorial do Rato. Avaliaram-se os níveis de expressão do NPY, em ambos os sexos, em animais controlo e noutros submetidos a infusão de neurotrofina *nerve growth factor* (NGF).

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Retrosplenial granular b cortex in normal and epileptic rats: A stereological study

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ABSTRACT

Human temporal lobe epilepsy and experimental models of this disease are associated with loss of neurons and other structural alterations in several limbic brain structures including the hippocampal formation and adjacent parahippocampal cortical areas. The goal of this study was to test the hypothesis that seizure activity can produce damage to the retrosplenial granular b cortex (Rgb) which is known to be strongly connected with other limbic structures implicated in epilepsy. To test this hypothesis, we estimated, using stereological methods, the volumes and total neuronal numbers in Rgb cortex of rats that had experienced prolonged status epilepticus induced by pilocarpine (350 mg/kg), rats treated with six electroshock seizures (the first five seizures were spaced by 24-h intervals, whilst the last two were only 2 h apart), and control rats. Adult male Wistar rats were used in this experiment. Status epilepticus produced significant loss of neurons in Rgb cortical layers IV (22%) and V (44%), which was accompanied by volume reductions in layers I (17%), IV (11%), V (18%) and VI (24%). In electroshock-treated rats, the volume of Rgb cortical layer VI was reduced by 17% and the number of neurons estimated in layer V was smaller by 16% relative to control rats. Thus, the finding that status epilepticus and administration of brief generalized seizures both lead to degenerative morphological alterations in Rgb cortex provides the first experimental support for the hypothesis that this cortical area can be involved in seizure activity, as suggested by its anatomical connections.

1. Introduction

Temporal lobe epilepsy (TLE) is often associated with progressive loss of neurons in the hippocampus proper, dentate gyrus, subicular and entorhinal cortices, which may lead to permanent cognitive impairments (Dawodu and Thom, 2005; Du et al., 1993; Engel, 1996; Fisher et al., 1998; Houser, 1999; Stafstrom, 2005). Neuronal death in TLE has been ascribed to sustained hyperactivity of brain circuits involved in either genesis or propagation of seizures, or both (McNamara, 1999; Meldrum, 1991; Pitkänen and Sutula, 2002; Salmenperä et al., 1998). This assumption is supported by animal studies in which prolonged seizures, induced by either chemoconvulsants, such as pilocarpine and kainic acid, or continuous electrical stimulation, produce a similar pattern of hippocampal neurodegeneration (Ben-Ari et al., 1980; Buckmaster and Dudek, 1997; Sloviter, 1987; Sloviter et al., 2003; Turski et al., 1983). It is further supported by evidence that, whereas single or widely spaced brief seizures do not cause considerable brain damage (Gombos et al., 1999; Vaidya et al., 1999), they certainly do so when administered at shorter intervals, particularly with respect to several seizure-vulnerable neuronal populations,

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such as hilar cells of the dentate gyrus and neurons of the entorhinal cortex (Cardoso et al., 2008; Cavazos and Sutula, 1990; Lukoyanov et al., 2004; Sutula et al., 1994).

The retrosplenial granular b cortex (Rgb) of the rat occupies the anterodorsal part of the retrosplenial granular area and lies ventral to the retrosplenial dysgranular cortex (Rdg) and caudal to the anterior cingulate cortex (Fig. 1; Vogt and Peters, 1981; Zilles and Wree, 1995). It is strongly interconnected with several other brain regions that are thought to be involved in seizure activity. More specifically, Rgb projects heavily to and receives afferent input from the hippocampal formation (Miyashita and Rockland, 2007; Van Groen and Wyss, 1990a, 1992b, 2003; Vogt and Miller, 1983; Wyss and Van Groen, 1992), mainly via the subicular complex (Finch et al., 1984; Meibach and Siegel, 1977; Van Groen and Wyss, 1990b, 2003; Wyss and Van Groen, 1992). In addition, Rgb is reciprocally connected with the anteroventral and anterodorsal thalamic nuclei (Shibata, 1998; Sripanidkulchai and Wyss, 1986; Van Groen and Wyss, 1992a), which are known to be specifically recruited in the propagation of limbic seizures within the Papez circuit (Dubé et al., 1998; Mirski et al., 2003; Mraovitch and Calando, 1999; Sherman et al., 1997). That Rgb area can be involved in epilepsy is supported by the results of a recent study of TLE patients with hippocampal sclerosis, which showed that hippocampal atrophy significantly correlates with loss of cortical gray matter in the retrosplenial cortex (Düzel et al., 2006). Furthermore, this possibility is also consistent with evidence from experimental studies in rats that generalized seizures produce a marked increase in blood oxygen level-dependent signal intensity in Rgb (Brevard et al., 2006) and that status epilepticus (SE) is associated with atrophic changes in the dendrites of Rgb pyramidal neurons (Ampuero et al., 2007). However, too few data are available at present to make conclusive statements relative to the implication of Rgb in epilepsy.

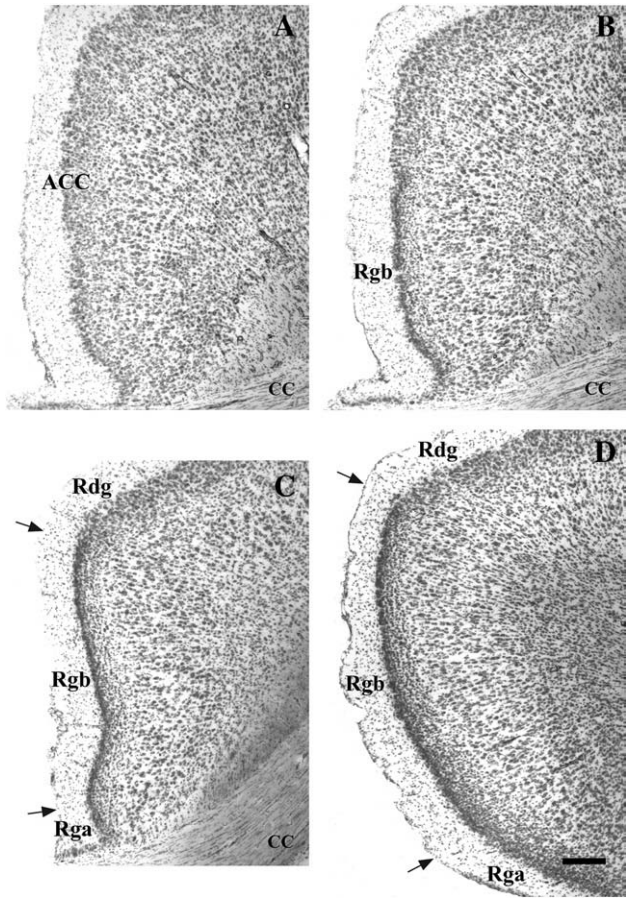

Fig. 1 – Photomicrographs of Nissl-stained coronal sections cut at the caudalmost level of the anterior cingulate cortex (A), and rostral (B), mid-rostrocaudal (C), and caudal (D) levels of the retrosplenial granular b (Rgb) cortex of a control rat. Note the high cell packing density in Rgb layers II/III (B) that distinguishes this retrosplenial area from the adjacent anterior cingulate cortex (ACC) in which layer II/III neurons are much more loosely packed (A). Rgb cortex borders dorsally the retrosplenial dysgranular (Rdg) cortex and joins caudally and ventrally the retrosplenial granular a (Rga) cortex, as indicated by arrows in C and D. CC, corpus callosum. Scale bar = 150 μm.

Whereas it is well documented that seizure activity can lead to degeneration of hippocampal and entorhinal neurons, the issue of what actually happens following seizures to neurons located in Rgb cortex has not yet been addressed. We hypothesized that, if Rgb cortex is indeed involved in epileptiform activity, as suggested by its strong connections with brain regions implicated in epilepsy, then, at least part of its neurons should be injured by the seizures and probably lost. To test this hypothesis, we estimated the total neuronal numbers in all Rgb cortical layers in control rats and compared them to the numbers found in rats that had experienced prolonged status epilepticus induced by pilocarpine. The volumes of the respective cortical layers were also estimated and compared between the groups. Furthermore, because the degenerative changes in the brain of pilocarpine-treated animals might be related to neurotoxic effects of this drug rather than to the seizures it induces, we also analyzed the morphology of Rgb cortex in rats following repeated administration of brief generalized seizures elicited by electroconvulsive shock (ECS). In doing so, we applied the treatment protocol that has been previously shown to produce small, but significant loss of seizure-vulnerable entorhinal and hilar neurons (Cardoso et al., 2008; Lukoyanov et al., 2004).

2 Results

2.1 Behavioral monitoring

From 8 rats treated with pilocarpine, 1 animal did not show any seizure-like activity and, therefore, was excluded from the study. Remaining 7 rats developed SE, but one of them died approximately 4 h after the treatment. After recovery, the 6 rats that survived in SE group went through a latent phase, during which they showed asymptomatic (seizure-free) behavior. Following this salient period lasting 2–3 weeks, spontaneous motor seizures of stage 3 or greater on the Racine scale (Racine, 1972) were repeatedly observed in all rats of this group. No behavioral alterations were detected in animals from the ECS-treated and control groups.

2.2. Qualitative morphological observations

The qualitative observation of the Giemsa-stained material obtained in this experiment revealed no gross abnormalities in the cytoarchitectonic organization of Rgb cortex of rats from either group (Fig. 2). However, the density of layer V neurons in Rgb of ECS-treated rats was somewhat reduced relative to control rats (Fig. 2A, C and D). In addition, inspection of these sections provided the impression that the volume of Rgb layer VI was reduced in ECS-treated rats when compared to controls. Similar changes in neuronal density in Rgb layer V and in the volume occupied by Rgb layer VI were observed in rats from SE group, but in this case they were much more evident (Fig. 2A and B). At the qualitative level, no treatment-related structural alterations were detected in Rgb layers I, II/III and IV.

2.3. Total volumes of Rgb cortical layers

The volume estimates of different layers of Rgb cortex are given in Table 1. These estimates show that the rat Rgb cortex has a mean volume of 4.4 mm^3, of which the heavily populated neuronal layers II and III collectively occupy only 8% and the cell-free layer I occupies 22%. The remaining volume of Rgb cortex was distributed between the thin layer IV (9%), layer V (39%), and layer VI (22%). Statistical analysis of these data confirmed the results of our qualitative observations by showing that there was a significant main effect of treatment on the volume of Rgb layer VI ($F_{(3,20)}=6.77$, $P<0.005$). The volume of this layer was significantly decreased relative to control rats in SE group (24%, $P<0.005$), DC-ECS group (17%, $P<0.05$) and AC-ECS group (18%, $P<0.05$). In addition, ANOVA revealed a significant main effect of treatment on the volume of layer I ($F_{(3,20)}=3.29$, $P<0.05$), layer IV ($F_{(3,20)}=4.96$, $P<0.05$) and layer V ($F_{(3,20)}=3.99$, $P<0.05$). However, post-hoc comparisons showed that these significant group effects were entirely due to the reduction of respective cortical volumes in the SE group ($P<0.05$ for all three layers), whereas no volume changes were detected in layers I, IV, and V in rats from DC-ECS and AC-ECS groups. The combined volumes of layers II and III did not differ significantly across the four groups. No significant differences were found between the SE, DC-ECS and AC-ECS groups.

Table 1 – Volumes of different layers of Rgb cortex in control, SE and ECS-treated rats

Rgb layers	Volume (mm^3)			
	Control	SE	DC-ECS	AC-ECS
I	0.976 (0.079)	0.813 (0.103)*	0.879 (0.115)	0.937 (0.082)
II/III	0.341 (0.042)	0.312 (0.030)	0.361 (0.012)	0.353 (0.039)
IV	0.371 (0.030)	0.327 (0.014)*	0.344 (0.024)	0.364 (0.029)
V	1.74 (0.175)	1.423 (0.167)*	1.612 (0.154)	1.603 (0.146)
VI	0.990 (0.106)	0.747 (0.127)**	0.833 (0.051)*	0.811 (0.088)*

Values are expressed as mean (SD), $n=6$ in each group.
* $P<0.05$ vs. control.
** $P<0.005$ vs. control.

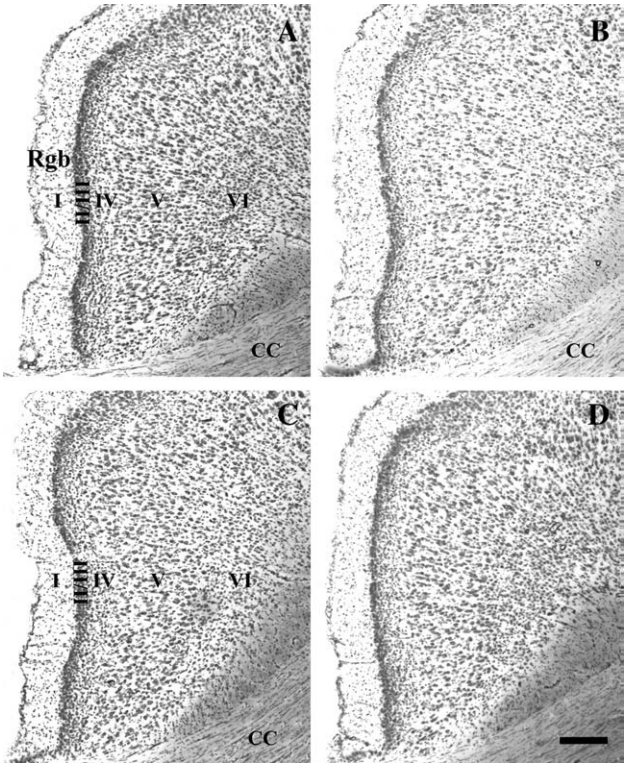

Fig. 2 – Photomicrographs of representative Nissl-stained coronal sections containing the retrosplenial granular b (Rgb) cortex of a control rat (A), a rat treated with pilocarpine (B), and rats from the DC-ECS (C) and AC-ECS (D) groups. Layers I to VI of the Rgb cortex are shown in A and C. Note that the density of neurons in the Rgb cortical layer V is dramatically reduced in the pilocarpine-treated animal (B) when compared to the control rat (A). In both rats treated with electroshock (C and D), the packing density of the Rgb layer V neurons is also somewhat reduced relative to the control rat. CC, corpus callosum. Scale bar = 150 μm.

2.4. Total number of neurons in Rgb cortex

The stereological estimates of the total number of neurons in Rgb cortex of rats used in this study are given in Table 2. These data show that the rat Rgb cortex contains approximately 600,000 neurons, of which 37% belong to the densely packed layers II and III, with the remaining neurons being relatively uniformly distributed between layers IV, V and VI. Statistical analysis of the estimates revealed no significant effect of SE or

Table 2 – Total number of neurons in different layers of Rgb cortex in control, SE and ECS-treated rats

Rgb layers	Total neuronal number (in thousands)			
	Control	SE	DC-ECS	AC-ECS
II/III	221.9 (23.0)	179.0 (28.7)	204.6 (40.5)	190.8 (30.1)
IV	92.6 (5.0)	72.5 (5.9)**	83.1 (12.5)	80.0 (11.4)
V	154.6 (23.9)	86.3 (6.4)***	128.4 (17.1)*	130.3 (7.9)*
VI	130.6 (10.7)	125.4 (9.4)	134.8 (12.1)	121.3 (8.6)

Values are expressed as mean (SD), $n=6$ in each group.
* $P<0.05$ vs. control.
** $P<0.01$ vs. control.
*** $P<0.001$ vs. all groups.

ECS-treatment on the total number of cells in the layers II/III and layer VI, suggesting that neurons located in these layers are not vulnerable to seizures. However, there was a significant main effect of treatment upon neuronal numbers found in layer V ($F_{(3,20)}=19.98$, $P<0.00001$). The number of neurons in this layer was significantly ($P<0.001$) and robustly (by 44%) lessened in rats from SE group relative to control group. Furthermore, significant ($P<0.05$) and selective loss of layer V neurons was also detected in both DC-ECS (17%) and AC-ECS (16%) groups. However, the loss of Rgb layer V neurons in ECS rats was significantly smaller than in the SE rats ($P<0.001$). ANOVA additionally revealed a significant main effect of treatment on neuronal numbers estimated in layer IV ($F_{(3,20)}=4.48$, $P<0.01$). However, the seizure-related loss of cells in this layer was less severe than in layer V, reaching only 22% in SE group ($P<0.01$ versus control group) and actually being nonsignificant in DC-ECS and AC-ECS groups.

3. Discussion

Retrosplenial granular b cortex is located within the transition zone between the three-layered hippocampal archicortex and neocortex (Lopes da Silva et al., 1990; Vogt and Peters, 1981) and has reciprocal connections with neighbouring hippocampal and parahippocampal regions (Van Groen and Wyss, 1990b, 2003; Vogt and Peters, 1981; Wyss and Van Groen, 1992). There is an increasing amount of evidence that Rgb cortex is implicated in brain functions such as recent memory and spatial navigation (Aggleton and Vann, 2004; Cooper and Mizumori, 2001; Dean and Platt, 2006; Lukoyanov et al., 2005; Van Groen et al., 2004), which have been traditionally viewed as dependent on the hippocampus (Morris, 1984) and adjacent subicular (Morris et al., 1990; Sharp and Green, 1994), entorhinal (Fyhn et al., 2004; Steffenach et al., 2005) and perirhinal (Liu and Bilkey, 2001) cortical areas. It is also becoming increasingly evident that the very same subdivisions of limbic cortex that are involved in cognitive functions additionally play important roles in the genesis and propagation of seizures, as it is the case of the hippocampus proper (Esclapez et al., 1999; Meldrum, 1991; Nadler et al., 1980; Siddiqui and Joseph, 2005), dentate gyrus (Buckmaster and Dudek, 1997; Ratzliff et al., 2002; Sloviter et al., 2003), subicular complex (Cavazos et al., 2004; Knopp et al., 2005; Stafstrom, 2005; Van Vliet et al., 2004), and entorhinal (Du et al., 1995; Kobayashi et al., 2003; Schwarcz et al., 2000) and perirhinal (Fukumoto et al., 2002) cortices. Yet, it has so far remained unclear to what extent, if any, this general rule can be extrapolated to the area Rgb. In the present study, we found that both prolonged SE and repeated brief generalized seizures lead to neuronal loss and volume reduction in specific layers of Rgb cortex, which may be considered as a first direct evidence for its involvement in seizure activity.

Animal models of SE resemble many of the features of human TLE, including degenerative changes in the hippocampal region (Ben-Ari et al., 1980; Buckmaster and Dudek, 1997; Sloviter, 1987; Turski et al., 1983), spontaneous recurrent seizures (Buckmaster and Dudek, 1997; Turski et al., 1983), and severe behavioural impairments (Kemppainen et al., 2006; Kotloski et al., 2002; Majak and Pitkänen, 2004). In particular, it has been reported that SE induced by either chemoconvulsants, such as pilocarpine and kainic acid, or electrical stimulation provokes marked neuronal loss in the entorhinal cortex (Du et al., 1995; Schwarcz et al., 2000), hilus of the dentate gyrus (Buckmaster and Dudek, 1997; Ratzliff et al., 2002; Sloviter, 1987; Sloviter et al., 2003), and CA3 and CA1 hippocampal pyramidal fields (Cavazos et al., 1994; Kotloski et al., 2002; Nadler et al., 1980). The SE-induced loss of neurons, albeit less severe, was also found in the subicular complex (Knopp et al., 2005; Van Vliet et al., 2004). Furthermore, morphological and physiological evidence indicates that prolonged seizures cause remodeling of neurites and reorganization of synaptic connections in the very same neuronal populations, i.e. in the dentate gyrus (Buckmaster and Dudek, 1997; Sloviter et al., 2003), hippocampus (Esclapez et al., 1999; Nadler et al., 1980; Siddiqui and Joseph, 2005), subicular (Cavazos et al., 2004; Knopp et al., 2005) and entorhinal (Kobayashi et al., 2003; Kumar et al., 2007) cortices. The present findings show that pilocarpine-induced SE leads to loss of neurons in layers IV and V of Rgb cortex, which is accompanied by a reduction in the volume of layers I, IV, V and VI. Notably, almost half of the Rgb layer V neurons were lost after SE, indicating that their vulnerability to seizures is comparable to that of the other susceptible neuronal populations, including layer III entorhinal (Du et al., 1995; Schwarcz et al., 2000), hilar (Buckmaster and Dudek, 1997; Ratzliff et al., 2002; Sloviter et al., 2003) and hippocampal pyramidal (Cavazos et al., 1994; Kotloski et al., 2002; Nadler et al., 1980) neurons. These results are consistent with previous studies that detected darkly stained, presumably degenerating cells in Rgb cortex of pilocarpine-treated rats using the silver impregnation technique (Covolan and Mello, 2000). Furthermore, the finding that SE caused conspicuous volume reduction in the Rgb molecular layer (I) as well as in its layer VI, where no neuronal loss was observed, is indicative of profound structural (and, likely, functional) reorganization in neuritic processes. This reorganization is likely to be prompted by the loss of layer V neurons, whose apical and basal dendrites extensively ramify in layers I and VI, respectively (Finch et al., 1984; Vogt and Peters, 1981). Such an interpretation is consistent with the results of another study reporting on dendritic atrophy and decreased spine density in layer V pyramidal neurons of Rgb cortex after kainic acid-induced SE (Ampuero et al., 2007).

Because the extent of neuronal damage produced by systemic administration of chemoconvulsants does not necessarily correlate with the frequency and severity of seizures they produce (André et al., 2000, 2007; Cadotte et al., 2003; Liu et al., 1994), it is possible that the loss of Rgb layer V neurons found in pilocarpine-treated rats is unrelated to their specific involvement in limbic seizures. To examine this issue, we also estimated in this study the total neuronal numbers and layer volumes in Rgb cortex of rats in which chronic seizures were induced using less invasive procedure — electroconvulsive stimulation. Furthermore, given the possibility that an electrical current of a certain intensity when passing through the brain can cause non-specific damage to some vulnerable neuronal populations (Sterling, 2000), we additionally compared two different ECS models in which seizures are elicited by applying currents with strikingly distinct electrical parameters, i.e. DC, 200 V versus AC, 60 mA. However, in both models, we found that administration of ECS seizures results in significant loss of Rgb layer V neurons and noticeable

reduction of the volume occupied by Rgb layer VI. Thus, the observation that the neuronal loss was fairly similar in both situations, 17% in DC-ECS group and 16% in AC-ECS group, is most likely to indicate that it was triggered by the ECS-induced seizures rather than by the electrical current per se. These data are fully consistent with experimental results discussed above, i.e. that pilocarpine-induced SE is associated with a loss of almost half of Rgb layer V cells, suggesting that similar seizure-related mechanisms may underlie Rgb pathology in either of the models employed in this study. Interestingly, the extent of ECS-induced loss of Rgb layer V neurons was similar to that previously found in this model for hilar (19%) and entorhinal layer III (21%) neurons (Cardoso et al., 2008; Lukoyanov et al., 2004), which are known for their heightened vulnerability to epileptic seizures (Buckmaster and Dudek, 1997; Du et al., 1995; Ratzliff et al., 2002; Schwarcz et al., 2000; Sloviter et al., 2003).

The present study was specifically designed to test the hypothesis that Rgb cortex is involved in seizure activity, as suggested by its anatomical connections. The finding that pilocarpine-induced SE and administration of brief ECS seizures both lead to degenerative morphological alterations in Rgb area provides the first experimental support for this hypothesis. In this regard, it is worth remembering that Rgb cortex has strong direct and also indirect connections with other brain regions implicated in epilepsy, namely the hippocampal formation, and anterodorsal and laterodorsal thalamic nuclei (Shibata, 1998; Sripanidkulchai and Wyss, 1986; Van Groen and Wyss, 2003; Vogt and Miller, 1983; Wyss and Van Groen, 1992). In addition, unlike adjacent cortical areas such as Rdg and Rga, Rgb receives strong afferent input from the anteroventral thalamic nucleus (Van Groen and Wyss, 2003; Wyss and Van Groen, 1992), which is suspected to play one of the essential roles in seizure propagation (Dubé et al., 1998; Mraovitch and Calando, 1999). Therefore, it appears plausible that Rgb cortex, being interposed between structures deeply implicated in epilepsy, may be a crucial neural hub involved in integrating thalamocortical activity during the initiation and/or propagation of generalized seizures (Brevard et al., 2006). This study additionally revealed that, among all Rgb neurons, those belonging to layer V are particularly vulnerable to seizure-induced injury. Interestingly, it has been shown that Rgb layer V neurons directly project to the brainstem, where their fiber terminals are distributed through the intermediate and deep layers of the superior colliculus and also present in the lateral periaqueductal gray (García Del Caño et al., 2000). Furthermore, these neurons massively project to both primary and secondary motor areas of the cortex (Shibata et al., 2004). In this context, the finding that Rgb layer V neurons participate in the genesis or propagation of seizures may provide a missing link helping to identify neuronal pathways involved in the transfer of epileptiform activity to the brainstem and frontal cortical areas related to motor control and consciousness.

4. Experimental procedures

4.1. *Animals and treatments*

A total of 26 male Wistar rats, obtained from Harlan Iberica (Barcelona, Spain), were used in this study. After arrival, they were maintained under standard laboratory conditions and had free access to food and water. At 2 months of age, the rats were randomly divided into four groups and submitted to the following protocols: pilocarpine-induced status epilepticus (group SE, $n=8$), repeated administration of a direct-current ECS (group DC-ECS, $n=6$), repeated administration of a standard alternating-current ECS (group AC-ECS, $n=6$), and no-treatment control group ($n=6$). Following the respective treatments, the rats were daily observed for spontaneous behavioral seizures at random times between 08:00 h and 20:00 h. All animals were killed at 5 months of age by transcardial perfusion. The handling and care of the animals were conducted according to the European Communities Council guidelines in animal research (86/609/UE). All efforts were made to minimize the number of animals used and their suffering.

4.2. *Pilocarpine model of status epilepticus*

Animals in SE group were pretreated with scopolamine methyl bromide (1 mg/kg, s.c.) in order to minimize peripheral cholinergic side effects of pilocarpine. Thirty min later, the rats received a single high dose of pilocarpine (350 mg/kg, i.p.) and were observed thereafter for signs of motor seizures. The onset of SE was defined as the appearance of behavioral symptoms corresponding to stage 4–5 seizures on the Racine scale (1972), i.e. rearing, falling and generalized convulsions, which persisted for at least 2 min. SE onset was usually detected 30–60 min following the pilocarpine injection. It has been previously reported that pilocarpine-induced SE, if lasting for several hours, can be associated with high mortality rate which ranges between 15% and 50% depending on the dose of pilocarpine and other experimental conditions (Glien et al., 2001; Goodman, 1998; Gröticke et al., 2007; Williams et al., 2002). Therefore, because animal mortality is a prominent cause of bias in quantitative evaluation of neuronal loss (Herguido et al., 1999), special efforts were made in order to improve the survival rate of the animals in SE group. In particular, two h after the beginning of SE, the rats were injected with diazepam (5 mg/kg, i.p.) in order to cease the convulsive manifestations of SE. However, seizure activity, albeit considerably reduced in severity, was not completely stopped by the single dose of diazepam. Thus, an additional dose of diazepam (2.5 mg/kg) was given to the rats 3 h after the onset of SE. Furthermore, the animals were periodically injected with saline (s.c.) during the first 12 h of the recovery period. On the following days, the rat diet was supplemented with apples that were sliced and left at the bottom of the cage.

4.3. *ECS models*

Animals in DC-ECS group received a course of 5 ECS seizures, administered on a 24-h schedule as previously described (Lukoyanov et al., 2004). Briefly, each stimulation (constant voltage of 200 V for 2 s) was delivered via ear-clip electrodes wired to a stimulus generator (model 215/IZ, Hugo-Sachs Elektronik, Germany). Two hours after the fifth stimulation, each of the animals received one additional ECS seizure. Animals in AC-ECS group received identical treatment except that an alternating current of 50 Hz (60 mA for 1 s) was used to induce seizures. In both models, ECS produced full tonic-clonic seizures with hind-limb extension lasting for 5–10 s.

4.4. *Tissue preparation*

4.4.1. *General procedures*

All rats were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and transcardially perfused with a fixative solution containing 1% paraformaldehyde and 1% glutaraldehyde in 0.12 M phosphate buffer at pH 7.4. The brains were removed from the skulls, weighed, separated by a midsagittal cut into right and left halves, codified to allow blind estimations, and placed in fresh fixative. The frontal pole was removed and the blocks of tissue containing the Rgb cortex were separated and processed for embedding in glycolmethacrylate. Because it is not known if Rgb cortex of rodents display right/left asymmetries, the blocks were alternately sampled from the right and left hemispheres, in order that Rgb areas from both sides were included in estimations.

4.4.2. *Glycolmethacrylate embedding*

After 30 days of post-fixation, the blocks of tissue containing the entire Rgb cortex were dehydrated through a graded series of ethanol solutions and embedded in glycolmethacrylate, as described in detail elsewhere (West et al., 1991). These blocks were then serially sectioned in the coronal plane at a nominal thickness of 40 μm using a Jung Multicut microtome. Every tenth section was collected using a systematic random sampling procedure (Gundersen and Jensen, 1987), mounted serially and stained with a modified Giemsa solution (West et al., 1991).

4.5. *Morphometric analysis*

4.5.1. *Anatomical definition of Rgb cortex*

Retrosplenial cortex (Brodmann's area 29) is located in the posteroinferior part of the cingulate gyrus caudal to the anterior cingulate cortex. It consists of two cytoarchitectonically distinct areas, the retrosplenial agranular and retrosplenial granular cortices (Brodmann, 1909; Paxinos and Watson, 1998; Vogt and Peters, 1981; Zilles and Wree, 1995). Because retrosplenial agranular area contains a rudimentary granular cell layer IV, it is often designated as retrosplenial dysgranular cortex (Rdg; Van Groen and Wyss, 1992b; Vogt and Peters, 1981). The retrosplenial granular cortex is located ventral to Rdg cortex and has a specific pattern of Nissl staining characterized by an extremely dense packing of darkly stained layer II/III cells that distinguishes it from the adjacent Rdg and anterior cingulate cortices (Fig. 1). The retrosplenial granular cortex is further subdivided into Rga and Rgb cortices (Paxinos and Watson, 1998; Van Groen and Wyss 1990a; Wyss and Sripanidkulchai, 1984). Rgb borders dorsally Rdg, joins caudally and ventrally Rga and corresponds to area 29c of Brodmann (1909) and Vogt and Peters (1981). Area Rga, in turn, joins caudally and ventrally the postsubiculum and corresponds to areas 29a and 29b. It continues rostrally between the corpus callosum and Rgb and has large layer V cells directly abutting layer IV (Vogt and Peters, 1981). The border between Rgb and Rga can be delineated taking into consideration that Rgb tends to have thinner layers II/III and IV in comparison to Rga (Vogt and Peters, 1981), and its layer II contains smaller and more darkly stained cells than that of Rga (Van Groen and Wyss, 2003). In addition, Rgb is characterized by the presence of a thin lamina dessicans between layer IV and superficial part of layer V, which is barely detectable in Rga.

4.5.2. *Estimation of layer volumes*

The volumes of Rgb cortical layers were estimated in Giemsa-stained coronal sections embedded in glycolmethacrylate by applying the principle of Cavalieri (Gundersen et al., 1988; Regeur and Pakkenberg, 1989). The boundaries of Rgb cortical layers were consistently defined on the basis of cell morphology and cytoarchitectonic criteria, as described elsewhere (Van Groen and Wyss, 2003; Vogt and Peters, 1981). Because it is difficult to delineate the boundary between layers II and III with certainty, the combined volumes of these laminae were determined. The border between layer VI and subjacent white matter can be relatively easily delineated in

Table 3 – Summary of the stereological parameters used for the estimation of volumes and neuronal numbers in Rgb cortical layers

	Rgb cortical layers				
	I	II/III	IV	V	VI
Number of sections	12	12	12	12	12
a (point) (mm^2)	0.017644	0.007528	0.007528	0.029407	0.007528
∑P	145	125	128	155	305
CE (P)	0.04	0.05	0.04	0.04	0.04
ssf	–	0.1	0.1	0.1	0.1
x-step (μm)	–	100	90	200	170
y-step (μm)	–	100	90	200	170
a (frame) (μm^2)	–	405	908	2219	1816
asf	–	0.0404	0.1121	0.0555	0.0419
h (μm)	–	10	10	10	10
tsf	–	0.3	0.28	0.28	0.28
$\sum Q^-$	–	225	245	192	154
CE (N)	–	0.07	0.07	0.07	0.08

a (frame): area of the counting frame, a (point): area per point in grid used for volume estimates, asf: area sampling fraction, CE (N) and CE (P) mean coefficients of error (for neuronal numbers and volumes of layers, respectively), h: height of the optical dissector, ssf: section sampling fraction, tsf: thickness sampling fraction, x-step and y-step predetermined distances used along the x and y-axes of the section to sample, ΣP: number of points on each neuronal layer, ΣQ^-: total number of neurons counted in each neuronal layer.

Rgb cortex, as it is characterized by a sharp reduction in cell size and packing density. Estimations were carried out using the Olympus C.A.S.T.-Grid System (Denmark) and a mean of 12 systematically sampled sections containing Rgb area was used per animal. In each section, the cross-sectional area of each cortical layer was estimated by point counting (Gundersen and Jensen, 1987), at a final magnification of 80×, using an adequate grid of test points. The volume of the layers was calculated from the total number of points that fell on each layer and the distance between the sections (Gundersen et al., 1988; Regeur and Pakkenberg, 1989). The coefficient of error (CE) of the individual estimates was calculated as shown by Cruz-Orive (1999) and the mean value was 0.04. The stereological parameters used for the estimations of cortical layer volumes are summarized in Table 3.

4.5.3. *Estimation of total neuronal numbers*

The total numbers of neurons in the Rgb cortex layers were estimated in the same Giemsa-stained coronal sections that were used for volume measurements, by applying the optical fractionator method (West et al., 1991). All the systematically sampled sections containing the Rgb region were used, which provided an average of 12 sections per each animal. The boundaries of Rgb cortical layers were defined as described above. No counts were performed in the relatively cell-free layer I. The counts of neurons belonging to layers II and III were made in the entire area occupied by both layers. Estimations were carried out using the Olympus C.A.S.T.-Grid System. Beginning at a random starting position, visual fields were systematically sampled along the *x* and *y* axes, using a raster pattern procedure. Neurons were counted in every frame using the optical disector at a final magnification, at the level of the monitor, of 2000×. The coefficient of error (CE) of the individual estimates was calculated according to Gundersen et al. (1999) and ranged between 0.07 and 0.08. The stereological parameters used for the estimations of cell numbers are summarized in Table 3.

4.6. *Statistical analysis*

The data were analyzed using analysis of variance (ANOVA) followed by post hoc Tukey's test, when appropriate. Results are expressed as the mean (SD). Differences were considered as significant at the $P<0.05$ level.

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Seizure-induced structural and functional changes in the rat hippocampal formation: Comparison between brief seizures and status epilepticus.

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Seizure-induced structural and functional changes in the rat hippocampal formation: Comparison between brief seizures and status epilepticus

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Purpose: Prolonged seizures produce death of hippocampal neurons, which is thought to initiate epileptogenesis and cause a disruption of hippocampally mediated behaviors. This study aimed to evaluate structural and functional changes induced in the hippocampal formation by brief seizures and to compare them with changes induced by prolonged seizures.

Methods: Adult rats were administered six brief seizures, elicited by electroshock (ECS), the first five of which were spaced by 24-h intervals, whereas the last two were only 2 h apart. Prolonged seizures (status epilepticus, SE) were elicited by intraperitoneal injection of pilocarpine (350 mg/kg). Two months later, the rats behavior was tested using the Morris water maze, passive avoidance and active avoidance tests. The effects of seizures on the number of neurons in the hippocampal formation were assessed using stereological methods.

Results: Administration of ECS seizures produced a loss of neurons, ranging between 14% and 26%, in the dentate hilus, subiculum, presubiculum, parasubiculum, and entorhinal layers III and V/VI. However, the loss of cells caused by SE in the same structures, as well as in the hippocampal CA3 and CA1 fields, ranged between 40% and 50%. SE additionally killed many neurons in the dentate granular layer, postsubiculum and entorhinal layer II. ECS treatment was associated with mild impairments in spatial learning and passive avoidance, but had no epileptogenic consequences. In contrast, SE produced a severe disruption of spatial learning, passive and active avoidance, and led to the development of spontaneous seizures.

Conclusions: The data obtained in this experiment show that both prolonged seizure activity and brief seizures result in structural and functional alterations in the temporal lobe circuits, but those caused by prolonged seizures are considerably more severe. Hippocampal damage elicited by brief seizures does not necessarily lead to epileptogenesis.

Key words: Electroconvulsive shock, pilocarpine, dentate gyrus, hippocampus proper, subicular cortex, entorhinal cortex, spatial memory, passive avoidance, active avoidance, stereology

Introduction

Growing evidence indicates that incident seizures can cause potentially epileptogenic brain damage and that uncontrolled seizure activity may lead to extension of the initial lesion, rendering thus the course of the disease progressive and ultimately resulting in various behavioral impairments (Kotloski et al., 2002; Pitkänen and Sutula, 2002; Sutula et al., 2003; Nearing et al., 2007; Williams et al., 2009). In particular, it is known that both febrile seizures (Cendes et al., 1993; Kuks et al., 1993) and status epilepticus (SE) caused, for example, by acute domoic acid intoxication (Teitelbaum et al., 1990; Cendes et al., 1995) may lead to the development of temporal lobe epilepsy (TLE), one of the most widespread seizure disorders in humans. Furthermore, it is also well documented that recurrent seizures in TLE patients are associated with progressive atrophic changes in the cerebral cortex which are particularly robust in the hippocampal and parahippocampal regions (Margerison and Corsellis, 1966; Pitkänen et al., 1998; Bernhardt et al., 2009). Consistent with these findings, studies in animals have shown that seizure activity elicited by either chemoconvulsants (Ben-Ari et al., 1980; Turski et al., 1983; Mello et al., 1993; Buckmaster and Dudek, 1997) or electrical stimulation (Sloviter, 1987; Kotloski et al., 2002; Gorter et al., 2003) induces neuronal death and that the pattern of seizure-induced neurodegeneration in animals resembles, partly at least, hippocampal sclerosis observed in human TLE.

Despite the large number of studies demonstrating neuropathological changes in TLE patients and respective animal models, the effects of seizures on brain structure and function are not yet completely understood. In particular, it is not clear whether repeated seizures of short duration are capable of

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affecting hippocampal circuits to such an extent that it would lead to epileptogenesis and/or to significant cognitive deficits. Indeed, the vast majority of studies related to this issue have used models in which animals are subjected to prolonged seizures, associated with large lesions (Ben-Ari et al., 1980; Turski et al., 1983; Mello et al., 1993; Pitkänen et al., 2002), whereas a few studies have focused on neuronal effects of brief seizures (Cavazos and Sutula, 1990; Cavazos et al., 1994; Kotloski et al., 2002; Lukoyanov et al., 2004; Cardoso et al., 2008a, 2008b, 2010). The present study, therefore, was specifically aimed at evaluating the effects of repeated brief seizures on the structural integrity of hippocampal circuits and hippocampal-dependent behaviors and at comparing them to the respective effects of prolonged seizures. To this end, we estimated, using stereological methods, the total neuronal numbers in the dentate gyrus, hippocampus proper, subicular complex and entorhinal cortex of rats which were either given a course of 6 seizures elicited by electroshock (ECS, model of brief seizures), treated with pilocarpine to induce SE (model of prolonged seizures), or given sham-treatments (controls). The behavioral profile of all rats was assessed with the Morris water maze, passive avoidance and active avoidance tests. The occurrence of spontaneous behavioral seizures was also monitored in all groups.

Methods

Animals and treatments

Male Wistar rats, maintained under standard laboratory conditions, were used in this study. At 10 weeks of age, they were randomly divided into four groups. In the ECS group (n = 10), rats received a course of five ECS seizures, administered on a 24-hour schedule. Each stimulus (50 Hz, 60 mA for 1 second) was delivered via ear-clip electrodes wired to a stimulus generator (Model 215/IZ, Hugo-Sachs Elektronik, Germany). Two hours after the fifth stimulation, each of the animals received one additional ECS seizure. ECS produced full tonic–clonic seizures with hindlimb extension lasting 5–10 seconds. In the SE group (n = 10), rats were pretreated with scopolamine methyl bromide (1 mg/kg, subcutaneously (s.c.), Sigma) to minimize peripheral cholinergic side effects of pilocarpine. Thirty minutes later, the rats received a single high dose of pilocarpine (350 mg/kg, intraperitoneally (i.p.), Sigma). The onset of SE was defined as the appearance of behavioral symptoms corresponding to stage 4 or 5 seizures on the Racine scale (Racine, 1972), i.e. rearing, falling and generalized convulsions. SE onset was detected usually 30-60 min following the pilocarpine injection. Two hours after the beginning of SE, the rats were injected with diazepam (2.5 mg/kg, i.p.) in order to cease the convulsive manifestations of SE. However, seizure activity, albeit considerably reduced in severity, was not completely stopped by the single dose of diazepam. Thus, an additional dose of diazepam (2.5 mg/kg) was given to the rats 30 min after the first injection. The animals were periodically injected with saline (s.c.) during the first 24 h of the recovery period in order to improve their survival rate. Five rats in the sham-ECS group received handling identical to that of ECS-treated rats, but were not stimulated. The other five rats (sham-SE group) received handling and treatment identical to that received by rats in the SE group, including injections of scopolamine and diazepam, but were not treated with pilocarpine. However, the animals from the latter two groups were pooled into a single control group (n = 10), because, in our previous studies, we have never found any behavioral or neuroanatomical differences between the sham-ECS groups and sham-SE groups.

Following the respective treatments, the rats were daily observed for spontaneous behavioral seizures at random times between 08:00 h and 20:00 h. The handling and care of the animals were conducted according to the “Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) and “European Communities Council guidelines in animal research” (86/609/UE). All efforts were made to minimize the number of animals used and their suffering.

Behavioral procedures

Behavioral testing began when animals were 4.5 months old. All behavioral assessments were conducted by experimenters blinded to the treatment received by the rats. Before testing, animals were handled for 5 consecutive days. Experiments were performed after at least 30-min habituation of animals to the testing room. Testing was done at the same time of day, beginning at 13:00 h.

Step-through passive avoidance test

The apparatus used in this task was comprised of two adjacent compartments separated by a guillotine door. The larger, open topped compartment (45 × 45 × 45 cm; opaque acrylic walls) was brightly lit by two 60-W fluorescent bulbs mounted on the ceiling of the testing room and the smaller compartment (30 × 16 × 16 cm; black acrylic walls and top) was dark. The floor of both compartments was composed of stainless steel bars, 0.5 cm in diameter and spaced 1.2 cm apart (center-to-center), but only the floor of the dark compartment was wired to the stimulus generator. On the first day, the rats were allowed to explore the apparatus with the guillotine door open for 5 minutes. The following day, each rat was placed into the brightly lit compartment facing away from the closed door.

When it turned around to face the dark compartment, the door was manually raised and the latency for the rat to enter the dark compartment was recorded. Upon entry into the dark compartment, the door was lowered and a 1-mA, 1-s footshock was delivered 3 times at 5-s intervals. Ten seconds after the last shock, the rat was removed from the apparatus and returned to its home cage. Twenty four hours later, this procedure was repeated, with the exception that no footshock was delivered, and the latency to enter the dark compartment was again recorded (up to a maximum of 5 minutes).

Morris water maze test

After the retention trial of the passive avoidance test, the rats were given a 3 day resting period prior to testing in the Morris water maze (Morris, 1984). The maze consisted of a black circular tank, 180 cm in diameter and 50 cm deep, and was located in a corner of a room containing extramaze cues, i.e., three posters of different size and shape, and a computer desk. The apparatus was filled with water at room temperature (21 ± 1 ºC) to a depth of approximately 35 cm. The water was made opaque by adding a non-toxic paint. The maze was divided, by imaginary lines, into four equal-size quadrants. A black escape platform, 10 cm in diameter, was placed in the center of one of the quadrants. It was located 2 cm below the surface of the water. The swim path was recorded by a computerized video-tracking system (EthoVision V3.0, Noldus, The Netherlands). In the place learning task, the animals were trained to find the submerged escape platform and to climb on it. For acquisition, rats were given two trials on each day for 14 consecutive days. Each rat was placed in the water facing the pool wall at one of the four starting points that were used in a pseudo-random order so that each position was used once in each block of four trials. If the rats did not find the escape platform within 60 s, the experimenter guided them to the platform where they were allowed to remain for 15 s. After the first daily trial, the animals were placed in a clean cage, and a 30-s interval was imposed before the beginning of the next trial. The platform location was not changed during the acquisition period. The swim path length in each trial was calculated.

One day after completion of the acquisition, animals were submitted to a single 60-s probe trial in which the platform was removed from the pool. The number of times the rats swam through the zone where the platform had been located (platform crossings) provided a measure of accuracy in recalling the former position of the platform. The percentage of time spent by rats swimming in the training and opposite quadrants (quadrant preference scores) were also recorded.

Performance of animals on the visible platform task was assessed during a 2-day period following the day on which the probe trials were carried out. In this task, the rats were given 1 block of 4 trials per day separated by 30-s inter-trial intervals. The platform, painted in white, was exposed 3 cm above the water surface. The position of the platform was different in each trial. The distances swum to locate the platform were recorded and averaged across 8 trials.

Two-way active avoidance test

Testing in the shuttle-box began 5 days following the visible platform task in the water maze. The box consisted of two equally sized compartments (25 × 23 × 24 cm), connected by an opening (10 × 14 cm). The lower 3.5 cm of the opening connecting the two compartments were closed with a 0.5-mm thin stainless hurdle, thus, preventing the rats from staying there for more than a few seconds (Jongen-Rêlo et al., 2002). Three walls and the ceiling of the compartments consisted of black acrylic and the front door consisted of clear acrylic. The floor was composed of stainless steel bars, 0.5 cm in diameter and spaced 1.2 cm apart (center-to-center), wired to the stimulus generator. Small halogen bulbs (10 W) were mounted on the ceiling of each compartment. The apparatus was located in a quiet experimental room which was dimly illuminated (indirect light from one 25-W fluorescent bulb mounted on the wall). On the first day of testing, the rats were familiarized with the apparatus for 15 minutes. One day later, each rat was placed into one of the compartments and left undisturbed for 5 minutes. After this period, 45 training trials were administered. Each trial consisted of a 6-s conditioned stimulus (light) followed by unconditioned footshock stimulus (0.5 mA). Both stimuli terminated simultaneously after 20 seconds. The intertrial interval varied randomly from 20 s to 60 s. Crossing into the opposite (dark) compartment during the first 6 seconds of the trial, i.e. before the onset of the shock, was registered as a correct avoidance response. In addition, a total time taken by the rat from the onset of the conditioned stimulus to enter the dark compartment (response latency) was measured.

Tissue preparation

Following the completion of the behavioral experiments, six animals in each group, selected at random, were deeply anesthetized with sodium pentobarbital and killed by transcardial perfusion with a fixative solution containing 1% paraformaldehyde and 1% glutaraldehyde in 0.12 M phosphate buffer. The brains were removed from the skulls, weighed, codified, and placed in fresh fixative for 60 days. The cerebral

hemispheres were then separated by a midsagittal cut and alternately sampled for further processing. After removal of the frontal and occipital poles, the blocks containing the hippocampal formations and the adjacent neocortical shell were dehydrated through a graded series of ethanol solutions and embedded in glycolmethacrylate (hydroxyethylmethacrylate; Technovit 7100, Kulzer and Co., Wehrheim, Germany), as described in detail elsewhere (West et al., 1991). These blocks were then sectioned in the coronal plane at a nominal thickness of 40 µm using a Jung Multicut microtome. Every tenth section was collected using a systematic random sampling procedure (Gundersen and Jensen, 1987), mounted serially and stained with a Giemsa solution modified for use in glycolmethacrylate-embedded material (West et al., 1991).

Table 1
Summary of the stereological parameters used for the estimation of neuronal numbers.

	No. sections	asf	h (µm)	ΣQ^-	CE (N)
Granular layer	12	0.00493	10	187	0.09
Hilus	12	0.14592	10	212	0.08
CA3	12	0.02522	10	195	0.08
CA1	12	0.01722	10	198	0.07
Subiculum	12	0.02322	10	224	0.07
Presubiculum	12	0.01681	10	175	0.09
Parasubiculum	12	0.03852	10	193	0.08
Postsubiculum	12	0.02235	10	214	0.07
Entorhinal layer II	12	0.07032	10	245	0.08
Entorhinal layer III	12	0.01622	10	175	0.08
Entorhinal layer V/VI	12	0.01392	10	183	0.08

No. sections – mean number of sections per animal, asf – area sampling fraction, h – height of the optical disector, ΣQ^- – total number of neurons counted in each neuronal layer, CE (N) – mean coefficient of error.

Estimation of Neuron Numbers

The total numbers of neurons were estimated on Giemsa-stained sections by applying the optical fractionator method (West et al., 1991). The boundaries of the granular layer of the dentate gyrus and its hilus, the CA3 and CA1 hippocampal fields, the subiculum, presubiculum, parasubiculum and postsubiculum, and the entorhinal cortex were consistently defined at all levels along the rostrocaudal axis of the brain on the basis of cell morphology and cytoarchitectonic criteria (Amaral and Witter, 1989, 1995; Witter et al., 1989; van Groen and Wyss, 1990; Insausti et al., 1997; Mulders et al., 1997). Neurons belonging to the CA2 hippocampal field were included in the CA3 region. The transition between the CA1 field and neuronal layer of the subiculum was defined at the point where the cell bodies of CA1 pyramids ceased to be closely packed and disposed in a palisade. The distal limit of the subiculum was traced at the point of transition of the subicular neurons to the smaller presubicular neurons, which is well defined. In the presubiculum and parasubiculum, cell counts were restricted to layers II/III. The boundaries of the postsubiculum were delineated according to van Groen and Wyss (1990). The entorhinal cortex was delineated medially by its border with the parasubiculum and laterally by the well-defined rhinal sulcus. The boundaries of the entorhinal layers were identified on the basis of their cytoarchitectonic features. No counts were performed in the relatively cell-free layers I and IV. Likewise, for the purposes of this study, we did not discriminate between the medial and lateral regions of the entorhinal cortex (Amaral and Witter, 1995; Mulders et al., 1997). Estimations were carried out using the C.A.S.T.-Grid System (Olympus, Denmark). Beginning at a random starting position, visual fields were systematically sampled along the x and y axes, using a raster pattern procedure. Neurons were counted in every frame using the optical disector at a final magnification of ×2,000. The nucleus of the neurons was used as the counting unit. The coefficient of error (CE) of the individual estimates was calculated according to Gundersen et al. (1999) and it ranged between 0.07 and 0.09. The stereological parameters used for the estimations of cell numbers are summarized in Table 1.

Statistical analysis

Data derived from the passive avoidance test, acquisition trials of the water maze task, and from the active avoidance test were analyzed using repeated measures ANOVA. Percentages of time spent swimming in the training and opposite quadrants of the water maze during the probe trial were analyzed with two-way ANOVA. The remaining data were analyzed using one-way ANOVA. Newman-Keuls post hoc test was used where appropriate. All behavioral data are presented as the mean ± SEM, while morphological results are expressed as the mean ± SD. Differences were considered as significant at the $P < 0.05$ level.

Results

Behavioral monitoring during the recovery period

No behavioral abnormalities were detected in animals from the ECS-treated group and sham-treated control groups. However, following a latent period lasting 1-3 weeks, spontaneous motor seizures of stage 3 or greater on the Racine scale, i.e. tremor, forelimb

myoclonus, rearing and falling (Racine, 1972) were repeatedly observed in all rats that experienced SE.

Passive avoidance

The results of the passive avoidance testing are shown in Fig. 1. Repeated measures ANOVA revealed a significant main effect of seizures on the performance of rats on this task ($F_{2,27}$ = 3.23, P < 0.05), a significant effect of training ($F_{1,27}$ = 118.43, P < 0.0001) and a significant interaction between these two effects ($F_{2,27}$ = 15.52, P < 0.0001). Further, although ECS-treated rats did not differ from control rats in the baseline latency to enter the dark compartment, on the retention trial, the entry latencies were significantly reduced in this group relative to controls (P < 0.01; Newman-Keuls post hoc test). In the SE group, the latencies to enter the dark compartment on the retention test were also considerably reduced when comparing to control group (P < 0.01). However, the baseline latency was likewise reduced in these rats relative to both control and ECS-treated groups (P < 0.001), suggesting that the impaired performance of SE rats on this task might be related to a decreased motivation to enter the dark compartment of the apparatus, for example, due to reduced levels of anxiety (Cardoso et al., 2009).

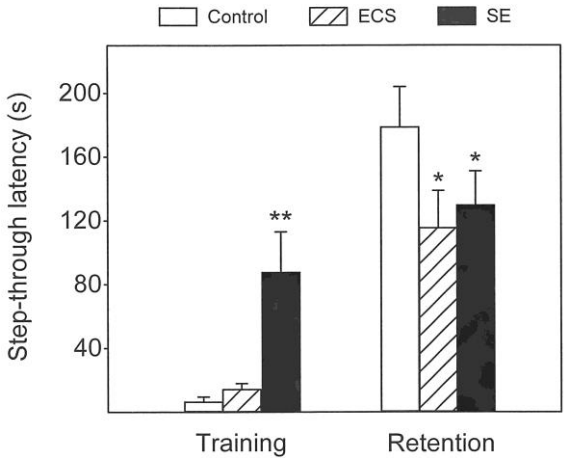

Fig. 1. Results from the passive avoidance test showing the mean (± SEM) step-through latencies during training as well as on the retention trial given 24 h later. Note that rats in the ECS group showed lower retention scores than did control rats. The performance of the rats in the SE group significantly differed from control rats during both training and retention sessions. *P < 0.01 and **P < 0.001 versus control group.

Water maze navigation

The mean distances swum by rats in the control, ECS and SE groups to locate the hidden platform in the place learning task are presented in Fig. 2. The statistical analysis by repeated measures ANOVA showed that the rats included in this experiment progressively improved their ability to find the platform over the 14 days of acquisition ($F_{6,162}$ = 18.49, P < 0.00001). However, the overall performance of the rats on this task differed among the groups studied, as indicated by a significant main effect of treatment ($F_{2,27}$ = 41.23, P < 0.00001) and significant treatment × trial block interaction ($F_{12,162}$ = 2.14, P < 0.01). Pairwise ANOVA revealed that animals from the ECS group performed the task less well than did control animals, as indicated by significant main effect of treatment ($F_{1,18}$ = 7.89, P < 0.05). On the other hand, analysis of the data showed that there was no significant interaction between the group effect and the course of training. This lack of significant interaction effects indicates that ECS-treated rats were equally impaired during the period of initial learning of the task and on the late phases of training. However, a visual inspection of the data presented in Fig. 2 clearly shows

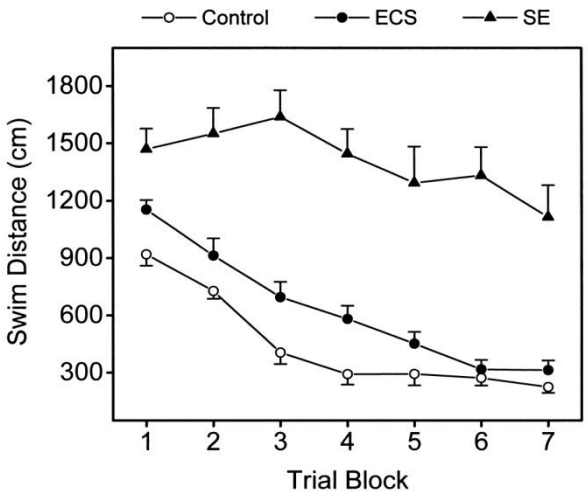

Fig. 2. The mean swim distance (± SEM) in centimeters (cm) to reach a hidden platform for each block of four consecutive trials throughout the acquisition of the water maze task. Note that the performance of SE rats on this task was extremely poor (P < 0.0001 versus control group). However, ECS-treated rats also performed less well than did control rats (P < 0.05).

that the performance of ECS-treated rats during the last four training days did not differ from that of control rats. In the SE group, the rats performed poorly during the entire period of training finding ($F_{1,18}$ = 53.78, P < 0.00001 for main effect of treatment and $F_{6,108}$ = 2.32, P < 0.05 for group × trial block interaction). Post hoc comparisons showed that the learning scores of SE rats for all trial blocks were significantly inferior to those of control rats (P < 0.0001).

Behavioral measures obtained on the probe trial are shown in Table 2. Two-way ANOVA revealed a significant effect of treatment on the total percentages of time spent swimming in the training and opposite quadrants ($F_{2,27}$ = 4.12, P < 0.05), a significant effect of quadrant ($F_{1,27}$ = 71.64, P < 0.00001) and a significant interaction between the two variables ($F_{2,27}$ = 10.13, P < 0.001). Post-hoc tests showed that both control and ECS-treated rats spent more time in the training

quadrant than in the opposite quadrant ($P < 0.0001$), suggesting that the animals from these groups used a spatial strategy when searching for the escape platform during the probe trial. In contrast, rats from the SE group spent similar percentages of time swimming in the training and opposite quadrants ($P = 0.25$). Furthermore, these rats spent less time in the training quadrant ($P < 0.005$) and crossed the former position of the escape platform less frequently ($P < 0.05$) than did control rats. However, the ECS group did not differ from the control group on the probe trial measures, thus, supporting the impression that, by the end of training, ECS-treated rats were able to perform the task as well as controls despite being significantly impaired on the initial acquisition trials.

Animals in all groups quickly learned to find the visible platform. The distances swum to locate the platform position, averaged over eight trials, were 238 ± 82 cm in the control group, 215 ± 95 cm in the ECS group and 305 ± 105 cm in the SE group. ANOVA failed to reveal a significant effect of treatment, indicating that all groups had comparable levels of performance on the cued platform task.

Table 2

The percentage of time spent by rats in the training quadrant of the water maze during probe trial and the number of times they swam through the zone where the platform had been located (platform crossings). For comparison, the percentage of time spent by rats in the opposite quadrant of the maze is also shown.

	Time (%) training quadrant	Time (%) opposite quadrant	Platform crossings
Control	52.6 ± 4.3$^{(\gamma)}$	8.9 ± 2.4	3.5 ± 0.4
Electroshock	56.4 ± 4.5$^{(\gamma)}$	9.4 ± 2.7	2.7 ± 0.6
Status epilepticus	31.2 ± 2.9$^{(\beta)}$	23.3 ± 2.2	1.3 ± 0.4$^{(\alpha)}$

Values are means ± SEM, n = 10 in each group.
$^{(\alpha)}$ – $P < 0.05$ and $^{(\beta)}$ – $P < 0.005$ versus control group
$^{(\gamma)}$ – $P < 0.0001$ versus opposite quadrant.

Active avoidance

Fig. 3A shows mean latencies to cross into the safe compartment after the light was presented, which were averaged over blocks of five consecutive trials each. Although the response latencies taken by rats decreased across the acquisition trials ($F_{8,216} = 64.91$, $P < 0.00001$ for the effect of trial blocks), the overall rate of response learning was different among the groups studied ($F_{16,216} = 8.54$, $P < 0.00001$ for the group × trial block interaction). Post hoc comparisons made for the

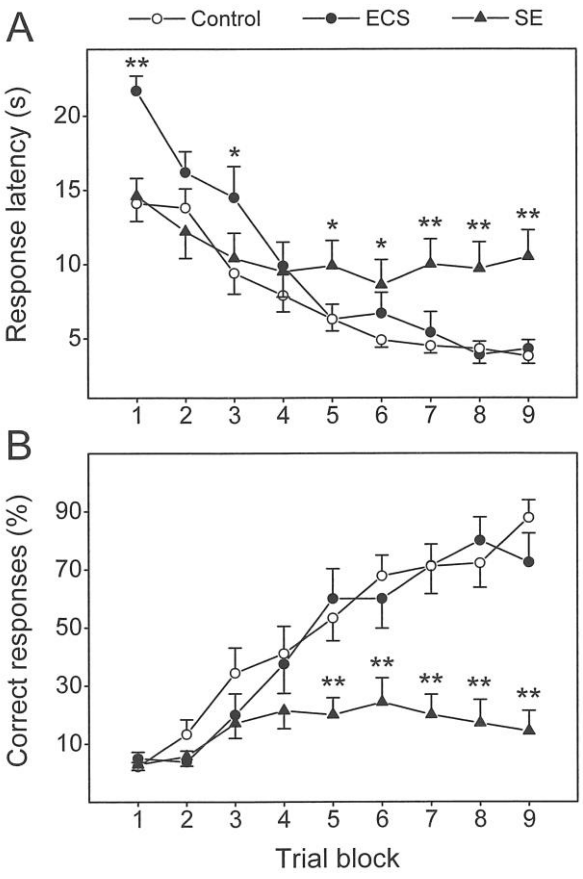

Fig. 3. Results from the active avoidance test showing the mean (±SEM) latency to cross to the safe compartment after the onset of the light stimulus (A) and the mean (± SEM) percentage of correct responses, that is crossings to the other side before the onset of the footshock (B). Both parameters were averaged over blocks of five consecutive trials each. Note that, relative to the control group, ECS-treated rats were slower in responding on the first and third trial blocks, but they did not differ from controls in the response accuracy throughout the entire course of training. In contrast, both the response latencies and the percentages of correct responses shown by SE rats during the last trial blocks were considerably inferior to the respective scores of control rats. *$P < 0.05$ and **$P < 0.0001$ versus control group.

group by trial block interaction showed that ECS-treated rats performed less well than controls in the beginning of training, i.e. on the first ($P < 0.0001$) and third ($P < 0.05$) trial blocks, while rats in the SE group were, in contrast, significantly impaired on the last 5 trial blocks ($P < 0.05$ for the fifth and sixth trial blocks, and $P < 0.0001$ for the seventh, eighth and ninth trial blocks). In fact, even at the end of training, the response latencies in this group remained superior to the 6-s "shock-free" interval, indicating that SE rats were only able to learn to cross into the safe compartment in response to footshock and showed no conditioning.

Fig. 3B shows the mean percentage of correct responses, i.e. crossings to the other side of the shuttle box before the onset of the footshock, which were averaged over blocks of five consecutive trials each. Although the response accuracy of the rats included in this experiment improved progressively across the acquisition trials ($F_{8,216}$=15.65, P < 0.00001 for the effect of trial block), this improvement was not uniform across the groups, as indicated by a significant main effect of treatment ($F_{2,27}$ = 10.79, P < 0.0001) and significant treatment × trial block interaction ($F_{16,216}$ = 6.95, P < 0.00001). In particular, starting from the fifth trial block, the animals from the SE group showed significantly less correct responses relative to both control group and ECS group (P < 0.0001). By the end of training, control and ECS-treated rats reached the level of approximately 80% of correct responses, whereas rats in the SE group remained at the level of 20%. No differences between the control group and ECS group were detected on this measure.

Neuron numbers

The estimates of the total numbers of neurons in various subdivisions of the hippocampal formation of the rats used in this study are shown in Table 3. Analysis of these data revealed that there was a significant main effect of seizures on cell numbers in the dentate hilus ($F_{2,15}$ = 69.85, P < 0.00001) and granule cell layer ($F_{2,15}$ = 10.29, P < 0.005), hippocampal pyramidal CA3 ($F_{2,15}$ = 25.46, P < 0.00001) and CA1 ($F_{2,15}$ = 84.97, P < 0.00001) fields, subiculum ($F_{2,15}$ = 17.90, P < 0.001), neuronal layers II/III of the presubiculum ($F_{2,15}$ = 35.04, P < 0.0001), parasubiculum ($F_{2,15}$ = 35.10, P < 0.00001), and postsubiculum ($F_{2,15}$ = 9.53, P < 0.001), and in layers II ($F_{2,15}$ = 6.88, P < 0.01), III ($F_{2,15}$ = 52.74, P < 0.00001) and V/VI ($F_{2,15}$ = 32.46, P < 0.00001) of the entorhinal cortex. Post-hoc comparisons showed that the administration of ECS seizures provoked a significant neuron loss in the dentate hilus (20%, P < 0.001), subiculum (14%, P < 0.05), presubiculum (19%, P < 0.05), parasubiculum (26%, P < 0.001), and entorhinal layers III (24%, P < 0.001) and V/VI (17%, P < 0.05). However, the changes in neuron numbers in the very same hippocampal and parahippocampal areas were even more robust after SE, reaching 50% in the dentate hilus (P < 0.0001), 40% in subiculum (P < 0.01), 49% in presubiculum (P < 0.001), 45% in parasubiculum (P < 0.001), and 56% in the entorhinal layers III (P < 0.0001) and 50% in layers V/VI (P < 0.0001). In these rats, post-hoc tests additionally revealed significant decline in the total neuron numbers in the dentate granule cell layer (22%, P < 0.01), hippocampal CA3 (40%, P < 0.0001) and CA1 (50%, P < 0.0001) fields, postsubiculum (33%, P < 0.001), and entorhinal layers II (21%, P < 0.01). Representative photographs of brain sections obtained from control, ECS-treated and SE rats are shown in Fig. 4.

Table 3
Total number of neurons in different layers of the hippocampal formation, subicular complex and entorhinal cortex of control rats, rats treated with electroshock, and rats that had experienced *status epilepticus*.

	Control	Electroshock	*Status epilepticus*
Granular layer	1160128 ± 95189	1090009 ± 151925	850206 ± 118501$^{(\beta)}$
Hilus	55241 ± 4574	44230 ± 3555$^{(\nu)}$	27369 ± 1367$^{(\delta)}$
CA3	238055 ± 17654	234228 ± 17069	153479 ± 30780$^{(\delta)}$
CA1	358513 ± 11918	347677 ± 30567	176301 ± 30719$^{(\delta)}$
Subiculum	316331 ± 28317	271018 ± 10608$^{(\alpha)}$	199451 ± 47816$^{(\beta)}$
Presubiculum	341480 ± 19384	278713 ± 40255$^{(\alpha)}$	175654 ± 21117$^{(\nu)}$
Parasubiculum	134449 ± 12531	100032 ± 9376$^{(\nu)}$	74602 ± 11405$^{(\nu)}$
Postsubiculum	380399 ± 71577	349236 ± 37467	257875 ± 27130$^{(\nu)}$
Entorhinal layer II	122247 ± 14952	130237 ± 17393	97199 ± 15852$^{(\beta)}$
Entorhinal layer III	294655 ± 29890	225089 ± 30415$^{(\nu)}$	130148 ± 22559$^{(\delta)}$
Entorhinal layer V/VI	347907 ± 39467	288273 ± 37485$^{(\alpha)}$	174630 ± 36533$^{(\delta)}$

Values are means ± SD, n = 6 in each group.
$^{(\alpha)}$ – P < 0.05, $^{(\beta)}$ – P < 0.01, $^{(\nu)}$ – P < 0.001, and $^{(\delta)}$ – P < 0.0001 versus control group.

Discussion

The main finding of the present study is that a few brief generalized seizures elicited by ECS were capable of producing significant loss of neurons in the hippocampal formation and long-lasting impairments of hippocampal-dependent behaviors. However, the effects of brief seizures on hippocampal structure and

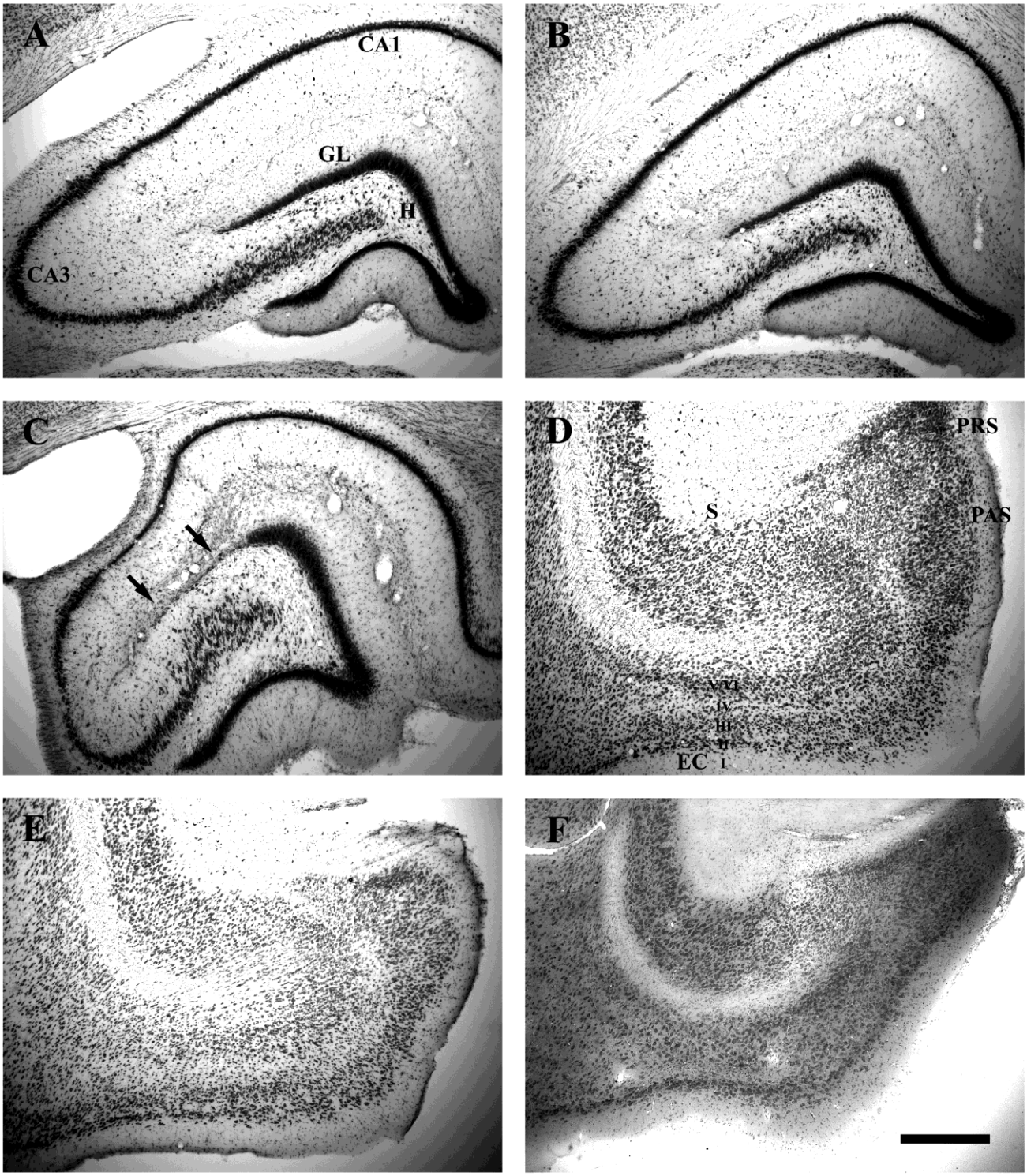

Fig. 4. Photomicrographs of representative Nissl-stained coronal sections containing the dentate gyrus, CA3 and CA1 hippocampal fields (A-C) and the subicular complex and entorhinal cortex (D-F) from a sham-treated control rat (A, D) and from rats treated with ECS (B, E) and pilocarpine (C, F). Note that the density of neurons in the dentate hilus, CA3 and CA1 hippocampal fields, and suprapyramidal blade of the dentate granular layer (arrows) is dramatically reduced in the pilocarpine-treated animal (C) when compared to the control rat (A). In the ECS-treated animal (B), the density of cells in the dentate hilus is also somewhat reduced relative to the control rat. Note also that the density of neurons in the subiculum, presubiculum, parasubiculum and entorhinal layers III and V/VI is markedly reduced in the pilocarpine-treated animal (F) compared with the control rat (D). In the rat treated with ECS (E), the packing density of the neurons in the subiculum, presubiculum, parasubiculum and entorhinal layer III also appears to be somewhat reduced compared to the control rat. GL, granular layer; H, hilus; CA3, pyramidal cell layer of the CA3 hippocampal field; CA1, pyramidal cell layer of the CA1 hippocampal field; S, subiculum; PRS, presubiculum; PAS, parasubiculum; EC, II, III, V/VI, entorhinal cortex and its layers. Scale bar = 500 μm.

function were not as disastrous as those of SE, which produced massive death of neurons in all the subdivisions of the hippocampal formation and severe disruption of hippocampally mediated behaviors. In

addition, the brain damage produced by SE was associated with the development of spontaneous recurrent seizures, whereas the more moderate ECS-induced neuronal loss had no epileptogenic consequences.

This is the first study to show that ECS seizures produce neuronal loss in an array of closely interconnected cortical regions, including the entorhinal cortex, dentate hilus, subiculum, presubiculum and parasubiculum, thus suggesting that these structures are strongly activated by propagating generalized seizures. It has been previously reported that this network can also include the retrosplenial granular b cortex (Cardoso et al., 2008b). The neuroanatomical changes induced by ECS seizures were accompanied by mild, but significant impairments of spatial learning and passive avoidance. These behavioral deficits are not surprising given that the structures in which ECS seizures caused neuronal loss are all implicated in spatial navigation and the formation of long-term memories (Morris, 1984; Squire, 1992; Eichenbaum, 1999). That the behavioral changes induced by ECS administration are due to cell loss in the hippocampal and parahippocampal cortical regions is indirectly supported by the finding that simple response learning of the active avoidance paradigm was unaffected in these rats. It can be still argued that ECS-induced neuropathology can alter the performance of rats in learning and memory tasks by producing some nonspecific behavioral abnormalities, such as changes in emotionality or state of arousal. This possibility is supported by the fact that the hippocampal formation is interconnected with many brain structures implicated in the regulation of emotionality including the amygdala, bed nucleus of the stria terminalis and prefrontal cortex (Jay and Witter, 1991; Ishikawa and Nakamura, 2006). Furthermore, it is involved in central regulation of the hypothalamo-pituitary-adrenocortical stress axis (Herman and Cullinan, 1997). Thus, the ECS-induced loss of hippocampal and subicular neurons may indeed lead to malfunctioning of the brain circuits regulating emotionality and stress responsiveness. In effect, we have recently reported that repeated administration of ECS seizures has an anxiogenic-like effect and is associated with elevated levels of general behavioral arousal (Cardoso et al., 2009). However, the increased levels of anxiety and behavioral arousal are rather predictive of facilitated learning in the aversively motivated tasks, such as the water maze and passive avoidance; in fact, it has been previously found that ECS treatment markedly facilitates fear conditioning (Cardoso et al., 2009). However, this was not the case in the present study, suggesting that the behavioral impairments observed in ECS-treated rats were caused by structural defects in the hippocampo-entorhinal memory network, which impeded normal information flow and/or encoding.

The present findings are in contrast with the results of several prior studies, in which no gross anatomical changes within the hippocampal formation were found following repeated administration of ECS (Devanand et al., 1994; Gombos et al., 1999; Vaidya et al., 1999). A possible reason for this discrepancy could be that the previous studies were carried out in rats treated according to a sparse stimulation protocol, whereas we used a specially designed protocol in which the last two seizures were set only 2 h apart. Differences in the methods of morphological analysis, as well as other methodological differences, could also contribute to this discrepancy. However, the current data are consistent with the profile of neuronal changes previously reported in rats (Lukoyanov et al., 2004), which were treated using a protocol similar to ours, except that seizures were elicited by applying 200-volt direct current (in the present study, rats were stimulated using an alternating 50-Hz current). Furthermore, our findings are quite compatible with the results of studies employing another model of brief seizures, electrically induced kindling, which showed that multiple secondary generalized seizures evoked by repeated stimulation of either the olfactory bulb or perforant pathway are associated with apoptosis and progressive neuronal loss in the dentate gyrus, hippocampus and subiculum (Cavazos and Sutula, 1990; Cavazos et al., 1994; Bengzon et al., 1997; Zhang et al., 1998; Haas et al., 2001; Kotloski et al., 2002). With respect to the behavioral consequences of seizures, it has been repeatedly reported that hippocampal kindling produces irreversible deficits in hippocampally mediated behaviors, including spatial memory (Lopes da Silva et al., 1986; Sutula et al., 1995; Gilbert et al., 2000; Hannesson et al., 2001). It is worth noting that the spatial memory deficits of kindled rats were demonstrated using both the Morris water maze (Gilbert et al., 2000; Hannesson et al., 2001) and the radial maze task (Lopes da Silva et al., 1986; Sutula et al., 1995), the latter not being dependent on aversive motivation. This provides additional support for the claim that brief seizures can produce brain changes that are directly responsible for the subsequent cognitive deficits, while that these deficits may show a high comorbidity with impairments of anxiety-related behaviors has never been questioned (Kalynchuk, 2000; Cardoso et al., 2009).

One of the goals of the present study was to directly compare the brain damage induced by brief seizures to that resulted from SE. The morphological analysis of brain sections obtained from rats in these groups have showed that a single episode of prolonged seizure activity killed approximately half of the neurons in the very same cortical regions in which ECS-induced neuronal loss barely exceeded 20%, i.e., in the dentate hilus, subicular complex and entorhinal layers III and V/VI. In addition, SE was associated with a massive loss

of neurons in the hippocampal pyramidal fields CA3 and CA1. Behaviorally, SE rats were not only unable to acquire the hippocampally mediated tasks, but were also severely impaired in response learning. These findings are in full agreement with prior results of other researchers, who reported marked neuronal loss and robust behavioral impairments in animals after SE induced by pilocarpine (Turski et al., 1983; Cavalheiro et al., 1991), kainic acid (Stafstrom et al., 1993; Buckmaster and Dudek, 1997), and electrical stimulation of the perforant pathway (Sloviter, 1987; Gorter et al., 2001) or amygdala (Nissinen et al., 2000; Kemppainen et al., 2006). Notably, the present experiment revealed that SE triggers cell loss, albeit moderate, in neuronal populations whose vulnerability to seizures has not yet been reported or remains a matter of controversy, that is, in the presubiculum and dentate granule cell layer. In particular, previous studies have shown that SE in rats produces neuronal loss in the parasubiculum, but not in the presubiculum (Pitkänen et al., 1995; van Vliet et al., 2004). Yet, in the present study SE was associated with neuronal loss both in the parasubiculum and presubiculum. This discrepancy is likely to be due to methodological differences between this study and the studies that were performed in other laboratories. For example, in the studies cited above SE was induced by either electrical stimulation of the perforant pathway (Pitkänen et al., 1995) and angular bundle (van Vliet et al., 2004) or systemic administration of the kainic acid (Pitkänen et al., 1995), whereas we used the pilocarpine model of SE. Nonetheless, the possibility of the involvement of presubicular neurons in epileptogenesis is supported by the results of immunocytochemical and electrophysiological studies (van Vliet et al., 2004; Peng and Houser, 2005; Tolner et al., 2005). With respect to the granule cells, our findings are consistent with those reported by Thind et al. 2010, who found that SE kills approximately 20% of granule cells in 35-day-old rats, but that their total number recovers quickly, probably, as a result of ongoing neurogenesis. In our experiment, this recovery did not occur, perhaps, because we used adult 10-week-old rats, in which neurogenesis is likely to be blunted. To the extent that this view is true, it is worth mentioning that the SE-induced granule cell loss found in the present study was particularly prominent in the suprapyramidal blade of the granular layer (Fig. 4), an area characterized by a reduced rate of neurogenesis in adult rats (Choi et al., 2007). In addition, granule neurons located in this area are likely to be more vulnerable to excessive excitation associated with seizures as their dendrites are longer and more spiny when compared to neurons located in other divisions of the granular layer (Desmond and Levy, 1982; Claiborne et al., 1990). In line with this, repeated administration of ECS seizures resulted in atrophic changes in the dendritic arborizations of neurons located in the suprapyramidal blade (Cardoso et al., 2008a). Taken together, the findings from this study and data previously reported by other researchers clearly show that both prolonged seizure activity and brief seizures produce significant structural and functional alterations in hippocampo-entorhinal circuits, but those caused by prolonged seizures are undoubtedly more profound.

Interestingly, superimposing the neurodegeneration patterns that resulted from treating rats with ECS and SE may help distinguish neuronal populations whose loss contributes to epileptogenesis from those whose loss does not. In this regard, it might be noted that in ECS model, which does not lead to spontaneous seizures, we observed no signs of cell death in the hippocampal pyramidal fields CA3 and CA1. Contrariwise, SE, while inducing the development of spontaneous recurrent seizures, kills approximately half of the hippocampal pyramidal neurons. This finding lends support to the idea that death of hippocampal pyramidal neurons, which may lead to the deafferentiation of the subiculum and other hippocampal targets, can be one of the triggering mechanisms of epileptogenesis. Another important difference between the two models is that ECS seizures kill 20% of cells in the dentate hilus, but spare many inhibitory interneurons, at least all of those that express neuropeptide Y and somatostatin, whereas SE is associated with a loss of majority of hilar interneurons (Cardoso et al., 2010). This result, in turn, extends the evidence in favor of the GABAergic dysfunction hypothesis of epileptogenesis (Sloviter, 1987; Buckmaster and Dudek, 1997; Gorter et al., 2001).

In summary, the present findings shed new light on cellular mechanisms of seizure-induced brain damage, dysfunction and epileptogenesis. These data show that brain damage elicited by repeated brief seizures, while not as widespread as observed after SE and not epileptogenic, can be, however, associated with noticeable behavioral impairments. The present findings underlie the importance of effective seizure management in preventing the development and progression of epilepsy. In addition, taken together with what is known from studies in humans (Sackeim et al., 2007), our study provides a cautionary note when considering electroconvulsive treatment in psychiatric patients, as this may be associated with enduring and probably irreversible cognitive deficits.

Acknowledgments

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Loss of Synapses in the Entorhinal-Dentate Gyrus Pathway Following Repeated Induction of Electroshock Seizures in the Rat

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The goal of this study was to answer the question of whether repeated administration of electroconvulsive shock (ECS) seizures causes structural changes in the entorhinal-dentate projection system, whose neurons are known to be particularly vulnerable to seizure activity. Adult rats were administered six ECS seizures, the first five of which were spaced by 24-hr intervals, whereas the last two were only 2 hr apart. Stereological approaches were employed to compare the total neuronal and synaptic numbers in sham- and ECS-treated rats. Golgi-stained material was used to analyze dendritic arborizations of the dentate gyrus granule cells. Treatment with ECS produced loss of neurons in the entorhinal layer III and in the hilus of the dentate gyrus. The number of neurons in the entorhinal layer II, which provides the major source of dentate afferents, and in the granular layer of the dentate gyrus, known to receive entorhinal projections, remained unchanged. Despite this, the number of synapses established between the entorhinal layer II neurons and their targets, dentate granule cells, was reduced in ECS-treated rats. In addition, administration of ECS seizures produced atrophic changes in the dendritic arbors of dentate granule cells. The total volumes of entorhinal layers II, III, and V–VI were also found to be reduced in ECS-treated rats. By showing that treatment with ECS leads to partial disconnection of the entorhinal cortex and dentate gyrus, these findings shed new light on cellular processes that may underlie structural and functional brain changes induced by brief, generalized seizures.

Key words: electroshock; hippocampal formation; granule cells; synaptic reorganization; stereology

Seizures provoked by electroconvulsive shock (ECS) provide an animal model for generalized epilepsy (Fisher, 1989). In humans, electroconvulsive therapy is regarded as one of the efficient, albeit controversial, treatments for otherwise intractable psychiatric disorders (Fink, 1999; Sterling, 2000). In animals, administration of ECS seizures has been shown to induce a number of metabolic and biochemical changes in the brain, including increased blood flow (André et al., 2002), variations in the synthesis and expression of neuropeptides (Mikkelsen and Woldbye, 2006), and changes in the levels of small-molecule neurotransmitters (Rowley et al., 1997). In addition, subtle structural changes, such as enhanced granule cell neurogenesis in the dentate gyrus (Scott et al., 2000) and sprouting of mossy fibers into its molecular layer (Gombos et al., 1999; Vaidya et al., 1999), were also found after ECS treatment.

Despite the widespread acceptance of the view that repeated ECS seizures can cause gross anatomical lesions in the brain (Sterling, 2000), this issue has not yet been thoroughly addressed. The main goal of the present study was, therefore, to obtain direct experimental evidence that might help to validate this hypothesis. To do so, we decided to focus this study on the effects of ECS on the entorhinal-dentate projection system, because there is strong evidence that both the entorhinal cortex and the dentate gyrus are particularly vulnerable to seizures (Sloviter et al., 1996; McNamara, 1999; Salmenperä et al., 2000). This choice is also based on our recent finding that repeated administration of ECS seizures in rats causes loss of approximately 17% of neurons in the hilus of the dentate gyrus (Lukoyanov et al., 2004). Indeed, because hilar neurons are directly involved in the regulation of the excitatory drive from the entorhinal cortex upon dentate granule cells (Buckmaster and Schwartzkroin, 1994; Vida and Frotscher, 2000), their loss is likely to be indicative of more devastating changes in the entorhinal-dentate pathway. Methodologically, we used a modified stimulation protocol such that the animals were administered six ECS seizures, the first five of which were spaced by 24-hr intervals, whereas the last

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two were only 2 hr apart. This protocol is based on the finding that repeated induction of five widely-spaced ECS seizures reduces the capacity of the brain amino acid reuptake system to maintain normal levels of glutamate for a minimum of 2 hr (Rowley et al., 1997), which renders neurons especially vulnerable to seizures elicited during this postictal period (Lukoyanov et al., 2004). Previous studies have shown that administration of ECS seizures at 24-hr or 48-hr intervals, i.e., on schedules similar to those commonly used in psychiatric practice, does not appear to cause significant neuronal loss (Gombos et al., 1999; Vaidya et al., 1999). For morphometric evaluation, we applied unbiased stereological approaches to compare quantitatively the total numbers of entorhinal and dentate gyrus neurons in rats repeatedly treated with ECS and in sham-treated rats. The effect of ECS seizures on the total number of excitatory synapses established between entorhinal neurons and dentate granule cells was assessed at the ultrastructural level by applying the physical disector method and the principle of Cavalieri. Golgi-stained sections of the hippocampal formation were used to analyze the dendritic arborizations of the dentate gyrus granule cells.

MATERIALS AND METHODS

Animals and Treatment

Thirty-six male Wistar rats were used in the present study. Animals were maintained under standard laboratory conditions and had free access to food and water. At 2 months of age, 18 rats were selected at random and received a course of five ECS seizures, administered on a 24-hr schedule as previously described (Rowley et al., 1997; Lukoyanov et al., 2004). Briefly, each stimulation (constant voltage of 200 V for 2 sec) was delivered via ear-clip electrodes wired to a stimulus generator (model 215/IZ; Hugo-Sachs Elektronik, Germany) and produced full tonic-clonic seizure with hind-limb extension lasting for 5–10 sec. Two hours after the fifth stimulation, each of the animals received one additional ECS seizure. The remaining 18 rats (control group) received handling identical to that of experimental rats, including attachment of ear-clip electrodes for 30 sec, but were not stimulated. All animals were killed at 3 months of age. From each group, 12 randomly selected rats were perfused with a mixture of paraformaldehyde and glutaraldehyde, and their brains were used either for embedding in Epon or glycolmethacrylate or for Golgi impregnation. The remaining six rats from each group were perfused with sodium sulfide solution, and their brains were used for Timm histochemistry as described below.

The handling and care of the animals were conducted according to the European Communities Council guidelines on animal research (86/609/UE), following the Portuguese regulations for the use of laboratory animals (Act 129/92). All efforts were made to minimize the number of animals used and their suffering.

Tissue Preparation: General Procedures

Twelve control and twelve ECS-treated rats were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and transcardially perfused with a fixative solution containing 1% paraformaldehyde and 1% glutaraldehyde in 0.12 M phosphate buffer at pH 7.4. The brains were removed from the skulls, weighed, codified to allow for blind estimations, separated by a midsagittal cut into right and left halves, and placed in fresh fixative. The frontal and occipital poles were removed, and the remaining blocks of tissue containing the hippocampal formations were separated. In total, 24 tissue blocks containing the hippocampal formation per group were obtained and processed for embedding in Epon (six per group) or glycolmethacrylate (12 per group) or for Golgi impregnation (six per group). Because the hippocampal formations of rodents display right/left asymmetries, the blocks were alternately sampled from the right and left hemispheres so that, whatever the procedure performed, hippocampal formations from both sides were included.

Epon Embedding

After 2 hr of postfixation, tissue blocks from six animals per group were selected at random, and the hippocampal formations were isolated. After resection of the septal and temporal poles, the remaining midseptotemporal parts of the hippocampal formation were sliced perpendicular to the septotemporal axis into 1-mm slabs on a tissue chopper. For each animal, three slabs were selected using a systematic random sampling procedure and processed for electron microscopy (Palay and Chan-Palay, 1974). Briefly, they were fixed for 2 hr in a 2% solution of osmium tetroxide, dehydrated through a graded series of ethanol solutions, and embedded in Epon according to the isector method in order to ensure isotropy (Nyengaard and Gundersen, 1992; Madeira et al., 1995). Two-micrometer-thick sections were cut from each slab, mounted on a glass slides, and stained with toluidine blue. Then, from each slab, 10–12 serial ultrathin sections containing the outer molecular layer (OML) of the dentate gyrus and 10–12 sections containing the inner molecular layer (IML) were cut. All ultrathin sections were collected on formvar-coated grids and double stained with uranyl acetate and lead citrate.

Golgi Impregnation

Blocks containing the hippocampal formation, also from six animals per group, were postfixed for an additional 15 days in the fixative solution that was used for perfusion. Impregnation was performed as described by Eckenhoff and Rakic (1984). After the silver nitrate reaction, the tissue was wrapped in a paraffin shell and sliced in the horizontal plane at a nominal thickness of 100 μm. The tissue slices were dehydrated, cleared in terpineol, and mounted on slides under Damar resin, with no coverslip.

Glycolmethacrylate Embedding

After 30 days of postfixation, the remaining blocks of tissue containing the entire hippocampal formations and the adjacent neocortical shell, from 12 animals per group, were dehydrated through a graded series of ethanol solutions and embedded in glycolmethacrylate, as described in detail elsewhere (West et al., 1991). These blocks were then serially

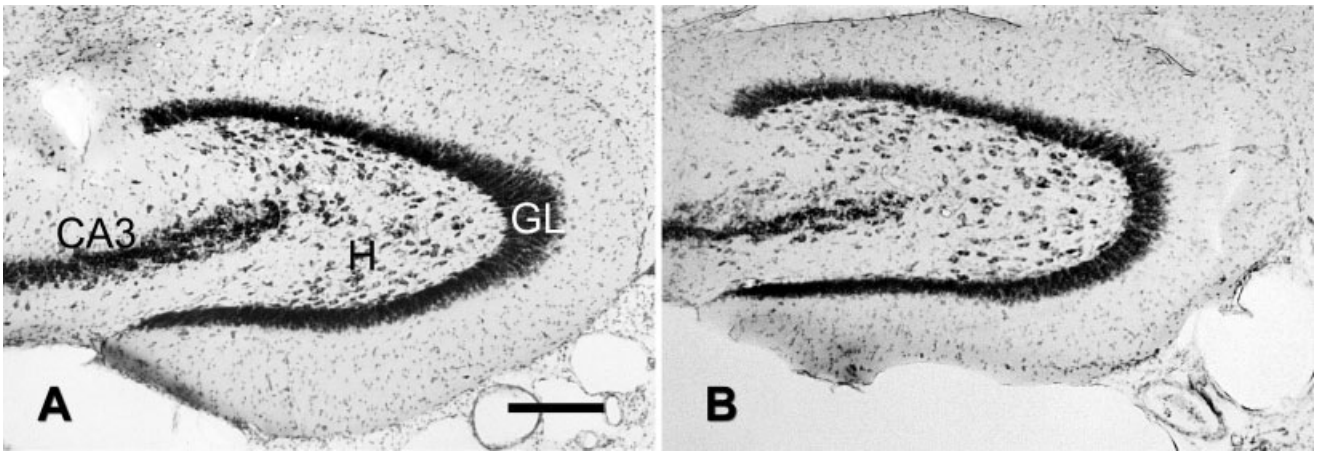

Fig. 1. Photomicrographs of Giemsa-stained horizontal sections of the dentate gyrus, cut at the midseptotemporal level, of a control rat (**A**) and of a rat that received a course of ECS treatment (**B**). The density of cells in the hilus of the dentate gyrus appears to be somewhat lower in the ECS rat than in the control rat. GL, granule cell layer; H, hilus; CA3, pyramidal cell layer of the CA3 hippocampal field. Scale bar = 220 μm.

sectioned in the horizontal plane at a nominal thickness of 40 μm using a Jung Multicut microtome. Every tenth section was collected using a systematic random sampling procedure (Gundersen and Jensen, 1987), mounted serially, and stained with a modified Giemsa solution (West et al., 1991).

Timm Staining

Six remaining animals from each group were used for histochemical staining with the Timm sulfide silver method (Danscher and Zimmer, 1978). Rats were anesthetized and transcardially perfused with a sodium sulfide solution (24 mM in phosphate buffer, pH 7.4), followed by 3% glutaraldehyde in Sörensen buffer (110 mM Na_2HPO_4, 40 mM KH_2PO_4, adjusted to pH 7.4). The brains were removed from the skulls, weighed, and codified to allow for blind estimations. Then, the hippocampal formations were isolated and immersed in a 30% sucrose solution, where they were stored overnight. After being frozen with gaseous carbon dioxide, the hippocampal formations, alternately sampled from the left and right hemispheres, were sectioned in the horizontal plane at a nominal thickness of 50 μm. Sections were serially mounted, subjected to physical development, and mounted in Damar resin.

Estimation of Neuron Numbers

The total numbers of neurons were estimated on glycolmethacrylate-embedded sections (from six rats per group) by applying the optical fractionator method (West et al., 1991). The boundaries of the granular layer of the dentate gyrus and its hilus were consistently defined at all levels along the septotemporal axis of the hippocampal formation on the basis of cell morphology and cytoarchitectonic criteria (Amaral and Witter, 1995; see Fig. 1). The entorhinal cortex was delineated medially by its border with the parasubiculum and laterally by the well-defined rhinal sulcus (see Fig. 2). The boundaries of the entorhinal layers were identified on the basis of their cytoarchitectonic features. No counts were performed in the relatively cell-free layers I and IV. Likewise, for the purposes of this study, we did not discriminate between the medial and the lateral regions of the entorhinal cortex (Amaral and Witter, 1995; Mulders et al., 1997). Estimations were carried out using the C.A.S.T.-Grid System (Olympus, Denmark), and a mean of 14 (dentate gyrus) or 12 (entorhinal cortex) sections was used per animal. Beginning at a random starting position, visual fields were systematically sampled along the x and y axes, using a raster pattern procedure. Neurons were counted in every frame using the optical disector at a final magnification of ×2,000. The coefficient of error (CE) of the individual estimates was calculated according to Gundersen et al. (1999) and ranged between 0.07 and 0.09. The stereological parameters used for the estimations of cell numbers are summarized in Table I.

Estimation of Volumes of Neuronal Layers

The total volumes of the different neuronal layers of the dentate gyrus and entorhinal cortex were estimated by applying the principle of Cavalieri (Gundersen et al., 1988; Regeur and Pakkenberg, 1989). All the sampled glycolmethacrylate-embedded sections that were used for cell counting (from six animals per group) were included in the analysis. In each section, the cross-sectional area of the neuronal layers was estimated by point counting (Gundersen and Jensen, 1987), at a final magnification of ×80, using an adequate grid of test points (C.A.S.T.-Grid System). The volumes of the neuronal layers were calculated from the total number of points that fell on each layer and the distances between the systematically sampled sections (Gundersen et al., 1988; Regeur and Pakkenberg, 1989).

Estimation of Synapse Numbers

The total numbers of synapses in the dentate gyrus molecular layer were determined by multiplying the numerical

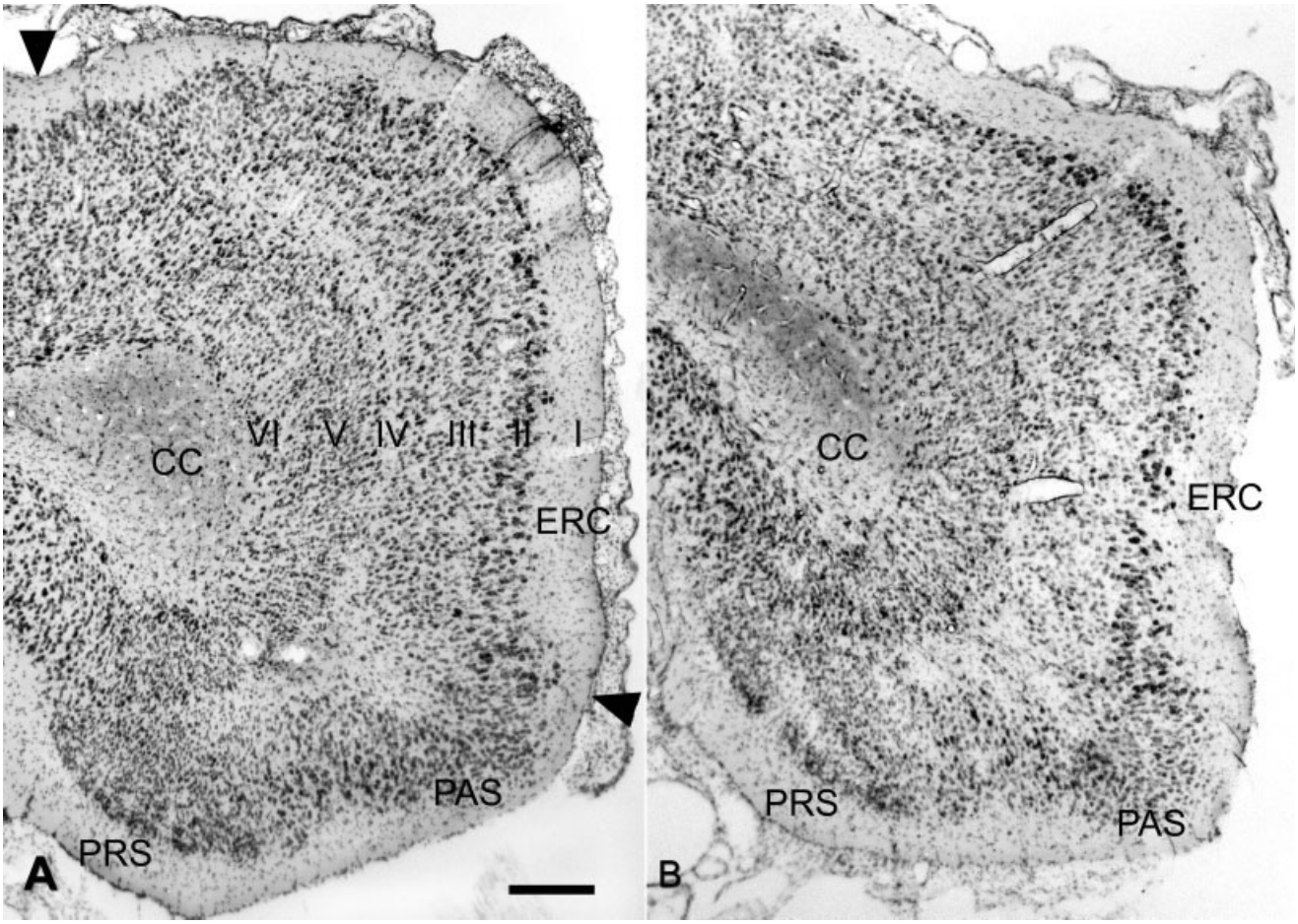

Fig. 2. Photomicrographs of Giemsa-stained horizontal sections containing the entorhinal cortex (ERC) of a control rat (**A**) and of a rat treated with ECS (**B**). Layers I–VI of the ERC are indicated in the left panel. Borders between the ERC and the parasubiculum (PAS) and between the ERC and the perirhinal cortex are also shown in A with arrowheads. Note that the density of neurons in the entorhinal layer III is reduced in the ECS-treated animal compared with the control rat. CC, corpus callosum; PRS, presubiculum. Scale bar = 220 μm.

TABLE I. Summary of the Stereological Parameters Used for the Estimation of Neuronal Numbers in the Dentate Gyrus and Entorhinal Cortex⋆

			Entorhinal cortex		
	Granular layer	Hilus	Layer II	Layer III	Layers V–VI
No. sections	14	14	12	12	12
asf	0.00493	0.0825	0.0703	0.0162	0.0139
h (μm)	10	15	10	10	10
ΣQ^-	172	220	263	125	130
CE	0.09	0.07	0.07	0.09	0.09

⋆asf, Area sampling fraction; h, height of the optical disector; ΣQ^-, total number of neurons counted in each neuronal layer; CE, mean coefficient of error.

density (N_V, number/mm^3) of the synapses found in the outer and inner subdivisions of the molecular layer by the total volume (mm^3) of that subdivision. From each set of serial ultrathin sections obtained from Epon-embedded material, 16–24 micrographs of randomly selected areas within IML (see Fig. 3) and within OML (see Fig. 4) were obtained at a primary magnification of ×5,400 and analyzed at a final magnification of ×16,200. The N_V of the synapses was estimated by applying the physical disector method (Sterio, 1984; see Figs. 3, 4). The thickness of the ultrathin sections was determined by using the minimal fold technique (Small, 1968). A mean section thickness of 70 nm was found based on measurements of 25 folds photographed at a magnification of ×10,000. Twenty-four to thirty-six disectors were made per each slab, providing a total of 72–108 disectors per animal. The synaptic contacts were considered as counting units when the pre- and postsynaptic elements were clearly identified and synaptic vesicles were visualized in at least one of the serial sections (Geinisman et al., 1996).

The volume of the dentate molecular layer was estimated as described above for neuronal layers. The estimations were carried out in glycolmethacrylate-embedded sections, which have shrinkage properties similar to those of Epon-

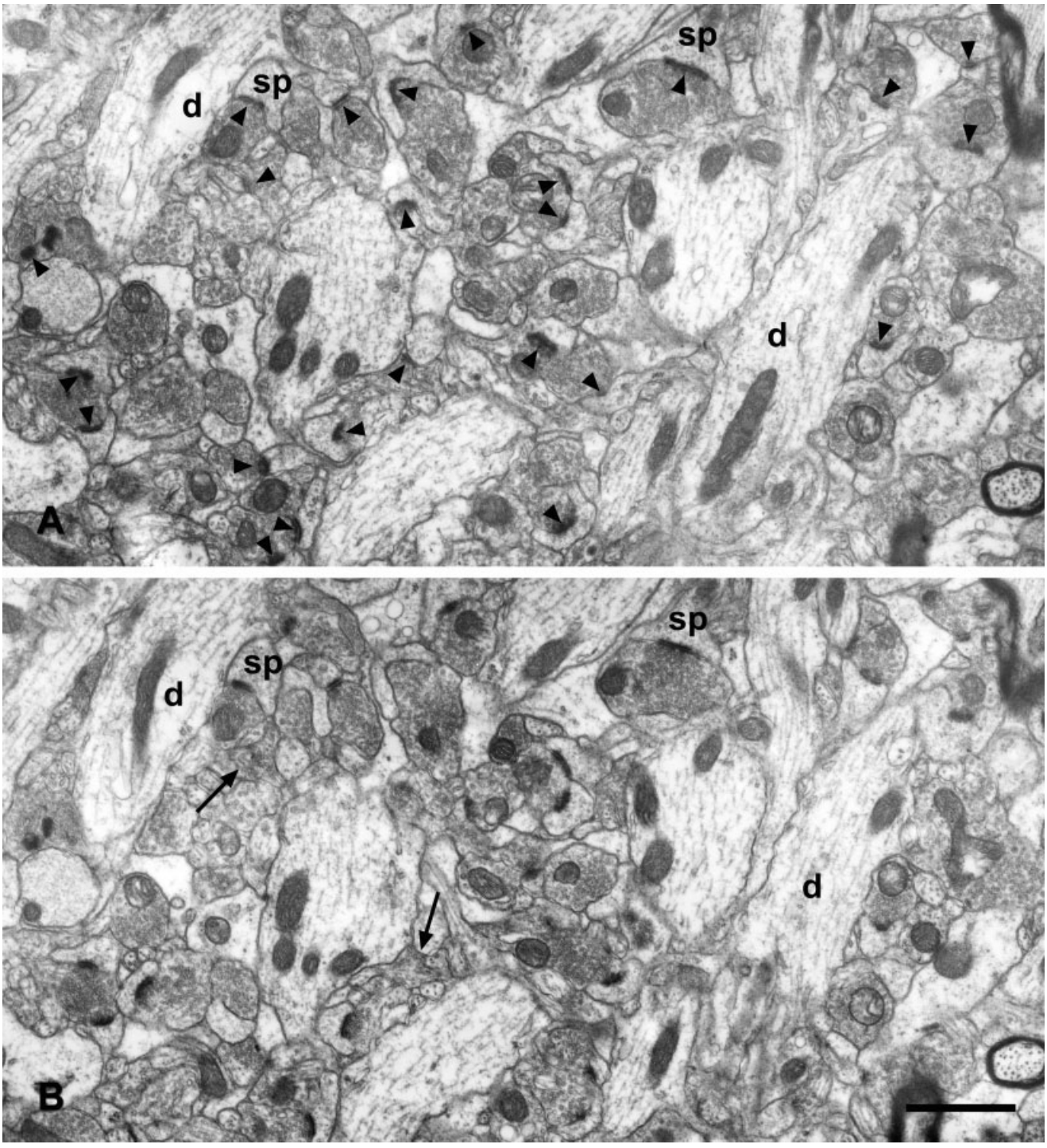

Fig. 3. Electron micrographs obtained from two serial ultrathin sections made through the inner molecular layer of the dentate gyrus of a rat treated with ECS. Photographs of adjacent sections similar to those shown in **A** and **B** were used for estimation of numerical density of axospinous and axodendritic synapses by applying the physical disector method. The axon terminals that contain clear round vesicles and form asymmetrical synapses with dendritic spines (sp) and dendritic shafts (d) are indicated in the reference section (A) by arrowheads. In the look-up section (B), arrows indicate locations where some of these synapses are no longer present. Scale bar = 1 μm.

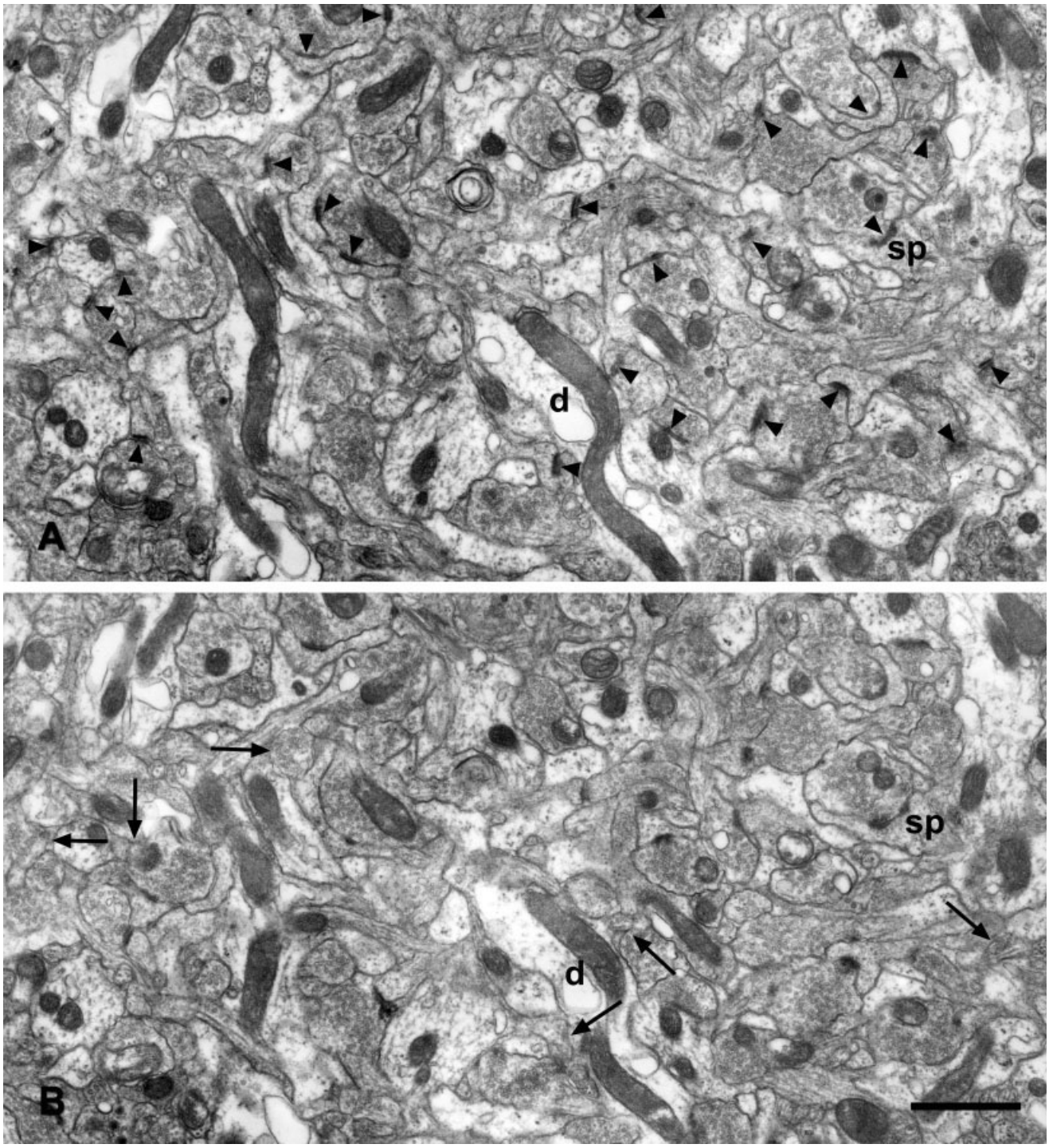

Fig. 4. Electron micrographs obtained from two serial ultrathin sections made through the outer molecular layer of the dentate gyrus of a control rat. Photographs of adjacent sections similar to these shown in **A** and **B** were used for estimation of numerical density of synapses. In B, arrows indicate locations where the synapses that were detected in the reference section (A, arrowheads) are no longer present in the look-up section. d, Dendritic shafts; sp, spines. Scale bar = 1 μm.

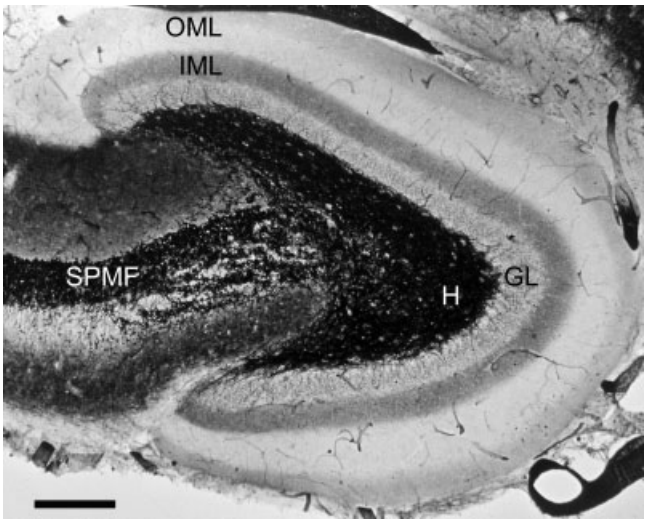

Fig. 5. Photomicrograph of a horizontal Timm-stained section made through the dentate gyrus of a control rat. Note that the boundaries of the inner and outer molecular layers (IML and OML, respectively) can be easily delineated. Timm-stained sections similar to this were used to estimate the relative proportion of the inner molecular layer vs. the outer molecular layer, and no difference between groups was found in this parameter. GL, granule cell layer; H, hilus; SPMF, suprapyramidal part of the mossy fiber system. Scale bar = 220 μm.

embedded material. Sections obtained from 10 rats in each group (14 sections per each animal) were included in the analysis. Because the boundaries between OML and IML are difficult to delineate in sections stained for Giemsa, the volumes of these subdivisions were estimated, using the same method, in Timm-stained material (see Fig. 5). Though not suitable for straightforward calculations of synaptic numbers (because shrinkage properties of Timm-stained sections differ from those of Epon-embedded material), these parameters were used to calculate the ratio of the volumes of OML and IML. The final values of OML and IML total volumes were thus calculated by dividing the volume of the entire molecular layer measured in glycolmethacrylate-embedded sections by the OML/IML volumes ratio determined from Timm-stained material. CE of the individual estimates was calculated as shown by Cruz-Orive (1999), and the mean value was 4.6%.

Analysis of Dendritic Trees

Level-matched Golgi-impregnated sections obtained from the midseptotemporal region of the dentate gyrus were analyzed. The quality of impregnation was judged on the basis of the discreteness of fine structures such as dendritic spines and terminal tips. Because there are variations in the morphology of granule cells as a function of their relative position within the granule cell layer (Green and Juraska, 1985), the neurons used for the analysis of the dendritic trees were sampled from the middle third of the suprapyramidal blade of the granule cell layer. The selection of neurons was done according to the criteria suggested by de Ruiter and Uylings (1987), and the first 10 cells visualized per animal fulfilling those criteria were drawn with the aid of a camera lucida at a magnification of ×640. The presence of cut terminal segments on a neuron was not considered as a criterion for its exclusion from the estimates, and the mean percentage of cut branches (approximately 26%) was similar in both groups. For the metric analyses, the two-dimensional lengths of all dendritic segments were measured with the aid of a graphic pen tablet and MOP-Videoplan computer software (Kontron Elektronik GmbH), and the total dendritic length was calculated as their sum. The total number of segments was also calculated.

The spine density was estimated separately on the proximal and distal dendrites of dentate granule cells. Dendritic segments interposed between the first and second or the second and third branching points (counting from soma) were considered as proximal, whereas those found between last bifurcations and terminal tips were regarded as distal. Dendritic segments were selected only when they were well impregnated and visualized within one plane of focus for at least 25 μm of length. Counting of spines was performed in five dendritic segments per cell (10 cells per animal) at a final magnification of ×640. No counts were performed at branching points, because it is known that there are marked variations in spine density at bifurcations (de Ruiter and Uylings, 1987). No attempts were made to correct for dendritic spines hidden either above or beneath the dendritic shafts.

Estimation of Mossy Fiber Sprouting

Mossy fiber synaptic reorganization was assessed by rating the distribution of Timm granules within the supragranular zone of the dentate gyrus. The density of the granules was rated semiquantitatively on a scale of 0–4 based on a previously validated scoring method (Cavazos et al., 1991): 0, no or only occasional granules; 1, sparse granules in a patchy distribution; 2, more numerous granules in a continuous distribution; 3, prominent granules in a continuous distribution with occasional patches of confluent granules; 4, prominent granules that form a confluent dense laminar band. Per each animal, eight sections were systematically sampled and analyzed by the reviewer, who was blind to treatment group. Because the extent of the seizure-induced mossy fiber sprouting is known to vary along the septotemporal axis (Cavazos et al., 1992; Buckmaster and Dudek, 1997; Pitkänen et al., 1999), the sections were sampled at matching levels. The eight scores were averaged to yield a mean Timm score for each animal.

Statistical Analysis

The data were analyzed via Student's *t*-test. Timm staining scores were compared by nonparametric Mann-Whitney U-test. Results are expressed as the mean (SD). Differences were considered as significant at the $P < 0.05$ level.

RESULTS

Total Neuronal Numbers and Volumes of Neuronal Layers

Qualitative observation of the Giemsa-stained material revealed that the density of cells in the hilus of the dentate gyrus and in the entorhinal cortex appeared to

TABLE II. Total Number of Neurons in Dentate Gyrus and Entorhinal Cortex of Control and ECS-Treated Rats[†]

	Control	ECS-treated
Dentate gyrus		
Granular layer	1,126,338 (92,417)	1,058,262 (147,500)
Hilus	55,065 (5,092)	44,542 (3,716)★
Entorhinal cortex		
Layer II	119,264 (15,700)	126,734 (16,596)
Layer III	290,872 (30,982)	228,851 (30,782)★
Layers V–VI	339,870 (51,657)	286,621 (37,112)

[†]Values represent mean (SD); $n = 6$ in each group.
★$P < 0.01$.

TABLE III. Numerical Density of Synapses (N_v, number/mm^3 × 10^9)[†] in Outer and Inner Molecular Layers of the Dentate Gyrus and Total Volumes of These Layers (mm^3)[‡]

	Control	ECS-treated
Inner molecular layer		
N_v of axospinous synapses	1.489 (0.145)	1.785 (0.194)
N_v of axodendritic synapses	0.195 (0.05)	0.153 (0.036)
Total volume	2.051 (0.27)	1.365 (0.169)★
Outer molecular layer		
N_v of axospinous synapses	2.247 (0.327)	2.180 (0.195)
N_v of axodendritic synapses	0.228 (0.05)	0.282 (0.034)
Total volume	4.201 (0.54)	2.874 (0.356)★
Molecular layer		
Total volume	6.252 (0.81)	4.238 (0.52)★

[†]Values represent mean (SD); $n = 10$ (Total Volumes).
[‡]$n = 6$; (Numerical Density of Synapses).
★$P < 0.0001$.

be somewhat smaller in ECS-treated rats than in controls (Figs. 1 and 2, respectively). Quantitative data (Table II) show that the total number of hilar cells, but not of dentate granule cells, was significantly reduced (by 19%; $P < 0.01$) in ECS-treated rats. However, the total volumes of the hilus and of the dentate granule layer did not differ between the groups (data not shown). ECS treatment was also associated with reduced numbers of neurons in the entorhinal layer III (21%; $P < 0.01$). In contrast, the total numbers of neurons in the superficial layer II and in deep layers V–VI did not differ significantly between the groups (Table II). There was a significant decrease in the volume of all measured layers of the entorhinal cortex in ECS-treated rats relative to controls, from a mean of 1.93 ± 0.15 mm^3 to a mean of 1.54 ± 0.16 for layer II ($P < 0.005$), from a mean of 3.23 ± 0.25 mm^3 to a mean of 2.16 ± 0.24 for layer III ($P < 0.001$), and from a mean of 2.65 ± 0.3 mm^3 to a mean of 1.94 ± 0.22 for layers V–VI ($P < 0.01$).

Total Numbers of Synapses in the Molecular Layer

Most synapses detected in the dentate molecular layer contained round vesicles in the presynaptic terminal and were asymmetric, i.e., presumptive excitatory, whereas the number of symmetric, presumptive inhibitory synapses was too small to quantify (Figs. 3, 4). The

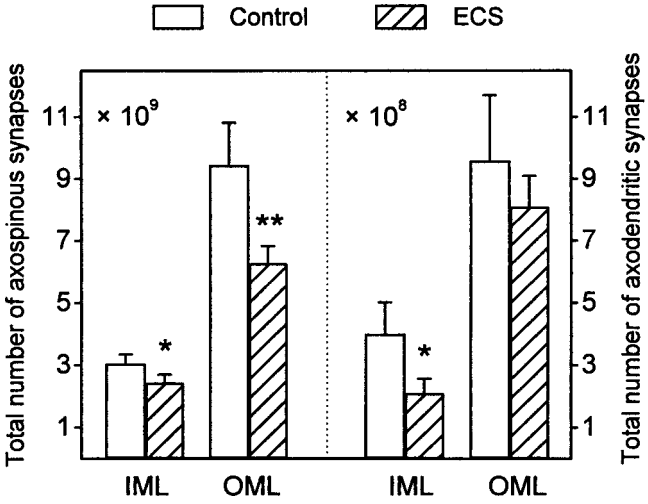

Fig. 6. Estimates of total synaptic numbers in the molecular layer of the dentate gyrus from control and ECS-treated rats. Note that ECS seizures produced a particularly robust reduction of the number of axospinous synapses in the outer molecular layer (OML). The seizure-induced loss of axodendritic synapses in the inner molecular layer (IML) was also evident. Columns represent means, and vertical bars represent 1 SD. *$P < 0.01$, **$P < 0.001$ vs. control group.

N_Vs of synapses in OML and IML are shown in Table III. In both of these subdivisions, N_V of axospinous synapses was found to be 9–10 times higher than that of axodendritic synapses. In addition, N_V of synapses of both types was somewhat higher in OML than in IML. However, the density of synapses of either type did not differ significantly among the groups. In contrast, analysis of Timm-stained sections revealed that both subdivisions of the dentate gyrus molecular layer were significantly decreased in ECS-treated rats relative to controls, from a mean of 3.12 ± 0.22 mm^3 to a mean of 2.56 ± 0.19 for OML ($P < 0.001$) and from a mean of 1.52 ± 0.10 mm^3 to a mean of 1.22 ± 0.09 for IML ($P < 0.001$). However, ECS treatment did not alter the ratio of the volumes of OML and IML, so that in both groups OML occupied approximately two-thirds of the total volume of the molecular layer (67.2% and 67.8% in control and ECS groups, respectively; Fig. 5). Analysis of hippocampal sections embedded in glycolmethacrylate confirmed these findings obtained in Timm-stained material by showing that the total volume of the molecular layer was significantly smaller (by approximately 33%) in ECS-treated rats than in control rats ($P < 0.0001$; Table III). Consequently, the volumes of OML and IML, calculated from the total volume of the molecular layer found in glycolmethacrylate-embedded sections and OML/IML volume ratios estimated in Timm-stained sections, were also reduced in ECS-treated rats (Table III; $P < 0.0001$).

The estimates of the total numbers of synapses in the dentate molecular layer are shown in Figure 6. As expected, the number of axospinous synapses, both in OML and in IML, was approximately 10 times higher

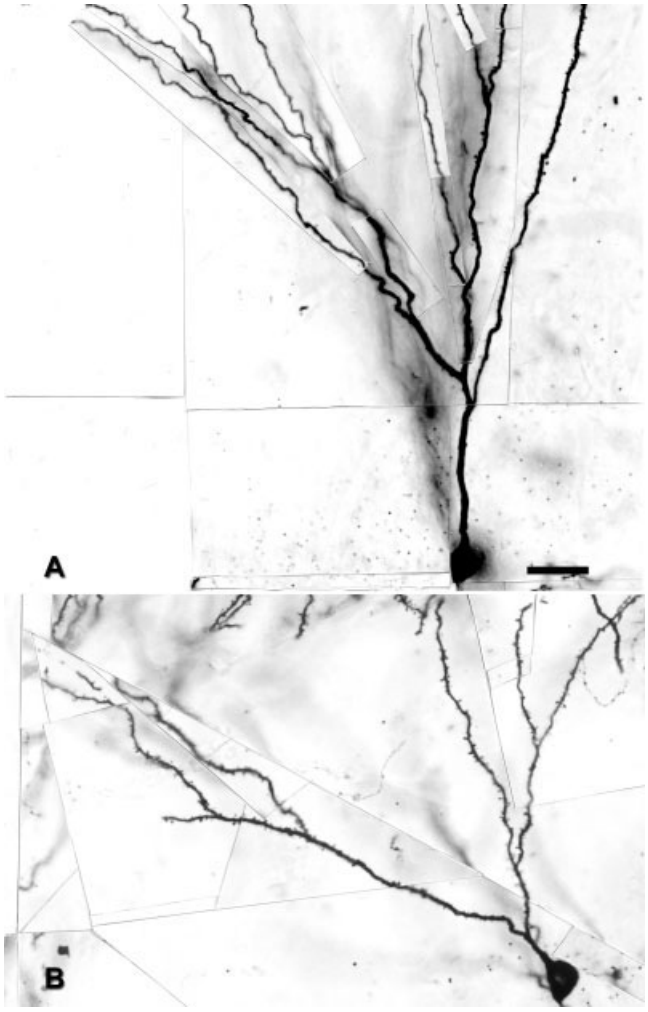

Fig. 7. Photomontages of representative Golgi-impregnated granule cells of a control rat (**A**) and a rat treated with ECS (**B**). Note that the dendritic tree of the granule cell from the control rat is more complex than that from the experimental rat. However, the proximal dendrites of the granule cell from ECS-treated animal display a higher number of dendritic spines than those from the control rat. Scale bar = 20 μm.

than that of axodendritic synapses. Furthermore, the number of synaptic contacts of both types estimated in OML was found to be 2.5–3 times greater than in IML. Importantly, OML of ECS-treated rats contained approximately 40% fewer axospinous synapses relative to control rats ($P < 0.001$). However, the loss of axodendritic synapses in OML was nonsignificant ($P = 0.11$). Seizure-related loss of synapses was also observed in IML (Fig. 6; $P < 0.01$ both for axospinous and for axodendritic synapses).

Granule Cell Dendritic Arbors

Dendritic arbors of many granule cells in ECS-treated rats appeared to have fewer segments relative to those in control rats (Fig. 7). In effect, the number of dendritic segments of granule cells was reduced in ECS-treated rats by approximately 20% (Table IV; $P < 0.05$). Moreover, the total dendritic length of the granule cells was also decreased by 18% ($P < 0.05$) following ECS treatment. Interestingly, seizures did not affect the number of spines per unit of dendritic length on the distal dendritic segments of granule cells (Table IV; Fig. 7). Therefore, it seems reasonable to conclude that the overall number of spines in the peripheral part of the dendritic arbors of granule cells, grossly corresponding to OML, was smaller in ECS-treated rats than in control rats, because both the number of dendritic segments and the total dendritic length were decreased. However, it is likely that the overall number of spines located proximally to the cell body, i.e., within IML, remained normal or even was augmented in ECS-treated rats, because their density on the proximal dendritic segments was found to be higher than in control rats ($P < 0.01$; Table IV).

TABLE IV. Metric Analysis of Dendritic Trees of Dentate Granule Cells[†]

	Control	ECS-treated
Total number of segments	15.28 (1.97)	12.47 (2.34)★
Total dendritic length (μm)	1049 (103)	863 (164)★
Spine density (number per 25 μm)		
Distal dendritic segments	8.72 (1.6)	9.3 (1.3)
Proximal dendritic segments	4.19 (0.5)	6.35 (0.6)★★

[†]Values represent mean (SD); $n = 6$ animals in each group. For each of the animals, the values were obtained by averaging the measurements derived from 10–12 granule cells.
★$P < 0.05$.
★★$P < 0.01$.

Mossy Fiber Morphology

The distribution pattern of the mossy fibers and their terminals within the dentate gyrus of control and ECS-treated rats was studied in Timm-stained sections (Fig. 8). In addition to the densely packed fiber plexus within the dentate hilus, in both groups, Timm granules were also observed within the granular and molecular layers. However, the density of these granules was greater in ECS rats than in control rats. This observation was confirmed by the results of the semiquantitative analysis, which showed that the density of the supragranular layer Timm granules, in arbitrary units, was increased in ECS-treated rats relative to control rats from a mean of 0.44 ± 0.15 to a mean of 0.96 ± 0.29 ($P < 0.05$).

DISCUSSION

The results of the present experiment confirm those prior studies demonstrating that brief, generalized ECS seizures, when elicited repeatedly, can produce noticeable structural alterations in the dentate gyrus (Gombos et al., 1999; Vaidya et al., 1999; Scott et al., 2000), including moderate cell loss (Lukoyanov et al.,

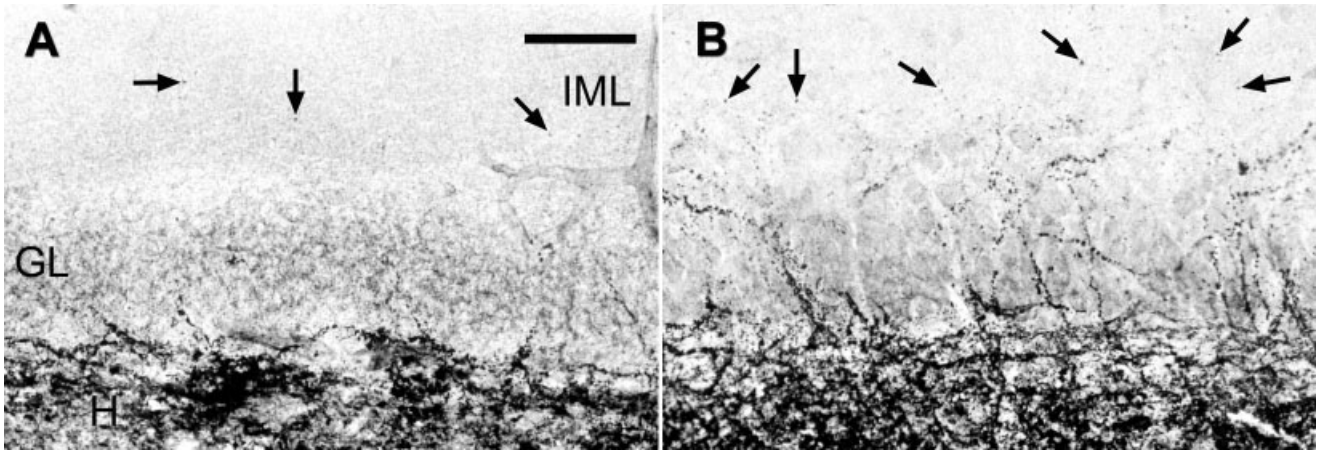

Fig. 8. Representative Timm-stained level-matched sections of the dentate gyrus (midseptotemporal region) of a control rat (**A**) and a rat treated with ECS (**B**). The arrows indicate Timm granules in the inner molecular layer (IML). Note that the density of these granules appeared to be higher in B than in A. GL, granule cell layer; H, hilus. Scale bar = 50 μm.

2004). These findings additionally show, for the first time, that ECS-induced damage extends beyond the dentate gyrus to include another seizure-vulnerable brain region, the entorhinal cortex. One of the findings of this study is that ECS seizures partially disconnected two temporal lobe structures, the entorhinal cortex and the dentate gyrus, as indicated by the significant loss of synapses and other impressive changes, atrophic in nature, that we observed in the receptive field of the dentate gyrus granule cells, i.e., in the molecular layer.

Temporal lobe epilepsy is often associated with structural changes in the dentate gyrus and hippocampus proper, such as synaptic rearrangements, loss of neurons, and morphological alterations in their dendritic arbors (Sutula et al., 1989; Mathern et al., 1995; Houser, 1999; McNamara, 1999; Maglóczky and Freund, 2005). In line with this, evidence from animal models shows that prolonged seizure activity provokes death of hippocampal neurons (Turski et al., 1983; Sloviter, 1987; Buckmaster and Dudek, 1997; Ben-Ari and Cossart, 2000), with hilar mossy cells and inhibitory interneurons being particularly vulnerable (Sloviter, 1987; Ratzliff et al., 2002; Sloviter et al., 2003). The results of this and previous studies show that brief repeated seizures also cause loss of hilar neurons, presumably mossy cells (Cavazos and Sutula, 1990; Cavazos et al., 1994; Lukoyanov et al., 2004). Furthermore, it is possible that the total number of neurons in the granular layer remained unchanged after ECS treatment, because granule cells that might have been lost were replaced by newly formed neurons as a result of neurogenesis that is known to be enhanced by ECS seizures (Scott et al., 2000). That the granule cells were also affected by ECS seizures is supported by the findings that both the total length of their dendrites and the number of dendritic segments per cell appear to be reduced in ECS-treated rats. The loss of synapses observed in IML indicates that commissural and associational connections of the dentate gyrus were severed by the seizures. However, and somewhat surprisingly, the density of spines on the proximal dendrites of granule cells was, in contrast, increased after ECS treatment, perhaps suggesting some degree of rearrangement of synaptic connections in IML. This view is supported by the observation of enhanced mossy fiber sprouting into this area in ECS-treated rats, a finding consistent with previous studies employing this model (Gombos et al., 1999; Vaidya et al., 1999; Lukoyanov et al., 2004) as well as other seizure models (Cavazos et al., 1992; Buckmaster and Dudek, 1997; Pitkänen et al., 1999). That this synaptic rearrangement is due at least partially to the death of mossy cell is suggested by the finding that the total number of hilar cells, but not of somatostatin-immunoreactive interneurons (Dalby et al., 1996; Lukoyanov et al., 2004), is reduced following ECS treatment. Interestingly, Kirov and Harris (1999) have shown that the removal of synaptic input from hippocampal neurons renders their dendrites more spiny. This effect has also been described, by Sotelo (1990), for cerebellar Purkinje cells. Moreover, it has recently been reported that epileptic activity in hippocampal slices induces a net loss of synapses on dendritic spines of pyramidal neurons, which is accompanied by the formation of new spine-like filopodia lacking postsynaptic densities (Zha et al., 2005). Therefore, it is possible that the increased density of spines on the dendrites of granule cells in ECS-treated rats can also be related to their capacity to generate new spine-like structures, possibly compensating for lost synaptic activity.

We found that synaptic loss in OML was even greater, in both relative and absolute terms, than that in IML. The vast majority of the excitatory synapses in OML is formed by the axons of stellate cells located in the layer II of the entorhinal cortex (Ruth et al., 1982; Amaral and Witter, 1995). The results of recent studies

suggest that their hyperexcitability plays an important role in epileptogenesis (Bear et al., 1996; Scharfman et al., 1998). However, histological evidence from patients with temporal lobe epilepsy and from animal models demonstrates preferential loss of neurons in entorhinal layer III, accompanied by less severe loss of layer II stellate cells (Du et al., 1993, 1995; Schwarcz et al., 2000). In our model, we found that the total number of neurons in entorhinal layer III was reduced in ECS-treated rats, whereas that in the layer II was unaffected. Because entorhinal layer III neurons are involved in the regulation of the excitability of neurons in nearby layers, probably by recruiting local circuit inhibitory interneurons (Kobayashi et al., 2003; Kumar and Buckmaster, 2006), it is possible that their loss can have deleterious effects on the layer II stellate cells. This possibility is supported by the finding that the volume of the entorhinal layer II was significantly reduced, by approximately 20%, after ECS treatment. Because the total number of neurons in this layer remained unchanged in ECS group, this finding suggests that either cell bodies or neuritic processes of these neurons became atrophic. Interestingly, the volume of layers V and VI, which contain many entorhinal afferents and efferents and where no significant neuronal loss was detected, was also reduced following ECS treatment. Considered together, these data lend support to the hypothesis that the ECS-induced loss of synapses established between the entorhinal cortex and the dentate gyrus may be attributable to morphologic and, probably, functional reorganization of neuritic processes, rather than to actual neuronal loss.

There are a number of caveats that must be kept in mind when interpreting the present findings. One of them is that the density of synapses, used to calculate the total synaptic numbers, was estimated only in the midseptotemporal region of the dentate gyrus. In other words, because the extent of the seizure-induced synaptic reorganization in the hippocampal formation varies along its septotemporal axis (Cavazos et al., 1992; Buckmaster and Dudek, 1997; Pitkänen et al., 1999), it is possible that synaptic changes at its septal and/or temporal poles were overlooked in the present study. However, given that the midseptotemporal part of the dentate gyrus, from which samples were taken, encompasses at least two-thirds of its entire volume, it seems improbable that analyzing additional samples from septal and temporal dentate regions would change the results appreciably. Another limitation of this study is that the numbers of synapses were assessed applying stereological methods, whereas dendritic spines were quantified using camera lucida drawings of Golgi-impregnated cells and expressed in units of the length density. It is possible, therefore, that the inconsistency between the loss of synapses in the IML and the preservation of spines on proximal dendrites of granule cells in ECS-treated rats can be explained by these methodological differences. Incomplete staining and/or reconstruction of dendrites and spines of granule cells can likewise contribute to this discrepancy. This later possibility is consistent with the fact that the dendritic measures herein reported, i.e., the total dendritic length, number of segments, and spine density, are all somewhat inferior to the values found in a number of prior studies that used other staining techniques and three-dimensional reconstruction of dendrites and spines (Seress and Pokorny, 1981; Green and Juraska, 1985; Hama et al., 1989; Claiborne et al., 1990). Thus, the hypothesis, mentioned above, that granule cells can respond to the seizure-induced decrease of synaptic input by forming new spines or spine-like filopodia remains to be further confirmed using more reliable cytological methods.

An important caveat of our results is that it is unclear to what extent they are relevant to the use of electroconvulsive therapy in psychiatry, because the protocol employed in this study is different from that used clinically. Evidence from previous studies (Gombos et al., 1999; Vaidya et al., 1999) and from our pilot experiments indicates that treating rats either with five to ten widely spaced ECS (at 24- or 48-hr schedules) or with two stimulations only 2 hr apart does not lead to loss of hippocampal neurons. However, we show here that, when the two stimulations spaced by 2-hr interval are preceded by a course of seizures administered on a 24-hr schedule, they produce significant neuronal damage. These data are consistent with the view that repeated administration of ECS can make neurons more vulnerable to subsequent excitotoxic stimuli, probably by decreasing the capacity of the excitatory amino acid reuptake system (Rowley et al., 1997). The current findings also suggest that the neuroanatomical sequelae of ECS seizures can become particularly severe under some pathological conditions involving changes in amino acid metabolism and/or physiology.

In conclusion, this study answers positively the question of whether repeated administration of ECS seizures can cause brain lesions. Our data are consistent with findings from other animal models and from human studies in showing that neurons located in the entorhinal cortex and in the hilus of the dentate gyrus are particularly vulnerable to repeated seizures. Plasticity changes in dendritic spines and synaptic contacts are known to be involved in both memory formation and epileptogenesis. The entorhinal cortex and the dentate gyrus are both implicated in these processes (Spencer and Spencer, 1994; Squire et al., 2004). Therefore, the finding that ECS seizures caused loss of synapses in the entorhinal-dentate pathway sheds new light on cellular processes that may underlie its amnestic effects as well as its capacity to raise seizure threshold and to retard the development of status epilepticus in animal models (Coffey et al., 1995; André et al., 2000). Furthermore, although not addressed in the present study, it is possible that ECS seizures produce loss of synapses in brain regions other than the hippocampal formation. Should this be the case, partial disconnection of the prefrontal cortex, for example, from diencephalic and temporal lobe limbic structures would provide a plausible explanation for the antidepressant properties of electroconvulsive therapy.

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Effects of repeated electroconvulsive shock seizures and pilocarpine-induced status epilepticus on emotional behavior in the rat

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ABSTRACT

Affective symptoms are frequently observed in patients with epilepsy. Although the etiology of these behavioral complications remains unknown, it is possible that brain damage associated with frequent or prolonged seizures may contribute to their development. To address this issue, we examined the behavioral sequelae of repeated brief seizures evoked by electroconvulsive shock (ECS) and compared them with those resulting from prolonged status epilepticus (SE) induced with pilocarpine. Using the open-field and elevated plus-maze tests, we detected the presence of behavioral alterations indicative of elevated levels of anxiety in rats that were administered a course of ECS seizures. Fear conditioning was also enhanced in these animals. However, the rats that had experienced SE exhibited less anxiety-like behavior than controls and were severely impaired in fear conditioning. These results support the notion that brain lesions caused by either brief repeated seizures or SE is sufficient to induce some affective disturbances.

1. Introduction

Anxiety, depression, and mood disorders are frequently observed in patients with epilepsy, but their causal relationship to seizures is uncertain [1–6]. It has been suggested that the same changes in the brain that cause epileptic seizures might additionally lead to affective disturbances [7]. However, it has also been hypothesized that the affective symptoms associated with epilepsy are secondary to seizures; that is, they are derived from the damage to specific brain regions caused by frequent or prolonged seizure activity [8]. Notably, if the second hypothesis is true, then it should be possible to prevent the occurrence of affective symptoms by controlling seizures with appropriate medication. Thus, given its clinical importance, this issue has been repeatedly addressed in studies in animals, which, however, have yielded evidence supporting both of these hypotheses [7–11].

Electroconvulsive therapy is regarded as one of the efficient, albeit controversial, treatments for otherwise intractable psychiatric disorders [12,13]. However, it has been recently demonstrated in animal studies that repeated administration of electroconvulsive shock (ECS) seizures produces noticeable structural changes in the brain, such as enhanced granule cell neurogenesis in the dentate gyrus [14], sprouting of mossy fibers into its molecular layer [15,16], and moderate neuronal loss in the hippocampal formation and adjacent cortical areas [10], that partly resemble those found in patients with epilepsy and in animal models of this disease. However, ECS seizures do not lead to the creation of epileptogenic foci in the brain but, on the contrary, can even increase neuronal threshold for seizure generation [17] and delay epileptogenesis [18]. Therefore, we found this model quite suitable for validation of the hypothesis that the moderate, nonepileptogenic brain lesions caused by brief seizures are sufficient to induce some affective symptoms in the rat, which would explain, at least partly, the occurrence of this type of psychopathology in patients with epilepsy [1–4,19]. Methodologically, we used a modified stimulation protocol in which the animals were administered six ECS seizures: the first five were spaced at 24-hour intervals, and the last two were only 2 hours apart. This protocol is based on the finding that repeated induction of five widely spaced ECS seizures reduces the capacity of the brain amino acid reuptake system to maintain normal levels of glutamate for a minimum of 2 hours [20], which renders neurons especially vulnerable to seizures elicited during this postictal period [10,21]. Previous studies have demonstrated that administration of 5 to 10 ECS seizures at 24- or 48-hour intervals, that is, on a schedule similar to those commonly used in psychiatric practice, does not appear to cause significant neuronal loss [15,16]. In addition, we also found it of interest to compare the behavioral changes provoked by ECS seizures with those derived from prolonged and uninterrupted seizure activity, which is known to produce much larger brain lesions when compared with ECS seizures [10,22–24]. For this purpose, we employed the pilocarpine-induced model of status epilepticus (SE) [25,26]. Behavioral changes were assessed using two common tests for anxiety, the

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open-field and elevated plus-maze tests [27,28], and the fear-conditioning test.

2. Methods

2.1. Animals and treatments

Male Wistar rats, 2 months of age, maintained under standard laboratory conditions were used in this study. In the first experiment, the rats (ECS group, $n = 12$) received a course of five ECS seizures, administered on a 24-hour schedule [22]. Each stimulus (50 Hz, 60 mA for 1 second) was delivered via ear-clip electrodes wired to a stimulus generator (Model 215/IZ, Hugo-Sachs Elektronik, Germany). Two hours after the fifth stimulation, each of the animals received one additional ECS seizure. ECS produced full tonic–clonic seizures with hindlimb extension lasting 5–10 seconds. Rats in the control group ($n = 12$) received handling identical to that of experimental rats, but were not stimulated. In the second experiment, animals from the SE group ($n = 12$) were pretreated with scopolamine methyl bromide (1 mg/kg, subcutaneously, Sigma) to minimize peripheral cholinergic side effects of pilocarpine. Thirty minutes later, the rats received a single high dose of pilocarpine (350 mg/kg, intraperitoneally, Sigma). The onset of SE was defined as the appearance of behavioral symptoms corresponding to stage 4 or 5 seizures on the Racine scale [29]. Two hours after the beginning of SE, the rats were injected with diazepam (Valium, 5 mg/kg, intraperitoneally) to end the convulsive manifestations of SE. However, seizure activity, albeit considerably reduced in severity, was not completely stopped by the single dose of diazepam. Thus, an additional dose of diazepam (2.5 mg/kg) was given to the rats 3 hours after the onset of SE. Control rats in the second experiment ($n = 8$) received the identical handling and treatment received by experimental rats, including injections of scopolamine and diazepam, but were not treated with pilocarpine. Following a latent (seizure-free) period lasting 2–3 weeks, spontaneous motor seizures of stage 3 or greater on the Racine scale [29] were observed in all rats treated with pilocarpine. No behavioral alterations were detected in animals from the ECS-treated group and both control groups. All animals were allowed to recover for 2 months before initiation of behavioral testing to ensure that reorganization of neuronal circuits induced by seizures was fully established [16,30,31]. The behavioral procedures used in the first and second experiments of this study were identical. The handling and care of the animals were conducted according to the *Principles of Laboratory Animal Care* (NIH Publication No. 86-23, revised 1985) and European Communities Council guidelines in animal research (86/609/UE). Animal protocols were approved by the Institutional Animal Care and Use Committee of the Center of Experimental Morphology, Porto Medical School. All efforts were made to minimize the number of animals used and their suffering.

2.2. Open-field and elevated plus-maze tests

Animals were handled 3 minutes per day for 5 days and subjected to the open-field and plus-maze tests [32]. The rats were counterbalanced so that, in each group, half received the open-field test first and the other half received the plus-maze test first. There was a 3-day interval between the tests.

The open field was a white acrylic arena measuring $100 \times 100 \times 40$ cm. The rats were placed in a corner of the apparatus and tested in 5-minute sessions. Distances traveled in the outer zone of the open field, defined as 20 cm from any wall, and in its inner zone, defined as the 60×60-cm square in the center of the arena, were measured using a computerized video-tracking system (EthoVision V3.0, Noldus, The Netherlands). At the end of each session, the urine deposited was collected using a filter paper. The difference between the weights of the paper before and after collecting the urine, in grams, was considered a measure of the amount of urine deposited during the session. The number of fecal boli deposited was also recorded, and the floor of the apparatus was thoroughly cleaned and dried.

The elevated plus maze was constructed of black acrylic and arranged as a cross with two opposite open and two opposite closed arms (50×12 cm), connected by a common central square (12×12 cm). The closed arms were enclosed by 50-cm-high walls. The rats were placed on the central square, facing one of the closed arms, and allowed to explore the apparatus for 5 minutes. Behavior of rats was recorded and analyzed using the computerized video-tracking system. Percentages of time spent and distances traveled by rats in the open arms, closed arms, and central square were calculated. At the end of each session, the number of fecal boli and the amount of urine deposited were recorded and the apparatus was thoroughly cleaned and dried.

2.3. Fear conditioning

All rats were given a single session of fear conditioning [32]. The conditioning chamber (San Diego Instruments, USA) consisted of a clear Plexiglas box ($26 \times 26 \times 18$ cm) equipped with a metal grid floor, wired to a stimulus generator (Hugo-Sachs Elektronik, Germany), and acoustic stimulus unit. The grid floor was composed of stainless-steel bars, 0.6 cm in diameter, spaced 1.4 cm apart (center-to-center). The chamber was scented with 1% acetic acid solution (placed in the base pan underneath the grid floor) to provide a distinct context odor. Rats were placed inside the apparatus and left undisturbed for 3 minutes. During the next 3-minute period they received five tone–footshock conditioning trials, at 30-second intervals. Each conditioning trial consisted of a 10-second tone conditioned stimulus (80 dB, 2.8 kHz), which co-terminated with a 1-second footshock unconditioned stimulus (0.8 mA) delivered via the grid floor. Thirty seconds after the last trial, the animals were removed from the apparatus and the grid floor was cleaned with 1% acetic acid.

One day later, all rats were submitted to retention tests. The order of testing was counterbalanced so that half of the rats in each group were tested for retention of the conditioned context by repeating the procedure employed during training (in the absence of the tone or footshock), whereas remaining rats were tested for retention of the conditioned tone in a different context. In the latter test, the rats were placed into a novel chamber located in a novel behavioral room [32]. The new chamber was composed of black Plexiglas, except for the top, which was translucent, and for the floor, which was composed of a piece of a black carpet. In addition, the chamber was scented with lemon instead of acetic acid. The animals remained in the chamber for a period of 6 minutes and the conditioned stimulus (10-second tone) was presented five times during the last 3 minutes of this period. Four hours later, these rats were tested for retention of the context as described above, whereas the animals from the context test were now subjected to the tone retention test.

All training and testing trials were recorded with a video camera connected to a SVHS recorder for subsequent analysis. Rat behavior was analyzed by observers blinded with respect to experimental group. Freezing (defined as the absence of all movement other than that required for breathing and associated with a crouching posture) was scored if the rat remained inactive for at least 3 seconds. The percentage of accumulated time spent freezing was calculated.

2.4. Statistical analysis

Data derived from the open-field and plus-maze experiments and from the context retention test were analyzed using Student's *t* test. The remaining data were analyzed using repeated-measures ANOVA followed by the Newman–Keuls post hoc test where appropriate. Differences were considered significant at the $P < 0.05$ level. Because some of the behavioral tests were presented in a counterbalanced manner, the data derived from these tests were collapsed across order of presentation.

3. Results

3.1. Behavioral effects of ECS seizures in the open field and elevated plus maze

As shown in Fig. 1a, administration of ECS seizures induced a significant decrease in overall locomotion of rats in the open field. In particular, animals from the ECS group showed two times less locomotion in the outer zone of the apparatus than did control rats ($P < 0.001$). In the inner zone, ECS-treated rats traveled approximately six times less distance when compared with control rats ($P < 0.05$). In addition, the two groups differed significantly with respect to defecation and urination scores (Fig. 2). Animals from the ECS group deposited in the open field more fecal boli ($P < 0.05$) and more urine ($P < 0.01$) relative to control rats.

Rats in both groups showed a strong preference for protected areas of the plus maze, as they spent considerably more time in the closed arms and central zone of the apparatus in comparison to its open arms (Fig. 1b). However, although the percentages of time spent by rats in the open arms did not differ between the two groups, ECS-treated rats exhibited a somewhat stronger preference for the closed arms when compared with control rats ($P < 0.01$). Consequently, they spent significantly less time than controls in the central square of the maze ($P < 0.05$). ECS-treated and control rats exhibited comparable levels of locomotor activity in the closed arms of the plus maze as indicated by the similar distances traveled (Fig. 1c). However, the activity of ECS-treated rats in the open arms and in the central square was somewhat reduced when compared with that of controls ($P < 0.01$ for both zones). In addition, animals from the ECS group deposited in the plus maze more fecal boli relative to the control group ($P < 0.05$) (Fig. 2). The two groups did not differ significantly in the plus maze with respect to their urination scores.

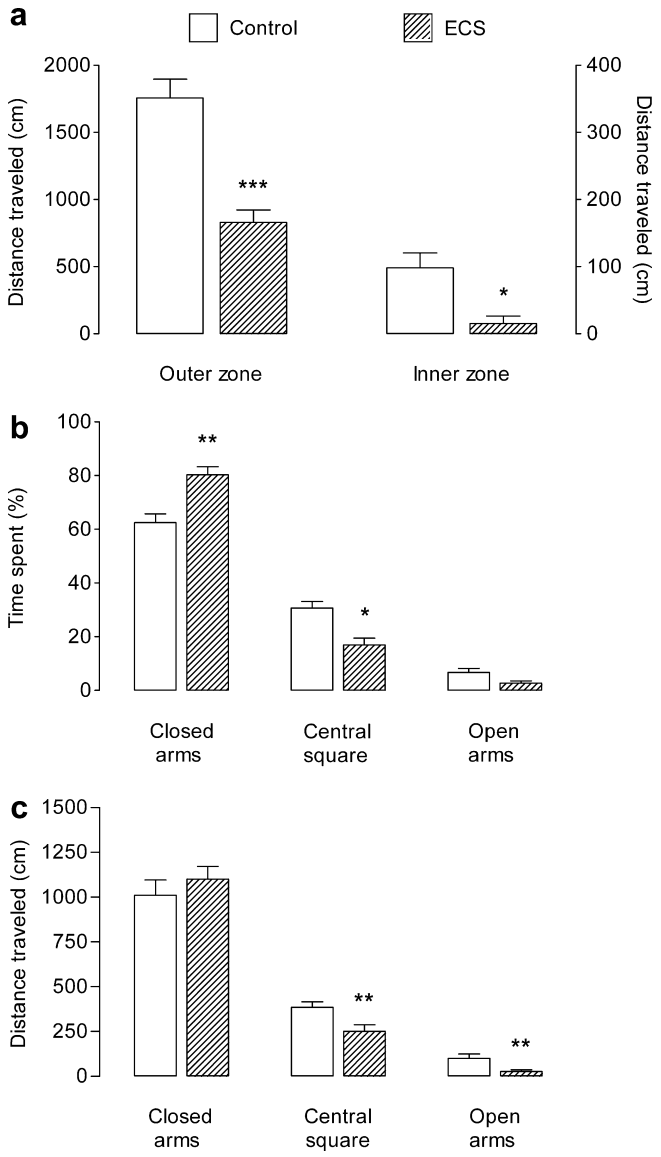

Fig. 1. Effects of repeated ECS-induced seizures on the behavior of rats in the open-field and elevated plus-maze tests. Distance traveled in the outer and inner zones of the open field (a) and percentage of time spent (b) and distance traveled (c) in the central square and closed and open arms of the plus maze. Data are presented as means ± SEM. $n = 12$ in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control group.

3.2. Effects of ECS seizures on conditioned freezing behavior

Conditioning produced an increase in the amount of freezing time in both the control and ECS groups ($F(1,22) = 230.24$, $P < 0.001$) (Fig. 3a). However, examination of the data revealed that rats treated with ECS froze somewhat more during both the first and the second halves of the training session (minutes 1–3 and 4–6, respectively) than did control rats. This was confirmed by a repeated-measures ANOVA that yielded a significant main effect of treatment on the freezing response ($F(1,22) = 9.81$, $P < 0.01$), but no significant interaction between treatment group and session interval. Thus, the finding that ECS-treated rats froze more than control rats during both the first and second halves of the acquisition session may suggest that these two groups had different levels of general behavioral arousal.

Once placed into the training chamber 1 day later, the animals from the control and ECS groups displayed increased levels of freezing behavior (Fig. 3b). However, similarly to what was observed during the training session, the amount of freezing differed

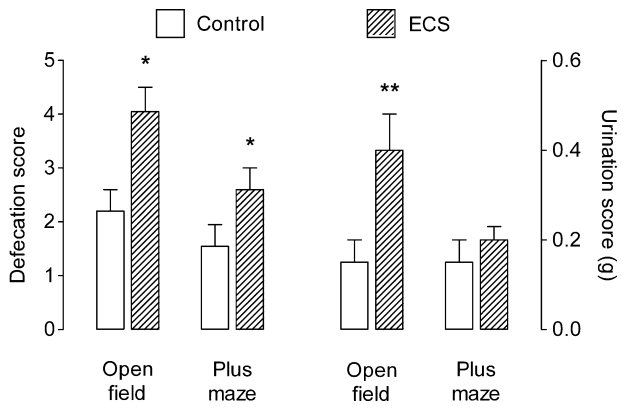

Fig. 2. Number of fecal boli and amount of urine deposited by rats during the open-field and elevated plus-maze tests. Data are presented as means ± SEM. $n = 12$ in each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group.

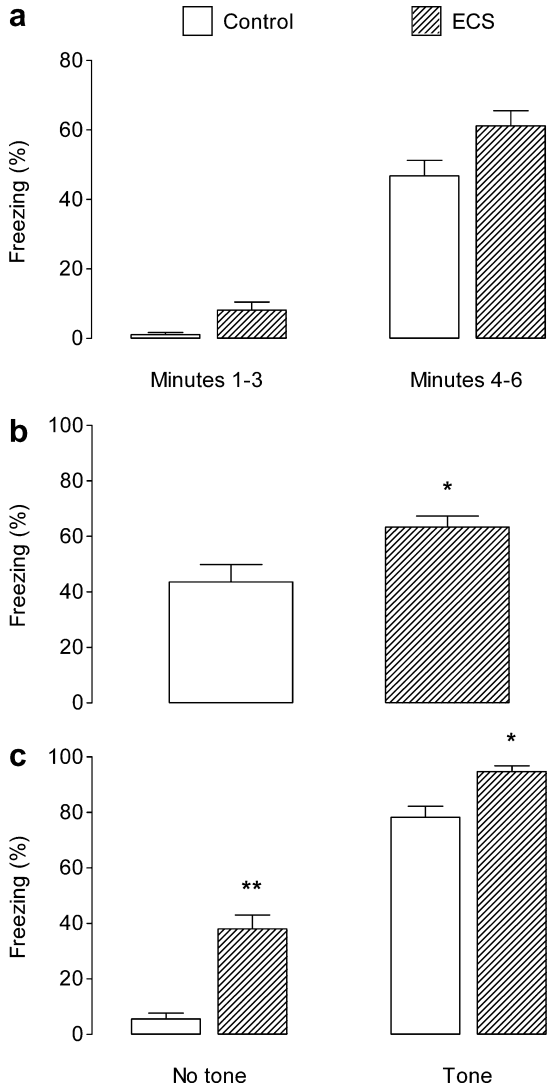

Fig. 3. Effects of repeated ECS-induced seizures on the acquisition and retention of conditioned fear. Percentage of freezing time in each of the 3-minute periods of the acquisition session (a), in the context retention test (b), and in each of the 3-minute periods of the tone retention test which was performed in a novel context (c). No tone or footshock was delivered during the first 3-minute periods of the acquisition session (a) and the tone retention test (c). Data are presented as means ± SEM. $n = 12$ in each group. $^{*}P < 0.01$ and $^{**}P < 0.001$ versus control group.

between the groups, so that rats treated with ECS froze to the familiar context more than control rats ($P < 0.01$). In the novel context (Fig. 3c), the levels of freezing displayed by control and ECS-treated rats in the absence of the tone were inferior to those observed during the tone presentation ($F(1,22) = 351.83$, $P < 0.001$). Repeated-measures ANOVA of these data additionally yielded a significant main effect of treatment ($F(1,22) = 59.96$, $P < 0.001$) and a significant treatment × tone presentation interaction ($F(1,22) = 5.47$, $P < 0.05$). ECS-treated rats froze more than control rats even in the absence of the tone ($P < 0.001$). During the tone presentation, they showed nearly maximum possible levels of fear (95 ± 2%), freezing significantly more than control rats ($P < 0.01$).

3.3. Behavioral effects of pilocarpine-induced SE in the open field and elevated plus maze

Pilocarpine-treated rats did not differ from control rats in the locomotion scores measured in the outer zone of the open field (Fig. 4a). Interestingly, the inner locomotion activity was higher in the SE group than in the control group ($P < 0.01$). No significant differences were found between the control and SE groups with respect to defecation and urination scores (Fig. 5).

Similarly to what was found in the first experiment, control rats spent most of their time (approximately 97%) within the protected closed arms and central square of the elevated plus maze (Fig. 4b). However, unlike controls, the rats in the SE group showed a reduced preference for the protected zones of the apparatus (Fig. 4b). In particular, they spent less time than control rats in the closed arms of the maze (36% vs 56%, respectively, $P < 0.01$). This tendency was also clearly seen in the data from the open arms of the apparatus, where rats in the SE group spent considerably more time than those from the control group ($P < 0.001$). In addition, the locomotor activity of SE rats was somewhat higher than that of control rats in the open arms of the maze ($P < 0.01$) and lower in the closed arms ($P < 0.001$) (Fig. 4c). Just like in the open-field test, no significant differences were detected between

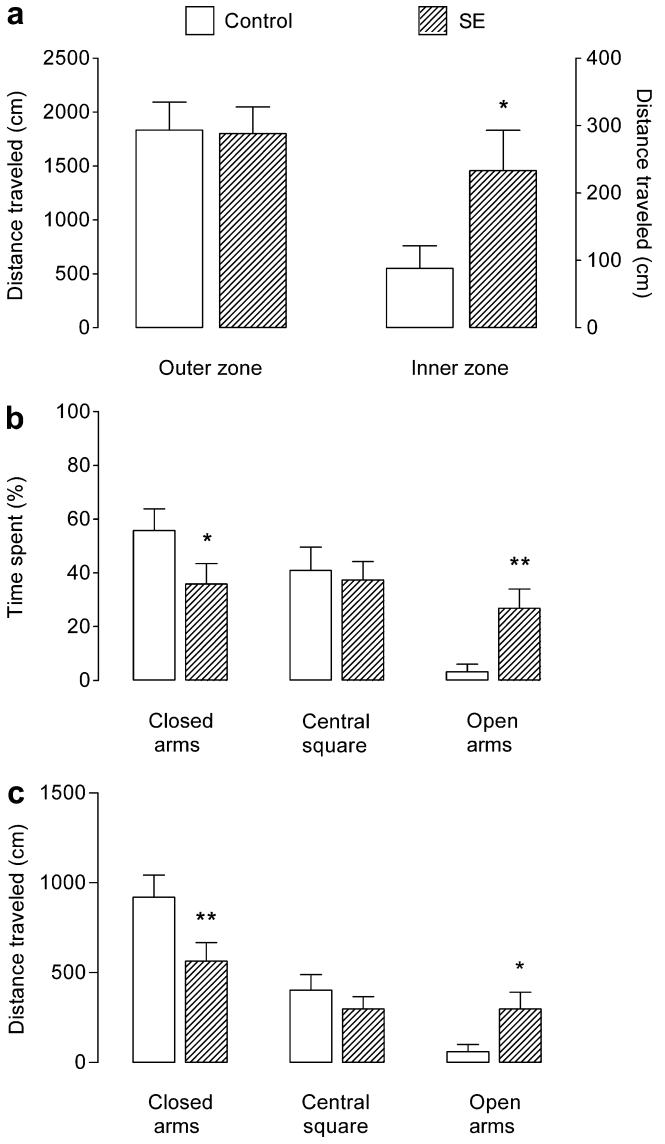

Fig. 4. Effects of SE on the behavior of rats in the open-field and elevated plus-maze tests. Distance traveled in the outer and inner zones of the open field (a) and percentage of time spent (b) and distance traveled (c) in the central square and closed and open arms of the plus maze. Data are presented as means ± SEM. $n = 12$ in SE group and $n = 8$ in control group. $^{*}P < 0.01$ and $^{**}P < 0.001$ versus control group.

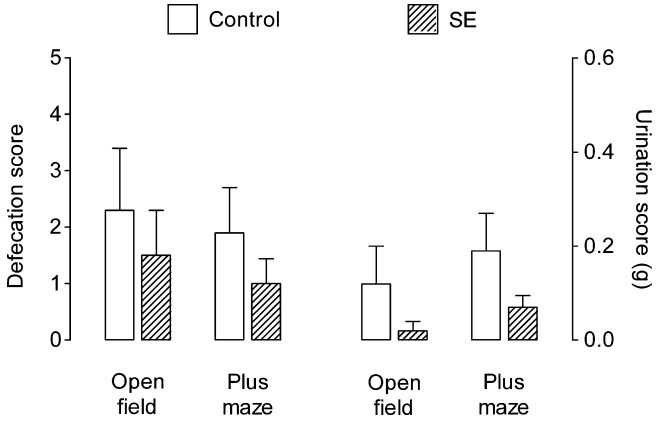

Fig. 5. Number of fecal boli and amount of urine deposited by rats during the open-field and elevated plus-maze tests. Data are presented as means ± SEM. $n = 12$ in the SE group and $n = 8$ in control group.

control and SE groups with respect to the defecation and urination episodes in the plus maze (Fig. 5).

3.4. Effects of pilocarpine-induced SE on conditioned freezing behavior

Conditioning produced an increase in the amount of freezing time in both the control and SE groups ($F(1,18) = 172.01$, $P < 0.001$) (Fig. 6a). Closer examination of the data, however, revealed that rats treated with pilocarpine froze less during the second half of the training session (minutes 4–6) than did control rats. This observation was confirmed by a repeated-measures ANOVA that yielded a significant interaction between treatment group and session interval ($F(1,18) = 9.48$, $P < 0.01$), but no significant main effect of treatment. Post hoc tests applied to this interaction revealed that rats treated with pilocarpine showed significantly less freezing than control rats during minutes 4–6 ($P < 0.001$). However, there were no effects of treatment on baseline freezing (minutes 1–3), suggesting that there were no differences between the two groups in general arousal behavior.

During the context retention test performed 1 day later, the animals from the control group displayed increased levels of freezing behavior (Fig. 6b). In contrast, SE rats exhibited marked context memory deficits relative to control rats ($P < 0.01$). In the novel context (Fig. 6c), the levels of freezing displayed by control and SE rats in the absence of the tone were inferior to those observed during the tone presentation ($F(1,18) = 75.03$, $P < 0.001$). Repeated-measures ANOVA of these data additionally yielded a significant treatment × tone presentation interaction ($F(1,18) = 4.33$, $P < 0.05$). The presence of this interaction (in the absence of a significant main group effect) suggests that SE animals exhibited less tone-elicited fear when compared with control rats.

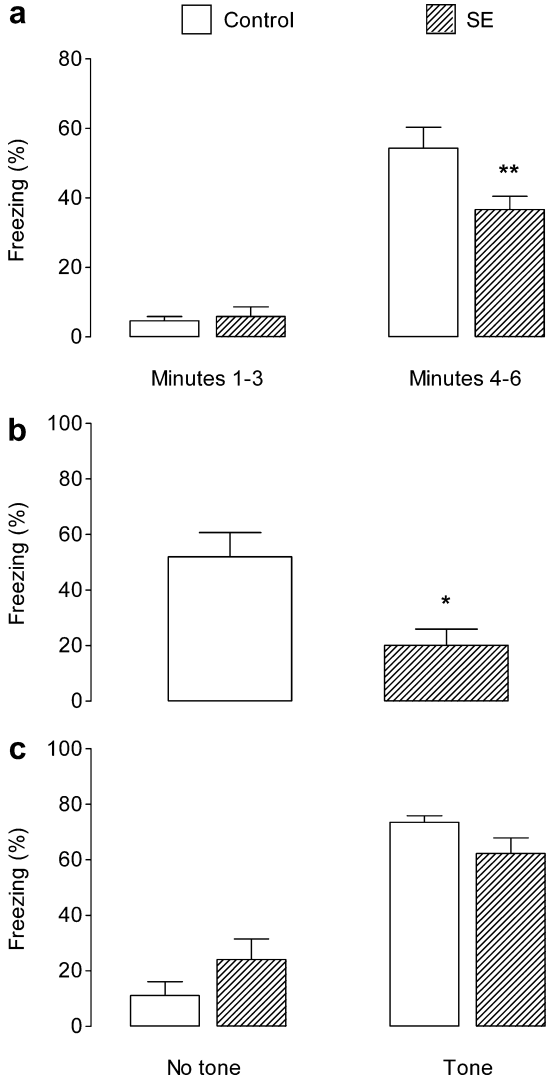

Fig. 6. Effects of SE on the acquisition and retention of conditioned fear. Percentage of freezing time in each of the 3-minute periods of the acquisition session (a), in the context retention test (b), and in each of the 3-minute periods of the tone retention test which was performed in a novel context (c). No tone or footshock was delivered during the first 3-minute periods of the acquisition session (a) and the tone retention test (c). Data are presented as means ± SEM. $n = 12$ in the SE group and $n = 8$ in the control group. $^{*}P < 0.01$ and $^{**}P < 0.001$ versus control group.

4. Discussion

The present findings demonstrate that repeated administration of brief seizures exerts an anxiogenic-like effect, as indicated by decreased locomotion of ECS-treated rats in the inner zone of the open field. This is corroborated by the results from the elevated plus-maze test, in which ECS rats spent more time in the enclosed arms than did control rats. In addition, rats from this group showed increased defecation and urination in the open field and increased defecation in the plus maze. All these indices of behavior are well-validated measures of anxiety in rodents [27,28,33] and have been successfully used by other authors to demonstrate changes in emotionality associated with seizures [9,11,23,24,34,35]. In these previous studies it was shown that repeated stimulation of one of the epileptogenic brain regions (a procedure known as *kindling*) results in similar behavioral changes [11,34,35]. Our findings are also consistent with existing evidence in showing that administration of ECS seizures enhances fear conditioning, just as has been previously found in kindled animals [36,37]. However, the behavioral mechanisms of this enhancement remain uncertain as our data additionally suggest that the state of general behavioral arousal of ECS-treated rats was different from that of control rats. Therefore, bearing in mind that increased arousal, at least in a potentially dangerous context, can be related to increased levels of emotional reactivity [38], it is possible that the enhanced fear conditioning in ECS rats rather reflects changes in anxiety than in emotional memory. This would be consistent with the open-field and elevated plus-maze data described above.

It has been previously reported that, depending on the location of the stimulation electrodes, kindling may be anxiogenic (right basolateral and anterior corticomedial nuclei of amygdala [9,11], perirhinal cortex [39]), may be anxiolytic (left basolateral amygdala [34,40]), or may have no effect on anxiety (caudate nucleus

[35] and dorsal hippocampus [41]). This focal nature of behavioral alterations induced by kindling is not surprising given that the brain regions chosen in these studies for stimulation play different roles in emotional processing. In contrast, ECS does not target any specific brain area, but instead activates a cascade of limbic structures involved in seizure propagation [10,22]. Furthermore, it has been reported that this activation, when excessive, may lead to structural changes in these regions. For example, repeated administration of ECS seizures according to the protocol employed in the present study causes a number of neuritic changes and moderate loss of neurons in at least three different regions of the limbic system, namely, dentate gyrus [21], entorhinal cortex [10], and retrosplenial cortex [22]. However, repeated administration of ECS seizures does not induce spontaneous epileptogenic activity in the brain; instead, it can even prevent or retard the occurrence of other types of seizures [17]. Therefore, the finding of the present study that ECS-treated rats exhibited elevated levels of anxiety can be taken as demonstrating a true behavioral effect of rather diffuse nonepileptogenic brain damage associated with generalized seizures.

Another finding of this experiment is that changes in emotional behavior resulting from SE appeared to be quite different from those observed after ECS seizures. Indeed, SE rats traveled more than controls in the central area of the open field and explored the open arms of the elevated plus maze as freely as the enclosed arms. These data are in accordance with several earlier studies in which similar behavioral changes were observed in rats after SE induced by either pilocarpine treatment [24,42,43] or continuous amygdala stimulation [23]. Moreover, although in this study we failed to detect any significant differences between the SE and control groups in defecation and urination scores, other authors have reported decreased novelty-induced defecation in SE rats [44]. The effects of SE on fear conditioning also differed from those of ECS seizures. In particular, we observed that SE rats showed somewhat diminished responsiveness to aversive stimuli during conditioning and impaired cued fear memory during the tone test. More impressively, memory for contextual information was lost almost entirely in the SE rats, as indicated by the fact that they exhibited levels of freezing similar to those of the original training context and the novel context. These results confirm previous reports that animals that have experienced prolonged seizure activity exhibit robust deficits in emotional memory [42–44]. The behavioral alterations induced by SE have been previously interpreted as an inability of subjects to properly evaluate and process emotionally arousing stimuli and ascribed to extensive damage to brain structures implicated in emotional functions [23,24,44]. In the same vein, it has been reported that SE may result in death of the majority of neurons (up to 90%) in the amygdala and hippocampal and several parahippocampal cortical areas [23,24,44,45]. For comparison, ECS-induced loss of cells known for their particular vulnerability to seizures, that is, neurons of the dentate hilus, entorhinal layer III, and retrosplenial layer V, was estimated to be no more than 20% [10,21,22]. With respect to the amygdala, there is so far no evidence that ECS seizures might cause any damage to this structure. Thus, the observation that behavioral changes resulting from SE were qualitatively and quantitatively different from those observed after ECS seizures is most likely due to the fact that, whereas brief seizures produce only mild neuronal injury, prolonged seizure activity is associated with profound structural alterations in several limbic regions critically involved in the regulation of emotional responses. In line with this view are the clinical data demonstrating that patients with early-onset temporal lobe epilepsy (indication of a high likelihood of amygdala and hippocampal damage) show both impaired facial emotion recognition [6,46] and impaired aversive conditioning [47,48].

There are at least two caveats that need to be kept in mind when interpreting the present findings. One of them is that the behavioral changes described above can be attributed to some nonspecific pathophysiological effects of seizures such as motor suppression, reduced arousal, and diminution of sympathetic tone. Indeed, ECS-treated rats showed decreased overall locomotor activity in the open field, and their behavior in the fear-conditioning test indicated that they might have changes in the general arousal state. However, the data on motor activity collected from the elevated plus-maze task do not support this possibility, as they showed that the locomotion scores of ECS-treated rats were selectively reduced only in the potentially "dangerous" open arms and central square of the apparatus, but not in the "safe" closed arms. Similarly, the decrease in closed-arm activity detected in the SE group was counterbalanced by a proportional increase in activity in the open arms of the maze, suggesting that changes in locomotion alone cannot explain the reduced preference for the closed arms over the open arms observed in this group. Nevertheless, more conclusive evidence is necessary to exclude the possibility that the behavioral changes detected in this study are related to some nonspecific long-term effects of seizures, which can be provided by employing behavioral tasks other than those used in our experiments, such as, for example, startle reactivity.

Another important caveat of our results is that it is unclear how the behavioral sequelae of seizures are related to the neuronal damage they produce. As noted above, evidence from previous studies [22,49–53] indicates that SE induces a diffuse loss of neurons in the hippocampal formation, amygdala, and retrosplenial, entorhinal, and piriform cortices, which can quite plausibly explain the reduced emotionality of SE rats described in this and prior [23,24,42–44] studies. There is much less evidence, however, about the structural changes in the brain associated with repeated administration of ECS seizures. It has been previously reported that ECS seizures, when administered using the present treatment protocol, can cause a moderate loss of retrosplenial, entorhinal, and dentate gyrus neurons, followed by remodeling of neuritic processes in several adjacent brain areas [10,22]. However, given the behavioral profile of ECS-treated rats revealed in the present experiment, it seems likely that administration of ECS seizures was associated with some important changes in other limbic structures, such as the amygdala and ventromedial prefrontal cortex, which are known to be critically involved in the regulation of anxiety [54–56]. These possibilities remain to be further investigated using quantitative neuroanatomical approaches.

The present findings confirm and extend previous reports demonstrating that epileptic seizures lead to significant emotion-like behavioral disturbances that can be further aggravated by prolonged or frequent seizures, such as those observed in patients with poorly controlled epilepsy. Extension of this research to the ECS model of seizures is important because it allowed us to demonstrate, for the first time, that the presence of an epileptogenic focus in the brain may not be a necessary prerequisite for the occurrence of these behavioral impairments. Indeed, it was found that even a few externally triggered generalized ECS seizures, which, unlike kindling, do not induce spontaneous seizures, are nevertheless sufficient to cause detectable changes in anxiety-related behaviors. We believe that these findings may shed more light on the role of seizures in the development of affective pathologies in patients with epilepsy and inspire future research aimed at identifying key neuroanatomical circuits implicated in this process.

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Seizure-induced changes in neuropeptide Y-containing cortical neurons: Potential role for seizure threshold and epileptogenesis.

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Seizure-induced changes in neuropeptide Y-containing cortical neurons: Potential role for seizure threshold and epileptogenesis

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ABSTRACT

Seizure activity induces transient changes in the levels of neuropeptide Y (NPY) and somatostatin (SS) in various brain regions, but it remains unclear whether this effect can persist for long periods and whether it is relevant to epileptogenesis. We report that brief seizures evoked by electroshock produced an increase in the number of NPY neurons in the dentate hilus and retrosplenial cortex, an effect that lasted 10 weeks. The number of hilar SS neurons remained unchanged. However, the pentylenetetrazole seizure threshold was somewhat decreased in electroshock-treated rats. Despite this, no spontaneous seizures were detected in this group. In contrast, status epilepticus (pilocarpine model) produced loss of the hilar NPY and SS cells. Moreover, all rats with status epilepticus showed spontaneous behavioral seizures and their seizure threshold was markedly decreased. These findings support the notion that sustained NPY overexpression induced by brief seizures can be important in preventing epileptogenesis.

1. Introduction

The etiology of temporal lobe epilepsy (TLE) remains poorly understood, but there is evidence that the epileptogenic process is associated with loss of neurons involved in the inhibitory modulation of hippocampal and parahippocampal neural networks, such as dentate gyrus mossy cells and local circuit GABAergic interneurons [1–7]. Furthermore, given the enhanced vulnerability of these neuronal populations to excitotoxicity, it is possible that their loss can, in turn, be triggered by prolonged seizure activity [8]. This possibility is consistent with the data for humans that both status epilepticus (SE) caused, for example, by acute domoic acid intoxication [9] and prolonged or repeated early life febrile seizures [10,11] may result in epileptogenesis. It is also consistent with the results of numerous studies of animal models of TLE demonstrating that SE induced by either neurotoxins, such as kainic acid and pilocarpine, or perforant pathway stimulation leads to progressive loss of neurons in the dentate hilus associated with the development of spontaneous seizures [2,3,12–14].

Despite the crucial role played by dentate mossy cells and GABAergic interneurons in maintaining the balance of excitation and inhibition in hippocampal networks [15,16], experimental evidence indicates that their loss, if not extensive, does not necessarily lead to epileptogenesis [17,18]. In line with this, data in humans show that febrile seizures persisting for relatively short periods, that is, presumably characterized by only moderate loss of neurons, do not result in epilepsy in later life [19,20]. These observations suggest that brain circuits, which can potentially be involved in the formation of epileptogenic foci, possess intrinsic mechanisms capable of attenuating the reduction in inhibitory transmission associated with moderate cell loss, thereby preventing or delaying epileptogenesis. Importantly, improved knowledge of these natural mechanisms that provide protection against seizure-induced neuronal injury could offer new targets for therapeutic intervention at the early stages of the epileptogenic process.

One candidate mechanism for attenuating inhibitory transmission following seizure-induced neuronal injury is enhancement of the release of neuropeptide Y (NPY), which is well known for its anticonvulsant properties [21–23]. Supporting this possibility are the findings from many studies showing that there is a strong increase in the expression of NPY in local circuit GABAergic interneurons during and shortly after seizures [24–26]. Furthermore, it has been reported that both SE and kindling seizures can induce NPY synthesis in glutamatergic dentate granule cells, an effect that may persist for very long periods [24,27–29]. However, it is less well established whether the seizure-induced NPY overexpression in GABAergic interneurons is also long-standing and whether it is specific to brain areas characterized by cell loss and implicated in epileptogenesis. To address this issue, we estimated the total number of NPY-immunoreactive (IR) cells in the rat dentate hilus 60 days after the administration of a course of six electroconvulsive shock (ECS) seizures according to a schedule described elsewhere [30]. It has previously been shown that treating rats according to this protocol

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produces moderate damage to the brain regions implicated in TLE, including the hilus of the dentate gyrus [31,32], but does not lead to the development of spontaneous seizures. Thus, we hypothesized that if adaptive changes in the NPY system are indeed partly responsible for the attenuation of inhibitory activity in brain regions in which seizures produce cell loss, then the number of hilar NPY-IR neurons would be increased after chronic ECS treatment. For comparative purposes, we tested the effects of ECS seizures on the number of hilar cells immunoreactive to another neuropeptide co-expressed by hippocampal GABAergic interneurons, somatostatin (SS), synthesis of which is also transiently enhanced by seizures [33]. However, the seizure-related changes in SS-IR cells, at least in the dentate hilus, appear to be less pronounced when related to NPY [28,34]. Also for the sake of comparison, we evaluated the effects of ECS seizures on the areal density of NPY-IR neurons in the retrosplenial granular b (Rgb) cortex, a brain region also known to be implicated in TLE [35], as well as in the primary somatosensory barrel field (S1bf) cortex, which appears not to be critically involved in this disorder. In parallel, morphometric analysis of immunostained brain sections obtained from rats that have experienced prolonged SE and developed spontaneous recurrent seizures was performed.

2. Methods

2.1. Animals and treatments

Male Wistar rats, 2 months of age, maintained under standard laboratory conditions, were used in this study. In the first experiment, the rats (ECS group, $n=12$) received a course of five ECS seizures, administered on a 24-hour schedule [31]. Each stimulus (50 Hz, 60 mA for 1 second) was delivered via ear-clip electrodes wired to a stimulus generator (Model 215/IZ, Hugo-Sachs Elektronik, Germany). Two hours after the fifth stimulation, each of the animals received one additional ECS seizure. ECS produced full tonic–clonic seizures with hindlimb extension lasting 5–10 seconds. Rats in the control group (sham-ECS group, $n=12$) received handling identical to that of experimental rats, but were not stimulated.

In the second experiment, animals from the SE group ($n=12$) were pretreated with scopolamine methyl bromide (1 mg/kg, subcutaneously, Sigma) to minimize peripheral cholinergic side effects of pilocarpine. Thirty minutes later, the rats received a single high dose of pilocarpine (350 mg/kg, intraperitoneally, Sigma). The onset of SE was defined as the appearance of behavioral symptoms corresponding to stage 4 or 5 seizures on the Racine scale [36], that is, rearing, falling, and generalized convulsions. SE onset was usually detected 30–60 minutes following the pilocarpine injection. It has been previously reported that pilocarpine-induced SE, if lasting several hours, can be associated with high mortality rates ranging between 15 and 50% depending on the dose of pilocarpine and other experimental conditions [37,38]. Therefore, because animal mortality is a prominent cause of bias in quantitative evaluations of neuronal loss [39], special efforts were made to improve the survival rate of the animals in the SE group. In particular, 2 hours after the beginning of SE, the rats were injected with diazepam (5 mg/kg, ip) to end the convulsive manifestations of SE. However, seizure activity, albeit considerably reduced in severity, was not completely stopped by the single dose of diazepam. Thus, an additional dose of diazepam (2.5 mg/kg) was given to the rats 3 hours after the onset of SE. Furthermore, the animals were periodically injected with saline (sc) during the first 24 hours of the recovery period. Control rats in the second experiment (sham-SE group, $n=12$) received handling and treatment identical to that received by experimental rats, including injections of scopolamine and diazepam, but were not treated with pilocarpine.

Following the respective treatments, the rats were observed daily for spontaneous behavioral seizures at random times between 08:00 and 20:00 hours. Ten weeks after the end of treatment, six animals in each group, selected at random, were killed by transcardial perfusion and their brains were processed for immunocytochemistry. Remaining rats were used for determination of the effects of the treatment on the susceptibility to seizures induced by a GABA(A) receptor antagonist, pentylenetetrazole (PTZ). The handling and care of the animals followed the *Principles of Laboratory Animal Care* (NIH Publication No. 86-23, revised 1985) and the *European Communities Council Guidelines in Animal Research* (86/609/UE). All efforts were made to minimize the number of animals used and their suffering.

2.2. Tissue preparation

Animals were deeply anesthetized with pentobarbital (90 mg/kg) and injected intracardially with 0.1 mL of a heparin solution, followed by 1 mL of 1% sodium nitrite in saline. Then, they were perfused transcardially with 150 mL of 0.1 M phosphate buffer (PB, pH 7.6) for vascular rinse, followed by 250 mL of a fixative solution containing 4% paraformaldehyde in PB. The brains were removed from the skulls, immersed for 2 hours in the fixative, and infiltrated overnight in 10% sucrose solution at 4 °C. After the frontal poles were trimmed away, the blocks were mounted on a vibratome and sectioned in the coronal plane at 40 μm, and the sections were collected in PB. All sections cut through the hippocampal formation, and Rgb and S1bf cortices, that is, between coronal planes corresponding to levels approximately 0.3 mm and 7.8 mm posterior to the bregma [40], were collected. From each brain, three adjacent series of sections were separately collected in phosphate-buffered saline (PBS) to be used for immunostaining for NPY and SS and for Nissl staining. The sections were stored until use at −20 °C in cryoprotectant (30% sucrose, 30% ethylene glycol, 0.25 mM polyvinylpyrrolidone in PBS).

2.3. Immunostaining for NPY and SS

Sections were washed twice in PB, treated with 3% H_2O_2 for 10 minutes to inactivate endogenous peroxidase, and incubated overnight at 4 °C with the primary polyclonal antibody against either NPY or SS (Bachem, Merseyside, UK; 1:10,000 dilution in PBS). Thereafter, the sections were washed twice and incubated with biotinylated anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA; 1:400 dilution in PBS). Sections were then treated with avidin–biotin–peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories; 1:800 dilution in PBS). In the two last steps, incubation was carried out for at least 1 hour at room temperature. Following treatment with the peroxidase complex, sections were incubated for 10 minutes in 0.05% diaminobenzidine (Sigma) to which 0.01% H_2O_2 was added. Sections were rinsed with PBS for at least 15 minutes between steps. To increase tissue penetration, 0.5% Triton X-100 was added to the PBS that was used in all immunoreactions and washes. Specificity of the immune reactions was controlled by omitting the incubation step with primary antiserum. All immunochemical reactions and washings described above were carried out in 12-well tissue culture plates, four sections in each well, to ensure that staining of the sections from all groups analyzed was performed in parallel and under identical conditions. Following termination of the staining procedures, sections were mounted on gelatin-coated slides and air-dried. They were then dehydrated in a series of ethanol solutions (50, 70, 90, and 100%) and coverslipped using Histomount (National Diagnostics, Atlanta, GA, USA).

2.4. Nissl staining

Sections were mounted serially on gelatin-coated slides. After being air-dried overnight at room temperature, they were stained with Giemsa [41], dehydrated, and coverslipped with Histomount.

2.5. Morphometric analysis

2.5.1. Determination of the total number of NPY-IR and SS-IR cells in the dentate hilus

Hilar neurons immunoreactive to NPY and SS were identified as darkly stained perikarya and on the basis of their location and morphology (Fig. 1). The total number of these neurons was estimated using the optical fractionator method [41]. The boundaries of the hilus of the dentate gyrus were consistently defined at all levels along the septotemporal axis of the hippocampal formation on the basis of cytoarchitectonic criteria [42] and using the rat brain atlas of Paxinos and Watson [40]. Neuron counting was carried out using the Olympus C.A.S.T.-Grid System (Denmark) and a mean of 11 systematically sampled sections were used per animal. Beginning at a random starting position, visual fields were systematically sampled along the *x* and *y* axes, using a raster pattern procedure. The neuronal nuclei were selected as a convenient counting unit. They were counted in every frame using the optical dissector at a final magnification, at the level of the monitor, of ×2000. The coefficient of error (CE) of the individual estimates was calculated according to Gundersen [43] and ranged between 0.08 and 0.10.

2.5.2. Determination of areal density of NPY-IR neurons in the Rgb cortex

Visual examination of the NPY-immunostained sections containing the Rgb cortex has shown that the boundaries of cortical layers are difficult to delineate with certainty in this material. On the basis of this observation and also taking into account that the distribution pattern of NPY cells in cortical structures is layer specific, it was concluded that unbiased stereological methods necessitating precise delineation of reference areas could not be used in this case. Therefore, we opted to analyze this material using the procedure previously developed for quantification of the areal density of immunostained cells in laminar cortical formations [44]. Because it had been previously reported that repeated ECS seizures do not affect the volumes of Rgb layers, it is likely that variations in the areal density of NPY-IR cells in each Rgb layer can reliably reflect the effect of ECS seizures on this neuronal population. In this procedure, brain sections containing the Rgb cortex were analyzed using a light microscope equipped with a camera lucida at final magnification of ×160. The boundaries of the Rgb cortex were consistently defined at all levels along the rostrocaudal axis of the brain as previously described in detail [35] and using the rat brain atlas of Paxinos and Watson [40]. From the NPY-stained sections obtained for each brain and containing the Rgb cortex, every sixth section was systematically sampled to yield a set of 12 sections, spaced 440 μm apart, to be included in the analysis. Level-matched sections were used for all groups. NPY-IR neurons were identified as darkly stained perikarya (Fig. 2), and were drawn unilaterally. Camera lucida diagrams of the boundaries of Rgb cortex with neighboring cortical regions and of its laminar organization [35] were drawn from the adjacent Nissl-stained sections. These diagrams were then overlaid on the images of NPY-positive perikarya, which allowed delineation of the boundaries of Rgb cortical layers in the immunostained material (Fig. 2). The neurons that fell on each layer were then counted from the drawings. The same camera lucida diagrams of Giemsa-stained sections were used for measuring the areas of the Rgb cortical layers. For this purpose, a transparent sheet bearing a test system composed of a set of regularly spaced points [45] was overlaid on the drawings, and the points that fell within the limits of each layer were counted. The area of each layer was then estimated by multiplying the number of points that fell on that layer by the area per point of the test system (0.0117 mm^2). Cell profile counts were performed in cortical layers II–IV, V, and VI, but not in layer I, where there were not enough stained neurons to take a reliable sample. The counts of neurons belonging to layers II–IV were made in the entire area occupied by all three layers, because it was difficult to delineate the boundary between them with certainty. The cell counts obtained were divided by the values of the corresponding laminar areas to yield the values of the areal densities (number/mm^2).

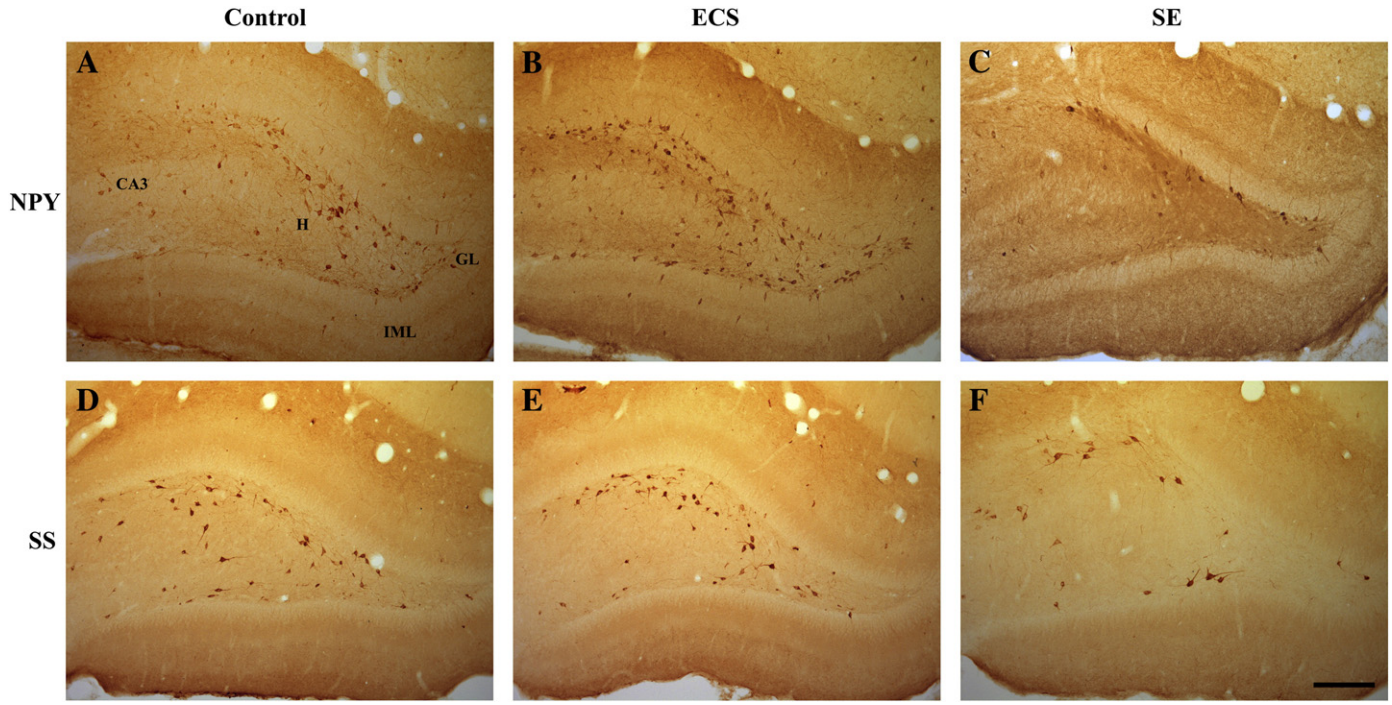

Fig. 1. Representative photomicrographs of level-matched coronal sections of the dentate gyrus from a sham-treated control rat (A, D) and from rats treated with ECS (B, E) and pilocarpine (C, F). Sections shown in (A), (B), and (C) were immunostained for NPY, whereas those shown in (D), (E), and (F) were immunostained for somatostatin (SS). Note that the density of NPY-IR cells in the hilus of the dentate gyrus is increased in the ECS-treated rat when compared with the control rat, whereas the density of SS-IR cells is similar in the two rats. The density of both NPY-IR cells and SS-IR cells is dramatically decreased in the rat that had experienced SE. Note also the presence of densely packed plexus of NPY-IR fibers in the dentate hilus and inner molecular layer of the animal from the latter group (C). CA3, pyramidal cell layer of the CA3 hippocampal field; H, dentate hilus; GL, granule cell layer; IML, dentate inner molecular layer. Bar = 200 μm.

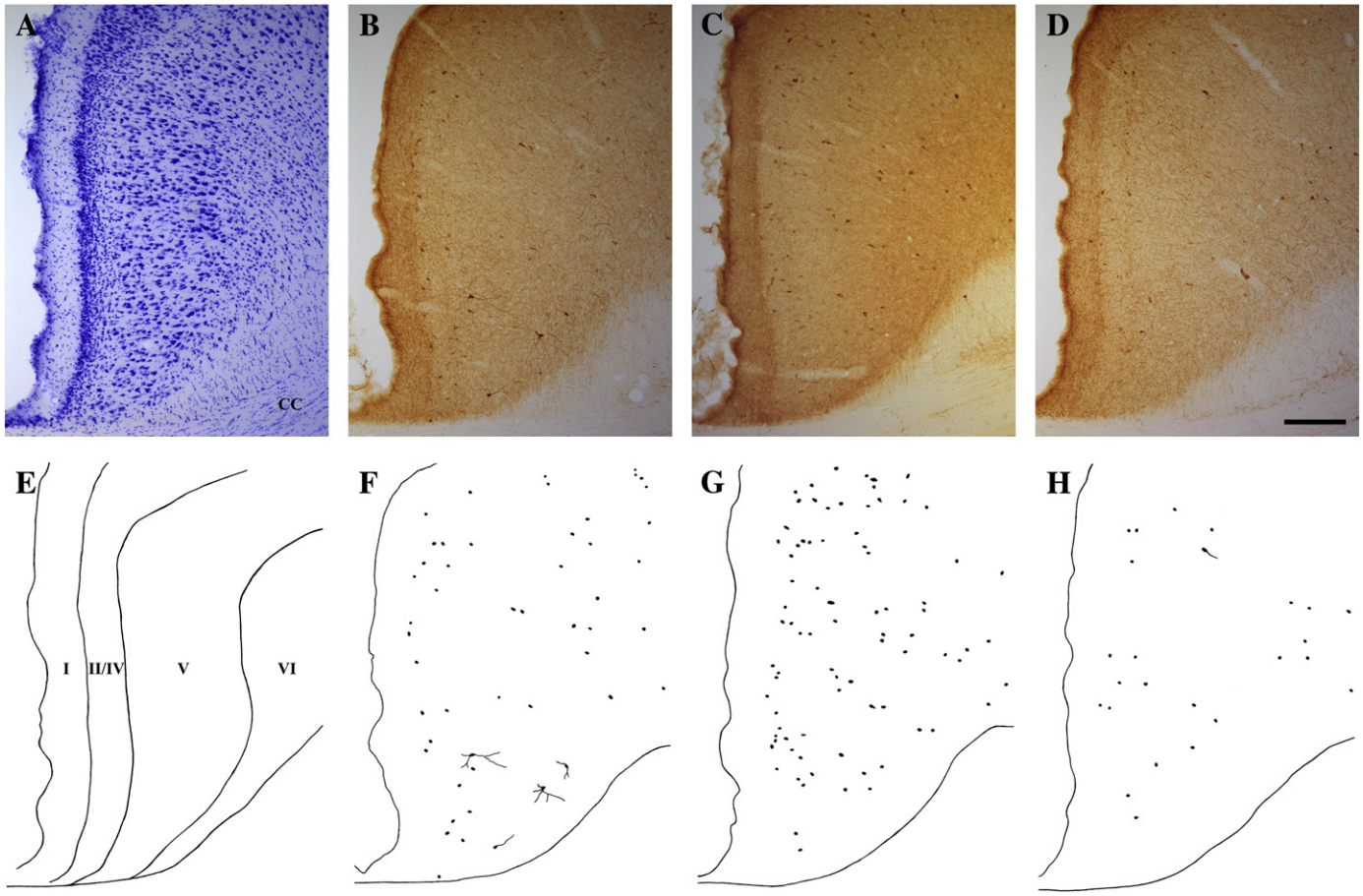

Fig. 2. Representative photomicrographs of level-matched NPY-immunostained coronal sections of the retrosplenial granular b (Rgb) cortex from a sham-treated control rat (B), and from rats treated with ECS (C) and pilocarpine (D). Camera lucida drawings of these sections are shown, respectively, in (F)–(H). Adjacent Nissl-stained sections, such as shown in (A), were used to delineate the boundaries of the cortical layers (E). Note that the Rgb cortex (layer V) of the ECS-treated rat has a higher density of NPY-IR neurons relative to that of the control rat. In contrast, the density of NPY-IR cells in the Rgb cortex (layers II–IV and VI) of the rat from the SE group (D, H) is reduced. CC, corpus callosum. Bar = 200 μm.

2.5.3. Determination of areal density of NPY-IR neurons in the S1bf cortex

For the reasons explained in the previous section, we opted to quantify the areal densities of NPY cells in each of the S1bf layers using morphometric procedures described elsewhere [44]. Briefly, from the NPY-stained sections obtained from each brain and contained in the S1bf cortical area, every sixth section was systematically sampled to yield a set of eight sections, spaced 440 μm apart. These sections were analyzed using a light microscope equipped with a camera lucida at final magnification of × 160, and level-matched sections were used for all groups. NPY-IR cells of the S1bf cortex were identified as darkly stained perikarya and on the basis of their location and morphology (Fig. 3). Camera lucida diagrams of these cells were drawn, unilaterally, from 700-μm-wide strips perpendicular to the pial surface and extending toward the cortical layer VI/white matter boundary (Fig. 3) as described previously in detail [44]. The intersection of the lateral border of the strip with the cortical surface was positioned 6 mm lateral to the midline. This position was chosen based on the rat brain atlas of Paxinos and Watson [40]. Camera lucida diagrams of the boundaries between different cortical laminae of the S1bf cortex were drawn from the adjacent Nissl-stained sections. These diagrams were then overlaid on the images of NPY-positive perikarya drawn from adjacent sections, which allowed delineation of the boundaries of S1bf cortical layers in the immunostained material (Fig. 3). The neurons that fell on each layer were then counted from the drawings. The same camera lucida diagrams were used for measuring the areas of the laminae as described in the previous section. Cell profile counts were performed in cortical layers II/III, IV, V, and VI, but not in layer I, where there were not enough stained neurons to take a reliable sample. The counts of neurons belonging to layers II and III were made in the entire area occupied by both layers, because it was difficult to undoubtedly delineate the boundary between the two laminae. The values of the areal densities were calculated as described above.

2.6. PTZ-induced seizures

Pentylenetetrazole (Sigma) was dissolved in saline (20 mg/mL) and injected intraperitoneally at a starting sub-threshold dose of 20 mg/kg body wt. Following this initial treatment, additional doses of 10 mg/kg PTZ were administered at 10-minute intervals until a generalized clonic seizure occurred. The behavior of the animals was monitored by two experimenters who were blind to treatment assignment. The latency to the onset of myoclonic jerks and the latency to the onset of generalized clonic convulsions with rearing and falling were registered.

2.7. Statistical analysis

All analyses were performed using Statistica 5.0 software (StatSoft, Tulsa, OK, USA). Before conducting statistical comparisons, data were tested for normality using the Kolmogorov–Smirnov one-sample test. Because all data samples passed the normality tests, they were analyzed for statistical significance using Student's *t* test. Differences were considered significant at the $P<0.05$ level. Results are expressed as means ± SD.

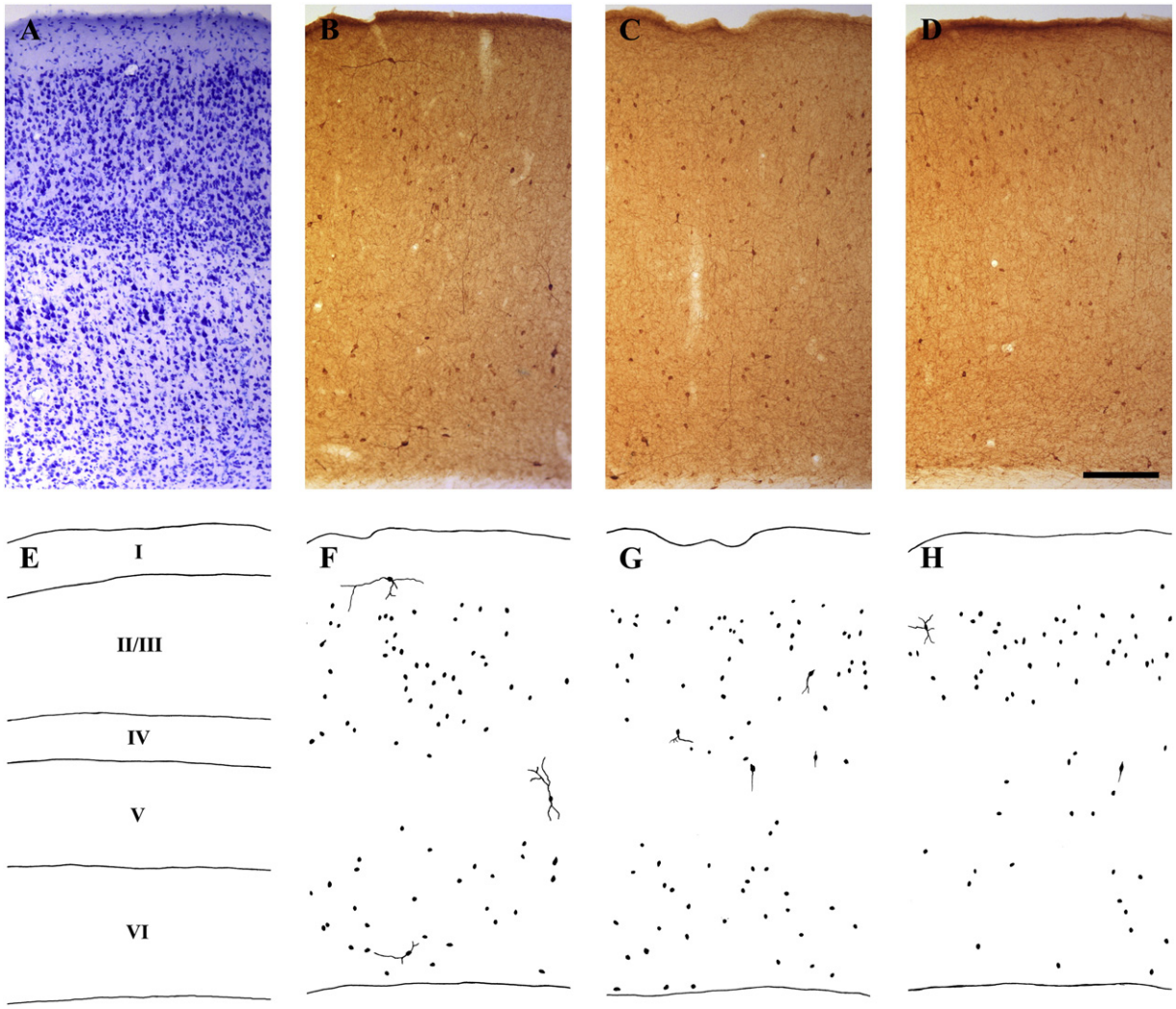

Fig. 3. Representative photomicrographs of level-matched NPY-immunostained coronal sections of the primary somatosensory barrel cortex (S1bf) from a sham-treated control rat (B) and from rats treated with ECS (C) and pilocarpine (D). Camera lucida drawings of these sections are shown, respectively, in (F)–(H). Adjacent Nissl-stained sections, such as shown in (A), were used to delineate the boundaries of the cortical layers (E). Note that the S1bf cortex (layer VI) of the rat from the SE group has a lower density of NPY-IR neurons relative to that of the control rat. Bar = 200 μm.

3. Results

3.1. Behavioral monitoring

No behavioral manifestations of seizures were detected in animals from the ECS-treated group and sham-treated control groups. However, following a latent period lasting 1–3 weeks, spontaneous motor seizures of stage 3 or greater on the Racine scale, that is, tremor, forelimb myoclonus, rearing, and falling [36], were repeatedly observed in all rats that experienced SE.

3.2. Qualitative morphological observations

Visual examination of the immunostained material obtained in this experiment showed that the density of NPY-IR neurons in the dentate hilus was somewhat increased in ECS-treated rats in comparison to sham-treated control rats, whereas it was markedly decreased in those animals that had experienced SE (Figs. 1A–C). In the latter group, examination of the sections additionally revealed densely packed plexus of NPY-IR fibers in the dentate hilus as well as in its inner molecular layer (Fig. 1C). The density of hilar SS-IR neurons in ECS-treated rats was similar to that in control rats, but in the SE group it was markedly decreased (Figs. 1D–F). In addition, we did not detect any increase in SS immunostaining in the molecular layer or cell-free areas of the dentate hilus of rats from the SE group (Fig. 1F). The qualitative observation also provided the impression that the density of NPY-IR neurons in Rgb layer V tended to be higher in ECS-treated rats when compared with respective controls (Figs. 2B, C). In addition, inspection of the sections revealed that the density of these neurons in Rgb layers II–IV and VI was considerably reduced in pilocarpine-treated rats relative to sham-treated control rats (Figs. 2B, D). At the qualitative level, no treatment-related structural alterations were detected in layers II–V of the S1bf cortex (Fig. 3). However, the density of NPY-IR neurons in S1bf layer VI appeared to be smaller in pilocarpine-treated rats than in controls (Figs. 3B, D).

3.3. Quantitative morphological estimates

The estimates of the total numbers of NPY-IR and SS-IR neurons in the hilus of the dentate gyrus of the rats used in this study are listed in Table 1. Analysis of these data confirmed the conclusions drawn from our qualitative observations by showing that chronic ECS treatment produced a significant, approximately 40% increase in the total number of hilar NPY-positive cells ($P = 0.001$). However, the number of hilar SS-IR cells remained unchanged following ECS treatment. In

Table 1
Effects of ECS seizures and SE on the total number of NPY-IR and SS-IR cells in the hilus of the dentate gyrus and on the areal density of NPY-IR cells in the Rgb and S1bf cortices.

	Sham-ECS ($n=6$)	ECS ($n=6$)	P	Sham-SE ($n=6$)	SE ($n=6$)	P
Dentate hilus						
NPY-IR cells (total number)	12,084 ± 1246	16,976 ± 1338	0.001	12,253 ± 1255	5958 ± 1716	0.001
SS-IR cells (total number)	11,816 ± 1359	12,375 ± 1704	NS	12,095 ± 840	7585 ± 1492	0.001
Rgb cortex						
NPY-IR cells (number/mm^2)						
Layers II–IV	99.9 ± 25.7	92.8 ± 19.9	NS	92.8 ± 20.6	51.4 ± 14.1	0.002
Layer V	30.4 ± 6.3	44.3 ± 9.8	0.017	28.4 ± 9.0	23.3 ± 6.1	NS
Layer VI	34.6 ± 9.3	32.9 ± 12.8	NS	36.6 ± 12.8	16.0 ± 4.6	0.002
S1bf cortex						
NPY-IR cells (number/mm^2)						
Layers II, III	183.7 ± 13.4	195.1 ± 27.2	NS	191.4 ± 20.8	164.6 ± 38.8	NS
Layer IV	96.9 ± 26.2	99.2 ± 20.6	NS	90.0 ± 22.0	98.3 ± 26.5	NS
Layer V	76.3 ± 18.4	82.1 ± 15.7	NS	78.4 ± 17.2	70.8 ± 24.8	NS
Layer VI	105.8 ± 18.9	115.7 ± 15.9	NS	112.6 ± 19.9	72.2 ± 23.0	0.009

Note. Values are means ± SD. P values were calculated using Student's t test versus respective sham-treated groups. NS, nonsignificant.

contrast, and also consistent with the qualitative observations, SE produced a strong decline in the total number of both hilar NPY-IR cells (50%, $P=0.001$) and SS-IR cells (40%, $P=0.001$).

The areal density of NPY-IR neurons in Rgb layer V was increased in ECS-treated rats by almost 50% relative to the sham-ECS group ($P=0.017$) (Table 1). However, no significant effect of ECS treatment on the densities of NPY-IR cells in Rgb layers II–IV and VI was observed. Again, changes in NPY staining produced by SE in this brain region were markedly different from those produced by ECS seizures (Table 1). In particular, SE produces no change in the areal density of NPY neurons in Rgb layer V. In addition, in Rgb layers II–IV and layer VI, that is, where ECS seizures showed no effects, SE was associated with dramatic loss of NPY-positive neurons (45%, $P=0.002$ for layers II–IV; 55%, $P=0.002$ for layer VI).

As expected based on the qualitative observations, the areal densities of NPY-positive cells in layers II/III, IV, V, and VI of the S1bf cortex of ECS-treated rats were similar to those found in sham-treated control rats (Table 1). Furthermore, although the density of NPY cells in S1bf layers II/III was somewhat decreased in the SE group relative to control values, the reduction was not statistically significant ($P=0.09$). However, SE was associated with significant loss of NPY-IR neurons in S1bf layer VI ($P=0.009$) (Table 1).

3.4. Susceptibility to seizures

Administration of PTZ induced a typical pattern of seizures in all rats in the present experiment, including characteristic myoclonic jerks and generalized clonic seizures accompanied by running, rearing, and falling. In some rats, generalized tonic–clonic convulsions with tonic hindlimb extension were observed. However, none of the rats that developed tonic seizures died during the experiment or shortly after it. With respect to the treatment groups (Table 2), the results showed that rats that had experienced SE had significantly shorter latencies to myoclonic seizures (80% reduction, $P=0.001$) and to generalized clonic seizures (50% reduction, $P=0.014$) than did sham-treated rats. Less expected, however, was that ECS-treated rats also had somewhat reduced latencies relative to the sham-ECS group regarding both myoclonic jerks (40% reduction, $P=0.036$) and generalized clonic convulsions (30% reduction, $P=0.031$).

Table 2
Latencies (in minutes) to myoclonic jerks and to generalized clonic seizures induced with pentylenetetrazole in ECS-treated rats and in rats that had experienced SE.

	Sham-ECS ($n=6$)	ECS ($n=6$)	P	Sham-SE ($n=6$)	SE ($n=6$)	P
Myoclonic jerks	17.7 ± 5.7	10.4 ± 4.6	0.036	18.8 ± 6.8	4.0 ± 2.4	0.001
Generalized seizures	29.3 ± 4.5	21.2 ± 6.5	0.031	25.3 ± 4.9	14.5 ± 7.9	0.014

Note. Values are means ± SD. P values were calculated using Student's t test versus respective sham-treated groups.

4. Discussion

The main findings of this study are as follows: (1) Repeated brief seizures elicited by ECS in rats caused an increase in the total number of NPY-IR cells in the dentate hilus and in the areal density of these cells in the retrosplenial cortex (area Rgb, layer V), but not in somatosensory (S1bf) cortex; however, administration of ECS seizures did not affect the total number of hilar SS-IR cells. (2) Pilocarpine-induced SE led to a loss of NPY-IR and SS-IR neurons in the dentate hilus; SE was also associated with a loss of NPY-IR cells in the Rgb cortex (layers II–IV and VI) and S1bf cortex (layer VI). (3) Spontaneous behavioral seizures developed in all rats that had experienced SE and in none of those treated with ECS. (4) SE and ECS seizure models were associated with increased levels of neuronal excitability, as indicated by the reduced latencies to PTZ-induced myoclonic jerks and generalized clonic seizures observed under both experimental conditions.

These results appear to support, albeit indirectly, the main hypothesis of this study in showing that repeated brief seizures produce chronic (lasting at least 10 weeks) increases in the expression of NPY in brain regions involved in the pathogenesis of TLE, that is, dentate hilus and Rgb layer V, but not in the S1bf cortex, the role of which in this process is not established. This effect seems to be specific to NPY because the total number of hilar cells immunoreactive to SS, a neuropeptide known to be co-expressed by the majority of hilar GABAergic interneurons including those that produce NPY, was unchanged following the administration of ECS seizures. The latter observation is consistent with the results of prior studies that showed that the number of hilar SSergic cells remains constant in rats after administration of as many as 110 ECS seizures [46], as well as in fully kindled animals [47]. Interestingly, we previously reported that ECS seizures produce moderate cell loss in the very same regions in which the increased numbers of NPY-expressing neurons were detected in the present study [31,32,35]. Therefore, it is possible that the increased numbers of hilar and Rgb layer V NPY-IR cells found in ECS-treated rats may reflect a plastic compensatory response of these cells to abnormal functioning of local neural circuits associated with their injury. If this assumption is true, the ECS-induced plasticity of NPY-containing neurons may well explain why repeated brief seizures do not induce any spontaneous seizure activity, despite producing significant damage to epileptogenic brain regions.

Previous studies have reported that multiple ECS seizures cause a strong increase in NPY mRNA in a number of limbic structures

including the hilus of the dentate gyrus [25,34,48]. Consistent with this increase in gene expression, parallel increases in NPY immunostaining of single neurons and in extracellular NPY levels have been observed in the hippocampal formation of rats following chronic treatment with ECS [49,50]. However, it was also reported that these effects, being maximal immediately after treatment termination, decay progressively and are practically undetectable 2–4 weeks later [49,51]. In contrast, the present findings show that the ECS-induced NPY overexpression in brain areas linked to epileptogenesis can last as long as 10 weeks. Possible sources of this disagreement may be differences in the methods used for morphometric analysis as well as in the protocols of ECS administration. Alternatively, the discrepancy between the results of this and prior studies can be attributed to the "biphasic" pattern of seizure-induced NPY overexpression. Indeed, it has recently been reported that hippocampal NPY is manyfold upregulated 1 day after SE induced by electrical stimulation of the hippocampus, returns to background levels 1 week later, and is upregulated again following a latent period of 3–4 months [52]. It is likely, therefore, that the prominent short-term increase in NPY synthesis (as shown by prior studies) refers primarily to a protective response capable of counteracting the acute deleterious effects of seizures, whereas the long-term NPY overexpression (as reported in this study) is rather indicative of enduring adaptive reorganization of inhibitory circuits that may contribute to repairing potentially epileptogenic lesions.

The pilocarpine model of SE resembles many of the features of human TLE, including degenerative changes in the hippocampal region and spontaneous recurrent seizures [13,53,54]. In line with this approach, we found that in all rats included in this experiment, pilocarpine-induced SE led to the development of spontaneous seizures a few weeks after treatment. Also consistent with previous reports, immunostaining revealed in these rats strong NPY overexpression in the granule cell mossy fibers, namely, within their terminal fields in the dentate hilus and inner molecular layer [24,29,55,56]. The present findings additionally show that SE was associated with a marked loss of NPY-IR neurons in two brain regions implicated in epileptogenesis, the dentate hilus and the Rgb cortex, as well as in the deep layer VI of the S1bf cortex known for its role in seizure propagation [57]. Moreover, approximately 40% of hilar cells immunoreactive to SS were also lost in these rats. These data are consistent with previous studies that showed that prolonged seizure activity kills many hippocampal GABAergic interneurons, including those that co-express NPY and SS, which in effect can contribute to epileptogenesis [1–3,5,14,29,58,59]. Striking evidence supporting this view comes from the studies of Brandt et al. [60] and André et al. [4], who reported that SE-induced death of hilar interneurons, even in the absence of significant cell loss in the principal neuronal populations of the hippocampal formation, is sufficient for the development of spontaneous seizures. Thus, the present finding that pilocarpine-induced SE causes a significant reduction in the number of interneurons immunoreactive to NPY and SS in epilepsy-related brain regions is in line with the hypotheses of causal relationships between dysfunction of GABAergic inhibition and the process of epileptogenesis.

It must be kept in mind when interpreting the present findings that the quantification of neurons in different brain areas was carried out using different morphometric methods. In particular, neurons in the dentate hilus were quantified using unbiased stereological methods, whereas in the Rgb and S1bf cortices they were counted using a simplified procedure based on camera lucida drawings and expressed in units of areal density. Yet, it is known that seizure activity can evoke changes not only in the density of neurons, but also in the volume of respective structures [35,61,62]. In other words, it is possible that seizure-induced variations in the density of any neuronal population can simply be due to changes in the reference volume. With respect to the effect of ECS seizures on NPY-IR cells in Rgb layer V, this possibility seems unlikely, because we previously found that the volume of this Rgb layer is unaffected by repeated administration of ECS seizures [35]. However, in the same study we also observed that pilocarpine-induced SE leads to significant shrinkage of Rgb layers I and IV–VI, suggesting that the actual loss of NPY-IR cells in the Rgb cortex of rats that experienced SE can be somewhat greater than reported in the present study. To the best of our knowledge, there are no quantitative data at present regarding the effects of seizures on the volume of the S1bf cortex or of its layers. Therefore, it remains unclear to what extent the current results can be used to provide insight into the effects of seizures on NPY cells located in this area. However, at the level of light microscopy, we did not observe any noticeable treatment-related morphological abnormalities in the region of the S1bf cortex that might indicate that its volume was altered considerably following ECS seizures or SE. Thus, it is probable that the findings herein can adequately describe, at least in qualitative terms, the effects of seizures on NPY-IR neurons in this cortical region.

Another finding of this experiment is that the latencies to the PTZ-induced myoclonic jerks and generalized clonic seizures observed in animals in the SE-model group were substantially, approximately twofold shorter when compared with those of control animals. This finding was not unexpected given the extensive and widespread neuroanatomical changes associated with this model [35,63,64] and, in particular, the profound loss of inhibitory interneurons observed in previous studies [65,66] as well as in the present study. However, despite the fact that the estimates obtained for NPY-IR neurons in the dentate gyrus and Rgb cortex (layer V) were increased in rats treated with ECS, these animals also showed reduced latencies to both types of convulsive activity. The present results differ from those of other studies that showed that prior administration of ECS seizures produces anticonvulsant effects in both kindling and SE models of epilepsy [25,67,68]. However, in these previous experiments, unlike in the present study, the induction of kindling or SE was initiated shortly after termination of ECS treatment, that is, when the extracellular NPY levels are transiently increased. The current findings appear to be more consistent with those previously reported by Kragh et al. [69], who showed that 12 weeks after the administration of a course of ECS seizures, animals have increased susceptibility to seizures induced by lidocaine. Taken together, these observations suggest that the neuronal damage caused by repeated ECS seizures, despite triggering sustained NPY overexpression capable of preventing the formation of epileptogenic foci in the brain, is nevertheless sufficient to enhance the susceptibility to seizures elicited by other factors.

In conclusion, the results of this study are consistent with the notion that the likelihood of the formation of an epileptogenic focus following an initial injury may partly depend on the capacity of the NPY-producing neuronal system to undergo plastic changes to maintain the excitability of local circuits at nearly normal levels. Reduced plasticity of NPY neurons may be associated with the heightened seizure susceptibility observed in several conditions including, for example, in aged subjects as well as in males as compared with females. Indeed, it has recently been reported that the density of cortical NPY cells in males is significantly inferior to that estimated in females and drops progressively with aging in both sexes [44]. Our findings are also consistent with those obtained in NPY knockout and transgenic rodent models that demonstrate that overexpression of this neuropeptide not only has an antiseizure effect, but also inhibits epileptogenesis [23,70,71]. In this study, we additionally show that seizures per se can trigger NPY synthesis in seizure-vulnerable neurons, even in those that do not normally express it, suggesting the existence of intrinsic defense mechanisms in the brain capable of repairing seizure-induced defects in neuronal circuits. However, it is worth noting that the current results, as well as the results of prior studies demonstrating seizure-induced changes in NPY expression, do not rule out the possibility that other neuronal

mechanisms are implicated in epileptogenesis. For example, it is well known that changes in the excitability of hippocampal neurons may be related to recurrent sprouting of their axon collaterals [3,72–74]. Furthermore, it is possible that the seizure-induced recurrent fiber sprouting and NPY overexpression are mediated by common signaling mechanisms, which may include the recruitment of locally produced neurotrophic factors [44,75–77] as well as of ascending cholinergic [44,78,79] and serotoninergic [80,81] pathways.

Acknowledgments

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Reduced density of neuropeptide Y neurons in the somatosensory cortex of old male and female rats: Relation to cholinergic depletion and recovery after nerve growth factor treatment.

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REDUCED DENSITY OF NEUROPEPTIDE Y NEURONS IN THE SOMATOSENSORY CORTEX OF OLD MALE AND FEMALE RATS: RELATION TO CHOLINERGIC DEPLETION AND RECOVERY AFTER NERVE GROWTH FACTOR TREATMENT

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Abstract—Synthesis of neuropeptide Y in the neocortex and activity of the basalocortical cholinergic system are both reduced in the aging brain. We hypothesized that, by stimulating the activity of the basal forebrain cholinergic neurons, nerve growth factor might also be capable of restoring the synthesis of neuropeptide Y in cortical neurons. Old male and female rats were intraventricularly infused with nerve growth factor for 14 days and their brains were analyzed in order to quantify the densities of neuropeptide Y-immunoreactive neurons and of fiber varicosities stained for vesicular acetylcholine transporter protein in layers II/III, V and VI of the primary somatosensory barrel-field cortex. The areal densities of neuropeptide Y neurons and of vesicular acetylcholine transporter protein varicosities in all cortical laminae were found to be dramatically decreased in old rats when compared with young rats. However, infusions of nerve growth factor, known to exert a powerful trophic effect upon cortically projecting cholinergic neurons, have led to considerable recovery of vesicular acetylcholine transporter protein-positive terminal fields, which was paralleled by complete restoration of function in neuropeptide Y-producing neurons. With respect to the gender differences, although the density of cortical neuropeptide Y neurons was found to be significantly higher in young females than in young males and the opposite was true for vesicular acetylcholine transporter protein-positive varicosities, the general pattern of age- and treatment-related changes in these neurochemical markers was similar in both sexes. Overall, the age- and treatment-related variations in the density of cortical neuropeptide Y cells were found to correlate with those observed in the density of vesicular acetylcholine transporter protein varicosities. These results lend support to the idea that there is a causal relationship between age-related changes in cortical cholinergic and neuropeptide Y-ergic neurotransmitter systems. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: aging, sexual dimorphism, interneurons, VAChT, neurotrophins.

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Abbreviations: ANOVA, analysis of variance; ChAT, choline acetyltransferase; IR, immunoreactive; NBM, nucleus basalis magnocellularis; NGF, nerve growth factor; NPY, neuropeptide Y; PB, phosphate buffer; PBS, phosphate-buffered saline; VAChT, vesicular acetylcholine transporter protein.

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Neuropeptide Y (NPY) is widely distributed in the CNS where it is involved in the regulation of various functions including circadian rhythms, feeding behavior, blood pressure, memory, and emotion (Morley and Flood, 1989; Wettstein et al., 1995). In the cortex, NPY is usually co-expressed with GABA and somatostatin (Chronwall et al., 1984; Hendry et al., 1984). Changes in the cortical levels of NPY and its receptors have been found to be related to several pathological conditions such as epilepsy, schizophrenia, and frontotemporal dementia (De Lanerolle et al., 1989; Frederiksen et al., 1991; Mathern et al., 1995). Furthermore, there is convincing evidence that the synthesis of this neuropeptide in a number of brain regions, including in the neocortex, undergoes a dramatic reduction with aging (Higuchi et al., 1988; Kowalski et al., 1992; Huguet et al., 1993; Cha et al., 1996, 1997; Huh et al., 1997). Thus, given the role played by NPY in the regulation of the excitability of cortical neuronal circuits (Bacci et al., 2002), it is likely that some of the functional alterations seen in the aging brain might be related to the reduced production of this neuropeptide.

The nucleus basalis magnocellularis of the basal forebrain (NBM) is the major source of the cholinergic innervation to the neocortex (Mesulam et al., 1983; Saper, 1984; Houser et al., 1985; Baskerville et al., 1993), which is known to play a crucial role in modulating cortical function (Kurosawa et al., 1989; Tremblay et al., 1990). Existing morphological evidence strongly indicates that aging leads to loss of NBM cholinergic cells, atrophic changes in the remaining neurons (Altavista et al., 1990; Fischer et al., 1992; Smith and Booze, 1995), and concomitant reduction in the cortical levels of the principal enzymes involved in the metabolism of acetylcholine (Alberch et al., 1991; Yufu et al., 1994; Zhang et al., 1998; Zambrzycka et al., 2002). Interestingly, it has been reported that the age-related decreases in the density of cortical NPY-producing neurons are correlated with corresponding changes in the markers of cholinergic activity (Zhang et al., 1998), suggesting that the integrity of this neuronal population is dependent on the trophic influence of the basal forebrain cholinergic afferents.

Nerve growth factor (NGF) is a member of the neurotrophin family that exerts powerful trophic effects on the basal forebrain cholinergic neurons by protecting them against atrophy and degeneration (Cuello et al., 1992; Fischer et al., 1994; Koliatsos et al., 1994; Gustilo et al., 1999; Cadete-Leite et al., 2003). Moreover, treatment with exogenous NGF has been repeatedly reported to restore the neurochemical phenotype of NBM neurons and to

augment the content of major biochemical markers of cholinergic activity in the aging brain (Williams and Rylett, 1990; Williams et al., 1991; Rylett et al., 1993; Niewiadomska et al., 2002). Taking into account that the synthesis of NPY in cortical neurons appears to be dependent on the trophic support from NBM, it seems plausible that, by stimulating the activity of the basal forebrain cholinergic neurons, NGF might also be capable of restoring the normal activity of cortical NPYergic neurons. However, to the best of our knowledge, no previous studies looked at the effects of NGF on NPY-producing neurons in the cortex of aged animals. Thus, the present study was designed to determine whether or not the administration of NGF would restore the number of neurons immunoreactive (IR) to NPY in the neocortex of aged rats. To address this issue, old rats were intraventricularly infused with NGF for 14 days and their brains were analyzed in order to quantify the densities of NPY-producing neurons in the principal cell-containing layers of the somatosensory barrel-field cortex as well as the corresponding densities of the cholinergic fiber varicosities. Furthermore, we found it of particular interest to perform this experiment in both males and females. Indeed, bearing in mind that the basal forebrain cholinergic and cortical NPYergic neurons are both responsive to circulating levels of gonadal steroid hormones (Toran-Allerand et al., 1992; Woller and Terasawa, 1994; Gibbs, 1998, 2003; Bora et al., 2005), it might be expected that aging will affect these neuronal populations in a gender-specific manner.

EXPERIMENTAL PROCEDURES

Animals and diet

A total of 20 young (6-month old; 10 males and 10 females) and 20 aged (24-month old; 10 males and 10 females) Wistar rats

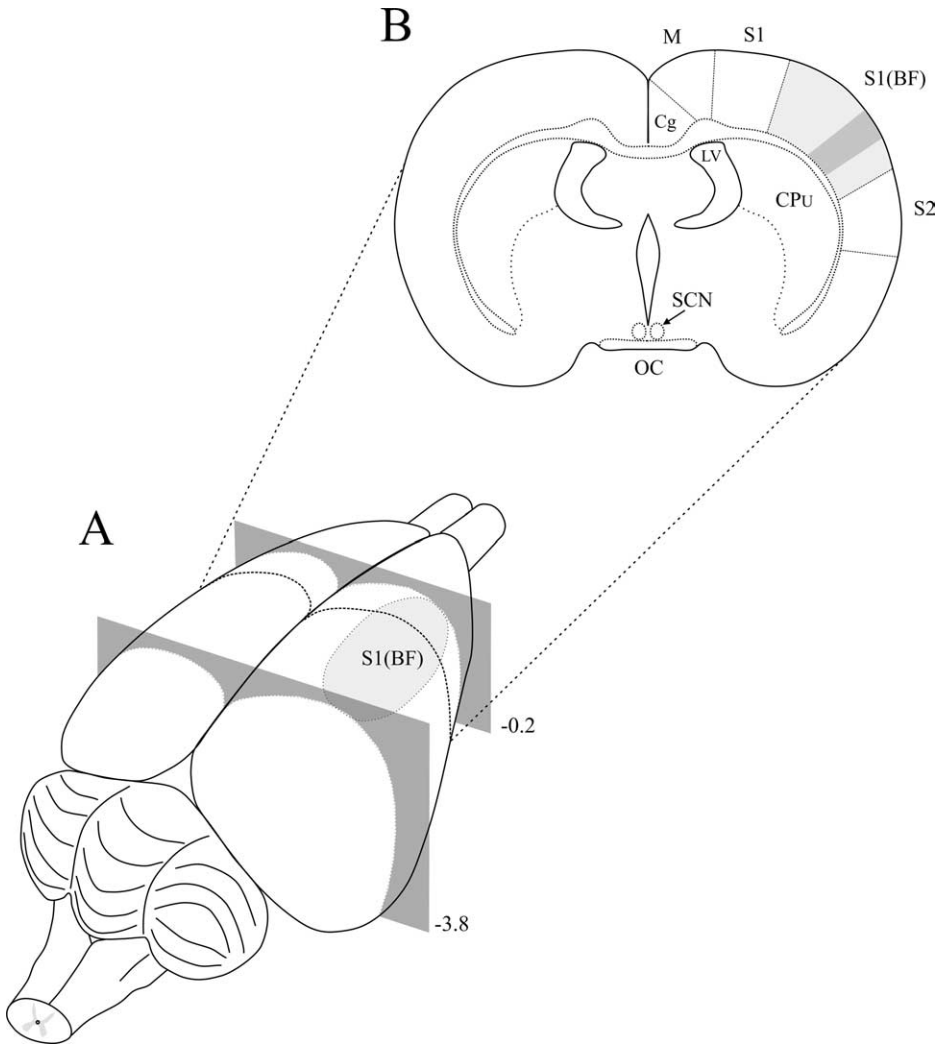

Fig. 1. Approximate location of the primary somatosensory barrel-field cortex in the rat brain is shown in (A) as a light gray area. Sections cut through this area, i.e. between coronal planes indicated on the drawing, were included in histological analysis. The numbers on the right indicate distances (in mm) from bregma. (B) Line drawing of a representative coronal section showing the location of the barrel-field cortex (light gray area) at the level of approximately −0.9 mm from bregma. The dark gray rectangle corresponds to the area of the barrel-field cortex that was subjected to morphometric analysis. Cg, cingulate cortex; CPu, caudate putamen; LV, lateral ventricle; M, motor cortex; OC, optic chiasm; S1, primary somatosensory cortex; S1(BF), primary somatosensory barrel-field cortex; S2, secondary somatosensory cortex; SCN, suprachiasmatic nucleus.

were used in the present study. They were individually housed in a temperature-controlled room (20–22 °C) under a 12-h light/dark cycle (lights on at 7:00 a.m.) and had *ad libitum* access to food and water. Half of the animals from all four groups were then randomly selected and submitted to treatment with NGF, whereas the remaining rats were sham-operated and served as controls. Each of the eight groups thus formed comprised five animals. In a separate experiment, morphological estimates obtained from the sham-operated rats were compared with those from rats that were not operated on. Because no differences in the density of NPY-stained cells were noted between the two control groups, the data from un-operated animals for simplicity are not shown. The experiments were performed in accordance with European Communities Council Directives of 24 November 1996 (86/609/EEC) and Portuguese Act n°. 129/92. All measures were taken to reduce animal suffering and numbers of animals in this study.

Surgical procedures and NGF treatment

Animals were anesthetized by subsequent injections of promethazine (10 mg/kg), xylazine (2.6 mg/kg), and ketamine (50 mg/kg) and placed in a Kopf stereotaxic apparatus. Permanent stainless steel cannulae (Alzet brain infusion kit) were stereotaxically placed in the right lateral ventricle at the following coordinates: 1.1 mm posterior to the bregma, 1.7 mm lateral to the midline, and 4.0 mm below the skull. The cannulae were connected to Methylene Blue (0.01%, Sigma) -filled Alzet osmotic minipumps (model 2002; Alza Corporation, Palo Alto, CA, USA) via sterile coiled polyethylene tubing (PE-60; Intramedic, Becton Dickinson, Sparks, MD, USA). This tubing was filled with air–oil spacer at the pump end and, according to the treatment protocol, with either vehicle alone (150 μl), composed of artificial cerebrospinal fluid supplemented with 0.1% bovine serum albumin (Sigma), or with 2.5S NGF (150 μg diluted in 150 μl of vehicle; Prince Laboratories Inc., Toronto, Canada). Osmotic minipumps were pre-tested to confirm their delivery rate and implanted s.c. in the neck. The incisions were closed with surgical stitches and treated with antiseptic. Fourteen days after the beginning of the infusions the animals were killed. The total infusion volume was 131.75± 14.12 μl per animal.

Tissue preparation

At the end of the experimental period, animals were deeply anesthetized with pentobarbital and injected intracardially with 0.1 ml of a heparin solution, followed by 1 ml of 1% sodium nitrite in saline. Then, they were perfused transcardially with 150 ml of 0.1 M phosphate buffer (PB, pH 7.6) for vascular rinse, followed by 250 ml of a fixative solution containing 4% paraformaldehyde in PB. The brains were removed from the skulls, immersed for 3 h in the fixative and infiltrated overnight in 10% sucrose solution at 4 °C.

After trimming away the frontal and occipital poles the blocks were mounted on a vibratome, sectioned in the coronal plane at 40 μm, and the sections were collected as free-floating. Only sections that were cut through the primary somatosensory barrel-field cortex, i.e. between coronal planes corresponding to the levels of approximately 0.2 mm and 3.8 mm posterior to the bregma (Paxinos and Watson, 1998; Fig. 1), were collected. From each brain, three adjacent series of sections were separately collected in phosphate-buffered saline (PBS) to be used, respectively, for immunostaining for NPY and vesicular acetylcholine transporter protein (VAChT), and for Nissl staining. The sections were stored until use at −20 °C in cryoprotectant (30% sucrose, 30% ethylene glycol, 0.25 mM polyvinylpyrrolidone in PBS).

Immunostaining for NPY

Sections were washed twice in PB and treated with 3% H_2O_2 for 10 min to inactivate endogenous peroxidase. The sections were then incubated overnight at 4 °C with the primary antiserum against NPY (Chemicon, Temecula, CA, USA; 1:10,000 dilution in PBS). Thereafter, the sections were washed twice and incubated with biotinylated anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA; 1:400 dilution in PBS). Sections were then treated with avidin–biotin peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories; 1:800 dilution in PBS). In the two last steps, the incubation was carried out for at least 1 h at room temperature. Following treatment with the peroxidase complex, sections were incubated for 10 min in 0.05% diaminobenzidine (Sigma) to which 0.01% H_2O_2 was added. Sections were rinsed

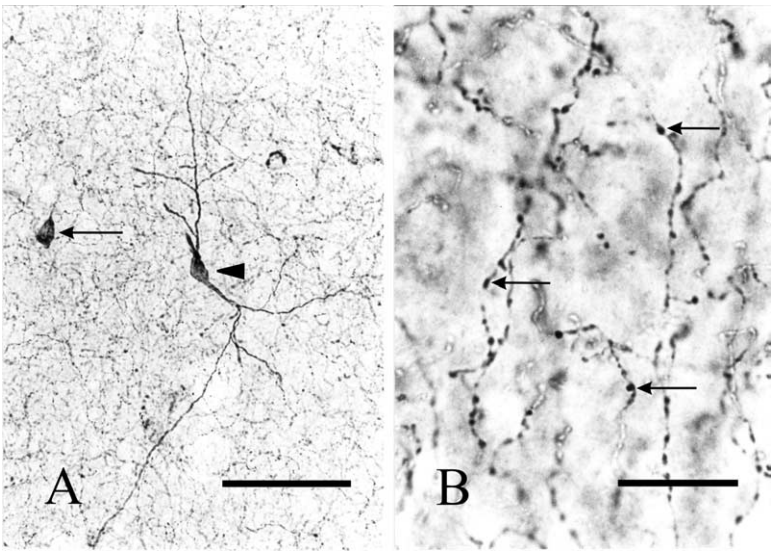

Fig. 2. Photomicrographs of brain sections of a female rat that were cut through the primary somatosensory barrel-field cortex and immunostained for NPY (A) and VAChT (B). Both images were taken from cortical layer VI. (A) Darkly stained cell bodies (indicated by arrow and arrowhead) were identified as NPY-IR neurons and were used for counting purposes. Neuronal arborizations were intensely stained in some of these cells (arrowhead). Scale bar=80 μm. (B) VAChT-containing fiber varicosities were defined as darkly stained axonal dilations with size greater than 0.25 μm^2 (shown by arrows). Scale bar=15 μm.

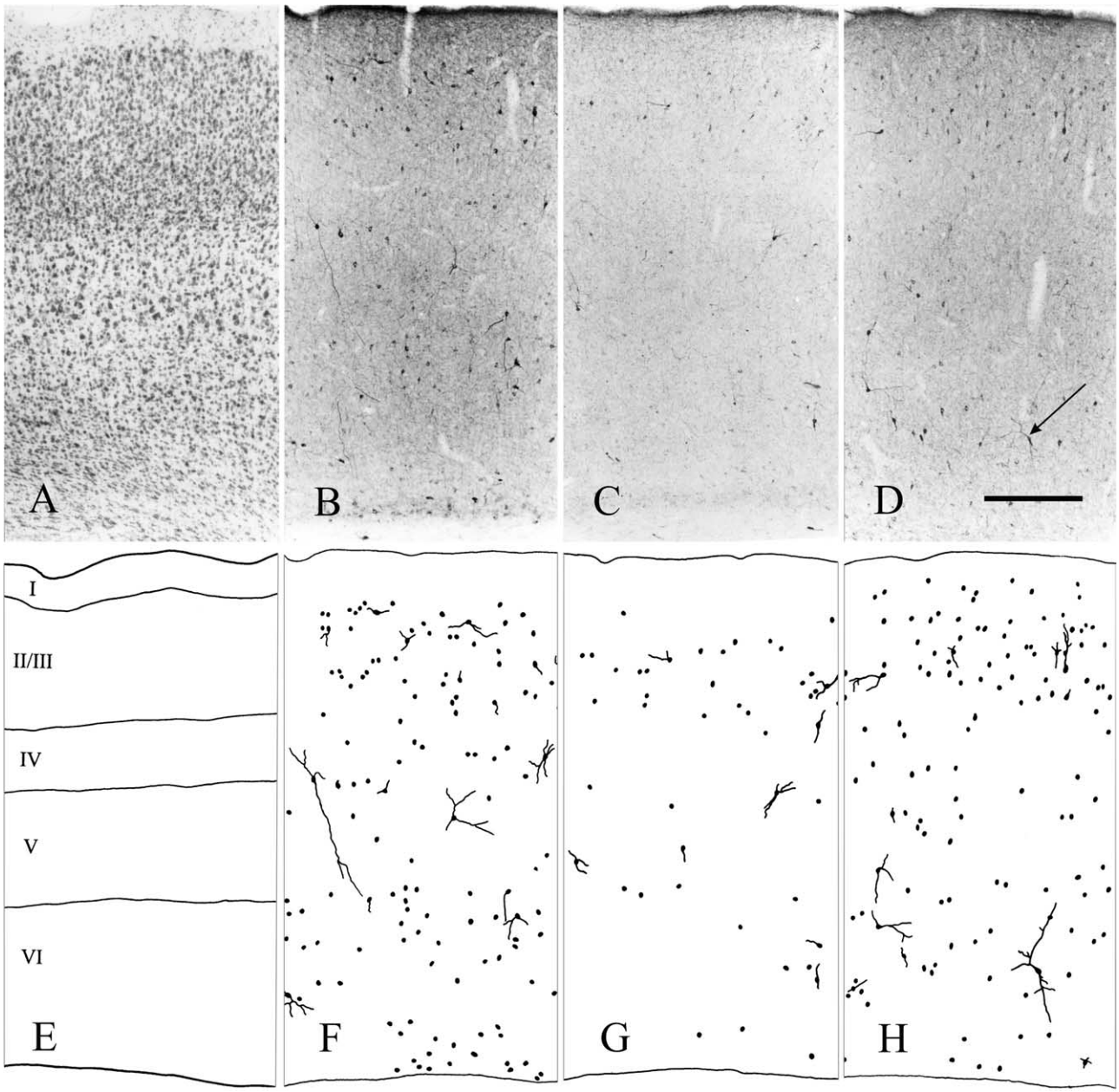

Fig. 3. Representative photomicrographs of level-matched NPY-immunostained brain sections obtained from three female rats: young (B), old (C), and old NGF-infused (D). Camera lucida drawings of these sections are shown, respectively, in (F–H). Adjacent Nissl-stained sections, such as shown in (A), were used to delineate the boundaries of the cortical layers (E). Note that the barrel cortex of the old rat has a lower density of NPY neurons relative to the young animal and that NGF treatment completely reversed this deficit. Scale bar=300 μm. A higher magnification of the neuron indicated in (D) by arrow is shown in Fig. 2A.

with PBS for at least 15 min between each step. To increase the tissue penetration 0.5% Triton X-100 was added to PBS that was used in all immunoreactions and washes. Sections were then mounted on gelatin-coated slides and air-dried. They were dehydrated in a series of ethanol solutions (50%, 70%, 90% and 100%) and coverslipped using Histomount (National Diagnostics, Atlanta, GA, USA). All procedures were performed on a rocking table.

Immunostaining for VAChT

Tissue sections were pretreated as shown above. Sections were then immersed in a 5% solution of rabbit normal serum in PBS (Vector Laboratories), for 30 min at room temperature. Thereafter, the sections were incubated for 72 h, at 4 °C, in a goat anti-VAChT polyclonal antibody (Chemicon; 1:15,000 dilution in PBS). Remaining steps were identical to those described above in the protocol for immunostaining for NPY.

Nissl staining

Sections were mounted serially on gelatin-coated slides. After air-drying overnight at room temperature, they were stained with Cresyl Violet, dehydrated, and coverslipped with Histomount.

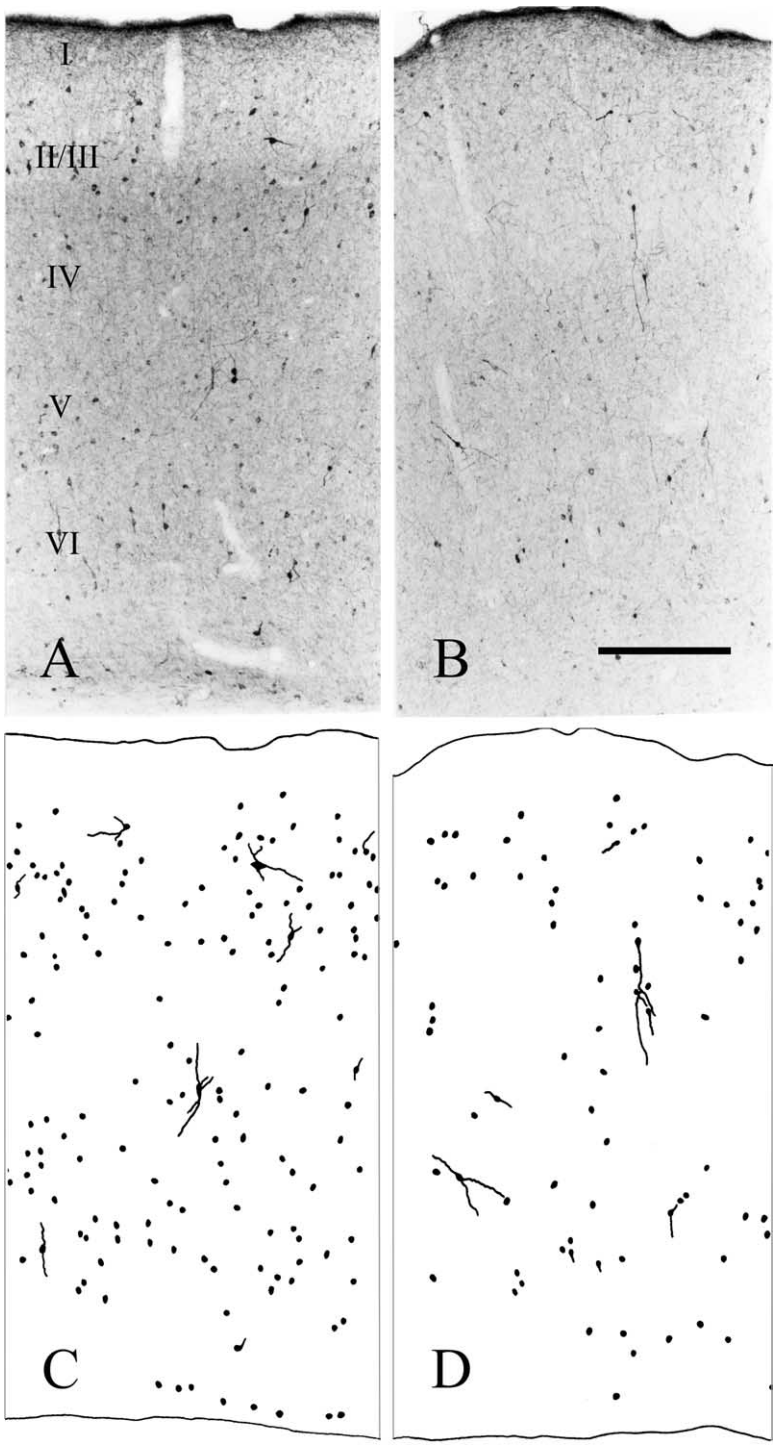

Fig. 4. Representative photomicrographs of level-matched NPY-immunostained brain sections obtained from a young female rat (A) and a young male rat (B). Camera lucida drawings of these sections are shown, respectively, in (C) and (D). Note that the female rat has more NPY-IR neurons in the somatosensory barrel-field cortex than does the male rat. Scale bar=300 μm.

Morphometric analysis

Brain sections immunostained for NPY were analyzed using a light microscope equipped with a camera lucida at final magnification of ×160. From the NPY-stained sections obtained for each brain, every third section was systematically sampled to yield a set of 10 sections, spaced 360 μm apart, to be included in the analysis. Level-matched sections were used for all groups. Camera lucida diagrams of NPY-IR perikarya were drawn, unilaterally, from 700-μm wide strips perpendicular to the pial surface and extending toward the cortical layer VI/white matter boundary (Fig. 1B). The intersection of the lateral border of the strip with the cortical surface was positioned 6 mm lateral to the midline. This

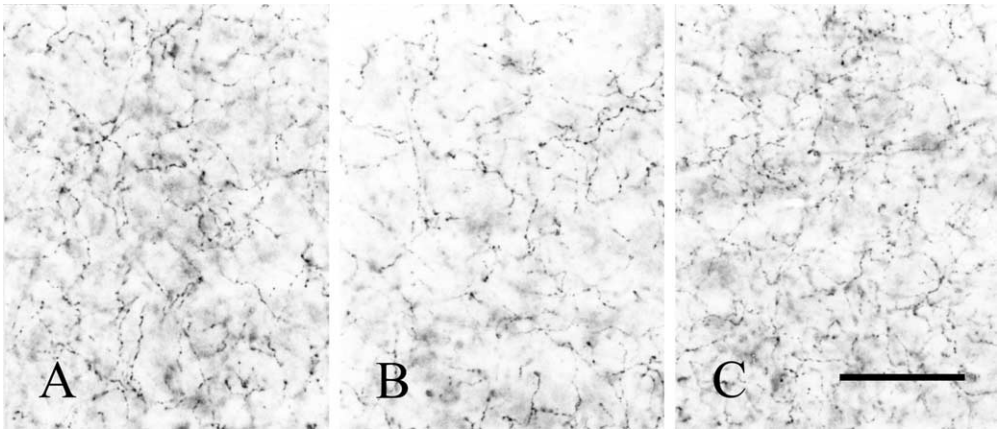

Fig. 5. Representative photomicrographs of VAChT-immunostained brain sections obtained from three female rats: young (B), old (C), and old NGF-infused (D). Images were taken from cortical layer VI. Note that the density of VAChT-IR varicosities in the cortex of the old animal is markedly lower when compared with both young rat and NGF-infused old rat. Scale bar=40 μm.

position was chosen based on the rat brain atlas of Paxinos and Watson (1998). Cortical NPY-IR neurons were identified as darkly stained perikarya and on the basis of their location and morphology (Fig. 2A). Camera lucida images of the adjacent Nissl-stained sections were then overlaid on the diagrams obtained from NPY-immunostained sections, which allowed to delineate the boundaries between different cortical laminae. The shrinkage of brain tissue along the radial axis of the cortex was found to be similar for sections stained for Nissl and for immunostained sections. Therefore, no effort was made to correct for possible differences in tissue shrinkage when overlaying the camera lucida images derived from adjacent sections processed by different methods. The number of neurons which fell on each layer was then counted from the drawings. The same camera lucida diagrams were used for measuring the areas of the cortical layers. For this purpose, a transparent sheet bearing a test system composed of a set of regularly spaced points (Gundersen et al., 1988) was overlaid on the drawings and the number of points that fell within the limits of each layer was counted. The area of each layer was then estimated by multiplying the number of points that fell on that layer by the area per point of the test system (0.0117 mm^2). Cell profile counts were performed in cortical layers II/III, V, and VI, but not in layers I and IV, where there were not enough stained neurons to take a reliable sample. The counts of neurons belonging to layers II and III were made in the entire area occupied by both layers, because it was difficult to delineate the boundary between the two laminae with certainty. The cell counts obtained were divided by the values of the corresponding laminar areas to yield the values of the areal densities (Number/mm^2).

The cholinergic varicosities stained with VAChT were counted using a computer-assisted image analyzer (Leica QWin) fitted with a Leica axioplan microscope and a Sony Hyper HAD Digital color video camera. For each animal, 10 VAChT-stained sections, adjacent to those used for counting NPY-IR cell profiles, were analyzed. Adjacent Nissl-stained sections were used to delineate the boundaries of the cortical layers in VAChT-stained sections. Measurements were performed separately in layers II/III, V and VI at a final magnification of ×2500. The varicosities were defined as darkly stained axonal dilations with size greater than 0.25 μm^2 (Wong et al., 1999; Fig. 2B). A sample frame (1.32×10^4 μm^2) was laid over each field of view and the number of varicosities falling within it was counted. Within each cortical layer, five different placements of the frame, each time at a randomly selected position, were used to obtain a mean count for that layer. The results were expressed as areal densities (Number/mm^2).

Statistical analysis

Analysis of variance (ANOVA) was used to analyze the effects of age, sex, and NGF treatment on the areal densities of NPY-IR neurons and VAChT-stained varicosities in the rat somatosensory cortex. When appropriate, post hoc Tukey's test was applied. Pearson's product moment regression model was used to analyze correlations between the areal densities of NPY-IR neurons and of cholinergic varicosities. Differences were considered to be significant if $P<0.05$.

RESULTS

Qualitative observations

In line with the results of previous studies, we found that, in the rat somatosensory cortex, NPY-stained neurons are abundant in laminae II/III, V, and VI, present in a smaller

Table 1. Summary of results of ANOVA on the areal densities of NPY-IR neurons in different layers of the somatosensory barrel-field cortex in young, young NGF-treated, old, and old NGF-treated rats of both sexes

Effect/interaction	Layers II/III		Layer V		Layer VI	
	$F(1,32)$	P	$F(1,32)$	P	$F(1,32)$	P
Sex	190.7	<0.001	71.97	<0.001	164.3	<0.001
Age	148.0	<0.001	79.37	<0.001	233.4	<0.001
Treatment	121.2	<0.001	55.51	<0.001	147.6	<0.001
Sex×age	28.23	<0.001	8.050	<0.05	37.71	<0.001
Sex×treatment	19.65	<0.001	10.68	<0.005	12.55	<0.005
Age×treatment	84.69	<0.001	22.63	<0.001	125.3	<0.001

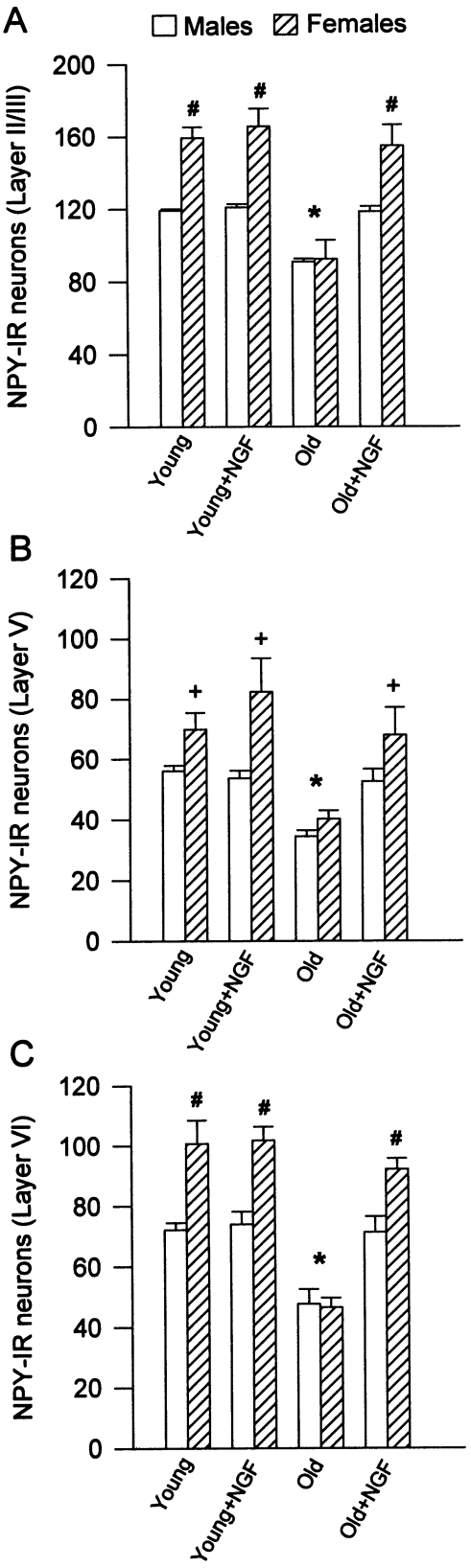

Fig. 6. Graphic representation of the areal density of NPY-IR neurons (Number/mm^2) in layers II/III (A), V (B) and VI (C) of the somatosensory barrel-field cortex of young (Young), young NGF-infused (Young+NGF), old (Old), and old NGF-infused (Old+NGF) rats. Note that old animals of both sexes had fewer NPY neurons in all cortical laminae and that NGF treatment ameliorated this deficit. The data also illustrate that the barrel cortex of young and NGF-infused old females has a higher density of NPY neurons than that of age- and treatment-matched males. * $P<0.001$ compared with young and NGF-treated old rats of both sexes; # $P<0.001$ compared with age- and treatment-matched males; + $P<0.05$ versus age- and treatment-matched males.

amount in lamina IV, and rarely found in lamina I (Figs. 3, 4). Although the general distribution pattern of NPY-IR neurons did not differ between the sexes, their density appeared to be higher in females than in males (Fig. 4). Examination of the brain sections obtained from aged animals showed that aging resulted in a dramatic loss of NPY-positive cells in all cortical layers and that NGF-treatment considerably reversed this effect (Fig. 3).

Consistent with prior studies, fiber varicosities IR to VAChT were most concentrated in the cell-free layer I of the somatosensory barrel cortex, but were also numerous in layers II/III, V and VI. In contrast, lamina IV was poorly stained. Although the distribution pattern of the cholinergic fiber varicosities was similar in animals of both sexes, their density appeared to be slightly lower in females than in males. Furthermore, just like in the case of the NPY-producing neurons, aging resulted in a striking reduction in VAChT immunoreactivity in all cortical layers. However, the cholinergic fiber network in the cortex of aged animals was considerably restored after NGF infusions (Fig. 5).

Areal density of NPY-IR neurons

Statistical analysis of the results of morphological measurements showed that there were significant effects of sex, age and NGF treatment on the areal densities of NPY-IR neurons in layers II/III, V and VI of the rat somatosensory cortex (Table 1; Fig. 6). In addition, ANOVA revealed that all three independent variables, i.e. sex, age, and treatment, significantly interacted with each other (Table 1). Confirming the qualitative observations given above, post hoc comparisons showed that the areal densities of cortical NPY-IR neurons were significantly higher in young females than in young males ($P<0.001$ for laminae II/III and VI and $P<0.05$ for lamina V) and that, independently of sex, these densities were significantly lower in old than in young animals ($P<0.001$ for all laminae). Moreover, these estimates did not differ between old males and old females. Thus, taking also into consideration the fact that the effect of gender significantly interacted with the aging effect, these findings suggest that the age-related loss of NPY neurons was greater in females than in males. No significant differences between young rats of both sexes and their NGF-infused counterparts were detected. However, the areal densities of NPY neurons were significantly higher in the cortex of NGF-treated old rats than in the cortex of age- and sex-matched control rats ($P<0.001$). Actually, the age-related deficits in the density of NPY-producing neurons were no longer detected in the cortex of old rats following NGF infusions (Fig. 6), suggesting that

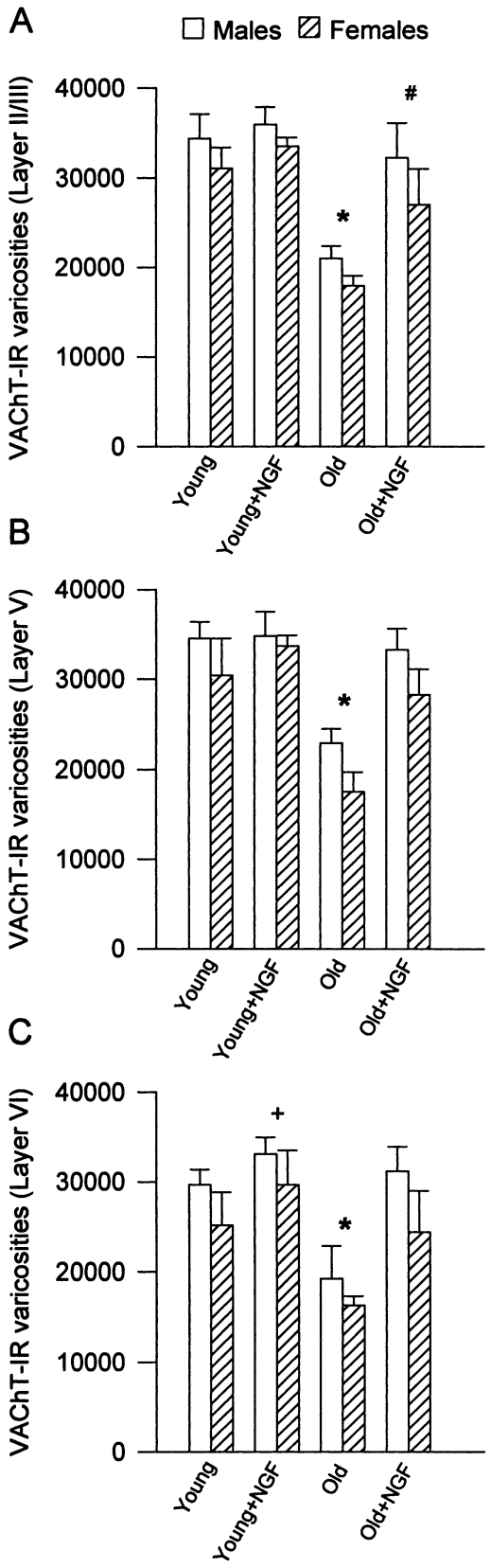

Fig. 7. Graphic representation of the areal density of VAChT-IR varicosities (Number/mm^2) in layers II/III (A), V (B) and VI (C) of the somatosensory barrel-field cortex of young (Young), young NGF-infused (Young+NGF), old (Old), and old NGF-infused (Old+NGF) rats. Note that old animals of both sexes had fewer cholinergic fiber terminals in all cortical laminae and that NGF treatment markedly reversed this decline. As additionally indicated in this figure, the density of the cholinergic varicosities remained slightly inferior to the control condition in NGF-treated old rats (significant for layers II/III), whereas it was superior to the control in NGF-treated young rats (significant for layer VI). * $P<0.001$ compared with young and NGF-treated old rats of both sexes; $^{\#}$ $P<0.05$ versus young rats; $^{+}$ $P<0.05$ versus young sham-operated control rats.

NGF treatment triggered a complete recovery of the synthesis of this neuropeptide in cortical interneurons.

Given that the cortical area where the measurements were performed is composed of both barrel and non-barrel regions, it was examined whether the densities of NPY cells could vary between the two regions. It was found that the coefficients of variation of the areal density of NPY-IR cells calculated for the series of 10 sections obtained at different rostrocaudal levels from the same animal usually ranged between 1 and 3% and never exceeded 5%, suggesting that the density of NPY neurons was not critically dependent on a relative percentage of barrel/non-barrel regions present. Furthermore, for some young animals of both sexes, the areal densities of NPY-IR cells were measured both in the barrel field cortex and in medially adjacent, non-barrel areas of the primary somatosensory cortex, and no differences between the two estimates were detected. Together these data suggest that the distribution pattern of NPY-IR cells does not vary considerably, at least in terms of the areal density, between barrel and non-barrel areas of the rat somatosensory cortex.

Areal density of VAChT-IR fiber varicosities

Quantitative estimates of the areal density of fiber varicosities obtained from VAChT-stained brain sections are shown in Fig. 7. ANOVA applied on these data showed that there were significant effects of sex, age and treatment on the density of VAChT-positive fiber varicosities in layers II/III, V and VI of the rat somatosensory cortex (Table 2). Although, unlike for NPY-IR cells, ANOVA failed to detect significant interactions between the effects of sex and age as well as between the effects of sex and treatment, there was a significant interaction between the effects of age and treatment (Table 2). These findings, thus, show that, contrary to what was found for NPY-containing neurons, the density of cholinergic varicosities, across all layers of the rat barrel cortex, was significantly superior in males than in females and that this difference was not dependent on the age of the animals (Fig. 7). Further inspection of the results of statistical analysis indicates that aging caused a significant depletion of VAChT-IR varicosities in the cortex ($P<0.001$ for either lamina), which was proportionally equal in both sexes. Post hoc comparisons also revealed that the areal densities of VAChT varicosities were significantly higher in the cortex of NGF-infused aged rats than in the cortex of their age-matched sham-operated controls ($P<0.001$ for either lamina), suggesting that the treatment was associated with significant recovery of cor-

Table 2. Summary of results of ANOVA on the areal densities of VAChT-IR fiber varicosities in different layers of the somatosensory barrel-field cortex in young, young NGF-treated, old, and old NGF-treated rats of both sexes

Effect/interaction	Layers II/III		Layer V		Layer VI	
	$F(1,32)$	P	$F(1,32)$	P	$F(1,32)$	P
Sex	19.15	<0.001	24.48	<0.001	20.31	<0.001
Age	131.7	<0.001	98.67	<0.001	45.68	<0.001
Treatment	57.46	<0.001	60.83	<0.001	51.28	<0.001
Sex×age	0.630	N.S.	2.585	N.S.	0.226	N.S.
Sex×treatment	0.157	N.S.	1.129	N.S.	0.490	N.S.
Age×treatment	25.95	<0.001	30.81	<0.001	9.603	<0.005

tical cholinergic functions. However, again unlike for the population of NPY neurons, the recovery detected in cortical cholinergic fibers appeared to be incomplete as indicated by the fact that the areal densities of VAChT varicosities in the cortex of aged NGF-treated rats still remained inferior to those found in young animals ($P<0.05$ for layers II/III; the difference did not reach the level of significance for layers V and VI; Fig. 7). Finally, analysis of the data shows that the density of cortical fiber varicosities IR for VAChT was higher in young NGF-infused rats than in young sham-operated rats ($P<0.05$ for lamina VI, non-significant for laminae II/III and V), suggesting that, even in normal conditions, exogenous NGF is capable of inducing significant hypertrophic changes in the basalocortical cholinergic projection system.

Similarly to the data from NPY-stained material, the coefficients of variation of the densities of VAChT-positive fiber varicosities were small and did not exceed 5%-value for the series of 10 sections obtained at different rostrocaudal levels from the same animal. Likewise, the densities of VAChT-stained terminals in medially adjacent, non-barrel areas of the somatosensory cortex, which were estimated in some young rats of both sexes for comparative purpose, did not differ from those found in the barrel field region. These observations suggest that the areal density of VAChT varicosities does not vary considerably between barrel and non-barrel areas of the rat somatosensory cortex.

Relationship between areal densities of VAChT-IR varicosities and of NPY neurons

Correlational analyses were conducted to determine whether individual variations in the density of NPY-synthesizing neurons in the rat somatosensory cortex are related to variations in the density of the cholinergic fiber varicosities. The analyses were conducted separately for each cortical layer and for each gender. Data obtained from young animals subjected to treatment with NGF were not included in the analysis. The results of these analyses, shown in Table 3, revealed significant correlations between the two morphological measures in both males and females, thus supporting the view that the synthesis of NPY in cortical interneurons is dependent on the cholinergic innervation.

DISCUSSION

It was found that the density of NPY-containing neurons in the somatosensory barrel-field cortex was dramatically decreased in old rats when compared with young rats. Further, it was observed that, commensurate with this loss of NPY neurons, the areal density of the fiber varicosities IR to VAChT protein, as assessed in the same cortical region, was also reduced in aged animals. However, the cortex of NGF-treated aged animals was found to have a normal density of cholinergic fibers and NPY-IR cells. Regarding the gender differences, the present results show that, although the areal density of cortical NPY neurons was significantly higher in young females than in young males and the opposite was true for VAChT-positive varicosities, the general pattern of age- and treatment-related changes in these neurochemical markers was similar in both sexes. Finally, the age- and treatment-related variations in the density of cortical NPY-IR cells were found to correlate, in animals of both sexes, with those in the density of VAChT-containing varicosities.

There have been few studies that have examined the effect of aging on cortical NPY-IR neurons, all of which reported a noticeable age-related decrease in the number of these cells in various regions of the neocortex, including the frontal cortex, frontoparietal and occipital cortices, as well as in the hippocampal formation (Cha et al., 1996, 1997; Huh et al., 1997; Zhang et al., 1998; Cadiacio et al.,

Table 3. Summary of results of correlational analyses between areal densities of VAChT-IR varicosities and of NPY neurons in different layers of the somatosensory barrel-field cortex of young, old, and old NGF-treated rats

Sex	Layers II/III		Layer V		Layer VI	
	r	P	r	P	r	P
Males ($n=15$)	0.89	<0.001	0.90	<0.001	0.83	<0.001
Females ($n=15$)	0.91	<0.001	0.90	<0.001	0.81	<0.001

The correlation coefficients (r) and the levels of statistical significance (P) were computed separately for male and female groups.

2003). The present findings fully support earlier research by showing that, in a relatively small and precisely delineated area of the rat somatosensory barrel cortex, the density of NPY neurons is strikingly reduced in aged subjects. The current results are also consistent with the data reported by other authors on the deleterious effect of aging on the basalocortical cholinergic system (Altavista et al., 1990; Fischer et al., 1992; Smith and Booze, 1995; Wellman and Sengelaub, 1995; Zhang et al., 1998), because we found that the density of VAChT-stained varicosities in all layers of the somatosensory cortex was reduced, by approximately 30–40%, in old rats when compared with young rats. Notably, Zhang et al. (1998) have reported that the loss of cortical NPY-IR neurons in aging is accompanied by a proportional decrease in the intensity of histochemical staining for acetylcholinesterase. It can be argued that the intensity of staining for acetylcholinesterase does not necessarily reflect the level of cholinergic activity because this enzyme is not directly involved in acetylcholine synthesis and/or release. In this study, we used as a marker VAChT protein, known to be present in the synaptic vesicles of cholinergic terminal fields, and detected a similar age-related reduction in the density of stained material.

Several lines of evidence suggest that acetylcholine exerts marked trophic effects in the target areas of the basal forebrain cholinergic projections, including the neocortex (Bear and Singer, 1986; Wettstein et al., 1995; Zhu and Waite, 1998). With respect to the synthesis of NPY, it has been shown that cortical neurons IR to this neuropeptide are both directly and indirectly innervated by cholinergic terminals (Davies et al., 1980; Finley et al., 1981; Lamour and Epelbaum, 1988; Wettstein et al., 1995) and that activation of cholinergic muscarinic receptors increases NPY gene expression and synthesis via protein kinase C transduction pathway (Magni et al., 1998). Furthermore, it has also been demonstrated that a selective cholinergic immunolesion of NBM leads to a striking decrease in the number of cortical NPY-containing neurons. In line with these observations are the results of the present study showing that intraventricular infusions of NGF, known to exert a powerful trophic effect upon cortically projecting cholinergic neurons in the basal forebrain (Hefti et al., 1989; Cuello et al., 1992; Heisenberg et al., 1994; Linke et al., 1995; Niewiadomska et al., 2002), have led to considerable recovery of VAChT-positive terminal fields in all layers of the cortex, which was paralleled by complete restoration of function in NPY-producing neurons. Taken together, these findings thus suggest that the loss of NPY neurons in the cortex of aged rats may be a consequence of the lack of trophic support otherwise provided by the cholinergic projections from NBM.

To our knowledge, the present study is the first that looked at gender differences in cholinergic activity and density of NPY-producing neurons in the rat cortex, and the results obtained were both surprising and expected. One surprising finding was that the density of VAChT-labeled fiber varicosities in the cortex was significantly higher, by approximately 15%, in males than in females. In a prior study, it was demonstrated that the expression of ChAT (choline acetyltransferase) mRNA and the density of ChAT-IR cells in the basal forebrain did not significantly differ between the sexes (Gibbs, 1998). Moreover, the activity of ChAT in the frontoparietal cortex was also found to be similar in males and females (Arters et al., 1998). The most likely reason for the discrepancy between our findings and those reported by others is that we compared the density of cholinergic fiber varicosities that were measured separately in each cortical layer, whereas in the previous studies the measurements were performed either in a homogenized cortex (Arters et al., 1998) or in the basal forebrain (Gibbs, 1998). The use of different markers of cholinergic activity, VAChT in our experiment versus ChAT in the above cited studies, might also have contributed to the difference in the results. Another unexpected finding of the present experiment was that females had a higher density of NPY-IR neurons, in either cortical layer, than did males. Bearing in mind that, in the same cortical area, VAChT staining in females was weaker than in males, this finding suggests that the synthesis of NPY in cortical neurons is not exclusively dependent on the trophic influence of cholinergic afferents, but can also be determined by other factors, such as the organizing effects of sex steroid hormones (Madeira and Lieberman, 1995). NPYergic neurons were recently shown to be capable of effectively decreasing the excitability of cortical circuits both by reducing excitatory postsynaptic current amplitude and by disinhibiting local circuit GABAergic interneurons (Bacci et al., 2002). In light of this, one intriguing interpretation of our results is that the cortical neuronal circuits in females are under a more powerful inhibitory control than those in males. Thus, if these results can be extrapolated to humans, they may help explain the lower incidence of certain neurological diseases, such as epilepsy and schizophrenia, in women compared with men. The expected result of this part of the experiment was that, in animals of both sexes, aging produced a profound decrease in the density of cortical NPY-containing neurons and of VAChT-positives varicosities, and that the treatment with NGF considerably, irrespective of gender, restored the neurochemical composition of this region of the cortex. Interestingly, we found that the densities of NPY neurons and of VAChT-positives varicosities, despite being different in absolute values in males and females, were highly intercorrelated in young, old and NGF-treated animals of both sexes. The latter result lends additional support to the idea that there is a causal relationship between age-related changes in cortical cholinergic and NPYergic neurotransmitter systems.

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DISCUSSÃO GERAL

A possibilidade das convulsões epilépticas poderem, por si só, induzir morte neuronal e alterações comportamentais é uma questão ainda controversa. De facto, existe ainda um amplo debate em torno da possibilidade das convulsões de curta duração serem ou não capazes de provocar lesões cerebrais. Numa série de experiências, cujos resultados se encontram descritos nas publicações incluídas na presente dissertação, tentou-se clarificar esta questão. Assim, demonstrou-se que, no Rato, as convulsões generalizadas de curta duração desencadeadas por ECS induzem morte de populações neuronais específicas, produzem modificações em redes neuronais potencialmente relevantes para o processo epileptogénico, alteram a expressão de neuropeptídeos e estão associadas a alterações cognitivas e afectivas.

Diversos estudos abordaram este tema procurando determinar as possíveis consequências neuroanatómicas e funcionais das convulsões de curta duração. Refira-se, a título de exemplo, os trabalhos de Cavazos e colaboradores (1990, 1994) que mostraram ser três convulsões de grau V, na escala de Racine (1972), induzidas por *kindling*, suficientes para reduzir a densidade neuronal no hilo da fáscia denteada. Contudo, estudos ulteriores (Adams et al., 1997; Tuunanen e Pitkänen, 2000) revelaram que as convulsões induzidas por *kindling* não provocam morte neuronal, e que a diminuição da densidade neuronal resulta apenas do aumento do volume do hilo da fáscia denteada resultante da expansão do seu neurópilo. Por sua vez, estudos que utilizaram o modelo de ECS demonstraram que a indução de cinco a dez convulsões a intervalos de 24 ou 48 horas também não provoca degenerescência neuronal significativa (Devanand et al., 1994; Gombos et al., 1999; Vaidya et al., 1999). Todavia, a possibilidade das convulsões de curta duração provocarem morte neuronal não pode ser afastada. Pelo contrário, é até apoiada pelo facto das convulsões desencadeadas por *kindling* e ECS promoverem *sprouting* das fibras musgosas (Gombos et al., 1999; Sutula et al., 1988; Vaidya et al., 1999) e activação e hipertrofia das células gliais da fáscia denteada (Jansson et al., 2009; Khurgel e Ivy, 1996; Wennström et al., 2003), fenómenos normalmente associados a processos neurodegenerativos. Por isso, é possível que a morte neuronal, nos trabalhos acima referidos, não tenha sido detectada devido à utilização de técnicas de quantificação menos precisas, isto é, não suficientemente sensíveis para detectar alterações moderadas. A este respeito, é também importante referir que nos modelos de *kindling* e de ECS as convulsões são tipicamente

administradas a intervalos de tempo relativamente grandes. Nestas condições experimentais, a activação do sistema endógeno de recaptação de aminoácidos induzida pela actividade convulsiva pode levar ao restabelecimento rápido dos níveis extracelulares de neurotransmissores excitatórios, impedindo que os danos neuronais se tornem evidentes e capazes de serem detectados por métodos de quantificação pouco precisos.

Uma parte importante da presente dissertação consistiu no desenvolvimento e caracterização de um modelo experimental que fosse capaz de avaliar os efeitos específicos das convulsões epilépticas de curta duração no cérebro do Rato. A pesquisa bibliográfica revelou a existência de um trabalho onde se demonstrou que os ECS repetidos provocam redução transitória, com duração de aproximadamente 2 horas, da capacidade de recaptação de aminoácidos no hipocampo (Rowley et al., 1997). Com base nesta observação, desenvolveu-se um modelo de indução de convulsões repetidas desencadeadas pela exposição a seis ECS, sendo os cinco primeiros administrados com 24 horas de intervalo e o último apenas 2 horas depois, isto é, quando os mecanismos responsáveis pela recaptação de aminoácidos se encontram comprometidos (Rowley et al., 1997). Tendo em conta que uma determinada corrente eléctrica pode provocar danos inespecíficos nas populações neuronais mais vulneráveis, decidiu-se comparar dois modelos de ECS que, embora administrados segundo protocolo idêntico, diferem quanto à intensidade e à voltagem. Foi assim possível verificar que as consequências neuroanatómicas e comportamentais das convulsões desencadeadas por ECS não dependem das características da corrente eléctrica. Por outro lado, os estudos comportamentais foram repetidos utilizando, para isso, diferentes coortes de ratos, tendo os resultados obtidos sido muito semelhantes. Por fim, para realizar os estudos morfométricos de forma a assegurar a precisão dos resultados optou-se pela utilização de métodos estereológicos *unbiased*, como o *fractionator* óptico e o princípio de Cavalieri (Gundersen et al., 1988; Regeur e Pakkenberg, 1989; West et al., 1991). Em suma, a utilização de dois modelos distintos de estimulação por electrochoques, a replicação dos estudos comportamentais e a aplicação de técnicas estereológicas *unbiased* permitiram assegurar que os resultados obtidos fossem indicadores fiáveis das alterações morfofuncionais induzidas no cérebro pelas convulsões de curta duração.

A análise estrutural realizada nos animais sujeitos a convulsões de curta duração ajudou a identificar as populações neuronais mais vulneráveis à actividade convulsiva e a compreender melhor os circuitos neuronais envolvidos na sua propagação. Mais concretamente, e em consonância com trabalhos realizados em modelos de *status epilepticus* (Buckmaster e Dudek, 1997; Gorter et al., 2003; Mello et al., 1993; Sloviter, 1987; Sloviter et al., 2003), verificou-se que as convulsões de curta duração desencadeadas por ECS estão associadas a morte neuronal no hilo da fáscia denteada, uma das estruturas cerebrais mais vulneráveis à actividade convulsiva (Margerison e Corsellis, 1966; Sloviter, 1987). A morte neuronal é selectiva para as células musgosas dado não se ter detectado morte de interneurónios GABAérgicos produtores de NPY ou somatostatina. Verificou-se, ainda, que as convulsões de curta duração provocam alterações degenerativas não só no hilo da fáscia denteada mas também no complexo subicular, no córtex entorrinal e no córtex retrosplénico e que estas alterações morfológicas estão associadas a défices comportamentais.

Em relação ao córtex entorrinal, diversos estudos em modelos experimentais de *status epilepticus* demonstraram que as convulsões prolongadas provocam morte neuronal nas suas camadas II e III (Du et al., 1995; Gorter et al., 2003; Kobayashi et al., 2003; Schwarcz et al., 2000). Curiosamente, com o recurso ao presente modelo de ECS foi possível demonstrar que os neurónios da camada III são mais vulneráveis às convulsões epilépticas que os da camada II. É importante salientar que as convulsões epilépticas provocam também alterações estruturais no córtex retrosplénico, uma região cerebral em relação à qual não havia indícios claros de estar implicada na epilepsia. Porém, o seu envolvimento na epilepsia não representa grande surpresa, uma vez que estabelece conexões directas e recíprocas com o complexo subicular e com o córtex entorrinal (Finch et al., 1984; van Groen e Wyss, 1990a, 2003; Wyss e van Groen, 1992). Convém, contudo, realçar que as convulsões induzidas por ECS provocaram também morte neuronal na camada V do córtex retrosplénico granular *b*, o que indica claramente que estes neurónios são, tal como os do hilo da fáscia denteada e os da camada III do córtex entorrinal, extremamente vulneráveis às convulsões epilépticas, mesmo de curta duração. Este achado está em concordância com o observado em estudos de ressonância magnética realizados em pacientes com ELT (Düzel et al., 2006). Para além das referidas conexões com o complexo subicular e o córtex entorrinal, o

córtex retrosplénico granular *b* estabelece também conexões particularmente fortes com o tálamo (Shibata, 1998; Sripanidkulchai e Wyss, 1986; van Groen e Wyss, 1992). Portanto, o facto das convulsões epilépticas, mesmo de curta duração, provocarem alterações neste córtex reforça a hipótese, ainda emergente, que sugere ser o tálamo uma estrutura central capaz de organizar e propagar as convulsões epilépticas devido ao estabelecimento de conexões com múltiplas regiões corticais implicadas na epilepsia (Bertram et al., 2001).

As lesões neuronais observadas nos animais sujeitos a convulsões de curta duração são genericamente do mesmo tipo que as observadas nos animais submetidos a *status epilepticus*, o que aponta para a existência de um circuito neuronal, relacionado com as convulsões epilépticas, comum a estes dois modelos. Refira-se, no entanto, que o *status epilepticus* provocou lesões consideravelmente mais graves e induziu alterações em estruturas que não foram afectadas pelo tratamento com ECS, nomeadamente os interneurónios GABAérgicos do hilo da fáscia denteada e os neurónios da camada II do córtex entorrinal, da camada IV do córtex retrosplénico granular *b* e, sobretudo, das áreas CA3 e CA1 do hipocampo. A comparação destes resultados sugere que as convulsões epilépticas quando prolongadas e espontâneas provocam morte neuronal em populações que são resistentes às convulsões de curta duração, como é o caso das células piramidais do hipocampo. Em alternativa, é possível que as convulsões de curta duração se propaguem através de um circuito neuronal relativamente restrito que inclui os córtices retrosplénico e entorrinal e o complexo subicular, enquanto as convulsões prolongadas activam também circuitos hipocampais. O facto do tratamento com ECS e do *status epilepticus* provocarem morte neuronal nas mesmas regiões cerebrais, excepto nas áreas CA3 e CA1 do hipocampo, pode ser importante para a compreensão dos mecanismos da epileptogénese. Contrariamente ao *status epilepticus*, as convulsões de curta duração, apesar de provocarem morte das células musgosas do hilo e de neurónios dos córtices retrosplénico e entorrinal bem como do complexo subicular, não conduzem ao desenvolvimento de convulsões espontâneas, o que sugere que a lesão dos neurónios CA3 e CA1 é crítica para o processo epileptogénico.

Os resultados dos estudos incluídos na presente dissertação revelam que existe uma base anatómica para as alterações comportamentais induzidas pelas convulsões de

curta duração. Na verdade, estudos prévios utilizando modelos de *kindling* e de ECS haviam já demonstrado que as convulsões de curta duração induzem alterações comportamentais irreversíveis (File e Green, 1984; Hannesson et al., 2001; Kotloski et al., 2002; Lopes da Silva et al., 1986; Nagaraja et al., 2007; Ripley et al., 2003). Paradoxalmente, os estudos morfológicos sugeriam que os mesmos tratamentos não provocavam alterações degenerativas significativas no cérebro, à excepção de *sprouting* das fibras musgosas das células granulares da fáscia denteada (Gombos et al., 1999; Vaidya et al., 1999), deixando assim em aberto uma explicação para os défices comportamentais. No entanto, utilizando o presente modelo de ECS foi possível demonstrar que apenas algumas convulsões de curta duração são suficientes para provocar morte neuronal numa série de estruturas cerebrais, nomeadamente no hilo da fáscia denteada, no complexo subicular, nas camadas III e V/VI do córtex entorrinal e na camada V do córtex retrosplénico granular *b*. A hipótese de que estas alterações estruturais possam ser responsáveis pelas alterações comportamentais observadas é suportada pelo facto das regiões afectadas estarem envolvidas em funções cognitivas e afectivas. Esta possibilidade é também apoiada, embora de forma indirecta, pelos resultados obtidos no modelo de *status epilepticus*, que mostraram estar as alterações degenerativas daquelas regiões associadas a perturbações da memória espacial e dos comportamentos emocionais. É de salientar, no entanto, que as alterações comportamentais resultantes do *status epilepticus* são qualitativa e quantitativamente distintas das observadas após tratamento com ECS. Com efeito, as convulsões de curta duração produzem apenas moderada degenerescência neuronal, enquanto a actividade convulsiva prolongada está associada à perda marcada de neurónios em várias regiões límbicas criticamente envolvidas na regulação da memória e das respostas emocionais, como é o caso do hipocampo, do córtex piriforme e dos núcleos central e basolateral da amígdala (não publicado).

Apesar de se saber que a actividade convulsiva esporádica produz alterações neuronais que podem, ulteriormente, progredir para epilepsia (Cendes et al., 1995; Mathern et al., 1995b; VanLandingham et al., 1998), os mecanismos precisos envolvidos no processo epileptogénico não estão ainda completamente percebidos. Dado que os doentes com epilepsia não demonstram convulsões espontâneas durante a fase latente

após a lesão inicial (French et al., 1993; Mathern et al., 1995b; Spencer e Spencer, 1994), tem sido sugerido que a degenerescência neuronal, por si só, não é suficiente para induzir convulsões e que outras alterações cerebrais, como por exemplo o desenvolvimento de novos circuitos neuronais através de *sprouting* axonal e da formação de novas sinapses (Herman, 2002; Sutula et al., 1989; Tauck e Nadler, 1985; Wuarin e Dudek, 2001) têm importância fundamental no desenvolvimento do processo epileptogénico. Todavia, existem dados, principalmente provenientes de estudos experimentais, que mostram que as alterações nos circuitos neuronais induzidas pelas convulsões epilépticas podem ser imediatamente epileptogénicas, sugerindo que os processos de reorganização sináptica a longo prazo podem não ser indispensáveis (Norwood et al., 2010; Sloviter, 2008). A este respeito, a comparação directa das alterações cerebrais induzidas pelas convulsões prolongadas, que resultam em epileptogénese, com as alterações não epileptogénicas induzidas pelas convulsões de curta duração pode ajudar a identificar os mecanismos neuronais responsáveis pela manifestação de convulsões espontâneas.

Nas experiências incluídas nesta dissertação verificou-se que apesar das convulsões de curta duração desencadeadas por ECS provocarem perda de aproximadamente 20% dos neurónios do hilo da fáscia denteada, do complexo subicular, das camadas III e V/VI do córtex entorrinal e da camada V do córtex retrosplénico granular *b*, tais alterações não contribuíram para a evolução do processo epileptogénico, uma vez que não se observaram convulsões espontâneas em nenhum dos animais sujeitos a ECS, pelo menos até seis meses após o término do tratamento. Outros autores demonstraram que a ablação selectiva das células musgosas do hilo não provoca hiperexcitabilidade das células granulares da fáscia denteada (Ratzliff et al., 2004). Quer isto dizer que a simples perda neuronal em algumas das regiões implicadas na epileptogénese acaba por, aparentemente, não produzir um foco epileptogénico. Pelo contrário, as convulsões prolongadas (*status epilepticus*) provocam lesões mais extensas no hilo da fáscia denteada, envolvendo não só as células musgosas mas também os interneurónios GABAérgicos, e induzem a formação de rearranjos sinápticos em várias regiões hipocampais e extra-hipocampais, seguido da manifestação progressiva de convulsões espontâneas. Outra diferença marcante entre estes dois modelos é que, contrariamente ao *status epilepticus*, as convulsões de curta duração

não provocam morte neuronal nas áreas CA3 e CA1 do hipocampo. A ausência de morte neuronal nestas áreas no modelo de ECS pode estar relacionado com o facto das convulsões de curta duração provocarem redução do número de sinapses excitatórias na camada molecular da fáscia denteada. Este resultado parece indicar que o tono excitatório das células piramidais do hipocampo, proveniente das células granulares da fáscia denteada, pode estar também reduzido. Curiosamente, um estudo recente demonstrou haver redução do número de sinapses excitatórias na camada molecular após tratamento com pilocarpina seguida de recuperação para o nível dos controlos, provavelmente devido ao *sprouting* das células sobreviventes da camada II do córtex entorrinal (Thind et al., 2010). Mais ainda, estas sinapses eram maiores que as dos controlos (Thind et al., 2010). Refira-se, no entanto, que apesar de se ter também verificado *sprouting* das fibras musgosas nos animais tratados com ECS, não se observou recuperação do número de sinapses, nem aumento do seu tamanho, apesar da análise ter sido realizada dois meses após tratamento. Por conseguinte, é possível que a desconexão parcial da fáscia denteada da projecção entorrinal, observada após convulsões provocadas por ECS, possa proteger os circuitos hipocampais dos fenómenos propiciadores do processo epileptogénico, como a neurodegenerescência e consequente reorganização dos circuitos locais.

A vulnerabilidade dos neurónios aos efeitos nocivos da actividade convulsiva parece ser dependente dos níveis de produção local de neuropeptídeos com propriedades neurotróficas e de neuroprotecção (Baraban, 2004; Sperk et al., 2007). A título de exemplo, refira-se que ratinhos *knockout* do gene NPY são mais susceptíveis às convulsões epilépticas (Baraban et al., 1997) e que a elevada expressão deste neuropeptídeo em ratos transgénicos (Vezzani et al., 2002a) e em ratos sujeitos a injecção, no hipocampo, de vector viral adeno-associado recombinante contendo o gene humano NPY recombinante (Richichi et al., 2004) está associada a efeitos anticonvulsivantes e antiepileptogénicos. Os resultados incluídos na presente dissertação apoiam o papel importante do NPY no processo epileptogénico, dado ter-se verificado que as convulsões de curta duração são capazes de induzir alterações duradouras, porventura permanentes, na expressão deste neuropeptídeo. Curiosamente, tais alterações ocorreram nas áreas cerebrais mais susceptíveis às convulsões epilépticas e onde as convulsões de curta duração provocaram moderada

morte neuronal, isto é, no hilo da fáscia denteada e na camada V do córtex retrosplénico granular *b*. É, pois, possível que o aumento de expressão do NPY nestas estruturas possa representar um mecanismo compensatório em resposta à actividade anormal dos circuitos neuronais em que ocorre degenerescência. Se tal hipótese for correcta, o aumento da actividade dos neurónios produtores de NPY pode justificar que as convulsões de curta duração não induzam actividade convulsiva espontânea, apesar de provocarem morte neuronal em regiões cerebrais epileptogénicas. Esta possibilidade é reforçada pelo facto das convulsões prolongadas, induzidas por *status epilepticus*, provocarem morte marcada dos neurónios produtores de NPY, reduzindo, assim, a potencial influência deste neuropeptídeo na prevenção da génese de focos epileptogénicos.

A redução da actividade dos neurónios produtores de NPY pode também ajudar a explicar a maior susceptibilidade às convulsões epilépticas observada nas pessoas idosas. Em consonância com essa possibilidade, verificou-se que a densidade dos neurónios NPYérgicos é significativamente inferior nos ratos velhos. Tendo em conta que o sistema NPYérgico diminui a excitabilidade dos circuitos corticais (Bacci et al., 2002; Baraban, 2004; Qian et al., 1997; Silva et al., 2001; Vezzani et al., 1999), é possível que estes circuitos estejam sobre menor controlo inibitório nas pessoas idosas, o que pode ajudar a explicar a grande incidência da epilepsia nessa população (Everitt e Sander, 1998; Ramsay et al., 2004; Sander, 2003). Curiosamente, diversos estudos têm evidenciado que a deaferenciação colinérgica aumenta a susceptibilidade às convulsões epilépticas (Ferencz et al., 1997; Silveira et al., 2000), facto que pode estar também relacionado com a redução da actividade dos neurónios produtores de NPY nessas circunstâncias. De acordo com essa possibilidade, verificou-se que o sistema NPYérgico é profundamente dependente do sistema colinérgico. Mais concretamente, verificou-se haver correlação entre a densidade de neurónios contendo NPY e a densidade de varicosidades colinérgicas no córtex cerebral. Em consonância, diversos trabalhos levados a cabo no Instituto de Anatomia, uns já publicados e outros ainda em curso, demonstraram haver correlação entre a actividade colinérgica e a expressão do NPY e de outros neuropeptídeos em diversas áreas do sistema nervoso central (Madeira et al., 2004; Paula-Barbosa et al., 2004; Pereira et al., 2005). Sendo assim, a redução da plasticidade do sistema NPYérgico pode contribuir para o aumento da susceptibilidade

às convulsões epilépticas em situações de deaferenciação colinérgica ou, ainda, em situações em que a função colinérgica esteja reduzida, como por exemplo no envelhecimento, no alcoolismo ou no stresse crónico. Por conseguinte, é importante conhecer os mecanismos moleculares envolvidos na regulação dos sistemas NPYérgico e colinérgico dado que a activação destes mecanismos pode aumentar a resistência dos neurónios às lesões induzidas por convulsões e assim prevenir a epileptogénese. A este respeito, para além das manipulações a nível genético, salienta-se o potencial papel dos factores neurotróficos, como por exemplo o NGF ou o BDNF, cuja expressão é aumentada pela actividade convulsiva (Sato et al., 1996; Takahashi et al., 1999). No caso da epilepsia, estes efeitos podem também estar associados ao aumento de expressão do NPY, dado saber-se que o receptor específico do BDNF (TrkB) co-localiza com este neuropeptídeo em muitos neurónios GABAérgicos (Gorba e Wahle, 1999) e que a infusão crónica de BDNF provoca aumento da expressão de NPY (Reibel et al., 2000). Refira-se, por fim, que o NGF pode ter também papel importante na expressão do NPY, e consequentemente na epilepsia, uma vez que a expressão do NPY nos neurónios corticais é modulada pela acção trófica desta neurotrofina sobre os neurónios colinérgicos do prosencéfalo basal.

CONCLUSÕES

Em suma, a análise dos resultados obtidos nos trabalhos que integram esta dissertação permite concluir que as convulsões de curta duração desencadeadas pelo modelo ECS, aqui pela primeira vez descrito, provocam morte neuronal e consequente reorganização dos circuitos neuronais em diversas estruturas cerebrais susceptíveis à actividade convulsiva, alteram a expressão de neuropeptídeos, induzem alterações comportamentais cuja função é dependente da integridade estrutural dessas estruturas e provocam redução do limiar convulsivo. Os presentes resultados contribuem assim para o aumento do conhecimento dos mecanismos da epileptogénese e dão sólido suporte para a hipótese de que a actividade convulsiva pouco controlada provoca, invariavelmente, danos cerebrais.

De forma mais específica, os principais resultados obtidos demonstram que:

As convulsões de curta duração desencadeadas por ECS provocam morte neuronal, aproximadamente de 20%, no hilo da fáscia denteada, no complexo subicular e nas camadas III e V/VI do córtex entorrinal. Adicionalmente, provocam também morte neuronal numa estrutura que se desconhecia estar implicada na epilepsia, o córtex retrosplénico granular *b* (camada V).

O *status epilepticus* provoca morte neuronal significativamente maior nas mesmas regiões lesadas pelo tratamento com ECS. Induz ainda morte neuronal noutras áreas do córtex límbico, nomeadamente nas áreas hipocampais CA3 e CA1, na camada II do córtex entorrinal e na camada IV do córtex retrosplénico granular *b*.

Os ECS induzem alterações significativas a nível dos neuritos, nomeadamente *sprouting* das fibras musgosas e redução do número de sinapses excitatórias na camada molecular da fáscia denteada. Todavia, o *sprouting* das fibras musgosas induzido pelo *status epilepticus* é consideravelmente superior ao observado após convulsões ECS e resulta principalmente no aumento da actividade sináptica na camada molecular da fáscia denteada. Para mais, o *status epilepticus* provoca morte neuronal nas células musgosas e nos interneurónios GABAérgicos do hilo, enquanto as convulsões desencadeadas por ECS provocam morte neuronal apenas das células musgosas.

Os ECS provocam alterações moderadas, embora significativas, nos testes da memória espacial, do *passive* e *active avoidance* e dos comportamentos afectivos, nomeadamente da ansiedade e do medo condicionado, enquanto o *status epilepticus* induz modificações comportamentais mais graves que parecem estar relacionadas com lesões estruturais mais extensas e profundas.

As convulsões desencadeadas por ECS apesar de provocarem alterações neuronais potencialmente epileptogénicas, como a morte neuronal, o *sprouting* das fibras musgosas e a diminuição do limiar convulsivo, não induzem a formação de focos epileptogénicos nem convulsões espontâneas. Pelo contrário, todos os animais sujeitos a *status epilepticus* desenvolveram consistentemente fortes e recorrentes convulsões espontâneas.

O aumento de expressão do NPY pode ter papel importante na prevenção do desenvolvimento do processo epileptogénico, uma vez que as convulsões de curta duração, não sendo epileptogénicas, estimulam a expressão deste neuropeptídeo especificamente nas regiões cerebrais onde a actividade convulsiva provoca neurodegenerescência (hilo e córtex retrosplénico). A somatostatina parece não ter papel tão relevante neste processo, dado que não se observou aumento da sua expressão no tratamento com ECS.

O envelhecimento provoca redução do número de neurónios produtores de NPY no córtex cerebral o que pode estar implicado na elevada susceptibilidade às convulsões epilépticas observada nessa situação. O sistema NPYérgico está na dependência do sistema colinérgico, uma vez que se verificou haver correlação entre a densidade de neurónios produtores de NPY e a densidade de varicosidades colinérgicas no córtex cerebral.

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RESUMO

São actualmente já relativamente numerosos os trabalhos que mostram serem as convulsões prolongadas causa de morte neuronal progressiva nas regiões hipocampal e parahipocampal acompanhada de reorganização dos circuitos neuronais locais e de alterações comportamentais. É, no entanto, ainda pouco claro se estas alterações podem também ser causadas por convulsões de curta duração. Por esse motivo, com o conjunto de trabalhos incluídos na presente dissertação pretendeu-se caracterizar as repercussões neuroanatómicas e funcionais das convulsões de curta duração induzidas por choques electroconvulsivos (ECS) e compará-las com as provocadas por *status epilepticus* (SE) induzido pela administração de pilocarpina.

Verificou-se que a administração de uma série de seis ECS conduzia à perda de cerca de 20% dos neurónios da fáscia denteada (células musgosas do hilo), complexo subicular, córtex entorrinal (camadas III e V/VI) e subdivisão granular *b* do córtex retrosplénico (camada V), e que a morte neuronal provocada nestas mesmas regiões por SE era cerca de duas vezes superior. Por outro lado, e ao contrário dos ECS, o SE também causava morte neuronal na camada granular da fáscia denteada, nas regiões CA3 e CA1 do hipocampo, na camada II do córtex entorrinal e na camada IV do córtex retrosplénico. Os estudos comportamentais realizados permitiram demonstrar que os ECS provocam alterações moderadas em comportamentos dependentes do hipocampo, tais como a memória espacial e *passive avoidance*, e exercem efeito ansiogénico. Pelo contrário, o SE altera marcadamente os comportamentos dependentes do hipocampo e a memória emocional, e possui efeito ansiolítico.

Estudaram-se ainda os efeitos dos dois tipos de convulsões na reorganização dos circuitos hipocampais potencialmente subjacentes à epileptogénese. Verificou-se que embora os dois modelos reduzissem o limiar convulsivo, apenas os ratos submetidos a SE desenvolviam convulsões recorrentes espontâneas. É possível que esta diferença se fique a dever à diversidade das alterações morfológicas induzidas por estes dois modelos experimentais de convulsões. De facto, observou-se que enquanto o SE induzia marcado crescimento axonal na camada molecular da fáscia denteada, os ECS reduziam o número de sinapses excitatórias nesta mesma área. Em sintonia com estas alterações, os ECS aumentavam o número de neurónios produtores de neuropeptídeo NPY no hilo e córtex retrosplénico, enquanto o SE reduzia para menos de metade o número destes neurónios, o que pode facilitar a reorganização neuronal.

Estes resultados indicam que as convulsões de curta duração podem causar alterações estruturais e funcionais de relevo nos circuitos que ligam os córtices hipocampal, entorrinal e retrosplénico, e que a degenerescência neuronal causada por convulsões, sobretudo se moderada, não conduz necessariamente a epileptogénese.

ABSTRACT

An increasing number of reports has indicated that prolonged seizures can cause progressive neuronal loss in hippocampal and parahippocampal regions accompanied by reorganization of local neuronal circuits and significant behavioral impairments. However, it is less well known whether and to what extent these neuronal changes may be induced by brief seizures. The aim of this thesis was to investigate the neuroanatomical and functional sequelae of repeated brief seizures induced by electroconvulsive shock (ECS) in rats and to directly compare them to those observed after a prolonged *status epilepticus* (SE) induced by pilocarpine.

Using unbiased stereological methods, it was first found that the administration of a course of six ECS seizures produced loss of approximately 20% of neurons in closely interconnected cortical regions including the dentate gyrus (hilar mossy cells), subicular complex, entorhinal cortex (layers III and V/VI) and retrosplenial granular *b* cortex (layer V). However, neuronal loss produced in the same brain regions by SE was at least twofold greater. In addition, SE killed many neurons in the dentate granular layer, CA3 and CA1 hippocampal fields, entorhinal layer II and retrosplenial layer IV, areas in which ECS seizures showed no effects.

Using a battery of behavioral tests, it was then showed that ECS treatment resulted in a moderate impairment of hippocampal-dependent behaviors, i.e. spatial learning and passive avoidance, and, in addition, exerted an anxiogenic-like effect. In contrast, SE resulted in a nearly complete disruption of hippocampal-dependent behaviors and emotional memory, and had a rather anxiolytic-like effect.

The remaining part of the thesis focused on the role played by seizures of both types in reorganization of hippocampal circuits that might underlie epileptogenesis. It was observed that, although both models were associated with reduced seizure thresholds, only rats that had experienced SE developed spontaneous recurrent seizures. This observation can be explained by the results of morphological analysis which showed that SE triggered a massive fiber sprouting in the dentate molecular layer, while ECS seizures resulted in a decrease of the number of excitatory synapses in this area. In line with this, ECS treatment produced an increase in the number of hilar and retrosplenial neurons expressing inhibitory neuropeptide NPY. For comparison, SE killed more than half of the NPY-expressing cells, an effect which may facilitate neuronal reorganization.

These results lend strong support to the hypothesis that brief seizures can cause noticeable structural and functional changes in the hippocampo-entorhino-retrosplenial circuits. They also show that generalized seizure-induced neurodegeneration, when moderate at least, does not necessary lead to epileptogenesis.