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EXPRESSÃO DOS RECEPTORES DE ESTROGÊNIO NO HIPOCAMPO E NEOCÓRTEX DE RATOS MACHOS SUBMETIDOS AO MODELO DE EPILEPSIA DE LOBO TEMPORAL DA PILOCARPINA

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RESUMO

Os receptores de estrogênio (RE) são difusamente distribuídos no sistema nervoso central e estão envolvidos com neuroplasticidade. O papel dos RE nos processos epileptogênicos ainda são desconhecidos. Nós investigamos a expressão dos RE por imunoistoquímica no hipocampo e no neocórtex de ratos machos adultos (n= 3-4 por grupo) submetidos ao modelo de *status epitepticus* (SE) induzido pela pilocarpina. Em comparação ao respectivo grupo controle, a expressão do RE aumenta significativamente nos neurônios das subregiões do hipocampo CA1, CA2, CA3 e giro denteado apos 1h (fase aguda), 12h (período tóxico) e 5 dias (período silente) apos a indução do SE. Em todas estas regiões, exceto em CA3, a expressão do RE retorna aos níveis do controle 50 dias após o SE. Na fase crônica, houve uma tendência ($p = 0,07$) de um leve aumento na expressão do RE na região CA3 de animais submetidos ao SE em comparação ao grupo controle. No neocórtex parietal a expressão dos RE encontra-se aumentada 1h e retorna ao nível do controle em 12h, 5 e 50 dias apos a indução do SE. O aumento da expressão do RE é variável de acordo com o tempo e a região do cérebro analisada. Adicionalmente, os neurônios fortemente marcados para RE não coraram para Fluoro-Jade (um marcador de degeneração neuronal), sugerindo uma associação entre RE e neuroproteção. Nossos achados demonstraram alterações na expressão do RE relacionadas ao tempo e a localização cerebral no hipocampo e neocórtex de animais submetidos ao modelo da pilocarpina.

Palavras chave: receptor de estrogênio; esclerose do hipocampo; epileptogênese; modelo da pilocarpina

ABSTRACT

The estrogen receptors (ERs) are widely distributed in the central nervous system and are involved with neuroplasticity. The ER role in epileptogenic processes are still unknown. We investigated the estrogen receptors expression by immunohistochemistry in the hippocampus and neocortex in adult male rats (n = 3-4 per group) submitted to the pilocarpine-induced *status epilepticus* (SE). In comparison to the respective control groups, the ER expression enhances significantly in neurons of the CA1, CA2, CA3 and DG sub regions of the hippocampus 1 h (acute phase), 12 h (toxic period) and 5 days (silent period) after the SE induction. In all these regions, except the CA3, the ER expression returns to the control levels 50 days after the SE. In the chronic phase, there was a trend (p = 0.07) for a slight enhancement in the ER expression in the CA3 regions of animals submitted to the SE in comparison to their controls. In the parietal neocortex the ER expression is enhanced 1 h and returned to the control level at 12 h, 5 and 50 days after the SE induction. The enhancement in the ER expression is variable according to the time and brain region analyzed. Additionally, the neurons strongly labeled for ER were not stained for Fluoro-Jade (a marker of neuronal degeneration) suggesting an association between ER and neuroprotection. Our findings demonstrated a time and brain region dependent changes in the ER expression in the hippocampus and neocortex in the pilocarpine model.

Key words: estrogen receptors; hippocampal sclerosis; epileptogenesis; pilocarpine model

SUMÁRIO

1. Introdução

1.1. Epilepsia

As epilepsias são transtornos neurológicos que atingem 50 milhões de pessoas no mundo, sendo 90% delas nos países em desenvolvimento (WHO 2009), e se caracteriza pela ocorrência de crises epilépticas espontâneas e recorrentes (Morrell and deToledo-Morrell 1999; Engel 2001). As crises epilépticas são distúrbios transitórios caracterizados por disfunção cerebral, devido à descarga neural excessiva, com manifestações clínicas que podem variar desde manifestações complexas de comportamento, sendo as últimas com grau variável de comprometimento da consciência (Lothman, Bertram et al. 1991; McNamara 1994; Delgado-Escueta, Wilson et al. 1999; McNamara 1999). A epilepsia de lobo temporal mesial associada à esclerose do hipocampo (ELTM-EH) é o tipo mais freqüente de epilepsia refratária ao tratamento farmacológico, sendo caracterizada pela ocorrência de crises parciais complexas (Kale 1997; Hauser, Rich et al. 1998). O sistema límbico, particularmente o hipocampo está envolvido com o início das crises epilépticas da ELTM-EH.

Em relação aos padrões neuroquímicos, a epilepsia de lobo temporal é caracterizada tanto por disfunções neuronais quanto das células gliais (astrócitos e microglias) (Connors and Gutnick 1990; Ullah, Cressman et al. 2009). As crises epilépticas são o resultado de uma atividade excitatória excessiva e síncrona anormal de neurônios do neocórtex ou estruturas límbicas como o hipocampo. (Coutinho-Netto, Abdul-Ghani et al. 1981; Reynolds 1995). Esta atividade anormal parece estar associada em parte a alterações nos sistemas de neurotransmissão, como o comprometimento da transmissão inibitória (mediada por GABA), e/ou estimulação da transmissão excitatória (glutamatérgica) (Dichter and Ayala 1987; Meldrum, Akbar et al. 1999; Coulter 2001). Conseqüentemente, o aumento dos níveis extracelulares de glutamato pode resultar em hiperexcitabilidade dos circuitos neuronais com

modificações, tanto estruturais como funcionais, no fenômeno de plasticidade sináptica, além de excitotoxicidade e perda neuronal no caso de crises epilépticas prolongadas, o chamado estado de mal epiléptico (Cavalheiro, Leite et al. 1991; Coulter 2001).

A avaliação histopatológica do hipocampo de pacientes com ELTM-EH evidencia uma perda neuronal seletiva, em especial na região CA1, CA3 e giro denteado (GD) associada com uma reorganização sináptica com brotamento das fibras musgosas (Murray, Lopez et al. 1994; Kale 1997). O grupo da UCLA (Califórnia), aplicando técnicas de quantificação patológica nas ressecções de lobo temporal realizadas para tratamento de epilepsia refratária realizadas de 1961 a 1992, expandiu estas associações clínico-patológicas. A perda neuronal severa (mais de 40% de neurônios no hipocampo) ocorre mais freqüentemente em pacientes com história de insulto precipitante inicial (IPI) (88,2%) em comparação à sua ausência (15,8%). Dentre os pacientes que apresentam ELTM-H, a história de IPI é bem freqüente quando comparado à população controle (sem epilepsia de lobo temporal) (Mathern GW 1997).

Estudos clínicos sugerem que a formação de um tecido capaz de gerar crises epilépticas espontâneas (processo denominado epileptogênese) na ELTM-EH é, em uma grande parte dos casos, deflagrada por um IPI na infância (crise epiléptica febril, crise epiléptica prolongada, meningite), seguido de um período silente variável, até o surgimento de crises recorrentes, em geral na segunda década de vida. Esta hipótese é corroborada por estudos experimentais em que uma crise epiléptica prolongada leva à crises espontâneas originadas no hipocampo de forma muito semelhante à ELTM-EH (ver abaixo o modelo da pilocarpina).

Lesões do hipocampo como a esclerose do hipocampo (Connors and Gutnick 1990; Prince and Tseng 1993; Hoffman, Salin et al. 1994) decorrente de crises epilépticas prolongadas (estado de mal epiléptico) estão associadas à modificações na fisiologia intrínseca

neuronal, levando ao surgimento de “neurônios epilépticos” hiperexcitáveis que associados a “redes neuronais epilépticas” são capazes de induzir à sincronização neuronal. Os mecanismos responsáveis por este processo de epileptogênese não são plenamente compreendidos (Cavalheiro, Leite et al. 1991). Inúmeras hipóteses têm sido sugeridas para explicar a fisiopatologia da doença, entre elas a que estabelece uma relação entre crises epilépticas, ativação e/ou inibição de diferentes vias de sinalização celular e dano neuronal.

1.2. Modelo da Pilocarpina

Um modelo clássico de indução de ELTM-EH é o induzido por injeção sistêmica de pilocarpina (Cavalheiro, Leite et al. 1991; Leite, Garcia-Cairasco et al. 2002) ou ácido caínico (Ben-Ari 1985). O uso desses modelos tem permitido a elucidação de possíveis mecanismos envolvidos na gênese, manutenção e evolução das diversas epilepsias (Bonan, Walz et al. 2000; Leite, Garcia-Cairasco et al. 2002; Walz, Castro et al. 2002).

O modelo animal de epilepsia induzida pela pilocarpina reproduz de forma bastante satisfatória as manifestações comportamentais, eletrofisiológicas e histopatológicas da ELTM-EH em humanos. Nesse modelo, o IPI é uma crise convulsiva prolongada ou estado de mal epiléptico (EME). Esse insulto promove modificações estruturais e funcionais no cérebro dos animais, os quais passam a apresentar crises epilépticas límbicas espontâneas (período crônico). O modelo é bastante útil para o estudo das diferentes fases do processo de epileptogênese induzido por uma crise epiléptica prolongada.

A pilocarpina é um agonista colinérgico que ao ser injetado em roedores induz a um estado epiléptico.(Cavalheiro, Leite et al. 1991; Leite, Garcia-Cairasco et al. 2002) Após a remissão espontânea os animais passam por um período “tóxico” que dura 2 a 3 dias, seguido de progressiva melhora até apresentarem-se com um comportamento aparentemente normal. A mortalidade é em torno de 30 a 50%. Entre 4 e 45 dias após o insulto, com uma média de 14,8

(+/- 3) dias, os animais passam a apresentar crises espontâneas recorrentes (2 a 4 por animal, por semana) sem remissão até pelo menos 6 meses após. As crises em geral iniciam no hipocampo com propagação para o córtex cerebral. As crises manifestam-se clinicamente por movimentos mastigatórios, piscamentos seguidos por movimentos clônicos da cabeça e crise motora límbica. O EME induzido pela plocarpina acarreta uma excitotoxicidade glutamatérgica (Cavalheiro, Leite et al. 1991), indução dos processos oxidativos celulares, necrose e/ou apoptose (Dal-Pizzol, Klamt et al. 2000).

Histopatologicamente observa-se uma perda neuronal predominantemente no hipocampo, mas também em amígdala, tálamo, córtex entorrinal, neocórtex e substância negra. Após o quarto dia identificam-se brotamentos supra-granulares de fibras musgosas, que atingem a máxima intensidade em 100 dias. A perda celular no hipocampo é mais significativa nas regiões CA1, CA3 e giro denteado (Cavalheiro, Leite et al. 1991; Leite, Garcia-Cairasco et al. 2002). Os achados lembram muito o que se observa em amostras de hipocampo de pacientes com ELTM-EH. (Mathern GW 1997).

1.3. Estrogênios e SNC

A possível relação entre os estrogênios e a excitabilidade neuronal tem sido observada e descrita há mais de 150 anos (Prince and Tseng 1993; Veliskova 2006). Desde então, inúmeros pesquisadores têm relatado a variação da freqüência de crises epiléticas em algumas mulheres durante o ciclo menstrual, condição referida como epilepsia catamenial e algumas características relativas à função de ativação dos estrogênios principalmente no que diz respeito à excitabilidade neuronal, modulação das crises epiléticas e o papel dessas moléculas no dano neuronal induzido pela epilepsia (Rune and Frotscher 2005).

Estudos anteriores têm demonstrado os efeitos do estrogênio na plasticidade cerebral através de modelos experimentais. Evidências farmacológicas e comportamentais sugerem

que a ativação dos receptores de estrogênio (RE) possui efeito regulatório na plasticidade sináptica do hipocampo, bem como um efeito benéfico nos mecanismos cognitivos de roedores (Arnold and Beyer 2009; Kelly and Ronnekleiv 2009).

Na década de 80, a hipótese de uma produção local de estrógenos no cérebro de mamíferos foi proposta por Baulieu e colaboradores, porém a demonstração direta desta esteroidogênese no cérebro não foi comprovada inicialmente, devido a níveis muito baixos destas substâncias no Sistema Nervoso Central (SNC) (Baulieu 1987; Warner and Gustafsson 1995). Posteriormente esta hipótese foi confirmada com a evidência da síntese de neuroesteróides em regiões como o hipocampo mesmo após a remoção de glândulas periféricas envolvidas na esteroidogênese (Kimoto, Tsurugizawa et al. 2001; Kawato, Hojo et al. 2002; Kawato 2003; Hojo, Hattori et al. 2004; Kretz, Fester et al. 2004).

A expressão da proteína regulatória aguda da esteroidogênese (StAR), e da enzima aromatase, duas moléculas essenciais à síntese de estrogênios já foram demonstradas no hipocampo de ratos adultos (Wehrenberg, Prange-Kiel et al. 2001). Vários estudos sugerem que além de efeitos excitatórios sobre o tecido nervoso, os hormônios sexuais têm papel na fisiopatologia da epilepsia do lobo temporal, uma das mais prevalentes síndromes epiléticas, freqüentemente associada à perda de neurônios hipocampais (Reddy 2009). Acredita-se que a principal função dos esteróides sexuais produzidos localmente é a modulação rápida da plasticidade sináptica em funções cognitivas, por este motivo, a sua investigação no hipocampo (Mukai, Takata et al. 2006; Mukai, Tsurugizawa et al. 2006; Hojo, Murakami et al. 2008).

Os receptores dos hormônios sexuais são amplamente distribuídos no SNC, sendo que os receptores de estrogênios foram identificados no citoplasma, quando inativos, e no núcleo, quando ativados ou inativados. Os efeitos dos hormônios estrogênicos no SNC são mediados pela ligação a receptores específicos ou por meio de efeitos diretos dos mesmos em receptores

de neurotransmissores das fendas sinápticas. Foram identificados receptores de estrogênio do tipo alfa e beta no SNC, ambos com uma estrutura similar, porém com distribuição e funções diferentes (McEwen 2002).

Entretanto, apesar de nos receptores de estrógeno serem amplamente expressos no hipocampo de ratos adultos, tanto machos quanto fêmeas (Sakuma, Tokuhara et al. 2009), sua função ainda permanecem desconhecidos. Conforme Green e colaboradores (2000) os estrogênios apresentam inúmeros efeitos celulares, incluindo ativação dos receptores nucleares específicos, aumento da expressão de proteínas anti-apoptóticas, interações com mecanismos intracelulares dependentes de segundo mensageiro, ativação glutaminérgica, além de manutenção da homeostase do cálcio intracelular e atividade antioxidante (Green and Simpkins 2000). Inúmeros desses efeitos estão associados com o aumento da sobrevida neuronal (Elzer, Muhammad et al. 2009). Vários estudos sugerem que uma das principais vias associadas à neuroproteção mediada pelos estrogênios envolve a ativação do AMPc/PKA/CREB (Kawano, Gunaga et al. 1975; Weissman, Daly et al. 1975; Szego, Barabas et al. 2006).

O aumento do AMPc com a subseqüente fosforilação do CREB contribuiria para os efeitos neuroprotetores do estradiol através do aumento da expressão da proteína BCL-2, da ativação da MAP quinase, ERK, ou inibição do raf-1. Essa via ativada é conhecida ainda por modular outros efeitos celulares incluindo despolarização de neurônios hipotalâmicos, crescimento dendrítico e sinapses excitatórias, aumentando a expressão dos receptores de NMDA, com aumento da potenciação sináptica no hipocampo (Mukai, Takata et al. 2006; Mukai, Tsurugizawa et al. 2006; Hojo, Murakami et al. 2008).

Considerando que RE estão amplamente distribuídos em diferentes estruturas do SNC, e que existe uma plausibilidade entre a ocorrência de crises epilépticas e modificações nos níveis do RE no hipocampo e neocórtex de animais (Veliskova 2006; Sakuma, Tokuhara et al.

2009), o presente estudo investigou os níveis de expressão deste receptor no hipocampo e neocórtex, nas diferentes fases do modelo de ELTM-EH induzido pela pilocarpina, e sua associação com a perda neuronal ocorrida neste modelo.

2. Objetivos

2.1 Objetivo Geral

Avaliar a expressão do RE em estruturas cerebrais de ratos submetidos ao modelo experimental da pilocarpina.

2.2 Objetivos Específicos

- Avaliar uma possível modificação no padrão de expressão do RE nas sub-regiões do hipocampo de animais submetidos ao modelo experimental de ELTM-EH induzido pela administração sistêmica de pilocarpina, por meio da técnica de imunoistoquímica.

- Avaliar uma possível modificação no padrão de expressão do RE no neocórtex de animais submetidos ao modelo experimental de ELTM-EH induzido pela administração sistêmica de pilocarpina, por meio da técnica de imunoistoquímica.

- Analisar uma possível associação entre a expressão de RE no hipocampo e neocórtex e os diferentes períodos do modelo na pilocarpina.

- Analisar a associação entre a expressão do RE e a degeneração neuronal, através da técnica de Fluoro-Jade no hipocampo e neocórtex de animais submetidos ao modelo da pilocarpina.

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4. Artigo Original

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Time-dependent enhancement of estrogen receptors expression in the hippocampus and neocortex of rats submitted to pilocarpine model

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ABSTRACT

The estrogen receptors (ERs) are widely distributed in the central nervous system and are involved with neuroplasticity. The ER role in epileptogenic processes are still unknown. We investigated the estrogen receptors expression by immunohistochemistry in the hippocampus and neocortex in adult male rats (n = 3-4 per group) submitted to the pilocarpine-induced *status epilepticus* (SE). In comparison to the respective control groups, the ER expression enhances significantly in neurons of the CA1, CA2, CA3 and DG sub regions of the hippocampus 1 h (acute phase), 12 h (toxic period) and 5 days (silent period) after the SE induction. In all these regions, except the CA3, the ER expression returns to the control levels 50 days after the SE. In the chronic phase, there was a trend (p = 0.07) for a slight enhancement in the ER expression in the CA3 regions of animals submitted to the SE in comparison to their controls. In the parietal neocortex the ER expression is enhanced 1 h and returned to the control level at 12 h, 5 and 50 days after the SE induction. The enhancement in the ER expression is variable according to the time and brain region analyzed. Additionally, the neurons strongly labeled for ER were not stained for Fluoro-Jade (a marker of neuronal degeneration) suggesting an association between ER and neuroprotection. Our findings demonstrated a time and brain region dependent changes in the ER expression in the hippocampus and neocortex in the pilocarpine model.

Key words: estrogen receptors; hippocampal sclerosis; epileptogenesis; pilocarpine model

Introduction

The gonadal steroid hormones modulate the development and neuroplasticity of the nervous system in physiologic and pathologic conditions (McEwen, 1991; Veliskova, 2006). The estrogen receptors (ERs) are widely distributed in the central nervous system (CNS) and mediate biological responses through a nuclear ER mechanism of action (Green e Simpkins, 2000), activating genes by directly binding to hormone-specific DNA regulatory elements (Evans, 1988).

Nuclear ERs activation modulate cellular mechanisms involved in excitotoxicity and epileptogenesis, including the expression of anti-apoptotic proteins, second messenger cascades, alterations in glutaminergic activation, maintenance of intracellular calcium homeostasis and antioxidant activities. Several of these effects have been shown to enhance neuronal survival; however, the exact role of each of these pathways in estrogen-enhancement of neuronal survival remains to be elucidated (Green e Simpkins, 2000). The possible link between estrogens and neuronal excitability was observed and described almost 150 years ago (Locock, 1857; Gowers, 1881) and several findings suggest that gonadal hormones play a role in epilepsies (Reddy, 2009) including the mesial temporal lobe epilepsy related to hippocampal sclerosis (MTLE-HS).

The MTLE-HS is the most common refractory epileptic syndrome in humans leading to complex partial seizures that can evolve to secondarily generalized seizures (Mathern et al., 1995; Engel, 2001). The pilocarpine model is a useful animal model to investigate the development epileptogenesis and neuropathology related to MTLE-HS (Babb TL, 1987) in rodents. In this model, the systemic admiministration of pilocarpine induces a prolonged *status epilepticus* (SE) resulting in neuronal loss, mossy fiber sprouting in hippocampus and spontaneous recurrent seizures (Ben-Ari, 1985; Leite et al., 1990; Cavalheiro et al., 1991; Mello et al., 1993; Bonan et al., 2000).

In order to provide new information about the role of estrogens receptor during the temporal and regional evolution of long-lasting changes in MTLE-HS, we have analysed the ER immunoreactivity in the hippocampus and cortex in different phases of the pilocarpine model in rats.

Materials and methods

Animal model

Adult male *Wistar* rats (age 70-90 days; weight 200-240 g) from our own colony were housed under optimum light, temperature, and humidity conditions (12/12 h light/dark cycle, 22 ± 1°C, under 60-80% humidity), with food and water provided *ad libitum*. All procedures within this study followed the "Principles of Laboratory Animal Care" from the National Institutes of Health's (NIH) publication number 85-23 and were approved by the Ethics Committee of our University.

The pilocarpine model has been previously described (Leite et al., 1990; Cavalheiro et al., 1991; Bonan et al., 2000). In summary, thirty minutes after subcutaneous pretreatment with scopolamine methyl nitrate 1 mg/kg (to minimize peripheral cholinergic effects), a single dose of pilocarpine (300 mg/kg, dissolved in saline) was injected intraperitoneally (i.p.). After the pilocarpine injection, the animal became hypoactive; generalized convulsions and limbic SE usually occurred 40-80 min after injection. A total of 25 rats received pilocarpine and monitored behaviorally for at least 6 h. Total mortality was 35% (n = 9), and 8% (n = 2) of the rats did not develop SE. Only animals that evolved to SE were studied. Control animals (n = 12) received vehicle (saline) and scopolamine and were sacrificed at similar times after infusions as their respective experimental groups (n = 3 per group).

The animals were sacrificed 1 h (acute phase), 12 h (toxic phase), 5 days (silent period) or 50 days (chronic period) after SE behavior beginning. After deeply anaesthetized with pentobarbital (70 mg/kg i.p.), animals were perfused transcardially with saline followed by 4% paraformaldehyde in phosphate buffer (PBS; 0.1 M, pH 7.4) for 5 min. The brains were rapidly removed, fixed overnight in 4%

paraformaldehyde (Sigma, CA) and submitted to conventional histological processing. Animals from the silent and chronic period were behaviorally evaluated 6 h before their sacrifice and had no epileptic seizure during the period of evaluation.

Immunohistochemistry

Immunohistochemistry for estrogen receptor (1:100, monoclonal antibody, clone 6F11, NovoCastra, United Kingdom) was carried out on 4 μm paraffin section and was applied to sections of each hippocampal specimen and positive control. After deparaffinization in xylene, the slides were progressively rehydrated. The endogenous peroxidase activity was blocked with a solution of 3% hydrogen peroxide in methanol for 20 min at room temperature (RT). Antigen retrieval was performed for 45 min at 95-98°C in 10 mM trisodium citrate buffer, pH 6.0. After 20 min at RT, the slides were washed in distilled water and transferred to standard PBS buffer. In order to detect the ER expression, the slides were incubated overnight at 2-8°C with the primary antibody. Binding of primary antibody was detected by the peroxidase-labelled amplification polymer method (EnVision™, Rabbit/Mouse, Dako Carpinteria, California, USA), using 3, 3´, diaminobenzidine (DAB) (DAKO). All specimens included in this study were stained under identical conditions. Control experiments included omission of primary antibody as well as replacement of the primary antibody for equivalent dilutions of nonimmune mouse IgG serum (DAKO), using the same staining protocol, and were devoid of specific immunoreaction product. Slides were counterstained with Harris hematoxylin, dehydrated and coverslipped. The presence of brown precipitate indicated positive labeling for the primary antibody.

Image analysis

The immunostaining was assessed in 4 μm sections of hippocampus and parietal neocortex localized between 4.0 and 4.2 mm posterior to the Bregma, according to the rat brain atlas of Watson and Paxinos (1986). For ER quantification, images of stained hippocampal CA1, CA2, CA3 and dentate gyrus (DG) subregions and the neocortex were acquired using a Sight DS-5M-L1 digital camera (Nikon, Melville, NY, USA) connected to an optical microscope (Eclipse 50i; Nikon, Melville, NY, USA) at 400x magnification. One image of each region per section (4 images per histological section) was captured. The threshold optical density (O.D.) that best discriminated staining from the background was obtained using the NIH ImageJ 1.36b imaging software (NIH, Bethesda, MD, USA). The total pixels intensity was determined and data were expressed as O.D. The data represent the average value obtained by the analysis of images of hippocampal CA1, CA2, CA3, DG subregions, and neocortex.

Combining Fluoro-Jade B with ER immunohistochemistry stainings

To further investigate the possible relation between ER expression and neuronal death associated with pilocarpine induced-SE, we performed a double stain of ER with Fluoro-Jade B and histochemistry (Schmued et al., 1997) 1 h and 12 h after SE onset and compared with their respective control group. The Fluoro-Jade (FJ) histochemistry was used as indicative of neuronal degeneration (Schmued et al., 1997; Poirier et al., 2000; Schmued e Hopkins, 2000; Wang et al., 2008). The ER immunohistochemistry was performed in paraffin-embedded sections from rat brain as described above. After development with chromogen solution, the slides were immersed in Fluoro-Jade B staining solution (10 mL of 0.01% Fluoro-Jade B dye

aqueous solution in 90 mL of 0.1% acetic acid in distilled water) for 30 min. After staining, the sections were rinsed three times with distilled water. Excess of water was drained off, and the slides were immersed in xylene and then coverslipped with D.P.X. (Aldrich Chem. Co., Milwaukee, WI) mounting media. Sections were examined with a confocal microscope (Leica DMI6000 B, Wetzlar, Germany) using a 514 nm excitation laser.

Statistical analysis

The statistical analysis used was one-way ANOVA, followed by post hoc Newmann Keuls test. The significant difference was considered when $p < 0.05$. Values represent the mean ± S.E.M.

Results

Expression of ER in the rat hippocampus and neocortex

In accordance with previous studies (Orikasa et al., 2000; Sakuma et al., 2009), the anti-ER antibody showed immunoreactivity in the nucleus and cytoplasm of the pyramidal cells. Such feature of labeling is similar to the one found in the positive control of gonadal cells of *Wistar* rats ovarian tissue (data not shown), confirming the ER recognition by the primary antibody used.

A time-dependent expression of ER, as evidenced by a high staining, was observed in the pyramidal cells in the hippocampal subfields (CA1, CA2, CA3 and DG) and parietal cortex layer IV and V after pilocarpine-induced SE. As expected, it was observed a weaker labeling for ER in the same regions of animals from the control group, indicating a low expression of such receptor. Because there were no differences in the ER expressions of controls in the different periods evaluated after SE induction (p = 0.9), only a representative control group were showed in the figures.

Figure 1 illustrates the intensity of labeled area relative to the expression of ER in the CA1 subregion of control animals and of the pilocarpine-induced SE obtained in different periods as follows: the acute phase (1 hour), toxic phase (12 hours), silent (5 days) and chronic period (50 days). The animals treated with pilocarpine notably showed a higher intensity of staining when compared to the control group (p = 0.005) (Fig. 1). The post-hoc analysis revealed significant difference in pilocarpine-treated animals 12 h (p = 0.02) and 5 days (p = 0.01) after epileptic insult when compared with control group. Considering CA1 region, the ER

expression 1 h or 50 days after SE induction did not differ from the respective control ($p > 0.3$).

As it was observed in the CA1 region, the Figure 2 illustrates a similarity in the ER immunoreactivity pattern observed in the CA2 region of hippocampus. However, there were a significant enhancement of ER expression 1 h and 12 h after SE induction. Animals submitted to SE and sacrificed after 1 h or 12 h presented a significant enhancement in the ER expression in the CA2 region ($p = 0.04$ and $p = 0.004$, respectively). Five days after SE induction there was a trend ($p = 0.06$) for a higher expression of ER in comparison to the respective control group (Fig 2). In the chronic period (50 days after the SE) the ER expression returned to the control level ($p = 0.8$).

The ER expression pattern in the hippocampal CA3 region is presented in the Figure 3. In comparison to the control group, there were a significant augmentation of ER expression of animals sacrificed 1 h ($p = 0.006$), 12 h ($p = 0.03$), and 5 days ($p = 0.003$) after the SE induction (Fig 3). However, fifty days after the SE there was a trend ($p = 0.07$) for higher ER expression in the pilocarpine-treated animals in comparison to the control group.

In the dentate gyrus (DG) region, the ER expression pattern is showed in the Figure 4. The ER expression enhanced 1 h ($p = 0.02$), 12 h ($p = 0.04$) and 5 days ($p = 0.007$) after SE induction (Fig. 4). In this structure, the ER expression of pilocarpine-treated animals returned to the control level 50 days after SE induction ($p = 0.19$).

Figure 5 illustrates the average of ER expression in the whole hippocampus (pooled sub regions CA1, CA2, CA3 and DG) showing the increase in ER expression 1 h ($p = 0.006$), 12 h ($p = 0.0003$) and 5 days ($p = 0.001$) after pilocarpine-induced

SE. Conversely, in the chronic period evaluated (50 days) there were a reduction in the ER expression ($p = 0.17$).

The expression of ER in the parietal cortex localized over the same coronal slices containing the dorsal hippocampus is showed in the Figure 6. In comparison to the respective control group, animals treated with pilocarpine showed a high staining intensity in the neocortex only in the first hour ($p = 0.003$), but not 12 h ($p = 0.15$), 5 days ($p = 0.25$) or 50 days after SE induction ($p = 0.32$).

The Fluoro-Jade positive cells did not express the ER in rat hippocampus

In accordance with previous study (Wang et al., 2008), the histochemical analysis performed 1 h (Fig. 7) and 12 h (data not shown) after the SE induction using Fluoro-Jade staining revealed an intense neuronal death. As demonstrated in Figure 7, the cells strongly labeled for ER were not stained for Fluoro-Jade.

Discussion

The present study demonstrated a time- and region-dependent enhancement of ER expression in the hippocampus and parietal cortex after the pilocarpine-induced SE in adult male rats. In the whole hippocampus the mean ER expression enhancement showed an inverted "U" shape at earlier times (1 h, 12 h and 5 days) after the SE induction returning to the control level in de chronic phase (50 days). The detailed subregion analysis demonstrated that in the CA1 and CA2 the ER expression increased up to de maximum level 12 h after the SE induction, decreasing its expression 5 days after, and returning to the control level in the chronic period. In the CA3 and DG subregions, the ER expression remains high 5 days after SE induction and decline in the chronic phase (50 days). The maximal level of ER expression of treated animals in comparison to the controls was greater in CA2 (12-fold), followed by CA1 and CA3 (8-fold) and DG (4-fold). In the neocortex, it was observed a 10-fold increase in the ER expression in the first hour, evolving to a progressive decrease reaching the control level 12 h, 5 days and 50 days after seizure induction. The ER expression decrease observed in the chronic phase after SE may be due, at least in part, to a reduction in the number of neuronal cells that is the hallmark of the hippocampal sclerosis.

In spite of the facts mentioned above, these findings may be related to differences in the acute and long-term consequences of pilocarpine-induced SE in different cortical and subcortical areas. Particularly in CA1, the most sensible hippocampus subregion, the enhancement of ER was not expressive in 1 h, but demonstrated an increase 12 h after SE, and decreased latter after the seizure induction in comparison to the most resistant region CA2. Taken together with the demonstration that neurons strongly labeled for ER were in degeneration, it is possible to suggest an association

between ER and neuroprotection, although the cause-effect relationship cannot be established by the present experimental design. The estrogens functions in hippocampus depend on their specific binding the respective receptors, represented by the two known isoforms (alfa and beta), both present in male and females *Wistar* rats (DeFranco et al., 1998; Gruber et al., 2002). Nonetheless, the mechanisms by which these hormones exert their neuroprotective functions are still unknown. According to Green and Simpkins, the estrogens present a plethora of cellular effects, including activation of specific nuclear receptors, increase of the expression of anti-apoptotic proteins, interactions with intracellular second messengers, glutamatergic activation, maintenance of the intracellular calcium homeostasis and antioxidant activity (Green e Simpkins, 2000). Several of these mechanisms are pointed to contribute to the increase of the neuronal survival and the increase in the ER expression after SE may be related to neuroprotection.

Rune and colleges (2002) reported that ER nuclear staining was stronger in neurons of CA3 than of CA1 subregion, and this was clearly seen in single sections containing both regions, and an increased presence of receptors in nuclei of CA3 neurons was indicative of activated receptors (Rune et al., 2002). In this study, the ER nuclear and cytoplasmatic staining were seen in all hippocampus regions (CA1, CA2, CA3 and DG) and the highest ER expression was found in CA3 region (Rune et al., 2002). The animals submitted to SE demonstrated an important increase in the RE expression in the acute and toxic phase, and silent period, which decreased in the chronic period of the model. These results corroborate the other studies, indicating that the increase in ER expression coincides with the induction of neurodegenerative processes that are more prominent in toxic and acute phases, mainly in CA1 and CA3 hippocampal subregions (Ciriza et al., 2004; Sakuma et al., 2009).

Modifications in the intrinsic physiology of neurons of animals submitted to pilocarpine-induced SE may be critical for the epileptogenic mechanisms in the hippocampus and seizure propagation in the neocortex. Sanabria and colleagues (Garrido et al., 1998) demonstrated that pilocarpine-induced SE induced changes in the electrophysiological characteristics in the hippocampus of the chronic epileptic animals. The CA1 region of these animals presented a significant proportion of neurons with intrinsic burst or spontaneous burst cell characteristics (not observed in controls) which are likely to be the triggers of spontaneous population bursts in epileptic slices. Non-genomic effects of estrogen on cell functions can be observed as picomolar concentrations of 17β-estradiol directly enhanced glutamatergic synaptic transmission in the CA1 pyramidal cells of rat hipocamppus (Terasawa e Timiras, 1968). The 17β-estradiol also induced a significant and persistent increase in the long-term potentiation induced in the CA1 hipocampus of male rats (Zadran et al., 2009). The same author also demonstrated a significant increase in intrinsic bursting neurons in the layer V of neocortex of chronic animals submitted to pilocarpine-induced SE (Garrido et al., 1998). Considering that ER activation modulates the excitability and neurodegenerative processes, the early increase (acute phase) of ER expression could be involved not only in neuroprotection but also in the epileptogenic long-term modifications of the intrinsic neuronal physiology and network observed in those animals.

Several studies have been exhaustively reporting the neuroprotective and/or pro-convulsivant role of estrogens, but the real role of these hormones still remains uncertain (Veliskova, 2006; Yadav et al., 2007; Frye et al., 2009). The histochemistry and immunohistochemical labellings found in the present study suggest an association between estrogen and neuroprotection (Zhou et al., 2007; Schauwecker et al., 2009).

An important pathway related to the estrogens neuroprotective action involves the activation of the AMPc – PKA – CREB (Green e Simpkins, 2000) modulating the BCL-2 protein expression and activation of the MAP kinase, ERK. In fact, Garrido et al. demonstrated a MAP kinase, ERK, activation in the first 12 h after SE induction (Garrido et al., 1998). This pathway is also known to mediate other cellular effects including hippocampal neuron despolarization and dendritic spine growth (Green e Simpkins, 2000; Yadav et al., 2007) that may contribute to the synaptic reorganization occurring in the cortical and subcortical regions.

Meantime, in the literature, there are conflicts about the effects of the estrogens in the hippocampus and how they influence seizures and neuronal excitability (Green e Simpkins, 2000; Veliskova, 2006). In spite of the evidences, the specific sign that leads to the increase in the ER expression in the pilocarpine experimental model, as well as the disparity of the immunoreactivity between the groups of control animals and the animals treated with pilocarpine, still needs to be elucidated and explained. In the same way, additional studies to evaluate the ER expression in other encephalic regions are essential, since the studied model also includes the modulator participation of other neocortical and limbic regions.

In summary, our findings demonstrated a time- and brain region-dependent changes in the ER expression in the hippocampus and neocortex in the pilocarpine model of mesial temporal lobe epilepsy related to hipocampal sclerosis. Changes in the ER expression are more obvious in the earlier times after SE induction by pilocarpine. The characterization of the genomic and non-genomic actions of estrogens in different epilepsy models are important challenges to understand the role of estrogen receptors in epileptogenic processes and may help to identify therapeutic targets for epilepsy treatment.

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LEGENDS

Figure 1 – ***Expression of estrogen receptor (ER) in granular cells of CA1 subregion of the hippocampus.*** Immunohistochemical analyses of ER were performed 1 h, 12 h, 5 days and 50 days after pilocarpine-induced *status epilepticus* (SE) and compared with the respective control group. Rats treated with pilocarpine showed an increased ER immunostaining in the CA1 region 12 h ($p < 0.05$) and 5 days ($p < 0.01$) after SE induction. (A) Representative images of ER immunostaining in the CA1 subregion of the hippocampus. Scale bar = 50 μm. (B) Graphic representation of the ER immunostaining average in the CA1 subregion of the hippocampus. Values represent the mean ± S.E.M. (n = 3 rats per group). *$p < 0.05$ compared to control group.

Figure 2 – ***Expression of ER in granular cells of CA2 subregion of the hippocampus.*** Immunohistochemical analyses of ER were performed 1 h, 12 h, 5 days and 50 days after pilocarpine-induced *status epilepticus* (SE) and compared with the control group. Rats treated with pilocarpine showed an increased ER immunostaining in the hippocampus CA2 subregion 1 h ($p < 0.05$) and 12 h ($p < 0.01$) after SE induction. (A) Representative images of ER immunostaining in the CA2 subregion of the hippocampus. Scale bar = 50 μm. (B) Graphic representation of the ER immunostaining average in the CA2 subregion of the hippocampus. Values represent the mean ± S.E.M. (n = 3-4 rats per group). *$p < 0.05$ and **$p < 0.01$ compared to control group.

Figure 3 – *Expression of ER in granular cells of CA3 subregion of the hippocampus.* Immunohistochemical analyses for ER were performed 1 h, 12 h, 5 days and 50 days after pilocarpine-induced *status epilepticus* (SE) and compared with the control group. Rats treated with pilocarpine showed an increased ER immunostaining in the CA3 subregion of the hippocampus 1 h ($p < 0.01$), 12 h ($p < 0.05$) and 5 days ($p < 0.01$) after SE induction. (A) Representative images of ER immunostaining in the CA3 subregion of the hippocampus. Scale bar = 50 μm. (B) Graphic representation of the ER immunostaining average in the CA3 subregion of the hippocampus. Values represent the mean ± S.E.M. (n = 3 rats per group). *$p < 0.05$ and **$p < 0.01$ compared to control group.

Figure 4 – *Expression of ER in granular cells of the dentate gyrus subregion of the hippocampus.* Immunohistochemical analyses of ER were performed 1 h, 12 h, 5 days and 50 days after pilocarpine-induced *status epilepticus* (SE) and compared with the respective control group. Rats treated with pilocarpine showed an increased ER immunostaining in the DG subregion of the hippocampus 1 h ($p < 0.05$), 12 h ($p < 0.05$) and 5 days ($p < 0.005$) after SE induction. (A) Representative images of ER immunostaining in the DG subregion of the hippocampus. Scale bar = 50 μm. (B) Graphic representation of the average immunostaining for ER evaluated in the DG sub-region of the hippocampus. Values represent the mean ± S.E.M. (n = 3 rats per group). *$p < 0.05$ and **$p < 0.01$ compared to control group.

Figure 5 – *ER expression in hippocampus subregions.* Immunohistochemical analyses of ER were performed 1 h, 12 h, 5 days and 50 days after pilocarpine-induced *status epilepticus* (SE) and compared with the respective control group. Rats

treated with pilocarpine showed an increased ER immunostaining in the whole hippocampus 1 h ($p < 0.005$), 12 h ($p < 0.001$) and 5 days ($p < 0.001$) after SE induction. Graphic representation of the ER immunostaining average of CA1, CA2, CA3 and DG hippocampus subregions. Values represent the mean ± S.E.M. (n = 3 rats per group). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to control group.

Figure 6 – *Expression of ER in neurons of neocortex.* Immunohistochemical analyses of ER were performed 1 h, 12 h, 5 days and 50 days after pilocarpine-induced *status epilepticus* (SE) and compared with the respective control group. Rats treated with pilocarpine showed an increased ER immunostaining in neurons of parietal cortex, mainly in layer IV, 1 h ($p < 0.001$) after the pilocarpine-induced SE. (A) Representative image of ER immunostaining in the cortex of animals, showing the different cortical layers (I, II, III, IV, V). Scale bar = 200 μm. (B) High magnification of the rat cortical layer IV. Scale bar = 100 μm. (C) Graphic representation of the ER immunostaining average of four different fields from layer IV of the parietal cortex. Values represent the mean ± S.E.M. (n = 3-4 rats per group). **$p < 0.01$ compared to control group.

Figure 7 – *The Fluoro-Jade positive cells did not express the ER receptor in the rat hippocampus.*

Double staining of ER through immunohistochemistry and Fluoro-Jade histochemistry (degenerate neurons) was performed 1 h after pilocarpine-induced *status epilepticus (SE)*. (A) Representative image of the Fluoro-Jade histochemically stained CA3 subregion of the hippocampus, and (B) the same field as in (A)

immunostained for ER, using confocal microscopy. The arrows indicate the negative and positive cells for Fluoro-Jade and ER, respectively.

FIGURES

Figure 1

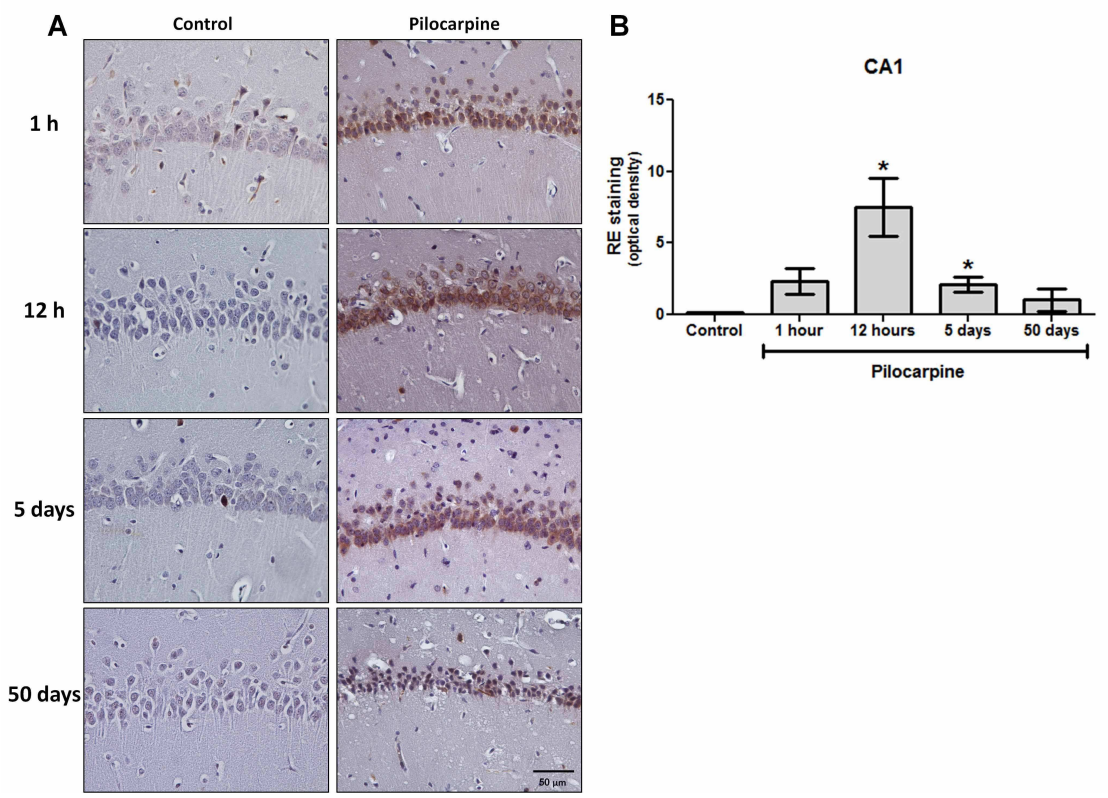


Figure 2

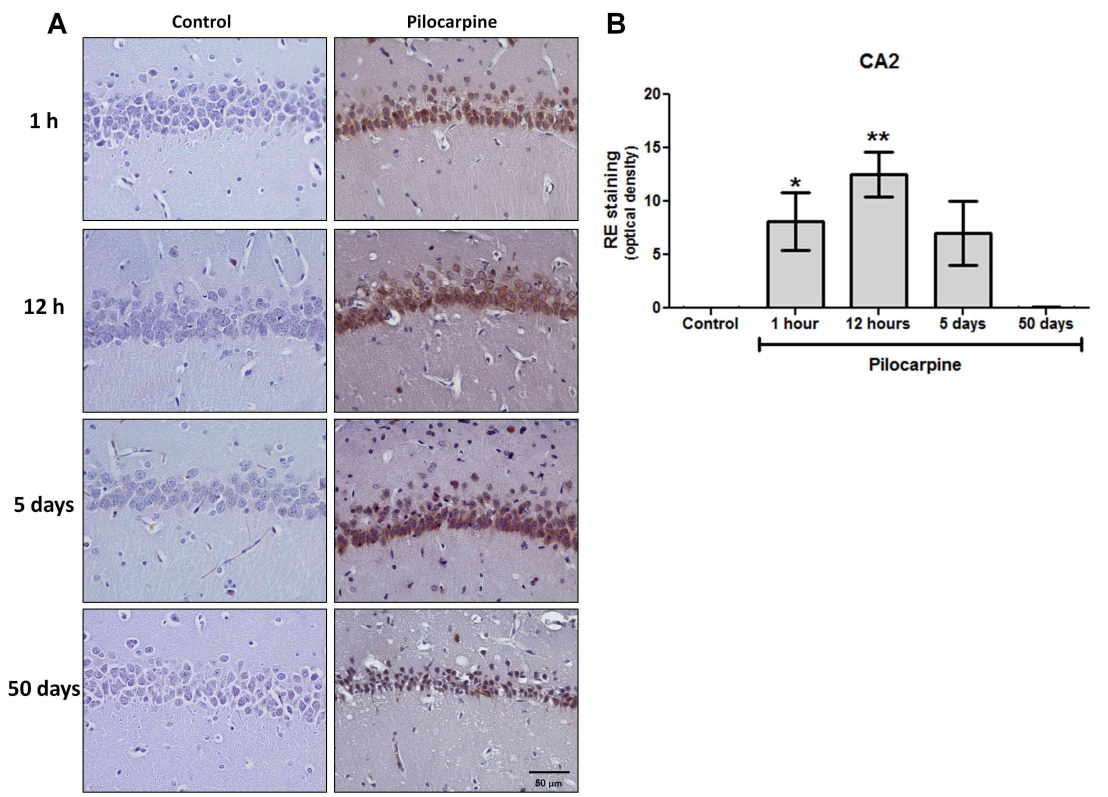

A
Control
Pilocarpine
1 h
12 h
5 days
50 days
50 µm
B
CA2
RE staining
(optical density)
20
15
10
5
0
*
**
Control
1 hour
12 hours
5 days
50 days
Pilocarpine

Figure 3

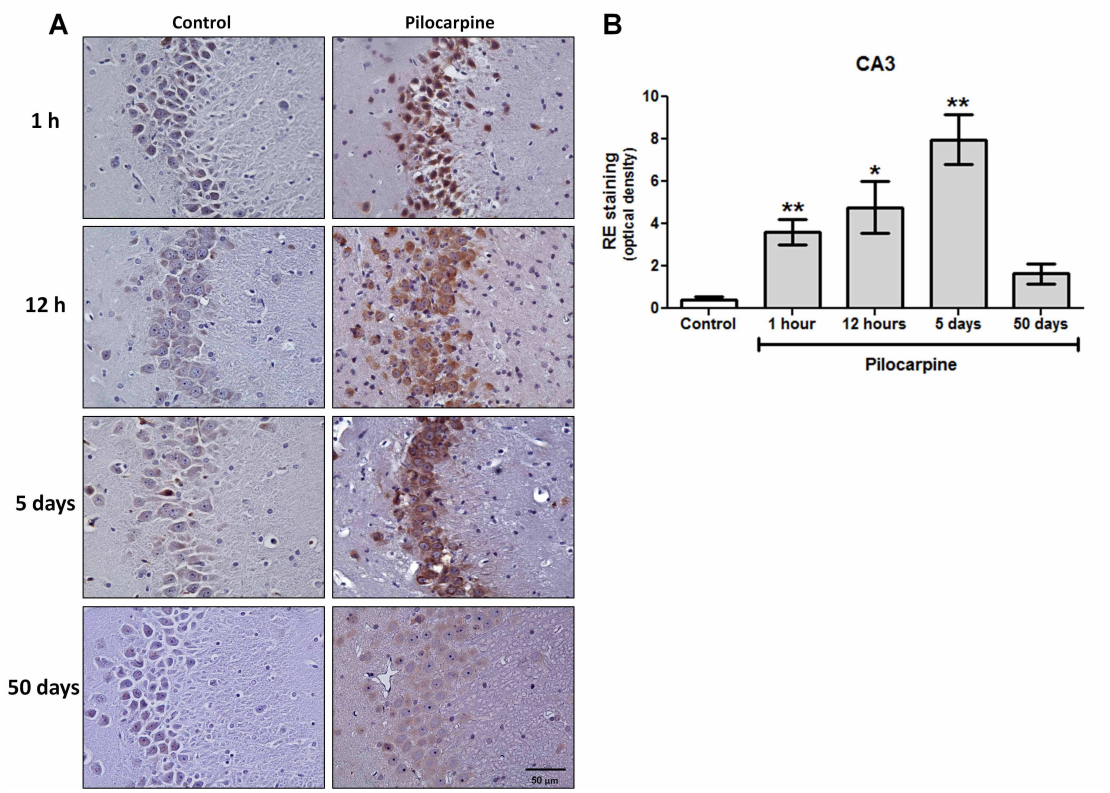

A
Control
Pilocarpine
1 h
12 h
5 days
50 days
50 µm
B
CA3
RE staining
(optical density)
0
2
4
6
8
10
**
*
**
Control
1 hour
12 hours
5 days
50 days
Pilocarpine

Figure 4

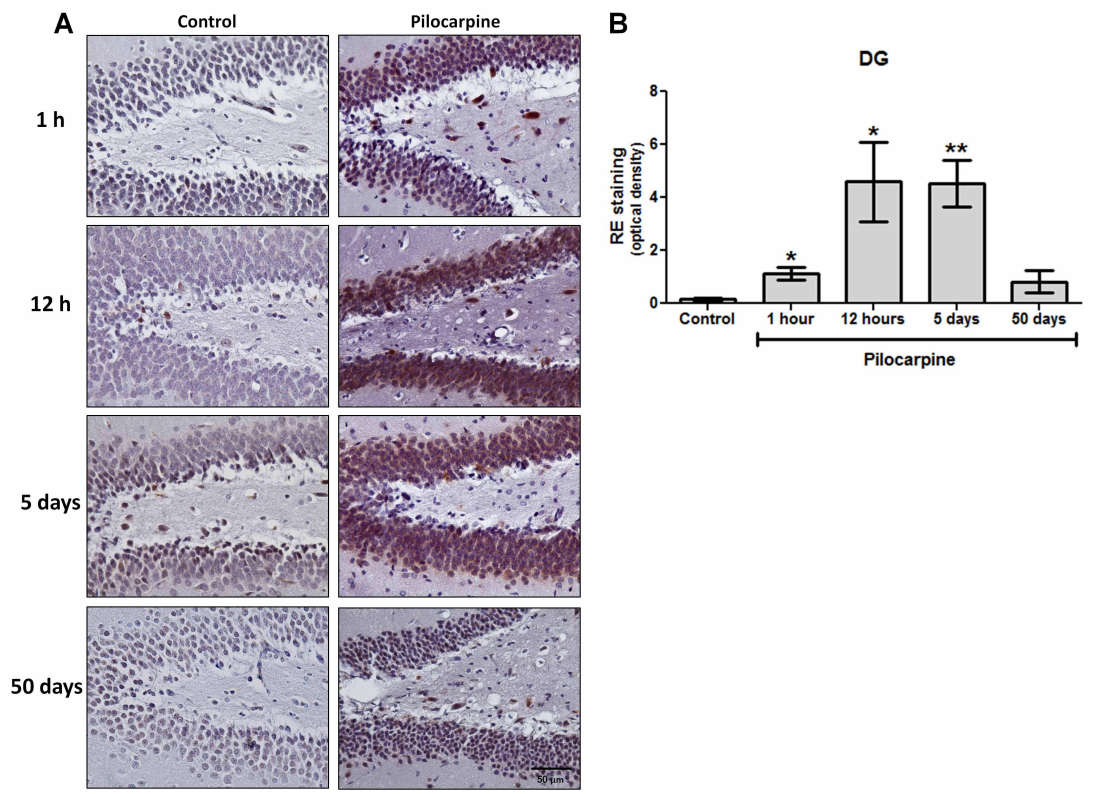

A
Control
Pilocarpine
1 h
12 h
5 days
50 days
B
DG
RE staining
(optical density)
8
6
4
2
0
*
*
**
Control
1 hour
12 hours
5 days
50 days
Pilocarpine

Figure 5

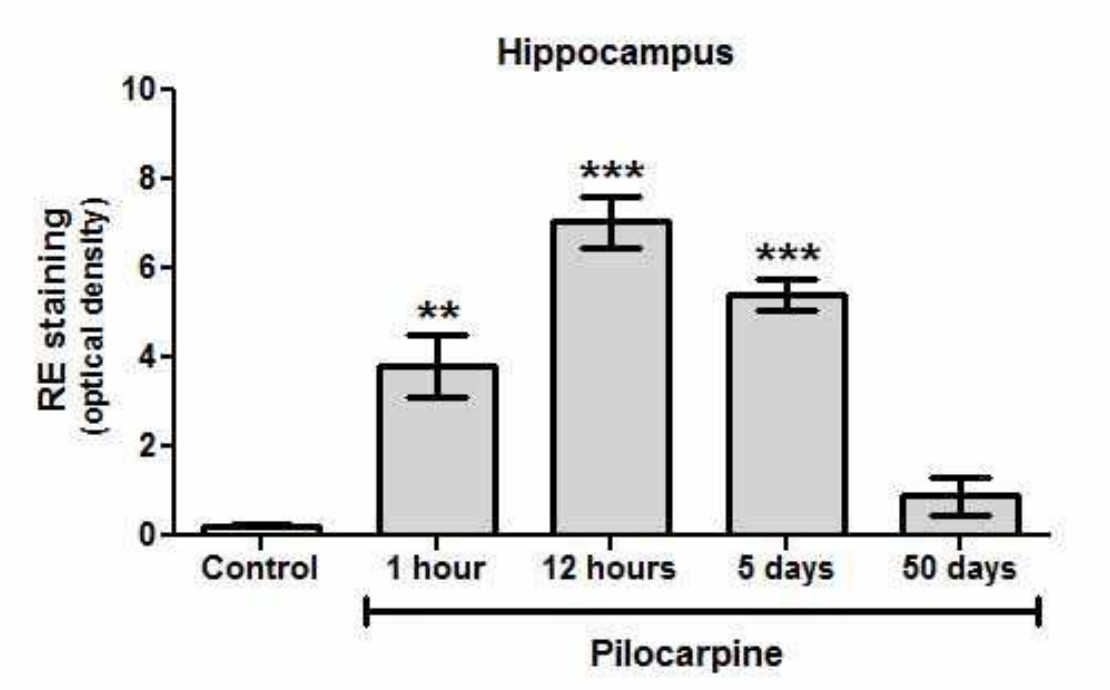

Hippocampus
RE staining
(optical density)
10
8
6
4
2
0
**
***
***
Control
1 hour
12 hours
5 days
50 days
Pilocarpine

Figure 6

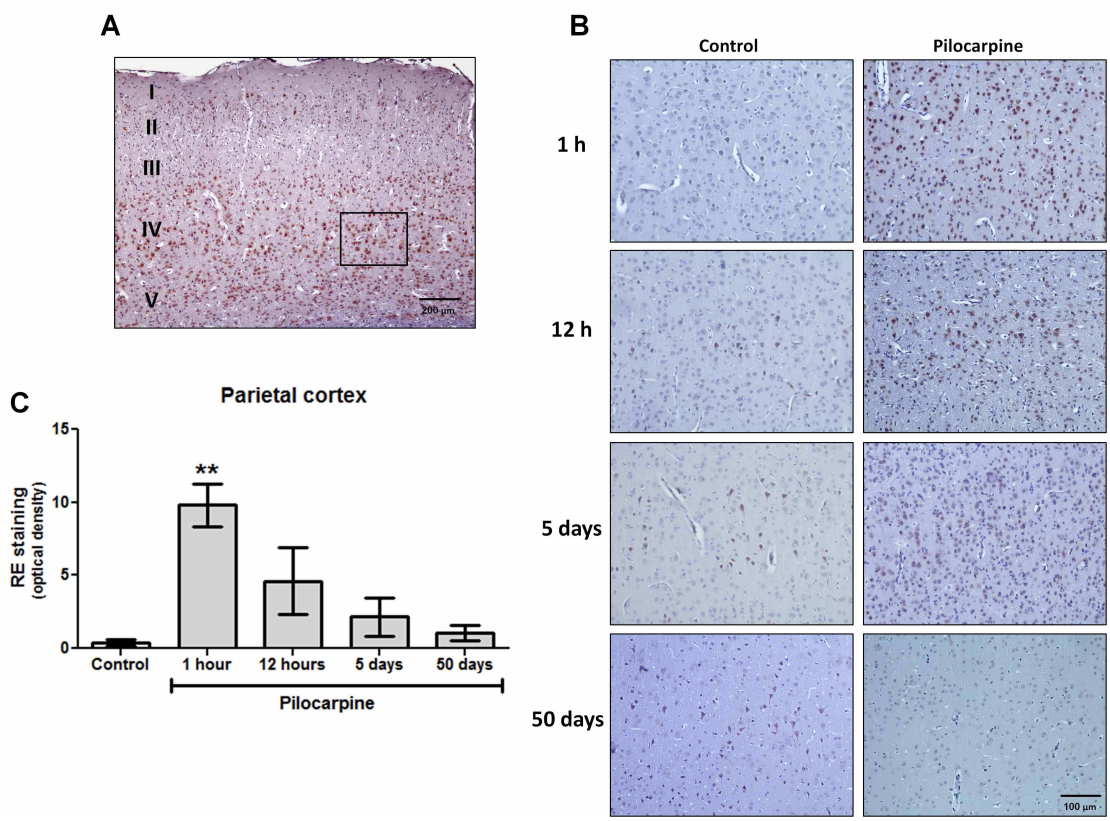
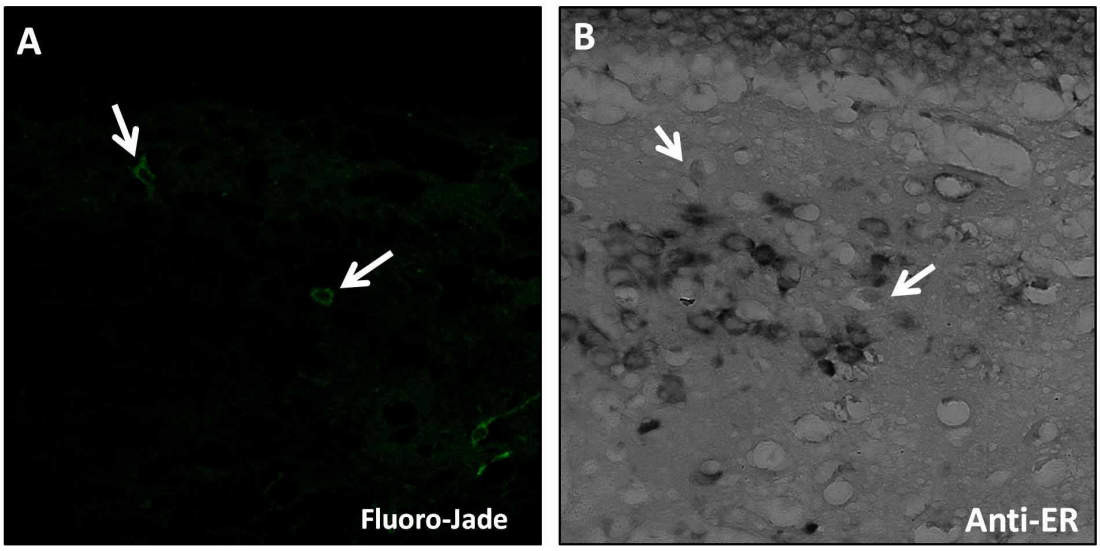

A
I
II
III
IV
V
200 µm
B
Control
Pilocarpine
1 h
12 h
5 days
50 days
100 µm
C
Parietal cortex
RE staining (optical density)
15
10
5
0
**
Control
1 hour
12 hours
5 days
50 days
Pilocarpine

Figure 7



7. Conclusões

Nossos achados demonstraram alterações na expressão dos receptores de estrogênio no hipocampo e neocórtex de ratos submetidos ao modelo de epilepsia do lobo temporal mesial da pilocarpina. Estes achados foram relacionados à localização e ao tempo. Alterações na expressão dos receptores de estrogênio são mais óbvias precocemente após a indução do *status epitepticus* pela pilocarpina. A caracterização de ações genômicas e não genômicas dos receptores de estrogênio nos processos de epileptogênese podem contribuir com a identificação de alvos terapêuticos no tratamento da epilepsia.