REGINA DE DEUS LIRA BENEVIDES

INFLUÊNCIA DOS HORMÔNIOS ANDROGÊNICOS SOBRE O DESENVOLVIMENTO NEURAL: ANÁLISE ELETROFISIOLÓGICA

RECIFE, 2015

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Dissertação apresentada para o cumprimento parcial das exigências para obtenção do título de Mestre em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco

Orientador: Dr. Rubem Carlos Araújo Guedes

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Regina De Deus Lira Benevides

“Influência de hormônios androgênicos sobre o desenvolvimento neural: análise eletrofisiológica”

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Á minha querida família, meu companheiro e meus amigos, pelo apoio e força

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Ao programa de Pós-Graduação em Bioquímica e Fisiologia.

RESUMO

O hormônio testosterona exerce um importante efeito sobre o desenvolvimento e funcionamento cerebral, incluindo modulação da atividade neuronal e excitabilidade. Neste estudo, nós investigamos em ratos adultos os efeitos do tratamento neonatal com testosterona, em três momentos distintos durante o desenvolvimento, sob o fenômeno relacionado à excitabilidade cerebral conhecido como Depressão Alastrante Cortical (DAC). Quatro grupos de ratos machos receberam diariamente injeções intraperitoneais na dose de 10 mg/kg/dia de propionato de testosterona na 2ª, 3ª ou 4ª , ou 2ª+ 3ª+ 4ª semana de vida (respectivamente grupos T2, T3, T4, eT2+3+4). Sob anestesia (1g/kg uretana + 40 mg/kg cloralose, i.p) nós deflagramos a DAC emintervalos de 20 min e a registramos em dois pontos da superfície cortical por 4 horas. A velocidade de propagação foi calculada baseada no tempo percorrido pela DAC em atravessar a distância entre os eletrodos. Comparados com os grupos controle tratados com veículo (azeite de oliva) nas correspondentes semanas do período pós-natal (grupos V2, V3 e V4), bem como um grupo ingênuo (não tratado), os animais que receberam testosterona apresentaram velocidades significamente menores ($p<0,05$). A média das velocidades variou de 2,76±0,05 para 2,99±0,29 mm/min nos grupos tratados com testosterona e de 3,13 ±0,03 para 3,37±0,07 mm/min nos animais controle. As velocidades baixas da DAC nos grupos tratados com testosterona foram acompanhadas por um aumento significante na duração da onda da DAC. Os dados indicam um novo efeito eletrofisiológico da testosterona no cérebro em desenvolvimento, provavelmente com importantes implicações em doenças neurológicas relacionadas a excitabilidade.

Palavras-chave: Testosterona. Excitabilidade cerebral. Desenvolvimento cerebral. Depressão alastrante cortical. Ratos.

ABSTRACT

Testosterone exerts important effects on brain development and functioning, including modulation of the neuronal activity and excitability. In this study, we investigated in adult rats the effects of neonatal treatment with testosterone, at three distinct time-points, on the brain excitability-related phenomenon known as Cortical Spreading Depression (CSD). Four groups of male Wistar newborn rats received daily intraperitoneal injections with 10 mg/kg/d propionate testosterone at the second, third, fourth, or second+third+fourth week of life. Under anesthesia (1g/kg urethane+40 mg/kg chloralose, i.p) we elicited CSD at twenty min intervals and recorded it at two points of the cortical surface during 4 hours. During the recording session, we measured the CSD parameters (velocity of propagation, amplitude and duration of the DC-negative CSD signal). Compared with control groups treated with the vehicle (olive oil) at the corresponding postnatal weeks, as well as with a naïve (not treated) group, testosterone-treated animals presented with significantly lower ($p<0.05$) CSD velocities. The CSD velocity ranged from 2.76±0.05 to 2.99±0.29 mm/min in the testosterone-treated groups, and from 3.13±0.03 to 3.37±0.07 mm/min in the control animals. The lower CSD velocity in the testosterone groups was accompanied by significantly longer duration of the CSD wave (range: 84.3±6.0s to 93.1±10.3s) compared with the vehicle and naïve controls (range: 73.3±5.3s to 76.4±4.7s). No amplitude difference was observed. Our data indicate a novel electrophysiological effect of testosterone in the developing brain, probably with important implications in excitability-related neurological diseases. Further studies shall investigate this hypothesis.

Keywords: testosterone, brain development, cortical spreading depression, cerebral excitability, rats.

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INTRODUÇÃO

A testosterona é o principal hormônio esteróide anabólico androgênico e apresenta um papel fundamental no desenvolvimento do cérebro. (Apostolinaset al., 1999) Este hormônio, predominante nos indivíduos do sexo masculino, apresenta numerosos papéis fisiológicos agindo sob o sistema nervoso central e seus tecidos-alvo periféricos (Li et al., 2012). Os efeitos da testosterona no sistema nervoso são cruciais para o desenvolvimento e comportamento sexual dos indivíduos, sendo responsável pelas diferenças entre os sexos (Janowsky, 2006). A síntese da testosterona, que éconsiderada um hormônio lipossolúvel, é feita a partir do colesterol. Sua lipo-solubilidade permite que a testosterona entre em todas as células do corpo, independente da existência de receptores androgênicos. Contudo, para modificar a função celular é preciso que a testosterona forme um complexo com seu receptor e influencie a síntese protéica. Ao final do processo de metabolização, a testosterona pode ser convertida em estradiol ou dihidrotestosterona. A conversão em estradiol ocorre pela ação da enzima aromatasee muitos dos seus efeitos neuroprotetores podem ser devidos a essa transformação (Galea, 2008).

Nos neurônios, a testosterona age como um neuroesteróide, podendo induzir mudanças em nível celular, que afetam o comportamento, memória, cognição e emoção (RaynaudandSchradin, 2014; Lacreuseet al., 2012; Seney, 2012). Alguns efeitos neuroprotetores da testosterona sob o sisteman nervosovêm sendo estudados (Aragnoet al., 2000; Fanaei, 2014). Alguns estudos em animais têm mostrado que, no período de desenvolvimento, uma exposição precoce aos hormônios testosterona e estrógeno pode causar alterações irreversíveis ao sistema nervoso (McCarthy et al., 2012). Esses efeitos irreversíveis ocorrem no chamado “período crítico” do desenvolvimento, que no caso do sistema nervoso parece ser uma “janela temporal” única (Pilgrim e Hutchison, 1994). Nesse período crítico define-se a distribuição dos receptores no cérebro, e a ação da testosterona pode alterar a expressão gênica desses receptores mais tarde, na idade adulta. Estudos envolvendo a testosterona no período pós-natal estão relacionados com desenvolvimento da linguagem e do comportamento. (Schaadt et al., 2015).

Um estudo realizado por Garcia-Segura et al. (1988) mostrou a relação entre os hormônios sexuais (durante o início da vida) e a modulação de células gliais. Nesse trabalho, foi observado

que ratos submetidos à orquidectomia no dia do nascimento possuiam menos marcação para GFAP, sugerindo que os hormônios gonadais durante o início podem modular o sistema glial.

É possível que os efeitos a longo prazo da testosterona sobre o desenvolvimento cerebral também envolvammodificações epigenéticas(Ghahramani et al., 2014). Os autores desse trabalho sugerem que a exposição à testosterona no início da vida afetam os padrões de metilação do cérebro durante a vida adulta.

Na Figura 1 pode-se comparar os processos de desenvolvimento do sistema nervoso, em humanos e ratos.

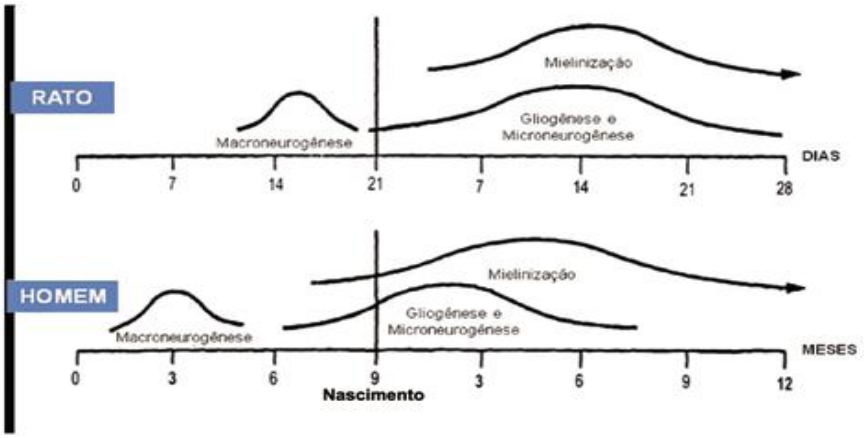


Figura 01: Comparação entre as etapas de desenvolvimento do sistema nervoso no homem e no rato. Adaptada de Morganeet al., 1993.

Os níveis de testosterona são aumentados substancialmente em indivíduos que utilizam doses elevadas de produtos anabólicos contendo esteroides (principalmente testosterona), para aumentar a massa muscular (Basualto-Alarconet al, 2013). Isso pode afetar negativamente o seu comportamento, como em alguns casos de suicídio(Thiblinet al, 1999; Zhang et al, 2015). Os mecanismos destes efeitos deletérios da testosterona ainda não estão totalmente elucidados. Com respeito às ações da testosterona sobre a excitabilidade neuronal, sabe-se que existe uma diferença entre sexos em relação à doença neurológica conhecida como Epilepsia. A incidência de epilepsia é aproximadamente 15% maior em homens do que em mulheres. Tem sido descrito, em homens com epilepsia, um desequilíbrio entre os níveis de testosterona e estrógenos (Herzog,1991; El-Khayat et al., 2003).

Em modelos experimentais de excitabilidade, a testosterona pode ter ação excitatória (Smith et al., 2002) ou inibitória (Eikermann-Haerter et al., 2009). Isto pode ser consequência do fato de que, no cérebro, a testosterona é convertida em metabólitos com atividades biológicas variadas. A aromatização da testosterona resulta na formação de estradiol, um hormônio que pode provocar convulsões (Herzog, 2008a). Por outro lado, a própria testosterona e seus metabólitos 5α- reduzidos podem inibir a atividade convulsiva, elevando os limiares de descarga no sistema límbico (Edwards et al., 1999), inibindo a excitação neuronal mediada pelo receptor-NMDA, e exercendo ação anti-convulsivante, possivelmente por efeitos diretos sobre receptores $GABA_{\mathbf{A}}$. As descargas convulsivas podem afetar o eixo hipotálamo-hipofisário e causar a liberação dos hormônios em momentos inadequados. Isso pode culminar em disfunção sexual, irregularidade menstrual e infertilidade. (Koppel e Harden, 2014). Por estas razões, o efeito da testosterona sob a excitabilidade cerebral merece ser mais investigado.

A diminuição da função sexual é comum em homens com epilepsia (Herzog, 2008b). Estudos anteriores têm sugerido que o tratamento, com testosterona, do hipogonadismo em homens epilépticos pode aumentar o controle das convulsões como também melhorar a função reprodutiva, através da adição de um inibidor de aromatase, para limitar a biossíntese de estradiol (Herzog, 1991; Herzog et al 1998; Reddy 2004). Sendo assim, a testosterona apresenta um grande potencial terapêutico para algumas doenças neurológicas.

Um modelo para o estudo das relações entre hormônios gonadais masculinos e excitabilidade cerebral constitui-se no fenômeno da depressão alastrante cortical (DAC), utilizado neste projeto. A DAC é um fenômeno eletrofisiológico caracterizado por uma onda de despolarização neuronal, em resposta à estimulação elétrica, mecânica, química, etc. de um ponto do córtex. O fenômeno se auto-propaga como uma onda com características eletrofisiológicas, iônicas, metabólicas e hemodinâmicas peculiares. É acompanhada por supressão transitória da atividade neuronal, plenamente reversível ao cabo de alguns minutos,. (Leão, 1944; Gorji, 2001; Lauritzenet al, 2011). Na Figura 2 (abaixo) podemos observar uma figura esquemática mostrando os eventos cíclicos da depressão alastrante cortical.

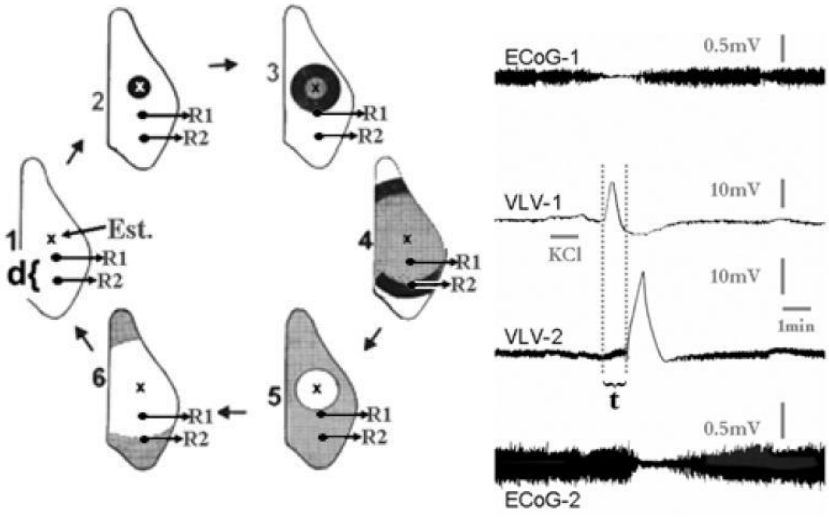


Figura 02: À esquerda observa-se a seqüência temporal cíclica dos eventos que ocorrem durante a propagação da DAC. R1 e R2 indicam pontos de registro. Um estímulo externo (x) deu início ao fenômeno (etapa 1) que se propaga de forma concêntrica (etapas 2-4). As áreas escuras (etapas 2, 3 e 4) representam áreas corticais na vigência do fenômeno, enquanto que as áreas quadriculadas (etapas 3 a 6) indicam o princípio da recuperação tissular. As áreas claras indicam o tecido recuperado (etapas 5-6), o que também ocorre de forma concêntrica, retornando à condição inicial (etapa 1). À direita observa-se o eletrocorticograma (ECoG) e a variação lenta de voltagem (VLV), esta última presente durante a DAC, quando o ECoG diminui sua amplitude. Tais registros, obtidos em nosso laboratório foram feitos simultaneamente nos pontos R1 e R2. Observe a recuperação do ECoG após a passagem do fenômeno (Guedes et al., 2004).

Estudos *in vitro* sugerem que a aplicação de testosterona em fatias de hipocampo de ratos gonadectomizados (tanto machos quanto fêmeas) aumenta a excitabilidade neuronal (Smith et al., 2002). Em ratos (FMH1) mutantes, cujo maior favorecimento à DAC é geneticamente induzido, mostrou-se que a testosterona agindo através de receptores de andrógenos, suprime este efeito facilitador daDAC. Portanto, os androgênios parecem poder modular a susceptibilidade à DAC, podendo assim fornecer uma nova estratégia profilática ou terapêutica para a enxaqueca

(Eikermann-Haerteret al, 2009). No entanto, inexistem informações sobre o efeito do excesso da testosterona atuando no cerébro em desenvolvimento sobre a DAC. Este é o objetivo central desta dissertação, como descrito mais abaixo.

O "Laboratório de Fisiologia da Nutrição Naíde Teodósio" (LAFINNT), do Departamento de Nutrição do CCS/UFPE, vem desenvolvendo uma linha de pesquisa em que se tem demonstrado que, assim como o estado nutricional, outras condições não nutricionais de importância clínica que influenciam a excitabilidade neuronal, como por exemplo a ação de certos hormônios, podem interferir com a incidência da DAC e sua propagação; este é o caso do hormônio tireoidiano (Guedes e Pereira-da-Silva, 1993) e da insulina (Costa-Cruz e cols, 2006). Estudos prévios têm mostrado que alterações hormonais durante o desenvolvimento influenciam a propagação da DAC (Guedes e Pereira-da-Silva, 1993; Santos, 2000)

Em um trabalho anterior realizado em nosso laboratório, nós estudamos o efeito da ovariectomia, realizada no início da vida, sobre a DAC avaliada em ratas adultas. Nesse trabalho, observou-se uma redução da velocidade de propagação do fenômeno (Accioly et al, 2012). Em trabalhos mais recentes, também realizados em nosso laboratório, a administração de glicocorticóides no período neonatal foi capaz de acelerar a velocidade de propagação da DAC, sendo revertida pelo efeito de vitaminas antioxidantes no cérebro. (Lopes-de-Morais, 2014).

As Tabelas 1 e 2 apresentam diversas condições, já estudadas, que podem dificultar ou facilitar a propagação da DAC.

Tabela 01: Algumas condições que <u>dificultam</u> a propagação da DAC

Condição experimental	**Autor/Ano**
Tratamento dietético com lítio	Guedes et al., 1989
Hiperglicemia	Ximenes-da-Silva e Guedes,1991; Costa-Cruz et al., 2001
Anestésicos	Guedes e Barreto, 1992
Hipotireoidismo	Guedes e Pereira-da-Silva, 1993

Envelhecimento	Guedes et al., 1996
Dieta hiperlipídica	Paixão et al., 2007
Epilepsia crônica provocada pela pilocarpina	Guedes e Cavalheiro, 1997; Costa-Cruz et al., 2006
Estimulação ambiental	Santos-Monteiro et al., 2000
Ativação do Sistema Serotoninérgico	Guedes et al., 2002; Amâncio-dos-Santos et al., 2006
Estimulação Elétrica Cerebral direta e trans-craniana	Fregniet al., 2005; 2007
Condições favoráveis de aleitamento	Rocha-de-Melo et al., 2006

Tabela 02: Algumas condições que facilitam a propagação da DAC

Condição experimental	**Autor/Ano**
Redução do Cloreto extracelular	Guedes e Do Carmo, 1980
Privação do sono paradoxal	Vasconcelos et al., 2004
Diazepam	Guedes et al., 1992
Etanol	Guedes e Frade, 1993; Bezerra et al., 2005
Deficiência nutricional pela DBR	Rocha-de-Melo e Guedes,1997
Hipertireoidismo	Santos, 2000
Hipoglicemia	Ximenes-da-Silva e Guedes,1991
Privação sensorial	Tenório et al., 2009
Arginina durante o desenvolvimento	Maia et al., 2009
Hipertermia ambiental	Farias-Santos et al., 2009
Glutamina durante o desenvolvimento	Lima et al., 2009
Uso de dipirona no início da vida	Amaral et al., 2009

Frente ao exposto, o presente trabalho se propôs a estudar o efeito do tratamento com testosterona durante o desenvolvimento cerebral sobre a DAC em ratos adultos. Este trabalho é a continuação de uma linha de pesquisa do "Laboratório de Fisiologia da Nutrição Naíde Teodósio" (LAFINNT) que utiliza o fenômeno da DAC para estudar o efeito de fatores nutricionais, ambientais, farmacológicos e hormonais sobre o cérebro em desenvolvimento

OBJETIVOS

Geral

Efetuar o tratamento precoce com testosterona (nas diferentes semanas do aleitamento) e avaliar, na idade adulta, os possíveis efeitos sobre o desenvolvimento eletrofisiológico do sistema nervoso, através da susceptibilidade cortical à DAC.

Específicos

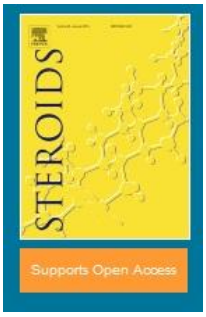
-Nas condições acima, avaliar a evolução ponderal, como indicador de efeitos sobre o desenvolvimento do organismo;

- Registrar eletrofisiologicamente a DAC, analisando-lhe alterações na velocidade de propagação, na amplitude e na duração da variação lenta de voltagem, que acompanha o fenômeno, como indicadores de alterações eletrofisiológicas cerebrais;

- Ao final do registro da DAC, avaliar o peso do cérebro, como indicador de alterações do seu desenvolvimento.

RESULTADOS – ARTIGO ORIGINAL

Submetido à revista STEROIDS



Title: Neonatal testosterone and brain development: electrophysiological effects on spreading depression in the adult rat cortex

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Abstract

Testosterone exerts important effects on brain development and functioning, including modulation of the neuronal activity and excitability. In this study, we investigated in adult rats the effects of neonatal treatment with testosterone, at three distinct time-points, on the brain excitability-related phenomenon known as Cortical Spreading Depression (CSD). Four groups of male Wistar newborn rats received daily intraperitoneal injections with 10 mg/kg/d propionate testosterone at the second, third, fourth, or second+third+fourth week of life. Under anesthesia (1g/kg urethane+40 mg/kg chloralose, i.p) we elicited CSD at 20min intervals and recorded it at two points of the cortical surface during 4 hours. During the recording session, we measured the CSD parameters (velocity of propagation, amplitude and duration of the DC-negative CSD signal). Compared with control groups treated with the vehicle (olive oil) at the corresponding postnatal weeks, as well as with a naïve (not treated) group, testosterone-treated animals presented with significantly lower ($p<0.05$) CSD velocities. The CSD velocity ranged from 2.76±0.05 to 2.99±0.29 mm/min in the testosterone-treated groups, and from 3.13±0.03 to 3.37±0.07 mm/min in the control animals. The lower CSD velocity in the testosterone groups was accompanied by significantly longer duration of the CSD wave (range: 84.3±6.0s to 93.1±10.3s) compared with the vehicle and naïve controls (range: 73.3±5.3s to 76.4±4.7s). No amplitude difference was observed. Our data indicate a novel electrophysiological effect of testosterone in the developing brain, probably with important implications in excitability-related neurological diseases.

Keywords: testosterone, brain development, cortical spreading depression, cerebral excitability, rats.

Highlights

Title:

Neonatal testosterone and brain development: electrophysiological effects on spreading depression in the adult rat cortex

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Highlights:

- We studied spreading depression in rats neonatally treated with testosterone for 7 days
- Neonatal testosterone decelerates cortical spreading depression (CSD) at adulthood
- Testosterone injected at postnatal week 2, 3 or 4 impaired equally CSD propagation
- Treatment during weeks 2+3+4 led to the same outcome suggesting no additive effect
- Data may help to understand testosterone-based mechanisms in excitability diseases

Introduction

During the brain development in mammals, circulating testosterone has organizational effects leading to permanent male phenotype [1]. Testosterone plays a substantial role in a number of physiological processes required for brain development and functioning [2-9] and this includes brain excitability[10]. However, the brain excitability effects of testosterone are still a matter of investigation. Several recent studies suggest that testosterone has neuroprotective effects, including prevention of neuronal death in experimental models that involve injuries of the nervous system [3,11-12]. On the hippocampus, testosterone has excitatory effects [13]. Such excitatory effects can enhance neuronal excitability, increasing circuit activity and seizure susceptibility [10] In order to evaluate the action of high levels of testosterone during distinct time-points within the critical period of brain development, we treated developing rats with testosterone at the second, third, or fourth week of postnatal life, and investigated, when the pups reached adulthood, the electrophysiological effects on the excitability-related brain phenomenon known as Cortical Spreading Depression (CSD).

CSD is a neural phenomenon, which is influenced by changes in the brain excitability. In the cerebral cortex, it is a responseconsequent to electrical, mechanical or chemical stimulation of one point of the tissue, consisting of a reversible and slowly propagating “wave” of reduction of the spontaneous and evoked cortical electrical activity, with a simultaneous DC slow potential change of the tissue [14,15]. It has been demonstrated in a number of vertebrate animals [16], including the human species [17]. Because CSD is a phenomenon related to neural excitability, several authors consider that CSD is important to study the pathophysiology of the brain, and have associated CSD to neurological diseases such as epilepsy, brain ischemia and migraine [18-20].

Our hypothesis is that theincreased testosterone levels, during the period of brain development, is causally associated in adulthood with impairment of CSD propagation.

Material and methods

Animals

The animals in this study (n=78) were handled in accordance with the norms of the Institutional Ethics Committee for Animal Research of our university (Approval protocol no. 23076.017033/2013-33). These norms comply with the "Principles of Laboratory Animal Care" (National Institutes of Health, Bethesda, USA). Animals were reared in polypropylene cages (51 cm x 35.5 cm x 18.5 cm) in an air-conditioned room maintained at 23±1°C with a 12h light/ 12h dark cycle (lights on at 7:00 a.m.), and free access to water and food. Eight groups of Wistar male newborn rats were used: naïve [Nv] rats without any treatment (n=9), three groups injected with 10 mg/kg/d testosterone propionate dissolved in olive oil during the second, or third, or fourth week of postnatal life (groups T2, T3 and T4; n=9, 11 and 10 respectively), three control groups injected with the vehicle (olive oil) during the second, or third, or fourth week of postnatal life (groups V2, V3 and V4; n=10, 12 and 9 respectively), and a group treated with testosterone during the second+third+fourth weeks of postnatal life (group T2+3+4; n=8). The dose and the timing of neonatal exposure to testosterone were based on data from the literature [21,22]. The distribution of the eight groups is shown in Table 1.

Please insert Table 1 about here

CSD recording

When the pups were 90 to 120 days old, they were anesthetized with 1 g/kg urethane plus 40 mg/kg chloralose, i.p., and three trephine holes (2–3 mm in diameter) were drilled on the right side of the skull (two at the parietal bone and one at the frontal bone). The three holes were aligned in the frontal-to-occipital direction and were parallel to the midline. CSD was elicited at 20 min intervals by 1-min application of a cotton ball (1–2 mm diameter), soaked in 2% KCl solution (approximately 0.27 M) to the anterior hole drilled at the frontal region. The slow DC potential change that is generated during CSD was recorded at the two parietal points on the cortical surface by using a pair of Ag-AgCl agar-Ringer electrodes. These electrodes consisted of plastic conic pipettes (5 cm length, 0.5 mm tip inner diameter), filled with Ringer solution solidified with the addition of 0.5% agar, into which a chlorided silver wire was inserted. The pair of recording pipettes was fixed with cyanoacrylate glue, so that the interelectrode distance was kept constant. For distinct pairs, the interelectrode distance ranged from 4.0 mm to 5.5 mm. A common reference electrode, of the same type, was placed on the nasal bones. The velocity of CSD propagation was calculated based on the time required for a CSD wave to cross the distance between the two recording electrodes. In the measurement of CSD velocities, the initial point of each DC negative rising phase was used as the reference point. We also measured the amplitude and duration of the DC potential change of CSD. During the recording session, rectal temperature was maintained at 37±1ºC by means of a heating blanket.

Statistics

Body-, brain-, testicles- and adrenal weights, and CSD parameters were compared between groups by Anova, followed by a post hoc (Holm–Sidak) test when indicated. The statistical software used was "Sigmastat® version 3.10. Differences were considered significant when $P \leq 0.05$.

Results

Weights

Body, brain and adrenal weights did not differ between the groups. In the T2+3+4 group, testicles presented with lower weights (2.52±0.29 g) compared with the other control groups, whose weights ranged from 2.92±0.19 g to 3.01±0.29 g.

CSD propagation

Figs. 1, 2 and 3 show recordings of the slow DC potential shift that is generated during CSD, corresponding to the treatment with testosterone and vehicle during the second, third and fourth weeks of postnatal life. As a rule, the 1-min stimulation with 2% KCl at one point of the frontal cortex elicited a single CSD wave that propagated without interruption and was recorded by the two electrodes located more posterior in the parietal cortex (see stimulation- and recording-points in the skull diagram of the figures).

Please insert Figures 1, 2 and 3 about here

In the testosterone groups, CSD propagated with significantly lower velocity (mean±standard deviation: 2.84±0.18mm/min, 2.99±0.29mm/min, 2.80±0.14mm/min and

2.76±0.05mm/min for the groups T2, T3, T4 and T2+3+4, respectively) compared to the Naïve and to the corresponding Vehicle controls (respectively 3.34±0.04mm/min, 3.35±0.07mm/min, 3.37±0.07mm/min and 3.31±0.03mm/min; $p<0.005$, ANOVA followed by Holm-Sidak test). No significant difference was observed between the distinct testosterone-treated groups (Figure 4).

Please insert Figures 4 about here

CSD amplitude and duration

Table 2 presents the mean values for the amplitudes and durations of the negative DC-slow potential shift of CSD in the control and experimental groups. Amplitudes were similar in all groups, whereas the durations were shorter ($P< 0.01$) in the experimental groups T3, T4 and T2+3+4 compared with the naïve group, and their corresponding vehicle-treated controls.

Please insert Table2 about here

Discussion

The main electrophysiological outcome of the present study was that increasing the testosterone levels during brain development reduced the brain capability to propagate CSD in adulthood, as indexed by its lower velocity of propagation, in comparison to the vehicle and naïve controls. The duration of the CSD wave in the testosterone groups was longer than in the controls (Table 2), and this also reinforces the above conclusion.

We chose the neonatal period to make this intervention because of the brain organizational actions of sex hormones early in life. As the present CSD effect was found at adulthood, we conclude that the early testosterone treatment had long lasting action on the brain.

The gonadal steroid hormones, especially testosterone, exert profound neuromodulatory actions upon neurons during development and in adulthood. Our results demonstrated for the first time that the treatment with testosterone propionate during the critical period of brain development is able to reduce the propagation of CSD in the rat, and this effect endures in adulthood. We also showed that prolonged administration of testosterone (group T 2+3+4) did not further reduce CSD velocity compared with the groups treated during one week, suggesting the absence of an additive effect on CSD propagation. However, it reduced the weights of testicles, but not of body and brain weights. The reduction in testicle weight confirms previous data [23]. According to these authors, the treatment with exogenous testosterone elevated the circulating levels of this hormone, leading to reduction of intrinsic testosterone production as reflected in a decreased testicular weight.

Several findings from others suggest a steroid/CSD relationship. For example, the CSD susceptibility in familial hemiplegic migraine type 1 knockin mice is higher in females than in males; ovariectomy reverses this gender difference, which is partially restored by estradiol replacement, suggesting that actually estrogens modulate CSD susceptibility [24]. In addition, removal of the ovaries during the early life impaired CSD at adulthood in rats [25], indicating that the decrease in ovarian hormones is capable to impair the CSD propagation. The sex differences in brain excitability appear to be associated with sex-related differences in the brain immune system; female rats on P30 have more microglia with an activated phenotype than males. Moreover, behavioral disorders such as depression and anxiety, that in males usually prevail at late ages, in females often appear earlier (in the adolescence) [26]. Accordingly, behavioral studies in early testosterone-treated male rodents reveal positive effects on anxiety, memory and

spatial learning [7, 27-28]. In male Rhesus monkeys, positive effects of testosterone on visual recognition memory have been reported even after acute testosterone treatment [8]

Although in this study we did not monitor blood testosterone levels, we consider reasonable to assume that the long-term treatment with 10 mg/kg/d testosterone might in all probability have imbalanced brain testosterone levels, due to the increase of testosterone levels in the blood, as previously demonstrated [29] These authors reported significantly increased blood levels of testosterone after treating rats with a testosterone dose that was ten times lower than the present one.

Several mechanisms could explain our findings. However, we hypothesize that the most likely mechanisms are based on the non-genomic actions of testosterone by activating the $GABA_A$receptors [30, 31]) and serotonin receptors [32]. The GABAergic function is modulated by steroids increasing $GABA_A$ receptor opening frequency and duration [33]. In mature neurons, the $GABA_A$ receptor mediates fast synaptic inhibition by gating the influx of chloride ions, thereby hyperpolarizing the cell and reducing action potential firing. We suggest that this would be a possible mechanism whereby testosterone could antagonize CSD.

The action of testosterone on the serotoninergic system includes antidepressant effects [34, 35]. The administration of this hormone increases the genomic expression of serotonin enzymes and transporters[36]. By this way testosterone could impair CSD propagation, as the pharmacological enhancement of serotoninergic activity [37-39], and the administration of the serotonin precursor tryptophan [40] impair CSD in rats. On the other hand, rats treated with the serotonin reuptake enhancer tianeptine present with increased CSD velocity as compared with vehicle-injected controls [41].

Another plausible mechanism to explain the testosterone effect on CSD is represented by the protective action of testosterone against oxidative stress, acting like antioxidant in the central nervous system [42]. A study in aged rats showed that chronic treatment with propionate testosterone activated antioxidant pathways and muscular strength [43] Several pieces of evidence convincingly demonstrate that antioxidant molecules counteract CSD in a dose-dependent manner [44, 45], whereas prooxidant conditions facilitate CSD elicitation and propagation [44,46]

The possible clinical implications of our findings deserve some comment. Processes such as stress, epilepsy, anxiety, depression, memory and myelin-degeneration suffer positive influence from testosterone[33, 47].The action of testosterone on CSD (present results) and on excitability-related neurological diseases [33,48] might share important relationship in terms of common mechanisms [20]. This raises the question on the potential therapeutic uses of testosterone, and maybe other neurosteroids, in certain neurological diseases, or genetic- and injury-induced disturbances [29].

We conclude that this study documents a novel electrophysiological action of testosterone on CSD in the rat brain. Data support the following four primary conclusions: first, treatment with testosterone for relatively short periods (7 days) within the critical period of brain development decelerates CSD; second, this effect was observed later in the adulthood, indicating that it is a long-lasting action of testosterone; third, treatments on the second, third and fourth weeks of life resulted in similar outcome, suggesting that the three periods are equally susceptible to the effects of testosterone on CSD; fourth, treatment during a longer period (second to fourth postnatal weeks) resulted in the same CSD effect as the treatments for one week, suggesting absence of additive effect.Our findings highlight the importance of further searching for the

molecular mechanisms underlying the action of testosterone on brain development and their electrophysiological properties.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Tables

Groups	Treatment Condition	Week of life
1 (Nv)	Naïve	no treatment (9)
2 (T2)	Testosterone	2nd (9)
3 (T3)	Testosterone	3rd (11)
4 (T4)	Testosterone	4th (10)
5 (V2)	Vehicle (oliveoil)	2nd (10)
6 (V3)	Vehicle (oliveoil)	3rd (12)

Table 1- Distribution of the 8 experimental groups according to treatment condition (Nv, naïve; T, testosterone; V, vehicle), and the week of life in which treatment was applied (2^{nd}, 3^{rd}, 4^{th} and 2^{nd}+3^{rd}+4^{th} weeks of life, respectively). Numbers in parentheses indicate the number of rats studied.

Amplitudes and durations of the CSD slow potential shifts in the Naïve (Nv), vehicle-treated (V2, V3 and V4) and testosterone-treated groups of rats (T2, T3, T4 and T2+3+4).

GROUP	AMPLITUDE (mV)	DURATION (s)
Nv (naïve)	9.31±2.42	73.27±5.34
V2 (vehicle at postnatal week 2)	9.41±3.45	76.42±4.73
V3 (vehicle at postnatal week 3)	9.36±3.12	75.41±3.06
V4 (vehicle at postnatal week 4)	9.29±3.84	75.50±6.09
T2 (testosterone at postnatal week 2)	9.88±3.54	84.30±5.99
T3 (testosterone at postnatal week 3)	12.98±5.46	92.40±11.98*
T4 (testosterone at postnatal week 4)	11.05±3.58	93.14±10.30*
T2+3+4 (testosterone at postnatal weeks 2+3+4)	11.38±4.59	89.37±7.10*

Data are expressed as mean ±standard deviation.

* Testosterone values that are significantly different from the corresponding vehicle and Naïve groups ($P< 0.01$; ANOVA plus Holm-Sidak test).

Figures

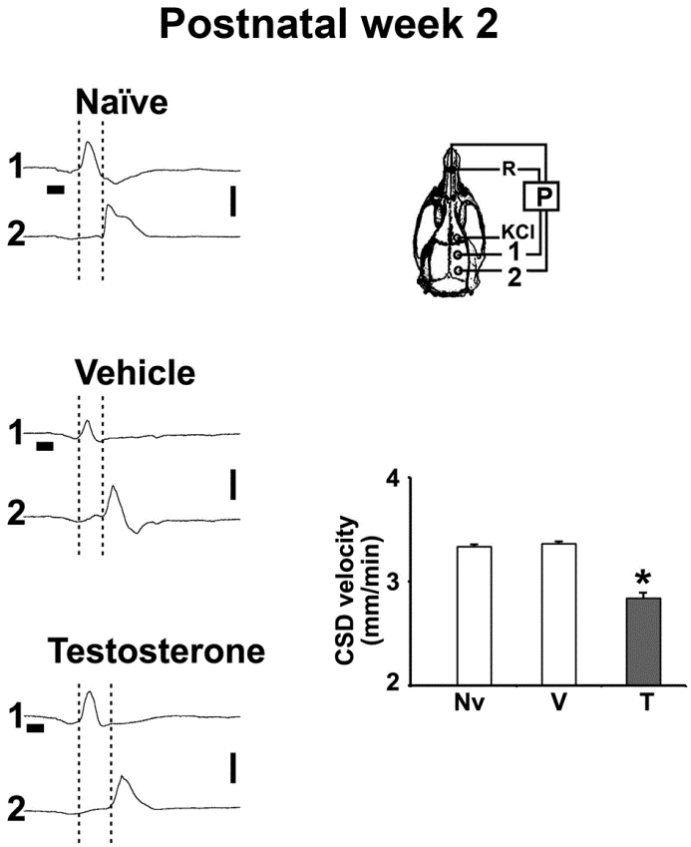

Fig.1. Slow potential change (P) recordings during cortical spreading depression (CSD) in the right hemisphere of three 90-120day-old rats, which received no treatment (naïve), or a daily intraperitoneal injection of vehicle (olive oil), or 10 mg/kg/d during the **second** week of postnatal life. The top-right drawing depicts the stimulation point (where 2% KCl was applied to elicit CSD) and the recording positions 1 and 2, as well as the position of the common reference electrode (R).The horizontal bars under the 1-traces indicate the period (1 min) in which KCl stimulation was applied to the frontal region of the same hemisphere.Vertical bars correspond to 10 mV (negative upwards).The vertical dashed lines indicate the latency for a CSD wave to cross the interelectrode distance. The latencyislonger in thegroups treated with testosteronewhen compared with the respective naïve and vehicle controls. The bottom-right bar graphic presents the mean±SEM velocity for each group. * lower velocity compared with the naïve and vehicle values (P<0.001 ANOVA followed by the Holm-Sidak test).

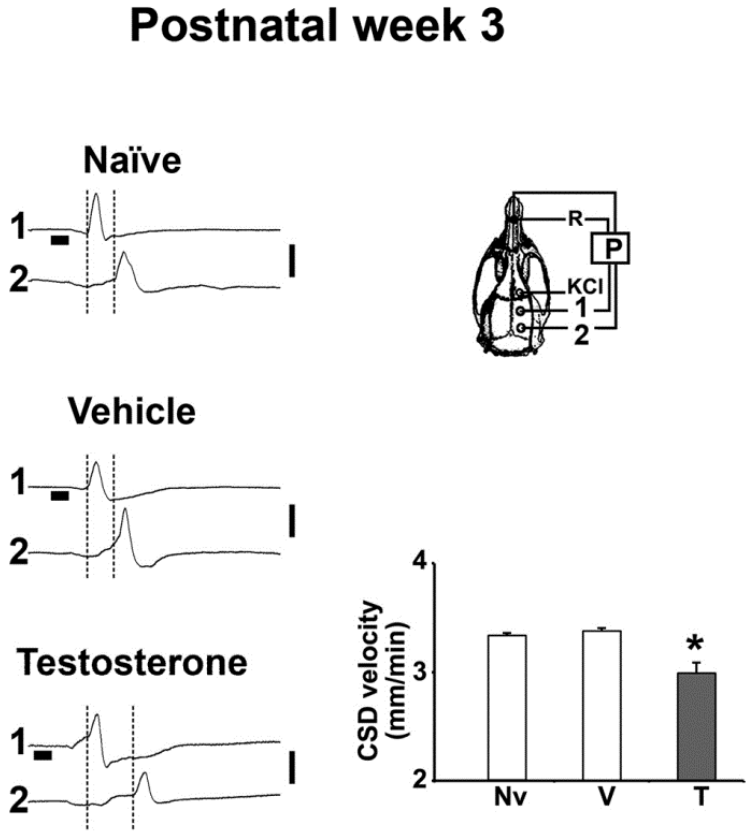

Fig.2. Slow potential change (P) recordings during cortical spreading depression (CSD) in the right hemisphere of three 90-120day-old rats, which received no treatment (naïve), or a daily intraperitoneal injection of vehicle (olive oil), or 10 mg/kg/d during the **third** week of postnatal life. The top-right drawing depicts the stimulation point (where 2% KCl was applied to elicit CSD) and the recording positions 1 and 2, as well as the position of the common reference electrode (R).The horizontal bars under the 1-traces indicate the period (1 min) in which KCl stimulation was applied to the frontal region of the same hemisphere.Vertical bars correspond to 10 mV (negative upwards).The vertical dashed lines indicate the latency for a CSD wave to cross the interelectrode distance. The latencyislonger in thegroups treated with testosteronewhen compared with the respective naïve and vehicle controls. The bottom-right bar graphic presents the mean±SEM velocity for each group. * lower velocity compared with the naïve and vehicle values (P<0.001 ANOVA followed by the Holm-Sidak test).

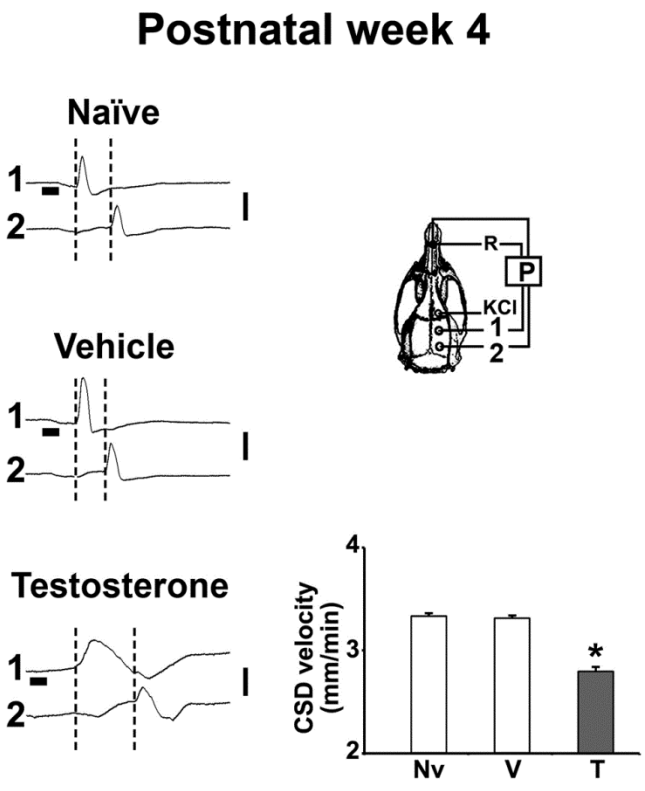

Fig.3. Slow potential change (P) recordings during cortical spreading depression (CSD) in the right hemisphere of three 90-120day-old rats, which received no treatment (naïve), or a daily intraperitoneal injection of vehicle (olive oil), or 10 mg/kg/d during the **fourth** week of postnatal life. The top-right drawing depicts the stimulation point (where 2% KCl was applied to elicit CSD) and the recording positions 1 and 2, as well as the position of the common reference electrode (R).The horizontal bars under the 1-traces indicate the period (1 min) in which KCl stimulation was applied to the frontal region of the same hemisphere.Vertical bars correspond to 10 mV (negative upwards).The vertical dashed lines indicate the latency for a CSD wave to cross the interelectrode distance. The latencyislonger in thegroups treated with testosteronewhen compared with the respective naïve and vehicle controls. The bottom-right bar graphic presents the mean±SEM velocity for each group. * lower velocity compared with the naïve and vehicle values (P<0.001 ANOVA followed by the Holm-Sidak test).

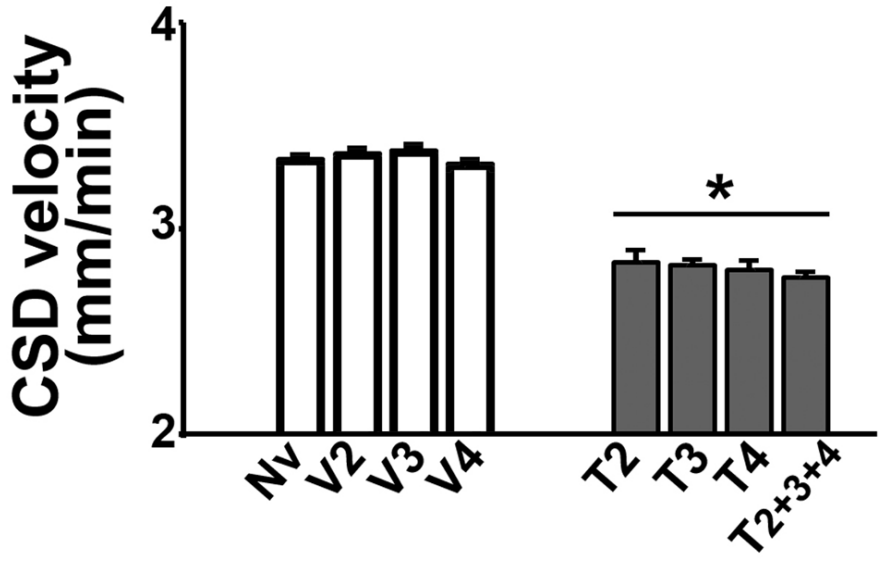

Fig.4. Comparison of the mean±SEM CSD velocities in the eight groups of this study. Note that the four groups treated with testosterone (right) display significantly lower CSD velocities as compared with the four control groups (left). * P<0.001; ANOVA followedbythe Holm-Sidak test).

CONCLUSÕES

A análise dos resultados desta dissertação permite as seguintes conclusões .

- O tratamento com testosterona por períodos relativamente curtos (7 dias) dentro do período crítico de desenvolvimento cerebral desacelerou a DAC, a julgar pela menor velocidade de propagação, e maior duração da variação lenta de voltagem, que acompanha o fenômeno, em comparação com os grupos controle;

- O efeito desacelerador da DAC, provocado pela testosterona, foi observado na idade adulta, indicando que se trata de um efeito duradouro;

- Os tratamentos na segunda, terceira e quarta semana de vida resultaram em efeitos semelhantes sobre a DAC, sugerindo que nos três períodos o cérebro é igualmente susceptível a essa ação da testosterona;
- O tratamento mais longo (da segunda até a quarta semana de vida) resultou no mesmo efeito sobre a DAC que o tratamento por apenas uma semana, sugerindo ausência de efeito aditivo;

- Sendo assim, nossos dados indicam um novo efeito eletrofisiológico no cérebro em desenvolvimento, provavelmente com importantes implicações em doenças neurológicas relacionadas à excitabilidade. Estudos futuros deverão investigar essa hipótese.

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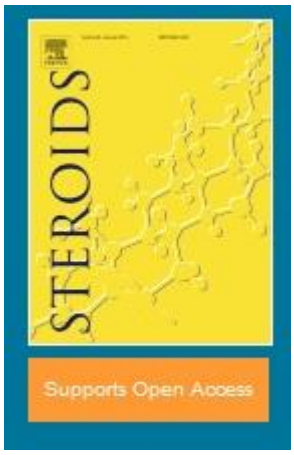
ANEXO 1: Guia para autores



STEROIDS

Article structure

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Essencial title page information

Title

Author names and affiliations

Corresponding author

Present/permanentaddress

References

ANEXO 2: Parecer do Comitê de Ética em Pesquisa

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Recife, 23 de Maio de 2013.

Ofício nº 572/13

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof Rubem Carlos Guedes**
Departamento de Nutrição
Universidade Federal de Pernambuco
Processo nº 23076.017033/2013-33

Os membros da Comissão de Ética no Uso de Animais do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEUA-UFPE) avaliaram seu projeto de pesquisa intitulado, **"Influência dos hormônios androgênicos sobre o desenvolvimento neural: análise eletrofisiológica".**

Concluímos que os procedimentos descritos para a utilização experimental dos animais encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo National Institute of Health Guide for Care and Use of Laboratory Animals as quais são adotadas como critérios de avaliação e julgamento pela CEUA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 11.794 de 08 de outubro de 2008, que trata da questão do uso de animais para fins científicos e didáticos.

Diante do exposto, emitimos **parecer favorável** aos protocolos experimentais a serem realizados.

Origem dos animais: Biotério do Departamento de Nutrição/UFPE. Animais: ratos; Linhagem: Wistar; Idade: 7-90 dias; Peso: 15-300g; Sexo: Machos; Nº total de animais: 70 ratos.

Atenciosamente,

Profa. Maria Teresa Jansen
Presidente do CEEA

ANEXO 3: Apresentação de trabalho em congresso

SBNeC

XXXVIII Reunião Anual da SBNeC

10 a 13 de Setembro de 2014, Búzios - RJ

Certificamos que

REGINA DE DEUS LIRA BENEVIDES participou da

XXXVIII Reunião Anual da Sociedade Brasileira de Neurociências e Comportamento - SBNeC, realizada no Centro de Convenções do Hotel Atlântico Búzios em Armação de Búzios - Rio de Janeiro, de 10 a 13 de setembro de 2014.

Cecília Hedin Pereira
Presidente da SBNeC

ANEXO 4: Comprovante de submissão do artigo

From: **Steroids**<steroids@elsevier.com>
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Subject: Submission Confirmation
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