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NUTRIÇÃO, HORMÔNIOS OVARIANOS E DESENVOLVIMENTO CEREBRAL: ANÁLISE ELETROFISIOLÓGICA PELA DEPRESSÃO ALASTRANTE CORTICAL

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Tese apresentada ao Programa de Pós-Graduação em Bioquímica e Fisiologia do Centro de Ciências Biológicas da Universidade Federal de Pernambuco para obtenção do título de Doutora em Fisiologia e Bioquímica.

Orientador: Prof. Dr. Rubem Carlos Araújo Guedes

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Noranege Epifânio Accioly

“Nutrição, hormônios ovarianos e desenvolvimento cerebral: análise eletrofisiológia pela depressão alastrante cortical”

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

Aprovado por:

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Dedico este trabalho à minha vó e aos meus pais como gratidão e reconhecimento por todo amor, ensinamentos, paciência e dedicação durante toda a minha vida.

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RESUMO

Há evidências clínicas e experimentais de que os hormônios ovarianos exercem profundas e duradouras ações sobre o cérebro, tanto durante o seu desenvolvimento, quanto após essa fase (cérebro adulto). Algumas dessas ações repercutem de forma importante sobre a excitabilidade cerebral. Em trabalho anterior, filhotes de ratas Wistar aos 7 dias pós-natais foram submetidas à ovariectomia bilateral (grupo ovx) ou pseudo-cirurgia de ovariectomia (grupo sham) ou nenhuma cirurgia (grupo ingênuo). Quando os filhotes alcançaram a idade adulta (90-120 dias) o grupo ovx e os dois grupos controles (sham e ingênuo-na fase proestro) foram submetidos ao registro da Depressão Alastrante Cortical (DAC), fenômeno que é influenciado pela excitabilidade do cérebro. No grupo ovx, a DAC se propagou com velocidades significativamente menores em comparação aos dois grupos controle. Neste trabalho continuou-se essa linha de investigação. Inicialmente, demonstrou-se que a ovariectomia na vida adulta não se acompanha dos efeitos, observados em ratas com ovariectomia precoce. Esses dados, juntados àqueles obtidos durante o mestrado, originaram um artigo publicado na revista “International Journal of Developmental Neuroscience”. Em seguida, estudou-se os efeitos, sobre a DAC, da interação entre a administração de hormônios ovarianos e condições desfavoráveis de lactação (amamentação em ninhadas de grande tamanho). Ratas Wistar foram tratadas com estradiol ou progesterona ou ambos dos 7 aos 21 dias de vida ou submetidas, na vida adulta, a aplicações tópicas, no córtex cerebral, de diferentes concentrações de estradiol ou progesterona durante o registro da DAC. Ao contrário do observado anteriormente em ratas ovariectomizadas, o tratamento sistêmico ou tópico com ambos os hormônios acelerou a propagação da DAC, em comparação com ratas controle, tratadas com o veículo. No caso da aplicação tópica, o efeito apresentou-se reversível e dependente da dose aplicada. Sugere-se que tais efeitos estão relacionados com a açzão dos hormônios ovarianos sobre a excitabilidade cerebral.

PALAVRAS-CHAVE: Hormônios ovarianos. Depressão alastrante cortical. Desenvolvimento cerebral. Ratas.

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ABSTRACT

There is experimental and clinical evidence that ovarian hormones exert profound and lasting action on the brain, both during its development, and after this phase (adult brain). Some of these actions have repercussions significantly on brain excitability. In previous work, offspring of female rats at 7 postnatal days were subjected to bilateral ovariectomy (OVX group) or ovariectomy pseudo- surgery (sham group) or no surgery (naive group). When the pups reached adulthood (90-120 days) the OVX group and the two control groups (sham and naive in the proestrus phase) were subjected to the recording of Cortical Spreading Depression (CSD), a phenomenon that is influenced by the excitability of the brain. In the OVX group, the CSD spread with significantly lower rates compared to the two control group.it was demonstrated that ovariectomy in the period of brain development reduced the spread of cortical spreading depression (CSD), a phenomenon which is influenced by brain excitability. This work continued this line of research. Initially, it was demonstrated that ovariectomy in adult life is not accompanied by the effects observed in rats with early ovariectomy. These data, coupled to those obtained during the master, gave an article published in the "International Journal of Developmental Neuroscience" journal. Then studied the effects on the CSD, the interaction between the administration of ovarian hormones and unfavorable conditions of lactation (suckling in large litters). Wistar rats were treated with estradiol or progesterone or both from 7 to 21 days or submitted in adult life, topical applications, the cerebral cortex, different concentrations of estradiol or progesterone during registration of CSD. Unlike the previously observed in ovariectomized rats, the systemic or topical treatment with both hormones accelerated the velocity of the CSD compared to control rats treated with the vehicle. In the case of topical application, the effect is reversible and showed dependent on the applied dose. It is suggested that these effects are related to the action of ovarian hormones on brain excitability.

Keywords: Ovarian hormones. Cortical spreading depression. Brain development. Female rats.

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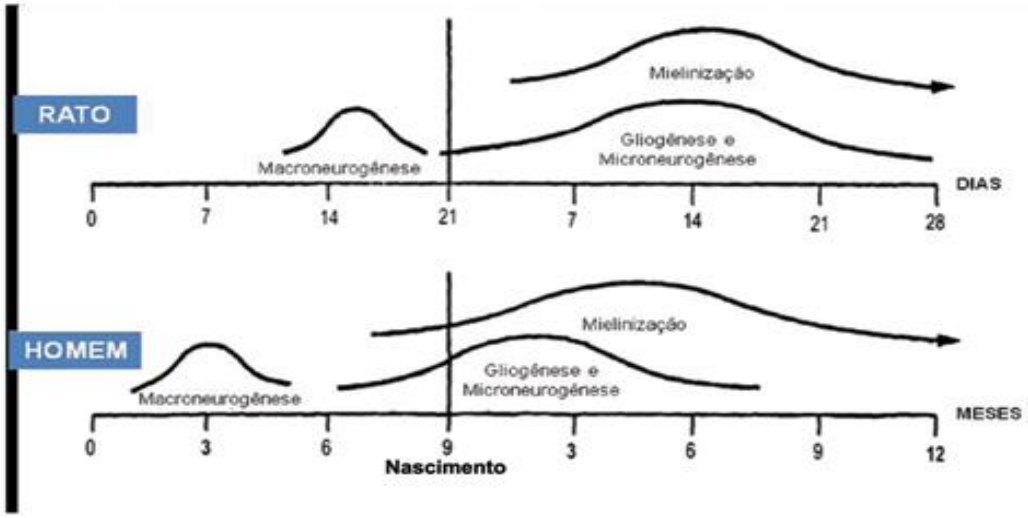
1- INTRODUÇÃO

O sistema nervoso central é um alvo importante para as ações dos hormônios ovarianos desde o desenvolvimento à vida adulta (MELCANGI et al., 2014; AREVALO et al., 2015). Variações sistêmicas dos hormônios ovarianos podem influenciar o cérebro, pois eles podem atravessar a barreira hematoencefálica e exercer efeitos profundos sobre a função cerebral (EIKERMANN-HAERTER et al., 2007). Durante o desenvolvimento, esses efeitos hormonais incluem vários mecanismos com importantes repercussões nas propriedades eletrofisiológicas do cérebro adulto (SCHARFMAN AND MACLUSKY, 2014).

No sistema nervoso embrionário, os processos de neurogênese, gliogênese e migração neuronal ocorrem mais intensamente na fase chamada de "período de crescimento rápido do cérebro" ou simplesmente "período crítico". Essa fase é considerada crítica para o perfeito desenvolvimento e funcionamento neurológico, sendo uma etapa de grande vulnerabilidade do cérebro a agressões internas e/ou externas (DOBBING, 1968). Tal fase acha-se compreendida entre o terceiro trimestre gestacional e o segundo ano de vida, no homem, e corresponde ao período do aleitamento, no rato (Fig. 1; MORGANE et al., 1993).

Figura 1 - Comparação entre as etapas de desenvolvimento do sistema nervoso no homem e no rato.



Adaptada de MORGANE et al., 1993.

Nos últimos anos, o conhecimento de como os hormônios ovarianos interferem no desenvolvimento das funções cerebrais dos mamíferos tem aumentado substancialmente. Sabe-se que os hormônios ovarianos exercem múltiplas ações regulatórias no sistema nervoso

durante o desenvolvimento, na idade adulta e envelhecimento e sob condições fisiológicas e patológicas (MELCANGI, et al., 2014; AREVALO et al., 2010).

Cérebros femininos de recém-nascidos estão expostos aos hormônios ovarianos originado de suas mães (lactação), também derivado suas próprias gônadas e sintetizado localmente no cérebro (McCARTHY, 2009). Tais hormônios são altamente lipofílicos e podem facilmente atravessar a barreira hematoencefálica, atingir alvos no cérebro e influenciar a excitabilidade neuronal e outras funções cerebrais (REDDY, 2014). Essas ações são, em parte, mediadas por efeitos diretos desses hormônios em neurônios. Além disso, tais hormônios podem regular a função do sistema nervoso por agir sobre as células gliais (MELCANGI et al., 2014; SCHUMACHER et al., 2014; AREVALO et al., 2015).

Os hormônios ovarianos podem agir através de ambos mecanismos genômicos e não-genômicos. Receptores para hormônios ovarianos são fatores de transcrição nucleares que regulam a expressão do gene, mas também têm ações sobre a membrana, incluindo a ativação de vias de transdução de sinal (MELCANGI et al., 2014; AREVALO et al, 2015).

Além disso, os hormônios ovarianos podem ser sintetizados *de novo* a partir do colesterol inteiramente dentro de regiões específicas do cérebro (AREVALO et al., 2015). Depois de ter sido metabolizado a progesterona, o colesterol pode ser posteriormente metabolizado para androstenediona e testosterona. Estes, por sua vez, através da aromatização, podem ser transformados em estradiol. As enzimas 5α-redutase e 3α-hidroxiesteroide são as enzimas mais importantes para que o cérebro possa sintetizar neurosteroides, sendo amplamente distribuídas no tecido cerebral. Os esteróides que são sintetizados no cérebro são também chamados de neuroesteróides; eles são precursores e metabólitos de hormônios esteróides e influenciam a excitabilidade neuronal principalmente através de mecanismos não genômicos. (AREVALO et al, 2015; FRYE and RHODES, 2009).

O estradiol é o composto biológico mais prevalente e potente da classe de hormônios esteróides chamados estrógenos e exerce efeitos de grande porte sobre o cérebro desde o seu desenvolvimento até a idade adulta (MCCARTHY, et al., 2009).

Clinicamente, o estradiol supostamente aumenta a excitabilidade cortical em humanos durante a estimulação magnética transcraniana (SMITH, 2004) e doses elevadas aumentam a incidência de migrânea com aura durante a terapia de reposição hormonal. Além disso, os níveis mais elevados de estradiol estão associados com aumento na frequência de convulsões em mulheres (KLEIN E HERZOG, 1998). Experimentalmente, os limiares convulsivos

diminuem durante os picos nos níveis de estradiol (WOOLLEY and TIMIRAS, 1962) e o kindling da amígdala é aumentado (EDWARDS et al., 1999). Em conjunto, estes resultados destacam o papel complexo dos hormônios ovarianos sobre a excitabilidade cerebral.

No cérebro em desenvolvimento, o estradiol atenua a excitotoxicidade mediada pelo glutamato diminuindo a regulação dos receptores metabotrópicos tipo 1 e diminuindo a quantidade de cálcio liberada do retículo endoplasmático (HILTON et al., 2006). Além disso, o estradiol marcadamente reforça as respostas GABAérgicas despolarizantes, específicas do período de desenvolvimento cerebral (NUNEZ et al., 2008; PERROT-SINAL et al., 2001).

No cérebro adulto, o estradiol exerce efeitos sobre a excitabilidade cerebral regulando "para cima" a expressão do gene da subunidade do receptor excitatório N-metil-d-aspartato (NMDA: Eikermann-Haerter et al., 2007; Martin e Behbehani, 2006) e diminuindo a atividade inibitória dos neurônios GABAérgicos (EIKERMANN-HAERTER et al., 2007). Tanto o β-estradiol quanto a progesterona podem aumentar a potenciação de longo termo (LTP) nos tecidos neocorticais (SACHS et al., 2007).

A progesterona, bem conhecida por seu papel benéfico na gestação, é sintetizada e ativamente metabolizada no sistema nervoso central e periférico (SCHUMACHER et al, 2014). Progestrona e/ou seus metbólitos exercem uma variedade de efeitos no cérebro agindo como reguladores fisiológicos do desenvolvimento e plasticidade neuronal e glial, participando de eventos e ações neroendócrinos (MELCANGI et al, 2014).

Estudos a partir de modelos animais indicam que o cérebro é realmente sensível à progesterona durante períodos críticos de desenvolvimento e maturação (LÓPEZ and WAGNER, 2009). Receptores de progesterona (PR) são transitoriamente expressos durante desenvolvimento fetal e neonatal (WAGNER, 2008) e amplamente distribuídos no cérebro adulto (SCHUMACHER et al, 2014). A progesterona é capaz de promover crescimento dendrítico, spinogênese e sinaptogênense nas células de Purkinje em desenvolvimento (TSUTSUI, 2008).

A literatura sugere que a progesterona exerce profundo impacto sobre convulsões (FRYE, 2010, SCHARFMAN e MACLUSKY, 2014; Reddy, 2014). Tanto a progesterona como seus metabólitos têm mostrado ter ações anticonvulsivantes em vários modelos animais (REDDY, 2014). O mecanismo amplamente aceito para o efeito anticonvulsivante da progesterona é via sua conversão ao neuroesteróide alopregnolona ao qual é um potente modulador alostérico de receptores $GABA_A$ (MUNOKA et al., 2010).

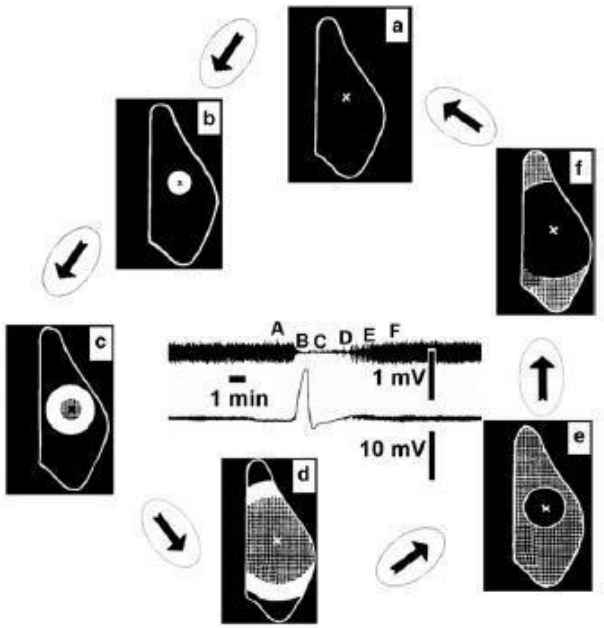
Vários efeitos da desnutrição sobre a estrutura e função neural têm sido descritos em seres humanos, bem como em animais de laboratório (MORGANE et al., 1993). A desnutrição nos estágios iniciais da vida é capaz de prejudicar o desenvolvimento cerebral normal podendo induzir alterações morfológicas e funcionais nas células neurais, incluindo déficits na atividade elétrica neural tanto ao nível do sistema nervoso periférico quanto central (ROCHA-DE-MELO e GUEDES, 1997; GUEDES, 2011).

A desnutrição é um problema abrangente que afeta milhões de seres humanos, durante o período de maior vulnerabilidade do sistema nervoso (MORGANE et al., 1993), seja na vida fetal, seja como recém-nascidos e crianças. O inadequado aporte nutricional continua sendo um dos principais fatores que afetam o desenvolvimento cerebral (ALAMY, 2012). Como o desenvolvimento do cérebro é tempo-dependente, uma vez que a sequência de eventos de crescimento ocorra, não poderá ser reiniciada, o que pode ter grande impacto sobre a função cognitiva na idade adulta. Por conseguinte, a nutrição ideal é fundamental para apoiar o crescimento neural até o cérebro atingir o seu máximo potencial de desenvolvimento (MORGANE et al., 1993).

Quando se aumenta artificialmente, a quantidade de filhotes por ninhada, (12 a 15 filhotes por ninhada - em comparação com ninhadas normais -6 a 9 filhotes por ninhada) há um aumento da competição pelo leite materno, resultando em desnutrição moderada a grave (HERNANDES et al., 2005; ROCHA-DE-MELO et al., 2006). Esta condição pode influenciar o desenvolvimento do SNC, alterando-o do ponto de vista eletrofisiológico, o que pode ser estudado por meio do fenômeno da depressão alastrante cortical DAC (MENDES-DA-SILVA et al., 2014). A DAC, que foi empregada no presente trabalho, é um modelo interessante para o estudo das relações entre hormônios ovarianos, nutrição e excitabilidade cerebral. Tem sido bem estabelecido que a desnutrição precoce aumenta a propagação da DAC (GUEDES, 2011), mas não há informações disponíveis acerca dos efeitos da associação entre desnutrição e tratamento com hormônios ovarianos, sobre os parâmetros da DAC. Esta é um fenômeno eletrofisiológico caracterizado por uma onda de excitação neuronal seguida de inibição. O fenômeno se auto propaga como uma onda de despolarização com características iônicas, metabólicas e hemodinâmicas peculiares, plenamente reversíveis ao cabo de alguns minutos, acompanhada por supressão transitória da atividade neuronal (LEÃO, 1944a, b). A propagação da onda de depressão da atividade eletroencefalográfica ocorre simultaneamente a uma variação lenta de voltagem (VLV) tecidual. Essa propagação se dá de forma concêntrica e reversível, a partir do ponto estimulado, numa velocidade entre 2 e 5 mm/min, sendo sua

latência de reversão de 10 a 15min (MARTINS-FERREIRA, 1983). A Fig. 2 ilustra o fenômeno.

Figura 2 - Esquema da sequência temporal cíclica de eventos da depressão alastrante cortical (DAC). Em "a", um córtex normal e nele o ponto estimulado (x), iniciando a DAC. Na sequência, indicada pelas setas, "b" a "d", está ilustrada a propagação concêntrica da DAC. As áreas em branco representam porções do tecido cortical invadidas pelo fenômeno em tempos sucessivos. As áreas quadriculadas, "c" a "f" indicam regiões que já sofreram a DAC e agora estão se recuperando (áreas refratárias a uma nova estimulação). De "b" a "f", observa-se que propagação (área branca) e recuperação (área escura) dão-se de forma concêntrica, sendo o ponto inicialmente estimulado o primeiro a se recuperar totalmente. Finalmente, em "a" todo o tecido se mostra completamente recuperado, retornando à condição inicial. No centro da figura, tem-se o eletrocorticograma (EcoG; traçado superior) e a variação lenta de voltagem (VLV; traçado inferior), a qual sempre aparece durante a DAC, quando o ECoG diminui sua amplitude. As letras "A" a "F" correspondem a sequência das etapas representadas nos desenhos externos.



Adaptada de Guedes, 2011

A DAC foi demonstrada em várias espécies de animais (Bures et al., 1974), incluindo o homem (MAYEVSKY et al., 1996). Evidências clínicas e experimentais têm demonstrado a relação do fenômeno da DAC com desordens na excitabilidade cerebral e suas doenças, como migrânea com aura (FERRARI et al., 2015), esclerose múltipla (PUSIC et al., 2015) e epilepsia (FABRICIUS et al., 2008; WEI et al., 2014).

Estudos experimentais prévios indicam que o tecido nervoso apresenta naturalmente uma resistência à passagem da DAC (GUEDES E DO-CARMO, 1980), e que esta resistência pode diminuir ou aumentar na vigência de alguns tratamentos, modificando assim a sua velocidade de propagação (ABADIE-GUEDES et al., 2008). Diversas modificações de condições sistêmicas podem alterar a propagação da DAC (GUEDES, 1984; GUEDES et al.,

1987; ANDRADE et al., 1990; GUEDES et al., 1992; ROCHA-DE-MELO e GUEDES, 1997). Tratamentos locais do tecido cortical podem também modificar a sua propagação (RICHTER et al., 2005; GUEDES et al., 1987; AMÂNCIO-DOS-SANTOS et al., 2006).

O fenômeno da DAC vem sendo utilizado pelo LAFINNT (Laboratório de Fisiologia da Nutrição Naíde Teodósio) como modelo experimental para avaliar o bom funcionamento do tecido neural. No LAFINNT, tem-se demonstrado que algumas condições nutricionais, hormonais, ambientais e farmacológicas podem modificar a suscetibilidade cortical ao fenômeno da DAC, algumas dificultando, e outras, facilitando sua propagação. As tabelas 1 e 2 listam algumas das condições, disponíveis na literatura, envolvidas na alteração da excitabilidade cortical à DAC, seja facilitando ou dificultando sua propagação.

Tabela 1- Algumas condições que dificultam 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., 2006
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	Fregni et al., 2005; 2007
Condições favoráveis de aleitamento	Rocha-de-Melo et al., 2006
Tratamento com triptofano (precursor da serotonina)	Trindade-Filho et al, 2009
Deficiência, na dieta, de ácidos graxos essenciais	Borba et al., 2010
Abolição da função ovariana (castração) no início da vida	Accioly et al, 2012
Tratamento com o antagonista opioide Naloxone	Guedes et al, 2013
Exercício físico	Lima et al., 2014
Tratamento com os aminoácidos taurina e alanina	Francisco e Guedes, 2015

Tabela 2- 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 administrado cronicamente	Guedes e Frade, 1993; Abadie-Guedes et al, 2012
Deficiência nutricional pela DBR*	Rocha-de-Melo e Guedes,1997
Hipertireoidismo	Santos, 2000
Hipoglicemia	Costa-Cruz e Guedes, 2001
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
Tratamento crônico com 60mg/kg de ác. Ascórbico	Monte-Guedes et al, 2011
Tratamento com o antagonista serotoninérgico Tianeptina	Amâncio-dos-Santos et al, 2013
Tratamento sistêmico com glutamato monossódico	Lima et al., 2013
Tratamento com o hormônio corticóide dexametasona	Lopes-de-Morais et al., 2014

A desnutrição, seja por manipulação da dieta ou do tamanho da ninhada, facilita a propagação da DAC (GUEDES et al., 2013; LIMA et al., 2009; MENDES-DA-SILVA et al, 2014; ROCHA-DE-MELO E GUEDES, 1997; ROCHA-DE-MELO et al., 2006). Estudos em roedores têm demonstrado que a ingestão insuficiente de nutrientes, especialmente se no início da vida, fase crítica do desenvolvimento do SNC, modifica a ação de certos compostos no cérebro, aumentando ou diminuindo as respostas ao fenômeno da DAC. Em animais com o estado nutricional comprometido, a pilocarpina (VASCONCELOS et al., 2004), a dipirona (AMARAL et al. 2009) e o lítio (DE AGUIAR et al., 2011) alteram a DAC mais intensamente do que o fazem em animais bem-nutridos. Outros compostos, como o citalopram (GUEDES et al., 2002), a fluoxetina (AMÂNCIO-DOS-SANTOS et al., 2006) e a glutamina (LIMA et al., 2009) influenciam a DAC de forma semelhante em animais nutridos e desnutridos. Por outro lado, o diazepam (GUEDES et al., 1992), a glicose (COSTA CRUZ e GUEDES 2001), a L-arginina (FRAZÃO et al., 2008), a tianeptina (AMÂNCIO-SANTOS et al., 2013), e o antagonista de opióides naloxona (GUEDES et al., 2013) afetam a DAC de forma menos intensa em animais desnutridos do que em bem-nutridos.

Tem sido postulado que os hormônios ovarianos podem modular a susceptibilidade à DAC (CHAUVEL et al., 2012). Em camundongos os limiares para DAC são menores em fêmeas que em machos (BRENNAN et al., 2007). O estradiol é considerado responsável por velocidades de propagação da DAC mais altas em ratas Wistar audiogênicas (Guedes et al., 2009). Em camundongos fêmeas “nockin” para migrânea hemiplégica familiar tipo 1, a susceptibilidade à DAC foi maior que nos machos na mesma condição e esse aumento foi abolido com a ovariectomia e parcialmente restaurado com tratamento com estradiol (EIKERMANN-HAERTER et al., 2009). Um estudo in vitro, em ratas adultas sugere uma possível influência facilitadora, dose-dependente, dos hormônios ovarianos sobre a DAC (SACHS et al., 2007).

Frente ao exposto, o presente trabalho se propôs a estudar os efeitos, sobre a DAC e o comportamento de ansiedade, da interação entre a administração de hormônios ovarianos e condições desfavoráveis de lactação (amamentação em ninhadas de grande tamanho).

2. OBJETIVOS

2.1. Gerais

Continuar o estudo do mestrado (ovariectomia no inicio da vida e DAC) avaliando os efeitos, sobre a DAC, da ovariectomia bilateral na idade adulta.

Avaliar, os efeitos, sobre o comportamento e sobre a DAC, da interação entre a administração de hormônios ovarianos e condições desfavoráveis de lactação (amamentação em ninhadas de grande tamanho).

2.2. Específicos

Nas condições de tratamento acima descritas:

- Analisar a evolução do peso corporal e de alguns órgãos (cérebro, ovários, útero, adrenais) como um indicador do desenvolvimento do organismo;

- Avaliar o desempenho no labirinto em cruz elevado e campo aberto como indicador do comportamento de ansiedade.

- Investigar as modificações de parâmetros da DAC (velocidade de propagação, bem como amplitude e duração de sua variação lenta de voltagem).

- Comparar os efeitos eletrofisiológicos e comportamentais de ansiedade do tratamento com hormônios ovarianos em animais submetidos a condições normais ou desfavoráveis de lactação.

3. REFERÊNCIAS

ABADIE-GUEDES R.; SANTOS S.D.; CAHÚ T.B.; GUEDES R.C.A.; BEZERRA R.S. Dose-dependent effects of astaxanthin on cortical spreading depression in chronically ethanol-treated adult rats. **Alcoholism: Clinical and Experimental Research**, v. 32, n. 8, p. 1417-1421, 2008.

ACCIOLY, N.E.; BENEVIDES, R.D.; DA COSTA, B. L.; GUEDES, R.C. Ovariectomy in the developing rat decelerates cortical spreading depression in adult brain. **International Journal of Developmental**, v. 30, n. 5, p. 405-10, 2012.

ALAMY, M.; BENGELLOUN, W. A.; Malnutrition and brain development: an analysis of the effects of inadequate diet during different stages of life in rat. **Neuroscience & Biobehavioral Reviews**, **v.** 36, n. 6, p. 1463-1480, 2012.

AMÂNCIO-DOS-SANTOS, A.; MAIA, L.M.S.S.; GERMANO, P.C.P.S.; NEGRÃO, Y.D.S.; GUEDES, R.C.A. Tianeptine facilitates spreading depression in well-nourished and early-malnourished adult rats. **European Journal of Pharmacology**, v. 706, p. 70-75, 2013.

AMÂNCIO-DOS-SANTOS, A; PINHEIRO, P. C. F; LIMA, D. S. C; OZIAS, M. G; OLIVEIRA, M. B; GUIMARÃES, N. X; GUEDES, R. C. A. Fluoxetine inhibits cortical spreading depression in weaned and adult rats suckled under favorable and unfavorable lactation conditions. **Experimental Neurology**, v. 200, n.2, p.275-282, 2006.

AMARAL, A.P.B.; BARBOSA, M.S.S.; SOUZA, V.C.; RAMOS, I.L.T.; GUEDES, R.C.A. Drug/nutrition interaction in the developing brain: dipyrone enhances spreading depression in rats. **Experimental Neurology**, v. 219, p. 492-498, 2009.

ANDRADE, A.F.D.; GUEDES, R.C.A.; TEODÓSIO, N.R. Enhanced rate of cortical spreading depression due to malnutrition: Prevention by dietary protein suplementation. Braz. **Journal Of Medical and Biological Research**, v. 23, 889-893, 1990.

AREVALO, M.A.; AZCOITIA, I.; GARCIA-SEGURA, L.M. The neuroprotective actions of oestradiol and oestrogen receptors. **Nature Reviews Neuroscience,** v.16, p. 17–29, 2015.

AREVALO, M.A.; SANTOS-GALINDO, M.; LAGUNAS, N.; AZCOITIA, I.; GARCIA-SEGURA, L.M. Selective estrogen receptor modulators as brain therapeutic agents. **Journal of Molecular Endocrinology**, v. 46, p. R1-R9, 2011.

BORBA, J. M.; ROCHA-DE-MELO, A. P.; DOS SANTOS, A. A.; DA COSTA, B. L.; DA SILVA, R, P.; PASSOS, P. P.; GUEDES, R. C. Essential fatty acid deficiency reduces cortical spreading depression propagation in rats: a two-generation study. **Nutritional Neuroscience**, **v.** 13, n. 3, p. 144-50, 2010.

BRENNAN, K.C.; REYES, M.R.; LÓPEZ-VALDÉS, H.E.; ARNOLD, A.P.; CHARLES, A.C. Reduced threshold for cortical spreading depression in female mice. **Annals of Neurology**, v.61, p. 603–606, 2007.

BUREŠ, J.; BURESOVÁ, O.; KRIVÁNEK, J. The mechanism and applications of Leão's spreading depression of electroencephalographic activity. **Academia, Prague**, 1974.

CHAUVEL, V.; SCHOENEN, J.; MULTON, S. Influence of ovarian hormones on cortical spreading depression and its suppression by L-kynurenine in rat. **PLoS One**, v. 8, p. 8–13, 2013.

COSTA-CRUZ, R.R.G.; AMÂNCIO-DOS-SANTOS, A.; GUEDES, R.C.A. Characterization of cortical spreading depression in adult well-nourished and malnourished rats submitted to the association of pilocarpine-induced epilepsy plus streptozotocininduced hyperglycemia. **Neuroscience Letters**, v. 401, p. 271-275, 2006.

COSTA-CRUZ, R.R.G.; GUEDES, R.C.A. Cortical spreading depression during streptozotocin-induced hyperglycemia in nutritionally normal and early-malnourished rats. **Neuroscience Letters**, v. 303, p. 177-180, 2001.

DE AGUIAR, M. J.; DE AGUIAR, C. R.; GUEDES, R. C. Lithium/nutrition interaction in the brain: a single lithium administration impairs spreading depression in malnourished, but not in well-nourished rats. **Nutritional Neuroscience**, **v.** 14, n. 4. P. 159-164, 2011.

DOBBING, J. Vulnerable periods in developing brain, in: **Applied Neurochemistry**, p. 287–316, 1968.

EDWARDS, H.E.; BURNHAM, W.M.; MENDONCA, A.; BOWLBY, D.A.; MACLUSKY, N.J. Steroid hormones affect limbic afterdischarge thresholds and kindling rates in adult female rats. Brain Research, v. 14, p. 136-150, 1999

EIKERMANN-HAERTER, K.; DILEKÖZ, E.; KUDO, C.; SAVITZ, S.I.; WAEBER, C.; BAUM, M.J.; FERRARI, M.D.; VAN DEN MAAGDENBERG, A.M.; MOSKOWITZ, M.A.; AYATA, A.C. Genetic and hormonal factors modulate spreading depression and transient hemiparesis in mouse models of familial hemiplegic migraine type 1. **Journal of Clinical Investigation**, v. 119, p. 99–109, 2009.

EIKERMANN-HAERTER, K.; KUDO, C.; MOSKOWITZ, M.A. Cortical spreading depression and estrogen. **Headache**, v 47, p. S79-S85, 2007.

FABRICIUS, M; FUHR, S.; WILLUMSEN, L.; DREIER, J.P.; BHATIA, R.; BOUTELLE, M.G.; HARTINGS, J.A.; BULLOCK, R.; STRONG, A.J.L.M. Association of seizures with cortical spreading depression and peri-infarct depolarisations in the acutely injured human brain. **Clinical Neurophysiology**, v. 119, p. 1973–1984, 2008.

FARIAS-SANTOS, R.C.; LIRA, M.C.A.; PEREIRA, D.E.; SÁ, I.R.; PIMENTEL, M.R.F.; ARAÚJO, L.L.; GUEDES, R.C.A. Exposure of developing well-nourished and malnourished rats to environmental heating facilitates cortical spreading depression propagation at adulthood. **Neuroscience Letters**, v. 454, n. 3, p. 218-222, 2009.

FERRARI, M.D.; KLEVER, R.R.; TERWINDT, G.M.; AYATA, C.; VAN DEN MAAGDENBERG, A.M.J.M. Migraine pathophysiology: lessons from mouse models and human genetics**. Lancet Neurolology**, v. 14, p. 65–80, 2015.

FRANCISCO, E. S.; GUEDES, R. C. A. Neonatal taurine and alanine modulate anxiety ‐like behavior and decelerate cortical spreading depression in rats previously suckled under different litter sizes. **Amino Acids**, v. 47, n. 11, p. 2437-2445, 2015.

FRAZÃO, M. F; MAIA, L. M. S. S; GUEDES, R. C. A. Early malnutrition, but not age, modulates in the rat the L-Arginine facilitating effect on cortical spreading depression. **Neuroscience Letters,** v.4, n.47, p.26-30, 2008.

FREGNI, F.; LIEBETANZ, D.; MONTE-SILVA, K.K.; OLIVEIRA, M.B.; AMANCIO-DOS-SANTOS, A.; NITSCHE, M.A.; PASCUAL-LEONE, A.; GUEDES, R.C.A. Effects of transcranial direct current stimulation coupled with repetitive electrical stimulation on cortical spreading depression. **Experimental Neurology**, v. 204, p. 462-466, 2007

FREGNI, F.; MONTE-SILVA, K.K.; OLIVEIRA, M.B.; FREEDMAN, S.; PASCUAL-LEONE, A.; GUEDES, R.C.A. Lasting accelerative effects of 1 Hz and 20 Hz electrical stimulation on cortical spreading depression: relevance for clinical applications of brain stimulation. **European Jouna of Neuroscience**, v. 21, p. 2278-2284, 2005.

GUEDES, R.C.A.; ROCHA-DE-MELO, A.; LIMA, K.R.; ALBUQUERQUE J.M.S.; FRANCISCO, E.S. Early malnutrition attenuates the impairing action of naloxone on spreading depression in young rats. **Nutritional Neuroscience**, v. 16, n. 4, p. 142-146, 2013.

GUEDES, R.C.A Cortical Spreading Depression: A Model for Studying Brain Consequences of Malnutrition. In: Victor R. Preedy, Ronald R Watson and Colin R Martin (eds.), **Handbook of Behavior, Food and Nutrition**, p. 2343-2355, 2011.

GUEDES, R.C.A., DE OLIVEIRA, J.A.C., AMÂNCIO-DOS-SANTOS, Â., GARCÍA-CAIRASCO, N. Sexual differentiation of cortical spreading depression propagation after acute and kindled audiogenic seizures in the Wistar audiogenic rat (WAR). **Epilepsy Research**, v. 83, p. 207–214, 2009.

GUEDES, R.C.A; AMANCIO-DOS-SANTOS, A; MANHÃES-DE-CASTRO, R; COSTA-CRUZ, R. R. G. Citalopram has an antagonistic action on cortical spreading depression in well-nourished and early-malnourished adult rats. **Nutritional Neuroscience**, v.5, n.2, p.115-123, 2002.

GUEDES, R.C.A.; CAVALHEIRO, E.A. Blockade of spreading depression in chronic epileptic rats: reversion by diazepam. **Epilepsy Research**, v. 27, p. 33-40, 1997.

GUEDES, R.C.A.; AMORIM, L.F.; TEODÓSIO, N.R. Effect of aging on cortical spreading depression. **Brazilian Journal of Medical and Biological Research**, v. 29, n. 10, p. 1407-1412, 1996.

GUEDES, R.C.A., FRADE, S.F., 1993. Effect of ethanol on cortical spreading depression. **Brazilian Journal of Medical and Biological Research**, v. 26, p. 1241-1244, 1993.

GUEDES, R.C.A.; BARRETO, JM. Effect of anesthesia on the propagation of cortical spreading depression in rats. **Brazilian Journal of Medical and Biological Research**, v. 25, n. 4, p. 393-397, 1992.

GUEDES, R.C.A.; CABRAL-FILHO, J.E.; TEODOSIO, N.R. GABAergic mechanisms involved in cortical spreading depression in normal and early malnourished rats. In: Do Carmo, R.J. (Ed.), Spreading Depression, Experimental Brain Research Series. **Springer, Berlin**, p. 17–26, 1992.

GUEDES, R.C.A.; AMORIM, L.F.; MEDEIROS, M.C.; SILVA, A.T.; TEODÓSIO, N.R. Effect of dietary Lithium on cortical spreading depression. **Brazilian Journal of Medical and Biological Research**, v. 22, n. 7, p. 923-925, 1989.

GUEDES, R.C.A.; ANDRADE, A.F.D.; CABRAL-FILHO, J.E. Propagation of cortical spreading depression in malnourished rats: facilitatory effect of dietary protein deficiency. **Brazilian Journal of Medical and Biological Research**, v. 20, p. 639-642, 1987.

GUEDES, R.C.A.; DO CARMO, R.J. Influence of ionic disturbances produced by gastric washing on cortical spreading depression. **Experimental Brain Research**, v. 39, n. 3, p. 341-349, 1980.

GUEDES, R.C.A.; PEREIRA-DA-SILVA, M.S. Effect of pre and postnatal propylthiouracil administration on propagation cortical spreading depression of adults rats. **Brazilian Journal of Medical and Biological Research**, v. 26, n. 10, p. 1123-1128, 1993.

HERNANDES, A.S.; FRANÇOLIN-SILVA, A.L.; VALADARES, C.T.; FUKUDA, M.T.H.; ALMEIDA, S.S. Effects of different malnutrition techniques on the behaviour of rats tested in the elevated T-maze. **Behavioural Brain Research**, v. 162, p. 240-245, 2005.

HILTON, G.D.; NUNEZ, J.L.; BAMBRICK, L.; THOMPSON, S.M.; MCCARTHY, M.M. Glutamate-mediated excitotoxicity in neonatal hippocampal neurons is mediated by mGluR-induced release of Ca++ from intracellular stores and is prevented by estradiol. **European Journal of Neuroscience**, v. 24, p.3008–3016, 2006.

LEÃO, A.A.P. Pial circulation and spreading depression of activity in the cerebral cortex. **Journal of Neurophisiology**, v. 7, p. 391-396, 1944b.

LEÃO, A. A. P. Spreading depression of activity in the cerebral cortex. **Journal of lima**

LIMA, D.S.C.; MAIA, L.M.S.S.; BARBOZA, E.A.; DUARTE, R.A.; SOUSA, L.S.; GUEDES, R.C.A. L-glutamine supplementation during the lactation period facilitates cortical spreading depression in well-nourished and early-malnourished rats. **Life Sciences,** v. 85, p. 241-247, 2009.

LIMA, C.B.; SOARES, G.S.F.; VITOR, S.M.; ANDRADE-DA-COSTA, B.L.S.; CASTELLANO, B.; GUEDES, R.C.A. Spreading depression features and Iba1 immunoreactivity in the cerebral córtex developing rats submitted to treadmill exercise after treatment with monosodium glutamate. **International Journal of Developmental Neuroscience**, v. 33, p. 98-105, 2014.

LÓPEZ, V.; WAGNER, C.K. Progestin receptor is transiently expressed perinatally in neurons of rat isocortex. **Journal of Comparative Neurology**, v. 512, p. 124–139, 2009.

LOPES-DE-MORAIS, A.A.C.; MENDES-DA-SILVA, R.F.; DOS-SANTOS, E.M.; GUEDES, R.C.A. Neonatal dexamethasone accelerates spreading depression in the rat, and antioxidant vitamins counteract this effect. **Brain Research**, v. 1591, p. 93-101, 2014.

MAIA, L.M.S.S.; AMANCIO-DOS-SANTOS A.; DUDA-DE-OLIVEIRA D.; ANGELIM M.K.C.; GERMANO P.C.P.; SANTOS S.F.; GUEDES R.C.A. L-Arginine administration during rat brain development facilitates spreading depression propagation: evidence for a dose- and nutrition-dependent effect. **Nutritional Neuroscience**, v. 12 (2), p. 73-80, 2009.

MARTINS-FERREIRA, H., 1983. Spreading depression in chick retina. In: Ookawa, T. (Ed.) The Brain and Behavior of the Fowl. **Japan Scientific Societies Press**, Tokyo, p 317-333, 1983.

MAYEVSKY, A.; DORON, A.; MANOR, T.; MEILIN, S.; ZARCHIN, N.; OUAKNINE, G.E. Cortical spreading depression recorded from the human brain using a multiparametric monitoring system. **Brain Research**, v. 740, p. 268-274, 1996.

McCARTHY, M.M., The two faces of estradiol: effects on deeloping brain. **Neuroscientist**, v. 88, p. 91–124, 2009

MELCANGI, R.C., GIATTI, S., CALABRESE, D., PESARESI, M., CERMENATI, G., MITRO, N., VIVIANI, B., GARCIA-SEGURA, L.M., CARUSO, D. Levels and actions of progesterone and its metabolites in the nervous system during physiological and pathological conditions. **Progress Neurobiology**, v. 113, p. 56–69, 2014.

MENDES-DA-SILVA, R.F.; LOPES-DE-MORAIS, A.A.C.; BANDIN-DA-SILVA, M.E.; CAVALCANTI, G.A.; RODRIGUES, A.R.O.; ANDRADE-DA-COSTA, B.L.S.; GUEDES, R.C.A. Prooxidant versus antioxidant brain action of ascorbic acid in well-nourished and malnourished rats as a function of dose: a cortical spreading depression and malondialdehide analysis. **Neuropharmacology**, v. 86, p. 155-160, 2014.

MONTE-GUEDES, C.K.R.; ALVES, E.V.; VIANA-DA-SILVA, E.; GUEDES, R.C.A. Chronic treatament with ascorbic with ascorbic acid enhances cortical spreading depression in developing wellnourished and malnourished rats. **Neuroscience Letters**, v. 496, p. 191-94, 2011.

MORGANE, P.J.; BRONZINO, J.D.; TONKISS, J.; DIAZ-CINTRA, S.; CINTRA, L.; KEMPER, T.; et al. Prenatal malnutrition and development of the brain. **Neuroscience and Biobehavioral Reviews**, v. 17, p. 91-128, 1993.

NUÑEZ, J.L; ABERDEEN, G.W.; ALBRECHT, E.D. M.M. Impact of estradiol on gamma-aminobutyric acid- and glutamate-mediated calcium responses of fetal baboon (Papio anubis) hippocampal and cortical neurons. **Endocrinology**, v. 149, p. 6433–6443, 2008.

PAIXÃO, A.D.O.; TRINDADE, A.S.; DANTAS, A.C.; BARRETO, I.S.S.; VIEIRA-FILHO, L.D.; MEDEIROS, M.C.; TEODÓSIO, N.R.; GUEDES, R.C.A. 2007. Impact of two early malnutrition models on renal and neural functions in rats. in: Vesler LW (Ed) Malnutrition in the 21st Century. **Nova Science Publishers**, Inc., N.York, Chapter 13, p 239-263, 2007.

PERROT-SINAL, T.S.; AUGER, A.P.; MCCARTHY, M.M. Excitatory actions of GABA in developing brain are mediated by l-type Ca2+ channels and dependent on age, sex, and brain region. **Neuroscience**, v. 116, p. 995–1003, 2003.

PUSIC, D.A.; MITCHELL, H.M.; KUNKLER, P.E.; KLAUER, N.; KRAIG, R.P. Spreading depression transiently disrupts myelin via interferon-gamma signaling. **Experimental Neurology** v. 264, p. 43-54, 2015.

REDDY, D.S. Neurosteroids and their role in sex-specific epilepsies. **Neurobiology of Disease**, v. 72, p. 198–209, 2014.

RICHTER, F.; LEHMENKÜHLER, A.; SCHAIBLE, H.G. Voltage-gated calcium channels are not involved in generation and propagation of spreading depression (SD) in the brainstem of immature rats. **Neuroscience Letters**, v. 390, p. 15-20, 2005.

ROCHA-DE-MELO, A. P; CAVALCANTI, J. B; BARROS, A. S; GUEDES, R. C. A. Manipulation of rat litter size during suckling influences cortical spreading depression after weaning and at adulthood. **Nutrition Neuroscience**, v.9, n.4, p.155-160, 2006.

ROCHA-DE-MELO, A.P.; GUEDES, R.C.A. Spreading depression is facilitaded in adult rats previously submitted to short episodes of malnutrition during the lactation period. **Brazilian Journal of Medical and Biological Research**, v. 30, n. 5, p. 663-669, 1997.

SACHS, M.; PAPE, H.C.; SECKMANN, E.J.; GORJI, A. The effect of estrogen and progesterone on spreading depression in rat neocortical tissues. **Neurobiolology of Disease**, v. 25, p. 27-34, 2007.

SANTOS, R.S. Nutrição, hipertireoidismo precoce e desenvolvimento cerebral: estudo em ratos recém-desmamados. Dissertação de Mestrado. Depto. De Nutrição, UFPE, 2000.

SANTOS-MONTEIRO, J.S.; TEODÓSIO, N.R.T.; GUEDES, R.C.A. Long-lasting effects of early environmental stimulation on cortical spreading depression in normal and early malnourished adult rats. **Nutritional Nerusocience**, v. 1, n. 1, p. 29-40, 2000.

SCHARFMAN, H.E.; MACLUSKY, N.J. Sex differences in the neurobiology of epilepsy: a preclinical perspective. **Neurobiolology of Disease**, v. 72, p. 180-192, 2014.

SCHUMACHER, M.; MATTERN, C.; GHOUMARI, A.; OUDINET, J.P.; LIERE, P.; LABOMBARDA, F.; SITRUK-WARE, R.; DE NICOLA, A.F.; GUENNOUN, R. Revisiting the roles of progesterone and allopregnanolone in the nervous system: resurgence of the progesterone receptors. **Progress in Neurobiology**, v. 113, p. 6-9, 2014.

SMITH SS, W.C. Cellular and molecular effects of steroid hormones on CNS excitability. **Cleveland Clinic Journal of Medicine**, v. 71, p. S4–S10, 2004.

TENÓRIO, A.S., OLIVEIRA, I.D.V.A., GUEDES, R.C.A., 2009. Early vibrissae removal facilitates cortical spreading depression propagation in the brain of well-nourished and malnourished developing rats. **International Journal of Development Neuroscience**, v. 27, p. 431–437, 2009.

TRINDADE-FILHO, E.M.; VASCONCELOS, C.A.; GUEDES, R.C.A. Acute tryptophan administration impairs cortical spreading depression propagation in RE sleep deprived and non-deprived adult rats. **Psychology Neuroscience**, v. 2, n. 2, p. 235-241, 2009.

WAGNER, C.K. Minireview: Progesterone receptors and neural development: A gap between bench and bedside? **Endocrinology**, v. 149, p. 2743–2749, 2008.

WEI, Y; ULLAH, G; SCHIFF, S,J. Unification of neuronal spikes, seizures, and spreading depression. **Journal of Neuroscience**, v. 35, p. 11733-11743, 2014.

WOOLLEY, D.E.; TIMIRAS, P.S. Estrous and circadian periodicity and electroshock convulsions in rats. **American Journal of Physiology**, v. 202, p. 379–382, 1962.

VASCONCELOS, C.A.; DE OLIVEIRA, J.A.F.; DE OLIVEIRA COSTA L.A.; GUEDES, R.C.A. Malnutrition and REM-sleep deprivation modulate in rats the impairment of spreading depression by a single sub-convulsing dose of pilocarpine. **Nutritional Neuroscience**, v. 7, n. 3, p. 163-170, 2004.

XIMENES-DA-SILVA, A.; GUEDES, R.C.A. Differential effect of changes in blood glucose levels on the velocity of propagation of cortical spreading depression in normal and malnourished rats. **Brazilian Journal of Medical and Biological Research**, v. 24, p. 1277-1281, 1991.

4. ARTIGO 1

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Ovariectomy in the developing rat decelerates cortical spreading depression in adult brain

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The brain of mammals is one important target organ for the action of gonadal steroids and, when occurring during development, this hormonal influence may result in important repercussion on the brain electro-physiological properties at adulthood, some of which depending on the brain excitability. Here we have characterized in early-ovariectomized adult rats the brain ability to propagate the excitability-related phenomenon known as cortical spreading depression (CSD), as an index of the cerebral electrophysi-ological effects of the early-induced absence of the ovarian hormones. Wistar female rat pups (7-day old) underwent bilateral ovariectomy (Ovx group; n = 21) or Sham surgery (Sham group; n = 22), or no surgery (Naive group; n = 22). When the pups became adult (90–130 days), they were submitted to the recording of CSD (electrocorticogram and slow DC-voltage variation) in two points of the cortical surface during 4 h. Compared with both Naïve and Sham controls, bilateral ovariectomy early in life resulted in significantly higher body weights (from days 50–65 onwards) and severely reduced uterus weights at adulthood. Furthermore, in the Ovx animals the amplitudes and durations of the DC-potential changes of CSD were higher, and the CSD propagation velocities were reduced. Another group of rats ovariectomized in adulthood did not present such CSD alterations. It is concluded that ovariectomy during brain devel-opment is causally associated with the CSD changes in the adult brain, indicating a long-lasting effect, which we suggest as being related to the long-term suppression of the action of the ovarian hormones on brain excitability.

1. Introduction

In mammals, the fetal environment is rich in estradiol and pro-gesterone derived from the maternal organism (McCarthy, 2009; Sanyal, 1978). In the female fetal brain of the rat, -fetoprotein (AFP), a steroid binding globulin, sequesters circulating estrogens to avoid their brain masculinizing effects early in life (Bakker et al., 2006; Gillies and MCarthur, 2010) and selectively deliver estra-diol to specific neuronal populations (Bakker and Baum, 2008; Bakker et al., 2006). As a result, the female fetal brain is exposed to lower levels of estradiol, as compared with the male fetal brain. Nevertheless, there is evidence that estradiol can be de novo syn-thesized (locally from cholesterol) directly in fetal and neonatal neurons during the female developing brain (Amateau et al., 2004; Bakker et al., 2002; McCarthy, 2008; Mellon and Vaudry, 2001). This hormonal scenario extends postnatally and in the offspring it influences the sexual differentiation of the developing brain (McCarthy and Konkle, 2005; Amateau et al., 2004).

The brain enzyme aromatase, that synthesizes estradiol, presents its highest activity in the immature brain as compared to the mature brain (McCarthy, 2009) and the AFP activity no longer plays a significant role postnatally when the ovaries start to pro-duce estrogens (Bakker and Baum, 2008). During this initial period of life, ovarian hormones can influence developmental processes in the brain (Bakker et al., 2002). Estradiol is capable of modulat-ing brain development by enhancing depolarizing GABA responses, which are specific of the neonatal period, causing a trophic effect (Perrot-Sinal et al., 2003) and preventing glutamate-induced cell death (Hilton et al., 2006). In addition, progesterone is capable of promoting dendritic growth, spinogenesis, and synaptogenesis in the developing Purkinje cell (Tsutsui, 2008).

In the fully developed brain, estrogen can exert effects on excitability by upregulating the gene expression of excitatory N-methyl-d-aspartate (NMDA) receptor subunit (Eikermann-Haerter et al., 2007; Martin and Behbehani, 2006) and by decreas-ing the inhibitory activity of -aminobutyric acid (GABA)-ergic neurons (Eikermann-Haerter et al., 2007). Both -estradiol and

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progesterone may enhance long-term potentiation (LTP) induction in neocortical tissues (Sachs et al., 2007). These data indicate a relationship between ovarian hormones and neuronal excitability, which can be experimentally explored by using the electrophysiological phenomenon denominated as cortical spreading depression (CSD). CSD is characterized by a wave of self-propagating depolarization with characteristic ionic, metabolic, and hemodynamic changes followed by transient suppression of neuronal activity (Leão, 1944a,b). In one in vitro study, ovarian hormones applied to neocortical slices obtained from adult rats facilitated CSD (Sachs et al., 2007). However, little attention has been paid to the effects of ovarian deficiency during brain development on CSD features in the cerebral cortex of adult rats. The functional impact of gender-related CSD effects resides in two lines of evidence. First, experimental findings suggest that CSD is involved in the produc-tion of the symptoms of migraine aura (Lauritzen et al., 2011), and that CSD and epilepsy mechanisms have some common features (Leão, 1944a; Guedes and Cavalheiro, 1997; Guedes et al., 2009). Second, ovarian hormones influence those neurological diseases (Klein and Herzog, 1998; Woolley and Timiras, 1962).

The present study aimed to address these issues in female rats that had been previously ovariectomized early in life, and to com-pare them with rats ovariectomized in adult age. Our hypothesis is that ovariectomy during the period of brain development is causally associated in adulthood with impairment of CSD propagation.

2. Materials and methods

2.1. Animals

Wistar female newborn rats (n = 65) from the colony of Departamento de Nutric¸ ão of Universidade Federal de Pernambuco (Brazil) were randomly distributed to three groups, submitted respectively on the postnatal day 7 to the following treat-ments: (a) bilateral ovariectomy (Ovx group; n = 21); (b) Sham surgery (n = 22); (c) no surgery (Naïve group; n = 22). Two additional groups of 13 female rats, one Sham (n = 7) and one ovariectomized (n = 6), were operated in adulthood (90 days of life), and submitted to CSD recording 30 days after operation.

The handling procedures involving the animals were in accordance with the Institution's guidelines, which comply with the Principles of Laboratory Animal Care (National Institutes of Health, Bethesda, USA). The experimental design was approved by the University Committee on Ethics in animal research, which complies with the "Principles of Laboratory Animal Care" (National Insti-tutes of Health, Bethesda, USA). Animals were reared in polypropylene cages (51 cm × 35.5 cm × 18.5 cm) in a room maintained at 22 ± 1 ° C with a 12 h light/12 h dark cycle (lights on at 7:00 a.m.) with free access to water and food.

2.2. Bilateral ovariectomy

Under deep surgical cryoanesthesia (Phifer and Terry, 1986; see also Tenório et al., 2009), the ovaries of the 7-day old rat pups were removed through a dor-sal midline incision on the lumbar region, as described elsewhere (Brouwer et al., 1980). Suture procedures were the same in both groups. After recovering from anes-thesia, the pups were returned to the maternal cage. After weaning, they were housed in cages similar to the maternal ones (3–4 rats per cage). Total surgery time was 10–15 min and the post-surgery mortality was very low (3 out of 46 operated pups). The group ovariectomized in adulthood was anesthetized with 250 mg/kg tribromoethanol, i.p., followed by bilateral dorso-lombar incision. After bilateral ovary exeresis, the sutured skin was covered with a local antiseptic and the animal was intramuscularly injected with 30,000 IU of benzatine Penicillin. In the groups Sham-operated early in life or in adulthood, all rats received the same incisions as the corresponding Ovx animals; the ovaries were identified and palpated, but not removed.

The effectiveness of the ovariectomy was confirmed on two occasions: at 60–90 days of life (in the early-ovariectomized group), by the atrophic pattern of the genital epithelium as well as by the delayed vaginal opening, as compared to the Sham and Naïve controls, and on the day of CSD recording (90–130 days of life), in both early- and late-operated rats, when the animal was killed and the uterus was removed, showing atrophy.

2.3. CSD elicitation and recording

When the pups were 90- to 130-day old, they were submitted to the CSD recording for a 4-h period. In the Sham and Naïve groups, the CSD recordings were performed only when the animals were in the proestrus phase of the estrous cycle, which was histologically confirmed on the day of the CSD recording.

Under anesthesia (1 g/kg urethane plus 40 mg/kg chloralose, i.p.), three trephine holes (2–3 mm in diameter) were drilled on the right side of the skull. The first hole (on the frontal bone) was used to apply the stimulus (KCl solution) to elicit CSD. The propagating CSD wave was then recorded on two points of the parietal cortex surface through the other two holes, drilled on the parietal bone. Rectal temperature was continuously monitored and maintained at 37 ± 1 ° C by a heating blanket. CSD was elicited at 20 min intervals by applying 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 for 1 min. The electrocorticogram (ECoG) and the slow DC potential change accompanying CSD were recorded simultaneously 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 and solidified with the addition of 0.5% agar, into which a chlorided silver wire was inserted. The pipettes were fixed together pair-wise with cyanoacrylate glue, so that the interelectrode distance was kept constant for each pair (range: 4–5.5 mm). Each pair of electrodes was connected to a lever that could be vertically moved by turning around a screw, so that the recording electrodes could be gently placed on the intact dura-mater, under low-power microscope control, without any excessive pressure on the cortical surface. A third electrode, of the same type, placed on the nasal bones, served as common reference electrode. 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.

2.4. Statistics

In the early-operated animals and their controls, body-, uterus-, adrenals-and brain-weights and CSD propagation rates were compared between groups by ANOVA, followed by a post hoc (Tukey–Kramer) test when indicated. In the two late-operated groups, body weights were compared with t-test. The statistical software used was "Sigmastat® version 3.10. Differences were considered significant when P ≤ 0.05. All values are presented in the text as mean ± standard deviations.

3. Results

3.1. Body weights

Fig. 1 shows the body weights of the early-operated rats and their controls, measured at the following age-intervals: 40–46 days, 50–65 days, 74–85 days and 100–120 days. Compared to the two control groups, bilateral ovariectomy early in life resulted in signif-icantly higher body weights from days 50 to 65 onwards (P = 0.006). The mean values (in g) ranged from 114.9 ± 22.2 to 224.0 ± 26.5 for the Nv group, from 119.2 ± 11.9 to 226.3 ± 26.1 for the Sham group and from 127.1 ± 12.7 to 262.8 ± 34.8 for the Ovx rats. In the two groups operated in adulthood, the mean weights at 120 days

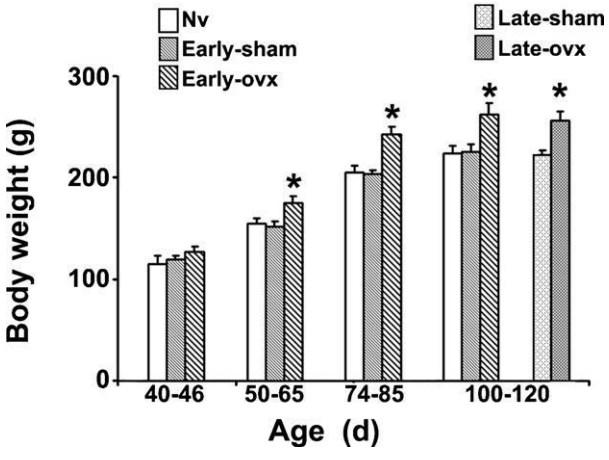

Fig. 1. Body weights (mean ± EPM) of rats submitted early in life (7-day old), or at adulthood (70–90 days) to bilateral ovariectomy (Ovx group), or to Sham operation (Sham group), or not submitted to any surgery (Nv; Naïve group). The weights were measured at the following age-intervals: 40–46 days, 50–65 days, 74–85 days and 100–120 days. The asterisks (*) indicate the Ovx body weights that are significantly higher than the corresponding Sham and Naïve values (P = 0.006; ANOVA followed by Tukey test).

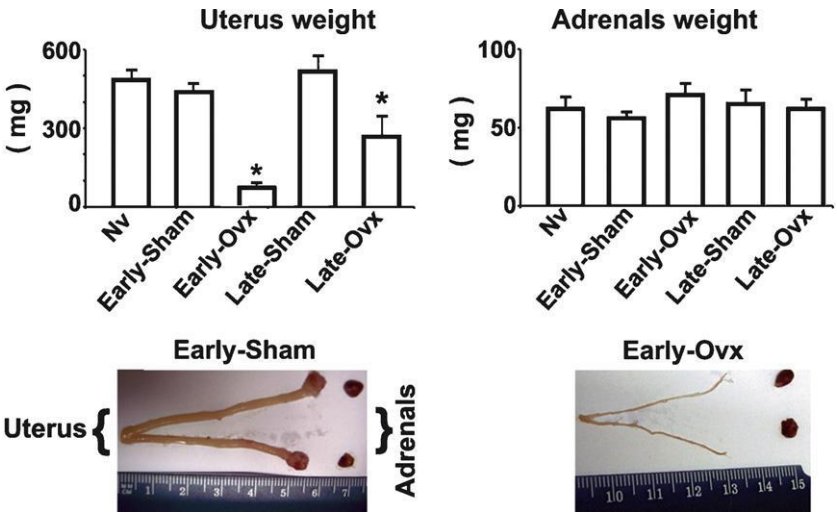

Fig. 2. Uterus and adrenal weights (mean ± EPM) of the Nv, Sham and Ovx groups, as defined in Fig. 1. **The** asterisks (*) in the upper-left panel indicate that the Ovx groups presents uterus weights significantly lower (P < 0.001) than those of the Sham and Naïve controls. The mean adrenal weights were comparable in all groups (upper-right panel). The two photographs of the lower part of the figure illustrate the uterus atrophy in the early-Ovx condition (right photograph), as compared to the Sham control (left photograph). The adrenal glands are also shown in the two photographs, and no macroscopically appreciable intergroup difference could be detected (ANOVA followed by Tukey test).

were 222.2 ± 14.0 and 249.6 ± 25.4 g, for the Sham and Ovx groups, respectively (P < 0.05; t-test).

3.2. Uterus and adrenal weights

As can be seen in Fig. 2 (upper-left panel), in the early-Ovx group the uterus weights were severely decreased (P < 0.001) (80 ± 27 mg), as compared with both Naïve and Sham controls (490 ± 88 mg and 447 ± 88 mg, respectively). The adrenal weights of the early-Ovx group (71 ± 27 mg) were slightly higher than the Sham (56 ± 12 mg) and the Nv group (62 ± 19 mg), but the differ-ence did not reach statistical significance (upper-right panel of Fig. 2). The two photographs in the lower part of Fig. 2 illustrate the easily recognizable uterus atrophy in the early-Ovx condition (right photograph), as compared to the Sham control (left photo-graph). The adrenal glands (also shown in the two photographs) were macroscopically comparable in the two groups. In the rats operated at adulthood, the mean uterus weights at 120 days were 570 ± 59 mg and 277 ± 181 mg (P < 0.05), for the Sham and Ovx groups, respectively, while the adrenal weights for the same respec-tive groups were 63 ± 19 mg and 57 ± 5 (not shown in Fig. 2).

3.3. CSD propagation

Fig. 3 shows a typical electrophysiological recording (slow DC-potential change and ECoG) in one Nv, one early-Sham and one early-Ovx rat. In all groups, the 1-min stimulation with 2% KCl at one point of the frontal cortex elicited a single CSD wave that propa-gated without interruption and was recorded by the two electrodes located more posterior in the parietal cortex (see stimulation- and recording-points in the inset of the figure). In each recording point the ECoG depression and the slow potential change confirmed the presence of CSD, after KCl application.

In the early-Ovx rats, CSD propagated with significantly lower velocities (mean ± standard deviation: 2.72 ± 0.24 mm/min), as compared to the Sham and to the Naïve control groups (respectively 3.36 ± 0.09 mm/min and 3.36 ± 0.10 mm/min; P < 0.001, ANOVA followed by Tukey test). In the two groups operated at adult-hood, the mean CSD velocities were comparable to the controls (3.30 ± 0.06 mm/min and 3.27 ± 0.03 mm/min for the Sham and Ovx animals, respectively). Measurement of the amplitudes and durations of the CSD DC-potential change revealed intergroup significant differences (Ovx > Nv = Sham). Fig. 4 (lower panel) and Table 1 show these CSD findings, as well as the brain weights (upper panel of Fig. 4), which were comparable between the five groups: the mean brain weights (in g) for the Nv, early- and late-Sham,

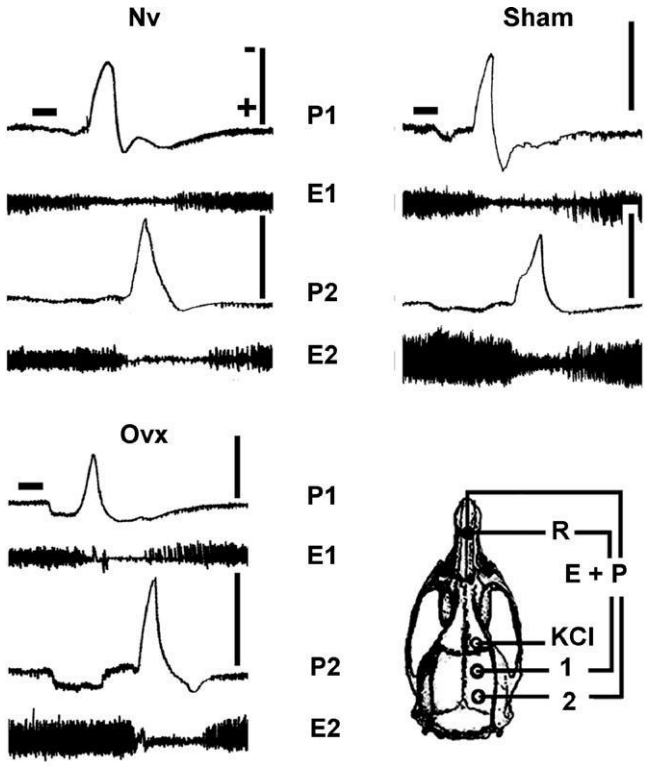

Fig. 3. Electrocorticogram (E) and slow DC-potential variation (P) recorded during the passage of cortical spreading depression (CSD) at two points (designated as 1 and 2) in the parietal cortex. The horizontal black bars above the P1 traces indicate the period (1 min) in which the chemical stimulus (2% KCl) was applied to trigger CSD, in the frontal region. The inset of the figure shows the position of the reference electrode (R), common to the two recording electrodes, as well as the point of KCl stimulation and the two recording sites. Vertical bars indicate −10 mV for P and −1 mV for ECoG.

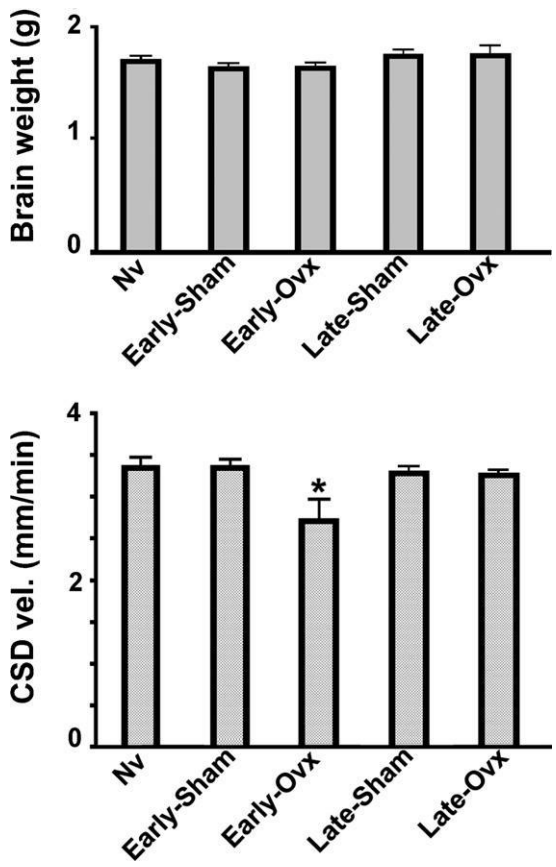

Fig. 4. Mean (±EPM) brain weights (upper panel) and CSD velocity of propagation (lower panel) of rats submitted early in life (7-day old), or at adulthood (70–90 days) to bilateral ovariectomy (Ovx group), or to Sham operation (Sham group), or not submitted to any surgery (Nv; Naïve group). The brain weights were comparable between the five groups. In the lower panel, the asterisk indicates that the propaga-tion of CSD in the early-Ovx group is significantly lower ($P < 0.001$) than the values of the other groups (ANOVA followed by Tukey test).

and early- and late-Ovx groups were respectively 1.703 ± 0.088, 1.633 ± 0.084, 1.746 ± 0.101, 1.642 ± 0.091 and 1.755 ± 0.147.

4. Discussion

4.1. Ovariectomy in developing rats

In this study we observed important developmental systemic and localized alterations resulting from bilateral ovariectomy, which was performed in the developing rats as early as the post-natal day 7. We have chosen this age because it corresponds to the time-point in which the ovaries become capable of secreting

Table 1
Amplitudes and durations of the CSD slow potential shifts in the Naïve (Nv), and early- and late-Sham ovariectomized (Ovx) groups. A and P refers to the anterior and posterior cortical recording points, respectively.

Group	Amplitude (mV)		Duration (s)	
	A	P	A	P
Nv	10.2± 4.1	12.1± 5.8	61.8 ± 11.4	76.5± 20.2
Early-Sham	10.1± 4.7	11.4± 4.6	62.2 ± 12.1	75.1± 12.6
Early-Ovx	18.2± 7.7*	21.8± 10.6*	77.3 ± 10.4*	76.2± 11.4
Late-Sham	11.2± 6.5	8.5± 3.2	67.0 ± 12.9	74.2± 11.5
Late-Ovx	7.21± 3.4	9.1± 5.7	70.2 ± 10.3	71.9± 4.6

Data are expressed as mean ± standard deviation.

* Early-Ovx values that are significantly different from the corresponding Sham and Naïve group ($P < 0.05$; ANOVA plus Tukey test).

significant amounts of their hormones (Lamprecht et al., 1976). In accordance with a previous report (Gitlin, 1974), the effectiveness of the ovariectomy was presently evidenced by the severe atrophic patterns of uterus- and genital-epithelium, delayed vaginal open-ing, and body weight increment, as compared to the controls. Regarding the ovariectomy-induced increase in body weights, it is known that the hypoestrogenic status participates in the increased food intake and the resulting higher body weight gain, as compared to the controls (Wade, 1975; Roesch, 2006). The post-ovariectomy deficiency in the uterus development would be expected, since it is well described in the literature; ovaries participate in the uter-ine growth observed between the second and the fourth postnatal weeks (Branham and Sheehan, 1995). Concerning adrenal weights the lack of intergroup statistically significant difference is in agree-ment with data of Ramaley (1973). Taken together, our data on the systemic and localized developmental effects seen in early ovariec-tomized rats, besides assuring the effectiveness of the ovariectomy procedure also supports the causal link between ovarian hormones deficiency and the here described brain CSD effects.

4.2. CSD propagation

The main electrophysiological finding of the present study was that chronic ovarian hormones deficiency, which was provoked during development, reduced brain capability to propagate CSD in adulthood, as indexed by its lower velocities in comparison to the velocities of the Sham and Naïve controls. The alterations in CSD amplitude and duration also reinforce this conclusion. The CSD effects could not be detected in the group ovariectomized in adult-hood, and this is also evidence in favor of a lasting, developmental brain effect. It is well established that the gonadal steroids exert some of its action on the central nervous system, which is one important target organ for their actions, both during development and in adult life (Genazzani et al., 2005; Kawata, 1995). Although the final mechanisms are yet unknown, based on evidence from the literature we hypothesized that the GABAergic and glutamater-gic systems are involved in the effects of early ovariectomy on CSD. In contrast to its role on the adult brain, in the developing brain GABA is the predominant source of excitation via membrane depolarization, and estradiol markedly enhance depolarizing GABA responses in neonatal neurons (Nunez et al., 2008; Perrot-Sinal et al., 2003). Furthermore, in the developing brain estradiol damp-ens glutamate-mediated excitotoxicity by down-regulating their receptors (mGluR1 and mGluR5) and by decreasing the amount of calcium released from the endoplasmic reticulum (Hilton et al., 2006). Therefore, it appears to us compelling to accept that ovariec-tomy early in life changes excitability of the developing brain, and our CSD findings support that assumption.

Considering that CSD is influenced by changes in the brain excitability (Leão, 1944a,b, 1972; Guedes and Cavalheiro, 1997; Guedes et al., 2009; Guedes, 2011), it is reasonable to raise the question of if, and how early ovarian deficiency (or ovarian absence, as in the ovariectomy paradigm) would modulate brain excitabil-ity during development, and therefore influencing phenomena like seizures and CSD at adulthood. One possibility to explain the effects of ovariectomy on the excitability of the adult brain would be based on the influence of ovarian hormones on the glutamatergic neuro-transmitter system. Estrogens can increase neuronal excitability via upregulating the gene expression of NMDA receptor subunit and by decreasing the inhibitory action of GABAergic neurons (Eikermann-Haerter et al., 2007; Martin and Behbehani, 2006). Estrogen also can inhibit l-glutamate uptake by astrocytes (Sato et al., 2003), and increases the number of dendritic spines, which are densely popu-lated with NMDA receptors (Woolley et al., 1997). As a rule, higher levels of estrogen are associated with an increased seizure fre-quency in females (Klein and Herzog, 1998) and seizure thresholds

are decreased during peak estrogen levels (Woolley and Timiras, 1962), while progesterone is associated with seizure control in catamenial epilepsy (Herzog, 2009). Application of exogenous estrogen to the cerebellum of female rats significantly potentiates the excitatory neuronal response of Purkinje cells to glutamate applied by iontophoresis (Smith et al., 1988). Besides the gluta-matergic, the GABAergic neurons also are strongly modulated by ovarian steroids. Estrogen can increase GABA release and upregu-late the number of GABA receptors (Shughrue and Merchenthaler, 2000). Progesterone has been shown to have depressant effects on CNS excitability via modulation of the gamma-aminobutyric acid type A (GABAA; Lambert et al., 2003). These, and several other pieces of evidence showing that gonadal hormones can affect brain excitability (Eikermann-Haerter et al., 2007, 2009; Sachs et al., 2007; Woolley et al., 1997; Martin and Behbehani, 2006; Scharfman and MacLusky, 2006; Scharfman et al., 2005), collectively suggest that ovarian hormones could have a critical role in epilepsy, and perhaps could also play a role in epilepsy treatment (Herzog, 2009). In this context, it is important mentioning a recent report showing effects of ovariectomy on the ratio between n-3 and n-6 polyun-saturated fatty acids synthesis in the brain (Alessandri et al., 2011). We think that this is important because of the evidence showing the relationship between those fatty acids and brain excitability (Borba et al., 2010).

Concerning the ovarian hormones/CSD relationship, several findings from others (Eikermann-Haerter et al., 2007, 2009) deserve comment. First, the CSD susceptibility in familial hemiplegic migraine type 1 (FHM1) knockin mice is higher in females than in males; second, ovariectomy reverses this gender difference; third, the CSD-effect of ovariectomy is partially restored by estradiol replacement. These pieces of evidence suggest that actually estro-gens can modulate CSD susceptibility (Eikermann-Haerter et al., 2009). Furthermore, the thresholds for CSD elicitation with KCl-and electrical-stimulation are lower in female mice compared to males (Brennan et al., 2007). In rat neocortical slices, applica-tion of both -estradiol and progesterone enhances CSD features (Sachs et al., 2007). This compelling evidence from the literature favors our initial hypothesis that ovariectomy during the period of brain development is causally associated in adulthood with CSD impairment, probably because of ovariectomy-induced changes in neuron/glia excitability.

In conclusion we demonstrated for the first time that the brains of adult rats that had been ovariectomized during their develop-ment are more resistant (or less sensible) to CSD propagation. We suggest that this effect is related to the long-term suppression of the physiological action of the ovarian hormones on synaptic transmis-sion with consequent modulation of brain excitability. Our findings document the importance of further searching for the molecular mechanisms underlying the role of the ovarian hormones on the brain development and their electrophysiological properties.

Acknowledgments

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References

Alessandri, J.-M., Extier, A., Al-Gubory, K.H., Langelier, B., Baudry, C., LePoupon, C., Lavialle, M., Guesnet, P., 2011. Ovariectomy and 17 -estradiol alter transcription of lipid metabolism genes and proportions of neo-formed n-3 and n-6 long-chain polyunsaturated fatty acids differently in brain and liver. J. Nutr. Biochem. 22, 820–827.

Amateau, S.K., Alt, J.J., Stamps, C.L., McCarthy, M.M., 2004. Brain estradiol content in newborn rats: sex differences regional heterogeneity, and possible de novo synthesis by the female telencephalon. Endocrinology 145, 2906–2917.

Bakker, J., Honda, S., Harada, N., Balthazart, J., 2002. The aromatase knock-out mouse provides new evidence that estradiol is required during development in the female for the expression of sociosexual behaviors in adulthood. J. Neurosci. 22, 9104–9112.

Bakker, J., Baum, M.J., 2008. Role for estradiol in female-typical brain and behavioral sexual differentiation. Front. Neuroendocrinol. 29, 1–16.

Bakker, J., De Mees, C., Douhard, Q., Balthazart, J., Gabant, P., Szpirer, J., Szpirer, C., 2006. Alpha-fetoprotein protects the developing female mouse brain from masculinization and defeminization by estrogens. Nat. Neurosci. 9, 220–226.

Borba, J.M.C., Rocha-de-Melo, A.P., Amâncio-dos-Santos, A., Costa, B.L.S.A., Silva, R.P., Passos, P.P., Guedes, R.C.A., 2010. Essential fatty acid deficiency reduces cor-tical spreading depression propagation in rats: a two-generation study. Nutr. Neurosci. 13, 144–150.

Branham, W.S., Sheehan, D.M., 1995. Ovarian and adrenal contributions to postnatal growth and differentiation of the rat uterus. Biol. Reprod. 53, 863–872.

Brennan, K.C., Reyes, M.R., Valdés, H.E.L., Arnold, A.P., Charles, A.C., 2007. Reduced threshold for cortical spreading depression in female mice. Ann. Neurol. 61, 603–606.

Brouwer, E.A., Dailey, R., Brouwer, J.B., 1980. Ovariectomy of newborn rats: a descriptive surgical procedure. Lab. Anim. Sci. 30, 546–548.

Eikermann-Haerter, K., Kudo, C., Moskowitz, M.A., 2007. Cortical spreading depression and estrogen. Headache 47 (Suppl. 2), S79–S85.

Eikermann-Haerter, K., Dileköz, E., Kudo, C., Savitz, S.I., Waeber, C., Baum, M.J., Ferrari, M.D., van den Maagdenberg, A.M.J.M., Moskowitz, M.A., Ayata, C., 2009. Genetic and hormonal factors modulate spreading depression and transient hemiparesis in mouse models of familial hemiplegic migraine type 1. J. Clin. Invest. 119, 99–109.

Genazzani, A.R., Bernardi, F., Pluchino, N., Begliuomini, S., Lenzi, E., Casarosa, E., Luisi, M., 2005. Endocrinology of menopausal transition and its brain implications. CNS Spectr. 6, 449–457.

Gillies, G.E., MCarthur, M.S., 2010. Estrogen actions in the brain and the basis for differential action in men and women: a case for sex-specific medicines. Pharmacol. Rev. 62, 155–198.

Gitlin, G., 1974. Vaginal opening and vaginal epithelium following ovariectomy in newborn rats. Acta Anat. 90, 117–132.

Guedes, R.C.A., 2011. Cortical spreading depression: a model for studying brain consequences of malnutrition. In: Preedy, V., Watson, R., Martin, C. (Eds.), Handbook of Behavior, Food and Nutrition. Springer, Berlin, pp. 2343–2355.

Guedes, R.C.A., Cavalheiro, E.A., 1997. Blockade of spreading depression in chronic epileptic rats: reversion by diazepam. Epilepsy Res. 27, 33–40.

Guedes, R.C.A., Oliveira, J.A.C., Amâncio-dos-Santos, A., García-Cairasco, N., 2009. Sexual differentiation of cortical spreading depression propagation after acute and kindled audiogenic seizures in the Wistar audiogenic rat (WAR). Epilepsy Res. 83, 207–214.

Herzog, A.G., 2009. Hormonal therapies: progesterone. Neurotherapeutics 6, 383–391.

Hilton, G.D., Nunez, J.L., Bambrick, L., Thompson, S.M., McCarthy, M.M., 2006. Glutamate-mediated excitotoxicity in neonatal hippocampal neurons is medi-ated by mGluR-induced release of Ca^{2+} from intracellular stores and is prevented by estradiol. Eur. J. Neurosci. 24, 3008–3016.

Klein, P., Herzog, A.G., 1998. Hormonal effects on epilepsy in women. Epilepsia 39, S9–S16.

Kawata, M., 1995. Roles of steroid hormones and their receptors in organization in the nervous system structural. Neurosci. Res. 24, 1–46.

Lambert, J.J., Belelli, D., Peden, D.R., Vardy, A.W., Peters, J.A., 2003. Neurosteroid modulation of GABAA receptors. Prog. Neurobiol. 71, 67–80.

Lamprecht, S.A., Kohen, F., Ausher, J., Zor, U., Lindner, H.R., 1976. Hormonal stimulation of estradiol-17 beta release from the rat ovary during early postnatal development. J. Endocrinol. 68, 343–344.

Lauritzen, M., Dreier, J.P., Fabricius, M., Hartings, J.A., Graf, R., Strong, A.J., 2011. Clinical relevance of cortical spreading depression in neurological disorders: migraine, malignant stroke, subarachnoid and intracranial hemorrhage, and traumatic brain injury. J. Cereb. Blood Flow Metab. 31, 17–35.

Leao, A.A.P., 1944a. Spreading depression of activity in the cerebral cortex. J. Neurophysiol. 7, 359–390.

Leão, A.A.P., 1944b. Pial circulation and spreading depression of activity in the cerebral cortex. J. Neurophysiol. 7, 391–396.

Leão, A.A.P., 1972. Spreading depression. In: Purpura, D.P., Penry, K., Tower, D.B., Woodbury, D.M., Walter, R.D. (Eds.), Experimental Models of Epilepsy. Raven Press, New York, pp. 173–195.

Martin, V.T., Behbehani, M., 2006. Ovarian hormones and migraine headache: understanding mechanisms and pathogenesis—part l. Headache 46, 3–23.

McCarthy, M.M., 2009. The two faces of estradiol: effects on the developing brain. Neuroscientist 15, 599–610.

McCarthy, M.M., 2008. Estradiol and the developing brain. Physiol. Rev. 88, 91–124.

McCarthy, M.M., Konkle, A.T.M., 2005. When is a sex difference not a sex difference? Front. Neuroendocrinol. 26, 85–102.

Mellon, S.H., Vaudry, H., 2001. Biosynthesis of neurosteroids and regulation of their synthesis. Int. Rev. Neurobiol. 46, 33–78.

Nunez, J.L., Aberdeen, G.W., Albrecht, E.D., McCarthy, M.M., 2008. Impact of estradiol on GABA- and glutamate-mediated calcium responses of fetal baboon (papio anubis) hippocampal and cortical neurons. Endocrinology 149, 6433–6443.

Perrot-Sinal, T.S., Auger, A.P., McCarthy, M.M., 2003. Excitatory GABA-induced pCREB in developing brain is mediated by L-type Ca^{2+} channels and dependent on age sex and brain region. Neuroscience 116, 995–1003.
Phifer, C.B., Terry, L.M., 1986. Use of hypothermia for general anesthesia in prewean-ling rodents. Physiol. Behav. 38, 887–890.
Ramaley, J.A., 1973. Adrenal function in female rats before puberty: the effects of gonadectomy. Steroids 22, 597–608.
Roesch, D.M., 2006. Effects of selective estrogen receptor agonists on food intake and body weight gain in rats. Physiol. Behav. 87, 39–44.
Sachs, M., Pape, H.C., Seckmann, E.J., Gorji, A., 2007. The effect of estrogen and pro-gesterone on spreading depression in rat neocortical tissues. Neurobiol. Dis. 25, 27–34.
Sanyal, M.K., 1978. Secretion of progesterone during gestation in the rat. J. Endocrinol. 79, 179–190.
Sato, K., Matsuki, N., Ohno, Y., Nakazawa, K., 2003. Estrogens inhibit l-glutamate uptake activity of astrocytes via membrane estrogen receptor alpha. J. Neurochem. 86, 1498–1505.
Scharfman, H.E., Goodman, J.H., Rigoulot, M.A., Berger, R.E., Walling, S.G., Mercurio, T.C., Stormes, K., Maclusky, N.J., 2005. Seizure susceptibility in intact and ovariec-tomized female rats treated with the convulsant pilocarpine. Exp. Neurol. 196, 73–86.
Scharfman, H.E., MacLusky, N.J., 2006. The influence of gonadal hormones on neu-ronal excitability seizures, and epilepsy in the female. Epilepsia 47, 1423–1440.
Shughrue, P.J., Merchenthaler, I., 2000. Estrogen is more than just a sex hormone: novel sites for estrogen action in the hippocampus and cerebral cortex. Front. Neuroendocrinol. 21, 95–101.
Smith, S.S., Waterhouse, B.D., Woodward, D.J., 1988. Locally applied estrogens poten-tiate glutamate-evoked excitation of cerebellar Purkinje cells. Brain Res. 475, 272–282.
Tenório, A.S., Oliveira, I.D.V.A., Guedes, R.C.A., 2009. Early vibrissae removal facili-tates cortical spreading depression propagation in the brain of well-nourished and malnourished developing rats. Int. J. Dev. Neurosci. 27, 431–437.
Tsutsui, K., 2008. Minireview: progesterone biosynthesis and action in the develop-ing neuron. Endocrinology 149, 2757–2761.
Wade, G.N., 1975. Some effects of ovarian hormones on food intake and body weight in female rats. J. Comp. Physiol. Psychol. 88, 183–193.
Woolley, C.S., Weiland, N.G., McEwen, B.S., Schwartzkroin, P.A., 1997. Estradiol increases the sensitivity of hippocampal CA1 pyramidal cells to NMDA receptor-mediated synaptic input: correlation with dendritic spine density. J. Neurosci. 17, 1848–1859.
Woolley, D.E., Timiras, P.S., 1962. Estrous and circadian periodicity and electroshock convulsions in rats. Am. J. Physiol. 202, 379–382.

5. ARTIGO 2 – EM VIAS DE SUBMISSÃO

Title-1: Neonatal treatment with ovarian hormones modulates cortical spreading depression, but not anxiety-like behavior, in adult female rats previously suckled under different litter sizes

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Abstract

Ovarian hormones and early malnutrition are two factors that exert profound effects on the developing brain, with repercussion on behavioral and excitability-dependent processes. In this study, we investigated in female rats suckled in litters with 6-9 and 12-15 pups (L_9 and L_{15} groups, respectively) the effect of treatment in early life with β-estradiol or progesterone on anxiety behavior (elevated plus maze [EPM] and open field [OF]) and the excitability-dependent phenomenon known as cortical spreading depression (CSD). From postnatal days (P) 7 to 21, the animals received 50μg/kg of β-estradiol or progesterone every other day. At P80-89 we tested the behavioral reactions. At P90-120, we recorded CSD on two cortical points over the course of four hours, and analyzed CSD velocity of propagation, amplitude, and duration of the CSD DC-shift. Regarding the behavioral parameters, no intergroup difference was observed. Both β-estradiol- and progesterone-treated groups displayed higher CSD velocities and amplitudes, and shorter durations, compared with naive and olive oil-treated controls ($p < 0.5$). Another set of six L_9 and six L_{15} groups received topic cortical application of three distinct solutions of β-estradiol (5 mg/ml, 10 mg/ml and 20 mg/ml) and progesterone (66 mg/ml, 131.5 mg/ml and 263 mg/ml). Topic application of both hormones produced reversible and dose-dependent CSD acceleration ($p < 0.5$). We concluded that treatment with the ovarian hormones during brain development is causally associated with CSD, but not behavioral, modulation in the adult brain, which suggests a long lasting effect that might be related to the lasting hormonal action on brain excitability.

Keywords: Estradiol; Progesterone; Brain development; Neural excitability; Cortical spreading depression; Anxiety behavior;

1. Introduction

In mammals, the proper brain development and functioning depends on an adequate nutrition early in life, such that the neonatal period is decisive for brain development. Processes such as neurogenesis, gliogenesis and neuronal migration occur most intensely in the phase known as “period of rapid brain growth” or simply “critical period” (Dobbing, 1968). This phase is considered critical for the perfect development and function of the brain, because it represents a vulnerable period for endogenous and exogenous brain challenges, such as that represented by the action of ovarian hormones. Thus, ovarian hormones (Calza et al, 2010) and early malnutrition (Guedes, 2011) can affect both behavior and brain excitability. However the possible interaction between these two factors has not been subject of much investigation. In the rat, pups suckled in large litters compete for the dam’s milk, and this unfavorable lactation condition results in a moderate state of malnutrition that can affect the animal’s behavior and the electrical activity of the brain (Franisco and Guedes, 2015).

The brain of mammals is a steroidogenic organ that is an important target for the physiological and pathological action of ovarian hormones (Wagner, 2008). In female rats, the newborn brain is exposed to ovarian hormones produced by their mothers (via maternal circulation/placenta/lactation). Also, the newborn’s gonads and brains sintethize such hormones, which contribute to the hormonal influence that acts on the newborn’s brain (Arevalo et al., 2015; McCarthy, 2009; Tanaka and Sokabe, 2012; Reddy, 2014). Accumulating evidence indicate that ovarian hormones can be synthesized de novo from cholesterol entirely within select regions of the brain. All of the synthetic enzimes required to complete the conversion of cholesterol to estradiol or progesterone have been identified in both the adult and developing brain (Arevalo et al., 2015). In the last decade, the interest in understanding the neural actions of ovarian hormones has increased substantially (Calza et al, 2010; Bakker and Baum, 2008; Hill et al, 2014; Wagner, 2008; Muneoka et al, 2010). It is now well established that some of the neural action of ovarian hormones involve brain excitability (Grassi et al, 2011; Reddy, 2013). This issue can be experimentally addressed by using the electrophysiological phenomenon denominated as cortical spreading depression (CSD; Chauvel et al, 2013; Eickermann-Haerter et al, 2009; Guedes et al., 2009; Sachs et al, 2007).

CSD is a reversible wave of self-propagating depolarization of the cerebral cortex, in response to electrical, chemical, or mechanical stimulation of one point of the cortical surface. The cerebral cortex response consists in a reducton of its spontaneous and evoked electrical

activity that spreads concentrically from the stimulated point. CSD presents characteristic ionic, metabolic, and hemodynamic changes that after a few minutes return to the pre-CSD levels (Leão, 1944a,b). Compelling clinical and experimental evidence has linked CSD to excitability disorders of the human brain and their diseases such as migraine with aura (Ferrari et al. 2015; Lauritzen et al., 2011), brain vascular disorders (Eickermann-Haerter, 2014; Lauritzen et al., 2011), and epilepsy (Fabricius et al. 2008; Wei et al. 2014). CSD-like waves were detected in human neocortex during the aura phase of migraine attacks (Hadjikhani et al., 2001).

The influence of ovarian hormones on CSD (Sachs et al., 2007) as well as relevant human neurological dieases, such as epilepsy and migraine has been documented (Taubøll et al., 2015; Borsook et al., 2014). In a previous study, we demonstrated that rats ovariectomized early in life, but not at adulthood, presented with reduced brain capability to propagate CSD in adult age (Accioly et al, 2012). In the present study, we demonstrated that treatment with β-estradiol and progesterone early in life modulates brain excitability, but not anxiety-like behavior. Using the CSD model, we could show the facilitating effect of β-estradiol and progesterone evaluated at adulthood. Furthermore, unfavorable conditions of lactation (see methods) modulate this effect.

2. Materials and methods

2.1. Animals

All experimental procedures were previously approved by the Institutional Ethics Committee for Animal Research of our University (Approval protocol no. 23076.010208/2012-09), whose norms comply with those established by the National Institutes of Health, Guide for Care and Use of Laboratory Animals (Bethesda, MD, USA). Wistar female newborn rats (n=204) from the colony of our department were randomly distributed to one of two different conditions of lactation: 1) normal lactation, in which the pups were suckled on litters formed by 6 to 9 pups (n=109), and 2) unfavorable lactation (litters with 12 to 15 pups; n=95). These two lactation conditions (designated as L_9 and L_{15} groups, respectively) have been shown to be nutritionally distinct (Francisco and Guedes, 2015), and this has been confirmed in this study (see results). The weaning occurred in all groups on postnatal (P) day 21, considering the day of birth as day 0. Dams and pups had free

access to water and a commercial lab chow (Purina, with 23% protein). They were housed in polyethylene cages (51 cm X 35.5 cm X 18.5 cm) under controlled temperature (23±1°C) with a 12-h light:12-h dark cycle (lights on at 6:00 AM).

2.2. Systemic hormonal treatment

From P7 to P21, L_9 and L_{15} pups (n = 25 and 22, respectively) received, every other day, one intraperitoneal injection containing 50 µg/kg β-estradiol (15 L_9 rats and 10 L_{15} rats), or 50 µg/kg progesterone (10 L_9 rats and 12 L_{15} rats). Both hormones (purchased from Sigma, St Louis, USA) were dissolved in 0.1 ml olive oil. These groups were compared to a vehicle-treated group (group V; 15 L_9 rats and 9 L_{15} rats) that received olive oil, and a naïve group (group Nv; 8 L_9 rats and 12 L_{15} rats) that received no treatment). Body weight was determined at P7, P21, P60 and P90, when we performed the electrophysiological recordings, just after completion of the behavioral tests.

2.3. Behavioral tests

When the animals reached 80-89 days of life, they were tested in the elevated plus-maze (EPM) and the open field (OF). This aims to evaluate the animal's ability, during the test, to recognize anxiogenic places (open arms in the EPM and center part of the OF), where it spends less time during the test (Calza et al, 2010). Each animal was tested once for 5 min in each behavioral test (first, in the EPM and 48 h latter in the OF). Animals were tested randomly between 7 am and 1 pm in a sound-attenuated room, under low light intensity.

The EPM (elevated 55 cm from the floor) comprised two open and two closed arms (each measuring 49 cm x 10 cm) connected by a central square (10 cm), where the animal was placed to start the test. Following 30-60 minutes adaptation period, the animal was placed on the central square facing a closed arm. We took the following measures in the EPM: travelled distance, immobility time, number of entries in the open arms, and time spent in the open arms. We considered that the animal entered one arm when its four paws entered the arm.

The OF consists of a circular arena with 89 cm in diameter. For the OF test, the animal was placed for 30-60 minutes in the OF to adapt to the environment. Following adaptation, OF activity was assessed for 5 minutes. The center of the OF was defined as an area with 62 cm diameter. We took the following measures: distance travelled, number of entries in the

center area, time spent in the center and the mean velocity of locomotion. We considered that the animal entered the center of the OF when its four paws entered the center.

In both OF and EPM tests, the videorecorded behavioral reactions were analyzed with the help of the AnyMaze™ software (version 4.99). Following each OF and EPM test the apparatuses were properly cleaned with 70% alcohol, to eliminate olfactory cues that could influence the following test.

2.4. CSD electrophysiological recording

Between 90-120 days of age, under anesthesia with a mixture of 1 g/kg urethane + 40 mg/kg alpha chloralose (ip; both purchased from Sigma, St. Louis, Mo, USA), three trephine holes were made at the right side of the skull, parallel to the midline, to expose portions of the cortical surface. One hole, which was used to elicit CSD, was on the frontal bone. The other two holes (on the parietal bone) were used to record the propagating CSD waves. During the recording period, the animal was maintained over an electric heater such that the rectal temperature remained stable (37 ± 1 ° C) and could be adjusted when necessary. The animals were subjected to a 4-h CSD recording session, as previously described (Mendes-da-Silva et al, 2014). Briefly, CSD was elicited at 20-min intervals by applying, for 1 min, a cotton ball (1-2 mm diameter) soaked in KCl solution (2 g KCl dissolved in 100 ml distilled water; approximately 0.27 M) to the anterior hole drilled at the frontal region. With a pair of Ag-AgCl agar-Ringer electrodes, the direct current (DC)-potential change typical of CSD was recorded at two parietal points on the cortical surface. A common reference electrode of the same type was placed on the nasal bones. The electrodes were connected to a digital data acquisition system (EMG Systems, São Paulo, Brazil). The recorded data were stored, visualized and analyzed in an IBM®-compatible computer. We calculated the velocity of CSD propagation, as well as the amplitude and duration of the negative slow potential shifts of the CSD waves, as previously reported (Mendes-da Silva et al, 2014). In order to calculate the velocity of CSD propagation, we measured the time required for a CSD wave to cross the distance between the two recording electrodes, taking the initial point of each DC negative rising phase as the reference point. In all experiments, we determined the estrous cycle status on the day of the CSD-recording. CSD was always recorded in the proestrous phase of the rat's estrous cycle. At the end of the 4 h CSD-recording session, we measured brain, adrenal, ovary and uterus weight.

2.5. Cortical topic treatment with estradiol or progesterone

A diagram of the whole process of topic cortical treatment with estradiol or progesterone is presented in Fig. 1. The anesthesia, trepanation, placement of the recording electrodes, CSD recording and calculation of CSD velocity were as described above for the systemic treatment (topic *2.4.*). In order to treat a circular cortical region (3–4 mm diameter) under one recording electrode (recording point 1 in Fig.4), water-soluble estradiol (5 mg/ml, 10 mg/ml and 20 mg/ml), or progesterone solutions (66 mg/ml, 131.5 mg/ml and 263 mg/ml) were topically applied over the intact dura-mater during the CSD recording session in six groups of adult rats (90–120 days; n = 7, 10 and 10, respectively, for estradiol, and n = 7, 9 and 10, respectively, for progesterone). Initially, we recorded four CSD episodes (baseline recording). Following that, estradiol or progesterone was topicly applied during the last 10 min of the 20-min interval between two consecutive KCl-stimulations. We applied only one hormone per rat. At the end of the 10 min topic treatment, the treated region was dried with a piece of cotton. This was followed by CSD elicitation with KCl. Hormone application was repeated three times at 20 min intervals. Following the hormone effect had been documented, the topicly treated region was dried with a piece of cotton, and four subsequent CSD episodes were recorded3 to document the recovery of the CSD-features. In the same animal, we compared CSD velocity, and amplitude and duration of the negative slow potential shift before and after hormone topic application. In some animals, we made a topic application of Ringer solution just before hormone treatment in order to have an additional control. Application of Ringer solution did not influence CSD. Following the topic hormonal application on the recording point 1 (described above), we repeated the application protocol on the recording point 2. In all experiments, the recordings of CSD were always made in the proestrous phase of the rat's estrous cycle.

2.6. Statistical analysis

For the weight, behavioral and CSD effects of the systemic hormone treatment, statistical analysis was performed by analysis of variance (ANOVA) including as factors the lactation condition (L_9 and L_{15}), and hormonal treatment (estradiol, progesterone, vehicle and naïve), followed by the Holm-Sidak test when indicated. For the CSD experiments on topic application of hormones, we used the paired t-test to compare, in the same animal, the CSD

velocity, and amplitude and duration of the DC-shift of CSD before and after topic application. Differences were considered statistically significant when $p < 0.05$.

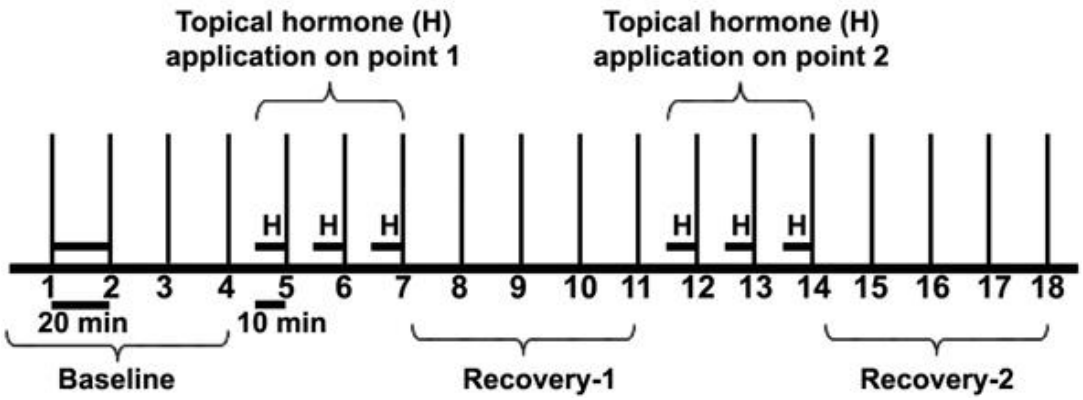

Figure 1- Time diagram of topic cortical hormonal (H; estradiol or progesterone) treatment. The shorter horizontal bars marked with H represents the estradiol or progesterone topic application during the last 10 min (shorter horizontal bars) of the 20-min interval (larger bar between 1 and 2) kept between two consecutive KCl-stimulations, necessary to elicit CSD. The numbers 1 to 18 indicate the eighteen CSD episodes elicited. The equidistant vertical dark lines indicate KCl stimulation at 20-min intervals. The same protocol extends to the recording point 2, with three topic applications of hormone followed by a recovery period (Recovery-2).

3. Results

3.1. Body- and brain-weights

ANOVA showed a main effect of the lactation condition for body weight. The Holm-Sidak test indicated that L_{15} animals presented with lower body- and brain-weights in comparison with the L_9 rats, in all ages ($p < 0.05$). Sistemic hormonal treatment did not significantly influence body weights. The mean body weight (± standard deviation) among the L_{15} groups ranged from 11.3±2.1 g to 11.7±1.5 g at P7, from 27.7±4.3 g to 30.0±4.3 g at P21, from 143.6±5.4 g to 174.8±22.7 g at P60 and from 176.0±13.3 g to 180.6±9.9 g at P90. In the

L_9 groups, the mean weight ranged from 13.0±1.8 g to 13.9±2.1 g at P7, from 34.9±1.4 g to 37.9±3.8 g at P21, from 158.1±14.1 g to 172.3±16.9 g at P60 and from 196.9±12.8 g to 203.6±13.5 g at P90 (Fig. 2, upper panel). Brain weights (Fig. 2, lower panel) were obtained from 5-9 L_9 rats and 5-10 L_{15} animals. The Holm-Sidak test indicated that in the L_{15} condition brain weights (ranging from 1.489±0.081 g to 1.569±0.019 g) were reduced in comparison with the L_9 groups (from 1.611±0.106 g to 1.652±0.039 g; $p < 0.05$).

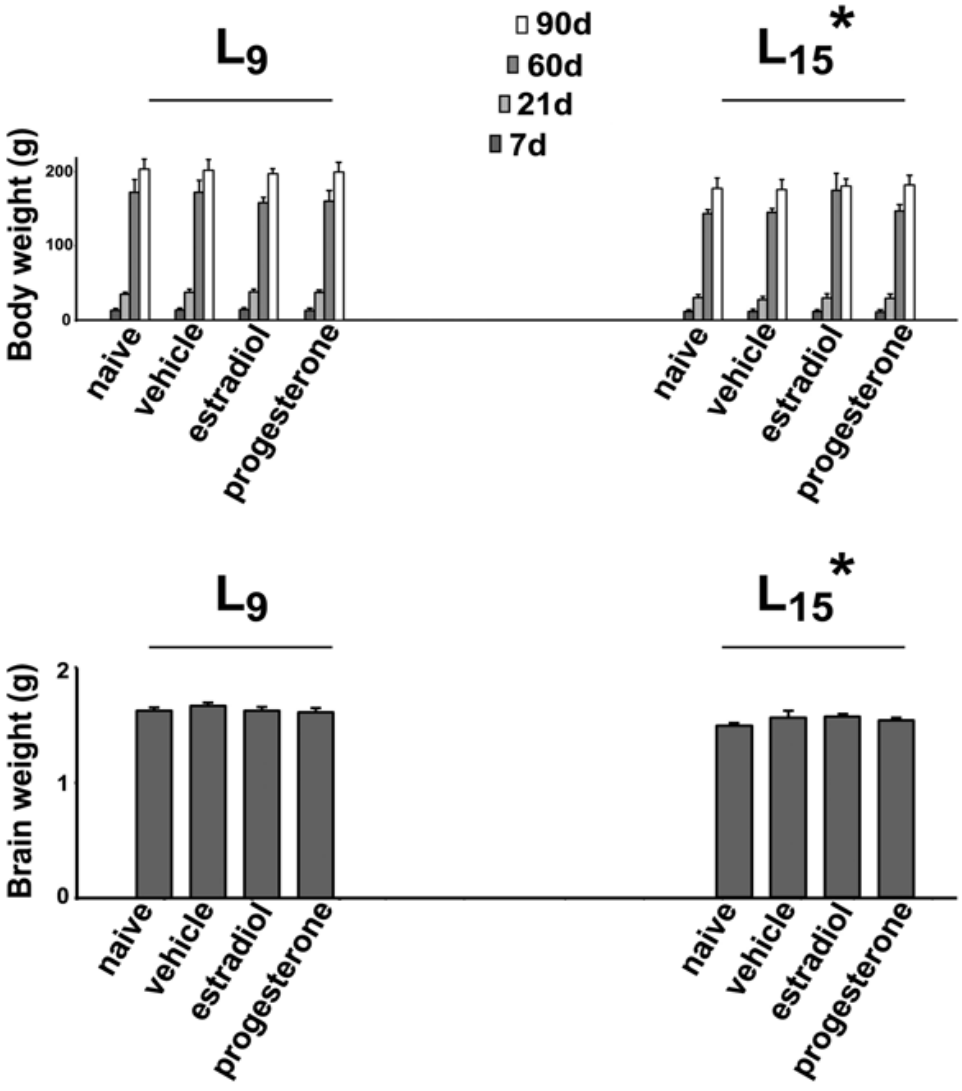

Fig. 2- Body weight (upper panel) and brain weight (lower panel) of female rats suckled in litters of 6-9 and 12-15 pups (respectively L_9 [left] and L_{15} condition [right]). Data are expressed as mean ± S.E.M. The asterisks indicate that L_{15} weights are significantly lower than the corresponding L_9 values ($p < 0.05$; one-way ANOVA followed by the Holm–Sidak test).

3.2. Ovaries, uterus and adrenal weights

As can be seen in Table 1, no intergroup difference was observed regarding the ovarian and adrenal weights. In the L_9 condition, both β-estradiol and progesterone treatment increased the uterus weight in comparison with the naïve and vehicle controls ($p < 0.05$). In the L_{15} condition, the progesterone treatment resulted also in higher uterus weights ($p < 0.05$). However, in the case of β-estradiol treatment the weight difference was significant only in comparison with the vehicle, but not the naïve, controls (Table 1).

Table 1- Weights of uterus, ovaries and adrenals of female rats treated in early life (from P7 to P21) with 50 μg/kg of β-estradiol or progesterone every other day. Data are mean ± standard deviation, with the number of animals in parentheses. *=different from the corresponding naïve and vehicle controls. #= different from the corresponding vehicle group ($p < 0.05$; ANOVA followed by the Holm-Sidak test). L9 and L15 are groups suckled under litters with 6-9 and 12-15 pups, respectively.

	UTERUS (mg)		**OVARIES (mg)**		**ADRENALS (mg)**	
GROUPS	**L9**	**L15**	**L9**	**L15**	**L9**	**L15**
Naïve	454.0 ± 60.0 (n=7)	445.8 ± 47.9 (n=5)	132.1 ± 11.4 (n=6)	113.0 ± 10.2 (n=9)	78.0 ± 11.1 (n=5)	68.8 ± 15.0 (n=9)
Vehicle	436.9 ± 1.3 (n=7)	415.7 ± 86.1 (n=6)	133.5 ± 8.3 (n=10)	113.0 ± 17.4 (n=5)	72.2 ± 10.1 (n=8)	68.4 ± 9.1 (n=5)
Estradiol	584.0 ± 95.0***** (n=5)	511.9 ± 43.5$^{\#}$ (n=6)	136.0 ± 12.0 (n=8)	150.9 ± 17.9 (n=6)	73.0 ± 22.0 (n=6)	74.4 ± 6.3 (n=7)
Progesterone	589.5 ± 97.2***** (n=5)	505.1 ± 61.3***** (n=5)	132.6 ± 9.2 (n=6)	130.5 ± 8.0 (n=5)	72.1 ± 21.0 (n=5)	77.1 ± 19.6 (n=7)

3.3. Behavioral tests

The behavioral parameters assessed in the EPM and OF are in Tables 2 and 3, respectively. Neonatal treatment with β-estradiol did not affect the behavioral parameters of L_9 and L_{15} female rats measured at adulthood in the EPM and OF tests.

Table 2- Elevated plus-maze parameters measured in 80-89 days-old female rats that received previously (from P7 to P21) one intraperitoneal injection, every other day, with 50 g/kg of β-estradiol, or progesterone, or vehicle (olive oil), or no treatment (naïve). Data are presented as mean ± standard deviation, with the number of animals in parentheses. No intergroup significant difference was observed.

GROUPS	**Travelled distance (m)**		**Immobility time (s)**		**No. Entries, open arms**		**Time, open arms (s)**	
	L_9	**L_{15}**	**L_9**	**L_{15}**	**L_9**	**L_{15}**	**L_9**	**L_{15}**
Naïve	11.0 ± 2.3 (n=14)	12.6 ± 3.1 (n=12)	89.9 ± 27.9 (n=14)	79.3 ± 34.9 (n=12)	11.5 ± 6.5 (n=13)	14.9 ± 6.8 (n=10)	58.7 ± 21.3 (n=9)	66.4 ± 22.3 (n=9)
Vehicle	12.5 ± 3.2 (n=8)	11.1 ± 3.8 (n=8)	75.9 ± 34.4 (n=8)	100.4 ± 48.1 (n=8)	12.8 ± 3.8 (n=8)	12.2 ± 5.3 (n=8)	71.4 ± 41.4 (n=8)	55.5 ± 35.5 (n=8)
Estradiol	11.1 ± 2.3 (n=6)	11.1 ± 2.9 (n=11)	89.3 ± 31.5 (n=6)	95.0 ± 26.9 (n=11)	15.5 ± 4.8 (n=6)	10.0 ± 2.5 (n=8)	64.3 ± 32.4 (n=6)	34.4 ± 16.3 (n=9)
Progesterone	12.8 ± 3.7 (n=8)	12.9 ± 3.7 (n=12)	80.5 ± 40.3 (n=8)	79.8 ± 41.3 (n=12)	15.6 ± 5.1 (n=8)	11.1 ± 5.6 (n=12)	79.2 ± 19.8 (n=7)	60.9 ± 18.8 (n=9)

Table 3- Open field parameters measured in 80-89 days-old female rats that received previously (from P7 to P21) one intraperitoneal injection, every other day, with 50 μg/kg of β-estradiol, or progesterone, or vehicle (olive oil), or no treatment (naïve). Data are presented as mean ± standard deviation, with the number of animals in parentheses. No intergroup significant difference was observed.

GROUPS	**Travelled distance (m)**		**No. entries (center)**		**Time in the center (s)**		**Mean speed (x1000) (m/s)**	
	L_9	**L_{15}**	**L_9**	**L_{15}**	**L_9**	**L_{15}**	**L_9**	**L_{15}**
Naïve	22.2 ± 2.1 (n=9)	23.2 ± 5.7 (n=9)	6.9 ± 3.2 (n=11)	11.8 ± 6.4 (n=12)	21.0 ± 10.7 (n=11)	28.0 ± 15.4 (n=12)	74.0 ± 7.0 (n=9)	81.0 ± 16.0 (n=8)
Vehicle	23.7 ± 4.6 (n=11)	21.3 ± 4.9 (n=11)	8.73 ± 5.8 (n=11)	8.7 ± 5.5 (n=11)	19.4 ± 11.8 (n=11)	21.3 ± 13.9 (n=11)	79.6 ± 15.7 (n=11)	71.0 ± 16.2 (n=11)
Estradiol	21.4 ± 5.0 (n=8)	24.8 ± 4.4 (n=10)	7.9 ± 5.2 (n=8)	7.3 ± 3.6 (n=10)	23.7 ± 13.2 (n=8)	15.4 ± 6.9 (n=10)	73.9 ± 17.6 (n=8)	84.4 ± 15.7 (n=10)
Progesterone	22.0 ± 6.0 (n=10)	26.7 ± 9.6 (n=10)	10.0 ± 5.5 (n=10)	9.9 ± 6,6 (n=10)	21.1 ± 8.9 (n=10)	28.3 ± 15.8 (n=10)	74.0 ± 20.0 (n=10)	90.6 ± 31.2 (n=10)

3.4. CSD parameters -systemic treatment

Fig. 3 shows representative CSD recordings of all groups that underwent the systemic treatment protocol. Stimulation with 2% KCl at one point of the frontal cortical surface for 1 min elicited a single CSD wave that propagated without interruption and was recorded at the two parietal recording points (Fig. 3; see the recording points 1 and 2 in the skull diagram). The regular appearance of the slow potential change after KCl stimulation confirmed the presence of CSD.

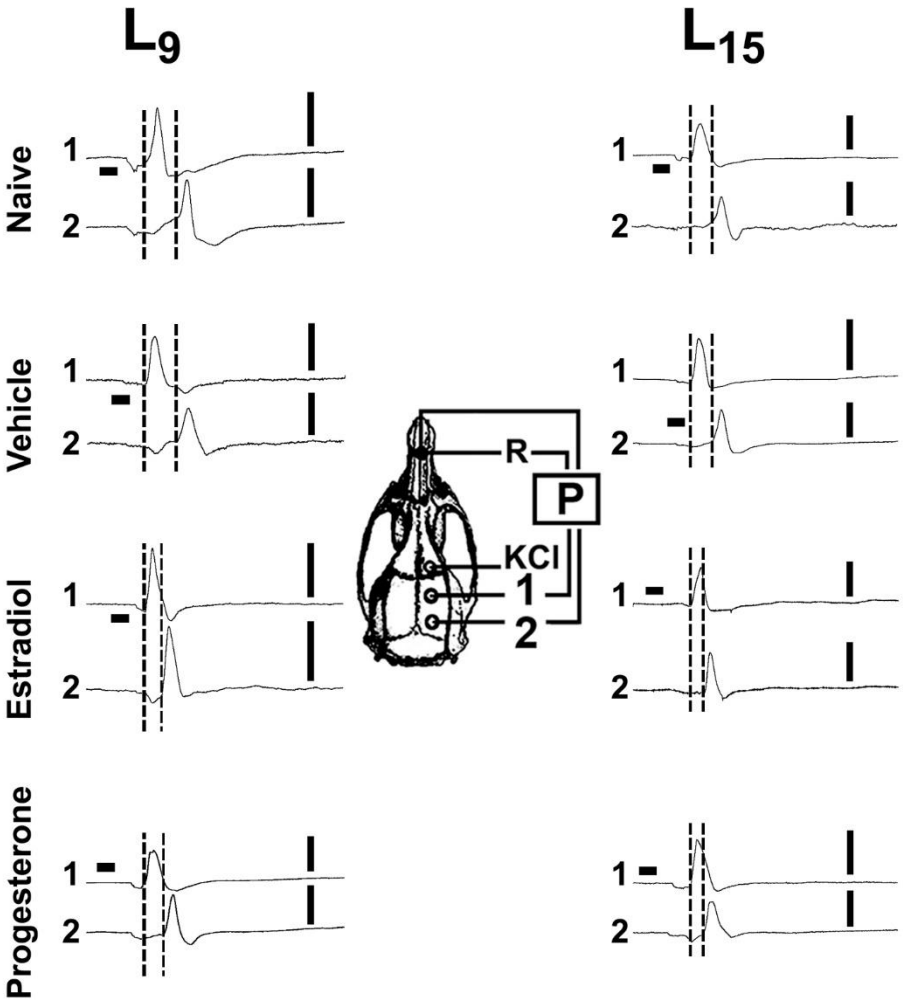

Figure 3. Slow potential changes (P) during cortical spreading depression (CSD), recorded at two cortical points (1 and 2) in three L_9 and three L_{15} adult female rats. The diagram of the skull shows the recording positions 1 and 2 from which the traces marked at the left with the same numbers were obtained. The position of the common reference electrode (R) on the nasal bones, and the application point of the CSD-eliciting stimulus (KCl) are also shown. The vertical bars indicate 10 mV for P (negative upwards). CSD was elicited in the frontal cortex by chemical stimulation (a 1–2 mm diameter cotton ball soaked with 2% KCl) applied for 1 min on the intact dura mater, as indicated by the horizontal bars. The vertical dashed lines indicate the latency for a CSD wave to cross the inter-electrode distance. Note the shorter latencies in the groups treated early in life (P7-P21) with β-estradiol or progesterone.

ANOVA indicated difference between the two lactation conditions, and *post hoc* (Holm–Sidak) test comparisons showed that the velocities were higher ($p < 0.05$) in the L_{15} rats in comparison with the L_9 animals.

Regarding β-estradiol or progesterone treatment, ANOVA detected a main effect, and the *post hoc* test revealed that treatment with β-estradiol or progesterone significantly enhanced the CSD propagation velocities ($p < 0.05$) in comparison with the naïve and vehicle controls in both lactation conditions. In the L_9 animals, the CSD velocity (mean ± SD) in the naïve and vehicle controls was respectively 3.28 ± 0.08 mm/min and 3.34 ± 0.09 mm/min; in the estradiol group and in the progesterone group the CSD velocity was significantly higher (4.25 ± 0.28 mm/min and 4.06 ± 0.20 mm/min, respectively). In L_{15} animals, the CSD velocities in the control groups were higher than in the L_9 rats (from to 4.37 ± 0.16 mm/min and 4.34 ± 0.18 mm/min for the naïve and vehicle groups, respectively). Treatment with β-estradiol or progesterone further increased ($p < 0.05$) CSD velocity in L_{15} animals to 4.70 ± 0.40 mm/min (estradiol) and 5.01 ± 0.44 mm/min (progesterone). The quantitative data of the CSD velocities are illustrated in Fig. 4.

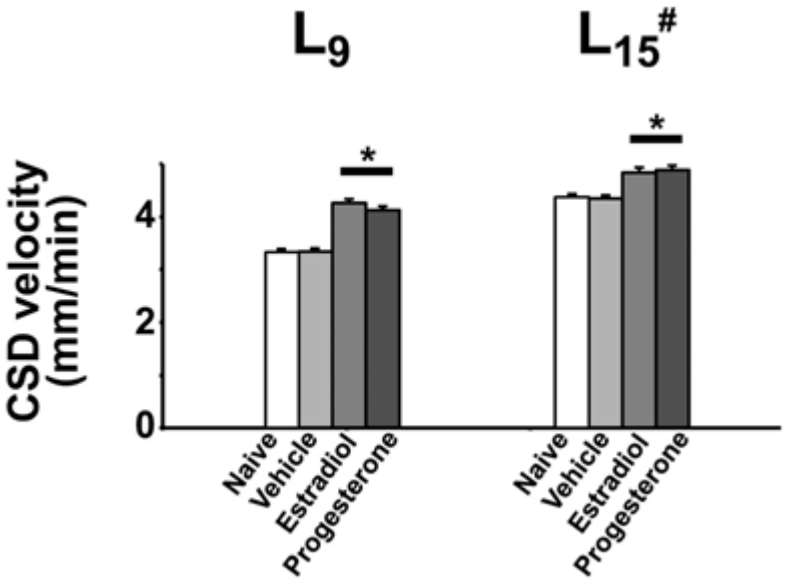

Fig. 4. CSD velocity of 90-120 days old female rats that were suckled in two distinct lactation conditions represented by litters with 6-9 and 12-15 pups (respectively well-nourished and malnourished groups). Data are mean ± S.E.M. Asterisks indicate that the hormone-treated groups are significantly different from the naïve- and vehicle-control groups. # indicates that all L_{15} groups were significantly different from the corresponding L_9 groups ($p < 0.05$; ANOVA followed by the Holm–Sidak test).

The amplitude (mean ± SD in mV) and duration (mean ± SD in s) of the negative slow potential shift, which is the hallmark of CSD, are shown in Table 4. ANOVA revealed a significant main effect of β-estradiol or progesterones on CSD amplitude and duration, which were respectively larger and shorter in the estradiol or progesterone groups in comparison with the corresponding vehicle and naïve controls.

Table 4- Amplitudes (mV) and durations (s) of the CSD slow potential change in L_9 and L_{15} female rats that received no treatment (naïve), vehicle (olive oil), β-estradiol and progesterone. Hormones and vehicle were administered systemically from P7 to P21, every other day, via intraperitoneal injection. Data are mean ± standard deviation with the number of animals in parentheses.

Groups	**Amplitude (mV)**		**Duration (s)**	
	L_9	**L_{15}**	**L_9**	**L_{15}**
Naive	9.0 ± 1.7 (n=7)	10.5 ± 1.1 (n=9)	64.2 ± 2.3 (n=8)	59.9 ± 2.3 (n=12)
Vehicle	8.8 ± 1.3 (n=14)	10.2 ± 1.5 (n=9)	64.2 ± 3.5 (n=12)	60.4 ± 2.2 (n=9)
Estradiol	11.3 ± 1.5 * (n=14)	12.7 ± 1.6* (n=8)	55.9 ± 2.3* (n=12)	55.9 ± 1.1* (n=8)
Progesterone	11.2 ± 1.3* (n=9)	12.5 ± 1.3* (n=12)	55.4 ± 2.6* (n=10)	54.2 ± 0.6* (n=12)

* $P < 0.05$ in comparison with the corresponding vehicle and naïve groups (ANOVA plus Holm-Sidak test).

3.5. CSD parameters - cortical topic treatment

To assess the effect of topical estradiol or progesterone on CSD parameters, in twelve groups of naïve female rats (six groups previously suckled under the L_9, and six under the L_{15} paradigm) we applied either hormone on a circular portion of the cortical surface (dura mater intact) during the CSD recording session. We tested three different estradiol concentrations (5 mg/ml, 10 mg/ml and 20 mg/ml) and three concentrations of progesterone (66 mg/ml, 131.5 mg/ml and 263 mg/ml).

Our results showed that in both nutritional groups the lowest tested concentration of estradiol (5 mg/ml) and progesterone (66 mg/ml) did not affect the velocity, amplitude and duration of CSD in any of the two recording points. However, 10 mg/ml and 20 mg/ml estradiol concentrations, as well as 131.5 mg/ml and 263 mg/ml progesterone solutions were effective in significantly and reversibly increasing the amplitude (Tables 5 and 6), decreasing the wave duration (Tables 7 and 8) and accelerating CSD (Fig. 5).

In the L9 groups, after estradiol application on the recording point 1(Estr-1), the baseline CSD velocity (3.25 ± 0.05 mm/min and 3.39 ± 0.04 mm/min for 10 mg/ml and 20mg/ml groups, respectively), increased to 3.56 ± 0.07 mm/min and 3.80 ± 0.08 mm/min, respectively ($P<0.05$). After the estradiol effect was documented, the topicaly treated region was dried with a piece of cotton and subsequent SD episodes confirmed the return of SD velocity to the basal levels (3.27 ± 0.04 mm/min and 3.39 ± 0.04 mm/min, respectively). Thereafter, a second application of estradiol on the recording point 2 (Estr-2) increased the CSD velocities in the 10mg/mg and 20mg/ml groups respectively to 3.68 ± 0.04 mm/min and 3.81 ± 0.04 mm/min, and the removal of estradiol was followed by returning the CSD velocities to the basal range (3.35 ± 0.05 mm/min and 3.46 ± 0.06 mm/min). The topic application of progesterone solutions (131.5 mg/ml and 263 mg/ml) also increased the CSD velocities from the respective basal values of 3.34 ± 0.14 mm/min and 3.49 ± 0.11 mm/min to 3.64 ± 0.24 mm/min and 4.15 ± 0.21 mm/min, respectively. After progesterone, the following four CSD episodes confirmed the return of the CSD velocities to the basal levels (respectively 3.28 ± 0.16 mm/min and 3.63 ± 0.14 mm/min). A second application of progesterone solutions, now on the point 2, increased the CSD velocity to 3.75 ± 0.25 mm/min and 4.17 ± 0.33 mm/min, respectively, and removal of the hormone resulted in return of the CSD velocity to the basal levels (respectively 3.38 ± 0.25 mm/min and 3.62 ± 0.16 mm/min).

In the L_{15} groups, after estradiol application on the recording point 1 (Estr-1), the basal CSD velocities (4.18 ± 0.09 mm/min and 4.20 ± 0.03 mm/min for 10 mg/ml and 20mg/ml

groups, respectively) increased to 4.55 ± 0.24 mm/min and 4.72 ± 0.17 mm/min, respectively ($P<0.05$). After the estradiol effect was documented, the topicly treated region was dried with a piece of cotton and subsequent SD episodes confirmed the return of SD velocity to the basal levels (4.16 ± 0.11 mm/min and 4.19 ± 0.03 mm/min, respectively). Thereafter, a second application of estradiol, now on the recording point 2 (Estr-2), increased the CSD velocities in the 10mg/mg and 20mg/ml groups respectively to 4.53 ± 0.14 mm/min and 4.75 ± 0.13 mm/min, and the removal of estradiol was followed by returning the CSD velocities to the basal range (4.15 ± 0.08 mm/min and 4.18 ± 0.02 mm/min). The topic application of progesterone solutions (131.5 mg/ml and 263 mg/ml) also increased the CSD velocities from the respective basal values of 4.09 ± 0.11 mm/min and 4.12 ± 0.12 mm/min to 4.32 ± 0.16 mm/min and 4.67 ± 0.20 mm/min, respectively. After progesterone, the following four CSD episodes confirmed the return of the CSD velocities to the basal levels (respectively 4.11 ± 0.04 mm/min and 4.16 ± 0.14 mm/min). A second application of progesterone solutions, now on the point 2, increased the CSD velocity to 4.35 ± 0.12 mm/min and 4.65 ± 0.13 mm/min, respectively, and removal of the hormone resulted in return of the CSD velocity to the basal levels (respectively 4.10 ± 0.07 mm/min and 4.10 ± 0.12 mm/min).

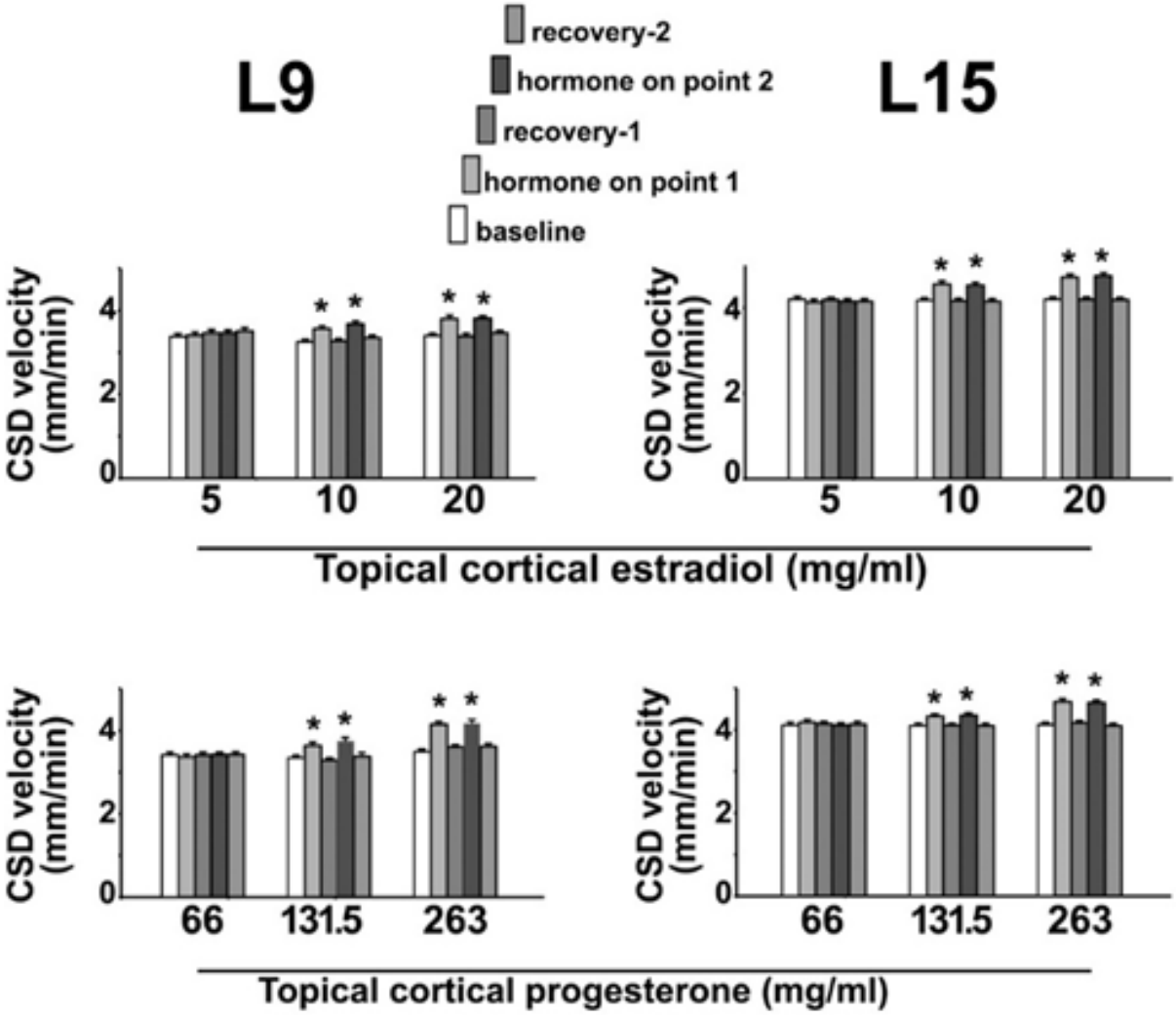

Figure 5: Mean velocity (± standard error of the mean) of CSD propagation in L_9 and L_{15} groups submitted to topic estradiol or progesterone. Asterisk indicates that "estradiol or progesterone apllication" values are significantly different from the corresponding controls. ($P < 0.05$; paired t test).

Table 5- Amplitude (in mV) of the negative DC slow potential shift of CSD in the female rat cortex. CSD was elicited before (baseline), during and after (recovery period) topic cortical application of 5 mg/ml, 10 mg/ml and 20 mg/ml solutions of water-soluble estradiol on the recording point 1. The same protocol was repeated on point 2. Each estradiol concentration was tested in a single group of female rats. Data are expressed as mean ± standard deviation.

		Estradiol concentration					
		5 mg/ml		**10 mg/ml**		**20 mg/ml**	
		L_9 (n=8)	L_{15} (n=5)	L_9 (n=9)	L_{15} (n=7)	L_9 (n=7)	L_{15} (n=8)
Baseline	Point 1	9.1 ± 3.3	9. 7 ± 3.2	8.6 ± 1.8	10.3 ± 3.3	7.8 ± 2.0	11.4 ± 4.0
	Point 2	9.1 ± 2.3	7.8 ± 3.1	9.3 ± 3.4	9.6 ± 3.1	8.5 ± 2.1	8.9 ± 4.9
Topical application point 1	Point 1	9.4 ± 4.2	11.1 ± 1.4	11.5 ± 3.5*	14.8 ± 3.6*	9.6 ± 2.4*	14.8 ± 5.4*
	Point 2	9.3 ± 1.8	7.9 ± 2.5	11.6 ± 5.9	10.0 ± 5.4	9.3 ± 1.1	9.7 ± 4.1
Recovery-1	Point 1	11.5 ± 2.9	11.1 ± 2.5	9.5 ± 2.3	12.4 ± 5.5*	8.8 ± 2.8	12.9 ± 4.9
	Point 2	9.1 ± 1.9	9.1 ± 2.9	8.9 ± 3.1	9.5 ± 6.6	8.8 ± 2.4	8.5 ± 3.2
Topical application point 2	Point 1	13.1 ± 4.6	11.3 ± 4.2	10.0 ± 2.2	12.0 ± 5.9	8.4 ± 2.8	12.8 ± 5.8
	Point 2	8.3 ± 2.8	9.3 ± 4.8	11.6 ± 4.8*	12.4 ± 8.5	9.9 ± 2.1*	11.4 ± 4.5
Recovery-2	Point 1	12.2 ± 4.0	8.8 ± 5.0	10.7 ± 4.4	13.6 ± 4.4	8.4 ± 3.4	13.2 ± 6.3
	Point 2	7.6 ± 2.8	10.6 ± 7.5	9.9 ± 3.2	11.7 ± 1	9.0 ± 2.3	8.1 ± 3.5

* Indicates values significantly higher than the corresponding ‘before’ value ($p < 0.05$; paired t-test).

Table 6- Amplitude (in mV) of the negative DC slow potential shift of CSD in the female rat cortex. CSD was elicited before (baseline), during and after (recovery period) topic cortical application of 66 mg/ml, 131.5 mg/ml and 263 mg/ml solutions of progesterone on the recording point 1. The same protocol was repeated on point 2. Each progesterone concentration was tested in a single group of female rats. Data are expressed as mean ± standard deviation

		Progesterone concentration					
		66 mg/ml		**131,5 mg/ml**		**263 mg/ml**	
		L_9 (n=7)	L_{15} (n=6)	L_9 (n=5)	L_{15} (n=5)	L_9 (n=7)	L_{15} (n=6)
Baseline	Point 1	8.6 ± 3.3	9.7 ± 2.4	8.3 ± 2.4	8.5 ± 2.6	9.0 ± 1.2	8.9 ± 2.2
	Point 2	10.7 ± 4.8	8.3 ± 5.7	8.5 ± 1.7	10.2 ± 5.2	7.9 ± 2.7	9.6 ± 4.1
Topical application point 1	Point 1	8.5 ± 2.3	10.3 ± 3.7	10.2 ± 4.8	12.1 ± 3.3*	11.6 ± 1.4*	12.3 ± 2.9*
	Point 2	13.3 ± 7.6	10.9 ± 11.2	7.8 ± 2.8	11.9 ± 12.8	8.9 ± 4.5	10.5 ± 4.6
Recovery-1	Point 1	8.4 ± 2.6	9.8 ± 1.6	7.8 ± 3.4	8.1 ± 4.2	8.7 ± 1.8	9.2 ± 2.1
	Point 2	10.9 ± 2.8	11.8 ± 9.1	6.0 ± 1.4	8.2 ± 8.3	7.8 ± 5.0	9.3 ± 4.1
Topical application point 2	Point 1	8.9 ± 4.5	9.8 ± 1.4	8.1 ± 6.0	9.7 ± 6.6	8.4 ± 1.6	8.9 ± 2.9
	Point 2	10.2 ± 3	8.0 ± 5.9	8.5 ± 4.5	10.5 ± 9.7*	10.7 ± 6.5*	12.2 ± 6.8*
Recovery-2	Point 1	9.2 ± 5.0	9.6 ± 2	10.1 ± 9.6	10.3 ± 9.7	8.3 ± 1.3	9.6 ± 3.2
	Point 2	9.7 ± 2.2	8.8 ± 7.2	5.7 ± 1.3	9.7 ± 10.9	6.1 ± 2.6	9.9 ± 6.2

* Indicates values significantly higher than the corresponding ‘before’ value ($p < 0.05$; paired t-test).

Table 7- Duration (in s) of the negative DC slow potential shift of CSD in the female rat cortex. CSD was elicited before (baseline), during and after (recovery period) topic cortical application of 5 mg/ml, 10 mg/ml and 20 mg/ml solutions of estradiol on the recording point 1. The same protocol was repeated on point 2. Each estradiol concentration was tested in a single group of female rats. Data are expressed as mean ± standard deviation.

		Estradiol concentration					
		5 mg/ml		**10 mg/ml**		**20 mg/ml**	
		L_9 (n=10)	L_{15} (n=7)	L_9 (n=10)	L_{15} (n=9)	L_9 (n=10)	L_{15} (n=8)
Baseline	Point 1	59.9 ± 4.4	58.6 ± 2.6	59.9 ± 3.7	58.3 ± 3.3	60.7 ± 2.4	57.8 ± 2.2
	Point 2	59.8 ± 1.6	57.7 ± 2.3	59.0 ± 4.0	57.6 ± 2.4	60.3 ± 2.2	57.7 ± 1.5
Topical application point 1	Point 1	57.4 ± 4.0	59.0 ± 4.1	54.5 ± 3.8*	52.4 ± 4.8*	55.3 ± 8.3*	55.4 ± 4.2*
	Point 2	59.2 ± 2.5	57.8 ± 3.3	59.7 ± 3.5	58.0 ± 2.4	58.6 ± 5.6	59.7 ± 3.1
Recovery -1	Point 1	58.3 ± 3.2	59.0 ± 5.4	59.2 ± 3.8	57.4 ± 4.3	58.5 ± 8.4	59.2 ± 3.8
	Point 2	58.8 ± 3.2	58.6 ± 3.0	59.6 ± 3.6	57.8 ± 2.4	58.7 ± 6.9	61.3 ± 5.1
Topical application point 2	Point 1	57.6 ± 6.0	58.9 ± 5.3	57.5 ± 6.0	57.7 ± 3.6	58.4 ± 6.0	57.7 ± 4.1
	Point 2	58.2 ± 5.7	58.8 ± 3.3	50.5 ± 4.2*	51.9 ± 6.4*	51.9 ± 6.5*	54.6 ± 7.9*
Recovery -2	Point 1	55.9 ± 9.8	58.0 ± 3.3	55.7 ± 6.3	55.9 ± 7.2	59.9 ± 4.4	58.6 ± 3.1
	Point 2	57.7 ± 5.9	58.5 ± 4.5	57.2 ± 9.8	58.7 ± 7.5	57.4 ± 6.0	60.0 ± 3.9

* Indicates values significantly lower than the corresponding ‘before’ value ($p < 0.05$; paired t-test).

Table 8- Duration (in s) of the negative DC slow potential shift of CSD in the female rat cortex. CSD was elicited before (baseline), during and after (recovery period) topic cortical application of 66 mg/ml, 131.5 mg/ml and 263 mg/ml solutions of progesterone on the recording point 1. The same protocol was repeated on point 2. Each progesterone concentration was tested in a single group of female rats. Data are expressed as mean ± standard deviation. * Indicates values significantly lower than the corresponding 'before' value ($p < 0.05$; paired t-test).

		Progesterone concentration					
		66 mg/ml		**131,5 mg/ml**		**263 mg/ml**	
		L_9 (n=9)	L_{15} (n=10)	L_9 (n=7)	L_{15} (n=7)	L_9 (n=9)	L_{15} (n=9)
Baseline	Point 1	59.7 ± 1.0	58.3 ± 3.6	60.0 ± 1.4	60.3 ± 2.9	64.6 ± 4.1	59.3 ± 2.9
	Point 2	60.0 ± 1.5	57.6 ± 2.3	58.5 ± 4.1	57.6 ± 3.4	59.6 ± 2.7	58.2 ± 1.6
Topical application point 1	Point 1	59.3 ± 1.0	58.8 ± 3.7	55.8 ± 3.9*	52.0 ± 5.2*	59.0 ± 5.0*	55.3 ± 5.1*
	Point 2	61.0 ± 1.4	59.3 ± 3.2	59.7 ± 3.5	55.7 ± 4.8	60.2 ± 2.4	58.9 ± 3.2
Recovery-1	Point 1	59.0 ± 1.9	57.0 ± 2.4	59.1 ± 2.9	59.5 ± 3.5	62.7 ± 4.7	59.1 ± 2.9
	Point 2	59.8 ± 3.3	58.8 ± 2.9	59.7 ± 2.7	57.9 ± 4.2	61.03 ± 6.7	58.6 ± 3.6
Topical application point 2	Point 1	58.6 ± 5.3	58.1 ± 2.3	59.5 ± 4.5	57.9 ± 2.3	60.2 ± 5.7	56.1 ± 8.7
	Point 2	59.0 ± 3.4	57.6 ± 2.5	53.8 ± 3.5*	51.8 ± 7.3*	55.6 ± 5.2*	52.8 ± 7.9*
Recovery-2	Point 1	59.1 ± 4.9	57.3 ± 6.7	56.6 ± 7.0	53.4 ± 6.9	59.1 ± 5.1	56.7 ± 4.4
	Point 2	60.8 ± 1.5	59.8 ± 5.6	59.9 ± 3.6	58.4 ± 4.4	59.9 ± 2.8	57.8 ± 4.5

4. Discussion

As the main finding of the present study, our data demonstrate that treatment of female rats with β-estradiol or progesterone over the course of the development increased the brain capability to propagate CSD in adulthood, as indicated by its higher velocities in comparison to the velocities of the vehicle and Naïve controls. Besides increasing the CSD velocity of propagation, hormone treatment also increased the amplitude of the CSD slow potential shift, and decreased its duration, in adult age (Fig. 4 and Table 4), which also reinforces the conclusion of a hormone effect on CSD. No difference in anxiety-like behavior was observed in these animals, in comparison with vehicle-treated controls (Tables 2 and 3), which suggests a differential effect of the ovarian hormones on brain electrophysiological activity. The lack of behavioral effects in the hormone-treated animals is in line with the findings of others, who reported that β-estradiol administration to female developing rats did not affect behavior in the EPM and OF in the adult age (Calza et al., 2010). Our CSD results under systemic hormone treatment were presently confirmed by another set of experiments on topic cortical application of estradiol and progesterone. Topic hormone application dose-dependently modified CSD parameters in a similar way as the systemic treatment, and the effects were reverted by the removal of the hormone (Fig. 5 and Tables 5 to 8). Our data collectively indicate that neonatal treatment with ovarian hormones influences the CSD phenomenon, but not anxiety behavior, in adult female rats previously suckled under different litter sizes. CSD features depend on oscilations in brain excitability (Guedes and Cavalheiro, 1997; Guedes et al., 2009). Because that, we have to immediately question how ovarian hormones would modify neuronal excitability, and consequently modulate CSD and excitability-related diseases.

Estradiol is the most biologically prevalent and potent compound of a class of steroid hormones denominated estrogens, which exerts wide-ranging effects on the developing- and developed brain. Estradiol has been established as important modulator of cortical excitablity (Grassi et al., 2011; Inghilleri et al., 2004). The excitatory glutamatergic neurotransmission suffers influence of estrogens, which upregulates NMDA receptor expression (Martin and Behbehani, 2006), downregulates glutamate uptake by astrocytes (Sato et al., 2003. Tang et al., 2008) and increases the number of dendritic spines, most of which contain a huge number of NMDA receptors (Smith and Woolley, 2004; Woolley and McEwen, 1992, 1993, 1994). Therefore, we consider reasonable to accept that treatment with ovarian hormone early in life

modifies the excitability of the developing brain, by acting on the glutamatergic and other neurotransmitter systems, and the present CSD findings support that assumption.

Although the final mechanisms underlying the effects of estradiol and progesterone on CSD are unknown, based on the above evidence we suggest that all of the neural effects of estradiol and progesterone might contibute to the influence of such hormones on CSD via glutamatergic neurotransmission. Estradiol can influence susceptibility to CSD probably through its effect on glutamate neurotransmission (Liu and Zhao, 2013), which underlies CSD generation (Shatillo et al., 2015). In fact, activation of NMDA receptors is critical for induction of CSD in diferent neuronal tissues. Administration of glutamate and NMDA can trigger CSD (Ayata and Moskowitz, 2006), and antagonists of NMDA receptors inhibit initiation and propagation of CSD (Nellgard and Wieloch, 1992).

Another possible mechanism underlying the effects of estradiol and progesterone on CSD would implicate the involvement of the GABAergic system, mainly over the course of the development, because in the developing brain GABA is a very important source of excitation via membrane depolarization; in neonatal neurons, estradiol markedly enhance GABA responses, which at that early age are depolarizing (Nunez et al., 2008; Perrot-Sinal et al., 2003).

Regarding the ovarian hormones/CSD relationship, our findings are in line with several findings from others. Interestingly, CSD thresholds are reduced by aproximately 50% in wildtype female mice compared with males (Brennan et al 2007), and Wistar audiogenic female rats show higher CSD propagation rates than males (Guedes et al., 2009). In addition, in a model of rat neocortical slices, application of estradiol or progesterone to the medium, besides enhancing LTP, also increases CSD amplitude and frequency (Sachs et al., 2007). Higher CSD susceptibility in females has been reported in familial hemiplegic migraine type 1 (FHM1) konockin mice. In this genetically modified animal, ovariectomy decreased that gender effect, which was partially restored by estradiol administration (Eikermann-Haeter et al., 2009). Finally, it is important to note that the present results are absolutely coherent with those of our previous study on ovariectomized rats that clearly demonstrated CSD impairment in the absence of the ovarian hormones (Accioly et al., 2012).

The relationship between disturbances in the production and brain action of ovarian hormones, on one hand, and certain human neurological diseases, on the other, deserve special attention. For example, ovarian hormones are important modulators of migraine (Borsook et al., 2014) and cortical excitability (Reddy, 2014). The migraine prevalence is

reportedly similar in boys and girls before puberty, but increases in women (about three times higher than in men) after puberty (see Eikermann-Haerter et al., 2009; Granella et al., 2000, for an overview). Several pieces of evidence clearly indicate a causal relationship between ovarian hormones and the incidence of epileptic seizures (Taubøll et al., 2015). Ovarian hormones are also reported to be beneficially involved on demyelinating disturbances (Acs et al., 2009; Melcangi et al., 2014; Taylor et al., 2010), neurodegenerative disorders (Melcangi et al., 2014), microglia-mediated inflammatory reaction (Barreto et al., 2014) and neurotrauma (Arevalo et al., 2011). In this context, the search for therapeutically active molecules that act on the neural receptors or metabolism of ovarian hormones may constitute an interesting therapeutic possibility to be further explored.

Conclusion

In conclusion, our study indicates a clear influence of exogenous estradiol and progesterone, both via systemic and topic (cortical) administration, on the excitability-related CSD phenomenon (both hormones facilitated CSD), but not on anxiety-like behavior of female adult rats. Furthermore, early nutritional deficiency modulated the hormonal effect on CSD. Translational research on this brain-hormone interface will help to further understand the molecular mechanisms of the presently demonstrated effects.

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References

Accioly, N.E., Benevides, R. de D.L., Costa, B.L. da S.A. da, Guedes, R.C.A., 2012. Ovariectomy in the developing rat decelerates cortical spreading depression in adult brain. Int. J. Dev. Neurosci. 30, 405–410. doi:10.1016/j.ijdevneu.2012.01.012

Acs P, Kipp M, Norkute A, Johann S, Clarner T, Braun A, Berente Z, Komoly S, B.C., 2009. 17beta-estradiol and progesterone prevent cuprizone provoked demyelination of corpus callosum in male mice. Glia 57, 807–814.

Arevalo, M.A., Santos-Galindo, M., Lagunas, N., Azcoitia, I., Garcia-Segura, L.M., 2011. Selective estrogen receptor modulators as brain therapeutic agents. J. Mol. Endocrinol. 46. doi:10.1677/JME-10-0122

Arevalo MA, Azcoitia I, G.-S.L., 2015. The neuroprotective actions of oestradiol and oestrogen receptors. Nat. Rev. Neurosci. 16, 17–29.

Ayata C, M.M., 2006. Cortical spreading depression confounds concentration-dependent pial arteriolar dilation during N-methyl-D-aspartate superfusion. Am. J. Physiol. Hear. Circ. Physiol. 290, 1837–1841.

Bakker, J., Baum, M.J., 2008. Role for estradiol in female-typical brain and behavioral sexual differentiation. Front. Neuroendocr. 29, 1–16.

Barreto, G.E., Santos-Galindo, M., Garcia-Segura, L.M., 2014. Selective estrogen receptor modulators regulate reactive microglia after penetrating brain injury. Front. Aging Neurosci. 6, 1–7. doi:10.3389/fnagi.2014.00132

Borsook, D., Erpelding, N., Lebel, A., Linnman, C., Veggeberg, R., Grant, P.E., Buettner, C., Becerra, L., Burstein, R., 2014. Sex and the migraine brain. Neurobiol. Dis. 68, 200–214. doi:10.1016/j.nbd.2014.03.008

Brennan, K.C., Reyes, M.R., López Valdés HE, Arnold, A.P., Charles, A.C., 2007. Reduced threshold for cortical spreading depression in female mice. Ann. Neurol. 61, 603–606. doi:10.1002/ana.21138

Calza, A., Sogliano, C., Santoru, F., Marra, C., Angioni, M.M., Mostallino, M.C., Biggio, G., Concas, A., 2010. Neonatal exposure to estradiol in rats influences neuroactive steroid concentrations, GABAA receptor expression, and behavioral sensitivity to anxiolytic drugs. J. Neurochem. 113, 1285–1295. doi:10.1111/j.1471-4159.2010.06696.x

Chauvel, V., Schoenen, J., Multon, S., 2013. Influence of ovarian hormones on cortical spreading depression and its suppression by L-kynurenine in rat. PLoS One 8, 8–13. doi:10.1371/journal.pone.0082279

Chauvel, V., Vamos, E., Pardutz, A., Vecsei, L., Schoenen, J., Multon, S., 2012. Effect of systemic kynurenine on cortical spreading depression and its modulation by sex hormones in rat. Exp. Neurol. 236, 207–214. doi:10.1016/j.expneurol.2012.05.002

Dobbing, J., 1968. Vulnerable periods in developing brain, in: Applied Neurochemistry. pp. 287–316. doi:10.1007/978-1-4471-1766-7_1

Eikermann-Haerter K, Dileköz E, Kudo C, Savitz SI, Waeber C, Baum MJ, Ferrari MD, van den Maagdenberg AM, Moskowitz MA, A.C., 2009. Genetic and hormonal factors modulate spreading depression and transient hemiparesis in mouse models of familial hemiplegic migraine type 1. J. Clin. Invest. 119, 99–109. doi:10.1172/JCI36059

Eikermann-Haerter, K., 2014. Spreading depolarization may link migraine and stroke. Headache. doi:10.1111/head.12386

Fabricius M, Fuhr S, Willumsen L, Dreier JP, Bhatia R, Boutelle MG, Hartings JA, Bullock R, Strong AJ, L.M., 2008. Association of seizures with cortical spreading depression and peri-infarct depolarisations in the acutely injured human brain. Clin Neurophysiol. 119, 1973–1984.

Ferrari, M.D., Klever, R.R., Terwindt, G.M., Ayata, C., van den Maagdenberg, A.M.J.M., 2015. Migraine pathophysiology: lessons from mouse models and human genetics. Lancet. Neurol. 14, 65–80. doi:10.1016/S1474-4422(14)70220-0

Francisco Eda S, G.R., 2015. Neonatal taurine and alanine modulate anxiety-like behavior and decelerate cortical spreading depression in rats previously suckled under different litter sizes. Amino Acids 47, 2437–45.

Granella, F., Sances, G., Pucci, E., Nappi, R.E., Ghiotto, N., Napp, G., 2000. Migraine with aura and reproductive life events: a case control study. Cephalalgia 20, 701–707.

Grassi, S., Tozzi, A., Costa, C., Tantucci, M., Colcelli, E., Scarduzio, M., Calabresi, P., Pettorossi, V.E., 2011. Neural 17β-estradiol facilitates long-term potentiation in the hippocampal CA1 region. Neuroscience 192, 67–73. doi:10.1016/j.neuroscience.2011.06.078

Guedes, R.C.A., 2011. Cortical Spreading Depression: A Model for Studying Brain Consequences of Malnutrition, in: Handbook of Behavior, Food and Nutrition. pp. 2343–2355.

Guedes, R.C.A., de Oliveira, J.A.C., Amâncio-dos-Santos, Â., García-Cairasco, N., 2009. Sexual differentiation of cortical spreading depression propagation after acute and kindled audiogenic seizures in the Wistar audiogenic rat (WAR). Epilepsy Res. 83, 207–214. doi:10.1016/j.eplepsyres.2008.11.003

Guedes RCA, C.E., 1997. Blockade of spreading depression in chronic epileptic rats: reversion by diazepam. Epilepsy Res. 27, 33–40.

Hadjikhani, N., Sanchez Del Rio, M., Wu, O., Schwartz, D., Bakker, D., Fischl, B., Kwong, K.K., Cutrer, F.M., Rosen, B.R., Tootell, R.B., Sorensen, A.G., Moskowitz, M.A., 2001. Mechanisms of migraine aura revealed by functional MRI in human visual cortex. Proc. Natl. Acad. Sci. U. S. A. 98, 4687–92. doi:10.1073/pnas.071582498

Hadkikhani N, SanchezdelRioM, WuO, E., 2001. Mechanisms of migraine aura revealed by functional MRI in human visual cortex. Proc. Natl. Acad. Sci. USA 98, 4687–4692.

Hill M, Pašková A, Kančeva R, Velíková M, Kubátová J, Kancheva L, Adamcová K, Mikešová M, Žižka Z, Koucký M, Šarapatková H, Kačer V, Matucha P, Meloun M, P.A., 2014. Steroid profiling in pregnancy: A focus on the human fetus. J. Steroid Biochem. Mol. Biol. 139, 201–222. doi:10.1016/j.jsbmb.2013.03.008

Inghilleri M, Conte A, Currà A, Frasca V, Lorenzano C, B.A., 2004. Ovarian hormones and cortical excitability. An rTMS study in humans. Clin. Neurophysiol. 115, 1063–1068.

Kaminski, R.M., Rogawski, M.A., 2011. 11??-Hydroxylase inhibitors protect against seizures in mice by increasing endogenous neurosteroid synthesis. Neuropharmacology 61, 133–137. doi:10.1016/j.neuropharm.2011.03.019

Lauritzen M, Dreier JP, Fabricius M, Hartings JA, G.R. and S.A., 2011. Clinical relevance of cortical spreading depression in neurological disorders: migraine, malignant stroke, subarachnoid and intracranial hemorrhage, and traumatic brain injury. J. Cereb. Blood Flow Metab. 31, 17–35.

Leão, A.A.P., 1944a. Pial circulation and spreading depression of activity in the cerebral cortex. J. Neurophysiol. 7, 391–396.

Leão, A.A.P., 1944b. Spreading depression of activity in the cerebral cortex. J. Physiol. 7, 359–390.

Liu, S. bing, Zhao, M. gao, 2013. Neuroprotective effect of estrogen: Role of nonsynaptic NR2B-containing NMDA receptors. Brain Res. Bull. 93, 27–31. doi:10.1016/j.brainresbull.2012.10.004

Martin, V.T., Behbehani, M., 2006. Ovarian hormones and migraine headache: Understanding mechanisms and pathogenesis - Part I. Headache 46, 365–386. doi:10.1111/j.1526-4610.2006.00309.x

Mccarthy, M.M., 2009. NIH Public Access. Physiology 88, 91–124. doi:10.1152/physrev.00010.2007.Estradiol

Melcangi, R.C., Giatti, S., Calabrese, D., Pesaresi, M., Cermenati, G., Mitro, N., Viviani, B., Garcia-Segura, L.M., Caruso, D., 2014. Levels and actions of progesterone and its metabolites in the nervous system during physiological and pathological conditions. Prog. Neurobiol. 113, 56–69. doi:10.1016/j.pneurobio.2013.07.006

Mendes-da-Silva RF, Lopes-de-Morais AA, Bandim-da-Silva ME, Cavalcanti Gde A, Rodrigues AR, Andrade-da-Costa BL, G.R., 2014. Prooxidant versus antioxidant brain action of ascorbic acid in well-nourished and malnourished rats as a function of dose: a cortical spreading depression and malondialdehyde analysis. Neuropharmacology. 86, 155–160.

Muneoka K, Kuwagata M, Ogawa T, S.S., 2010. Sex-specific effects of early neonatal progesterone treatment on dopamine and serotonin metabolism in rat striatum and frontal cortex. Life Sci. 87, 738–742.

Nellgard B, W.T., 1992. NMDA-receptor blockers but not NBQX, an AMPA-receptor antagonist, inibit spreading depression in the rat. Acta Physiol. Scand. 146, 497–503.

Nuñez JL, Aberdeen GW, Albrecht ED, M.M., 2008. Impact of estradiol on gamma-aminobutyric acid- and glutamate-mediated calcium responses of fetal baboon (Papio anubis) hippocampal and cortical neurons. Endocrinology 149, 6433–6443. doi:10.1210/en.2007-1720

Perrot-Sinal, T.S., Auger, A.P., McCarthy, M.M., 2003. Excitatory actions of GABA in developing brain are mediated by l-type Ca2+ channels and dependent on age, sex, and brain region. Neuroscience 116, 995–1003.

Reddy, D.S., 2014. Neurosteroids and their role in sex-specific epilepsies. Neurobiol. Dis. 72, 198–209.

Sato k, Matsuky N, Ohno Y, N.K., 2003. Estrogens inhibit l-glutamate uptake activity of astrocytes via membrane estrogen receptor alpha. J. Neurochem. 86, 1489–1505.

Shatillo, A., Salo, R.A., Giniatullin, R., Gröhn, O.H., 2015. Involvement of NMDA receptor subtypes in cortical spreading depression in rats assessed by fMRI. Neuropharmacology 93, 164–170. doi:10.1016/j.neuropharm.2015.01.028

Smith SS, W.C., 2004. Cellular and molecular effects of steroid hormones on CNS excitability. Cleve. Clin. J. Med. 71, S4–S10.

Tanaka, M., Sokabe, M., 2012. Continuous de novo synthesis of neurosteroids is required for normal synaptic transmission and plasticity in the dentate gyrus of the rat hippocampus. Neuropharmacology 62, 2372–2386. doi:10.1016/j.neuropharm.2012.02.007

Tang B, Ji Y, T.R., 2008. Estrogen alters spinal NMDA receptor activity via a PKA signaling pathway in a visceral pain model in rat. Pain 137, 540–549.

Taubøll, E., Sveberg, L., Svalheim, S., 2015. Interactions between hormones and epilepsy. Seizure 28, 3–11. doi:10.1016/j.seizure.2015.02.012

Wagner, C.K., 2008. Minireview: Progesterone receptors and neural development: A gap between bench and bedside? Endocrinology 149, 2743–2749. doi:10.1210/en.2008-0049

Woolley CS, M.B., 1994. Estradiol regulates hippocampal dendritic spine density via an N-methyl-D-aspartate receptor-dependent mechanism. J. Neurosci. 14, 7680–7687.

Woolley CS, M.B., 1993. Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. J. Comp. Neurol. 336, 293–306.

Woolley CS, M.B., 1992. Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. J. Neurosci. 12, 2549–2554.

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6. ARTIGO 3- Short Comunication- EM VIAS DE SUBMISSÃO

Characterization of spreading depression in adult female rats previously treated with the association of estradiol plus progesterone and suckled under different litter sizes

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Abstract

Clinical and experimental evidence indicates that ovarian hormones exert deep and lasting influence on brain development. This influence includes several mechanisms with important repercussion on brain excitability. Cortical spreading depression (CSD) is a phenomenon that is influenced by brain excitability. In a previous study, we demonstrated that ovariectomy during the period of fast brain development reduced, in adulthood, the propagation of CSD. In this study we investigated the effect of treatment in early life, with the association of estradiol plus progesterone on CSD in female rats suckled in litters with 6-9 and 12-15 pups (L_9 and L_{15} groups, respectively). From postnatal days (P) 7 to 21, the animals received 50μg/kg of β-estradiol plus progesterone every other day. At P90-120, we recorded CSD on two cortical points over the course of four hours, and analyzed CSD velocity of propagation. Compared with vehicle (olive-oil)-injected and naïve controls, CSD velocity of propagation in the group treated with the mixture of both hormones was significantly higher ($p<0.05$).

We concluded that treatment with the association of estradiol plus progesterone during brain development increased the velocity of CSD propagation. This result emphasizes that ovarian hormones can modulate susceptibility to CSD, probably via hormonal action on neuro-glial excitability.

Keywords: Ovarian Hormones; Brain development; Neural excitability; Cortical spreading depression.

1. Introduction

Ovarian hormones are highly lipophilic and can readily cross the blood-brain barrier and reach targets in the brain, where they can influence neuronal excitability and other brain functions (Reddy, 2014). These actions are in part mediated by direct effects of the hormones on neurons. In addition, the ovarian hormones can also regulate the function of the nervous system by actions on glial cells (Melcangi et al., 2014; Schumacher et al., 2014; Arevalo et al., 2015).

Ovarian hormones can act through both genomic and nongenomic mechanisms. Receptors for ovarian hormones are nuclear transcription factors that regulate gene expression but also have actions on membrane, including activation of signal transduction pathways (Melcangi et al, 2014; Arevalo et al, 2015).

Moreover, ovarian hormones can be synthesized *de novo* from cholesterol entirely within select regions of the brain (Arevalo et al., 2014). Once having been metabolized to progesterone, cholesterol can be further metabolized to androstenedione and testosterone. These, in turn, through aromatization can be transformed to estradiol. The enzymes 5a-reductase and 3a -hydroxysteroid, the most important enzymes enabling the brain to produce neurosteroids, is widely distributed in the brain. Steroids that are synthesized in the brain are also called neurosteroids; they are precursors and metabolites of steroid hormones influencing neuronal excitability, mainly through non-genomic mechanisms. (Arevalo et al., 2015).

The brain excitability can be experimentally explored though of the electrophysiological phenomenon denominated as cortical spreading depression (CSD).

Cortical spreading depression (CSD) comprises a fully reversible reduction in the spontaneous and evoked electrical activity of the cerebral cortex in response to a mechanical, electrical, or chemical stimulation of one point of the cortex (Leão 1944). It has been demonstrated in many animal species, including humans (Hadjikhani et al., 2001). CSD represents a transient neuronal depolarization accompanied by a negative shift in the DC (direct current) potential and a depression on electroencephalography. Thus, CSD is an interesting and useful experimental model for evaluating the proper functioning of neural tissue (Lauritzen et al., 2011).

Malnutrition can cause structural, neurochemical and functional changes of organs and tissues, especially in the nervous system (Morgane et al, 1993). It has been well established

that early malnutrition increase CSD propagation (Guedes, 2011), but no information is available regarding their association treatment with estradiol plus progesterone effects on CSD.

In the present work, we used electrophysiological recording of CSD, in order to address the following two questions in the brain of female adult rats that were previously subjected to different litter sizes during lactation: (i) how does intraperitoneal administration of estradiol plus progesterone during the brain development affect CSD propagation, and (ii) if so, how would this effect be influenced by the previous brain nutritional condition. We were able to demonstrate a CSD facilitation effect of estradiol plus progesterone treatment that is appreciably modified by malnutrition.

In the present study we have therefore investigated the modulating effect of association of treatment with estradiol plus progesterone on CSD velocity of propagation in female adult rats previously suckled in normal and unfavorable conditions.

2. Materials and methods

2.1. Animals

All experimental procedures were previously approved by the Institutional Ethics Committee for Animal Research of our University (Approval protocol no. 23076.010208/2012-09), whose norms comply with those established by the National Institutes of Health, Guide for Care and Use of Laboratory Animals (Bethesda, MD, USA). Wistar female newborn rats (n=58) from the colony of our department were randomly distributed to one of two different conditions of lactation: 1) normal lactation, in which the pups were suckled on litters formed by 6 to 9 pups (n=32), and 2) unfavorable lactation (litters with 12 to 15 pups; n=26). These two lactation conditions (designated as L_9 and L_{15} groups, respectively) have been shown to be nutritionally distinct (Francisco and Guedes, 2015), and this has been confirmed in this study (see results). The weaning occurred in all groups on postnatal (P) day 21, considering the day of birth as day 0. Dams and pups had free access to water and a commercial lab chow (Purina, with 23% protein). They were housed in polyethylene cages (51 cm X 35.5 cm X 18.5 cm) under controlled temperature (23±1°C) with a 12-h light:12-h dark cycle (lights on at 6:00 AM).

2.2. Systemic hormonal treatment

From P7 to P21, female L_9 and L_{15} pups (n = 9 and n = 5, respectively) received one every other day intraperitoneal injection containing 50 µg/kg β-estradiol plus 50 µg/kg progesterone. Both hormones, purchased from Sigma (St Louis, USA), were dissolved in 0.1 ml olive oil. These groups were compared to a vehicle-treated group (group V; 15 L_9 rats and 9 L_{15} rats) that received olive oil, and a naïve group (group Nv; 8 L_9 rats and 12 L_{15} rats) that received no treatment. Body weight was determined at P7, P21, P60 and P90, when we performed the electrophysiological recordings.

2.3. CSD electrophysiological recording

On 90-120 days of age, under anesthesia with a mixture of 1 g/kg urethane + 40 mg/kg alpha chloralose (ip; both purchased from Sigma, St. Louis, Mo, USA), three trephine holes were made at the right side of the skull, parallel to the midline, to expose portions of the cortical surface. One hole, which was used to elicit CSD, was on the frontal bone and the other two holes (on the parietal bone) were used to record the propagating CSD waves. During the recording period, the animal was maintained over an electric heater such that the rectal temperature remained stable (37 ± 1 ° C) and could be adjusted when necessary. The animals were subjected to a 4-h CSD recording session, as previously described (Mendes-da-Silva et al, 2014). Briefly, CSD was elicited at 20-min intervals by applying, for 1 min, a cotton ball (1-2 mm diameter) soaked in KCl solution (prepared by dissolving 2 g KCl in 100 ml distilled water; approximately 0.27 M) to the anterior hole drilled at the frontal region. With a pair of Ag-AgCl agar-Ringer electrodes, the direct current (DC)-potential change typical of CSD was recorded at two parietal points on the cortical surface. A common reference electrode of the same type was placed on the nasal bones. The electrodes were connected to a digital data acquisition system (EMG Systems, São Paulo, Brazil). The recorded data were stored, visualized and analyzed in an IBM^{TM}-compatible computer. We calculated the velocity of CSD propagation, as well as the amplitude and duration of the negative slow potential shifts of the CSD waves, as previously reported (Mendes-da Silva et al, 2014). In order to calculate the velocity of CSD propagation, we measured the time required for a CSD wave to cross the distance between the two recording electrodes, taking the initial point of each DC negative rising phase as the reference point. In all experiments, we

determined the estrous cycle status on the day of the CSD-recording. CSD was always recorded in the proestrous phase of the rat's estrous cycle. At the end of the 4 h CSD-recording, we measured brain, adrenal, ovary and uterus weight.

2.6. Statistical analysis

For the weight and CSD effects of the systemic hormone treatment, statistical analysis was performed by a two-way analysis of variance (ANOVA) including as factors the lactation condition (L_9 and L_{15}), and hormonal treatment (β-estradiol, progesterone, vehicle and naïve), followed by the Holm-Sidak test when indicated. The statistical software used was Sigmastat™, version 3.10. Differences were considered statistically significant when $p < 0.05$. All values are presented in the text as mean ± standard deviations.

2. Results

3.1. Body weights

L_{15} animals presented with lower body weights in comparison with the L_9 rats, in all ages ($p < 0.05$). Systemic hormonal treatment did not significantly influence body weights. (Table. 1).

Table 1- Body weight of female rats suckled in litters of 6-9 and 12-15 pups.

		P7	P21	P60	P90
Naïve	**L_9**	13.4 ± 0.2	34.9 ± 0.5	172.3 ± 6.4	203.6 ± 4.8
	L_{15}	11.3 ± 0.3*	30 ± 1.3*	143.6 ± 1.9*	177.5 ± 4.0*
Vehicle	**L_9**	13.8 ± 0.6	37.4 ± 1.2	172.1 ± 1.2	201.9 ± 4.1
	L_{15}	11.7 ± 0.6*	27.7 ± 1.4*	145.5 ± 1.6*	176.0 ± 5.0*
Estradiol + Progesterone	**L_9**	14.4 ± 0.2	37.7 ± 0.5	161.8± 3.2	200.3 ± 3.5
	L_{15}	11.3 ± 0.5*	29.6 ± 1.0*	148.3 ± 1.4*	179.0 ± 1.6*

Data are expressed as mean ± S.E.M. The asterisks indicate that L_{15} weights are significantly lower than the corresponding L_9 values ($p < 0.05$; two-way ANOVA followed by the Holm–Sidak test).

3.2. CSD propagation

The CSD velocities in the L_{15} rats were higher ($p < 0.05$) in comparison with the L_9 animals.

Regarding β-estradiol plus progesterone treatment, the hormone treatment significantly enhanced the CSD propagation velocities ($p < 0.05$) in comparison with the naïve and vehicle controls in both lactation conditions. In the L_9 animals, the CSD velocity (mean ± SD) in the naïve and vehicle controls was respectively 3.28 ± 0.08 mm/min and 3.34 ± 0.09 mm/min, whereas in the β-estradiol plus progesterone group the CSD velocity was significantly higher (4.34 ± 0.31 mm/min; $p < 0.05$). In L_{15} animals, the CSD velocities in the control groups were higher than in the L_9 rats (4.37 ± 0.16 mm/min and 4.34 ± 0.18 mm/min for the naïve and vehicle groups, respectively). Treatment with β-estradiol plus progesterone further increased ($p < 0.05$) CSD velocity in L_{15} animals to 4.78 ± 0.21 mm/min. The quantitative data of the CSD velocities are illustrated in Fig. 1.

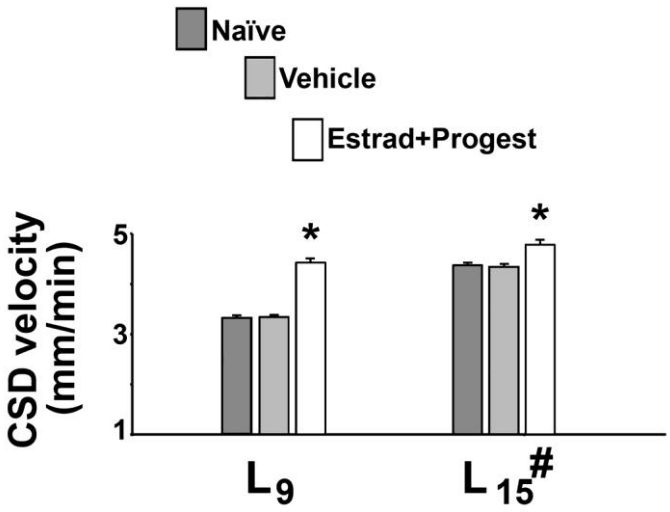

Fig.1. CSD velocity of 90-120 days old female rats that were suckled in two distinct lactation conditions represented by litters with 6-9 and 12-15 pups (respectively well-nourished

and malnourished groups). Data are mean ± S.E.M. Asterisks indicate that the hormone-treated groups are significantly different from the naïve- and vehicle-control groups. # indicates that all L_{15} groups were significantly different from the corresponding L_9 groups ($p < 0.05$; ANOVA followed by the Holm–Sidak test).

Discussion

Our data establish the importance of ovarian hormones as modulator of the brain susceptibility to CSD, as judged from the higher speed in adulthood in the group treated with estradiol plus progesterone in the development in comparison with the controls. Data also indicate that malnutrition induced by suckling in large-size litters (L_{15} condition) does not impede the hormone effect on CSD.

The mechanisms involved in the excitatory effect of estrogens are complex. An important point for the generally excitatory effect of estrogens, is the ability of neurons to respond rapidly to the excitatory effect of glutamate, as estrogens seem to have a glutamatergic action, A number of estradiol effects can increase neuronal excitability. For example, in the developing brain, estradiol markedly enhances depolarizing GABA responses, which are specific of the neonatal period. Estradiol can potentiate glutamate responses by up-regulating NMDA receptor activity. The actions of estradiol on excitability in the developed brain include enhancement of the excitatory activity of glutamate, impairment of the inhibitory activity of GABA, facilitation of the long-term potentiation (LTP) in the hippocampus (Grassi et al., 2011) and neocortical tissues (Sachs et al., 2007) and changes in brain morphology by increasing dendritic spine density (Smith and Woolley, 2004).

Progesterone and/or its metabolites exert a variety of effects on the brain acting as regulators of physiological development and glial and neuronal plasticity, participating in events beyond neuroendocrine effects (Melcangi et al, 2014). Steroidal hormones are reported to have neuroprotective potential and are increasingly being investigated in seizure disorders. Progesterone is an important hormone in epilepsy research due to its modulating actions in brain (Reddy et al., 2010). Progesterone targets multiple molecular and cellular mechanisms that are relevant to epileptogenesis. While it's cellular actions are mediated via progesterone receptors (Brinton et al., 2008), its antiseizure effects are attributed to its conversion to allopregnanolone, which is a potent positive modulator of $GABA_A$ receptors (Frye, 1995).

Regarding the ovarian hormones/CSD relationship, our findings are in line with several findings from others (Sachs et al., 2007; Chauvel et al, 2013) and coherent with those of our previous study on ovariectomized rats that clearly demonstrated CSD impairment in the absence of the ovarian hormones (Accioly et al., 2012).

Clinically, estrogen reportedly increases cortical excitability in humans during transcranial magnetic stimulation (Smith et al., 2002) and high doses increase the incidence of aura during hormone replacement therapy. Moreover, higher levels of estrogen are associated with an increase in seizure frequence in females (Klein and Hergoz, 1998). Experimentally, seizure thresholds are decreased during peak estrogen levels (Woolley and Timiras, 1962), and amygdala kindling is increased (Edwards et al., 1999). Taken together these results underscore the complex role of ovarian hormones in CSD.

We conclude that treatment of female rats with the association of estradiol plus progesterone during bran development is causally related with CSD acceleration in the adult brain, indicating a long-term facilitating effect on CSD.

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References

Accioly, N.E., Benevides, R. de D.L., Costa, B.L. da S.A. da, Guedes, R.C.A., 2012. Ovariectomy in the developing rat decelerates cortical spreading depression in adult brain. Int. J. Dev. Neurosci. 30, 405–410. doi:10.1016/j.ijdevneu.2012.01.012

Arevalo MA, Azcoitia I, G.-S.L., 2015. The neuroprotective actions of oestradiol and oestrogen receptors. Nat. Rev. Neurosci. 16, 17–29.

Chauvel, V., Schoenen, J., Multon, S., 2013. Influence of ovarian hormones on cortical spreading depression and its suppression by L-kynurenine in rat. PLoS One 8, 8–13. doi:10.1371/journal.pone.0082279

EDWARDS, H.E.; BURNHAM, W.M.; MENDONCA, A.; BOWLBY, D.A.; MACLUSKY, N.J. Steroid hormones affect limbic afterdischarge thresholds and kindling rates in adult female rats. Brain Research, v. 14, p. 136-150, 1999

Francisco Ed a S, G.R., 2015. Neonatal taurine and alanine modulate anxiety-like behavior and decelerate cortical spreading depression in rats previously suckled under different litter sizes. Amino Acids 47, 2437–45.

Grassi, S., Tozzi, A., Costa, C., Tantucci, M., Colcelli, E., Scarduzio, M., Calabresi, P., Pettorossi, V.E., 2011. Neural 17??-estradiol facilitates long-term potentiation in the hippocampal CA1 region. Neuroscience 192, 67–73. oi:10.1016/j.neuroscience.2011.06.078

Guedes, R.C.A., 2011. Cortical Spreading Depression: A Model for Studying Brain Consequences of Malnutrition, in: Handbook of Behavior, Food and Nutrition. pp. 2343–2355.

HadkikhaniN,SanchezdelRioM,WuO, etal, 2001. Mechanisms of migraine aura revealed by functional MRI in human visual cortex. Proc. Natl. Acad. Sci. USA 98, 4687–4692.

Lauritzen M, Dreier JP, Fabricius M, Hartings JA, G.R. and S.A., 2011. Clinical relevance of cortical spreading depression in neurological disorders: migraine, malignant stroke, subarachnoid and intracranial hemorrhage, and traumatic brain injury. J. Cereb. Blood Flow Metab. 31, 17–35.

Leão, A.A.P., 1944a. Pial circulation and spreading depression of activity in the cerebral cortex. J. Neurophysiol. 7, 391–396.

Leão, A.A.P., 1944b. Spreading depression of activity in the cerebral cortex. J. Physiol. 7, 359–390.

Melcangi, R.C., Giatti, S., Calabrese, D., Pesaresi, M., Cermenati, G., Mitro, N., Viviani, B., Garcia-Segura, L.M., Caruso, D., 2014. Levels and actions of progesterone and its metabolites in the nervous system during physiological and pathological conditions. Prog. Neurobiol. 113, 56–69. doi:10.1016/j.pneurobio.2013.07.006

Morgane, P.J., Austin-La France, R., Bronzino, J., Tonkiss, J., Díaz-Cintra, S., Cintra, L., Kemper, T. and Galler, J.R. Prenatal malnutrition and development of the brain. Neurosci. Biobehav. Rev. 17 (1993) 91–128.

Muneoka, K., Kuwagata, M., Ogawa, T., & Shioda, S., 2010. Sex-specific effects of early neonatal progesterone treatment on dopamine and serotonin metabolism in rat striatum and frontal cortex. Life Sci. 87, 738–742.

Reddy, D.S., 2014. Neurosteroids and their role in sex-specific epilepsies. Neurobiol. Dis. 72, 198–209.

Sachs, M., Pape, H.C., Seckmann, E.J., Gorji, A., 2007. The effect of estrogen and progesterone on spreading depression in rat neocortical tissues. Neurobiol. Dis. 25, 27-34

Smith SS, W.C., 2004. Cellular and molecular effects of steroid hormones on CNS excitability. Cleve. Clin. J. Med. 71, S4–S10.

7. CONSIDERAÇÕES FINAIS

- A ovariectomia na vida adulta não se acompanha dos efeitos sobre a DAC, observados previamente em ratas com ovariectomia precoce, sugerindo que este último tratamento opera sobre a DAC mediante mecanismos que atuam durante o desenvolvimento.

- O tratamento sistêmico com estradiol, ou progesterona, ou ambos durante o período do desenvolvimento cerebral está associado com a aceleração da DAC no cérebro adulto, independentemente das condições de lactação (lactação normal versus desfavorável), indicando um efeito facilitador de longo prazo.

- O tratamento tópico cortical com estradiol ou progesterona está também associado à aceleração da DAC no cérebro adulto de forma reversível e dose-dependente, independentemente das condições de lactação (lactação normal versus desfavorável). Este efeito sugere um segundo mecanismo de ação hormonal sobre a DAC, que independeria do desenvolvimento cerebral.

- Sugerimos que estes efeitos de longa duração podem estar relacionados com a ação hormonal duradoura, direta ou indireta, sobre a excitabilidade cerebral.

8.ANEXO - Parecer do Comitê de Ética em Pesquisa

Universidade Federal de Pernambuco
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Recife, 08 de março de 2012

Oficio 420/12

Comissão de Ética em Experimentação Animal (CEEA) da UFPE
Para: **Prof. Rubem Carlos Araújo Guedes**
Departamento de Nutrição - UFPE
Processo nº 23076.046121/2011-81

Os membros da Comissão de Ética em Experimentação Animal do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEEA-UFPE) avaliaram seu projeto de pesquisa intitulado: ***"Nutrição, hormônios ovarianos e desenvolvimento cerebral: avaliação eletrofisiológica pela depressão alastrante."***

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 CEEA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 9.605 – art. 32 e Decreto 3.179-art 17, de 21/09/1999, que trata da questão do uso de animais para fins científicos.

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

Atenciosamente,

Observação: Origem dos animais: Biotério do Departamento de Nutrição - UFPE; Animais: Ratos, Linhagem: Wistar; Sexo: Fêmeas; Idade: 90-120 dias; Peso: 250g a 350g; Nº de Animais previsto no projeto: 80 animais.

Profa. Maria Teresa Jansen
Presidente do CEEA