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Plasticidade dos espinhos somáticos e dendríticos e implicações funcionais da amígdala medial póstero-dorsal em ratos

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RESUMO

O subnúcleo póstero dorsal da amígdala medial (MePD) é uma área sexualmente dimórfica, com alta concentração de receptores para hormônios gonadais e capaz de modular comportamentos sociais em ratos. Fêmeas apresentam uma variação dinâmica de espinhos dendríticos ao longo do ciclo estral, indicando a ocorrência de plasticidade neural dependente dos níveis fisiológicos dos hormônios ovarianos em circulação para controle da secreção neuroendócrina e da emissão de comportamentos femininos relacionados com a reprodução. Comparativamente com núcleos hipotalâmios interconectados, nada se sabe sobre as sinapses feitas nos corpos neuronais no MePD de ratas durante as diferentes fases do ciclo ovulatório. Em machos, o MePD se relaciona com o comportamento sexual masculino e a quantidade de espinhos dendríticos fica reduzida após a castração de animais adultos. Não se conhece o que ocorre com a morfologia dos espinhos dendríticos no MePD após castração de longo tempo em ratos. De forma inédita, a presente dissertação teve como objetivos: (1) estudar a forma como ocorre a cobertura glial e axonal do corpo neuronal, a possível modificação cíclica nas sinapses axo-somáticas e nos contatos gliais sobre a membrana do corpo neuronal local e a presença e variação na densidade e morfologia dos espinhos somáticos no MePD de ratos e ratas ao longo das diferentes fases do ciclo estral empregando-se microscopia eletrônica de transmissão; e (2) estudar a densidade e a morfologia de espinhos dendríticos de ratos machos adultos submetidos à castração de longa data (3 meses) quando comparados a respectivos grupos controle empregando-se microscopia confocal. Para o primeiro objetivo, foram utilizados ratos Wistar adultos (3 meses de idade, n= 6 machos e 22 fêmeas), mantidos em condição padrão de biotério e sob cuidados éticos. As fêmeas foram estudadas nas fases de diestro, proestro (manhã e noite) e estro. Fotomicrografias da ultraestrutura do MePD foram reconstruídas, mensurou-se o perímetro do pericário e calculou-se o percentual da membrana do corpo celular coberta por processos gliais, axônio e dendritos. A densidade de espinhos somáticos (total e por tipo diferente morfológico) foi calculada dividindo-se o número total de espinhos pelo perímetro somático. Os resultados demonstraram que a membrana somática dos neurônios do MePD é recoberta, em sua maior parte, por processos gliais, porém não houve diferença estatisticamente significativa entre os grupos estudados. No entanto, encontrou-se

maior quantidade de espinhos somáticos em fêmeas no final do proestro que em estro e tais espinhos somáticos em proestro apresentaram-se com forma variável, incluindo-se espinhos achatados, espessos, finos, em formato de cogumelo, ramificados, com aspecto transicional entre formatos ou com morfologia atípica. Para contemplar o segundo objetivo foram utilizados 15 ratos Wistar adultos distribuídos nos grupos: não manipulados experimentalmente (“intactos”, n= 6), submetidos à castração fictícia (Sham= 4) ou castrados (n= 5) e estudados 90 dias depois da remoção cirúrgica dos testículos. A seguir, os encéfalos foram processados histologicamente e empregou-se o corante fluorescente DiI para aquisição e reconstrução tridimensional de imagens dos espinhos dendríticos proximais do MePD por microscopia confocal. Machos castrados apresentaram uma diminuição na densidade de espinhos dendríticos, com diminuição dos valores para os espinhos dos tipos finos, cogumelos e ramificados ao mesmo tempo em que aumentou a densidade de espinhos dendríticos do tipo achatado e espesso quando comparados aos grupos controle. Estes resultados contribuem para o entendimento da plasticidade morfológica dos neurônios do MePD e do dimorfismo sexual local com possível implicação na modulação de comportamento reprodutivo dependente de ações estáveis e/ou de variações dinâmicas dos hormônios gonadais em circulação em ratos.

ABSTRACT

The dorsal posterior subnucleus of the medial amygdala (MePD) is a sexually dimorphic area with a high concentration for gonadal hormones receptors and able to modulate social behavior in rats. Females have a dynamic range of dendritic spines during the estrous cycle, indicating the occurrence of neural plasticity dependent on physiological levels of ovarian hormones in circulation to control the neuroendocrine secretion and the issue of women's behaviors related to reproduction. Compared with interconnected hypotalamic nucleus, nothing is known about the synapses made in neuronal bodies in MePD of rats during different phases of the ovulatory cycle. In males, MePD is related to male sexual behavior and the amount of dendritic spines is reduced after castration of adult animals. It is not known what happens to the morphology of dendritic spines in MePD long term after castration in rats. In an unprecedented way, this dissertation aimed to: (1) study how occurs the glial and axonal coverage of the neuronal body, the possible cyclical change in axo-somatic synapses and glial contacts on the membrane of the local neuronal body and the presence and variation in density and morphology of somatic spines on MePD males and females during the different phases of the estrous cycle employing transmission electron microscopy; and (2) study the density and morphology of dendritic spines of adult male rats subjected to long-term castration (3 months) when compared to respective control groups employing confocal microscopy. For the first aim adult Wistar rats were used (3 months of age, n = 6 males and 22 females), kept under standard vivarium conditions and under ethical guidelines. The females were studied in the diestrus phase, proestrus (morning and evening) and estrus. Photomicrographs of the ultrastructure of MePD were reconstructed, measured to the perimeter of the soma and calculated the percentage of cell body membrane covered by glial processes, axon and dendrites. The density of somatic spines (total and by different morphological type) was calculated by dividing the total number of spines by somatic perimeter. The results showed that the somatic membrane of neurons MePD is covered, for the most part by glial processes, but there was no statistically significant difference between groups. However, we found a greater amount of somatic spines in females at the end of proestrus to estrus and such somatic spines in proestrus were presented with variable form, including stubby, wide, thin, mushroom, branched, with transitional aspect between formats or with atypical

morphology. To address the second objective were used 15 Wistar adult rats distributed in the following groups: non-manipulated experimentally ("intact", n = 6) underwent sham castration (Sham = 4) or castrated (n = 5) and studied 90 days after the surgical removal of the tests. Subsequently, the brains were processed histologically and used the fluorescent dye DiI for acquisition and reconstruction of three-dimensional images of dendritic spines of proximal MePD by confocal microscopy. Castrated males showed a decrease in the density of dendritic spines, with decreased values for spines of thin, mushrooms and branched at the same time increased the dendritic spines density of the stubby and wide type when compared to control groups. These results contribute to the understanding of morphological plasticity of neurons MePD and local sexual dimorphism with possible involvement in modulating the reproductive behavior depending on stable shares and/or dynamic variations of gonadal hormones in circulation in rats.

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LISTA DE ABREVIATURAS E SIGLAS

AMPA	Alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiónico
ACe	Amígdala central
BDNF	Fator neurotrófico derivado do encéfalo
DiI	Percloreto de 1,1'-dioctadecil-3,3,3',3'-tetrametil-indocarbocianina
GFAP	Proteína ácida fibrilar glial
GnRH	Hormônio liberador de gonadotrofinas
IGF-1	Fator de crescimento semelhante à insulina
LTP	Potenciação de longa duração
MeA	Amígdala medial
MeAD	Amígdala medial ântero- dorsal
MeAV	Amígdala medial ântero- ventral
MePD	Amígdala medial póstero- dorsal
MePV	Amígdala medial póstero- ventral
MePDi	Porção intermediária da amígdala medial póstero- dorsal
MePDl	Porção lateral da amígdala medial póstero- dorsal
MePDm	Porção medial da amígdala medial póstero- dorsal
NCAM	Molécula de adesão neuronal
NMDA	N-metil-D-aspartato
PSD	Densidade pós sináptica
REL	Retículo Endoplasmático Liso
SNC	Sistema Nervoso Central
TO	Trato óptico

SUMÁRIO

1. Introdução

1.1 Amígdala

A amígdala ou complexo amigdaliano é uma estrutura complexa composta por núcleos e subnúcleos localizados no lobo temporal, lateral ao hipotálamo e ventral ao estriado no prosencéfalo basal de mamíferos[1,2,3]. Em primatas, é caracterizada como uma estrutura ovóide de substância cinzenta subcortical rostral ao hipocampo[1,4]. Em ratos, localiza-se anteriormente ao hipocampo, ventral e lateral ao trato óptico (TO) ao longo de toda sua extensão rostro-caudal[2,5,6].

O termo amígdala foi empregado, previamente para se referir a uma região do cérebro de mamíferos constituída por um conjunto de núcleos em forma de amêndoa, de onde surgiu seu nome[7]. Conforme Kamal e Tömböl (1975)[8], foram os estudos de Johnston, Humphrey e Crosby que descreveram primeiramente a estria terminal, que representa a maior via de ligação entre a amígdala e as regiões médio-basais do telencéfalo além da organização citoarquitetônica desta estrutura e suas divisões. Considerava-se a amígdala dividida em uma região córtico- medial e outra basolateral; a primeira composta pelos núcleos basal e lateral, representando a região mais recente na escala evolutiva e a segunda constituída pelos núcleos central, medial (MeA) e cortical, representando a área filogeneticamente mais antiga. Além desses núcleos, foi descrita a existência de um terceiro grupo, denominado de amígdala anterior, do qual faziam parte os núcleos laterais do trato olfativo, áreas de transição e núcleos não classificados.

Mais recentemente a classificação da amígdala de ratos divide-a em quatro regiões que, segundo a sua citoarquitetura e hodologia são: 1) amígdala “expandida”, assim chamada por se estender além de seus limites anatômicos. Subdivide-se em núcleos central e MeA e estruturas anatomicamente correlacionadas; 2) amígdala com características corticais, subdividida em porção basolateral e em porções que se ligam às vias olfativas e vomeronasal; 3) região de transição, situada entre a porção ventral dos núcleos da base e a amígdala “expandida”; e 4) núcleos ainda não classificados, constituídos por um grande grupo de células dispersas na substância branca e no interior do núcleo próprio da estria

terminal[1,9]. Os limites anatômicos precisos da amígdala e a classificação plena de todas as suas subdivisões permanecem, no entanto, ainda controversos[2,7].

A amígdala de ratos compreende subnúcleos que formam uma complexa rede estrutural inter-relacionada e multifuncional, uma vez que está envolvida na modulação de diversos comportamentos sociais[1,2,10,11,12]. Os núcleos amigdalianos integram vias preservadas filogeneticamente, como as aferências que recebe do bulbo olfatório principal e acessórios e as eferências que envia para os diversos núcleos e subsistemas funcionais do hipotálamo[13,14]. Para realizar suas funções, a amígdala recebe diferentes informações sensoriais, tanto interoreceptivas quanto exteroceptivas, que acabam modificando sua atividade e estimulando diversas regiões do sistema nervosos central (SNC) em resposta ao estímulo inicial com relevância social para o indivíduo e sua espécie[15,16,17]. À vista disso, sabe-se, presentemente, que a amígdala não tem uma função unitária simples, mas um papel de integração de atividades comportamentais, endócrinas e simpático/parassimpáticas[10,18]. Atribui-se à amígdala funções como: percepções de estímulos olfativos e hormonais (como o dos esteroides sexuais e os glicocorticoides), interpretação e respostas comportamentais a estímulos gerados por medo e ansiedade, modulação do comportamento alimentar, reprodutivo, maternal e agressivo, e participação na aquisição de memória e aprendizado condicionados[10,11,12,15,16,17,19,20,21,22,23].

Alguns núcleos da amígdala são sexualmente dimórficos em ratos. Por exemplo, diferenças entre machos e fêmeas são evidenciadas quando compara-se a área total da MeA, maior em ratos a partir da terceira semana de vida pós-natal e mais notável após a puberdade[24,25,26,27]. Tal fato está na dependência da ação dos hormônios gonadais em ambos os sexos[26,28,29,30]. A MeA possui neurônios que apresentam receptores para os esteroides sexuais testosterona, estradiol e progesterona[27,31,32]. De modo geral, devido à sua capacidade de gerar alterações nos circuitos neurais, esses hormônios influenciam a plasticidade do SNC e causam alterações morfológicas como: modificação no número de neurônios, promoção da maturação neuronal e crescimento de neuritos, determinação da sinaptogênese e formação de circuitos neurais específicos[33,34], alteração no comprimento dendrítico, na arborização dendrítica e no número de espinhos dendríticos[33,35,36,37,38]. Tais

efeitos sobre dendritos e espinhos dendríticos variam de um sexo para outro e de uma região do SNC para outra[5,6,29,38]. O tema desta dissertação envolve as alterações morfológicas na membrana do soma neuronal, na densidade e na forma dos espinhos dendríticos de subnúcleo da MeA relacionadas com variações normais ou induzidas por hormônios gonadais em ratos

1.2 Amígdala Medial

1.2.1 Localização e divisão

A MeA é um dos núcleos superficiais do complexo amigdaloide, ocupando seu aspecto rostromedial. É formada por uma coluna de células que surgem em justaposição à superfície lateral das fibras que ascendem pelo TO[1,2]. Inicia medial e posteriormente ao núcleo do trato olfativo estendendo-se caudalmente até o surgimento do corno temporal do ventrículo lateral. Nesta altura, a MeA localiza-se dorso-medialmente ao polo cefálico da área de transição amígdalo-hipocampal, formando a região medial da parte anterior desta porção do ventrículo. Ao longo de toda sua extensão, a MeA encontra-se lateralmente com a região ventro-lateral do TO. Seus limites rostral com a amígdala anterior e lateralmente com as camadas profundas do núcleo cortical anterior não estão claramente elucidados[1,22].

Dorso-lateralmente, a MeA é separada da porção medial da ACe por uma região pobre em células, que é substituída mais ventral e caudalmente pelo núcleo próprio da estria terminal. Caudalmente e na posição dorsal, grupos de fibras que ascendem dentro da estria terminal estão interpostos entre a MeA e outros grupos amigdaloides[1,39].

Pitkänen (2000)[22] sugeriu que a MeA fosse composta por 3 partes: rostral, central (dorsal e ventral), e caudal. Alheid et al (1995)[1] e Paxinos e Watson (1998)[39] dividem-na da forma como tem sido mais utilizada e nos seguintes subnúcleos: ântero-dorsal (MeAD), ântero-ventral (MeAV), póstero-dorsal (MePD) e póstero-ventral (MePV). O MePD pode ser dividido conforme a disposição colunar de seus neurônios em uma porção mais superficial e medial (MePDm), uma intermediária com menos células agrupadas (MePDi) e uma porção lateral (MePDl) novamente com mais células justapostas[2,23,40,41]. Ademais, o MePD, área de estudo deste trabalho, separa-se do TO por uma camada com escassos corpos celulares, a qual

torna-se estreita em direção rostral e dorsal e desaparece completamente próximo ao MeAD[2,9]. Esta região deve ser considerada, preferentemente, como o local de passagem de axônios procedentes do núcleo próprio da via olfativa acessória para transmissão de informação vomeronasal, e não como uma dita “camada molecular” própria do MePD[2,42].

Em função da neurogênese na MeA em ratos ocorrer aproximadamente no 16º dia de vida intra-uterina e se iniciar antes nas regiões anterior e ventral concomitantemente ao estabelecimento de conexões com o sistema olfativo[43], Wood e Newman (1995)[21] sugeriram que os subnúcleos anteriores da MeA estariam sendo influenciados especialmente por informações olfativas e feromonais enquanto a região posterior seria a preferentemente afetada por hormônios gonadais. Tal divisão anátomo- funcional não é absolutamente exata pois há ações dos esteroides sexuais nos quatro subnúcleos da MeA e conexões sinápticas recíprocas entre todas as partes[21,22,31,32].

No entanto, a distribuição de receptores para hormônios sexuais na MeA não é uniforme. Neurônios com receptores para hormônios gonadais estão presentes em todos os subnúcleos da MeA, mas particularmente os maiores acumulados estão no MePD e MePV de machos[31,44,45,46]. De fato, é no MePD onde se encontra a maior quantidade de receptores para testosterona na MeA[31,47]. Aromatase, a enzima que converte testosterona em estradiol, também é encontrada na MeA[48,49]. No MePD, 80 a 90% dos neurônios que possuem receptores para estrógeno também possuem para andrógenos, enquanto o contrário acontece somente em cerca de 30% dos casos[50]. É notável que no MePD são encontrados neurônios com receptores do tipo α e β para estradiol[30,32,43,51,52,53] sendo que a concentração de receptores é tão marcada no MePD que chega a ter a mesma intensidade que nos diversos núcleos hipotalâmicos relacionados com a secreção neuroendócrina do hormônio liberador de gonadotrofinas (GnRH) e que modulam o comportamento reprodutivo masculino e feminino[31,49,53]. O MePD também apresenta receptores para a progesterona[46,54,55]. Em conjunto, esses dados sugerem fortemente que hormônios sexuais masculinos e femininos são capazes de modular a morfologia e a função das células presentes neste subnúcleo de machos e fêmeas.

1.2.2 Hodologia e Função da MeA

A MeA de ratos possui uma vasta rede de conexões neurais entre seus subnúcleos, com outros núcleos da amígdala e também com núcleos extra-amigdaloides[13,56]. Suas principais aferências são provenientes do córtex cerebral (da área pré-límbica, córtex entorrinal, infralímbico e perirrinal dorsal), de núcleos do hipotálamo (por exemplo, da área pré-óptica), da área septal, do tronco encefálico (núcleo dorsal da rafe e núcleo parabraquial) e da via olfativa e estruturas interconectadas (córtex piriforme, bulbo olfativo acessório e núcleo endopiriforme)[57]. Dentre as aferências intra-amigdalianas destacam-se as da área de transição amigdalo-hipocampal, dos núcleos basal e basal acessório e as dos núcleos corticais anterior, posterior, lateral e medial[22,57].

As eferências do MePD incluem, dentre as mais significativas e estudadas, as hipotalâmicas (para o núcleo periventricular ântero- ventral, para a área pré-óptica e para o núcleo pré-mamilar ventral, dentre outros), as corticais (para a área entorrinal lateral, área de transição pós-piriforme e região CA1 hipocampal), as para o tronco encefálico (para a área tegmentar dorsal e a substância cinzenta periaqueductal) e as para outras regiões como, por exemplo, o núcleo próprio da estria terminal (parte ântero-dorsal e posterior principal) e para a substância “inominata” sublenticular[13,56,58,59]. Dentre as eferências intra-amigdalianas destacam-se aquelas para os núcleos central e cortical póstero-lateral e póstero-medial[13].

O MePD participa da interpretação de informações sensoriais interoceptivas e exteroceptivas[60,61,62], na regulação de comportamentos sociais[63,64] incluindo-se os comportamentos agressivo[6], sexual de machos e fêmeas[19,23,65], maternal[66] e na modulação da memória condicionada e do aprendizado onde componente emocional esteja envolvido[10]. Este último achado foi observado em testes de esquiva inibitória[67] em respostas neuroendócrinas frente a um evento estressor[68,69] e em vários testes envolvendo comportamentos sociais, tais como o defensivo e o agressivo[61] ou com estímulo causador de medo[70]. Para tanto, o MePD também desencadeia ajustes cardiovasculares simpáticos e parassimpáticos muito provavelmente relacionados à gênese e à modulação de comportamentos sociais[71,72,73].

Dentre todos estes comportamentos, é importante destacar o papel na gênese e regulação de comportamentos reprodutivos em ratos e hamsters[15,21,74,75]. Por existirem aferências oriundas do bulbo olfatório, do órgão vomeronasal[76] e de diversos núcleos do hipotálamo[57], o MePD está em posição estratégica para modular comportamentos que requisitam estimulação quimiossensorial para sua ocorrência, como a atividade sexual[76]. Em ratos, a MeA como um todo facilita as respostas aos estímulos sexuais, enquanto nas fêmeas este parece ser um componente neural do sistema inibitório de regulação do comportamento de cópula[74]. Lesões principalmente da parte posterior da MeA de ratos adultos resultou na diminuição na frequência de ejaculações, aumento no número de intromissões penianas que precedem a primeira ejaculação e aumento do intervalo entre as intromissões, quando comparados com animais sem lesão[74,77].

Estudos mais detalhados sugerem que os subnúcleos da MeA possuem diferentes atribuições referentes à regulação do comportamento sexual masculino[23,63,76]. Isto foi observado em ratos com lesão no MePD que reduziram o comportamento copulatório e perderam a ereção peniana provocada pelo odor da fêmea em estro[75]. De outro modo, a investigação olfativa genital, feita por machos em fêmeas, leva a um aumento da proteína Fos no MePD, o que igualmente ocorre após a ejaculação neste subnúcleo[65]. Isso coloca o MePD como importante área de estudo da morfologia dos neurônios locais em machos e fêmeas e, a possível modulação feita pelos hormônios gonadais em estrutura sexualmente dimórfica, relevante para a organização de comportamentos necessários para a reprodução e perpetuação de uma espécie animal. No presente estudo, focaremos nos dados existentes até o presente momento obtidos em ratos e, adicionalmente, na contribuição que a presente dissertação apresenta para a literatura pertinente.

1.3 Ação dos hormônios gonadais na plasticidade neural do MePD de ratos: dados sobre membrana somática e espinhos dendríticos

Em mamíferos, as diferenças sexuais determinadas geneticamente ou por fatores epigenéticos são capazes de alterar a morfologia neuroglial e o funcionamento de circuitos neurais caracteristicamente em machos e fêmeas[25,28]. Isso decorre em grande parte pela ação dos hormônios gonadais no SNC ao longo do desenvolvimento intrauterino e em diferentes períodos pós-natais, incluindo a puberdade e a vida adulta[20,79]. O MePD não só possui neurônios e astrócitos que possuem receptores e respondem aos hormônios gonadais como também está incluído em circuitos cujas aferências são igualmente moduladas por tais esteroides. Neste sentido a modulação das aferências para o MePD e a própria ação dos hormônios sexuais podem modificar sítios pós-sinápticos neste subnúcleo[5,20]. De fato, o MePD apresenta diferenças morfológicas, neuroquímicas e nas conexões de seus neurônios quando comparam-se machos e fêmeas[5,6]. Em hamsters, há diminuição do comprimento dendrítico total e do percentual de neurônios com ramos dendríticos terciários na parte posterior da MeA[28] sugerindo que o neurópilo possa ser o local preferencial de mudanças morfológicas mediadas por esteroides sexuais nos neurônios do MePD[29]. Não obstante, há efeito da variação cíclica dos hormônios ovarianos no volume do corpo celular dos neurônios do MePD[79]. Ou seja, embora machos e fêmeas em diestro apresentem resultados similares, ambos os grupos têm valores maiores do que fêmeas em proestro e estro[79].

Esses dados no MePD de ação hormonal no corpo neuronal são importantes porque remetem a achados prévios no núcleo arqueado hipotalâmico relevante para a secreção neuroendócrina cíclica e comportamento reprodutivo em fêmeas, como será descrito a seguir e que serviu para a elaboração do primeiro artigo da presente dissertação. Ou seja, plasticidade sináptica evidente foi encontrada previamente no núcleo arqueado hipotalâmico de ratos, área envolvida na liberação cíclica e tipicamente feminina de GnRH e de prolactina, e onde ocorre modificação no número de sinapses axo-somáticas e na proporção da membrana celular do soma neuronal recoberta por processos gliais em ratas ao longo do ciclo ovulatório[80,81]. Isto é, em níveis fisiológicos, a variação hormonal presente ao longo das fases do ciclo estral da rata (diestro, proestro, estro e metaestro) é capaz de modificar a

morfologia e a funcionalidade dos neurônios e dos astrócitos hipotalâmicos[82]. Durante a fase de estro há tanto uma redução no percentual de membrana celular somática estabelecendo contatos sinápticos com terminais axonais pré-sinápticos quanto um aumento no percentual desta membrana recoberta por prolongamentos gliais[83]. Esse remodelamento funcional dinâmico da membrana celular é absolutamente importante para o estabelecimento ou o deslocamento mecânico de contatos inibitórios GABAérgicos que controlam a secreção cíclica de GnRH e, consequentemente, a reprodução em fêmeas[81,84]. Da mesma forma, diferenças entre os sexos e ao longo do ciclo estral foram encontradas na imunorreatividade para a proteína fibrilar ácida glial (GFAP), constituinte de filamentos intermediários do citoesqueleto de astrócitos maduros, sendo maior em fêmeas em proestro do que em machos e, notavelmente, no MePD[85,86]. Esses achados passaram a ter maior relevância uma vez que existem evidências indicando a possível interrelação funcional entre neurônios e células da glia no MePD e que, adicionalmente, este subnúcleo participa de circuitos integrados para a secreção neuroendócrina e emissão de comportamento reprodutivo em ratos. Não se sabe, no entanto, o que ocorre com a ultraestrutura da cobertura axonal e glial dos somas neuronais do MePD de machos e fêmeas ao longo do ciclo estral, tema desta dissertação e, para o que, emprega-se técnica de microscopia eletrônica de transmissão.

Adicionalmente, plasticidade neuronal vinculada aos esteroides sexuais foi detalhadamente descrita para os dendritos e seus espinhos, incluindo-se achados especificamente no MePD de ratos[5,6,40,41,87], o que serviu para embasar o segundo artigo da presente dissertação. Os espinhos dendríticos têm recebido atenção devido à sua relevância e correlação funcional com a modulação de informações sinápticas aferentes[6,88] podendo servir como um quarto elemento importante para a atividade celular[89,90,91,92,93]. Espinhos podem ser encontrados no soma e no segmento inicial do axônio de vários neurônios, mas são encontrados em muito maior quantidade em ramos dendríticos[29,90,94,95]. Todos os vertebrados e alguns invertebrados possuem espinhos dendríticos e o seu tamanho varia ao longo das diferentes regiões nervosas ou dentro de uma mesma área, assim como entre diferentes espécies[96]. O aspecto padrão de um espinho é definido pela presença de

uma “cabeça” (com volume de aproximadamente 0,001 a 1 μm³) conectada ao neurônio por um “pescoço” (com diâmetro menor que 0,1 μm³)[97,98].

Considera-se como base que um espinho usualmente representa uma sinapse e, assim, a determinação do número de espinhos por segmentos dendrítico tem servido para estimar a menor ou maior ocorrência de sinapses locais[97,99]. Os espinhos dendríticos formam pequenos compartimentos multifuncionais especializados para a integração da transmissão sináptica e a atividade eletrofisiológica neuronal[89,100] com receptores para neurotransmissores capazes de modular propriedades dendríticas passivas e ativas e sequências bioquímicas intracelulares locais e ao longo do neurônio[95]. Desta maneira, os espinhos podem representar os principais sítios celulares para as funções conectivas e integrativas dos neurônios relevantes para a formação e a estabilização de circuitos neurais[6,92,101,102,103,104]. Os receptores para neurotransmissores estão no espinho em justaposição ao elemento pré-sináptico represetado geralmente por um botão axonal terminal[105]. No espinho, a área pós-sináptica é reconhecida pela densidade pós-sináptica (PSD), zona de material elétron-denso formada por receptores, canais iônicos, actina e sistemas de sinalização intracelulares implicados na transmissão sináptica e no acoplamento da atividade sináptica à atividade pós-sináptica[95]. Usualmente, a PSD no espinho tem aspecto assimétrico em relação ao elemento pré-sináptico e são consideradas tais sinapses como excitatórias[90,96,106]. Muito embora não possuam mitocôndrias e microtúbulos, os maiores espinhos contêm retículo endoplasmático liso (REL), os quais podem formar uma estrutura denominada “aparelho do espinho”[90,107] cuja função deve ser a manutenção de cálcio intracelular em níveis não patológicos, o que se associa ao processo de neuroproteção[108].

Os receptores ionotrópicos ou metabotrópicos na PSD geram fluxo iônico através da membrana celular, resultando em mudança da voltagem local e possibilitando demais respostas pós-sinápticas sequenciais[95,106]. A transdução deste sinal, por propiciar variabilidade na compartimentalização bioquímica, pode ser afetada pela morfologia dos espinhos[89,96]. Por exemplo, espinhos com pescoço estreito podem não gerar uma elevação significativa do cálcio no interior dendrítico, compartimentalizando-o na cabeça do espinho e reduzindo o tempo e a amplitude

da alteração de voltagem induzida por neurotransmissor excitatório; alternativamente, em espinhos com pescoço largo e menor resistência, o aumento da concentração de cálcio por ativação sináptica pode ser mais prontamente disperso para dentro do dendrito adjacente e pode atingir outros espinhos próximos[107,109]. Isso pode favorecer a alteração da corrente elétrica local, como detector de coincidência de atividade e sincronização de voltagem, e contribuir para a ocorrência de atividade potencializada de longo prazo (LTP)[89,103]. De outra forma, os espinhos também podem atuar como subcompartimentos elétricos, onde a geometria do pescoço do espinho pode exercer resistência à passagem de corrente elétrica para o dendrito. Desse modo, há uma diferença de potencial elétrico localmente que pode ativar canais iônicos dependentes de voltagem ou estabelecer uma diferença de capacitância com toda a árvore dendrítica conforme as propriedades ativas ou passivas existentes no local[95]. Com isso, a morfologia do espinho gera um nível adicional de complexidade para o processamento da informação sináptica e com um grau de modulação maior do que poderia ser obtido com sinapses feitas diretamente sobre troncos dendríticos. De fato, faz parte da plasticidade sináptica que alguns espinhos possam variar dinamicamente sua forma, em um processo que envolve o rápido rearranjo do citoesqueleto de actina no seu interior, e que pode ocasionar mudança na estrutura e no número de espinhos[100,110].

"Filopódio" é o nome que se atribui à protrusão dendrítica que não tem cabeça definida e é fino e comprido; acredita-se que ou seja a forma precursora dos espinhos em busca do elemento pré-sináptico ou a forma de retração do espinho que está involuindo. Já os espinhos tem sido classificados segundo sua morfologia com a seguinte nomenclatura: "fino" (do inglês, *thin*), apresenta pescoço fino e geralmente comprido que se termina em uma cabeça definida; "espesso ou achatado" (do inglês, *wide or stubby*), o qual não apresenta um pescoço delimitado e mostra-se com uma elevação bulbosa a partir do contorno dendrítico; em forma de "cogumelo" (do inglês *mushroom*), como se infere de sua nomenclatura, com uma cabeça bem mais volumosa que seu pescoço e que parece ser o tipo mais estável no que diz respeito a mudanças numéricas e contatos sinápticos duradouros; e, "ramificado" onde um mesmo pescoço pode originar mais de uma cabeça no mesmo

espinho[90,93,111]. Há formas intermediárias entre esses tipos e que se caracterizam como um *continuum* entre as diferentes classificações[107,112,113].

Sabe-se que diferenças no tamanho da cabeça do espinho são diretamente relacionadas com o tamanho da PSD[106,110] e a forma dos espinhos pode afetar a estabilidade e a função sináptica[96,106,114]. Com isso, o padrão de desenvolvimento, a forma e a função dos espinhos dendríticos modifica de forma marcante a excitabilidade neuronal e, por sua vez, a demanda sináptica pode modificar o número e a forma dos espinhos, o que torna o estudo de sua morfologia tema importante para a compreensão das bases celulares de funcionamento do sistema nervoso[5,6,38,59,96,106,114]. Em neurônios em cultura a ativação de receptores glutamatérgicos do tipo N-metil-D-aspartato (NMDA) provoca aumento rápido e transitório no tamanho dos espinhos pré-existentes e gradativa formação de novos espinhos dendríticos[32,115]. Embora haja controvérsias, sugere-se que espinhos com cabeças maiores são mais estáveis, expressam maior número de receptores do tipo AMPA e contribuem para atividades sinápticas mais vigorosas enquanto espinhos com cabeças pequenas são mais instáveis e contribuem para conexões sinápticas que podem ser do tipo “silenciosas” envolvendo receptores NMDA que necessitam de ativação por ligante após alteração da voltagem local e desbloqueio, causado pelo deslocamento do magnésio, deste receptor[95].

No MePD de ratos, dados de imunomarcação para sinaptofisina, proteína presente no elemento pré-sináptico, e microscopia confocal permitiram identificar que a maioria dos espinhos dendríticos parece estabelecer conexões[112.] Dados ultraestruturais anteriores mostraram a ocorrência de sinapses unitárias formadas por um axônio terminal e um espinho como elemento pós-sináptico[116] o que se confirmou com reconstrução tridimensional da ultraestrutura dos espinhos dendríticos[93]. Desta forma, o estudo do número e da forma dos espinhos dendríticos constitui-se importante para a compreensão das bases celulares de funcionamento do sistema nervoso[5,6,38,111] e, no presente caso, do MePD de ratos.

Utilizando-se a técnica de Golgi descobriu-se que machos possuem maior densidade de espinhos dendríticos no MePD do que fêmeas ao longo do ciclo estral, preferentemente após a fase de diestro[20]. Ademais, a diminuição no número de espinhos dendríticos no MePD de fêmeas em proestro é coincidente com a redução

na imunorreatividade para sinapsina, proteína presente em terminais pré-sinápticos, o que pode estar associado com o estabelecimento de um menor número de conexões neste momento[117] e a modulação que o MePD faz na ocorrência da secreção de GnRH e do comportamento sexual feminino[5,6]. Estes achados são interessantes porque sugerem uma possível relação entre os diferentes níveis de hormônios gonadais em circulação, dimorfismo sexual na estrutura e remodelamento do citoesqueleto neuronal e mudanças sinápticas rápidas e fásicas no aporte sináptico no MePD de ratos, machos e fêmeas, em idade adulta. Em ratos, observou-se diferenças eletrofisiológicas relacionadas com potenciais pós-sinápticos excitatórios entre ratos pré-púberes machos e fêmeas[33,118]. Machos apresentam mais desta atividade no lado esquerdo do que fêmeas[33,118], provavelmente envolvendo uma maior quantidade de espinhos dendríticos no MePD esquerdo. Em idade adulta, a castração reduz a densidade de espinhos dendríticos em machos coincidindo com o declínio evidente do comportamento sexual desses animais[87].

Não obstante, ainda não é conhecida a modificação da morfologia dos espinhos dendríticos que ocorre concomitante à redução da densidade no MePD de ratos submetidos à castração em idade adulta[87]. Para tanto, a técnica de reconstrução tridimensional dos espinhos empregando-se o corante fluorescente do grupo das carbocianinas denominado DiI e visualizados por meio da microscopia confocal constitui-se uma das melhores abordagens metodológicas disponíveis na atualidade[112,119,120] a qual foi empregada na presente dissertação.

Baseado nesses dados precedentes, estudou-se, de forma inédita, a ultraestrutura das sinapses axo-somáticas e da cobertura glial na membrana do corpo celular de neurônios do MePD de ratos adultos machos e de fêmeas ao longo do ciclo estral, buscando um possível dimorfismo sexual e o efeito dos hormônios ovarianos na conectividade e na plasticidade desta importante área encefálica.

Da mesma forma, buscou-se avançar os conhecimentos a respeito da redução na densidade de espinhos dendríticos no MePD de ratos castrados em idade adulta[87] e demonstrar qual é a modificação na morfologia dos espinhos dendríticos que ocorre de forma concomitante com a diminuição desses locais de contato pós-sináptico. Atentou-se para possíveis diferenças hemisféricas

relacionadas com a funcionalidade do MePD[118,121 122] uma vez que já foram descritos registros eletrofisiológicos de correntes despolarizantes e a ocorrência de sinapses excitatórias nos neurônios locais de modo diferente no MePD de cada hemisfério cerebral de machos e fêmeas[118].

Com base nisso, elaboraram-se os objetivos a seguir para contribuição de forma inédita com conhecimentos sobre o MePD de ratos, machos e fêmeas ao longo do ciclo ovulatório, e sua correlação funcional plausível com tais dados experimentais.

2 Objetivos

2.1 Objetivos Gerais

Caracterizar a ultraestrutura de sinapses axo-somáticas, dos contatos gliais e axonais na membrana do corpo neuronal e a presença e morfologia de espinhos somáticos no MePD de ratos adultos, machos e fêmeas ao longo do ciclo estral, empregando-se microscopia eletrônica de transmissão.

Estudar a plasticidade na densidade e na morfologia dos espinhos dendríticos em neurônios do MePD de ratos submetidos à castração de longa data empregando-se microscopia confocal.

2.2 Objetivos específicos

Estudar a ultraestrutura, a ocorrência e possíveis mudanças na cobertura somática neuronal relacionadas à variação fisiológica dos níveis de hormônios gonadais, no que diz respeito à presença de prolongamentos gliais, de axônios e dendritos, no MePD de ratos e em ratas ao longo do ciclo ovulatório normal.

Descrever os efeitos dos esteroides ovarianos no padrão de cobertura glial do soma neuronal e comparar os resultados com os de outras áreas cerebrais que estão envolvidas na secreção neuroendócrina e na modulação de comportamentos sexuais em ratas.

Demonstrar a presença e descrever a morfologia de espinhos somáticos no MePD de machos e fêmeas ao longo do ciclo estral.

Determinar o efeito da retirada dos hormônios gonadais sobre a densidade e a morfologia dos espinhos dendríticos proximais no MePD de ratos machos castrados em idade adulta, comparando resultados prévios com o método de Golgi.

3 Materiais, Métodos e Discussão

3.1 Artigo I

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Original Research

GLIAL AND AXONAL PERIKARYAL COVERAGE AND SOMATIC SPINES IN THE POSTERODORSAL MEDIAL AMYGDALA OF MALE AND CYCLING FEMALE RATS

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3.1.1 Abstract

The posterodorsal medial amygdala (MePD) is a sex-steroid sensitive area that modulates reproductive behavior in rats. The volume of the neuronal cell body, density of dendritic spines, and glial fibrillary acidic protein-immunoreactivity are sexually dimorphic or affected by gonadal hormones in the MePD. Here, we add new data to this panorama and describe the ultrastructure of the glial and axonal coverage of the perikaryal membrane and the somatic spines in the MePD of males and cycling females (in diestrus, early proestrus, late proestrus, and estrus). Transmission electron microscopy data (mean values from 7-11 neurons per rat, 5-6 animals per group) showed that the rat MePD has most of the perikaryal membrane covered by glial processes and a relatively high amount (up to 40%) of axonal processes contacting the neuronal cell body. No statistically significant difference was found between groups for these somatic coverages ($p > 0.5$). However, the density of somatic spines along the length of the perikaryal membrane was higher in the late proestrus than in estrus ($p < 0.05$), and somatic spines in early and late proestrus showed variable shapes with stubby/wide, thin, mushroom-like, ramified, transitional or atypical aspects. These findings add to the rapid adjustable synaptic changes in the MePD and in the integrated neural circuits that control neuroendocrine secretion and the hormonally modulated timely display of social behaviors in rats.

3.1.2 Introduction

The posterodorsal medial amygdala (MePD) assembles few subpopulations of neurons (Choi et al., 2005; García-López et al., 2008; Carney et al., 2010; Bupesh et al., 2011) with a general morphology of multipolar bitufted or stellate cells (de Castilhos et al., 2008; Niimi et al., 2012; Rasia-Filho 2012b; Bian, 2013) in the "extended amygdala" of the rat basal forebrain (de Olmos et al., 2004; Dall'Oglio et al., 2008). Local astrocytes, identified with glial fibrillary acidic protein-immunoreactivity (GFAP-ir), have rod to stellate cell bodies with radiating branched processes (Rasia-Filho et al., 2002; Salazar et al., 2014) of varying complexity (Johnson et al., 2008; 2013).

The MePD serves as an interface with olfactory and pheromonal sensorial cues (Meredith and Westberry, 2004; Pereno et al., 2011), mating-related genitosensorial stimulation (Pfaus and Heeb, 1997; Polston et al., 2001), and the actions of gonadal hormones (Gréco et al., 1998; Isgor et al., 2002; Gréco et al., 2003; Simerly, 2004; Blake and Meredith, 2011) for the modulation of emotionally-loaded information and social behaviors in male and female rats (Newman, 1999; Choi et al., 2005; Rasia-Filho et al., 2012b). The MePD has one of the highest expressions of androgen receptors, α- and β-estrogen receptors, and progesterone receptors in the brain, which resemble levels found in different hypothalamic nuclei (Simerly et al., 1990; Österlund et al., 1998; Phillips-Farfán et al., 2007). Local neurons also coexpress both estrogen receptors, α and β, or estrogen and progesterone receptors (Gréco et al., 1998; 2001, 2003).

As part of an interconnected sex-steroid responsive network, the rat MePD has dense projections to the hypothalamic medial preoptic area (MPOA), the anteroventral periventricular nucleus, the ventral premammillary nucleus, and, to a lesser extent, to the ventrolateral part of the ventromedial nucleus (VMH), and the arcuate nucleus (ARC; Dong et al., 2001; Petrovich et al., 2001). In males, these connections modulate the occurrence of intromission and ejaculation (Coolen et al., 1997; Coolen and Wood, 1999; Newman, 1999; Rasia-Filho et al., 2012b). In females, they coordinate neuroendocrine secretion of both gonadotrophin releasing hormone (GnRH) and prolactin and the display of reproductive behaviors (Beltramino and Taleisnik, 1978; Newman, 1999; Polston et al., 2001; Rasia-Filho et al., 2004; Simerly, 2004; de Castilhos et al., 2008; Lin et al., 2011). The rat MePD shows a sexual dimorphism evidenced by a higher nuclear volume in males than in females (Mizukami et al., 1983; Hines et al., 1992; Morris et al., 2008). At the cellular level, males have a higher volume of the neuronal cell body (Hermel et al., 2006b) and a higher density of dendritic spines, considered as multifunctional integrative postsynaptic units for excitatory inputs (GarcíaLópez et al., 2010; Segal, 2010; Yuste, 2013), than proestrus or estrus females (Rasia-Filho et al., 2004; 2012a). The decrease in dendritic spine density in the MePD of females in proestrus is coincident with reduced synapsin reactivity, associated with synaptic pruning (Oberlander and Erskine, 2008), and with increased GFAP-ir (Martinez et al., 2006). This suggests a

dynamic synaptic organization in the female MePD, involving plastic and inversely related responses of local neurons and glial cells along the estrous cycle. Furthermore, the intensity of GFAP-ir is subregion-specific (higher in the MePD than in the other medial amygdala subnuclei), and is affected by sex steroids since diestrus females display higher levels than males, proestrus females have the highest levels along the estrous cycle and, in ovariectomized females, estradiol alone or estradiol plus progesterone increased GFAP-ir in the MePD (Rasia-Filho et al., 2002; Martinez et al., 2006).

There is no current report on the glial coverage or synaptic features on the perikaryal membrane of MePD neurons. Neuronal membrane remodeling and synaptic turnover was previously found in the hypothalamic ARC, an area involved in the regulation of GnRH surge and prolactin secretion (Garcia-Segura et al., 1994; GarciaSegura and McCarthy, 2004). In the ARC of estrus females, the amount of perikaryal membrane covered by presynaptic terminals decreases due to the close apposition of glial processes, which reduce the number of GABAergic axo-somatic contacts. These synaptic changes may be involved in the control of the cyclic secretion of GnRH, allowing the peak of GnRH for ovulation, and, ultimately, species reproduction (Olmos et al., 1989; Párducz et al., 1993; Garcia-Segura et al., 1994). Similar plastic changes may occur in the female MePD, although it is also likely that glial heterogeneity can define different brain areas (Emsley and Macklis, 2006), and the cross-talk between neurons and glia can have region specific features even in functionally integrated circuits of the rat brain (Garcia-Segura and McCarthy, 2004 and references therein).

Here, we describe for the first time the ultrastructure of the glial and axonal coverage of the perikaryal membrane as well as the presence and morphology of somatic spines in the MePD of males and cycling female rats.

3.1.3 Materials and Methods

3.1.3.1 Animals

Adult Wistar rats (3-month-old, n= 6 males and 22 females) were housed under standard laboratory conditions with free access to food and water, room temperature around 21°C, and a 12-h light/dark cycle (lights on at 6 h). Daily vaginal

smears were obtained along 2 consecutive weeks prior to the beginning of the experiment. The regularity of the estrous cycle was determined according to cytological criteria (Singletary et al. 2005). Here, the term estrous means the cycle itself whereas diestrus, proestrus, and estrus refer to each phase (Rasia-Filho et al., 2004). Normally cycling females were studied between 10:30 and 12:30 h of diestrus (n= 6), proestrus ("early proestrus", n= 5) or estrus (n= 5), and between 18 and 20 h on proestrus ("late proestrus", n= 6; based on Csakvari et al., 2007). We studied females during two periods of the proestrus phase (morning and night) corresponding to the estrogen surge and, afterwards, progesterone surge in circulation (Anselmo-Franci and Szawka, 2005).

Rats were manipulated according to international laws and guidelines for the care and use of laboratory animals (European Communities Council Directive of 24 November 1986, 86/609/EEC) and the Animal Ethics Committee of the UFCSPA (Brazil; approved protocol no. 248/13).

3.1.3.2 Transmission Electron Microscopy

Rats were deeply anesthetized with intraperitoneal injections of ketamine and xylazine (80 mg/kg and 10 mg/kg, respectively). Perfusion through the left cardiac ventricle was done with a single injection of heparin (1000 IU) and 500 ml of 1% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (0.1 M, pH 7.4; PB) at room temperature (RT). Using a peristaltic pump (Control Company, Brazil), flow was rapid in the beginning (50 ml during the first 90 s after chest opening) and, then, slowed to last for additional 30 min (15 ml/min flow) to maintain fine ultrastructural integrity (cf. Tao-Cheng et al., 2007). Brains were removed and immersed in the same fixative solution for 90-150 min, rinsed in PB, and coronally sectioned using a Vibratome (VT 1000S, Leica Microsystems, Germany). The MePD was studied from 3.0 to 3.4 mm posterior to the bregma, lateral to the optic tract and ventral to the stria terminalis (Paxinos and Watson, 2008; Figure 1A). The left MePD was selected for further study because of its higher local astrocyte complexity in males (Morris et al., 2008; Johnson et al., 2008; 2013) and because the electrochemical stimulation of the left medial amygdala increases LH secretion in female rats (Carrer et al., 1978). The number of excitatory synapses and the frequency of miniature excitatory postsynaptic currents are also higher in the left

MePD of young males than in females, with no difference in the right MePD (Cooke and Woolley, 2005).

We dissected the MePD from one single 400-µm-thick coronal slice and the tissue obtained had an isosceles triangle shape, base down, taking the caution of considering the differences between males and females in the apparent MePD volume (Figure 1B and C). Samples were taken from the intermediate to lateral part of the MePD (de Olmos et al., 2004; Dall'Oglio et al., 2008), avoiding the medial "molecular layer" which is a cell-sparse region close to the optic tract mainly formed by axons from the accessory olfactory path (Scalia and Winans, 1975; Pro-Sistiaga et al., 2007; Figure 1B and C). Each tissue block was post-fixed in the same perfusion solution for additional 18 h, washed in phosphate-buffered solution (PB, 0.1 M, pH 7.4), and postfixed in 1% osmium tetroxide (Sigma Chemicals Co., USA) for 1 h at RT. Thereafter, sections were washed in PB, dehydrated in a graded series of alcohol and propylene oxide (Electron Microscopy Sciences, USA), embedded in resin (Durcupan, ACM-Fluka, Switzerland), left in vacuum for 24 h, and put onto slides with resin to polymerize for 48 h at 60 °C.

Ultrathin sections (65-70 nm) containing the MePD were obtained with an ultramicrotome (Leica EM UC6, Austria). Sections were mounted on copper grids (200 mesh) and stained with 1% uranyl acetate (Merck, Germany) followed by 1% lead citrate (Merck, Germany). We examined the ultrathin sections with a transmission electron microscope (JEM-1200EX II, Jeol, Japan) operated at 80 kV. The images were recorded with a camera SIS MegaView III CCD (Germany).

3.1.3.3 Morphological Data Acquisition

To avoid the possible bias introduced by the observer, no attempt of selection was made when the perikarya were photographed, except that only perikaryal profiles showing the cell nucleus were included in the study. Therefore, all the neuronal perikarya profiles that were encountered in a given section were photographed first at 6,000 X magnification and then at 20,000 X. Multiple adjacent and partially overlapping microscopic fields were photographed to include the complete perikaryal profile. Three or more sections were studied for each rat until photographs from 7-11 complete neuronal perikaryal profiles were obtained. Images

were saved with the maximum resolution available (originally at 1.42 Mpx and 24 bits per pixel). Photographs were printed on paper and complete profiles of the perikarya were reconstructed using paper photomontages. A cartographer's curvimeter wheel was used to assess the perykarial perimeter and the length of the cell body plasma membrane covered by: 1) ensheating glial processes; 2) axon terminals, assumed as axosomatic contacts when the axonal bouton and the perikaryal membrane were in direct contact and have synaptic vesicles present in the presynaptic element (Párducz et al., 1993), as well as small boutons containing synaptic vesicles that appeared as non-terminals thin axons apposed on the perikarium; or 3) dendritic processes. The ultrastructural aspect of these cellular elements was in accordance with the characteristic morphological features described elsewhere (Peters et al., 1991). Those few cellular components that were not clearly identifiable were also counted and classified as ''unknown.'' The percentual values for the average length of perikaryal membrane covered by each element was calculated and divided by the number of neurons studied per rat in each group and, from these data, the glial:axonal ratio for the somatic coverage in males and females was estimated.

We also evaluated the occurrence, number, and shape of somatic spines randomly found during data collection. The identification of these spines was based on their ultrastructural membrane and cytoplasmic features. We recorded the length of the spine (L), the diameter of the neck (DN), the number of protrusions from a single stalk, and the diameter of the head (DH). Somatic spines were classified according to their apparent shape as: stubby/wide, thin, mushroom-like, and ramified. That is, morphological criteria for assigning spines to shape classes were: stubby/wide spines had DH higher or equal to L, thin spines had L higher than DH and DH higher than DN, mushroom-like spines had a characteristic large head and DH higher than DN, and ramified spines had a branched neck with two or more heads (adapted from Harris et al. 1992; Arellano et al., 2007; Brusco et al., 2010; 2014). Some spines could not be reliably included in one of the above mentioned types, although they were also counted to provide mean values per rat and compared between groups. These latter spines included transitional shapes and "atypical" protrusions with unusual complex aspects of neck and head.

Axo-somatic and axo-spine synapses were identified by the apposition of presynaptic and postsynaptic membranes, synaptic cleft, aspect and thickness of presynaptic and postsynaptic densities, and clustering of synaptic vesicles near the presynaptic terminal. The general density of somatic spines was calculated dividing the number of all spines by the perykarial perimeter (in μm) measured as mentioned above. Average values were obtained per rat in each group. The number of somatic spines of each shape class was counted and the respective spine density was calculated using the mean perykarial perimeter in each group.

Images had final fine adjustments of sharpness, brightness, and contrast made in Photoshop CS3 (Adobe Systems, Inc., USA) without altering their content.

3.1.3.4 Statistical Analysis

The Kolmogorof-Smirnof test was used to determine data normality and the Bartlett's test to assess for the homogeneity of variances. The "n" for the statistical analysis was the number of animals studied in each group. Percentage data of the perikaryal membrane coverage and density of somatic spines were submitted to square root and root transformations, respectively. Results were compared between groups using the one-way analysis of variance (ANOVA) test followed by the Tukey test. The statistically significant level was set a priori at $p < 0.05$.

3.1.4 Results

The general ultrastructural appearance of the MePD neuronal perikarya is similar to that described previously for adult male rats (Hermel et al., 2006a). However, we found that the MePD cell bodies had glial processes in close apposition to the perikaryal membrane, some with evident continuous glial processes covering the perikarya while others were intermingled among axonal boutons (Figures 2A and B, 3). Layers of glial processes were also observed at a position adjacent to the perikaryal membrane (Figure 2C) or ensheathing axons and isolating them from the somatic membrane (Figure 2D from a late proestrus female). Axon terminals with synaptic vesicles were found in direct contact upon the neuronal cell body membrane, either isolated or as clusters of bulbous processes. It was unusual to observe a well-developed postsynaptic density in these axo-somatic (Figure 2B) or in some axo-spine contacts (Figure 4) and we could not, therefore, determine the

percentage of symmetric or asymmetric synapses. Dendrites were not usually found in direct contact with the perikarya (Figure 2A).

Percentual values for the perikaryal coverage are shown in Figure 3A. In males and cycling females, glial ensheating of the neuronal cell body membrane ranged from 55% to 62% whereas axonal contacts accounted for 34% to 40%. Perikaryal coverage by dendrites varied from 0.1% to 0.7% (Figure 3A). When data were compared between groups, no statistically significant difference was found for the glial [$F(4,23) = 0.45$, $p = 0.76$], the axonal [$F(4,23) = 0.54$, $p = 0.70$] or the dendritic coverage [$F(4,23) = 0.65$, $p = 0.62$] on the cell bodies of the MePD neurons. The glial:axonal ratio for the perikaryal membrane coverage ranged from 1.4 in the estrus to 2.1 in the late proestrus. These data would be suggestive of a proportionally higher glial coverage in the latter group, but values did not reach statistical significance [$F(4,23) = 0.66$, $p = 0.63$].

From all studied animals, one male and one estrus female did not show somatic spines in the sampled neurons. A total of 162 somatic spines were counted in males and cycling females. The density of spines along the perikaryal membrane length was different between groups [$F(4,21) = 3.34$, $p = 0.02$] and the Tukey's test showed that the late proestrus females had higher values than the estrus ones ($p < 0.05$; Figure 3B).

We observed somatic spines as a continuum of shapes and sizes in all studied groups. Females in the late proestrus phase showed highest densities of somatic spines of all different shapes (Figure 3B and C). Representative examples of somatic spines with the apparent diameter of the spine head and neck and the spine length are shown in Figure 4. Spines were classified as stubby/wide (Figure 4A and B), thin (Figure 4C), mushroom-like (Figure 4D), ramified (Figure 4E), with a transitional aspect between these shapes (Figure 4F) or with atypical aspects of elongated or undefined shapes (Figure 4G and H). These somatic spines were found either isolated or intermingled with other types along adjacent parts of the perikaryon (Figure 4G). A total of 101 somatic spines could be classified according to their morphological aspect. Spines in male neurons usually had a thin shape (40%) and in females in diestrus 53% were stubby/wide. The shape diversity increased in the night of proestrus with 33% of stubby/wide spines, 22% of thin spines, and 36% with a

ramified, transitional or atypical aspect. In estrus, somatic spines were 84% of the stubby/wide type. The densities of the differently shaped somatic spines in each group are shown in Figure 3C.

In some cases somatic spines had synaptic contacts at their base, head or neck and putative asymmetric (Figure 4A, D, I) or symmetric aspect (Figure 4E). We also found spinules emerging from the head of somatic spines (Figure 4 I).

3.1.5 Discussion

Our findings indicate that the adult rat MePD has most of the perikaryal membrane covered by glia and a relatively high amount (up to 40%) of the neuronal somatic membrane in contact with axonal processes. No apparent effect of sex steroids was found in the percentage of perikaryal coverage by neuro-glial processes, which contrasts with data from an anatomically and functionally interconnected area, the ARC (Olmos et al., 1989; Párducz et al., 1993; Garcia-Segura et al., 1994). Nevertheless, some observations suggest the existence of a phasic synaptic remodeling during the ovarian cycle. These include the already described sex difference and plastic change in the volume of the neuronal cell body in the MePD of cycling females (Hermel et al., 2006b), and the change in the density and shape of somatic spines in proestrus compared to males and females along the other estrous phases. Our data have also to be adjoined with the plastic, reversible synaptic changes that occur for axonal contacts made directly on dendritic shafts and on dendritic spines in the MePD of cycling females. Altogether, these modifications may be important for the processing and integration of sensorial inputs, for the modulatory effects of gonadal hormones, and for the display of reproductive behavior within a physiologically relevant time frame that are organized differently in male and female rats.

At first glance, our present study did not identify a sexual dimorphism or cycling changes in the perikaryal coverage of MePD neurons. However, another interpretation is plausible. That is, proestrus females did have a local reduction in somatic volume and a higher GFAP-ir although showing no changes in the cell body surface covered by glial or axonal processes. This suggests that glial processes can dynamically modify their extension, possibly restructuring the astrocytic cytoskeleton

and folding properties to accompany the changeable available surface of perikaryal membrane. In proestrus, a decreased surface would obligate glial processes to partially retract and condense, possibly contributing to increase the GFAP-ir in the MePD neuropil. In addition, the MePD neuronal activity in the late proestrus phase would involve the intrinsic biophysical properties of the perikaryon for computing synaptic inputs and generate somatic action potentials associated with the modulatory function of new somatic spines with increased density and different shapes. In estrus, when ovarian hormones drop in circulation, this same condition no longer occurs.

Considering the number of synapses along the available membrane, it could be assumed that the perikaryon is not the principal site of synaptic input to nerve cell. However, axo-somatic synapses generate important consequences for the neuronal output (Spruston et al., 2013). Furthermore, the emergence of perisomatic axo-spine synapses provide an additional amount of perikaryal surface to establish dynamically regulated contacts, in a place closer to the axon hillock, that can strategically influence the neuronal output (Peters et al., 1991). This was evident in late proestrus females with a higher density of spines along the perikaryal perimeter, which could represent the type of labile spines with plastic capacities for local information processing and behavior modulation along the estrous cycle. The existence of spinules is also suggestive of plastic properties for the somatic spines in the MePD of proestrus females. Spinules were associated with an enhanced cellular activity since they are rapidly formed after synaptic stimulation (Applegate and Landfield, 1988; Schuster et al., 1990; Tao-Cheng et al., 2009; Brusco et al., 2014), serve for transferring large molecules between pre and postsynaptic elements (Tarrant and Routtenberg, 1977), and for intercellular signaling between active spines and the presynaptic element (Spacek and Harris, 2004).

Moreover, somatic spines in the MePD showed a variety of shapes, which is at the current scholarly debate about the functional impact of synapses made on spines with different geometries (Kasai et al., 2010; Segal, 2010; Chen et al., 2011; Chen and Sabatini, 2012; Yuste, 2013). Considering the morphological features of stubby/wide spines, thin spines with long necks, and large head, mushroom-like spines, it is likely that each spine class can differ in the available area for the

postsynaptic density and receptor trafficking, the local electrical resistance, the degree of biochemical compartmentalization and ultimately, synaptic strength, integration, and enduring effects (Arellano et al., 2007; Yuste, 2013; Tonnesen et al., 2014). Synaptic processing might also occur in microdomains in ramified spines or in more complex and atypical forms (Verzi and Noris, 2009). In the MePD, somatic spines appeared as postsynaptic elements, but additional efforts are required to obtain 3D-reconstructions and electrophysiological recordings to determine the functional properties of these spines in males and cycling females.

In addition, it is noteworthy that sex steroids can remarkably alter the function of the MePD cells by concomitantly affect the number of dendritic spines (reviewed in Rasia-Filho et al., 2012a) and the synaptic inputs in both dendritic shafts and spines of cycling female rats (Brusco et al., 2014). That provides the female MePD with a changeable somatic and dendritic processing of the incoming synaptic activity and a local switch in the neurotransmission depending on the levels of the ovarian hormones (Micevych et al., 1988; Oro et al., 1988; Polston and Erskine, 2001; Lehmann and Erskine, 2005; Lehmann et al., 2005; de Castilhos et al., 2008). We hypothesize that the morphological and synaptic rearrangement in the left MePD can change the inhibitory impact of output projections on hypothalamic circuits during the proestrus phase to disinhibit both the activity in the VMH, involved in lordosis behavior, and in the MPOA, for the display of proreceptive behavior (Pfaus and Heeb, 1997; see additional data and comments in Rasia-Filho et al., 2012a; Brusco et al., 2014). One possible reason for this hemispheric lateralization is that the left medial amygdala would be specialized for chemosensory and/or steroid negative feedback regulation of the secretion of hypothalamic luteinizing hormone (discussed in Cooke and Woolley, 2005). These changes in the MePD occur during the proestrus phase and, therefore, some hours prior to those reported in the ARC of estrus females relevant for the GnRH surge and ovulation. This is in quite contrast with the dendritic spine and ultrastructural data from males (see Arpini et al., 2010; Brusco et al., 2014) that indicate redundancy, but not amplification, of the androgenic effects in the medial amygdala of both brain hemispheres (Coolen and Wood, 1999).

3.1.6 Concluding remarks

Present ultrastructural findings indicate that the MePD of rats have neurons whose perikaryal membrane can have plastic properties to modulate synaptic inputs along the estrous cycle. This composes an intriguing new area of research aiming to establish the relationship between spine structure and the synaptic information processing. The somatic spines in MePD neurons can compute different phasic afferents that potentially change the activity of a sex-steroid sensitive circuit involved with the neuroendocrine secretion and the display of reproductive behavior, with contrasting features in males and in normally cycling females.

3.1.7 Conflict of Interest

The authors declare no conflict of interest regarding the present project and results.

3.1.8 Role of authors

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: MZ, TMS, LMGS, AARF.

Acquisition of data: MZ, TMS, MIM, LMGS, AARF.

Analysis and interpretation of data: MZ, ADO, MIM, LMGS, AARF.

Drafting of the manuscript: MZ, ADO, TMS, MIM, LMGS, AARF.

Critical revision of the manuscript for important intellectual content: MZ, ADO, TMS, MIM, LMGS, AARF.

Statistical analysis: MZ, LMGS, AARF. Administrative, technical, and material support: TMS, MIM, LMGS

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3.1.10 Legends

Figure 1 – (A) Schematic diagram of the ventral aspect of a coronal brain section showing the posterodorsal medial amygdala (MePD; in this case at – 3.0 mm posterior to the bregma) close to the optic tract (opt) and the stria terminalis (st) in the rat brain. Spatial coordinates correspond to: D, dorsal; L, lateral; M, medial; V, ventral. Scale bar = 0.5 mm. Adapted from Paxinos and Watson (2008). **(B, C)** Microscopic images of hematoxylin and eosin stained matched coronal brain sections exemplifying, at higher magnification, the location of the MePD from where tissue samples (limited by doted lines) were obtained for ultrastrutural studies in males and females, respectively. ml, molecular layer. Scale bar = 0.1 mm.

Figure 2 – Digitized photomicrographs of the ultrastructure obtained from female posterodorsal medial amygdala in late proestrus females showing **(A)** glial processes (highlightened in blue) engulfing a neuronal perikaryal membrane and **(B)** terminal axonal boutons and various small boutons containing synaptic vesicles (green) in direct contact with the somatic membrane. **(C)** Perikaryal membrane in direct contact to various glial processes (arrows), and **(D)** an axonal bouton ensheathed by various glial concentric layers (arrow) isolating it from the perikaryal membrane. den: dendrite. Fine adjustments were made in Photoshop CS3 (Adobe Systems, Inc., USA). Scale bar = 1 μm.

Figure 3 – Untransformed values (mean and standard deviation) of **(A)** the percentual coverage of neuronal cell body membrane accomplished by glial

processes, axons, dendrites or unclassified (“unknown”) elements, **(B)** density of somatic spines along perykarial perimeter (length in µm), and **(C)** histogram of the density of differently shaped somatic spines in the neurons of the rat posterodorsal medial amygdala (MePD) of males and cycling females in diestrus, early (morning) and late (night) proestrus or estrus. In **A**, no statistically significant difference was found between groups. In **B** *$p < 0.05$ compared to females in estrus (one way ANOVA test and Tukey post hoc test).

Figure 4 – Digitized photomicrographs of the ultrastructure of the posterodorsal medial amygdala showing representative somatic spines (highlighted in yellow) observed in female rats in the late proestrus. Somatic spines were assigned to different classes according to their shapes: stubby **(A)**, wide **(B)**, thin **(C)**, mushroom-like **(D)**, ramified **(E)**, with a transitional aspect **(F)** or with an “atypical” and more complex appearance (**G** and **H**). The presence of spinule (I) was also observed (arrow). See text for details. Fine adjustments were made in Photoshop CS3 (Adobe Systems, Inc., USA). Scale bar = 0.5 µm.

Figure 1

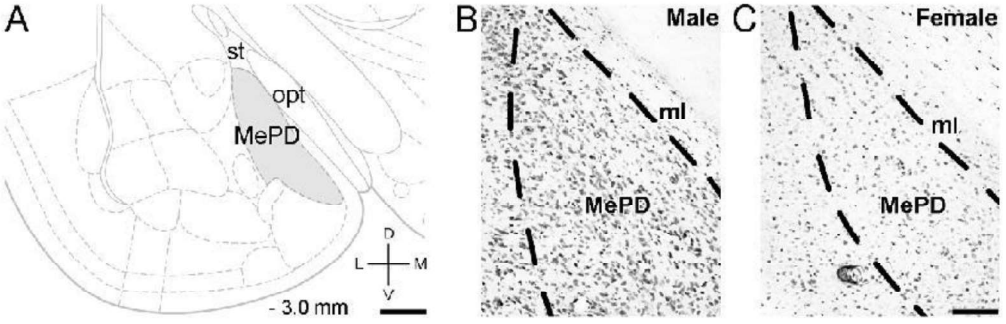


Figure 2

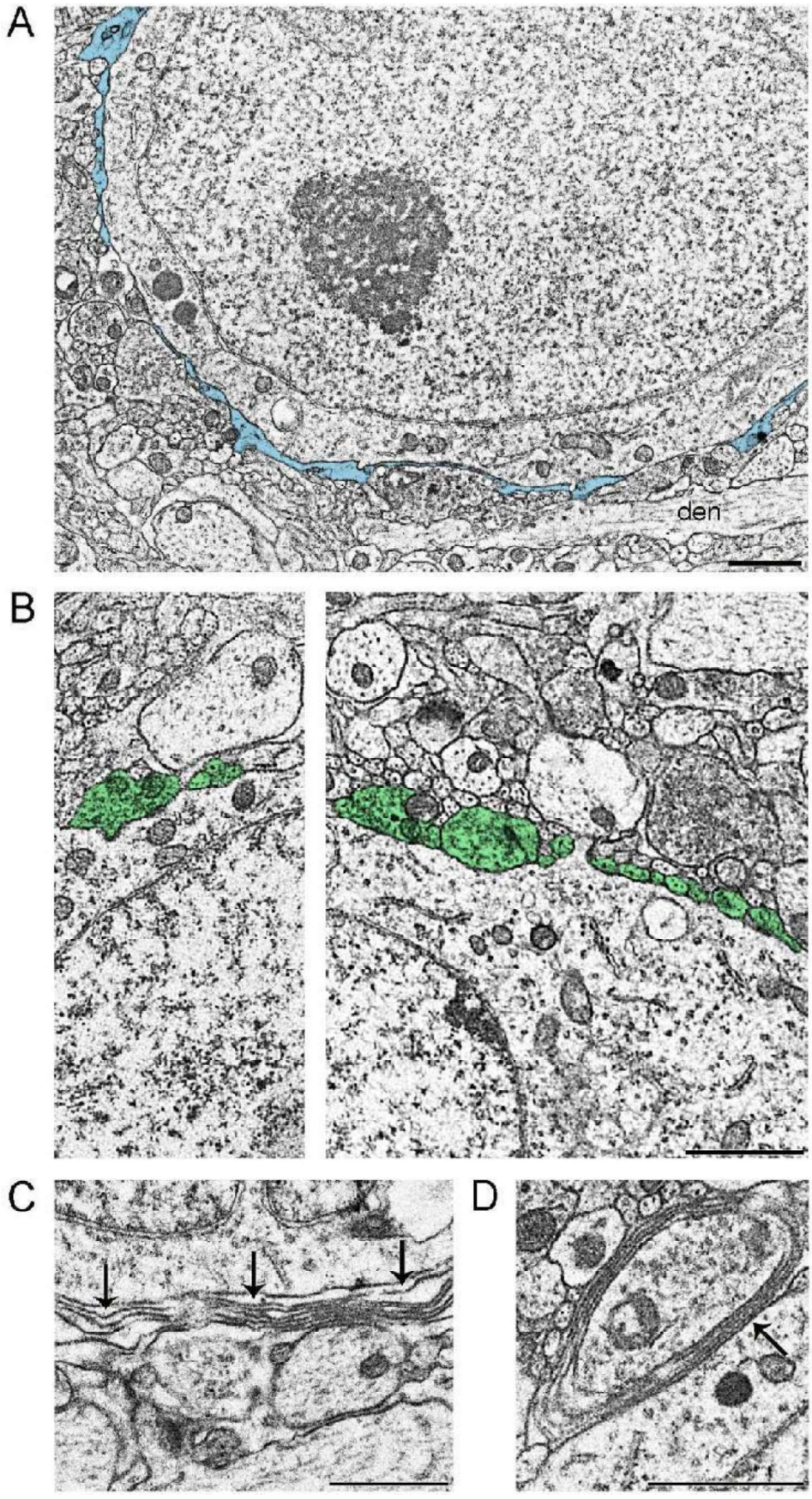


Figure 3

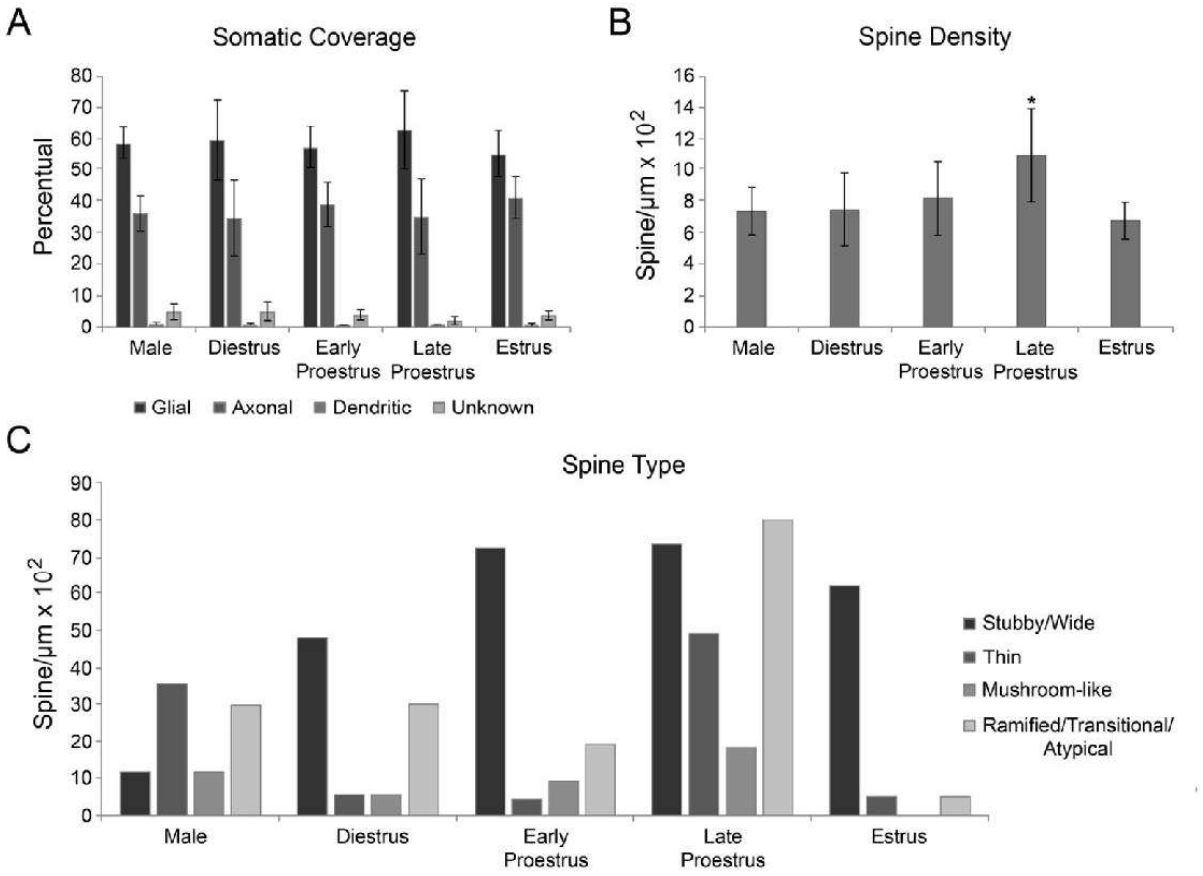
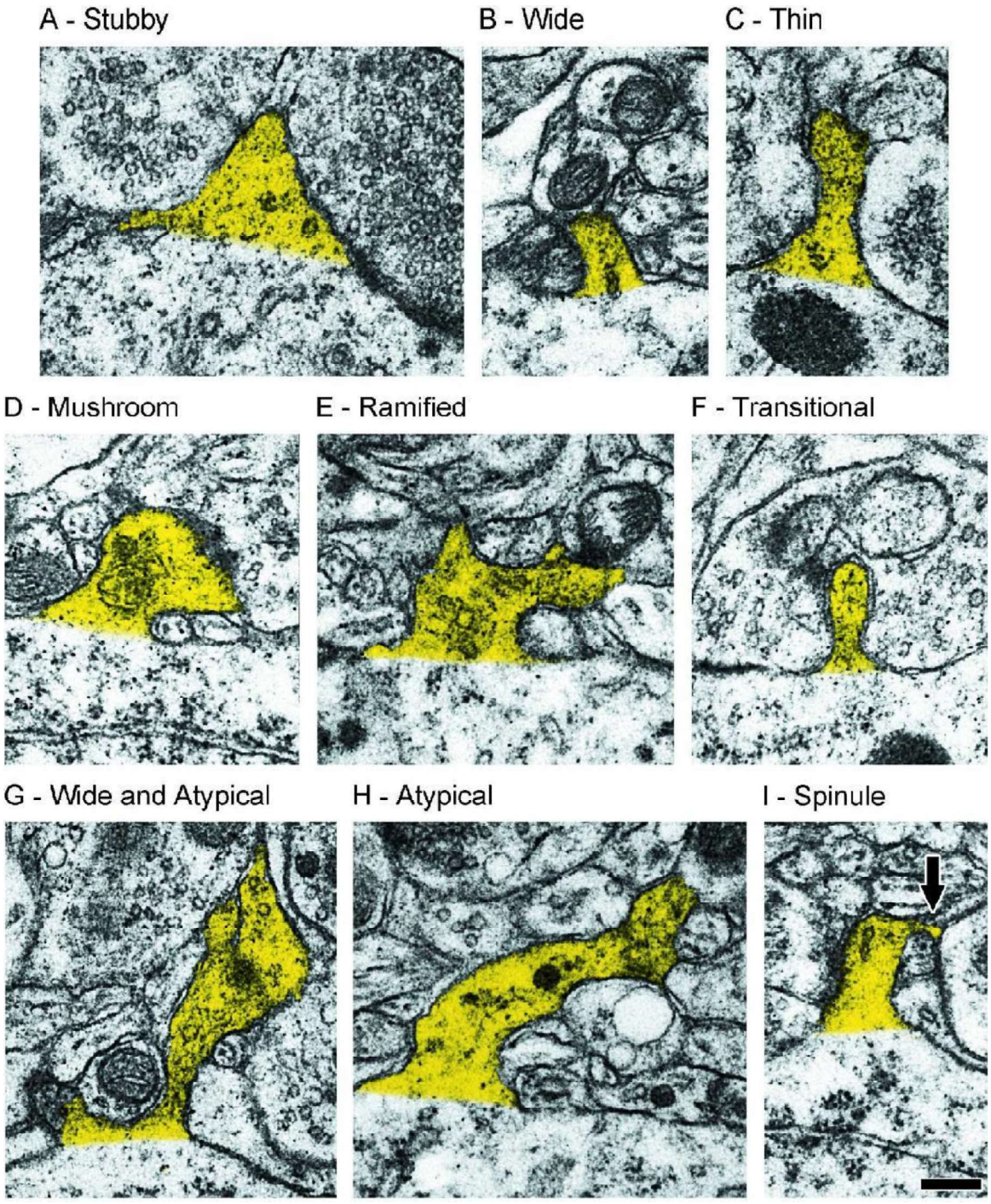

A
Somatic Coverage
Percentual
80
70
60
50
40
30
20
10
0
Male
Diestrus
Early Proestrus
Late Proestrus
Estrus
Glial
Axonal
Dendritic
Unknown
B
Spine Density
Spine/µm x 10-2
16
14
12
10
8
6
4
2
0
*
Male
Diestrus
Early Proestrus
Late Proestrus
Estrus
C
Spine Type
Spine/µm x 10-2
90
80
70
60
50
40
30
20
10
0
Male
Diestrus
Early Proestrus
Late Proestrus
Estrus
Stubby/Wide
Thin
Mushroom-like
Ramified/Transitional/ Atypical

Figure 4


A - Stubby
B - Wide
C - Thin
D - Mushroom
E - Ramified
F - Transitional
G - Wide and Atypical
H - Atypical
I - Spinule

3.2 Artigo II

Original Research – A ser encaminhado à *The Anatomical Record*

Castration modifies the density and shape of dendritic spines in the posterodorsal medial amygdala of male rats

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3.2.1 Abstract

The posterodorsal medial amygdala (MePD) is a sex-steroid responsive area involved in sensorial stimuli processing in the social behavior neural network of male rats. At the cellular level, spines serve as specialized postsynaptic elements relevant for synaptic plasticity. Here, we add new data to the effects of long-term gonadectomy (GDX) on the density and shape of dendritic spines in the right and left MePD of adult male rats using DiI dye, tridimensional image reconstruction, and confocal microscopy. Adult male rats were studied as nonmanipulated ("Intact", n = 6), submitted to a sham GDX ("Sham", n = 4) or castrated and studied 90 days after GDX (n = 5). In intact males, there were 1.1 spines/dendritic μm, and thin spines were the most abundant type found (~48% of the total), followed by the mushroom-like (~25%), and stubby/wide ones (~20%) in proximal branches. Results showed that GDX males had a decrease in the density of spines when compared to intact males ($p < 0.05$). Furthermore, males submitted to long-term GDX showed marked reductions in the density of thin, mushroom-like, and ramified spines at the same time that there was a marked increase in the density of the stubby/wide spines in the right MePD when compared to control groups ($p < 0.05$ in all cases). Similar results were obtained in the left MePD, with the exception of no significant effect for ramified spines. These data indicate that GDX induces a reduced synaptic input on spines by reducing their number and the remodeling of differently shaped spines with no hemispheric lateralization differences in the MePD of adult male rats. It is likely that the MePD remodeling after GDX can have implications in the impairment of sexual behavior display that is also observed in long-term castrated rats.

Keywords: Extended amygdala, androgen withdrawal, spine shape, synaptic plasticity.

3.2.2 Introduction

Several lines of connectivity, neurochemical, and functional evidence (Veinante and Freund-Mercier, 1997; Newman, 1999; Rochefort and Konnerth, 2012; Rasia- Filho et al., 2004; Brusco et al., 2014; Kasay et al., 2010) indicate the posterodorsal subnucleus of the medial amygdala (MePD) as an important area in the subcortical network that modulates social behaviors in rats (Newman, 1999; Rasia-Filho et al., 2012b). The MePD serves as an interface to integrate chemosensory cues and the effects of gonadal hormones (Blake and Meredith, 2011) for the interpretation of the social relevance of olfactory and vomeronasal stimuli (Meredith and Westberry, 2004; Baum and Bakker, 2013; Petrulis, 2013), the genitosensorial stimulation associated with the occurrence of mating behavior (Coolen et al., 1997; Dass and Vyas, 2014), and the occurrence of ejaculations (Phillips-Farfán and Fernández-Guasti, 2007; Veening and Coolen, 2014). The rat MePD assembles coexisting subpopulations of multipolar neurons with a general bitufted or stellate appearance (reviewed in Rasia-Filho et al., 2012). Some neurons co-express the inhibitory markers calbindin, neuronal nitric oxide synthase (a marker also for projection neurons), and somatostatin (Carney et al., 2010) that underlie the connectivity and functions of MePD interneurons and projection neurons (Bian et al., 2008; Niimi et al., 2012). Near 80% of all the mice MePD neurons are transcription factor Lhx6-immunoreactive cells (Choi et al., 2005) and are connected to specific hypothalamic nuclei to modulate reproduction (Petrovich et al., 2001; Choi et al., 2005; Cavalcante et al., 2006). That is, by projecting to the anteroventral periventricular nucleus, the medial preoptic area, and the ventral premamillary nucleus, the MePD neurons links the perception of sexually relevant stimuli with the display of sexual behavior (Dominguez and Hull, 2001; Cavalcante et al., 2014; Will et al., 2014). Lesions of the MePD impair penile erection (Kondo and Sachs, 2002) and, involving other adjacent areas, also the occurrence of ejaculation in rats (reviewed in Meisel and Sachs, 1994; Petrulis, 2013; Will et al., 2014).

The MePD expresses one of the highest concentration of androgen receptors and is a sexually dimorphic area in rats (Blake and Meredith, 2011; Simerly et al., 1990; Sheridan et al, 1979; Gréco et al., 1998). Males and females differ in the MePD volume, neuron and glial cells number, spatial domain of the dendritic

branches, synaptic connectivity with functional hemispheric lateralization, neurotransmitter binding sites, and expression of neuropeptides (reviewed in Rasia-Filho et al., 2012; Brusco et al., 2014; and references therein). Sex steroids alter the function of the MePD cells as implanted estradiol restores mounting behavior following gonadectomy (GDX) in male rats (Rasia-Filho et al., 1991) or by testosterone or estradiol in male hamsters (Wood, 1996). Furthermore, a higher number of double-labeled Fos and androgen receptors cells were found in the MePD of male hamsters after exposure to reproductively-related odour from sexually receptive females (Blake and Meredith, 2011). After mating stimuli in rats, the MePD c-fos expression increases in neurons that co-express androgen receptors (Gréco et al., 1996; 1998), with clusters of neurons activated concomitantly to intromission or ejaculation (Coolen et al., 1997). An increased density of estrogen receptors α in the male rat MePD is also associated with ejaculation and sexual satiety (Phillips-Farfán et al., 2007).

The sex steroid-mediated actions in the MePD can involve direct actions on the morphology and function of local cells as well as in interconnected brain areas that induce synaptic changes relevant for the display of male behavior. In this regard, androgen influences on dendritic spines of MePD neurons have been currently studied (Cunningham et al., 2007; Cooke and Woolley, 2009; Rasia-Filho et al., 2012) since they have connectional and functional implications for the occurrence of complex behaviors (Rasia- Filho et al., 2004; Gréco et al., 2003; Simerly 2004). Spines are postsynaptic elements with a range of critical properties for synaptic establishment, strength, and plasticity (Nimchinsky et al., 2002; Segal, 2005; DonCarlos et al., 2006; Nuriya et al., 2006; Vasudevan and Pfaff, 2007). The processing demand placed on dendrites influences the number of spines (Jacobs et al., 1997; Segal, 2010). In this regard, each dendritic spine in the male rat MePD is usually innervated by one axon terminal with ultrastructural excitatory features (Hermel et al., 2006a; see complementary data in Brusco et al., 2014). Pleomorphic spines are found in the MePD of male rats with thin spines as the most abundant type (~53%), followed by mushroom-like (~21% each, a value almost twofold more than females), and stubby/wide ones (Rasia-Filho et al., 2012). Males also have a higher proximal dendritic spine density than females (Rasia-Filho et al., 2004)

suggesting a different synaptic organization and sexual dimorphism in the circuits and inputs that reach the MePD of rats (Dall´Oglio et al., 2008a; Rasia-Filho et al., 2012a,b; Brusco et al., 2014). Moreover, long-term GDX causes a significant decrease in the density of Golgi-impregnated MePD spines at the same time that occurs strikingly reductions in the display of intromission and ejaculatory behavior (Rasia-Filho et al., 1991; de Castilhos et al., 2006; Rasia- Filho and Lucion, 1996), in the MePD volume, and in local substance P immunoreactivity (Malsbury and McKay, 1994). These findings reinforce the proposition that androgens influence the synaptic organization and neuronal activity in the MePD of adult male rats.

Here, we describe the effects of long-term GDX on the density and shape of dendritic spines in the MePD of adult male rats using tridimensional (3D) image reconstruction and confocal microscopy. We also looked for differences in the left and right MePD. Our findings confirm the reduction of spine number after testes removal (de Castilhos et al., 2008) and add new data regarding the change in the geometry of local spines, with variable results for differently shaped spines.

3.2.3 Material And Methods

Methodological approach was based on de Castilhos et al. (2008) and Brusco et al. (2010). Age-matched, adult male male Wistar rats (N= 15, 3 months old at the beginning of the experiment) were housed in groups in standard laboratory conditions, with food and water *ad libitum,* room temperature kept at 22 ◦C, and a 12 h light-dark cycle.

All efforts were made to minimize the number of animals studied and their suffering. Rats were manipulated according to the international laws for the ethical care and use of laboratory animals (European Communities Council Directive of 11.24.1986, 86/609/EEC) and the Animal Ethics Committee of the UFCSPA (Brazil; approved protocol no. 227/13).

Animals were divided randomly into 3 experimental groups: (1) nonmanipulated intact rats (“Intact”, n = 6), (2) rats submitted to a sham GDX and studied 90 days following this procedure (“Sham”, n = 4), and (3) castrated rats studied 90 days after GDX (n = 5). For these two latter groups, males were anesthetized with ketamine and xilazine (80 and 10 mg/kg, i.p. injected, respectively)

and submitted to GDX. Males were castrated via an incision in the scrotum to remove both testes. Sham GDX was performed in males under the same anesthetical and surgical procedure, except that the gonads were not removed. Animals showed an adequate recovery from the surgical procedure and no apparent sign of illness.

Following 3 months, all animals were anesthetized as above mentioned and submitted to transcardiac perfusion with a single injection of heparin (1000 IU) and immediately afterwards with 400 ml of 1.5% paraformaldehyde in phosphate buffer solution 0.1M and pH 7.4 (PBS) at room temperature (RT), and using a peristaltic pump (Control Company, Brazil). Flow was initially rapid (50-100 ml during the first 90 s after chest opening) and, then, slowed to last for additional 20 min (15 ml/min flow) in order to maintain fine ultrastructural integrity (Tao-Cheng et al., 2007). The brains were removed and maintained in the same fixative solution for 1 h and coronally sectioned (200 µm-thick) using a Vibratome (VT 1000S, Leica Microsystems, Germany). The MePD was studied from 3.0 to 3.4 mm posterior to the bregma, lateral to the optic tract and ventral to the stria terminalis (ST; Paxinos and Watson, 2008; Figure 1). The MePD of both brain hemispheres were studied apart to allow comparisons about a possible effect of laterality.

Afterwards, sonicated, fine powdered DiI (cat. no. D-282, Molecular Probes, USA; cf. Rasia-Filho et al., 2010) was applied on the surface of the ST, brain slices were covered with PBS, and DiI was allowed to diffuse to the MePD for 20 h at RT. Additional fixation was done with 4% paraformaldehyde in PBS for 30 min, slices were washed with PBS, mounted with an anti-fading media (Fluoromount EMS, USA) under coverslips, and images of the MePD neurons were obtained along 1 week. Bitufted and stellate multipolar neurons were observed in the MePD, but randomly sampled bitufted cells accounted for more than 94% of all studied neurons in the 3 experimental groups.

Proximal dendritic spines were imaged using a confocal microscope (Zeiss LSM 700, Germany), with a plan-apochromat 63×/1.25 oil-immersion lens, spectral detectors adjusted to capture emission to laser 555 nm of wavelength, z-stack acquisition at 0.1 µm step size, 1024×1024 pixel resolution per frame, and avoiding over and undersaturated pixels. One dendritic branch/neuron was imaged on 96 different neurons in intact rats (48 in both the right and left MePD), 48 neurons in the

Sham group (23 in the right and 25 in the left MePD, and 76 neurons in the GDX group (37 in the right and 39 in the left MePD). A maximum of 8 neurons was studied per hemisphere and per rat. Spines were imaged in proximal dendritic shafts of primary to secondary order and along 30-40 μm (based on Rasia-Filho et al., 2004; de Castilhos et al., 2006; 2008). The 3D reconstruction of each dendritic segment and spines was made with the software Carl Zeiss Zen 2012 SP 1 (Germany).

The morphological criteria for assigning dendritic spine shapes to different types were based on Harris et al. (1992) and Arellano et al. (2007), considering spine length, the diameters of the head and neck, and the number of protusions from a single stalk. The diameters of the spine neck and head were measured on the stack that had the maximal central diameter in the plane of section (Harris et al., 1992). Spines were classified as thin, mushroom-like, stubby/wide, ramified or atypical (when with a transitional aspect between classes or an unusual shape). In principle, a salient round broadening on a dendritic shaft, resembling a spine head without a visible neck, was considered as a stubby spine. Examples are shown in Figure 2 and additional description of each shape can be found elsewhere (Peters and Kaiserman-Abramof, 1970; Fiala and Harris, 1999; Brusco et al., 2010; 2014). Each spine was visualized in the z planes to avoid misinterpretation for the shape classification and/or spine quantifications. The same classification criteria were used for all experimental groups. In few cases, some spines could not be reliably included in one of the above mentioned types and were named “nonclassified”. Images had final fine adjustments of sharpness, brightness, and contrast made in Photoshop CS3 (Adobe Systems, Inc., USA) without altering spine counting or classification.

From the original 3D images, spine density was calculated as the number of spines per micrometer of dendritic segment, as previously employed (Canteras et al., 1995; Carrillo et al., 2007; Rasia- Filho et al., 2004). This was done to obtain data of overall spine density (i.e., total counts including all spine types) in the right and left MePD of each experimental group and to to compare data with a previous report from de Castilhos et al. (2008). We also calculated the density of each type of spine per hemisphere and per experimental group. Mean values per rat were used for statistical comparisons.

Data (presented as mean ± standard deviation) were tested for the normality distribution and homocedasticity using the Kolmogorov and Smirnov test and the Bartlett test, respectively. The overall density of dendritic spines were submitted to a two-way analysis of variance (ANOVA) test for repeated measures (comparing experimental groups, brain hemispheres, and interaction of these factors) followed by the Bonferroni test. The density of each class of dendritic spine per brain hemisphere and per experimental group was submitted to an one-way ANOVA and the Tukey multiple comparisons *post hoc* test. Data were not parametric only for the density of ramified and atypical spines and, then, values were compared using the Kruskal-Wallis test and the Dunn multiple comparison test. Statistical level of significance was set as $p \leq 0.05$.

3.2.4 Results

DiI dye allowed the visualization of cell bodies, dendritic shafts, pleomorphic spines and thin varicose axons in the MePD of both hemispheres and in the 3 studied groups. Confocal microscopy images could be reconstructed and dendritic spines were found either isolated or in clusters along the dendritic shafts. These spines showed a *continuum* of different shapes and sizes (Figure 2A-C).

As an overall control result, there were approximately 1.1 spines/dendritic µm in the MePD of intact males (Figure 3A), and thin spines were the most abundant type found (~48% of the total), followed by the mushroom-like (~25%), and stubby/wide ones (~20%). Spine shapes including ramified and, to a lesser extent, other atypical or nonclassified shapes accounted for the remaining values (Figure 3 B and C).

The overall density of dendritic spines in the MePD showed a statistically significant difference when data were compared between the experimental groups [$F(2,12) = 4.307$, $p = 0.03$], but there were no difference due to hemispherical laterality [$F(1,12) = 2.778$, $p = 0.12$] nor in the interaction of these two factors [$F(2,12) = 0.05$, $p = 0.94$]. The Bonferroni *post hoc* test showed that GDX males had a decrease in the density of spines when compared to intact males ($p < 0.05$; Figure 3A).

Furthermore, the density of the differently shaped spines in both the right and left MePD were modified after GDX. In the right MePD (Figure 3B), compared to the control intact and sham GDX data, males submitted to long-term GDX showed marked significant reductions in the density of the thin [~40% less; $F(2,12) = 84.612$, $p < 0.01$; Tukey post hoc test, $p < 0.01$ compared to both control groups], the mushroom-like [40% to 25% less; $F(2,12) = 23.337$, $p < 0.01$; Tukey post hoc test, $p < 0.01$ and $p< 0.05$ compared to intact and sham GDX, respectively], and the ramified spines [~40% less; $F(2,12) = 4.002$ $p = 0.04$; Tukey post hoc test, $p < 0.05$ compared to sham GDX] whereas there was a marked increase in the density of the stubby/wide ones in the GDX group [~125% more; $F(2,12) = 113.96$, $p < 0.01$; Tukey post hoc test, $p < 0.01$ compared to both control groups]. The density of atypical spines was not different between groups ($p = 0.19$). Intact and sham GDX showed similar results ($p > 0.05$ in all cases), with the exception of the density of mushroom-like spines (higher in intact males, $p < 0.05$).

In the left MePD (Figure 3C), compared to the control intact and sham GDX data, males submitted to long-term GDX showed marked significant reductions in the density of the thin [~40% less; $F(2,12) = 46.98$, $p < 0.01$; Tukey post hoc test, $p < 0.01$ compared to both control groups], the mushroom-like [40% to 25% less; $F(2,12) = 14.096$, $p < 0.01$; Tukey post hoc test, $p < 0.01$ and $p< 0.05$ compared to intact and sham GDX, respectively], and the atypical spines [Kruskal-Wallis test, $p = 0.01$; Dunn post hoc test, $p < 0.05$ compared to sham GDX] whereas there was a marked increase in the density of the stubby/wide ones in the GDX group [~135% more; $F(2,12) = 290.59$, $p < 0.01$; Tukey post hoc test, $p < 0.01$ compared to both control groups]. The density of ramified spines was not different between groups ($p = 0.09$). Intact and sham GDX showed similar results ($p > 0.05$ in all cases), with the exception of the density of atypical spines (lower in intact males, $p < 0.05$).

3.2.5 Discussion

Our data reinforces previous reports by demonstrating that: 1) the values obtained for the density of proximal dendritic spines and the percentage of differently shaped spines in the adult male rat are similar to that described by Brusco et al. (2010), 2) the density of Golgi-impregnated proximal dendritic spines do not show lateralization differences in the MePD of adult male rats (Arpini et al., 2010), which

add to the idea that androgenic effects are redundant, but not additive, in the MePD of both hemispheres (Coolen and Wood, 1999). And, 3) long-term GDX induces a significantly reduction in the density of Golgi-impregnated dendritic spines in the MePD of adult male rats (de Castilhos et al., 2008). Furthermore, we found that the shape of dendritic spines in this sex steroid-responsive brain area is affected by GDX. In the MePD, castrated males had ~20 % less proximal dendritic spines than intact animals and in, both right and left hemispheres, there were decreases in thin and mushroom-like shaped spines whereas stubby/wide spines increased locally. Ramified spines had a reduced density in the right MePD. Present results exemplify the neural morphological reorganization in the nervous tissue that accompany the long-term reduction of testosterone and metabolites in circulation and for which other sources of sex steroids, such as the adrenal ones, cannot compensate for the androgen drop in GDX males (Thompson et al., 1976).

Currently it is not possible to affirm that spine number reduction and spine shape change in the MePD of castrated males promote the marked sexual behavior in these rats. However, there are relevant data that directly links the MePD with male sexual arousal and performance, including Fos expression (Coolen et al., 1997) and electrophysiological recordings (Smock et al., 1992; Stark et al., 1998). Specific lesions of the MePD decrease the preference for opposite-sex odors, but copulatory activities can still occur (Veening and Coolen, 2014). Unilateral MePD microinjection of a 5-HT1A receptor agonist decreased the latencies to intromission and ejaculation and reduced the duration of the postejaculatory refractory period, the same effect obtained after systemic injections in rats (de Castilhos et al., 2006). In addition, in the rat MePD, unilateral microinjections of glutamate and histamine reduced the latency to ejaculation whereas GABA decreased the latency for the first intromission, probably due to a local disinhibitory effect (Rasia-Filho et al., 2012b). In the MePD of gerbils, glutamate accounts for ~70% of all ejaculation-activated cells and GABAergic neurons for ~50% of the activated cells following mating (Simmons and Yahr, 2003). Then, the synaptic input and plasticity of MePD neurons can be related to sexual behavior in male rats, with a complex processing of social cues and memories of contextual processing of sexual encounters and the sexual experience of the animal (Kondo, 1992; Stark et al, 1998; Stark, 2005). This allows the MePD to be part of a

dynamic, sex-steroid sensitive and neurochemically-modulated neural network with local cells integrating different demands from specific sensorial pathways that controls the social demands to the animal at each moment (Newman, 1999; Rasia-Filho et al., 2012b; Baum and Bakker, 2013; Petrulis, 2013).

Different microscopic approaches provided insights for the dendritic spines on the synaptic organization and plasticity of the adult rat MePD (Rasia-Filho et al., 2004; Brusco et al., 2010; 2014). Previously, it was demonstrated that most MePD spines were associated with immunolabeling for the pre-synaptic protein synaptophysin either with a single punctum close to the spine head and neck or with multiple puncta suggestive of multisynaptic sites (Brusco et al., 2010). Therefore, sex steroid-induced modifications in the density and shape of dendritic spines alter the number of available postsynaptic sites and, consequently, synaptic coding, and excitability of MePD neurons. Proximal spines are strategically located to modulate synaptic inputs to affect cell body voltage and neuronal action potentials discharge (Rasia-Filho et al., 2004; de Castilhos et al., 2006; Marcuzzo et al., 2007; de Castilho et al., 2008). Moreover, it is highly likely that spiny neurons have functions set at each single spine level and dependent on each brain region (Scalia and Winans, 1975; Nishizuka and Arai, 1983; Dielenberg and McGregor, 2001). Here, we found that more than a general effect of reduced androgenic support on local dendrites, which would equally decrease the number of all classes of spines, GDX promoted a spine-specific change in the male MePD. The remaining density of spines in GDX males probably reflects the local subpopulation of spines that are more stable and/or not sensitive, directly or indirectly via synaptic inputs of interconnected circuits, to the levels of plasma androgens. It was suggested that stable spines provide steady properties in sexually dimorphic neural circuits needed for sex-specific behavioral displays, whereas labile spines have intrinsic plastic properties to adapt their number and function for the ongoing synaptic processing and behavior modulation (Brusco et al., 2010; Rasia-Filho et al., 2012b). This could reflect the need for a balance between stability and plasticity in neural circuits (Spruston et al., 2013), with a high importance in nodal points related with social adaptation and species reproduction, such as the MePD.

Our MePD data also showed that structure of dendritic spines can be varied even on a single neuron (Spruston et al., 2013). The shape of a dendritic spine depends on the specific synaptic demand upon it (Segal, 2010) and, vice versa, the the spine geometry can impact on the synaptic processing, local electrical compartmentalization and coupling to the adjacent dendritic voltage (Spruston et al., 2013; Yuste, 2013). There can be a direct structure-function coupling for differently shaped dendritic spines (Bupesh et al., 2011; Dall'Oglio et al., 2008b), but this issue has not reached a consensus for all the studied brain areas (Nimchinsky et al., 2002; Yuste, 2013). Depending on the geometry and spacing along the dendrites, spines can either amplify or filter synaptically-mediated membrane potentials, associate ionic currents and postsynaptic potentials with those of other adjacent spines and dendritic shafts, activate intracellular biochemical cascades, and induce long-term changes in neuronal excitability (Rasia- Filho et al., 2010; Toni et al., 1999; Nimchinsky at al., 2002; Kasai et al., 2010; Stuart et al., 1999; Arellano et al., 2007; Rochefort and Konnerth, 2012). In this regard, thin spines can be more plastic and transient protrusions, have more NMDA-type glutamate receptors (Bourne and Harris, 2007), and their long necks can induce biochemical and electrical compartmentalization of synaptic inputs in the spine head (Yuste, 2013). Mushroom spines can be more stable (Bourne and Harris, 2007), have a characteristic large spine head and, accordingly, their postsynaptic densities are largest and have more AMPA-type glutamate receptors (Spruston et al., 2013). Stubby/wide or ramified spines were considered "immature" shapes (Bourne and Harris, 2007) and it is likely that stubby spines impose a relatively low resistance to synaptic input and voltage propagation to the adjacent dendrite (Spruston et al., 2013) whereas branched spines can have different microdomains for synaptic integration (Verzi and Noris, 2009). In conjunction, our present data indicate that GDX change the number of synaptic input made by axo-spine contacts and, likely, the synaptic strength and excitatory responses in the MePD. It is notably that stubby/wide spines are twofold more abundant in GDX than in control males, but the reduction in the other spine types led to decreased density of spines following castration. These data address further research on the presence and function of androgen receptors within and outside classical nuclear sites, including dendrites (Sarkey et al., 2008), and how testosterone can modulate cytoskeletal molecules for dendritic structural flexibility

(Takagi and Kawashima, 1993). It could be suggested that the lack of androgenic support in the MePD neurons makes some thin and mushroom-like spines lose their enlarged shape and begin to shrink, dismantle, and some disappear, showing a stubby/wide appearance and even reducing total number of spines. Other studies are needed to determine the role of each spine class on the neuronal activity and their relationship with socially-relevant sensorial inputs in the MePD. Electrophysiological recordings can demonstrate whether androgens influence the activity in the outuput projections from the MePD of intact and GDX males that reach interconnected hypothalamic nuclei for the display of sexual behavior (Choi et al., 2005; Will et al., 2014).

In conclusion, adult males submitted to long-term GDX have a significant reduction in the density and shape of proximal dendritic spines in the MePD compared to control animals. Castration induce a remodeling of differently shaped spines, decreasing thin, mushroom-like, and ramified spines and increasing stubby/wide spines with basically the same results in the right and left hemispheres. These data are important for the neuronal structural modulation made by sex steroids in the social behavior network in the adult male brain.

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3.2.7 Conflict of Interest

Authors declare no actual or potential conflict of interest for the present work.

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3.2.9 Legends

Figure 1. Schematic diagram of the ventral aspect of a coronal brain section showing the posterodorsal medial amygdala (MePD; at – 3.3 mm posterior to the bregma) lateral to the optic tract (opt) and ventral to the stria terminalis (st) in rats. Adapted from Paxinos and Watson (2008).

Figure 2. Digitized fluorescent images of spiny neurons labeled with DiI reconstructed by confocal microscopy. Proximal dendritic branches showed pleomorphic spines in the posterodorsal medial amygdala of intact, sham operated or long-term castrated (GDX) adult male rats. Corresponding samples with higher magnification in the left. Note the reduction in the density of dendritic spines, but the predominance of spines with a stubby/wide shape in GDX males compared to controls. Spines were classified as thin (t), stubby (s), wide (w), mushroom-like (m), ramified (r) or atypical (a). Scale bar = 10 μm for left images and 2 μm for the higher magnification ones at the right μm.

Figure 3. Values (mean and standat deviation) of **(A)** the density of dendritic spine in the posterodorsal medial amygdala (MePD) of intact (n= 6), sham operated (n= 4), or long-term castrated (n= 5) adult male rats. *p< 0.05 compared to intact male data (ANOVA test followed by Bonferroni test). The density of differently shaped dendritic spine was significantly different in the right **(B)** and **(C)** left MePD of GDX males, with both reduction and increased in this values, depending on the morphology of the spine. See text fot details. **p<0.01 and *p<0.05 compared to control data (ANOVA test and Tukey post hoc tes).

Figure 1

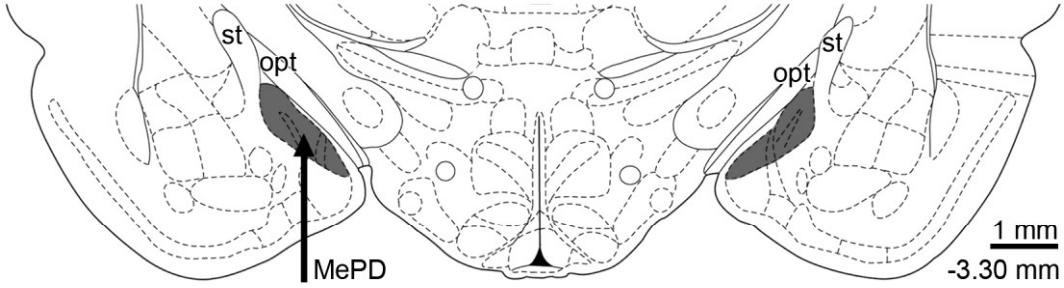


Figure 2

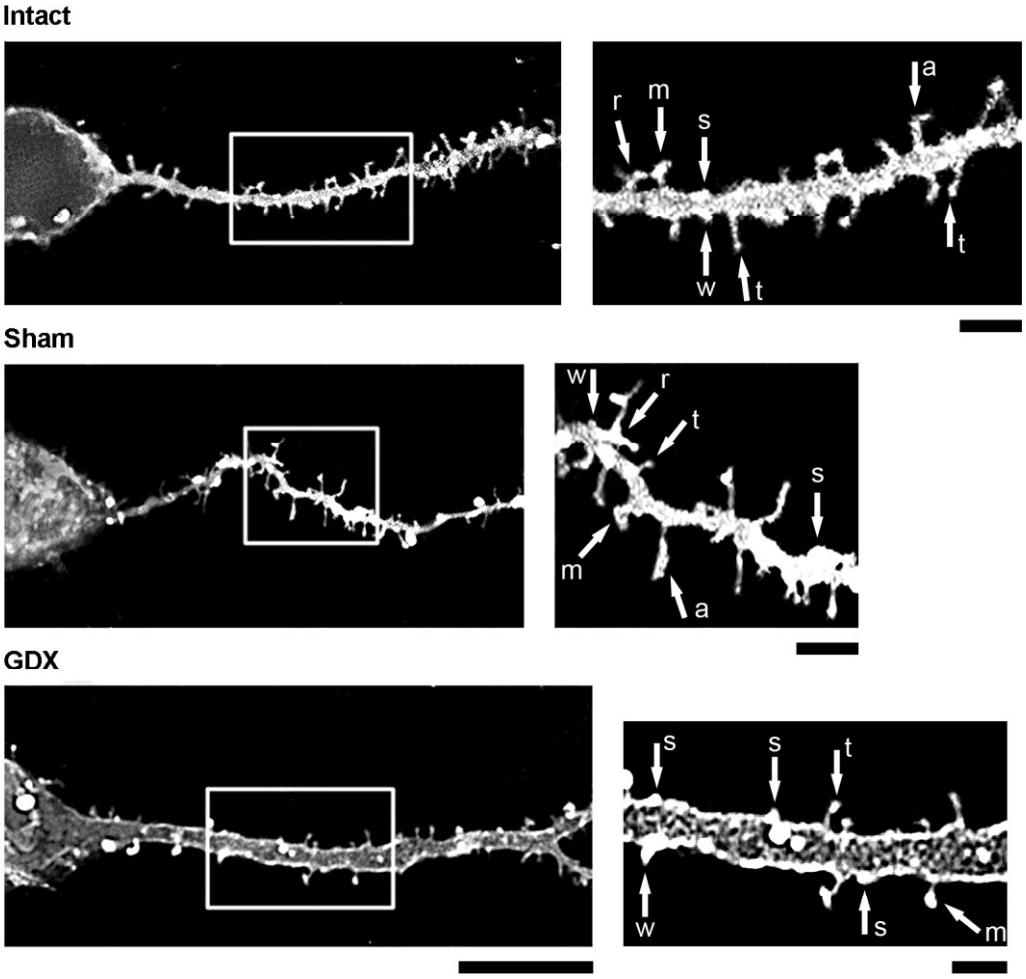
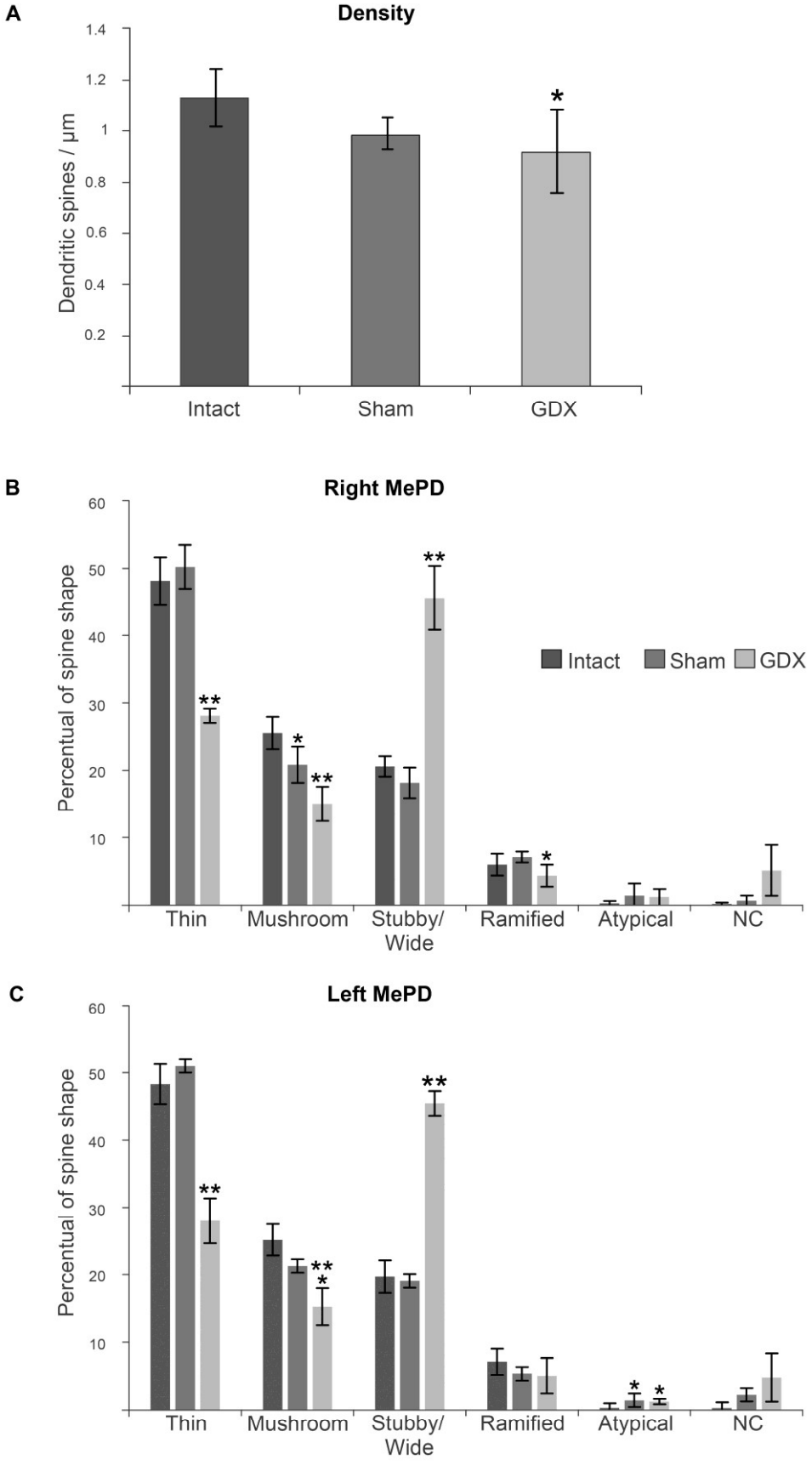


Figure 3


A
Density
Dendritic spines / µm
1.4
1.2
1
0.8
0.6
0.4
0.2
*
Intact
Sham
GDX
B
Right MePD
Percentual of spine shape
60
50
40
30
20
10
Intact
Sham
GDX
**
*
**
**
*
Thin
Mushroom
Stubby/ Wide
Ramified
Atypical
NC
C
Left MePD
Percentual of spine shape
60
50
40
30
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10
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*
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*
Thin
Mushroom
Stubby/ Wide
Ramified
Atypical
NC

4 Conclusões

Os dados da microscopia eletrônica revelaram que a membrana somática do MePD dos ratos é, em sua maior parte, coberta por processos gliais, e possui um percentual relativamente alto de processos axonais em contato com o corpo celular neuronal. Não houve diferença estatisticamente significativa entre machos e fêmeas ao longo do ciclo estral nesses parâmetros no MePD. Não obstante, ocorreu uma maior densidade de espinhos somáticos em fêmeas na fase final do proestro em comparação com fêmeas em estro. Tais espinhos somáticos apresentaram-se com formas mais variáveis durante o proestro, indicando uma plasticidade no processamento sináptico do MePD de ratas coincidente com os momentos de secreção neuroendócrina e emissão de comportamentos relacionados com a reprodução.

Os dados da microscopia confocal mostraram que a castração foi capaz de reduzir a densidade de espinhos dendríticos proximais nos neurônios do MePD de ambos os hemisférios cerebrais em ratos adultos. Ademais, a remoção dos testículos e queda da ação androgênica induziu o remodelamento da morfologia dos diferentes tipos de espinhos locais, com diminuição de espinhos dendríticos dos tipos fino e cogumelo, e notável aumento dos espinhos dendrítcos dos tipos achatado e largo, quando comparados aos grupos controle. Os espinhos do tipo ramificado tiveram sua densidade diminuída no MePD direita do grupo castrado.

Em conjunto, esses dados experimentais contribuem de forma inédita para avanço do entendimento da plasticidade morfológica e das possíveis implicações funcionais do MePD em ratos, machos e fêmeas ao longo do ciclo ovulatório, na modulação de comportamento reprodutivo dependente da ação contínua ou da secreção cíclica e dinâmica dos hormônios gonadais em circulação.

5 Perspectivas

Visando dar continuidade a estes achados e estudar novos aspectos do dimorfismo sexual é interessante que sejam feitos estudos onde se relacione a presença e ação locais do fator neurotrófico derivado do encéfalo (BDNF), da molécula de adesão celular neuronal (NCAM) e do receptor do fator de crescimento semelhante à insulina (IGF-1) na plasticidade numérica e na forma de espinhos dendríticos. Dados na literatura apontam para isso, sem que existam dados para o MePD, haja vista que esses fatores estão intimamente relacionados com a densidade de espinhos dendríticos[123], eventos subcelulares mediados por hormônios gonadais[5,6] e remodelamento da morfologia neuronal[124]. Técnicas de imunohistoquímica e *Western blot* deverão ser utilizadas para tanto, comparando-se machos e fêmeas ao longo do ciclo estral e quantificando os dados de imunohistoquímica regional e celular por densitometria óptica. Isso já faz parte de projeto de doutorado a ser desenvolvido em nosso meio, com projeto aprovado pelo CEUA-UFCSPA.

6 Referências Bibliográfica

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7 Anexos

A- Parecer da Comissão de Ética no uso de Animais

CEUA –COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

1) PROTOCOLO Nº:139/13 Parecer 248/13

2) DATA DO PARECER: 15/01/2014

3) TÍTULO DO PROJETO:

Sinapses axo-somáticas e contatos gliais na membrana do pericário de neurônios da amígdala medial póstero-dorsal de ratos e ratas ao longo do ciclo estral

4) PESQUISADOR RESPONSÁVEL:

Alberto A Rasia-Filho

5) RESUMO DO PROJETO:

A variação ao longo do ciclo estral na densidade de espinhos dendríticos sugere que possa ocorrer diferença entre machos e fêmeas e uma modificação dinâmica relacionada aos hormônios ovarianos em circulação nos neurônios e na glia da amídala medial póstero-dorsal.

6) OBJETIVOS DO PROJETO:

Estudar a presença e possível modificação cíclica nas sinapses axó-somáticas e na extensão dos prolongamentos gliais sobre a membrana do corpo celular de neurônios da amídala medial póstero-dorsal de ratas adultas ao longo do ciclo estral e ratos

7) FINALIDADE DO PROJETO: [ ] Ensino [x] Pesquisa

8) ITENS METODOLÓGICOS E ÉTICOS DO PROJETO:				
Título	x	Adequado		Comentários
Introdução	**x**	Adequada		Comentários
Objetivos	x	Adequados		Comentários
Relevância e Justificativa	x	Adequados		Comentários
Materiais e Métodos	x	Adequados		Comentários

Cronograma para execução da pesquisa	[x]	Adequado	[ ]	Comentários
Orçamento e fonte financiadora	[x]	Adequados	[ ]	Comentários
Referências Bibliográficas	[x]	Adequadas	[ ]	Comentários

9) O PROJETO ESTÁ ADEQUADO À LEGISLAÇÃO VIGENTE:

[x] Sim [ ] Não

10) INFORMAÇÕES RELATIVAS AOS ANIMAIS:

Grau de dor/estresse: **B** [ ] **C** [ ] **D** [x] **E** [ ]

Justifique:

Espécie: Ratos Wistar **Número Amostral:** 30

Redução Amostral: [ ] Sim [x] Não

Justifique:

Substituição de Metodologia: [ ] Sim [x] Não

Se achar necessário, justifique e sugira uma nova metodologia:

Aprimoramento da Metodologia: [ ] Sim [x] Não

Se achar necessário, justifique e sugira aprimoramentos da metodologia:

Acomodação e manutenção dos animais: [x] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias:

Manipulação dos animais: [x] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias:

Analgesia dos animais (se aplicável): [x] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com analgésico substituto:

Anestesia dos animais (se aplicável): [x] Adequada [ ] Inadequada

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Se achar inadequada cite abaixo as melhorias necessárias com metodologia substituta:

Local de Realização (Biotério/Laboratório):

Laboratório de Fiisiologia-UFCSPA

Outra instituição. Qual? Instituto de Ciências Básicas, UFRGS

11) CRONOGRAMA DE UTILIZAÇÃO DE ANIMAIS

Data	Espécie	Sexo	Quantidade
Março	Ratos	M	06
Março	Ratos	F	24

12) RECOMENDAÇÃO:

[X] Aprovado

[ ] Com Pendência

[ ] Não aprovado

Data de início 17/01/14 Data de Término 31/07/2014

Comentários gerais sobre o projeto:

Projeto aprovado.

Informação relativa ao preenchimento do formulário denominado Protocolo para uso de animais na Pesquisa - CEUA – UFCSPA.

No referido formulário, o item 7.1 refere-se as substâncias que serão administradas aos animais, bem como dose, volume, via, intervalo, duração, quando as mesmas são objetos (intervenção, modulação) de estudo ou fazem parte do modelo. No caso de analgésicos, anestésicos e outros fármacos com propósitos de minimizar os danos, a dor, etc estes deverão ser descritos no item 7.3

B- Parecer da Comissão de Ética no uso de Animais

CEUA –COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

1) PROTOCOLO Nº: 116/13 Parecer 186/13

2) DATA DO PARECER: 08/05/2013

3) TÍTULO DO PROJETO:

Influência da gonadectomia na morfologia dos espinhos dendríticos na amígdala medial póstero-dorsal de ratos adultos

4) PESQUISADOR RESPONSÁVEL:

Alberto Antônio Rasia Filho

5) RESUMO DO PROJETO:

O projeto se baseia na sensibilidade das células localizadas no subnúcleo póstero-dorsal da amígdala medial de ratos aos esteróides sexuais. Alterações nos níveis hormonais promovem mudanças plásticas nas células e modulação do comportamento social. Serão utilizados cortes histológicos previamente processados pela técnica de Golgi. A hipótese do trabalho é que a castração dos animais por longo tempo cause uma alteração na morfologia dos espinhos dendríticos, tornando-os menos complexos.

6) OBJETIVOS DO PROJETO:

O objetivo geral é estudar a plasticidade de neurônios quanto à sua morfologia e modulação por hormônios gonadais. O objetivo específico é determinar o efeito da retirada dos hormônios gonadais sobre a morfologia de espinhos dendríticos proximais no MePD de ratos machos castrados em idade adulta.

7) FINALIDADE DO PROJETO: [ ] Ensino [X] Pesquisa

8) ITENS METODOLÓGICOS E ÉTICOS DO PROJETO:

Título	x	Adequado		Comentários
Introdução	**x**	Adequada		Comentários
Objetivos	x	Adequados		Comentários
Relevância e Justificativa	x	Adequados		Comentários
Materiais e Métodos	x	Adequados		Comentários
Cronograma para execução da pesquisa	**x**	Adequado		Comentários
Orçamento e fonte financiadora	**x**	Adequados		Comentários
Referências Bibliográficas	**x**	Adequadas		Comentários

9) O PROJETO ESTÁ ADEQUADO À LEGISLAÇÃO VIGENTE:

X Sim ☐ Não

10) INFORMAÇÕES RELATIVAS AOS ANIMAIS:

Grau de dor/estresse: **B** ☐ **C** ☐ **D** ☐ **E** ☐

Justifique:

Não se aplica

Espécie: **Número Amostral:**

Redução Amostral: ☐ Sim ☐ Não

Justifique:

Não se aplica

Substituição de Metodologia: ☐ Sim **x** Não

Se achar necessário, justifique e sugira uma nova metodologia:

Aprimoramento da Metodologia: ☐ Sim **x** Não

Se achar necessário, justifique e sugira aprimoramentos da metodologia:

Acomodação e manutenção dos animais: ☐ Adequada ☐ Inadequada

Se achar inadequada cite abaixo as melhorias necessárias:

Não se aplica

Manipulação dos animais: ☐ Adequada ☐ Inadequada

Se achar inadequada cite abaixo as melhorias necessárias:

Não se aplica

Analgesia dos animais (se aplicável): ☐ Adequada ☐ Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com analgésico substituto:

Não se aplica

Anestesia dos animais (se aplicável): ☐ Adequada ☐ Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com anestésico substituto:

Não se aplica

Eutanásia dos animais (se aplicável): ☐ Adequada ☐ Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com metodologia substituta:

Não se aplica

Local de Realização (Biotério/Laborarório): Laboratório de Fisiologia (306) – prédio principal – UFCSPA

11) CRONOGRAMA DE UTILIZAÇÃO DE ANIMAIS

Data	Espécie	Sexo	Quantidade
--	--	--	--
--	--	--	--

12) RECOMENDAÇÃO:

☒ Aprovado

☐ Com Pendência

☐ Não aprovado

Comentários gerais sobre o projeto:

O projeto analisado envolve a utilização de material biológico animal previamente processado em cortes histológicos armazenados de outro projeto de pesquisa. Não haverá manipulação ou qualquer uso de animais.

UFCSPA

COMISSÃO CIENTÍFICA E COMISSÃO DE PESQUISA E ÉTICA EM SAÚDE

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA
UFCSPA

A Comissão de Ética no uso de Animais, analisou o Projeto:

Projeto: 13-116 **Versão do Projeto:** **Versão do TCLE:**

Pesquisadores:
ALBERTO ANTÔNIO RASIA FILHO
MARIANA ZANCAN

Título: INFLUÊNCIA DA GONADECTOMIA NA MORFOLOGIA DOS ESPINHOS DENTRÍTICOS NA AMÍGDALA MEDIAL PÓSTERO-DORSAL DE RATOS ADULTOS.

Este projeto foi aprovado em seus aspectos éticos e metodológicos. Todo e qualquer alteração do projeto, assim com eventos adversos graves, deverão ser comunicados a esta CEUA.

Porto Alegre, 18 de outubro de 2013.

Katya V. Rigatto
Coordenadora do CEUA
UFCSPA

C - Instrução aos autores da The Anatomical Record

The Anatomical Record

Edited By: Kurt H. Albertine, PhD Online ISSN: 1932-8494

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Kelley RO. 1970a. An electron microscopic study of mesenchyme during developme nt of interdigital spaces in man. Anat Rec 168:43-54.

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Bräuer G, Collard M, Stringer C. 2004. On the reliability of recent tests of the Out of Africa hypothesis for modern human origins. Anat Rec 279:701-707.

Broadfield DC, Holloway RL, Mowbray K, Silvers A, Yuan MS, Márquez M. 2001. Endocast of Sambungmacan 3 (Sm 3): a new Homo erectus from Indonesia. Anat Re c 262:369-379.

Bruner E, Manzi G. 2005. CT-based description and phyletic evaluation of the archai c human calvarium from Ceprano, Ital. Anat Rec 285:643-657.

Bush EC, Simons EL, Allman JM. 2004. High-resolution computed tomography study of the cranium of a fossil anthropoid primate, Paraphithecus grangeri: new insights int o the evolutionary history of primate sensory systems. Anat Rec 281:1083-1087.

Martin RD, MacLarnon AM, Phillips JL, Dobyns WB. 2006. The Flores hominid: new species or microcephalic dwarf? Anat Rec 288:1123-1145.

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CP Cortical Plate

Smc Primary somatosensory cortex

V Ventral

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