UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM NEUROCIÊNCIAS

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ORGANIZAÇÃO E PLASTICIDADE SINÁPTICAS NA AMÍGDALA MEDIAL PÓSTERO-DORSAL DE RATOS: MODULAÇÃO ONTOLÓGICA E SEXUAL DOS ESPINHOS DENDRÍTICOS, EXPRESSÃO DE CONEXINAS E DE FATORES NEUROTRÓFICOS LOCAIS

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Tese apresentada ao Programa de Pós-Graduação em Neurociências do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de doutora em Neurociências.

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Porto Alegre

2019

Aos meus pais, Aneila e Hélio,
exemplos de força, caráter, amor e
dedicação.

Agradecimentos

Ao meu orientador e amigo, prof Rasia, pelas oportunidades concedidas, pelas lições humanas e científicas. Fica aqui meu respeito e admiração!

À minha família, pai, mãe, Mateus e Letícia, por entenderem as minhas ausências, permitirem eu alçar vôos mais altos, estarem do meu lado em todos os momentos.

Às técnicas do Laboratório de Fisiologia da UFCSPA, Carmem e Alexandra, pelo suporte no desenvolvimento experimetal desta tese, pelas conversas e momentos de descontração.

Aos colegas de laboratório Francine, Francisco, Fernando, Nivaldo, Paulo, Edson, Aline, Letícia, Camila, Carlos, Débora, por compartilharem angústias e tornarem tudo mais fácil e leve.

Às colegas da PUCRS, Marina, Laura e Franciele, pela parceria que firmamos nesses anos.

Às amigas e colegas de pesquisa, Ana Carolina e Brenda, pelas inúmeras trocas e pelos cafés regados de muito afeto.

Às minhas amigas de alma, Patrícia e Dandara. Só posso dizer: vocês são sensacionais!

À grande família Albarello, que sempre me apoiou emocionalmente ao longo destes anos.

Aos colegas do Laboratório de Genética Toxicológica da UFCSPA

Ao CNPq e ao PPG Neurociências da UFRGS, pelo apoio financeiro e oportunidade.

SUMÁRIO

LISTA DE ABREVIATURAS

ABPs	Proteínas de ligação à actina
ACe	Amígdala central
AMbl	Amígdala basolateral
AMe	Amígdala medial
AMPA	Alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiónico
AOB	Bulbo olfatório acessório
Arc	Núcleo arqueado do hipotálamo
Arc	Proteína associada ao citoesqueleto
AVPV	Núcleo periventricular ântero-ventral
BST	Núcleo próprio da estria terminal
BDNF	Fator neurotrófico derivado do encéfalo
CaM	Calmodulina
CaMKII	Proteína quinase II dependente de cálcio/calmodulina
Cdc42	Proteína homóloga de controle da divisão celular 42
CREB	Proteína ligante de elemento responsivo ao cálcio
Cx36	Conexina 36
Cx43	Conexina 43
Cx45	Conexina 45
ERs	Receptores para estrogênio
ER-α	Receptor do tipo α para estrogênio
ER-β	Receptor do tipo β para estrogênio
GABA	Ácido γ-aminobutírico
GAPs	Proteínas ativadores de GTPases
GDP	Difosfato de guanosina
GTP	Trifosfato de guanosina
GEFs	Fatores de troca do nucleotídeo guanina
GluR1	Receptor para glutamato tipo 1
GluR2	Receptor para glutamato tipo 2
GnRH	Hormônio liberador de gonadotrofina
GTPase	Guanidina trifosfato com atividade enzimática
IGF-1	Fator de crescimento semelhante à insulina tipo 1
LIMK	Domínio K da proteína quiinase LIM

LTP	Potenciação de longa duração
MAPs	Proteínas associadas a microtúbulos
MAPKs	Proteína-quinases ativadas por mitógenos
RNAm	Ácido ribonucleico mensageiro
MeAD	Subnúcleo ântero-dorsal da amígdala medial
MeAV	Subnúcleo ântero-ventral da amígdala medial
MePD	Subnúcleo póstero-dorsal da amígdala medial
MePDi	Porção intermediária do subnúcleo póstero-dorsal da amígdala medial
MePDl	Porção lateral do subnúcleo póstero-dorsal da amígdala medial
MePDm	Porção medial do subnúcleo póstero-dorsal da amígdala medial
MePV	Subnúcleo póstero-ventral da amígdala medial
MPOA	Área pré-óptica medial do hipotálamo
NMDA	N-metil D-Aspartato
Pat	Ativador do plasminogênio tecidual
PI3K/Akt	Fosfoinositida 3-quinase/ proteína quiinase
PKC	Proteína quinase C
PMV	Núcleo pré-mamilar ventral
POA	Área pré-óptica
PSD-95	Proteína 95 da densidade pós- sináptica
PSA-NCAM	Forma polisialilada da molécula de adesão celular neuronal
PVH	Região hipotalâmica paraventricular
SNC	Sistema nervoso central
ST	Estria terminal
TO	Trato óptico
TrKB	Receptor B tirosina quinase
VMH	Núcleo ventromedial do hipotálamo
WAVE	Proteína homóloga à verprolina
WAS	Proteína da síndrome WisKott-Aldrich

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RESUMO

A amígdala medial póstero-dorsal (MePD) é estrutura cerebral sexualmente dimórfica, dependente dos níveis de hormônios gonadais em circulação e relacionado com o comportamento reprodutivo em ratos. A plasticidade sináptica local se relaciona com os espinhos dendríticos, locais de modulação da atividade pós-sináptica, relacionados com o funcionamento dos neurônios da MePD e as diferenças entre machos e as fêmeas ao longo do ciclo ovulatório. Apesar da prevalência de sinapses químicas, discretas sinapses elétricas já foram reportadas entre células gliais, mas não entre neurônios, na MePD. Nem se sabe a variação de fatores neurotróficos que pode ocorrer entre machos e fêmeas ao longo do ciclo estral. Os objetivos deste trabalho foram: (1) comparar número e estrutura dos espinhos dendríticos na MePD de ratos machos e fêmeas pré-púberes e, a seguir, entre machos pós-púberes com e sem experiência sexual empregando-se ténica com microscopia confocal; (2) estudar a ultraestrutura da MePD procurando pela presença de junções comunicantes e, a seguir, estudar a presença das conexinas 36, 43 e 45 por imunofluorescência nessa mesma estrutura; e, (3) estudar o possível dimorfismo sexual e variação ao longo do ciclo estral na expressão local de BDNF (fator neurotrófico derivado do encéfalo), IGF-1 (fator de crescimento semelhante à insulina tipo 1), PSA-NCAM (forma policialidade da molécula de adesão celular neuronal) e Efrina-A4, fatores neurotróficos envolvidos com a plasticidade sináptica, empregando-se RT-PCR e técnica imunohistoquímica com fluorescência. Os resultados evidenciaram (1) um dimorfismo sexual em espinhos dendríticos da MePD em animais pré-púberes e um remodelamento dos espinhos causado pela puberdade e pela experiência sexual; (2) presença de escassas junções comunicantes entre dendritos e a expressão mais evidente de conexinas 36, 43 e 45 na MePD; e, (3) não haver diferença estatisticamente significativa na expressão de BDNF, IGF-1, PSA-NCAM e Efrina-A4, nem na expressão gênica de BDNF, NCAM e Efrina-A4, entre machos e fêmeas ao longo do ciclo estral. No entanto, há diferença estatisticamente significativa e maior quantidade de puncta imunorreativa para BDNF em fêmeas em proestro do que em machos e maior de IGF-1 em fêmeas em proestro de que em diestro. Os presentes dados indicam que, na MePD, há dimorfismo sexual e notável reorganização e plasticidade sináptica relacionada com os espinhos dendríticos, que conexinas são mais abundantes que junções comunicantes locais sugerindo haver forma de comunicação intercelular adicional à sinapse química e que duas neurotrofinas (BDNF e IGF-1) podem estar participando da modulação da forma neuronal e da plasticidade sináptica que ocorre em fêmeas especificamente na fase de proestro, quando estrógeno e progesterona estão nos maiores níveis circulantes.

Palavras-chave: amígdala expandida, dimorfismo sexual, sinapse elétrica, plasticidade sináptica

ABSTRACT

The postero-dorsal medial amygdala (MePD) is a sexually dimorphic cerebral structure, dependent on the levels of circulating gonadal hormones and related to the reproductive behavior in rats. Local synaptic plasticity is related to the dendritic spines, modulation sites of postsynaptic activity, related to the functioning of MePD neurons and the differences between males and females throughout the ovulatory cycle. Despite the prevalence of chemical synapses, discrete electrical synapses have been reported between glial cells, but not between neurons, in MePD. Nor is it known the variation of neurotrophic factors that can occur between males and females throughout the estrous cycle. The aims of this study were: 1) to compare number and structure of the dendritic spines in the MePD of prepubertal male and female rats, then between postpubertal males with and without sexual experience using confocal microscopy; 2) to study the ultrastructure of MePD looking for the presence of gap junctions and then to study the presence of connexins 36, 43 and 45 by immunofluorescence in the same structure; e, 3) to study possible sexual dimorphism and estrous cycle variation in local expression of BDNF (Brain-Derived Neurotrophic Factor), IGF-1 (insulin-like growth factors 1), PSA-NCAM (polysialylated neuronal cell adhesion molecule) and Ephrin-A4, neurotrophic factors involved in synaptic plasticity, using RT-PCR and fluorescence immunohistochemical technique. The results showed (1) a sexual dimorphism in MePD dendritic spines in prepubertal animals and a remodeling of the spines caused by puberty and sexual experience; (2) presence of sparse gap junctions between dendrites and the most evident expression of Cx36, Cx43 and Cx45 in MePD; and, (3) there was no statistically significant difference in the expression of BDNF, IGF-1, PSA-NCAM and Ephrin-A4, nor in the BDNF, NCAM and Ephrin-A4 gene expression between males and females throughout the estrus cycle. However, there is a statistically significant difference and a greater quantity of immunoreactive puncta for BDNF in females in proestrus than in males and higher in IGF-1 females in proestrus than in diestrus. The present data indicate that in MePD there is sexual dimorphism and remarkable reorganization and synaptic plasticity related to dendritic spines, connexins are more abundant than local gap junctions suggesting that there is a form of intercellular communication in addition to the chemical synapse and two neurotrophins (BDNF and IGF-1) may be involved in the modulation of neuronal form and synaptic plasticity that occurs in females specifically in the proestrus stage, when estrogen and progesterone are at the highest circulating levels.

Key-words: extended amygdala, sexual dimorphism, electrical synapse, synaptic plasticity.

INTRODUÇÃO

1.1 Amígdala

A amígdala (ou preferentemente, complexo amigdaliano; Rasia-Filho e Hilbig, 2005) de ratos é formada por um conjunto de núcleos e subnúcleos localizados no telencéfalo basal, subcortical no lobo temporal anterior, lateral ao hipotálamo e ventral ao estriado (Alheid *et al*., 1995; Canteras *et al*, 1995; Swanson e Petrovich, 1998). Em ratos, localiza-se anteriormente ao hipocampo, ventralmente e lateralmente ao trato óptico (TO) ao longo de toda sua extensão rostro-caudal (de Olmos *et al.*, 2004; Rasia-Filho *et al.*, 2012a). Parte do complexo amigdaliano possui particularidades estruturais e estende-se além de seus limites anatômicos, como será tratado a seguir (Alheid *et al.*, 1995; Swanson e Petrovich, 1998; de Olmos *et al.*, 2004).

Como a amígdala não é nem uma unidade anatômica e também não tem uma única função, mas um papel de integração de diversas atividades comportamentais, endócrinas e simpáticas ou parassimpáticas (Rasia- Filho *et al*., 2000), torna-se obrigatório estudar de maneira individual e detalhada cada um de seus componentes e subcomponentes além de circuitos neurais específicos (Canteras *et al*., 1995; Rasia- Filho *et al*., 2004; Dall´Oglio *et al*., 2008a,b; Quagliotto *et al.,* 2008). Com base nisso, é consenso atualmente a existência de quatro principais regiões amigdalianas, de acordo com características citoarquitetônicas, imunohistoquímicas e hodológicas: 1) amígdala “expandida”, a qual se estende além de seus limites anatômicos e é formada pelos núcleos medial (MeA) e central (ACe); 2) amígdala com características corticais, subdividida em porção basilar lateral (AMBl) e porções que se conectam às vias olfatórias e vomeronasal; 3) áreas de transição, localizadas entre a porção ventral dos núcleos da base e a amígdala “expandida”; e 4) núcleos não classificados, constituídos por grupo de células dispersas na substância branca e no interior do BNST (Alheid *et al*., 1995; Swanson e Petrovich, 1998; de Olmos *et al*., 2004; Quagliotto *et al*., 2006).

Há ocorrência de dimorfismo sexual amigdaliano, ou seja, diferenças entre machos e fêmeas quando se compara a área total da AMe (Hines *et al*., 1992; Morris *et al*., 2008). A AMe possui maior volume em ratos a partir da terceira semana de vida pós-natal (Nishizuka e Arai, 2012) e mais notável após a puberdade (Cooke, 2011). Tal fato está na dependência da ação dos hormônios gonadais em ambos os sexos (de Castilhos *et al*., 2008). De fato, os subúcleos da AMe possuem neurônios que apresentam receptores para os esteroides sexuais testosterona, estradiol e progesterona (de Olmos *et al*., 2004; de Vries e Simerly, 2002; Gréco *et al.*, 2003; Frankiensztajn *et al.*, 2018). Pela capacidade desses hormônios gerarem alterações nos circuitos neurais, acabam por influenciar a plasticidade neural e causam alterações morfológicas como: modificação no número de neurônios, promoção da maturação neuronal e crescimento de neuritos, determinação da sinaptogênese (Gréco *et al.,* 2003), alteração no comprimento dendrítico, na arborização dendrítica e no número de espinhos dendríticos e somáticos (Hermel *et al*., 2006a, Zancan *et al*., 2015, Zancan *et a*l., 2017). Os efeitos sobre ramos dendríticos e espinhos dendríticos variam de um sexo para outro e de uma região nervosa específica a outra (Blake *et al.*, 2011; Bian *et al*., 2008).

1.2 Amígdala Medial: localização, citoarquitetura e divisão

A AMe é um dos componentes da denominada “amígdala expandida” e um dos núcleos superficiais amigdalianos (Alheid *et al.*, 1995; de Olmos *et al.*, 2004). Participa da modulação e integração de informações olfativas, vomeronasais e genitosensoriais (Pfaus e Heeb, 1997; Meredith e Westberry, 2004; Pro-Sistiaga *et al.*, 2007; Cádiz-Moretti *et al.*, 2014; Keshavarzi *et al.*, 2015); da ansiedade, medo inato e condicionado (Adekunbi *et al.*, 2018; Lopes *et al.*, 2018); do processamento emocional, das respostas neuroendócrinas a estímulos estressores (Marcuzzo *et al.*, 2007; Singewald *et al.*, 2008; Masini *et al.*, 2009; Shemesh *et al.*, 2016); de ajustes cardiovasculares relacionados à gênese e à modulação de comportamentos sociais (Quagliotto *et al.*, 2008; Quagliotto *et al.*, 2012; De Almeida *et al.*, 2015); da atividade sexual de machos e fêmeas e do comportamento maternal (Newman, 1999; Pardo-Bellver *et al.*, 2012; Rasia-Filho

et al., 2012a; Cádiz-Moretti *et al.*, 2014; McCarthy *et al.*, 2017); dos comportamentos agressivo e defensivo (Newman, 1999; Rasia-Filho *et al.*, 2012b; Ruiz-Reig *et al.*, 2018); e do aprendizado social (Stetzik *et al.*, 2018).

A AMe é formada por uma coluna de células que surgem em justaposição à superfície lateral das fibras que ascendem pelo TO (Alheid *et al*., 1995, de Olmos *et al*., 2004). Inicia medial e posteriormente ao núcleo do trato olfativo estendendo-se caudalmente até o surgimento do corno temporal do ventrículo lateral. Nesta altura, a AMe situa-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 AMe encontra-se em posição lateral à região ventro-lateral do TO (Alheid *et al*., 1995, de Olmos *et al*., 2004).

Alheid et al (1995) e Paxinos e Watson (1998) dividem a AMe nos seguintes subnúcleos: ântero-dorsal (MeAD), ântero-ventral (MeAV), póstero-dorsal (MePD) e póstero-ventral (MePV; Figura 1). A MePD, foco de estudo desta tese, separa-se do TO por uma camada com escassos corpos celulares, originalmente denominada “camada molecular”, a qual se torna estreita em direção rostral e dorsal e desaparece completamente próximo á MeAD (de Olmos *et al*., 2004). Ainda, a MePD pode ser subdividida conforme a disposição colunar de seus neurônios em uma porção densa mais superficial ou medial (MePDm) separada por uma coluna intermediária com menos células agrupadas (MePDi) e uma porção lateral (MePDl) novamente com mais células justapostas (de Olmos *et al*., 2004; de Castilhos *et al*., 2006; Dall'Oglio *et al*., 2008b).

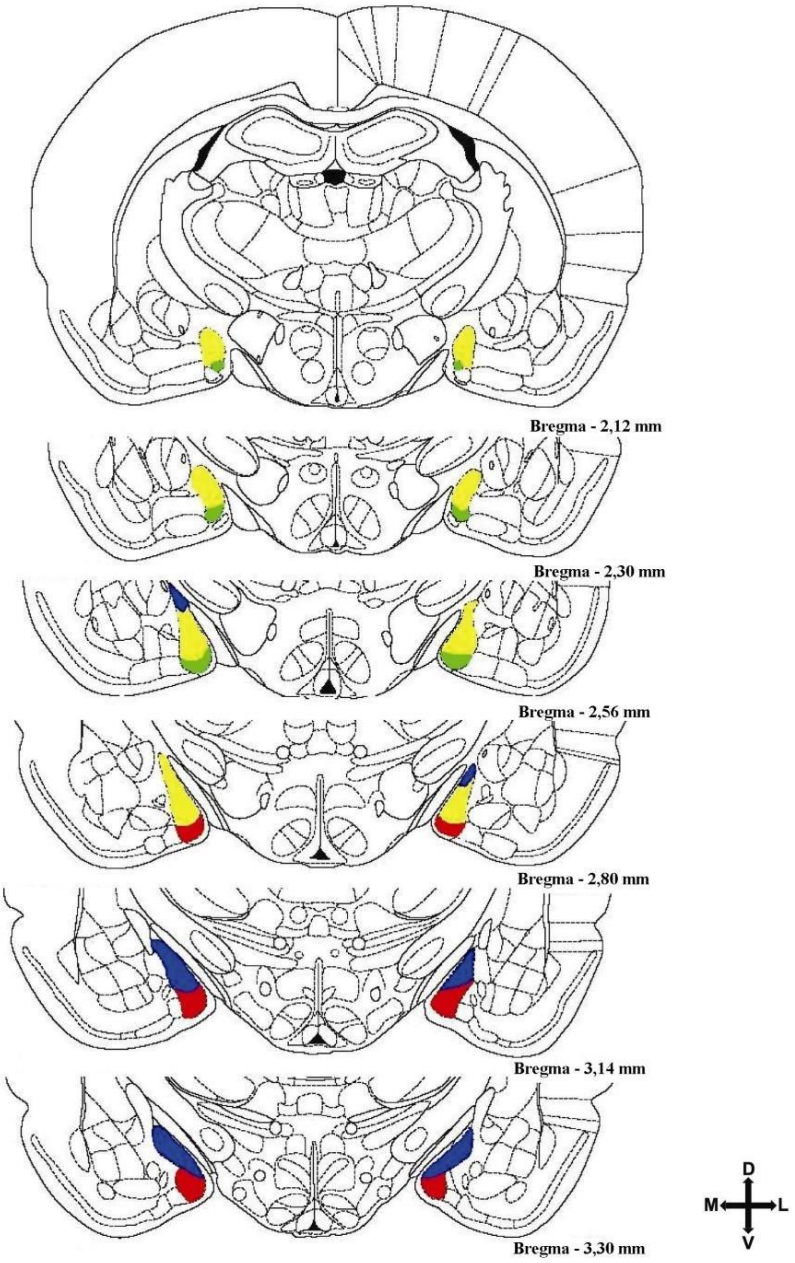

Figura 1. Representação esquemática de cortes coronais do encéfalo de rato, parte ventral, demonstrando a localização dos quatro subnúcleos do núcleo medial da amígdala, a saber: ântero-dorsal (MeAD, em amarelo), ântero-ventral (MeAV, em verde), póstero-dorsal (MePD, em azul) e póstero-ventral (MePV, em vermelho). Os valores em mm colocados no lado direito das imagens referem-se à distância posterior ao bregma. As coordenadas espaciais referem-se ao hemisfério direito e são: dorsal (D), ventral (V), medial (M) e lateral (L). Figuras adaptadas do atlas do encéfalo do rato de Paxinos e Watson (1998) e conforme apresentado originalmente por Quagliotto (2006).

1.2.1 Hodologia e função

A AMe de ratos possui uma vasta rede de conexões neurais entre seus subnúcleos, com outros núcleos do complexo amigdaliano e também com núcleos extra-amigdalianos subcorticais e áreas corticais (Canteras *et a*l., 1995). Por exemplo, as principais aferências são provenientes do córtex cerebral (da área pré-límbica, córtex entorrinal, infralímbico e perirrinal dorsal), de núcleos do hipotálamo (como da área pré-óptica), da área septal, do tronco encefálico (núcleo dorsal da rafe e núcleo parabraquial) e da via olfatória e estruturas interconectadas (córtex piriforme, bulbo olfatório acessório e núcleo endopiriforme). Dentre as aferências intra-amigdalianas destacam-se as da área de transição amígdalo-hipocampal, dos núcleos basal e basal acessório e as dos núcleos corticais anterior, posterior, lateral e medial (McDonald, 1999). As eferências da AMe incluem projeções para o sistema olfatório (principal e acessório), para a formação hipocampal (ventralmente), estriado ventral, globo pálido ventral e BNST no telencéfalo basilar, para vários núcleos hipotalâmicos, para a porção medial do tálamo, para a substância cinzenta periaquedutal e para os núcleos da rafe mesencefálica (Canteras *et al*., 1995; Petrovich *et al*., 2001).

Especificamente, a MePD possui como aferências mais estudadas as hipotalâmicas (área hipotalâmica anterior; áreas pré-ópticas medial e lateral; núcleo arqueado; núcleos dorsomedial, posterior, lateral, pré-mamilar ventral, supra-óptico, tuberal e ventromedial, VMH), as do córtex cerebral (área pré-límbica, córtex entorrinal, infralímbico e perirrinal dorsal), as talâmicas (núcleo medial, parafascicular, paraventricular, posterior, dentre outros), as do tronco encefálico (especialmente do núcleo dorsal da rafe e núcleo parabraquial), da via olfatória (córtex piriforme, bulbo olfatório acessório e núcleo endopiriforme) e de outras regiões como o núcleo próprio da estria terminal e da substância *innominata.* Existem também aferências intra-amigdalianas como as da área amígdalo-hipocampal, dos núcleos basal e basal acessório, dos núcleos corticais anterior, posterior, lateral e medial. Dentre as aferências inter-amigdalianas encontram-se as provenientes do córtex periamigdaliano, núcleos basal acessório, cortical posterior e do trato olfatório lateral (McDonald, 1998).

As eferências da MePD incluem as hipotalâmicas (para os núcleos hipotalâmicos periventricular e ântero-ventral, área pré-óptica medial e núcleo pré-mamilar ventral), as corticais (principalmente para a área entorrinal lateral, área de transição pós-piriforme, CA1 do hipocampo e subículo), as do tronco encefálico (área tegmental dorsal e substância cinzenta periaquedutal) e para outras regiões como o núcleo próprio da estria terminal e substância *innominata.* Além disso, existem eferências intra-amigdalianas como para os núcleos central e cortical póstero-lateral e póstero-medial (Canteras *et al*., 1995; Petrovich *et al*., 2001).

Com base em tal circuitaria neural, a MePD tem participação na interpretação de informações sensoriais interoceptivas e exteroceptivas (Meredith & Westberry, 2004), na regulação de comportamentos sociais (Newman, 2012) incluindo-se os comportamentos agressivo (Rasia-Filho *et al*., 2012b), sexual de machos e fêmeas (Rasia- Filho et al., 1991; de Castilhos et al., 2006), maternal (Rasia-Filho *et al*., 2004) e na modulação da memória condicionada e do aprendizado onde componente emocional esteja envolvido (revisado em Rasia-Filho *et al*., 2000). Ainda, a MePD desencadeia ajustes cardiovasculares simpáticos e parassimpáticos muito provavelmente relacionados à gênese e à modulação de comportamentos sociais (Quagliotto *et al*., 2008; Quagliotto *et al*., 2012; Neckel *et al*., 2012).

1.3 Organização e plasticidade sinápticas na MeP

Os hormônios gonadais induzem modificações na forma e função dos neurônios e células da glia no sistema nervoso central (SNC) e periférico (Rasia-Filho *et al*., 2004; Garcia-Segura *et al*., 1994; Garcia-Segura e McCarthy, 2004; Tetel e Acharya, 2013; Zancan *et al*., 2015; Zancan *et al*., 2015; Zancan *et al.*, 2018), atuando no tecido nervoso em período inicial organizacional a fim de promover o desenvolvimento de mudanças bioquímicas, fisiológicas e na estrutura de circuitos sexualmente dimórficos a longo prazo (Nishizuka e Arai, 1981; Rasia-Filho *et al.*, 2004; Cooke e Woolley, 2005; Bergan *et al.*, 2014; Zancan *et al.*, 2018). Na fase adulta, esses esteroides estimulam circuitos já formados, ativando-os e promovendo funções até então não desempenhadas,

como é o caso da gametogênese, ciclo estral e experiências sexual e reprodutiva (Herting e Sowell, 2017).

As ações neurotróficas da testosterona, do estradiol e da progesterona na MePD ocorre por ação em receptores específicos (revisado em Rasia-Filho *et al*., 2012a; Petrulis, 2013; Frankiensztajn *et al*., 2018). Na MePD há uma grande densidade de receptores para hormônios gonadais comparável àquela encontrada em núcleos do hipotálamo envolvidos com a reprodução (Simerly *et al*., 1990). Há receptores para testosterona e receptores do tipo α e ß para estradiol (ER-α e ER-ß; de Vries e Simerly, 2002; Gréco *et al*., 2003; Frankiensztajn *et al*., 2018) e para progesterona (De Vries e Simerly, 2002).

O papel integrado da ação dos hormônios gonadais e a ciruitaria da MePD pode ser compreendido pela modulação do comportamento reprodutivo. Em ratos, a MePD está relacionada com a ocorrência de penetração peniana e de ejaculação (de Castilhos *et al*., 2006; Veening e Coolen, 2014) ou na percepção da estimulação vaginocervical (Pfaus e Heeb, 1997; Lehmann *et al*., 2005; Oberlander e Erskine, 2008). Em machos com lesão na MePD há uma redução do comportamento de cópula e perda da ereção peniana quando expostos ao odor de uma fêmea em estro (Kondo *et al*., 1998; Kondo e Sachs, 2002). Porém, a atividade sexual copulatória aumenta após a microinjeção unilateral ou bilateral de testosterona na MePD de hamsters adultos castrados (Coolen e Wood, 1999). O estímulo percebido a partir de feromônios faz aumentar a atividade de neurônios que expressam também receptores para androgênios na MePD de hamsters machos (Blake e Meredith, 2011). Neurônios da MePD de ratos machos e fêmeas que possuem receptores para estrogênio (ERs) igualmente expressam o gene de resposta imediata c-fos após a percepção de estímulo olfativo e da atividade copulatória (Coolen *et al*., 1997; Gréco *et al*., 2003). Ademais, o aumento da densidade de ER-α na MePD de ratos machos também está associado com a ejaculação e a saciedade sexual (Phillips-Farfán *et al*., 2007). Em fêmeas, há uma alta concentração de ER-α e ER-β na MePD (Gréco *et al*., 2001; Gréco *et al*., 2003; Phillips-Farfan *et al*., 2007) e a expressão de RNAm para ER-β local é proporcional ao nível de progesterona em circulação (Isgor *et al*., 2002). A MePD controla o momento fisiológico durante o ciclo estral para a secreção

neuroendócrina hipotalâmica do hormônio liberador de gonadotrofina (GnRH) e a ovulação (Beltramino e Taleisnik, 1978; Carney *et al*., 2010; Lin *et al*., 2011). Isso envolve o processamento integrado da MePD com o núcleo pré-mamilar ventral (PMV; Cavalcante et al., 2006) e pelo núcleo periventricular ântero-ventral (AVPV; de Vries e Simerly, 2002) e, adicionalmente, a liberação de prolactina pelo núcleo arqueado (Arc; Gu e Simerly, 1997).

Diferenças entre machos e fêmeas na citoarquitetura e funcionamento de áreas nervosas podem ocorrer antes da puberdade (Nishizuka e Arai, 1981; Zancan e*t al*., 2018). A seguir, porém, o aumento dos hormônios sexuais plasmáticos após ativação do eixo hipotálamo-pituitária-gônada que levam à iniciação da ovulação e da espermatogênese marcam a ocorrência da puberdade e da maturação sexual (Cooke e Wooley, 2009; Cooke, 2011; Bergan *et al*., 2014). As alterações físicas concomitantes e que envolvem mudanças nos comportamentos social e emocional estão relacionadas a remodelamento da forma neuronal e de circuitos neurais em áreas envolvidas com a reprodução (Cooke, 2011; Bergan *et al*., 2014).

Nesse contexto, a MePD pode estar envolvida com as mudanças centrais desencadeadas pela puberdade devido à sua relação com a reprodução e com a ação dos hormônios gonadais, ambos interrelacionados com esse período. A MePD apresenta dimorfismo sexual mesmo antes da puberdade, sendo maior no hemisfério esquerdo de machos do que em fêmeas pré-púberes (Cooke *et al*., 2007). Durante a puberdade, com o aumento de 5-10 vezes no nível de testosterona circulante, há aumento no volume da MePD (Ballard e Wood, 2007), além de haver um aumento significativo de vGlut2 e PSD-95, marcadores de sinapses excitatórias glutamatérgicas nesse subnúcleo (Cooke, 2011). Também os astrócitos na MePD são afetados pela puberdade (Johnson et al., 2013). Machos apresentam maior quantidade de astrócitos, bem como processos astrocitários mais elaborados no MePD direito e esquerdo, respectivamente, quando comparados com fêmeas, efeito que não ocorre em ratos que possuem disfunção do receptor para andrógenos (Johnson *et al*., 2008). Além de dimorfismo sexual antes e durante a puberdade, refinamento funcional dependente da experiência também pode ocorrer na MePD de ratos. A MePD

poderia, então, servir para a integração da ação dos hormônios gonadais e a codificação sináptica de todas as etapas do comportamento sexual ao mesmo tempo em que a experiência copulatória poderia gerar efeitos duradouros na atividade neuronal nessa área.

De fato, é provável que o fato de o animal apresentar experiência sexual possa modificar a atividade neuronal e as conexões estabelecidas em áreas relevantes para a reprodução (Kollack-Walker e Newman, 1997; Coolen e Wood, 1998; Cooke e Woolley, 2009). Em hamsters, sinais quimiossensoriais advindos da cópula ou da exposição a feromônios são capazes de aumentar a expressão da proteína Fos e atividade neuronal em diversas regiões do encéfalo, como na via vomeronasal, na área pré-óptica medial e na AMe de machos (Westberry e Meredith, 2003). Nesse circuito, a MeAD identifica a relevância social do estímulo feromonal e estimula a MePD para gerar a ativação dos núcleos hipotalâmicos relacionados com a excitação sexual (Westberry e Meredith, 2003; Hosokawa e Chiba, 2005). Ratos machos pós-púberes apresentam liberação de hormônio luteinizante dentro de 15 a 30 minutos após perceberem odores de fêmeas em período de ovulação, o que é seguido de aumento dos andrógenos circulantes (Bonilla-Jaime *et al*., 2006). Essa ação ocorre tanto em machos com experiência sexual quanto naqueles sem experiência sexual. No entanto, machos com experiência sexual são mais atraídos pelo odor de fêmeas em estro do que em diestro, sendo que essa preferência não ocorre com ratos sem experiência sexual (Agmo, 2003). Ratos com lesão na MePD apresentam menor atividade sexual, com aumento significativo da latência da ejaculação e dos intervalos entre ejaculações (revisado em Rasia-Filho *et al*., 2012b). Já estimulação elétrica da AMe como um todo facilita a ocorrência do comportamento sexual masculino em ratos sem experiência sexual embora, surpreendentemente, ratos com experiência sexual não apresentem diferença nessa atividade (Stark, 2005). Ademais, ratos com experiência sexual com lesão ampla da MeA apresentam apenas pequena redução da atividade sexual, enquanto ratos sem experiência sexual apresentam perda notável da atividade sexual (Kondo, 1992). Isso indica um papel preponderante da modulação da atividade sináptica na MePD relacionado com a ocorrência da experiência sexual em machos. Esse é um dos temas principais do primeiro artigo a ser apresentado adiante.

Adicionalmente, está bem descrito que a MePD de machos possui maior volume e que, em seu parênquima, há igualmente maior soma neuronal e estrutura do neurópilo diferente (revisado em Rasia-Filho *et al*., 2012a,b). Por exemplo, a orientação espacial dendrítica é diferente entre os sexos (machos apresentam-na preferentemente direcionada medialmente), embora o padrão de ramificação dendrítica seja a mesma em neurônios de machos e fêmeas (em diestro) (Dall'Oglio *et al*., 2008a). Um dos locais de marcante plasticidade neuronal dependentes dos esteroides sexuais são os dendritos e seus espinhos, o que será detalhado aqui.

Espinhos são unidades integradoras multifuncionais que formam compartimentos especializados pós-sinápticos com receptores para neurotransmissores capazes de modular propriedades eletrofisiológicas passivas e ativas e sequências bioquímicas intracelulares locais e ao longo de diferentes segmentos dendríticos (Nimchinsky *et al.*, 2002; Segal, 2005). São, portanto, os principais sítios celulares para as funções conectivas e integrativas dos neurônios e relevantes para a formação e estabilização de circuitos neurais e da plasticidade sináptica (Alvarez e Sabatini, 2007; Bourne e Harris, 2007; Yuste, 2011; de Vivo *et al*., 2013; Hill e Zito, 2013).

Os espinhos dendríticos são estruturas heterogêneas tanto morfologicamente quanto funcionalmente (Lee *et al.*, 2012; Rochefort e Konnerth, 2012). Dependendo de sua forma, podem aumentar a área disponível da densidade pós-sináptica e modular a voltagem relacionada com a sinapse realizada, afetando sua ação no dendrito, estabilidade e função integrada (Rochefort e Konnerth, 2012; Yuste, 2013). Uma vez que a maioria dos espinhos é conectada com axônio pré-sináptico pode-se dizer que seu número representa uma estimativa dos contatos realizados em cada célula (Arellano *et al*., 2007). O estudo dos espinhos dendríticos é, portanto, importante para o entendimento das bases celulares do funcionamento do sistema nervoso, sendo que a determinação da morfologia e da densidade desses espinhos tem servido para estimar tanto a ocorrência quanto a intensidade de sinapses (Sala e Segal, 2014). Isso fica ainda mais evidente pelo fato de que os espinhos dendríticos recebem a maioria dos terminais axonais contendo glutamato, tornando-se, dessa forma, um quarto

elemento celular para a modulação da atividade sináptica e da excitabilidade neuronal (Bourne e Harris, 2007; Lai e Ip, 2013; Brusco *et al*., 2014).

As diferenças no comprimento do pescoço e diâmetro da cabeça dos espinhos dendríticos sugerem que formas distintas podem impor processamentos biofísicos diversos para voltagem geradas pelas sinapses locais e a resistência do meio onde se encontram (Segev e Rall, 1998). Os espinhos dendríticos podem ser classificados como de tipo fino, achatado/espesso, com formato de cogumelo ou com outras formas menos frequentes, como com múltiplas ramificações a partir de um único pescoço ou formas atípicas (Peters e Kaiserman-Abramof, 1970; Woolley e McEwen, 1993; Wearne *et al*., 2005; Arellano *et al.*, 2007; Kim *et al*., 2007; Brusco *et al*., 2008; Brusco *et al*., 2010; Dall'Oglio *et al*., 2010, 2015). Formas intermediárias também podem ser visualizadas como um "*continuum*" entre as diferentes classificações (Arellano *et al*., 2007; Brusco *et al.*, 2010; Zancan *et al*., 2017). Essa característica de possuir uma variedade de formatos e tamanhos, sugere uma diversidade funcional para os espinhos dendríticos (Segal, 2010; Rasia-Filho *et al*., 2012a; Rochefort e Konnerth, 2012). De fato, as diferenças no tamanho da cabeça do espinho são correlacionadas com diferenças no tamanho da densidade pós-sináptica (Holtmaat e Svoboda, 2009; McKinney, 2010), o tipo e a distribuição de receptores na membrana pós-sináptica (como os receptores para glutamato do tipo AMPA e NMDA), a permeabilidade iônica seletiva dos receptores ionotrópicos e a alteração de voltagem após sua ativação (Kasai *et al*., 2010; Yuste, 2013). Espinhos dendríticos do tipo cogumelo têm maior densidade pós-sináptica à microscopia eletrônica, maior quantidade de mitocôndrias e apresentam polirribossomos para síntese proteica local na base do seu pescoço (Peters et al., 1991). O comprimento do pescoço do espinho pode gerar alta resistência local, limitando a difusão de íons, isolando eletricamente a cabeça do espinho do ramo dendrítico adjacente (Tonnesen *et al*., 2014) e criando uma compartimentalização dos processos bioquímicos nesse espinho (Segal, 2005; Alvarez e Sabatini, 2007; Rochefort e Konnerth, 2012). Essa propriedade também preservaria o dendrito de elevações nos níveis intracelulares de cálcio após a atividade sináptica mais intensa, protegendo contra a chance de morte neuronal por causa excitotóxica ainda que em situações fisiológicas (Segal, 2010). Adicionalmente, os espinhos podem ser afetados pela voltagem do

dendrito adjacente e modificar a atividade de receptores para glutamato do tipo NMDA, retirando seu bloqueio pelo magnésio (Kasai *et al*., 2010). Espinhos com localizações mais proximais ou mais distais nos ramos dendríticos podem impactar diferentemente na voltagem neuronal ou sofrer maior efeito da impedância dada pela estrutura dendrítica como um todo (Spruston *et al*., 2013). E, finalmente, podem servir como detectores de coincidência e sincronização de atividade sináptica aferente no tempo e no espaço para iniciar a ativação de sequências bioquímicas intracelulares que podem persistir por muito mais tempo que a atividade sináptica e, assim, formar memória celular, como é o caso da potenciação de longa duração (do inglês, 'LTP'; Bourne e Harris, 2007; Fiala e Harris, 2007; Kasai *et al*., 2010).

Na MePD, os dendritos apresentam uma quantidade moderada de espinhos e sua distribuição é homogênea ao longo do comprimento dendrítico (Marcuzzo *et al.*, 2007). Não há diferença na densidade de espinhos nos primeiros 40 µm dendríticos entre as colunas celulares medial e lateral da MePD (de Castilhos *et al.*, 2006) nem entre a MePD dos hemisférios direito e esquerdo de ratos machos adultos (Arpini *et al*., 2010). A densidade de espinhos dendríticos em machos é maior do que em fêmeas virgens em proestro, estro e metaestro ou que passaram pela experiência da maternidade e se encontram em diestro (Rasia-Filho *et al*., 2004; Rasia-Filho *et al*, 2012a). Isto é, machos apresentam cerca de 40% mais espinhos dendríticos do que fêmeas em proestro, estro ou metaestro; já as fêmeas, quando passam da fase de diestro para proestro, reduzem a densidade de espinhos dendríticos em 35% (Rasia-Filho *et al.*, 2004; Rasia-Filho *et al.*, 2012a). A porcentagem de espinhos dendríticos do tipo cogumelo em macho é duas vezes maior quando comparado com fêmeas em diestro e proestro (Rasia-Filho *et al.*, 2012a). A castração reduz a densidade de espinhos em machos, coincidindo no tempo com a redução evidente do comportamento sexual desses animais (Rasia-Filho *et al*., 1991; Rasia-Filho e Lucion, 1996; de Castilhos *et al.*, 2008, Zancan *et al*., 2017). A diminuição no número de espinhos dendríticos na MePD de fêmeas em proestro, quando os níveis de estrógeno e progesterona estão altos, é coincidente com a redução na imunorreatividade para sinapsina, o que pode estar associado com o estabelecimento de um menor número de contatos sinápticos em espinhos (Oberlander e Erskine, 2008), mas ainda com

maior ocorrência de contatos inibitórios no tronco dendrítico (Brusco *et al*., 2014). A atuação dos esteróides sexuais pode também alterar as regiões de aferências para a MePD e modificar sítios sinápticos, como os espinhos dendríticos, tornando-os sexualmente dimórficos quanto à densidade e forma (Rasia-Filho *et al*., 2004; Rasia-Filho *et al*., 2012a).

Neste contexto, a variação de número e de forma dos espinhos na MePD poderia estar relacionada com diferenças entre os sexos e de fatores sabidamente envolvidos na plasticidade sináptica, tema do primeiro artigo. Ademais, processos fisiológicos, como a puberdade e a aquisição de experiência sexual, poderiam ser capazes de modular a atividade sináptica de áreas nervosas específicas com alterações na estrutura e quantidade desses espinhos (Cooke, 2011), tema igualmente abordado no primeiro artigo.

1.4 Sinapses elétricas e conexinas na MePD

Na MePD de ratos, dados anteriormente descritos apontam para a prevalência de sinapses químicas como a base de sua organização estrutural e funcional. Não obstante, estudo prévio sugeriu a existência de sinapses elétricas entre células gliais nesse subnúcleo (Brusco *et al*., 2014). Há verdadeira importância da descrição de sinapses elétricas na MePD porque o acoplamento elétrico pode induzir respostas eletrofisiológicas em circuitos divergentes que são relevantes para gerar respostas rápidas frente a estímulos percebidos ou, alternativamente, servem para sincronizar atividade neuroglial e gerar atividade eferente para a ocorrência de comportamentos sociais (Rela e Szczupak, 2004; Petrulis, 2013; Rasia-Filho *et al*., 2012a).

O acoplamento elétrico intercelular que surge durante o desenvolvimento e persiste em animais adultos exige a interação coordenada de várias conexinas e pode: 1) auxiliar na sincronização neuronal (Christie *et al*., 2005); 2) atuar no desenvolvimento de circuitos neuronais (Yu *et al*., 2012); 3) fornecer uma via de baixa resistência para a transmissão rápida de íons difusíveis que alteram o potencial elétrico (Langer *et al*., 2012; Posłuszny, 2014); 4) levar à sincronização no disparo entre as células envolvidas no circuito neural (Wang *et al*., 2010); 5)

mudar a liberação de neurotransmissores, como o glutamato (Chever *et al*., 2014); 6) participar na propagação de sinais em redes neurogliais (Pannasch *et al*., 2012; Bukalo *et al*., 2013; Anders *et al*., 2014) e 7) promover oscilações nos circuitos neurais que estão relacionadas com a ocorrência de comportamentos que envolvem aprendizado e memória (Posłuszny, 2014; Mercer, 2012). O acoplamento elétrico dendrítico ou astrocitário pode gerar compartimentalização no processamento de informações em dendritos na MePD e/ou poderia sincronizar funções associadas dos neurônios usando a glia para transmitir essas informações e integrá-las em microdomínios (baseado em Rela e Szczupak, 2004). Em combinação com as sinapses químicas, o acoplamento celular elétrico poderia produzir funções complexas na MePD e poderia ser vantajoso para o funcionamento celular local porque auxiliaria a atividade neuronal sequencial e sincronizada para os machos identificarem as pistas olfativas/vomeronasais socialmente relevantes de fêmeas receptivas, para a ocorrência da intromissão, ejaculação, tempo do período pós-ejaculatório e saciedade sexual (Rela e Szczupak, 2004; Petrulis, 2013; Rasia-Filho *et al*., 2012a).

Para tanto, as gap junctions (denominadas como junções comunicantes, a partir deste ponto da tese) podem ser formadas por diferentes tipos de conexinas (Connors and Long, 2004), permitem a passagem de íons e moléculas de baixo peso molecular entre os elementos envolvidos, acoplando eletricamente células adjacentes e permitindo seu disparo sincrônico de atividade (Maeda e Tsukihara, 2011). As conexinas podem formar hemicanais nas células com possibilidade de se difundirem na membrana, atracar e combinar com outras conexinas para estabeler contato com a membrana de outra célula, embora somente uma pequena fração de todas as conexinas venha a formar um acoplamento funcional (Bennett e Zukin, 2004). Neste contexto, a conexina 36 (Cx36) é a mais abundante das conexinas acoplando neurônios (Schoenfeld *et al*., 2014). A conexina 43 (Cx43) é muito frequente no tecido nervoso e está presente no acoplamento entre astrócitos (Chever *et al*., 2014) e, no hipocampo, está co-localizada com a proteína ácida fibrilar glial (GFAP, Wu *et al*., 2015). Os astrócitos são acoplados através destes canais que formam uma rede fornecendo suporte metabólico e trófico aos neurônios liberando ATP (Franco-Perez *et al*., 2012). Ademais, a comunicação célula a célula pode estar organizada por canais

homotópicos, onde a mesma conexina forma ambos hemicanais, ou heterotópica onde diferentes conexinas formam os hemicanais. Esses últimos variam muito em condutância, permeabilidade, seletividade e propriedades de excitação (Harris, 2001), o que se reflete, por exemplo, no acoplamento entre um hemicanal formado por Cx36 e outro por conexina 45 (Cx45). A Cx45 tem sensibilidade à voltagem média e alta condutância de um único canal e facilita a sincronização de disparos celulares por elevar a condutância do seu canal quando as células estariam disparando simultaneamente (Söhl *et al*., 2005). Comparativamente, a sensibilidade à voltagem e a condutância são menores em um hemicanal formado por Cx36 e tais propriedades divergentes influenciam o comportamento rítmico das células (Söhl *et al*., 2005).

A expressão de diferentes conexinas na MePD poderia sugerir implicações adicionais funcionais e plásticas para esse subnúcleo. O estudo descritivo da expressão e dos achados de imunofluorescência para Cx36, Cx43 e Cx45, bem como a apresentação de imagens de junções comunicantes por microscopia eletrônica de transmissão na MePD de ratos, compõe o segundo artigo desta tese.

O conhecimento sobre sinapse elétrica na MePD é relevante na modulação integrada de estímulos neuroquímicos com os hormonais para os comportamentos sociais relacionados à reprodução e sobrevivência da espécie.

1.5 Papel dos fatores neurotróficos na plasticidade sináptica

Os hormônios gonadais podem atuar de forma direta para modular a estrutura neuroglial e/ou podem afetar a síntese e ação de fatores neurotróficos no SNC. É o caso das ações neurais de BDNF (fator neurotrófico derivado do encéfalo), IGF-1 (fator de crescimento semelhante à insulina tipo 1), PSA-NCAM (forma polisialilada da molécula de adesão celular neuronal) e Efrina-A4 (Luine e Frankfurt, 2013; O'kusky *et al*., 2003; Varea *et al*., 2007; Lippman and Dunaevsky, 2004).

O BDNF pode modular a estrutura neuronal e a plasticidade sináptica (Luine and Frankfur, 2013). Esse processo gradual requer a mobilização de proteínas

intracelulares, especialmente a actina, notavelmente concentrada nos espinhos dendríticos (revisado por Penzes and Rafalovich, 2012). Existem duas formas como o BDNF atua nos espinhos dendríticos: por efeitos rápidos na membrana celular ou de forma mais lenta envolvendo regulação da transcrição pela via CREB (proteína ligante de elemento responsivo ao cálcio; Srivastava *et al*., 2013). A ligação do BDNF ao seu principal receptor, TrKB, resulta na ativação de diversas vias de sinalização para a plasticidade sináptica (Santos *et al*., 2010) onde RNAm são desinibidos, os receptores TrKB são inseridos na membrana plasmática, pró-BDNF e PAt (ativador do plasminogênio tecidual, que converte plaminogênio em plasmina) são elaborados para serem liberados na fenda sináptica (Luine and Frankfur, 2013; Srivastava *et al*., 2013). Dessa forma, pró-BDNF é convertido em BDNF pela plasmina, BDNF se liga ao TrKB na membrana dendrítica local; a ativação do TrKB pelo BDNF aumenta a translação de CaMKII (proteína quinase dependente de cálcio/calmodulina), GluR1 (proteína integral de membrana da família dos canais iônicos modulados por glutamato e componente do receptor do tipo AMPA), Arc (proteínas associadas ao citoesqueleto), e LIMK1 (domínio k da quinase Lim), induzindo aumento da formação do receptor para glutamato de tipo AMPA e sua inserção na membrana e, por fim, aumento da polimerização de actina (Luine e Frankfurt, 2013; Santos *et al*., 2010). A sinalização via TrkB também induz à fosforilação de receptores para glutamato de tipo NMDA, sinapsina-1, quinase ativada por p21 (PacK) e cofilina, aumentando assim a atividade destes receptores para glutamato, fusão da membrana plasmática vesicular e a liberação de neurotransmissores e a polimerização da actina, respectivamente (Waterhouse e Xu, 2009). Nesse contexto, a cofilina, regulador da polimerização da actina, e o próprio receptor de tipo AMPA também podem alerar a estrutura e função dos espinhos dendríticos (Ferri *et al*., 2014).

O IGF-1, por sua vez, é uma citocina solúvel com efeitos parácrinos tróficos nas células neuronais e com efeitos como hormônio na secreção hipotalâmica (Fernandez–Galaz *et al*., 1997). No Arc, área sexualmente dimórfica, os níveis de IGF-1 são diretamente regulados pelo estradiol e, dessa forma, ao mesmo tempo que há um aumento nos níveis de IGF-1 (quando do aumento nos níveis de estrógeno em circulação) ocorre remodelamento dos contatos sinápticos e dos processos gliais em seu entorno evidente em fêmeas em proestro e estro

(Fernandez-Galaz *et al*., 1999). Ou seja, fêmeas na tarde do proestro apresentam alta imunorreatividade para IGF-1, quando há o pico de estrógeno plasmático, permanecem alto na manhã do estro e retornando para níveis basais quando em metaestro. Nesse período há reestruturação sináptica e funcional no Arc para participação integrada no mecanismo de secreção neuroendócrina para a ovulação e o comportamento sexual feminino (Fernadez-Galaz, *et al*., 1997; Fernandez-Galaz *et al.,* 1999;; Azcoitia *et al*., 2002).

A molécula de adesão neural (NCAM) é uma das macromoléculas da superfície celular envolvida na sinaptogênese, regeneração e adesão celular (Tan *et al*., 2009). A adição de um homopolímero, o PSA, ao NCAM facilita processos de migração celular, crescimento neurítico e plasticidade sináptica (Naftolin et al., 2007). Especificamente na MePD, o PSA-NCAM é necessário para a emergência da atração sexual por fêmeas e aumento da atividade sexual de machos durante a puberdade (Cooke e Job, 2015). Surpreendentemente, não está ligada aos níveis de testosterona, indicando que na puberdade a elevação dos níveis de PSA-NCAM é controlada por outros fatos independentemente da ação direta de andrógenos (Cooke e Job, 2015).

A Efrina A4 pertence à família dos receptores tirosina quinase (Takasu *et al*., 2002), possui importante propriedade de modificar a forma e dinâmica celular ao promover a reorganização do citoesqueleto, especificamente a actina. Por mecanismos ainda não elucidados, onde protusões dendríticas longas e finas são substituídas por espinhos dendríticos cuja forma passa a ser variada, o receptor para Efrina-A4 é um potente regulador da plasticidade de espinhos da area CA1 do hipocampo (Murai *et al*., 2003). A interação da Efrina-A4 com a Efrina-A3, localizadas em astrócitos peri-sinápticos, contribui para a manutenção e estabilização dos espinhos dendríticos no hipocampo (Murai e Pasquali, 2011).

Sabe-se que a atuação dos esteróides sexuais pode alterar as regiões de aferências com o MePD e, desta forma, modificar sítios pós-sinápticos, como os espinhos dendríticos, tornando-os sexualmente dimórficos quanto à sua densidade em ramos proximais (Rasia-Filho et al., 2004; Rasia-Filho et al., 2012a). A densidade de espinhos dendríticos é maior em machos do que em fêmeas virgens em proestro, estro e metaestro ou que passaram pela experiência

da maternidade e se encontram em diestro (Rasia-Filho et al., 2004). Machos apresentam cerca de 40% mais espinhos dendríticos do que fêmeas em proestro, estro ou metaestro; já as fêmeas, quando passam da fase de diestro para proestro, reduzem a densidade de espinhos dendríticos em 35% (Rasia-Filho et al., 2004; 2012a). Ademais, a castração reduz a densidade de espinhos em machos, coincidindo no tempo com a redução evidente do comportamento sexual desses animais (de Castilhos et al., 2008; Zancan et al., 2017). E a reposição hormonal com estradiol e progesterona, após a castração de fêmeas, faz aumentar o número de espinhos dendríticos (de Castilhos et al., 2008). Não se sabe, no entanto, se há envolvimento de fatores neurotróficos, como o BDNF, IGF-1, PSA-NCAM e Efrina A4, na modulação da estrutura e função da MePD. De fato, não há dados na literatura evidenciando o papel desses fatores neurotróficos com a plasticidade sináptica na MePD, nem de possível dimorfismo sexual ou da variação dos níveis desses fatores ao longo do ciclo estral e sua possível relação com as modificações na estrutura neuronal local em ratos. Dessa forma, a análise da expressão gênica e protéica de BDNF, IGF-1, PSA-NCAM e Efrina A4 em machos e fêmeas ao longo do ciclo estral compõe o terceiro artigo desta tese.

Os dados desta tese são relevantes para as áreas de Morfologia e Neurociências e contribuem para os esforços que estão sendo realizados para descrever a organização e a plasticidade sinápticas dos neurônios da MePD de ratos, como modelo animal. Contribuem também para compreender as características morfológicas e funcionais dos neurônios da MePD e sua implicação funcional integrada nos circuitos envolvidos com a modulação de diferentes comportamentos sociais.

2. OBJETIVOS

2.1 Objetivos gerais

Estudar a organização e a plasticidade estruturais e sinápticas na MePD de ratos e possível dimorfismo sexual e efeito da puberdade e experiência sexual nos espinhos dendríticos, presença de sinapses elétricas e diferentes conexinas como seus componentes protéicos e a expressão de diferentes fatores neurotróficos locais em machos e em fêmeas ao longo do ciclo estral.

2.2 Objetivos específicos

Estudar a presença, o número e a forma de cada tipo de espinho dendrítico na MePD de ratos machos e de fêmeas pré-púberes empregando-se técnica com corante fluorescente DiI e microscopia confocal.

Estudar a presença, o número e a forma de cada tipo de espinho dendrítico na MePD de ratos pós-púberes sem experiência sexual e com experiência sexual, empregando-se técnica com corante fluorescente DiI e microscopia confocal.

Identificar a presença de junções comunicantes na MePD de ratos adultos utilizando microscopia eletrônica de transmissão.

Estudar a presença das conexinas 36, 43 e 45, componentes protéicos de junções comunicantes em neurônios e células da glia, na MePD de ratos adultos empregando-se técnica imunohistoquímica e de fluorescência.

Estudar o dimorfismo sexual e a variação do ciclo estral na expressão gênica, por técnica de qRT-PCR, e a presença, número de puncta ou células imunomarcadas e a intensidade de fluorescência, por técnica imunohistoquímica para BDNF, IGF-1, PSA-NCAM e Efrina-A4 na MePD de ratos adultos.

3. MÉTODOS E RESULTADOS

3.1 Artigo Científico I

European Journal of Neuroscience – Impact factor 2.832

Received: 21 December 2017 | Revised: 18 April 2018 | Accepted: 13 June 2018

DOI: 10.1111/ejn.14052

RESEAR CHREPORT

WILEY EJN European Journal of Neuroscience FENS

Remodeling of the number and structure of dendritic spines in the medial amygdala: From prepubertal sexual dimorphism to puberty and effect of sexual experience in male rats

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Funding information
Conselho Nacional de Desenvolvimento Científico e Tecnológico, Grant/Award Number: 141867/2015-9, 306594/2016-1 and 306644/2016-9

Abstract

The posterodorsal medial amygdala (MePD) is a sexually dimorphic area and plays a central role in the social behavior network of rats. Dendritic spines modulate synaptic processing and plasticity. Here, we compared the number and structure of dendritic spines in the MePD of prepubertal males and females and postpubertal males with and without sexual experience. Spines were classified and measured after three-dimensional image reconstruction using DiI fluorescent labeling and confocal microscopy. Significantly differences are as follows: (a) Prepubertal males have more proximal spines, stubby/wide spines with long length and large head diameter and thin and mushroom spines with wide neck and head diameters than prepubertal females, whereas (b) prepubertal females have more mushroom spines with long neck length than age-matched males. (c) In males, the number of thin spines reduces after puberty and, compared to sexually experienced counterparts, (d) naive males have short stubby/wide spines as well as mushroom spines with reduced neck diameter. In addition, (e) sexually experienced males have an increase in the number of mushroom spines, the length of stubby/wide spines, the head diameter of thin and stubby/wide spines and the neck diameter of thin and mushroom spines. These data indicate that a sexual dimorphism in the MePD dendritic spines is evident before adulthood and a spine-specific remodeling of number and shape can be brought about by both puberty and sexual experience. These fine-tuned ontogenetic, hormonally and experience-dependent changes in the MePD are relevant for plastic synaptic processing and the reproductive behavior of adult rats.

KEYWORDS
dendritic spine density, dendritic spine morphometry, dendritic spine shape, sex difference, social behavior network, synaptic plasticity

Abbreviations: ANOVA, analysis of variance; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; HD, head diameter; i.p., intraperitoneal; IU, international unit; MeA, medial amygdala; MePD, posterodorsal medial amygdala; NAc, nucleus accumbens; ND, neck diameter; NL, neck length; PB, phosphate buffer; RT, room temperature; *SD*, standard deviation; SL, spine length; ST, stria terminalis; 3D, three-dimensional.

†These authors contributed equally to the present work.

Edited by Marco Capogna. Reviewed by Emma Louth and Maya Frankfurt.

All peer review communications can be found with the online version of the article.

1 | INTRODUCTION

The posterodorsal medial amygdala (MePD), part of the extended amygdala (de Olmos, Beltramino, & Alheid, 2004), is an important site for the modulation of social behaviors in rats (Newman, 1999; Rasia-Filho et al., 2012a, 2012b). This sexually dimorphic area has one of the highest density of receptors for androgens, estrogen and progesterone in the brain (De Vries & Simerly, 2002; Gréco, Edwards, Michael, & Clancy, 1996; Gréco, Edwards, Zumpe, Michael, & Clancy, 1998; Simerly, Swanson, Chang, & Muramatsu, 1990). Gonadal hormones acting on neurons and glial cells modulate the MePD synaptic organization in males and females (Brusco et al., 2014; Cooke, Stokas, & Woolley, 2007; Cooke & Woolley, 2009a, 2009b; Dall'Oglio, Gehlen, Achaval, & Rasia-Filho, 2008; Johnson, Breedlove, & Jordan, 2013; Malsbury & McKay, 1994; Rasia-Filho, Fabian, Rigoti, & Achaval, 2004; Zancan et al., 2015). One of the effects of these hormones is the induction of the masculine differentiation of the MePD during the early postnatal period, when increase both the nuclear volume and the occurrence of local axodendritic synapses (Mizukami, Nishizuka, & Arai, 1983; Nishizuka & Arai, 1981).

Several lines of evidence demonstrated the MePD activation by socially relevant environmental cues (Bergan, Ben-Shaul, & Dulac, 2014; Kondo & Sachs, 2002; Li et al., 2017; Newman, 1999). The MePD forms a network involving the accessory olfactory bulb and the vomeronasal inputs (Bian, Yanagawa, Chen, & Luo, 2008; Hashikawa, Hashikawa, Falkner, & Lin, 2016; Pereno, Balaszczuk, & Beltramino, 2011), specific parts of the bed nucleus of the stria terminalis (Dong, Petrovich, & Swanson, 2001) and reproduction-related hypothalamic nuclei (Choi et al., 2005; Petrovich, Canteras, & Swanson, 2001). Then, the MePD can serve as an interface for the processing of chemosignals and the actions of sex steroids to elaborate proper reproductive behavior display (Baum & Bakker, 2013; Blake & Meredith, 2011; Petrulis, 2013; Rasia-Filho et al., 2012a, 2012b; Wood & Coolen, 1997). Indeed, the MePD has a marked Fos activation after exposure to conspecific pheromones and genital sensory stimuli or the occurrence of intromission, ejaculation and, depending on the species, sexual satiety (Coolen, Peters, & Veening, 1997; Hashikawa et al., 2016; Kollack-Walker & Newman, 1997; Phillips-Farfán & Fernández-Guasti, 2009).

Puberty is an ontogenetic period of the development when young animals become sexually mature adults, showing significant changes in their physical structure, endocrine secretion, emotional and social behavior (Bergan et al., 2014; Cooke & Woolley, 2009a; Cooke, 2011). The hypothalamic–pituitary–gonadal axis is activated and increases the secretion of sex steroids needed for ovulation and spermatogenesis. Gonadal hormones modify previously established neural circuits, redefine synaptic transmission and the display of adult-typical social behaviors (Bergan et al., 2014; Cooke & Woolley, 2009b; Johnson et al., 2013; Koss, Belden, Hristov, & Juraska, 2014; Zehr, Todd, Schulz, McCarthy, & Sisk, 2006). The pubertal male rat MePD undergoes a synaptic remodeling process as the volume of this nucleus further enhances and shows a significant increase in vesicular glutamate transporter-2 and postsynaptic density 25, two markers of glutamatergic synaptic transmission (Cooke, 2011). Glutamate accounts for ~70% of all ejaculation-activated cells following mating in the MePD of gerbils (Simmons & Yahr, 2003). Glutamate microinjected in the MePD facilitates ejaculation in adult male rats (Rasia-Filho et al., 2012b). Besides the execution of innate behaviors, experience-dependent functional refinements can also occur in the male MePD. That is, the MePD is shaped for the integration of gonadal hormones and the synaptic coding of sexual activity at the same time that experience and its lasting effects change the neuronal activity in this area. For example, electrical stimulation of the medial amygdala (MeA) of male rats leads to an increase in the frequency of mounting and in the investigative behavior of males to other conspecific animals, but only animals with no prior sexual experience show these effects (Stark, 2005). In the hamster MePD, the pattern of Fos expression reflects the conditioned responses to environmental cues associated with the sexual experience pretests and the occurrence of mating to different endpoints of copulation (i.e., intromissions or one to five ejaculations; Kollack-Walker & Newman, 1997). In conjunction, these data indicate that pubertal and sexual experience-dependent changes are relevant for the development of the reproduction-related neural circuit in the male MePD.

At the cellular level, dendritic spines are specialized components for most excitatory postsynaptic processing, integration and strength (Bourne & Harris, 2007; Hayashi-Takagi et al., 2015; Sala & Segal, 2014; Spruston, Häusser, & Stuart, 2013; Yuste, 2013). Dendritic spines can be modulated by genetic programming and hormonally mediated development as well as provide experience-dependent plasticity to neural circuitries (Bourne & Harris, 2007; Nishizuka & Arai, 1983; Pitchers et al., 2010; Simerly, 2000). The number of dendritic spines in the MePD is sexually dimorphic, affected by circulating levels of sex steroids (Rasia-Filho et al., 2004, 2012a; Zancan, Dall'Oglio, Quagliotto, & Rasia-Filho, 2017) and reduced after castration in prepubertal males (Cooke, 2011; Cooke & Woolley, 2009a) and in adult male and female rats (de Castilhos, Forti, Achaval, & Rasia-Filho, 2008; Zancan et al., 2017). Spines are classified in different types which, based on region-specific characteristics, can reflect their synaptic functions (Bourne & Harris, 2007; Harris, Jensen, & Tsao, 1992; Spruston et al., 2013; Tønnesen & Nägerl, 2016; Yuste, 2013). Dendritic spines in the rat MePD are pleomorphic (Brusco et al., 2010), and detailed morphometric data

have recently unraveled additional features regarding the modifications in the spine number, shape and structure after adult male castration (Zancan et al., 2017). No such detailed data are currently available for the dendritic spines comparing the prepubertal differences between sexes or after puberty and sexual experience in the male rat MePD.

Therefore, the aims of the present work were twofold: (a) to compare the number and structure of dendritic spines in the MePD of prepubertal males and females and (b) the dendritic spines in the MePD of prepubertal males and both postpubertal näive or sexually experienced males. We used 1,1′-di octadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) dye fluorescent labeling, confocal microscopy and three-dimensional (3D) image reconstruction to depict prepubertal sex differences and the effects of both puberty and sexual experience in the density, shape and morphometric parameters of dendritic spines of different classes in the rat MePD.

2 | MATERIALS AND METHODS

The present methodological approach is similar to that reported by Zancan et al. (2017 and references therein).

2.1 | Animals

Animals were Wistar rats from a local breeding facility (Federal University of Health Sciences-UFCSPA, Porto Alegre, Brazil), housed in groups (no isolation, and up to three animals per standard cage of 41 × 34 × 16 cm) under laboratory conditions with food and water ad libitum, room temperature at 22–24°C and light/dark cycle of 12 hr.

Males ($N = 18$) and females ($N = 6$) were used. Females were studied at the prepubertal period (30 days old). Males were randomly divided into three experimental groups ($n = 6$ each): (a) prepubertal (30 days old), which served for the comparisons with prepubertal females and also with both groups of adult males; (b) postpubertal (90 days old) without sexual experience, that is, rats that were reared only with other males in the same cage; (c) postpubertal with sexual experience, that is, rats that were maintained with females during 3 weeks prior to the beginning of the experiment. These females became pregnant.

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 (National Institutes of Health Guide for the Care and Use of Laboratory Animals - DHEW Publication 80-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205) and the local Animal Ethics Committee (UFCSPA, Brazil; protocol no. 163/15).

2.2 | Experimental procedure

Rats were anesthetized with xylazine (10 mg/kg, i.p.) and thiopental (120 mg/kg, i.p.). Transcardiac perfusion was carried out after heparin (1,000 IU) injection in the left ventricle using 200 ml of 1.5% formaldehyde diluted in phosphate buffer (PB; 0.1 M, pH 7.4) at room temperature (RT). Using a peristaltic pump, perfusion flow was initially rapid (90 s) and, then, slowed to last for additional 20 min in order to maintain fine ultrastructural synaptic integrity (Tao-Cheng, Gallant, Brightman, Dosemeci, & Reese, 2007). Brains were kept in fixative solution for one additional hour and then washed with PB. Coronal sections (200-μm thick) were performed with a vibrating microtome (VT 1000S, Leica Microsystems, Germany). The MePD was localized in the ventral part of the telencephalon at the coordinates 3.0 to 3.4 mm posterior to the bregma, lateral to the optic tract and ventral to the stria terminalis (ST;

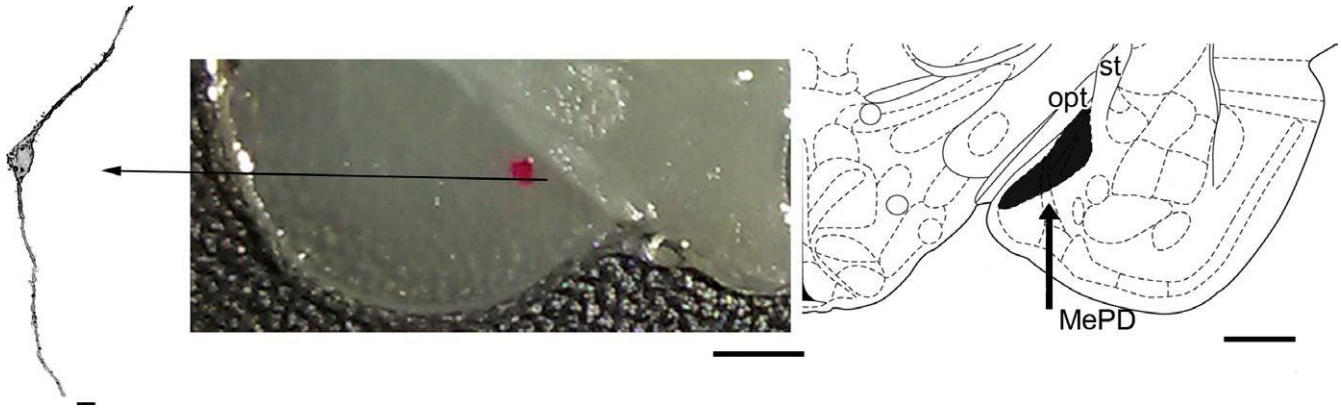

FIGURE 1 Left: Ventral aspect of a coronal rat brain section showing DiI dye powder (dot) placed to diffuse to the adjacent posterodorsal medial amygdala (MePD). Scale bar = 1 mm. Labeled spiny neurons were imaged as the representative bitufted one reconstructed by confocal microscopy. Scale bar = 10 μm. Right: Schematic diagram of a matched brain section showing the MePD (at −3.3 mm posterior to the bregma) marked in black, lateral to the optic tract (opt) and ventral to the stria terminalis (st). Adapted from the rat brain atlas of Paxinos and Watson (2008) Scale bar = 1 mm. [Colour figure can be viewed at wileyonlinelibrary.com]

Paxinos & Watson, 2008; Figure 1). Fine-powdered carbocyanine fluorescent dye DiI (Molecular Probes, USA) was applied on the ST and laterally to the MePD using the tip of a fine needle (Figure 1). DiI was initially maintained on the brain slices for 18 hr with PB at RT. The sections were then postfixed using a 4% formaldehyde solution for 30 min and washed with PB again. Sections were placed onto slides using "Fluoromount G" ("antifading medium solution", refractive index = 1.4, Electron Microscopy Sciences, USA) and were imaged along 7 days after slides were mounted (Brusco et al., 2010; Rasia-Filho, Brusco, Rocha, & Moreira, 2010). We used a confocal microscope (Leica TCS SP8, Germany) with a plan apochromatic 63×/1.4 water-immersion objective lens. Spectral detectors were adjusted to capture emission from lasers wavelength of 555 nm. The *z*-stack acquisition was performed at 0.1 μm using a resolution of 1,024 × 1,024 pixels with four times zooming, providing a voxel size of approximately 55 × 55 × 300 nm (Brusco et al., 2010).

Including criteria for selecting and studying neurons and their proximal spiny dendrites in all experimental groups were as follows: (a) Cell must be within the boundaries of the MePD and relatively distant from all its ultimate limits; (b) near the middle third of the section and have high quality fluorescent dendrites with, at least, initial 40-μm-length segments and on; (c) have well-defined borders and clearly distinguishable spines compared with the background; and, (d) be relatively isolated from neighboring cells to avoid "tangled" dendrites. The first neurons that randomly fulfilled these criteria were imaged and coded for further study. Right and left hemispheres were studied apart. Using this approach, the MePD usually provides homogeneous morphological data which are obtained almost exclusively from local bitufted multipolar neurons (see additional data and comments in Arpini, Menezes, Dall'Oglio, & Rasia-Filho, 2010; de Castilhos et al., 2006; Rasia-Filho et al., 2012a, 2012b; Zancan et al., 2017). The mean number and range (minimum and maximum values, respectively) of different MePD neurons studied from each rat and each experimental group were as follows: prepubertal females (12, 9–14), prepubertal males (12, 7–16), postpubertal naive males (11, 9–13) and postpubertal males with sexual experience (9, 7–11).

Spines were counted and measured along the proximal 40 μm dendritic branches (as previously done by Rasia-Filho et al. (2004) and de Castilhos et al. (2008); Figure 2). For this purpose, each acquired sequence of *z* stacks was summed and aligned to compose 3D images. The same sampling density for pixel size and *z*-step advance and the same image acquisition procedures were performed for all brain sections and experimental groups (based on Heck, Betuing, Vanhoutte, & Caboche, 2012). The blurring and the "pixelated" aspect of the borders of the spines were kept to as minimal as possible (Zancan et al., 2017). The 3D reconstruction of the dendritic segments and their pleomorphic spines was performed using the software Image

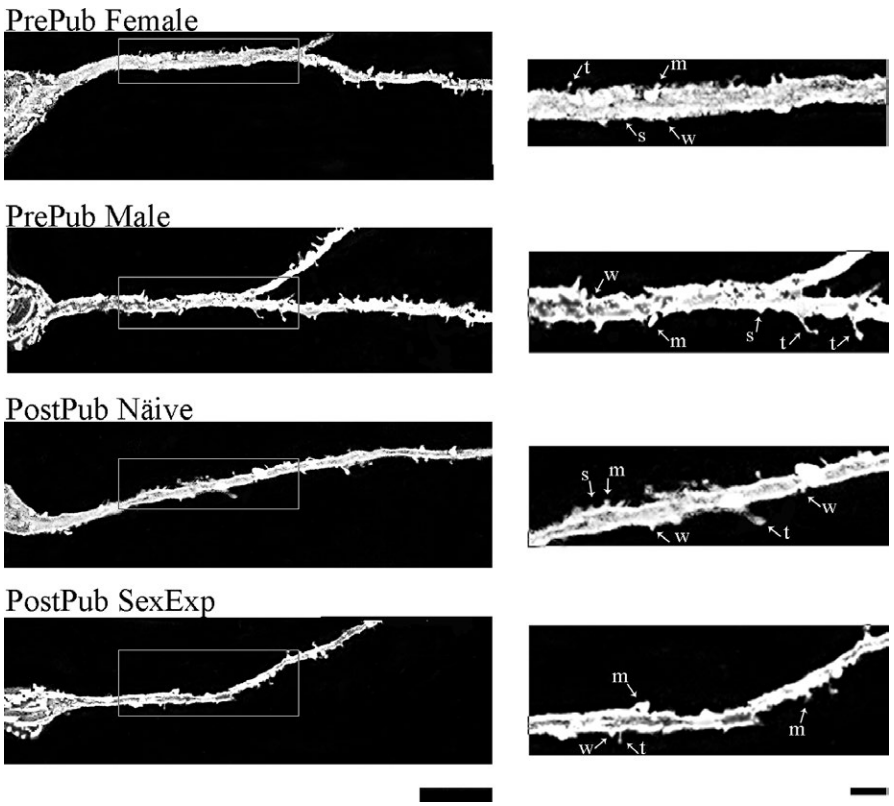

FIGURE 2 Digitized fluorescent images of neurons labeled with DiI and reconstructed by confocal microscopy. Representative proximal dendritic branches showed pleomorphic spines in the posterodorsal medial amygdala of rats from groups of prepubertal females, prepubertal males, postpubertal males without sexual experience (näive) and postpubertal males with sexual experience. Scale bar = 10 μm for images in the left and 5 μm for those in the right

Pro Plus 7.0 (Media Cybernetics, USA). Spine images were then further enhanced by 200× or 400× and each reconstructed spine was counted, classified and measured, as described below, by two independent researchers. As a rule, the coincidence for the results between researchers was near 100% and, when necessary, a third researcher, blind to the experimental condition, proceeded to determine final values.

2.3 | Dendritic spine counting, classification and measurements

Dendritic spine density was calculated as the total number of spines divided by the length of the dendrite studied (Rasia-Filho et al., 2004).

Each dendritic spine was visualized along the *z*-stack microscopic planes and the length, width and general aspect were used for its classification. Length was defined as the distance from the base at the dendritic shaft to the top of the spine or its neck; diameter was defined as the maximum distance perpendicular to the long axis of the spine and was measured for the neck or and the head of the spine (Ryu et al., 2006). Then, from the 3D reconstructed images, spines were classified and counted according to morphological criteria based on spine length (SL), neck length (NL), neck diameter (ND), head diameter (HD) and the number of protrusions from a single stalk (Dall'Oglio, Dutra, Moreira, & Rasia-Filho, 2015; Zancan et al., 2017 and references therein). Based on their shapes, spines were classified into the following: (a) thin (when SL > HD and HD > ND), (b) mushroom-like (HD ≫ ND), (c) stubby/wide (HD > SL), (d) ramified (with a single stalk that branches in two heads) or (e) atypical (when showing a transitional aspect between classes or an unusual shape not classified in the other classes (based on Arellano, Benavides-Piccione, DeFelipe, & Yuste, 2007; Brusco et al., 2010, 2014; Dall'Oglio et al., 2015; Harris et al., 1992; Stewart, Popov, Kraev, Medvedev, & Davies, 2014; Zancan et al., 2017 and references therein). Representative examples are shown in Figures 2 and 3. Images had final fine adjustments of contrast made in Photoshop CS3 (Adobe Systems, Inc., USA) without altering spine counting or morphological classification.

The mean number and range (minimum and maximum values, respectively) of dendritic spines studied from each rat and each experimental group were as follows: prepubertal females (222, 165–283), prepubertal males (245, 150–297), postpubertal naive males (245, 188–259) and postpubertal males with sexual experience (245, 161–294). Morphometric data from up to 15 spines of each type in each studied dendrite were obtained after further enhancing (400×) the 3D reconstructed images. Similar sampling density (pixel size and *z*-step) and image acquisition procedures were held constant, and both blurring "pixelated" aspects of the spine borders were kept to as minimal as possible (Zancan et al., 2017).

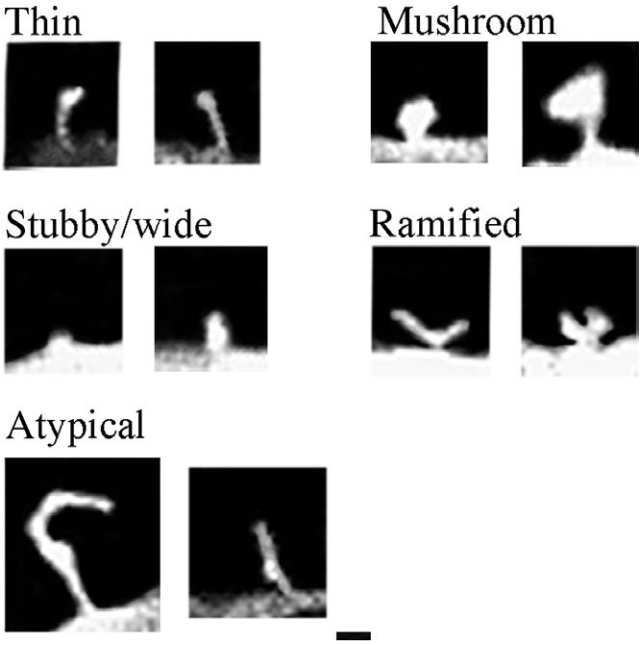

FIGURE 3 Three-dimensional reconstructed images of dendritic spines from the rat posterodorsal medial amygdala imaged by confocal microscopy. Representative examples of each spine type classified as thin, mushroom-like, stubby/wide, ramified or atypical. Scale bar = 1 μm

Measurements (in micrometers) were made on the "z" stack images that provided the best visualization of well-defined borders of the spine and its components using the software Image Pro Plus 7.0 (Media Cybernetics, USA).

2.4 | Statistical analysis

Average values for each animal and experimental group were used for the statistical analyses. Data are presented as mean ± standard deviation (*SD*) and scatter plots for the data of each variable from each rat and experimental group according to the recommendations found in Rousselet, Foxe, and Bolam (2016). The Kolmogorov–Smirnov test and the Bartlett test were used to determine data normality and homocedasticity, respectively. The density of dendritic spine was submitted to the *t* test to compare prepubertal male and female data and to a two-way analysis of variance (ANOVA) test to compare the three groups of male rats (prepubertal, postpubertal naive and sexually experienced) for the effects of experimental condition and of hemispheric laterality. Data for the number of each spine type per group and their corresponding morphometric values were compared by the *t* test between prepubertal males and females and by a one-way ANOVA test followed by the Tukey test among the three male groups. When nonparametric, data were compared by the Kruskal–Wallis test and the Dunn post hoc test. In all cases, the statistical significance level was set as $p < 0.05$. We used the GraphPad InStat version 3.0 (GraphPad Software, USA).

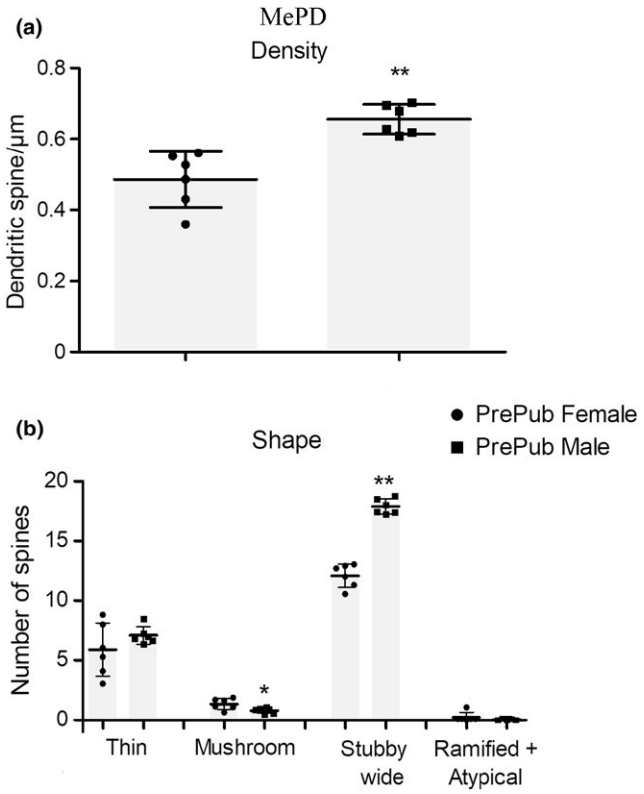

FIGURE 4 Values (mean ± *SD*) for (a) the density of overall proximal dendritic spines and (b) the number of each type of dendritic spine (classified by their shapes) in the rat posterodorsal medial amygdala (MePD) of prepubertal females and males ($n = 6$ in both groups). Symbols (●■) represent the mean values for each studied rat per group, some data overlapping. *$p < 0.05$, **$p < 0.01$ in each comparison indicated

3 | RESULTS

Dendritic spines showed a *continuum* of shapes and sizes (Figure 2) and were counted, classified and measured in proximal dendritic branches of all experimental groups (Figures 4–7). Thin, mushroom-like and stubby/wide spines were the most common types found in the MePD of males and females (Figures 4 and 6).

The comparisons of prepubertal male and female data are shown in Figures 4 and 5. No statistically significant difference was found for the density of dendritic spines in the right and left MePD [$t(5) = 1.893$; $p = 0.116$ for males and $t(5) = 1.990$; $p = 0.103$ for females]. Then, data from both hemispheres were pooled together and compared between sexes. High significant differences were found for the density, number of each type of spine and their morphometric details between prepubertal groups. That is, compared to females, prepubertal males have (a) more proximal spines [$t(11) = 5.37$; $p < 0.001$; Figure 4a], most of them are stubby/wide shaped [$t(11) = 8.627$; $p < 0.001$; Figure 4b] with a longer spine length [$t(11) = 8.346$; $p < 0.001$; Figure 5a], (b) both thin and mushroom spines with large neck diameters [$t(11) = 10.44$; $p < 0.001$ and $t(10) = 4.825$; $p < 0.001$, respectively; Figure 5c] and (c) thin, mushroom and stubby/wide spines with large head diameters [$t(11) = 10.58$; $p < 0.001$; $t(10) = 2.321$; $p = 0.042$; and, $t(11) = 3.037$; $p = 0.011$, respectively; Figure 5d]. On the other hand, prepubertal females have more mushroom spines [$t(11) = 2.426$; $p = 0.033$; Figure 4b] with longer neck length than male counterparts [$t(10) = 2.808$; $p = 0.018$; Figure 5b]. The slight occurrence of ramified and atypical spines precluded statistical comparisons.

The comparisons of prepubertal and postpubertal male data are shown in Figures 6 and 7. There were no statistically significant difference in the density of proximal dendritic spines in the MePD of males from the groups prepubertal, postpubertal without sexual experience and postpubertal with sexual experience [$t(2,15) = 0.30$; $p = 0.74$; Figure 6a], nor were differences in the hemispheric laterality [$F(1,15) = 0.01$; $p = 0.97$] or in the interactions of these two factors [$F(2,15) = 1.90$; $p = 0.18$]. Because no hemispheric difference was found, data were pooled together to be further analyzed among these groups.

Marked differences were found in the number of each type of spine in the MePD of the three male experimental groups. Data are shown in Figure 6b. That is, for the number of proximal spines of males: (a) Thin spines were higher in prepubertal males than in postpubertal sexually experienced ones [$F(2,33) = 3.93$; $p = 0.02$; and, post hoc $p < 0.05$]; (b) mushroom spines were higher in postpubertal sexually experienced males than in the two other groups (KW $p < 0.01$; and, post hoc $p < 0.01$ in both cases), (c) stubby/wide spines did not differ between groups taking the statistical level set a priori [$F(2,33) = 3.05$; $p = 0.06$]; and, (d) ramified and atypical spines were not different among groups (KW $p = 0.36$), but their occurrence was so slight that no further conclusions were taken for these two last classes studied here.

In addition, the following results were obtained for the morphometric data: (a) Thin spines did not differ between male groups in total length [$F(2,33) = 1.73$; $p = 0.19$] and neck length (KW $p = 0.37$), but neck diameter [$F(2,33) = 3.76$; $p = 0.03$] and head diameter [$F(2,33) = 4.51$; $p = 0.01$] showed higher values in postpubertal males with sexual experience than in prepubertal rats ($p < 0.05$ in both cases; Figure 7c,d, respectively); (b) mushroom spines did not differ between groups in total length [$F(2,32) = 0.19$; $p = 0.31$], neck length [$F(2,32) = 0.44$; $p = 0.64$] and head diameter [$F(2,32) = 1.82$; $p = 0.17$], but neck diameter (KW $p < 0.01$) showed higher values in postpubertal males with sexual experience than in postpubertal males without sexual experience ($p < 0.01$) or prepubertal rats ($p < 0.05$; Figure 7c); (c) stubby/wide spines also showed statistically significant differences between groups. Their spine length (KW $p < 0.01$)

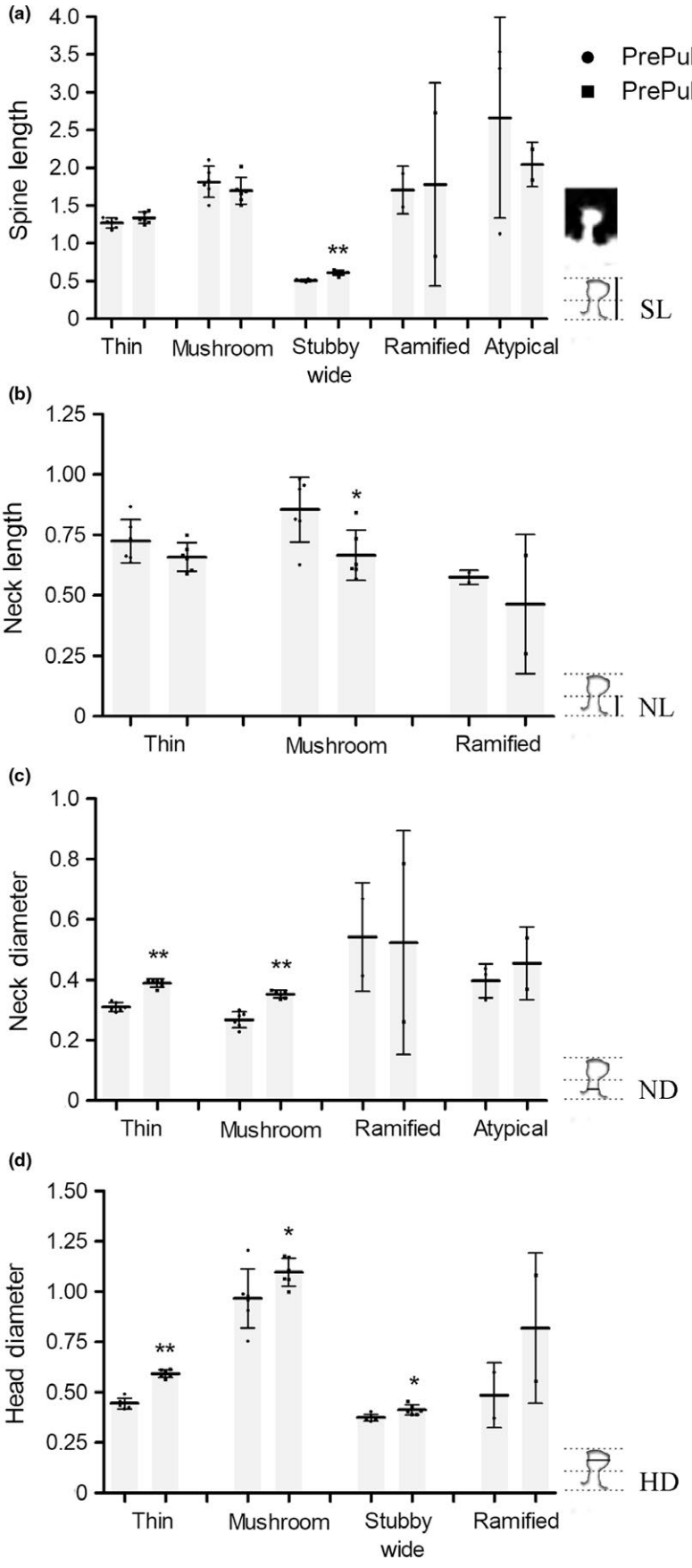

FIGURE 5 Values (mean ± *SD*, in μm) of the (a) spine length (SL), (b) neck length (NL), (c) neck diameter (ND) and (d) head diameter (HD) for the different types of dendritic spines in the rat posterodorsal medial amygdala of prepubertal females and males ($n = 6$ in both groups). Symbols (●■) represent the mean values for each studied rat per group, some data overlapping. Right images exemplify each morphological parameter measured. *$p < 0.05$, **$p < 0.01$ in each comparison indicated

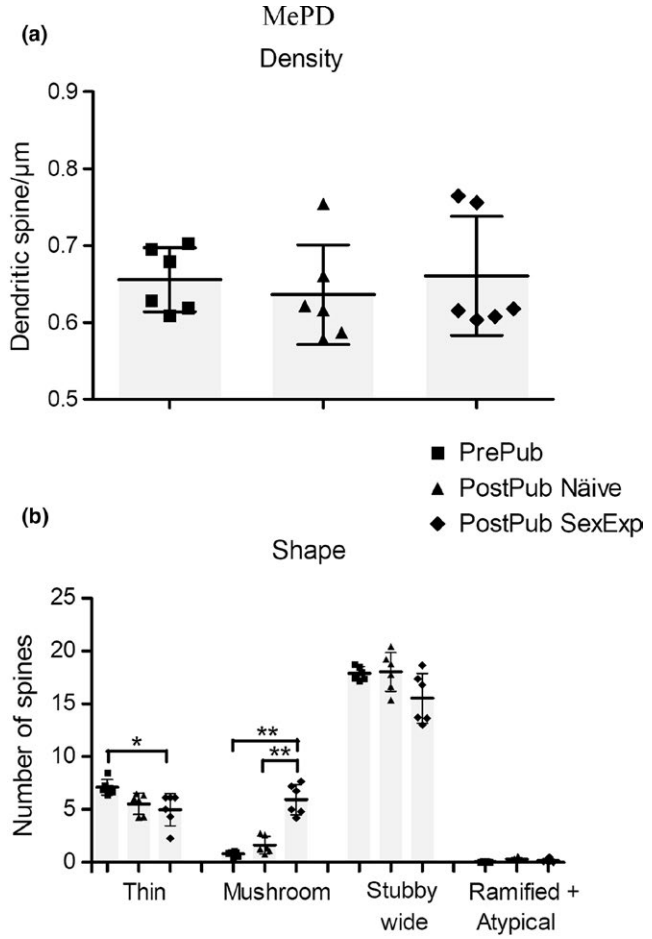

FIGURE 6 Values (mean ± *SD*) for (a) the density of overall proximal dendritic spines and (b) the number of each type of dendritic spine (classified by their shapes) in the rat posterodorsal medial amygdala (MePD) of prepubertal males, postpubertal males without sexual experience and postpubertal males with sexual experience ($n = 6$ in all groups). Symbols (■▲♦) represent the mean values for each studied rat per group, some data overlapping. *$p < 0.05$, **$p < 0.01$ in each comparison indicated

showed higher values in postpubertal males with sexual experience than in postpubertal males without sexual experience ($p < 0.05$) or prepubertal rats ($p < 0.01$; Figure 7a). In addition, the head diameter of stubby/wide spines were higher in postpubertal males with sexual experience than in both postpubertal males without sexual experience and prepubertal rats [$F(2,33) = 24.73$, $p < 0.01$; Figure 7d], and (d) ramified and atypical spines showed a scarce occurrence that precluded further statistical analyses.

4 | DISCUSSION

Our present results showed that, in the MePD, (a) Prepubertal males had a higher density of proximal spines whose morphological features were different from those of females in various structural parameters. Considering the spine type, males had more stubby/wide shaped ones, but females had more mushroom spines; (b) no statistical difference was found in the overall density of dendritic spines among prepubertal and postpubertal naive or sexually experienced males. Nevertheless, (c) the number of thin spines was higher in the prepubertal group when compared to the postpubertal group with sexual experience, whereas the number of mushroom spines increased in the postpubertal group with sexual experience when compared to the other male groups. In addition, (c) there were evident structural changes, as evaluated by the measured morphometric parameters of SL, NL, HD and ND in the different types of spines in the postpubertal group with sexual experience.

Reproductive behavior display requires the integration of chemosensory information, the neural effects of sex steroids and the processing of dynamic synaptic inputs in interconnected areas of the social behavior brain network (Becker, Rasia-Filho, & Giovenardi, 2017; Li et al., 2017; Newman, 1999; Westberry & Meredith, 2003). It is conceivable that part of the synaptic contacts was developed to establish "hardwired" connections and sex-specific features relevant for species reproduction and survival. This "reference point" for the density and shape of dendritic spines with specific functions in the neural circuitry can serve as a substrate for further effects of sex steroids on adult social behavior and for the timing regulation of neuroendocrine secretion (Cooke & Woolley, 2005; Rasia-Filho, Brusco, & Moreira, 2009; Rasia-Filho et al., 2004, 2012a, 2012b). Indeed, the MePD of males and females display differences in the density and fine details of dendritic spine shape at prepubertal age. Male to female differences in the dendritic spines of prepubertal rats would suggest that the MePD has a sex-specific synaptic processing and function determined genetically and/or due to gonadal hormones effects during the intrauterine period and along the early postnatal development (Cooke & Woolley, 2009a; Cooke et al., 2007; Nishizuka & Arai, 1981). Independently of their types, dendritic spines in the MePD are mainly contacted by excitatory axon terminals and form asymmetric synapses (Brusco et al., 2014). Therefore, differences in dendritic spine density likely reflect differences in the density of excitatory synapses on dendrites of MePD neurons (Cooke & Woolley, 2009a). Morphological and electrophysiological data demonstrated that a sexual dimorphism could be found in the MePD of prepubertal male and female Sprague Dawley rats (Cooke & Woolley, 2005; Cooke et al., 2007). Prepubertal males have approximately 80% more excitatory synapses per MePD neuron than females (Cooke & Woolley, 2005). Male prepubertal gonadectomy impaired the expression of juvenile rough-and-tumble play, a sexually dimorphic behavior, and reduced both the excitatory synaptic transmission and the density of dendritic spines, without affecting overall dendritic length or branching, in MePD neurons

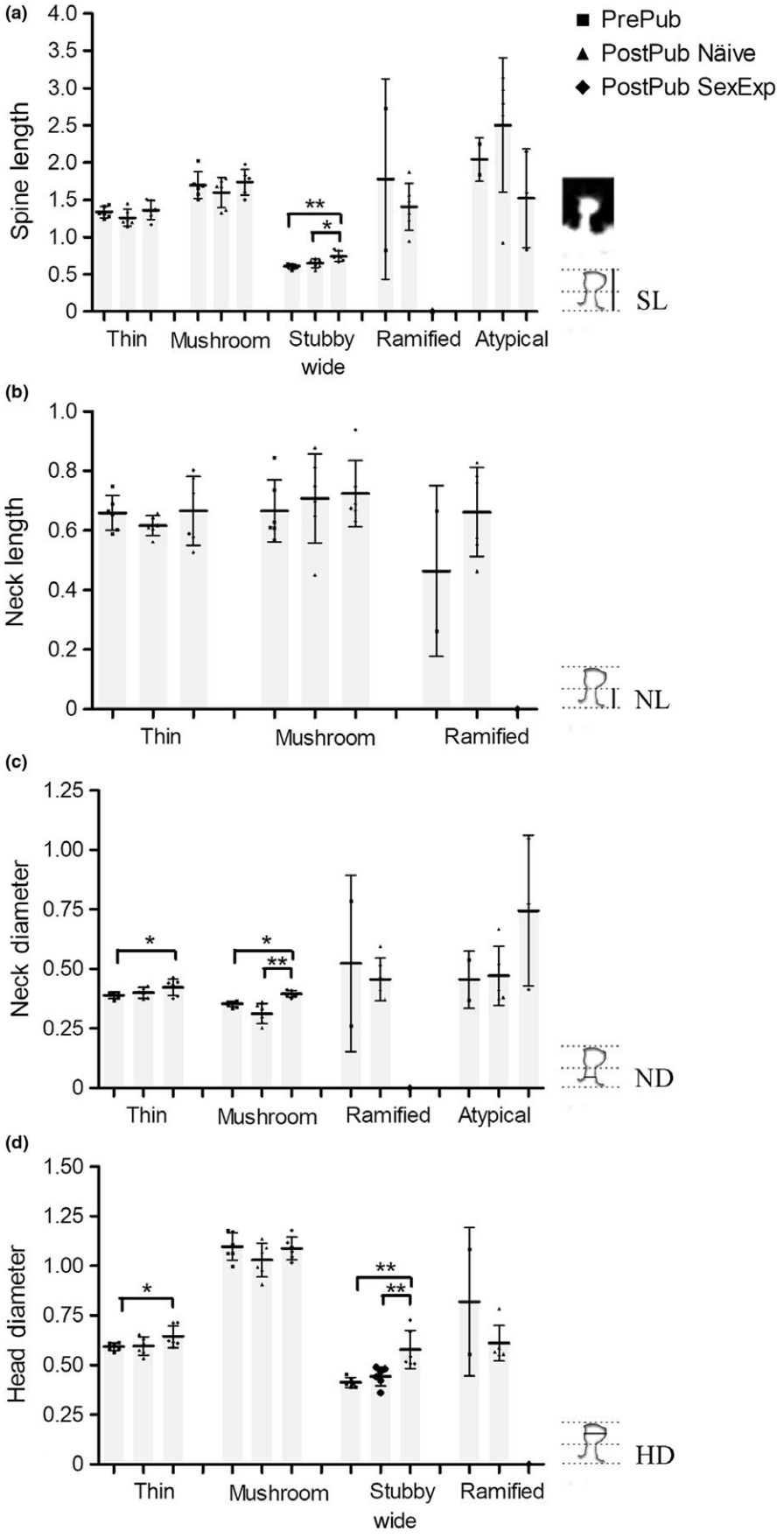

FIGURE 7 Values (mean ± *SD*, in μm) of the (a) spine length (SL), (b) neck length (NL), (c) neck diameter (ND) and (d) head diameter (HD) for the different types of dendritic spines in the rat posterodorsal medial amygdala of prepubertal males, postpubertal males without sexual experience and postpubertal males with sexual experience ($n = 6$ in all groups). Symbols (■▲♦) represent the mean values for each studied rat per group, some data overlapping. Right images exemplify each morphological parameter measured. *$p < 0.05$, **$p < 0.01$ in each comparison indicated

(Cooke & Woolley, 2009a). Male Sprague Dawley rats castrated before puberty had a reduced dendritic spine number (~30%–45% lower values than control data) along the first 70 μm of primary dendrites or along the last 70 μm of terminal dendrites (Cooke & Woolley, 2009a). Our prepubertal data about density and type of dendritic spines in the MePD of males and females indirectly indicate how densely are the inputs and the likely postsynaptic processing at this early age in Wistar rats.

It was highly expected that plastic adjustments could occur following an increase in sex steroid levels during puberty and/or by the various stimuli related to the animal experience of social interaction and sexual intercourse (Cooke, 2011; Koss et al., 2014; Morrison, Rodgers, Morgan, & Bale, 2014; Pitchers et al., 2010; Stark, 2005; Vigil et al., 2011). Indeed, the MePD is part of the sex steroid-sensitive neuronal ensemble that encodes social cues to trigger species-specific social behaviors (Bergan et al., 2014; Fewell & Meredith, 2002; Griffiths & Brennan, 2015; Li et al., 2017; Malsbury & McKay, 1994; Newman, 1999; Rasia-Filho et al., 2012b). At least in females, the MePD regulates the timing of puberty independently of changes in body weight and caloric intake, with local glutamatergic actions advancing the timing of puberty onset (Li et al., 2015). In adulthood, sex differences in the density of dendritic spines (~30% higher in males than in cycling females in proestrus) and effects of ovarian hormones fluctuations (~30% reduction from diestrus to proestrus) are evident in the MePD of Wistar rats (Rasia-Filho et al., 2004). Considering the MePD activity and connections in both sexes, these data can be related to the sequencing expression of the copulatory behavior in males and, in females, the disinhibition of sexual behavior concomitant with the increase in gonadotrophin-releasing hormone secretion for ovulation (as reviewed in Rasia-Filho et al., 2012a, 2012b and references therein; Zancan et al., 2017). It is noteworthy that the density of proximal spines in the MePD reduces following castration of postpubertal males (~20% after 90 days of the testes removal; de Castilhos et al., 2008). That value would indicate the percentage of "labile" spines modulated by sex steroids compared with the percentage of remaining spines that would be more "stable" in the male MePD. This balance is critical in areas related to social behavior, reproduction and adaptation, and where some stable spines could provide steady properties for the neural circuit, whereas others are plastic and adapted to additional synaptic demands.

There was no statistically significant difference in the overall density of proximal dendritic spines in the MePD of prepubertal or postpubertal males. The density of proximal dendritic spines showed no hemispheric lateralization in the MePD of prepubertal and postpubertal Wistar male rats. This agrees with a previous report obtained with the Golgi method in adult males of this same strain (Arpini et al., 2010). On the other hand, the spine density finding in the MePD contrasts with the reported increase in dendritic spine density in the basolateral amygdala throughout puberty (Koss et al., 2014), which indicates a region-specific modulation of the hormonally mediated effects in the different nuclei of the amygdala. It is also important to note that changes in the adult male MePD were found in the structure of the dendritic spines. That is, the type of spine and the morphometric features of the spine head and neck were more susceptible to both postpubertal and sexual experience-dependent modifications in the MePD.

The shape of MePD dendritic spines can be related to different synaptic processing, strength and plasticity for or caused by the display of sexual behavior in males. Indeed, in the male MePD, sexual experience plays an essential role in inducing a larger fraction of neurons to be stable and selectively activated by female cues (Li et al., 2017). Naive males have comparatively less pronounced sexual behavior than sexually experienced animals (Swaney, Dubose, Curley, & Champagne, 2012). Then, the synaptic inputs that reach the MePD neurons code the processing of learned social cues and the contextual memories of sexual encounters of the animal (Becker et al., 2017; Stark, 2005; Stark et al., 1998). This means that the MePD can change the execution of an innate behavior by undergoing adaptive modulatory changes evidenced as an experience-dependent lasting structural remodeling of local dendritic spines. As previously indicated (Rasia-Filho et al., 2004), "It has… been proposed that the execution of sexual reflexes can promote learning and a positive effect on reinforcement (Ågmo, 1999). Indeed, the MeA of adult male mice plays an essential role for the acquisition of memories after social contacts that are relevant for female recognition (Ferguson, Aldag, Insel, & Young, 2001). The rat MeA projections to the entorhinal area and to the postpiriform transition area would represent an alternative route for pheromonal influences to affect the hippocampal circuit (Petrovich et al., 2001)… It is also possible that the MeA innervation to the subiculum could be prioritizing the temporal organization of motivated behaviors (Petrovich et al., 2001). This mechanism is still not fully understood, but activity-dependent facilitation of hippocampal LTP by MeA neurons would also serve as another approach for revealing the plastic synaptic mechanisms underlying memory related to emotional arousal stimuli (Abe, 2001; Ikegaya, Abe, Saito, & Nishiyama, 1995; Roozendaal & McGaugh, 1996). Dendritic spines in the MeA subnuclei would be in an initial stage of this synaptic processing.". Based on the present morphological data, two additional nonexcluding functional possibilities could be proposed for the MePD of males: (a) As the MeA activity can process higher level categorical information, remodeling spines in the MePD of postpubertal sexually experienced males can be a way to control the flow of synaptic information to further organize the functional representation of sensory stimuli and selectivity in the sexually dimorphic circuit for reproduction (Bergan et al., 2014). And, (b) considering

that the MeA is part of a network that includes the nucleus accumbens (NAc) for social bonding (Atzil et al., 2017), and the number of dendritic spines in the shell and core of the NAc increases 1 week after sexual experience (Pitchers et al., 2010), we also hypothesize "that the sex behavior-induced alterations regulate the reinforcing components of sexual behavior and thus might be critical for positive reinforcement of rewarding behavior in general" (Pitchers et al., 2010).

Proximal dendritic spines are at a strategical site to modulate the cell body and axonal voltage and the output activity of the MePD. The structure–function coupling of spines and the activity-driven changes related to synaptic demand, stability and plasticity can be set at the level of each single spine (Arellano et al., 2007; Becker, Dall'Oglio, Rigatto, Giovenardi, & Rasia-Filho, 2015; Chen, Leischner, Rochefort, Nelken, & Konnerth, 2011; Dall'Oglio et al., 2015; Dalpian, Brusco, Calcagnotto, Moreira, & Rasia-Filho, 2015; Hansberg-Pastor, González-Arenas, Piña-Medina, & Camacho-Arroyo, 2015; Rochefort & Konnerth, 2012; Stewart et al., 2014). Interestingly, prepubertal males have more stubby/wide spines than prepubertal females. Stubby/wide spines could more directly affect the parent dendritic shaft voltage without a neck to promote an electrical and/or biochemical compartmentalization (Spruston et al., 2013). It is possible that input information coming to this kind of spine in the male MePD neurons would promote faster somatic responses (based on Segal, 2010) and help to build an initially different masculine pattern of neural activity to social stimuli, which will be further developed after puberty. Furthermore, there is a decrease in the number of thin spines and an increase in the number of mushroom spines following sexual experience, both findings suggesting a remodeling of synaptic connections in the MePD. This is consistent with the possibility that thin spines are related to "learning" processes, showing a labile aspect prone to changes in the synaptic processing, whereas mushroom spines are related to "memory" elaboration, showing a higher stability, great postsynaptic density with glutamatergic receptors and strong synaptic responses (Bourne & Harris, 2007; but see Segal, 2010). Spines classified as atypical might represent transient forms at varying stages of development or retraction and can have, as the ramified ones, functional microdomains in the same spine (Chen & Sabatini, 2012; Dall'Oglio et al., 2015 and references therein). These data indicate that the synaptic input and strength can be fine regulated at small segments of the MePD dendrites. This interpretation gets even more complex when considering that there are species differences when comparing rats and mice for the type of spines that are modified following male sexual experience (see detailed data in Becker et al., 2017).

The morphometric analyses of each class of spine complement the data obtained for the number of different spines, reinforcing the idea that the synaptic remodeling in the MePD occurs throughout the processes of puberty and after acquisition of sexual experience. That is, each type of spine had its shape altered and had at least one of its measures increased in the group of postpubertal males with sexual experience. The length and width of the spine compartments alter its biophysical properties, as occurs for the length and diameter of the neck of thin spines that impose local resistance and compartmentalization of the biochemical processes in the spine head (Spruston et al., 2013; Tønnesen & Nägerl, 2016; Yuste, 2013). The spine neck geometry is determinant of the spine Ca^{2+} signaling to the parent dendrite (Yuste, 2013). Larger necks permit greater efflux of Ca^{2+} into the dendritic shaft, whereas smaller necks increase calcium concentration in the spine (Noguchi, Matsuzaki, Ellis-Davies, & Kasai, 2005). The head volume is directly related to the number of presynaptic vesicles, the extent of the postsynaptic density and the different presence and amount of AMPA and/or NMDA glutamate receptors (Yuste, 2013). Notably, males with sexual experience had higher values of both head and neck diameters of thin spines, higher neck diameter of mushroom spines and higher spine length and head diameter of stubby/wide spines compared to prepubertal or naive animals. These data would suggest that synaptic contacts are more effective to affect the neuronal voltage in the MePD of males that underwent sexual experience than before puberty or in postpubertal naive rats. Our results open a new avenue for further studies, including optogenetic approaches, to determine the role of each spine type on the processing of socially relevant sensorial inputs, the impact of each type of spine on the neuronal activity and the formation of task-specific synaptic ensembles in the rat MePD (based on Hayashi-Takagi et al., 2015). Electrophysiological recordings would also demonstrate how androgens influence the MePD neuronal activity and the output projections that reach interconnected hypothalamic nuclei for the display of sexual behavior (Choi et al., 2005; Petrovich et al., 2001). The same can be proposed for the female MePD where clear cyclic changes are brought about after puberty, including epigenetic actions that can alter neuronal structure and function (Hirsch et al., 2018; Morrison et al., 2014, and references therein).

In conclusion, our data demonstrate spine-specific differences between prepubertal males and females and notable changes following puberty and sexual experience in the MePD of male rats. Postsynaptic plasticity evidenced by structural remodeling occurs in this sex steroid-responsive and experience-dependent area with a fine-tuned aspect at each type of spine level. These results address the importance of the MePD in the central organization of social behaviors, its sexual dimorphism, the structural transformation along the ontogenetic process of puberty and following sexual experience acquisition by revealing changes in the number, type and shape of local dendritic spines. There are likely functional implications for the synaptic refinement in the MePD for the reproductive behavior of adult rats.

ACKNOWLEDGEMENTS

Mariana Zancan receives a grant from the Brazilian "Conselho Nacional de Pesquisa e Desenvolvimento" (CNPq, grant no. 141867/2015-9). Alberto A. Rasia-Filho (grant no. 306594/2016-1) and Léder L. Xavier (grant no. 306644/2016-9) are CNPq researchers.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

DATA ACCESSIBILITY

Data are public, available via Figshare, https://doi.org/10.6084/m9.figshare.5728452.

AUTHOR CONTRIBUTIONS

MZ designed the study, collected and interpreted the data and elaborated the manuscript. RSRC designed the study and collected and interpreted the data. FS collected and interpreted the data. LLX interpreted the data and elaborated the manuscript. AARF designed the study, collected and interpreted the data and elaborated the manuscript.

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How to cite this article: Zancan M, da Cunha RSR, Schroeder F, Xavier LL, Rasia-Filho AA. Remodeling of the number and structure of dendritic spines in the medial amygdala: From prepubertal sexual dimorphism to puberty and effect of sexual experience in male rats. *Eur J Neurosci*. 2018;48:1851–1865. https://doi.org/10.1111/ejn.14052

3.2 Artigo Científico II

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GAP JUNCTIONS AND EXPRESSION OF Cx36, Cx43 AND Cx45 IN THE POSTERODORSAL MEDIAL AMYGDALA OF ADULT RATS

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Abbreviated title: MePD gap junctions and connexins

Keywords: extended amygdala, connexin, transmission electron microscopy, immunofluorescence.

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Acknowledgements: Grants from Brazilian Agency National Council for Scientific and Technological Development to MZ and AARF (CNPq; no. 141867/2015- 9 and 6594/2016-1), and CAPES.

Abstract

The posterodorsal medial amygdala (MePD) has an adapted synaptic organization that dynamically modulates reproduction and other social behaviors in rats. Discrete gap junctions between glial cells were previously reported in the MePD neuropil. Connexins (Cx) are components of gap junctions and indicative of cellular electrical coupling. Here, we report the ultrastructural occurrence of gap junctions between neurons in the MePD and demonstrate the expression and immunofluorescent labeling of Cx36, Cx43 and Cx45 in this subcortical area of adult male rats. Few neuronal gap junctions were found in the MePD and, when identified, occurred between dendrites. On the other hand, there is a diffuse presence and distribution of punctate labelling for the tested Cxs. Puncta were visualized isolated or forming clusters in the same focal plane of cell bodies or along the MePD neuropil. The Cx36 puncta were found in neurons, Cx43 in astrocytes and Cx45 in both neurons and astrocytes. Our data indicate the presence of few gap junctions and different Cxs composition in the MePD. Because Cxs can assemble, form hemichannel units and/or serve as transcriptional regulator, it is likely that additional modulation of intercellular communication can occur besides the chemical transmission in the MePD of adult rats.

Introduction

The posterodorsal medial amygdala (MePD) has a subpallial origin and is one of the “extended amygdala” subcortical nuclei in the rat basal forebrain (de Olmos et al., 2004; Olucha-Bordonau et l., 2015). Two main subpopulations of subcortical multipolar neurons were reported in this area, classified as bitufted or stellate cells (Rasia-Filho et al., 2012a), with distinct embryonic origins and phenotypic expressions (Choi et al., 2005; Bupesh et al., 2011). The MePD is a highly plastic area that serves as an interface for the actions of gonadal hormones (DeVries and Simerly, 2002; Gréco et al., 2003), the sensorial processing coming from main olfactory and vomeronasal pathways (Meredith and Westberry, 2004; Pereno et al., 2011; Petrulis, 2013) or mating-related genitosensorial stimuli (Coolen et al., 1997; Pfaus and Heeb, 1997). It then densely projects to hypothalamic nuclei (Petrovich et al., 2001) for the modulation of neuroendocrine secretion and reproductive behavior in both males and females (Newman, 1999; Simerly, 2004; Rasia-Filho et al., 2012b; Hull and Rodríguez-Manzo, 2017).

The MePD receives afferent synaptic inputs whose transmission, processing, and plasticity are modulated by glutamate, GABA, histamine, serotonin, and various neuroactive peptides (e.g., see Micevych et al., 1988; Rasia-Filho et al., 2012b; Quagliotto et al., 2015). Besides the chemical transmission, a previous ultrastructural report suggested the existence of discrete gap junctions between glial cells in the rat MePD (Brusco et al., 2014). Here, we further addressed the ultrastructural occurrence of gap junctions between neurons at the same time that we describe for the first time the pattern of

expression of connexins (Cx) in the MePD of adult rats. In this regard, gap junctions/electrical synapses are complex multimolecular structures formed by different Cxs (Rackauskas et al., 2007; Pereda et al., 2013; Griemsmann et al., 2015; Pereda, 2016; Nagy et al., 2018). Cx36-containing gap junctions establish electrical synapses between neurons (Baude et al., 2007; Schoenfeld et al., 2014). The widely expression of Cx36 in the brain suggests that not yet discovered electrical synapses can be part of distributed neural circuits (Connors and Long, 2004: Nagy et al., 2018). Cx43 is the major astroglial Cx (Theis and Giaume, 2012), occurs in coupled astrocytes (Chever et al., 2014) and co-localizes with glial fibrillary acidic protein (GFAP) in the hippocampus (Wu et al., 2015). Cx45 is present in neuronal gap junctions, primarily at "mixed" glutamatergic/electrical synapses likely between mitral/tufted cell dendrites in the olfactory bulb (Rash et al., 2005). It can be co-localized with Cx36-puncta as well as along Bergmann glial processes adjacent to Cx43-puncta in the cerebellar molecular layer (Nagy and Rash, 2017). We checked for the immunofluorescence labeling of Cx36 in neurons, Cx43 in astrocytes, and Cx45 in both neurons and astrocytes to provide additional data for the likely occurrence of thee constitutive proteins of gap junctions in the MePD.

Materials and Methods

Animals

Adult male Wistar rats (3-month-old) were housed under standard laboratory conditions with food and water *ad libitum*, room temperature around 21°C, and a 12-h light/dark cycle (lights on at 6 h). 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 study was approved by the Animal Ethics Committee of Federal University of Health Sciences of Porto Alegre (protocols no. 248/13 and 310/15).

Transmission Electron Microscopy

Tissue processing was carried out exactly as reported previously (Zancan et al. 2015). Rats (n= 9) were deeply anesthetized with ketamine and xylazine (intraperitoneal injections, 80 mg/kg and 10 mg/kg, respectively). Transcardiac perfusion was carried out after heparin (1,000 IU) injection in the left ventricle using 500 ml of 2% formaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer solution (0.1 M, pH 7.4; PBS) at room temperature (RT). Peristaltic pump (Control Company, Brazil) flow was initially rapid (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 tissue fine ultrastructural integrity (Tao-Cheng et al., 2007; Brusco et al., 2014).

Brains were removed and immersed in the same fixative solution for 90-150 min, rinsed in PBS, and coronally sectioned using a vibrating microtome (VT 1000S, Leica Microsystems, Germany). The MePD was identified from 3.0 to 3.4 mm posterior to the bregma, laterally to the optic tract (opt) and ventrally to the stria terminalis (Figure 1; Paxinos and Watson, 2008). The MePD was dissected from one single 400-μm-thick coronal slice. Samples were taken from the intermediate to lateral cellular parts of the MePD[1]. We avoided gathering data from the medial cell-sparse rim “molecular layer” close to the opt.

Each tissue block was post-fixed in the same perfusion solution for additional 18 h, washed in PBS and postfixed in 1% osmium tetroxide (Sigma Chemicals Co., USA) for 1 h at RT. Thereafter, sections were washed in PBS, 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) from the whole block 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 (TEM; JEM-1200EX II, Jeol, Japan) operated at 80 kV. The images were recorded with a camera SIS MegaView III CCD (Germany). The cellular components of the MePD were photographed first at 6,000 X magnification and then at 20,000 X and 60,000 X. When needed, we checked for their aspect with magnifications up to 300,000 X. As a rule, we observed the gap junctions at the limit of our TEM equipment resolution. Images were saved with the maximum resolution available (at 1.42 Mpx and 24 bits per pixel).

The ultrastructural aspect of neuronal gap junctions is in accordance with the characteristic morphological features described previously (Peters et al., 1991; Pannese, 2015). Dendrites were identified by the ultrastructural aspect of mitochondria, microtubules, and agranular endoplasmic reticulum (Pannese, 2015). Gap junctions in the adult rat MePD resembled those previously described in various brain nuclei of different species (Peters et al., 1991; Horiguchi et al.,

2011; Brusco et al., 2014; Figure 2). Specifically, we looked for close membrane-to-membrane appositions between neurons. We carefully observed the aspect of the apposed structurally symmetric cell membranes, the narrow synaptic cleft formed by an array of apparently layered aspect of tight intercalated dense and lighter lines, and a lack of dense material associated with the cytoplasmatic faces of plasma membranes (Peters et al., 1991; Pannese, 2015). In addition, the space between the apposed membranes should not be obliterated by an apparent fusion of the outer leaflets of the plasma membranes as a zonula occludens (Peters et al., 1991) and should not show symmetrical thick and electron-dense plaques at both sides of cytoplasmic face of the plasma membranes and fine filaments converging upon these plaques as a puncta adhaerentia (Sätzler et al., 2002; Pannese, 2015).

Immunofluorescence Procedure

Rats (n= 4) were deeply anesthetized as above-mentioned and perfused with 400 ml of 4% formaldehyde in PBS. Brains were submitted to post-fixation during 2 h in the same fixative solution, cryoprotected using a 30% sucrose solution diluted in PBS, and stored at 4°C for 24–48 h.

Frozen cryostat sections (10 μm thick, Leica/CM 3050S, Germany) were collected on gelatinized glass slides, fixed and permeabilized by cold acetone for 10 min and dried. Acetone was the substance that provided the best results for the MePD. Although acetone could cause extraction of Cxs and under-represent actual values, it did not lead to a failure of their detection in the aimed area.

Afterwards, the sections were washed with PBS and delimited with a hydrophobic barrier pen (Vector Laboratories, USA). Then, the slides were air dried once again and the samples were incubated in 10% fetal bovine serum prepared in PBS containing 0.05% Triton X-100 for 1 h at RT. Afterwards, sections were washed using PBS and incubated with the following primary antibodies: rabbit monoclonal anti-Cx36 (1:200; catalog no. 701630, Thermo Fisher Scientific, USA); mouse monoclonal anti-Cx43 (1:200; catalog no. 138300, Invitrogen, USA); mouse monoclonal anti-Cx45 (1:500; catalog no. MAB3100-C, Millipore, USA); mouse monoclonal anti-βIII-tubulin conjugated with eFluor 570 (1:200; catalog code 41-4510-80, eBioscience, USA), and mouse monoclonal anti-GFAP conjugated with Alexa Fluor 488 (1:200; catalog no. 131-17719; Invitrogen, USA). βIII-tubulin served as a neuronal marker and GFAP to visualize astrocytes to test their co-localization with Cxs. All antibodies were diluted in 2 % fetal bovine serum prepared in PBS containing 0.05 % Triton X-100 overnight using a shaker for gentle continuous agitation at 4ºC, except for the anti-GFAP and anti-βIII-tubulin which were incubated for 2 h. After subsequent rinsing with 0.05 % Triton X-100 diluted in PBS and only PBS, sections were incubated with the following secondary antibodies for 120 min at RT: goat anti-mouse IgG3 conjugated with Alexa Fluor 555 (1:1000; catalog code ab98707, ABCAM, USA) and goat anti-rabbit IgG conjugated with Alexa Fluor 488 (1:1000; catalog code ab150077, ABCAM). After rinsing with PBS, sections were counterstained with the fluorescent dye Hoechst 33342 (4 µ/ml; catalog code H3570, Thermo Fischer) for 10 min, washed again with PBS, and covered with mounting media. Hoeschst served to identify the nuclei of both neurons and glia cells in the MePD.

The antibody specificity was checked by Western blot (data not shown). Non-specific binding of the secondary antibody was controlled by omitting the primary antibody and replacing it by PBS. No reaction was observed in this condition. The pattern of immunofluorescent expression of each studied Cx was specific in fixed tissues (validated with different methodological approaches, as depicted in Nagy and Rash, 2017), following the recommendations of Saper (2005), similar to previous report (e.g., Nagy and Rash, 2017) and according to the manufacturer´s data sheet, as follows:

Cx36: https://www.thermofisher.com/antibody/product/Connexin-36-Antibody-clone-12H11L18-Monoclonal/701630

Cx43: https://www.thermofisher.com/antibody/product/Connexin-43-Antibody-clone-CX-1B1-Monoclonal/13-8300

Cx45 http://www.merckmillipore.com/BR/pt/product/Anti-Connexin-45-Antibody-near-CT-cytoplasmic-clone-8A11.2-Ascites-Free,MM_NF-MAB3100-C?ReferrerURL=https%3A%2F%2Fwww.google.com.br%2F&bd=1).

The MePD was identified as described above. Images were captured using a fluorescence-inverted microscope (Olympus IX51 U-RFL-T, Olympus Corporation, USA) with plan semi-apochromat objective lens (×20, Olympus UPLFL, 0.5 N.A.). The Olympus DP controller 3.3.1292 software was used for image acquisition. At least two different images were obtained from each section, and six sections were obtained from each animal. All image acquisition procedures were held constant when gathering data for the different Cxs, βIII-tubulin, and GFAP. Hoescht was excited at 350 nm, FITC was excited at 488 nm (for βIII-tubulin and GFAP) and TRITC was excited at 555 nm (for Cx36, Cx43,

and Cx45). All images had the same size (4080 x 3072 pixels). In each focal plane, results were visualized isolated or merged for the expression of: (1) Cx36 and βIII-tubulin to evaluate concomitant presence in neurons, (2) Cx43 and GFAP to evaluate concomitant presence in astrocytes, and (3) Cx45 and both βIII-tubulin and GFAP to evaluate concomitant presence in neurons and astrocytes.

Images had final fine adjustments of sharpness, brightness, and contrast made in Photoshop CS3 or CS5 (Adobe Systems, USA) without altering their content.

Results

Scarce gap junctions between neurons were found in the MePD neuropil (Figure 2). Few of them could be observed along several ultrathin sections from all the studied animals. We looked for such junctions between all parts of the neurons. Local gap junctions had the characteristic close apposition of cellular membranes and a narrow synaptic cleft with intercalated electron-lucent and electron-dense layers. They were identified between close neuronal segments (two dendrites), as shown in Figure 2A and B. Empirically, we found gap junctions only between dendrites. We could not reliably observe them between cell bodies or axons.

The MePD expresses the three studied Cxs. Punctate labelling and representative images for Cx36, Cx43, and Cx45 results are shown in Figure 3. Immunolabeled puncta were observed isolated or forming clusters. They had round or elongated aspects and were small, but usually variable in size. We

identified immunfluorescent puncta in the cell body or in the neuronal and glial prolongments.

The immunofluorescent puncta for each Cx were visualized in the same focal plane of cell bodies and along the neuropil. For all Cxs, there were puncta that did not match exactly with the markers for neurons or glia, although it was possible to identify Cx36 puncta associated with β–III tubulin, Cx43 with GFAP, and Cx45 with both β–III tubulin and GFAP. Taking the Cx45 immunolabeling as an example, there were a consistent occurrence of puncta along neuronal and astrocytic branches. However, these puncta showed a selective distribution in these cellular prolongments, i.e., there was neither a homogenous presence of this Cx along the entire extension of a cellular process nor all branches showed the same occurrence of immunolabeled punca in each of them.

Discussion

Our findings indicate the occurrence of gap junctions between neurons and the evident expression of Cxs in neurons and glia cells in the MePD of adult rats. The morphological and functional implications of these findings are depicted below.

First, in our samples, the search for gap junctions in the MePD using TEM was a laborious procedure. This agrees with the same concern elaborated by Brusco et al. (2014), as follows: “TEM also revealed gap junctions between glial cells in the MePD neuropil... No such structures could be found on MePD neurons, either because these junctions are rare, have a restricted distribution, or have a modified structure that cannot be identified readily”. We have studied

adult male Wistar rats and used a similar procedure for obtaining sample sections for TEM as these authors did. Although scarce, we could identify gap junctions between dendrites in the MePD neuropil. Interestingly, data were obtained in a mature rat brain area whose cells and circuits retain structural and functional plasticity after critical developing periods (Rasia-Filho et al., 2012a; Zancan et al., 2015, 2018). The combination of TEM with other techniques would provide additional direct data on gap junction composition, but the total number of these gap junctions in the MePD was very low and variable within each animal and between animals that precluded additional efforts.

The reasons for the presence of gap junctions in the adult MePD are not currently known. To the best of our knowledge, there are no reports with electrophysiological recordings of electrical synapses in the MePD of adult male Wistar rats. Nevertheless, gap junctions and electrical synaptic coupling exist between neural cells in an activity-dependent manner and for cellular functional synchronization (Bennett and Zukin, 2004; Pereda et al., 2013; Nagy et al., 2018; Pernelle et al., 2018). Gap junction transmission can be modulated by the activity of the neural network and can affect awake active behaviors (Posłuszny, 2014). It is possible that coupling dendrites of adjacent neurons would provide the MePD with a possibility of compartmentalization of information processing within cellular microdomains (based on Rela and Szczupak, 2004). Some electrical coupling might also produce complex input–output functions in combination with chemical transmission (Langer et al., 2012; Postuszny, 2014; Rela and Szczupak, 2004), even that gap junctions form transient cellular ensembles (Pannasch et al., 2012; Bukalo et al., 2013; Anders et al., 2014; Pogoda et al., 2016) for short-term events (Allen et al., 2011).

In addition, we found that different Cxs are expressed in the adult rat MePD, a finding that has not been addressed directly before. Rash et al. (2004), when studying the basomedial and basolateral amygdala, provided an image for the expression of Cx36 in the medial amygdala likely in its anterior aspect in mice (inferred from Figure 5). Moderate levels of Cx36 mRNA and protein were reported in the medial amygdala of adult rats, but it is not possible to determine exactly the rostrocaudal level or the dorsoventral position where data were obtained in this nucleus and/or from a specific subnucleus (Figures 6 and 10 in Belluardo et al., 2000).

The presence of various Cx puncta contrast with the few TEM data of gap junctions in the MePD. However, the identification of Cx36 immunofluorescence is well correlated with its localization in neuronal gap junctions (Nagy et al., 2018). We found Cx36 co-expressed with βIII-tubulin in MePD neurons, as expected for the cell specificity of this Cx, suggestive of the existence of gap junctions in this brain area. In addition, Cx43 was associated with local GFAP-immunomarked astrocytes. Cx45 was detected with both βIII-tubulin and GFAP, i.e., in both neurons and astrocytes. This latter finding is interesting because Cx45 is largely absent in the adult nervous tissue, except for few brain areas (Bennett and Zukin, 2004). The rat MePD has now to be included in the group of areas with Cx45 expression at adulthood as well.

The consistent expression of different Cxs in the adult MePD cells would have functional implications. We hypothesize some possibilities. For example, Cxs serve for the modulation of the dynamic cellular activity and the information processing plasticity (Nagy et al., 2018). Assembled Cxs, besides transferring ions and synchronize activity, can also allow the exchange of small metabolites

and intracellular signaling molecules between cells (Bennett and Zukin, 2004). Cx36 binding sites also have a phosphorylation-dependent interaction and promote activation of Ca^{++}-calmodulin-dependent kinase II, which correlates with the activation of glutamate NMDA receptors associated with adjacent chemical synapses (Pereda et al., 2013). Cx43 indirectly regulate cell–cell adhesion as well as provide astrocytes with a region-specific and activity-mediated physiological heterogeneity for intercellular communication (Theis et al., 2012; see additional data in Kovacs et al., 2017). Recently, it was demonstrated that Cx43 can also regulate gene transcription and N-cadherin expression (Kotini et al., 2018).

In addition, it has been reported that Cxs can be part of hemichannels, which can migrate in the membrane, dock and combine with other compatible Cxs to establish a contact with the apposed membrane of a neighbor cell (Pogoda et al., 2016). We can not identify specific hemichannels from our immunofluorescent data. Nevertheless, this possibility could not be dismissed when expression of different Cx are found in the MePD. Cx hemichannels can form large poorly selective pores (Tong et al., 2015), be functional units *per se,* have their permeability modulated by phosphorylation/dephosphorylation and various cytosolic redox agents (Pogoda et al., 2016). Hemichannels-dependent ATP release promotes a signaling pathway by propagated intercellular Ca^{++} waves and activation of purinergic receptors in neighboring cells *in vitro* (Bader et al., 2012 and references therein). Hemichannels can open and connect the cell interior with the extracellular space, release and uptake diffusing ions and molecules up to 1.2 kDa, including metabolites and signaling molecules (Bader et al., 2012). For example, at least for the rat basolateral amygdala, the release of

gliotransmitters through Cx43 hemichannels is necessary for memory consolidation (Stehberg et al., 2012). The occurrence of hemichannels composed by Cx in the MePD has still to be demonstrated with additional experimental approaches.

In conclusion, our findings indicate that the rat MePD has few gap junctions identified using TEM, but has an evident occurrence of immunofluorescence puncta for Cx36, Cx43 and Cx45 in local neurons and astrocytes. These morphological data address further connectional and intercellular interactions in the MePD with likely functional implications for the local neuroglial elaboration of complex social behaviors in adult rats.

Conflict of Interest

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

Role of authors

All authors contributed to study concept and design, analysis and interpretation of data, and critical revision of the manuscript. Acquisition of immunofluorescent data: MZ, DJM, AMM, and LS.

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Legends

Figure 1 - (**Left**) Schematic diagram of the ventral aspect of a coronal brain section showing the posterodorsal medial amygdala (MePD, marked in black; in this case at -3.3 mm posterior to the bregma) lateral to the optic tract (opt) and ventral to the stria terminalis (st). Adapted from Paxinos and Watson (2008). (**Center**) Microscopic image of a matched coronal section of the MePD (scale= 200 µm) to demonstrate where ultrastructural data (top, scale= 1 µm) and immunofluorescence for connexins (bottom, scale = 20 µm) were obtained.

Figure 2 - (**A** and **B**) Digitized electron micrographs of the ultrastructure of the posterodorsal medial amygdala of adult male rats showing a gap junction (arrow) between two dendrites. **A'** and **B'** are corresponding images at higher magnification. Den: dendrite; m: microtubule; mit: mitochondria; er: endoplasmic reticulum. Fine adjustments of brightness and contrast were made in Photoshop CS3 (Adobe Systems, Inc., USA). Scales= 70 nm (A and B) and 20 nm (A' and B').

Figure 3 - Digitized images of representative immunofluorescent results for connexin (Cx) expression in the posterodorsal medial amygdala of adult male rats. Results are presented for the cell body identification (Hoechst), βIII-tubulin to identify neurons, glial fibrillary acidic protein (GFAP) to identify astrocytes, and merged corresponding images. Immunolabeled puncta for Cx36, Cx43, and Cx45 (indicated by arrows) are all present and broadly distributed in the MePD. Fine adjustments of brightness and contrast were made in Photoshop CS3 (Adobe Systems, Inc., USA). Scale= 20 µm.

Figure 1

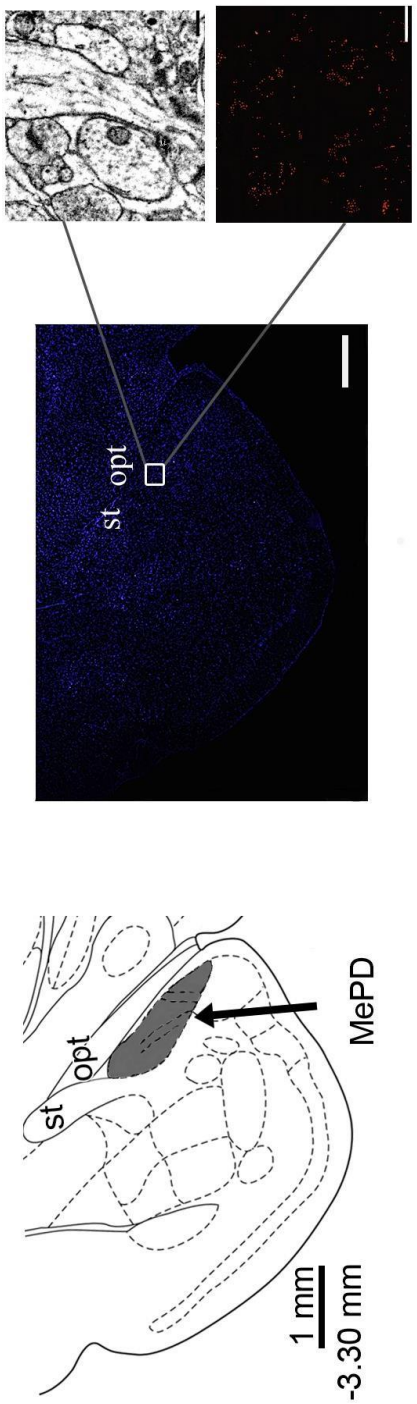


Figure 2

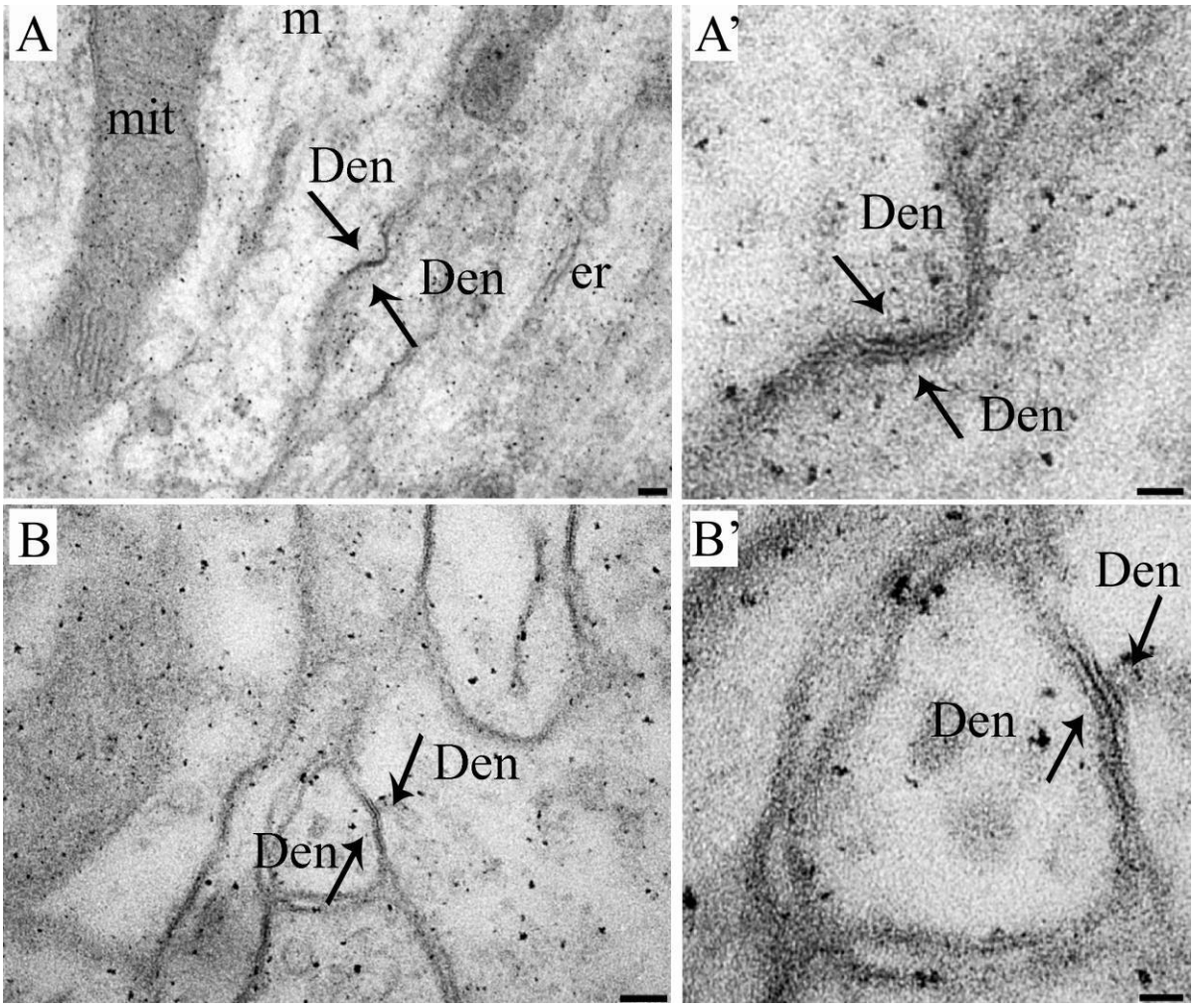
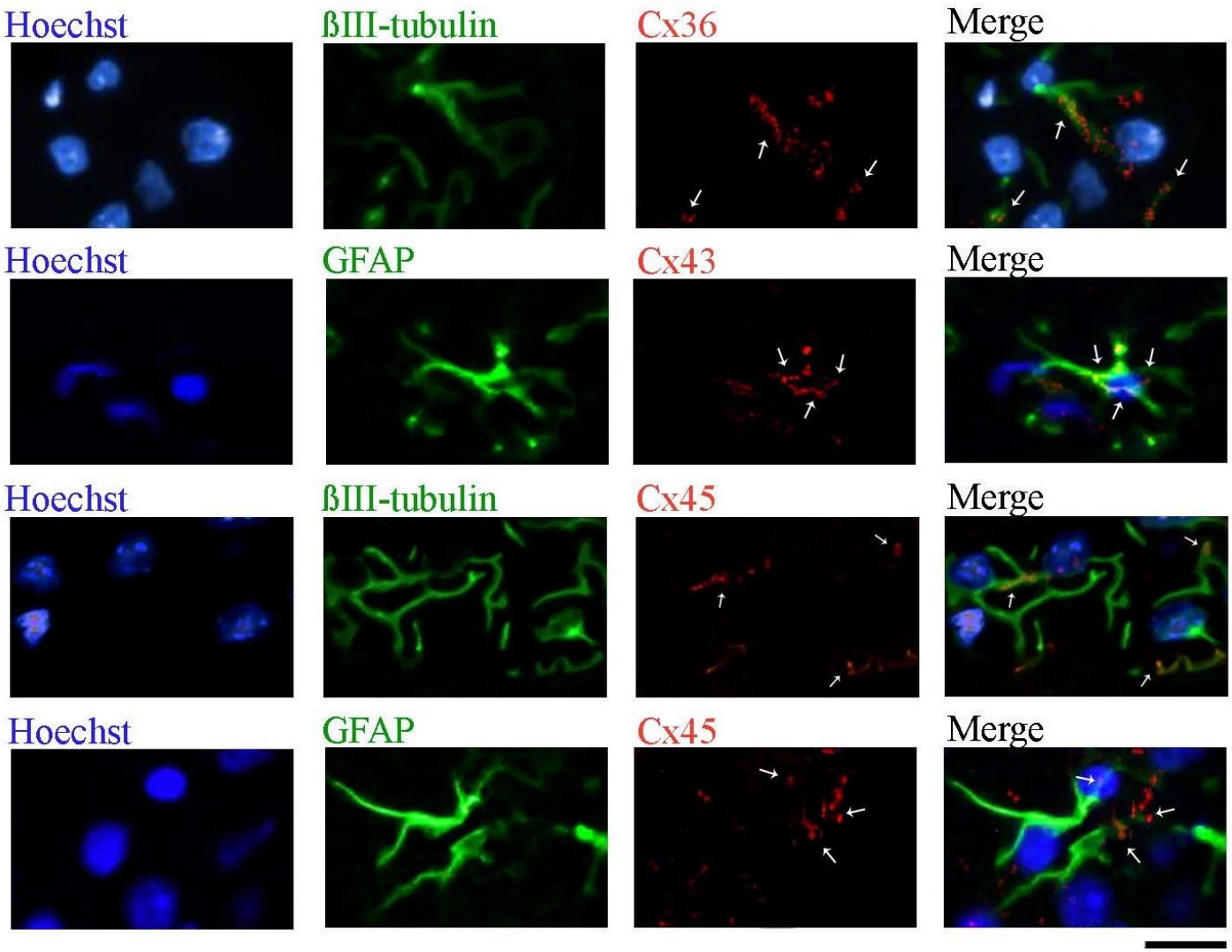


Figure 3


Hoechst
ßIII-tubulin
Cx36
Merge
Hoechst
GFAP
Cx43
Merge
Hoechst
ßIII-tubulin
Cx45
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Hoechst
GFAP
Cx45
Merge

3.3 Artigo Científico III

Submetido na revista Neuroscience – Fator de Impacto 3.382

Dear Dr. Mariana Zancan,

You have been listed as a Co-Author of the following submission:

Journal: Neuroscience
Title: NEUROTROPHIC FACTORS IN THE POSTERODORSAL MEDIAL AMYGDALA OF MALE AND CYCLING FEMALE RATS
Corresponding Author: Alberto Rasia-Filho
Co-Authors: Mariana Zancan, M.Sc., Ph.D; Dinara J Moura, Ph.D; Ana M Morás, M.Sc.; Luiza Steffens, M.Sc.; Ana C de Moura, Ph.D; Márcia Giovenardi, Ph.D;

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Original Research

Neurotrophic factors in the posterodorsal medial amygdala of male and cycling female rats

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Abbreviated title: Neurotrophic factors in the MePD

Keywords: extended amygdala, BDNF, Ephrin-A4, IGF-1, PSA-NCAM, sex steroid effects.

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Acknowledgements: Authors are deeply thankful to Prof. Rochelle Cohen (University of Illinois at Chicago, USA) for her contributions and critical review to this manuscript. Grants from Brazilian Agency National Council for Scientific and Technological Development to MZ and AARF (CNPq; no. 141867/2015- 9 and 6594/2016-1), and CAPES.

Abstract

The posterodorsal medial amygdala (MePD) has a high concentration of receptors for gonadal hormones, is a sexually dimorphic region and dynamically controls the reproductive behavior of both males and females. Structural and synaptic plasticity can be modulated by neurotrophic factors to promote dendritic spine remodeling and change synaptic input strength in a region-specific manner. In the present study, we analyzed the gene and protein expression of brain-derived neurotrophic factor (BDNF), insulin-like growth factor-I (IGF-1), polysialylated neural cell adhesion molecule (PSA-NCAM) and Ephrin-A4 in the MePD of males and females in diestrus, proestrus and estrus using real-time PCR (RT-qPCR) and fluorescent immunohistochemistry. These latter studies revealed that BDNF, IGF-1, PSA-NCAM and Ephrin-A4 are expressed in the MePD of the adult rat and RT-qPCR showed their amplification except for IGF-1. Average immunofluorescence for protein expression of these neurotrophic factors showed no differences between groups whereas, proestrus females displayed a higher number of labelled puncta than males for BDNF expression and diestrus females for IGF-1 expression in the MePD. These data contribute to our understanding of gonadal steroid action on the modulation and timing of structural and functional changes during the estrous cycle and their possible effects on sexual dimoprphism in the adult rat MePD. The observed changes in BDNF and IGF-1 in proestrus are coincident with dendritic and somatic spine density changes in the MePD, the disinhibition of gonadotrophin secretion for ovulation and female receptive sexual behavior.

Key words: BDNF, Ephrin A4, extended amygdala, IGF-1, PSA-NCAM, sex steroid effects.

1. Introduction

The posterodorsal medial amygdala (MePD), a component of the “extended amygdala”, is a sexually dimorphic region implicated in the dynamic control of reproductive behavior in both males and females (Newman, 1999; de Olmos et al., 2004, reviewed in Rasia-Filho et al., 2012a,b). Neurons in the rat MePD have a high concentration of receptors for gonadal hormones (Simerly et al., 1990; Gréco et al., 1998; De Vries and Simerly 2002; Isgor et al., 2002). In females, this subcortical area shows a complex local organization and dynamic changes in the number and structure of synapses during the estrous cycle (Rasia-Filho et al., 2004; Zancan et al., 2015; Dalpian et al., 2019). Indeed, sex steroids modulate the MePD volume and the number of neurons and glial cells, the neuronal somatic volume, the length and spatial orientation of the dendritic branches and the density and shape of somatic and dendritic spines, the synaptic input, and the frequency of excitatory and inhibitory postsynaptic currents, among other features (Hines et al., 1992; Dall´Oglio et al., 2008; Hermel et al., 2008; Morris et al., 2008; Rasia-Filho et al., 2012a,b; Zancan et al., 2015, 2017, 2018; Dalpian et al., 2019 and references therein)

Plastic changes are found in the number and structure of dendritic spines, postsynaptic elements involved in excitatory transmission, in the adult rat MePD (Rasia-Filho et al., 2004; de Castilhos et al., 2008; Zancan et al., 2018). Spines can establish both stable and plastic contacts that will be integrated to determine the neuronal activity within neural networks (Dall´Oglio et al., 2008; Brusco et al., 2014; Tønnesen and Nägerl, 2016; Zancan et al., 2018). In the MePD, dendritic spines show a notable sexual dimorphism and are affected by normally circulating levels of gonadal hormones or by castration and hormonal replacement therapies (Rasia-Filho et al., 2004; Cooke and Woolley, 2005; de Castilhos et al., 2008; Rasia-Filho et al., 2012a; Zancan et al., 2017). Males have a higher density of proximal dendritic spines than females in proestrus, estrus or metaestrus, but not in diestrus (Rasia-Filho et al., 2004; Rasia-Filho et al., 2012a). Furthermore, females in proestrus have a higher density and more variable shapes of somatic spines than in estrus (Zancan et al., 2015), and more inhibitory contacts on dendritic shafts than males or females in the other phases of the estrous cycle (Brusco et al., 2014). These data were recently supported by

electrophysiological recordings in the MePD of adult males and females along the estrous cycle (Dalpian et al., 2019).

Marked structural and synaptic plasticity can be modulated by neurotrophic factors (Park and Poo, 2013; Dyer et al., 2016) associated with neural gonadal steroid actions in the central nervous system (CNS) (Luine and Frankfurt, 2013; McEwen and Milner, 2017). The release and action of these factors can promote dendritic spine remodeling and change the synaptic input and strength with brain region-specific features (Rasia-Filho et al., 2012a; Srivastava, 2012; Hansberg-Pastor et al., 2015). For example, estrogen can influence protein and/or mRNA levels of brain-derived neurotrophic factor (BDNF) and alter dendritic spine density by both rapid membrane-associated second-messengers and genomic mechanisms (Luine and Frankfurt, 2013). In the CA1 hippocampal subfield and in the ventromedial hypothalamic nucleus (VMN), estrogen and BDNF increase the number of dendritic spines in proestrus female (Luine and Frankfurt, 2013). Also, insulin-like growth factor-I (IGF-1) has neuroplastic effects on dendritic spines (Dyer et al., 2016), and associates with increased levels of estrogen in the hypothalamic arcuate nucleus (Dueñas et al., 1994) linked to the synaptic remodelling for the cyclic release of gonadotropin releasing hormone (GnRH) (Cardona-Gómez et al., 2000). The polysialylated neural cell adhesion molecule (PSA-NCAM) is involved in structural synaptic remodeling and facilitates morphological and glial plasticity (Parkash and Kaur, 2005). In the median eminence-arcuate nucleus of the hypothalamus, PSA-NCAM expression during the proestrus phase relates to the structural reorganization of GnRH neuron terminals for neuroendocrine secretion (Parkash and Kaur, 2005). Ephrin-A4 modifies neuronal shape by reorganizing integrin activity and downstream signaling molecules that stabilize dendritic spines (Bourgin et al., 2007), reduces spine length and density (Murai et al., 2003) and, also relevant, induces axonal guidance and the establishment of connections (Liebl et al., 2003; Richter et al., 2007). There are no current data about sex differences or effects of normally occurring fluctuations of ovarian steroieds on the expression of these neurotrophins in the MePD of adult rats.

The aim of the present study was two-fold: (1) to determine gene and protein expression of BDNF, IGF-1, PSA-NCAM and Ephrin-A4 in the MePD of

adult males and in females along the estrous cycle (in diestrus, proestrus and estrus) using real-time PCR (RT-qPCR) and fluorescent immunohistochemistry for confocal microscopy; and (2) to determine if there are sex differences or differences in estrous cycle stages between experimental groups. Differences in these parameters may reflect sex steroid effects on the existence and variable expression of neurotrophic modulators of synaptic plasticity.

2. Materials and Methods

2.1. Animals

Adult Wistar rats (3 months-old, n = 10 males and 26 females for the RT-qPCR study, and n= 6 males and 17 females for the immunofluorescence study) were obtained from a federal facility (UFCSPA), and housed in groups in standard laboratory conditions with food and water *ad libitum*. Room temperature (RT) was maintained around 22ºC in a 12 h light-dark cycle. The estrous cycle regularity, according to cytological criteria (Singletary at al., 2005), was monitored along 3 consecutive weeks prior to the beginning of the experiment. Only normally cycling females were studied when in the diestrus, proestrus or estrus. For the RT-qPCR and immunofluorescence studies, we used males (n = 8 and 6) and females in diestrus [n= 4-7 (minimum and maximum per gene tested) and 5], proestrus (n= 4 and 6), estrus [n= 5-7 (minimum and maximum per gene tested) and 5-6 (minimum and maximum per antibody tested)].

All efforts were made to minimize the number of animals and their suffering. Rats were manipulated according to international laws for the ethical care and use of laboratory animals (National Institutes of Health Guide for the Care and Use of Laboratory Animals (DHEW Publication 80-23, Revised 1985, Office of Science and Health Reports, DRR/ NIH, Bethesda, MD 20205). This work was approved by the Animal Ethics Committee of UFCSPA (Brazil; protocol no. 162/15 and 227/18).

2.2. RT-qPCR Procedure

2.2.1. Sample Obtention, RNA Extraction Procedure and cDNA Synthesis

Animals were anesthetized using ketamine and xylazine (80 mg/kg and 20 mg/kg i.p. injected, respectively), decapitation was performed, brain was quickly removed from the skull, put on a cold plate and the right MePD was immediately dissected with a surgical blade 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 1). The right hemisphere was chosen because of its sexual dimorphism, evidenced by transmission electron microscopy, synaptic plasticity-related expression of microRNA, and electrophysiological differences between males and females along the estrous cycle (Brusco et al., 2014; Hirsch et al., 2018; Dalpian et al., 2019). The MePD was then placed in tubes with RNA-later (Ambion, USA) for 24 h at 4ºC. Afterwards, the RNA-later was removed and the samples were stored at -80ºC until the next experimental procedure (approximately 1 month later).

The RNA was extracted using TRIzol (Invitrogen, USA) according to manufacturer's guidelines. Each brain piece was homogenized in TRIzol, followed by cloroform addition (1:5, v/v) and an aqueous phase was obtained by centrifugation (12,000 x g, 15 min). The RNA was precipited with isopropanol (15 min) at room temperature (RT), followed by an additional centrifugation period (12,000 x g for 10 min), isopropanol was discarded and the pellets were resuspedend in 0.1% DEPC-treated water. The concentration of total RNA was determined by measuring the optical density at 260 nm, and the RNA purity was assessed based on the 260nm/280nm ratio (BioSpec-nano, Shimadzu, USA).

Total RNA was used as a template to synthesize cDNA. RNA was first incubated with 1 µL oligo (dT) (0.5 µg/lL, Invitrogen, Brazil), 1 µL 10 mM dNTPs, and DEPC-treated water to a final volume of 12 µL, for 5 min at 65ºC and, then, for 1 min in ice. The following reagents were then added to reach a final volume of 19 µL: 4 µL of RT buffer (50 mM Tris–HCl, pH 8.3; 75 mM KCl, 3 mM $MgCl_2$), 2 µL of 0.1 M DTT, and 1 µL of RNaseOUT (40 U/µL, Invitrogen, Brazil). After 2 min incubating at 37ºC, 1 µL of M-MLV-Reverse Transcriptase (200 U/lL,

Invitrogen, Brazil) was added and cDNA synthesis was performed at 50ºC for 1 h. The reaction was inactivated by incubation at 70ºC for 15 min.

2.2.2. Real Time qPCR

Table 1 shows all primer sequences used in this study, which were designed using the software Primer-3 (Rozen and Skaletsky 2000) and based on rat mRNA sequences in the GenBank database (Benson et al. 2013). The specificity of the primers was checked using BLAST search against nucleotide collection (nr) of the NCBI database. All primers were obtained from Invitrogen (Brazil). The Norm Finder software (Department of Molecular Medicine Aarhus University Hospital, Denmark) was used to determine the best housekeeping gene, if Act-B, UbC or CypA.

Using the SYBR® Green PCR Master Mix (Applied Biosystems, Brazil), the cDNA (1 µL) was subjected to qPCR in a StepOnePlus™ thermocycler (Applied Biosystems, USA). The amplification of all genes was carried out using 7.5 µl of SYBR Green PCR Master Mix, 0.5 µL of forward and reverse primers (0.33 µM each), 100 ng of cDNA and nuclease-free water providing a total volume of 15 µL. Reactions were performed in an optical 96-well plate using a StepOnePlusTM thermocycler (Applied Biosystems, USA). An initial denaturation step at 95ºC for 10 min was performed, followed by an amplification in 40 cycles of denaturation at 95ºC for 30 s, annealing at 60ºC for 40 s, and extension at 72ºC for 40 s. Amplification was followed by a melting curve analysis to confirm PCR product specificity. No signals were detected in non-template controls (data not shown). The experimental cycle threshold (Ct) was calculated using the algorithm enhancement provided by the equipment. All samples were run in duplicate and mean values were used for the further steps and calculations. The Ct value of each reaction was used to calculate the level of mRNA expression of each specific gene, after normalizing it to the expression of the control housekeeping gene Act-B, analyzed in parallel in the same reaction plate. Gene expressions were calculated with the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001). Finally, the IGF-1 gene amplification was additionally tested in the rat dorsal hippocampus using the same protocol to check and control for its absence

in the MePD. IGF-1 gene expression was evident in the hippocampus (data not shown).

2.3. Immunofluorescence Procedure

2.3.1. Tissue Processing and Antibodies Used

Rats were deeply anesthetized with xylazine (10 mg/kg) and thiopental (120 mg/kg) and transcardially perfused, after the injection of heparin (1000 IU), using 4% formaldehyde diluted in 0.1 M phosphate buffer solution (PBS, pH 7.4) at RT. The brains were removed and kept at the same fixation solution for 24 h, cryoprotected in 15% and 30% sucrose solutions, quickly frozen in liquid nitrogen and maintained at -80ºC until further processing. Brains were sectioned using a cryostat (Leica, Germany) providing rostrocaudal serial coronal sections of 10 µm-thick each. The right MePD was examined.

Samples were then fixed and permeabilized in cold acetone at RT for 10 min, washed with PBS and delimited with a hydrophobic barrier pen (Vector Laboratories, USA). The sections were blocked with 10% fetal bovine serum in PBS containing 0.05% Triton X-100 for 1 hour at RT, rinsed in PBS and incubated with primary monoclonal antibody for (1) mouse anti-BDNF (Thermo Fisher Scientific, USA; catalog code MA5-23665) diluted 1:300 in 2% BSA/0.05% PBS-Triton X for 24 h (this same medium solution for each antibody tested), (2) mouse anti-IGF-1 (Thermo Fisher Scientific, code MA5-18035) diluted 1:200, (3) anti-PSA-NCAM (1:200; Thermo Fisher Scientific, code 14-1918-80) and (4) anti-EphrinA4 conjugated with fluorescent Alexa Fluor 488 (Santa Cruz Biotchnology, code 365503 FITC). Samples were rinsed with PBS-Triton X twice, three times with PBS, and incubated with the secondary polyclonal antibody goat anti-mouse IgG3 heavy chain fluorescent FITC (ABCAM, USA, catalog code ab98707) diluted 1:1000 in 2% BSA/0.05% PBS-Triton-X for 2 h. Afterwards, samples were rinsed with PBS-Triton X twice and three times with PBS. Sections were counterstained along 10 min with the nuclear dye Hoescht 33342 (4 µ/ml; Thermo Fischer Scientific, catalog code H3570). After being washed in PBS, the sections were mounted with the antifading mounting “Fluoromount G” (“antifading

medium solution", refractive index = 1.4, Electron Microscopy Sciences, USA) and coverlips.

To control non-specific binding, the primary antibody was omitted and replaced by PBS. No reaction was observed. In order to minimize diferences in staining and in background levels, the brains in all experimental groups were fixed and postfixed in identical solution for the same time, processed at the same time and incubated in the same immunostaining medium for the same period of time (Saur et al., 2014).

The antibody specificity was ascertained by Western blot (data not shown). Nonspecific binding of the secondary antibody was controlled by omitting the primary antibody and its replacement by PBS. No reaction was observed in this condition (data not shown). The pattern of immunofluorescent expression of each antibody studied was previously reported in fixed tissues (Job and Cooke, 2015; Rosenberger et al., 2014; Wang et al., 2014), and according to the manufacturer´s data sheet, as follows:

BDNF: https://www.thermofisher.com/antibody/product/BDNF-Antibody-clone-35909-Monoclonal/MA5-23665
IGF-1: https://www.thermofisher.com/antibody/product/IGF1-Antibody-clone-7973-Monoclonal/MA5-18035
PSA-NCAM: https://www.thermofisher.com/antibody/product/PSA-NCAM-Antibody-clone-12E4-Monoclonal/14-9118-80
Ephrin-A4 : https://www.scbt.com/scbt/product/epha4-antibody-d-4

2.3.2. Immunofluorescence Image Analysis

Fluorescence images were obtained using a fluorescence-inverted microscope (Olympus IX51 U-RFL-T, Olympus Corporation, USA) with plan semi-apochromat objective lens (×40, Olympus UPLFL, Olympus UPLFL, 0.55 N.A.). The 510-550 wavelength laser was used to excite the samples and the Olympus DP controller 3.3.1292 software was used for imaging. The best exposure time for each antibody was tested and maintained constant for gathering data in all groups as follows: 1.2 s for anti-BDNF, 5 s for anti-IGF-1, and 1.5 s, for PSA-NCAM 2.5 s, and for anti-Ephrin A4.

For each tested antibody, we studied 6 sections containing the MePD along the rostrocaudal axis from each animal. Four squares (named “areas of interest”, AOI, each side 84,5 µm, total 7056 μm^2, with two including and two excluding borders) were randomly overlaid on the image of the MePD in each section, totalizing 24 AOI per rat in each group. The fluorescence intensity in each AOI was assessed using the Image J software (Rasband 1997-2016) with the plugin ‘‘Intensity for Background Subtraction’’ (available at http://www.loci.wisc.edu/software). This plugin allows for both uniform background subtraction across all images and measurement of fluorescence intensity puncta in the sampled AOI. Average fluorescence intensity was calculated for each rat and experimental group. Because Ephrin-A4 data was seen in individual cells, we also evaluated the fluorescence intensity from the whole AOI and, afterwards, from each whole cell body and somatic cytoplasm sampled. This was done for all the cells that are in the included borders of the AOI. Mean values were calculated for each rat and used for further statistical analyses between groups. All image analysis was performed on raw image data. Final image adjustments of brightness were done using the Adobe Photoshop CS5 (Adobe Systems, USA) and equally apllied to all groups as shown in the figures below.

For the stereological estimation, the numerical densities of immunofluorescent puncta for all tested antibodies and of cells (specific for the Ephrin-A4 results) were estimated using the following formula:

$$Nv = (1/[a/f.h]) \,.\, (\Sigma Q/\Sigma P)$$

where Nv= estimated numerical density, a/f= area of the counting frame, h= disector height; ΣQ= sum of cells (puncta or cells) counted, and ΣP= sum of analyzed counting frames. The postprocessing thickness of each brain slice was measured, and the section height was used as the disector height (Costa Ferro et al., 2010).

2.4. Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM) for the relative gene expressions and as mean $\pm$ standard deviation (SD) for each tested immunofluorescent antibody results in each experimental group. Values were

tested for normality using the Kolmogorov-Smirnov test and for homogeneity of variance using the Bartlett test. To fulfill parametric requirements, data were transformed and results were submitted to the one-way analysis of variance (ANOVA) test and, when indicated, to the Bonferroni *post hoc* test for multiple comparisions. Statistical level of significance was set as $P < 0.05$ in all cases.

3. Results

The qRT-PCR results show the gene expression of BDNF, PSA-NCAM and Ephrin-A4 in the MePD of males and females along the phases of the estrous cycle (Figure 2 A-C). No amplification was detected for IGF-1 in the MePD even after repeating tests and cheking for the amount of tissue available for testing. On the other hand, the same samples of IGF-1 were amplified in the hippocampus, indicating that its expression in the MePD was indeed absent or too low to be detected (data not shown).

There were no statistically significant difference between groups in the gene expression of BDNF [$F(3,24) = 1.649$, $p = 0.208$; Figure 2A], NCAM [$F(3,25) = 1.056$, $p = 0.387$; Figure 2B] or Ephrin-A4 [$F(3,18) = 0.927$, $p = 0.451$; Figure 2C].

Immunofluorescent puncta for neurotrophins occurred either isolated or in clusters, and at the same focal plane of cell bodies or along the neuropil of the MePD of males and cycling females (Figures 3-6). Representative data are shown for the aspect of the overall, regional immunolabeled puncta in the experimental groups for BDNF (Figure 3A), IGF-1 (Figure 4A) and PSA-NCAM (Figure 5A). In addition to the overall, regional immunolabeling, the fluorescence cellular aspect for Ephrin-A4 cells was found in cell bodies and their proximal prolongments in the MePD of males and cycling females (Figure 6A).

Quantifications for these four neurotrophic factors and comparisons between groups showed no statistically significant differences for the average fluorescence intensity of BDNF [$F(3,22) = 0.760$, $p = 0.972$; Figure 3B], IGF-1 [$F(3,22) = 0.134$, $p = 0.939$; Figure 4B] or PSA-NCAM [$F(3,22) = 1.929$, $p = 0.159$; Figure 5B]. Nor were there statistical differences for the average fluorescence intensity of the overall, regional Ephrin-A4 [$F(3,22) = 0.231$, $p =$

0.874; Figure 6B] or the somatic Ephrin-A4 immunoreactivity [F(3,22) = 2.174, p = 0.125; Figure 6C] and the cytoplasmic Ephrin-A4 immunoreactivity [F(3,22) = 2.174, p = 0.124; Figure 6D] in the MePD of males and cycling females.

On the other hand, there were statistically significant differences in the stereological estimation of the relative number of immunolabeled puncta for BDNF [F(3,21) = 3.671, p = 0.031; proestrus > males, *post hoc* test p = 0.037; Figure 3C] and IGF-1 [F(3,21) = 3.895, p= 0.026; proestrus > diestrus, *post hoc* test p = 0.037; Figure 4C] in the rat MePD. No statistically significant difference between groups was detected for the numer of immunolabeled puncta for PSA-NCAM [F(3,20) = 1.579, p = 0.231; Figure 5C] or Ephrin-A4 [F(3,20) = 2.077, p = 0.141; Figure 6E] in this brain area and studied groups.

4. Discussion

Our main finding indicates differences in immunolabed puncta for BDNF between proestrus females and males and for immunolabeled puncta for IGF-1 between females in proestrus and diestrus. To dMales and females in diestrus, proestrus and estrus did not differ in the gene expression of BDNF, PSA-NCAM and Ephrin-A4 whereas IGF-1 could not be detected in the MePD. Males and females in diestrus, proestrus and estrus did not differ in the protein expression assessed as the overall, regional immunofluorescence intensity of BDNF, IGF-1, PSA-NCAM and Ephrinn-A4 in the MePD.

We observed an absence of IGF-1 gene expression, but not IGF-1 protein detection in the MePD of both sexes. Quantification of mRNA and corresponding protein levels are complementary approaches for the identification of likely candidates to modulate trophic actions on the cellular structure and function (Greenbaum et al., 2003). The mRNA is eventually translated into specific proteins, but some discrepancies between data provided by RT-qPCR and the corresponding immunofluorescent protein detection are not uncommon to occur in the nervous tissue (Gazzaley at al., 1996; Reyna- Neyra et al., 2002). This can be attributed to: (1) various complex posttranscriptional mechanisms involved in turning mRNA into protein that are not be completely known (Greenbaum et al., 2002; Maier et al., 2009); (2) gene expression does not always reflect protein

expression, stability or turnover (Bethea and Reddy, 2012). Proteins can differ substantially in their *in vivo* half-lives as a result of variable synthesis and degradation processes along time, depending on the balance between local stimulatory and inhibitory factors (Glickman and Ciechanover, 2002; Rizzo et al., 2014). And, (3) there can be region-specific and cell-specific features that contribute to the heterogeneity of experimental results using different techniques (Hansberg-Pastor et al., 2015, Popovic et al., 2018). For example, the MAP2, a protein mainly expressed in dendrites, is increased in the hipocampus of ovariectomized rats treated with estrogen, but the mRNA expression for MAP2 did not change concomitantly in these rats (Reyna-Neyra et al., 2002). In addition, there are no changes in the MAP2 expression in the medial preoptic area of rats along pregnancy, when sex hormones are notably increased, suggesting that tissue-specific factors are also involved in local protein expression (González-Arenas et al., 2014). Likewise, IGF-I gene expression varies regionally and developmentally in the central nervous system with a marked decrease after 2-3 months of age in the olfactory bulb and cerebral cortex, but not in the hypothalamus and cerebellum of rats (Bach et al., 1991). These possibilities may explain the absence of IGF-1 gene expression, but not IGF-1 protein detection in the MePD of both sexes in the present study. The MePD may be included in brain areas where actions of IGF-1 continue at adulthood, likely mediating changes in morphology and synaptic efficacy in the developing CNS (Dyer et al., 2016).

It was initially hypothetized that structural effects of sex steroids in MePD cells would involve the concomitant synthesis and actions of neurotrophic factors (based on Zhou et al., 2005; Rasia-Filho et al., 2012a; Hansberg-Pastor et al., 2015). The male MePD neuropil has a characteristic synaptic organization of axonal inputs (Nishizuka and Arai, 1981, 1983; Cooke and Woolley, 2005) ending on dendritic spines (Hermel et al., 2006; Brusco et al., 2014) whose number is higher than in females and change shape following puberty and sexual experience (Rasia-Filho et al., 2012a; Zancan et al., 2018; Dalpian et al., 2019). Indeed, stubby/wide, thin and mushroom-like dendritic spines show particular morphological features in prepubertal males that differ from those of age-matched females (Zancan et al., 2018). At adulthood, the number of thin spines

decreases whereas the number of mushroom-like ones increases in the MePD of males (Zancan et al., 2018). On the other hand, in proestrus females, there is a decrease in dendritic spines, an increase in the number and variability in the shape of somatic spines, and an increase of synapses made directly on dendritic shafts (Rasia-Filho et al., 2004; Brusco et al., 2014; Zancan et al., 2015). These data indicate a complex modulation of the dendritic spine number and shape and the synaptic organization modulated by gonadal hormones under physiological conditions in the MePD of both sexes (Rasia-Filho et al, 2004, 2012a; Zancan et al., 2015, 2018; Dalpian et al., 2019). Synaptic plasticity in the MePD may, therefore, depend on neurotrophic factors to account for the sexually dimorphic genetic determinantion of its structure, as well as the activational effects of gonadal hormones on local cells in both sexes.

Estrogen induces trophic effects on cellular and synaptic growth, development and connectivity in functionally integrated networks for both reproductive and non-reproductive behaviors, such as mood and cognition (Pfaff and Cohen, 1987; Lucion et al., 1996; Spencer-Segal et al., 2012; Hansberg-Pastor et al., 2015; McEwen and Milner, 2017; Sheppard et al., 2019). Sex steroid effects can be elicited by direct actions on cells or indirectly via second-messenger pathways and the regulation of gene products (Woolley and Cohen 2002; Srivastava et al., 2011; Hansberg-Pastor et al., 2015). The estrogenic effects can be due to binding on classical nuclear receptors or on membrane receptors to promote cell proliferation, growth and survival (Srivastava et al, 2011). Particularly important are proteins involved in the regulation of actin, the main cytoskeletal component of spines and which appears to determine their number and shape (Matus, 2000; Ethell and Pasquale, 2005; Kennedy et al., 2005; Gordon-Weeks and Fournier, 2014; Nakahata and Yasuda, 2018). The present sex-specific results suggest the participation of BDNF and IGF-1 in the complex scenario of synaptic remodelling in the MePD of proestrus female rats. The proestrus phase shows the highest plasma levels of ovarian steroids due to an increased secretion of GnRH, the occurrence of ovulation and the disinhibition of female receptive, sexual behavior (reviewed in Simerly, 2004; Rasia-Filho et al., 2012b; Rasia-Filho and Zancan, 2016). Interestingly, the MePD has relevant connections with the hypothalamic nuclei that control all these timely and

dynamic physiological events (Petrovich et al., 2001; Simerly, 2004; Zancan et al., 2015).

In this regard, the estradiol upstream effectors Akt, LIMK, TrkB and the expression of the pre- and postsynaptic markers synaptophysin and PSD-95 are activated in the mice hippocampus during proestrus (Spencer et al., 2008). Estradiol upregulates BDNF in female mice (Spencer et al., 2008) and this neurotrophin increases the number of dendritic spines in the hippocampus of ovariectomized female rats (Kramár et al., 2012). In the medial amygdala, long-term estrogen treatment increased gold immunolabeling and mRNA levels evidenced by *in situ* PCR of BDNF in ovariectomized rats (Zhou et al., 2005). Higher levels of BDNF in the MePD occurs during the proestrus and may also be associated with dynamic changes in the number of somatic and dendritic spines, the remodeling of axo-spine and direct axo-dendritic synapses, and the higher astrocytic expression of GFAP (Rasia-Filho et al., 2004; Martinez et al., 2006; Rasia-Filho et al., 2009, 2012a; Zancan et al., 2015). Furthermore, IGF-1 is required for neuroplasticity (Dyer et al., 2016) and the maintenance of dendritic spine size and density in adult rats (Nieto-Bona et al., 1997). IGF-1 also increases spine dynamics affecting the spine length, the volume of the spine head (Landi et al., 2011) and the postsynaptic density (Dyer et al., 2016 and references therein). Higher values of IGF-1 were also found during the proestrus in the female MePD. The cellular effects of both BDNF and IGF-1 have now to be examined in proestrus for signal transduction cascades that can lead to local protein synthesis and/or rearrangement of the cytoskeleton, including actions on the cyclic AMP response element-binding protein (CREB)-related gene products and phosphorylated forms, calcium/calmodulin-dependent protein kinase type IV, and the activity-regulated cytoskeleton-related protein (Zhou et al., 2005; Rasia-Filho et al., 2012a and references therein).

Finally, it was recently demonstrated that microRNA (miRNA) expression is also a main target element modulated by gonadal hormones in the MePD of males and cycling females (Hirsch et al., 2018). miRNAs can be localized at dendritic spines and the postsynaptic density (Im and Kenny, 2012). In the rat MePD, the expression of (1) miR138-5p was higher in males than in females along the estrous cycle, (2) miR181a was higher in males than in females in

diestrus and estrus, (3) miR25-3p was higher in diestrus females than in proestrus ones, and (4) mi25-3p, mi181a-5p and 195-5p were higher in diestrus females than in estrus females (Hirsch et al., 2018). Moreover, estrogen is a strong regulator of the activity of small GTPases (Kramár et al., 2009; Babayan and Kramár, 2013). The GTPase family of proteins has a role in neuronal structural plasticity, linking extracellular signals and direct regulators of actin dynamic structural changes to modulate the formation and development of dendritic spines (Bethea and Reddy 2012; Saneyoshiand and Hayashi, 2012; Woolfrey and Srivastava, 2016; Nakahata and Yasuda, 2018). Cortical neurons exposed to 17β-estradiol show a rapid increase in a specific smal GTPase Rap1 concomitant with an increase in dendritic spine number (Srivastava et al., 2008). These data suggest that the structural and synaptic plasticity modulated by ovarian steroids in the MePD may involve BDNF and IGF-1 as local neurotrophic factors, the actions of different miRNA and, as a possibility to be further addressed experimentally, estrogen-stimulated small GTPases.

In conclusion, our results indicate that neurotrophic factors are not homogeneously expressed in the adult rat MePD, being evident for BDNF, PSA-NCAM and Ephrin-A4, but not for IGF-1. Average immunofluorescence for the protein expression of these neurotrophic factors showed no differences in the MePD of males and females, but proestrus rats showed a higher number of labelled puncta compared to males (for BDNF) and diestrus females (for IGF-1). These data add to our knowledge of the complex modulation of structure and function in the MePD of adult rats, which likely involve local neural gonadal steroid actions and the time-dependent modulation of synaptic function in females along the estrous cycle.

Conflict of Interst

Authors declare no real or potential conflict of interest.

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Legends

Figure 1. **(Left)** Schematic diagram of the ventral aspect of a coronal brain section showing the posterodorsal medial amygdala (MePD, filled in gray; in this case at -3.0 mm posterior to the bregma) lateral to the optic tract (opt) and ventral to the stria terminalis (st). Adapted from Paxinos and Watson (2008). (**Right**) Microscopic fluorescent image of a matched coronal section of the MePD (indicated by an arrow) to demonstrate where gene and protein expressions were studied. Fluorescence using the dye Hoescht 33342 to visualize cell nuclei in the brain section. Scale= 200 µm.

Figure 2. Data are mean ± standard error of the mean for the RT-qPCR for the gene expression of BDNF, NCAM and Ephrin-A4 in the posterodorsal medial amygdala of males (n= 8, 8 and 6) and females in diestrus (n= 6, 7 and 4), proestrus (n= 4 in all experiments) and estrus (n= 7, 7 and 5), respectively. No amplification was found for IGF-1. Symbols represent values for each rat studied in each group. No statistical differences were found between groups ($p > 0.05$ in all cases).

Figure 3. (A) Digitized images for the immunofluorescence presence and quantity of BDNF in the posterodorsal medial amygdala (MePD) of males (n= 6) and females in diestrus (n= 5), proestrus (n= 6) and estrus (n= 6). The dye Hoescht 33342 served to visualize cell nuclei and to compose merged images showing immunofluorescent BDNF puncta at the same focal plane as cell bodies and in the MePD neuropil. **(B)** Data are mean ± standard deviation(SD) for the average immunofluorensce intensity (AU) related to the protein expression of BDNF in these four experimental groups. Symbols represent values for each rat studied in each group. No statistical differences were found between groups ($p > 0.05$ in all cases). **(C)** Mean ± SD for the number of immunofluorescent BDNF puncta in the MePD of these rats. Proestrus females showed higher values than male rats ($*p < 0.05$).

Figure 4. (A) Digitized images for the immunofluorescence presence and quantity of IGF-1 in the posterodorsal medial amygdala (MePD) of males (n= 6)

and females in diestrus (n= 5), proestrus (n= 6) and estrus (n= 6). The dye Hoescht 33342 served to visualize cell nuclei and to compose merged images showing immunofluorescent IGF-1 puncta at the same focal plane as cell bodies and in the MePD neuropil. **(B)** Data are mean ± standard deviation (SD) for the average immunofluorensce intensity (AU) related to the protein expression of IGF-1 in these four experimental groups. Symbols represent values for each rat studied in each group. No statistical differences were found between groups ($p > 0.05$ in all cases). **(C)** Mean ± SD for the number of immunofluorescent IGF-1 puncta in the MePD of these rats. Proestrus females showed higher values than diestrus females ($*p < 0.05$).

Figure 5. (A) Digitized images for the immunofluorescence presence and quantity of PSA-NCAM in the posterodorsal medial amygdala (MePD) of males (n= 6) and females in diestrus (n= 5), proestrus (n= 5) and estrus (n= 6). The dye Hoescht 33342 served to visualize cell nuclei and to compose merged images showing immunofluorescent PSA-NCAM puncta at the same focal plane as cell bodies and in the MePD neuropil. **(B)** Data are mean ± standard deviation(SD) for the average immunofluorensce intensity (AU) related to the protein expression of PSA-NCAM in these four experimental groups. Symbols represent values for each rat studied in each group. **(C)** Mean ± SD for the number of immunofluorescent PSA-NCAM puncta in the MePD of these rats. No statistical differences were found between groups in both comparisons ($p > 0.05$ in all cases).

Figure 6. (A) Digitized images for the immunofluorescence presence and quantity of Ephrin-A4 in the posterodorsal medial amygdala (MePD) of males (n= 6) and females in diestrus (n= 5), proestrus (n= 6) and estrus (n= 6). The dye Hoescht 33342 served to visualize cell nuclei and to compose merged images showing immunofluorescent Ephrin-A4 cell bodies and prolongments at the same focal plane in the MePD neuropil. **(B)** Data are mean ± standard deviation(SD) for the average immunofluorensce intensity (AU) related to the protein expression of Ephrin-A4 in these four experimental groups. Symbols represent values for each rat studied in each group. In addition, data are mean ± SD for the **(C)** regional, **(D)** whole cell body and **(E)** somatic cytoplasm immunofluorescence for

Ephrin-A4 in the MePD of these rats. No statistical differences were found between groups in all comparisons done ($p > 0.05$ in all cases).

Table 1. Primers sequences related to the genes estudied in posterodorsal medial amygdala of male and female rats.

Gene[a]	ID[b]	Primer F	Primer R
Bdnf	24225	5'GATGAGGACCAGAAGGTTCG3'	5'GATTGGGTAGTTCGGCATTG3'
IGF-1	24482	5'CGTACCAAAATGAGCGCACC3'	5'TAGCCTGTGGGCTTGTTGAA3'
NCAM	24586	5'TAGAGGTCCGAACGGAGGAG3'	5'TCTACAGGACCCTTCTCGGG3'
EphA4	31643	5'CCACTAACCCCAGGTTGCTT3'	5'TAATGCAAACGTTTCCGGGC3'
ActB	81822	5'TATGCCAACACAGTGCTGCTGG3'	5'TACTCCTGCTTGATCCACAT3'

[a] Abbreviations: Bdnf,brain-derived neurotrophic factor; IGF-1, insulin-like growth factor 1; NCAM, neural cell adhesion molecule; EphA4, Ephrin-A4, ActB, beta-actin

[b] Gene Identification Number, available at: http://www.ncbi.nlm.nih.gov/gene.

Figure 1

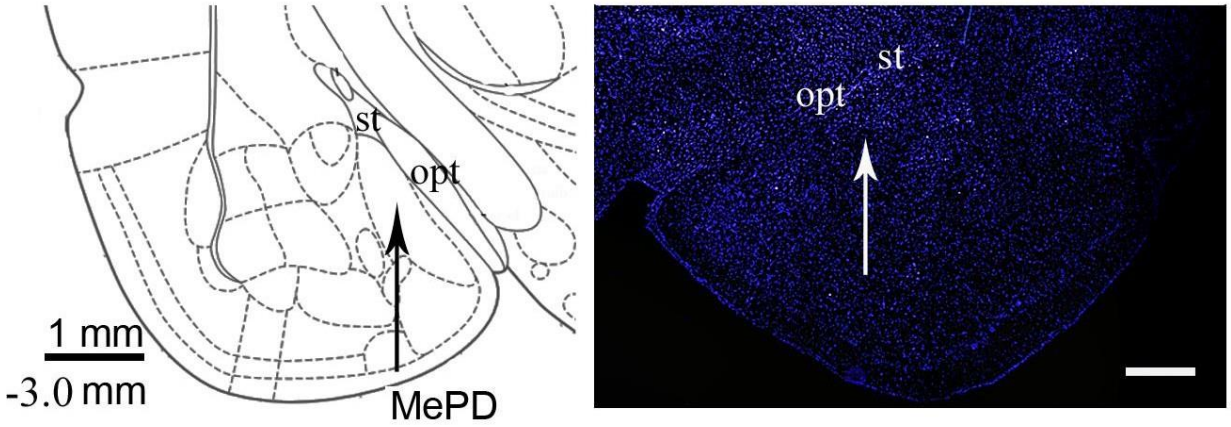


Figure 2

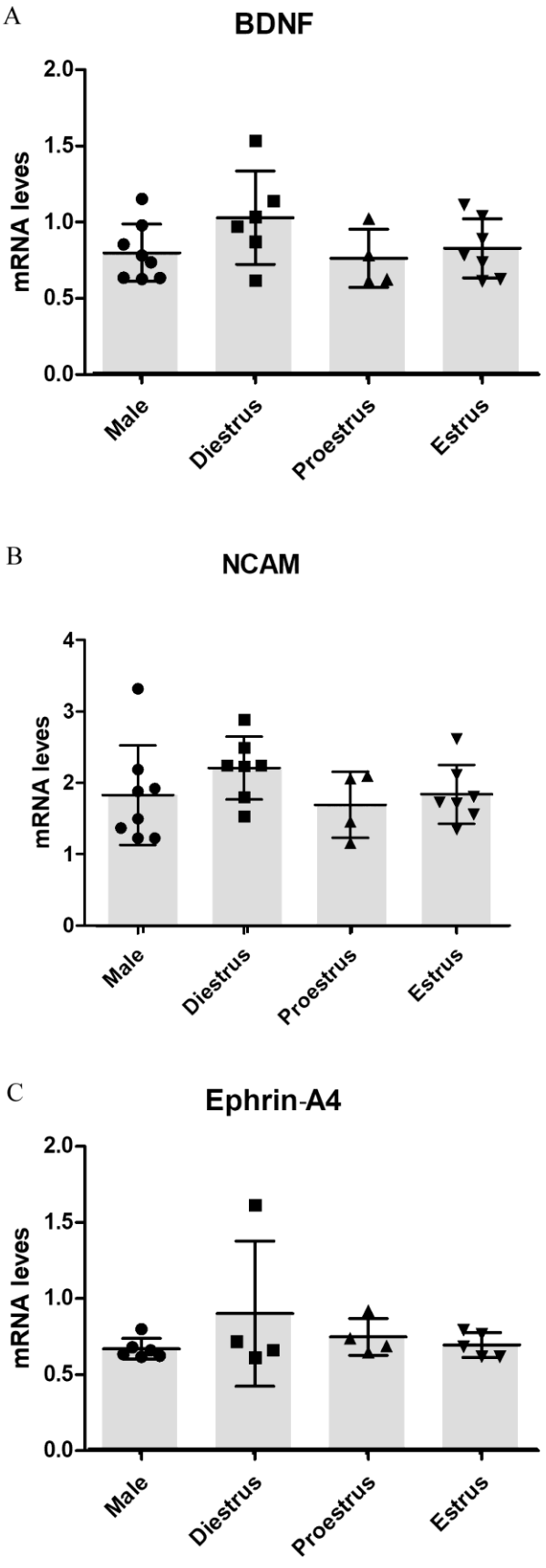

A
BDNF
mRNA leves
2.0
1.5
1.0
0.5
0.0
Male
Diestrus
Proestrus
Estrus
B
NCAM
mRNA leves
4
3
2
1
0
Male
Diestrus
Proestrus
Estrus
C
Ephrin-A4
mRNA leves
2.0
1.5
1.0
0.5
0.0
Male
Diestrus
Proestrus
Estrus

Figure 3

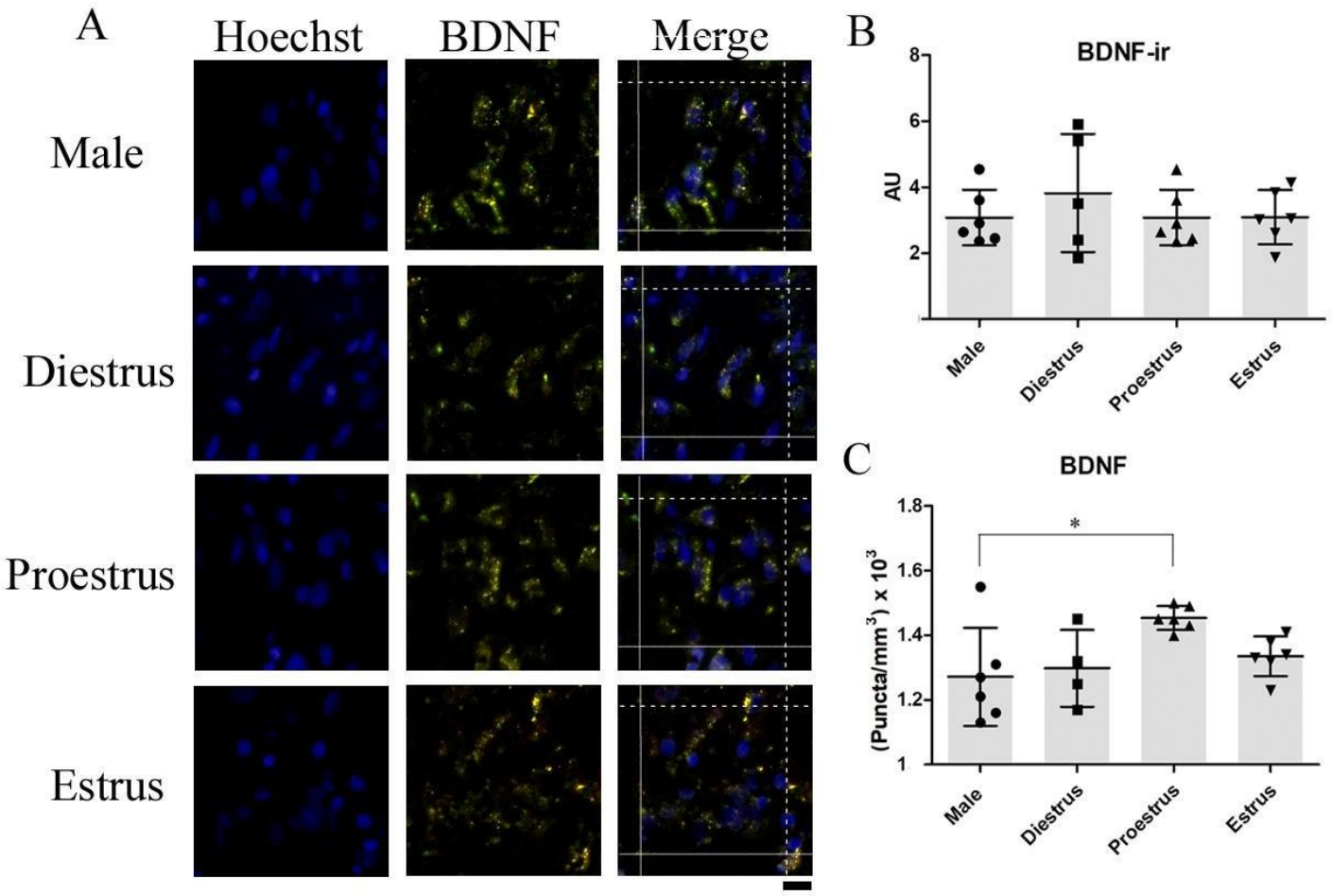

A
Hoechst
BDNF
Merge
Male
Diestrus
Proestrus
Estrus
B
BDNF-ir
AU
8
6
4
2
Male
Diestrus
Proestrus
Estrus
C
BDNF
(Puncta/mm3) x 10^3
1.8
1.6
1.4
1.2
1
*
Male
Diestrus
Proestrus
Estrus

Figure 4

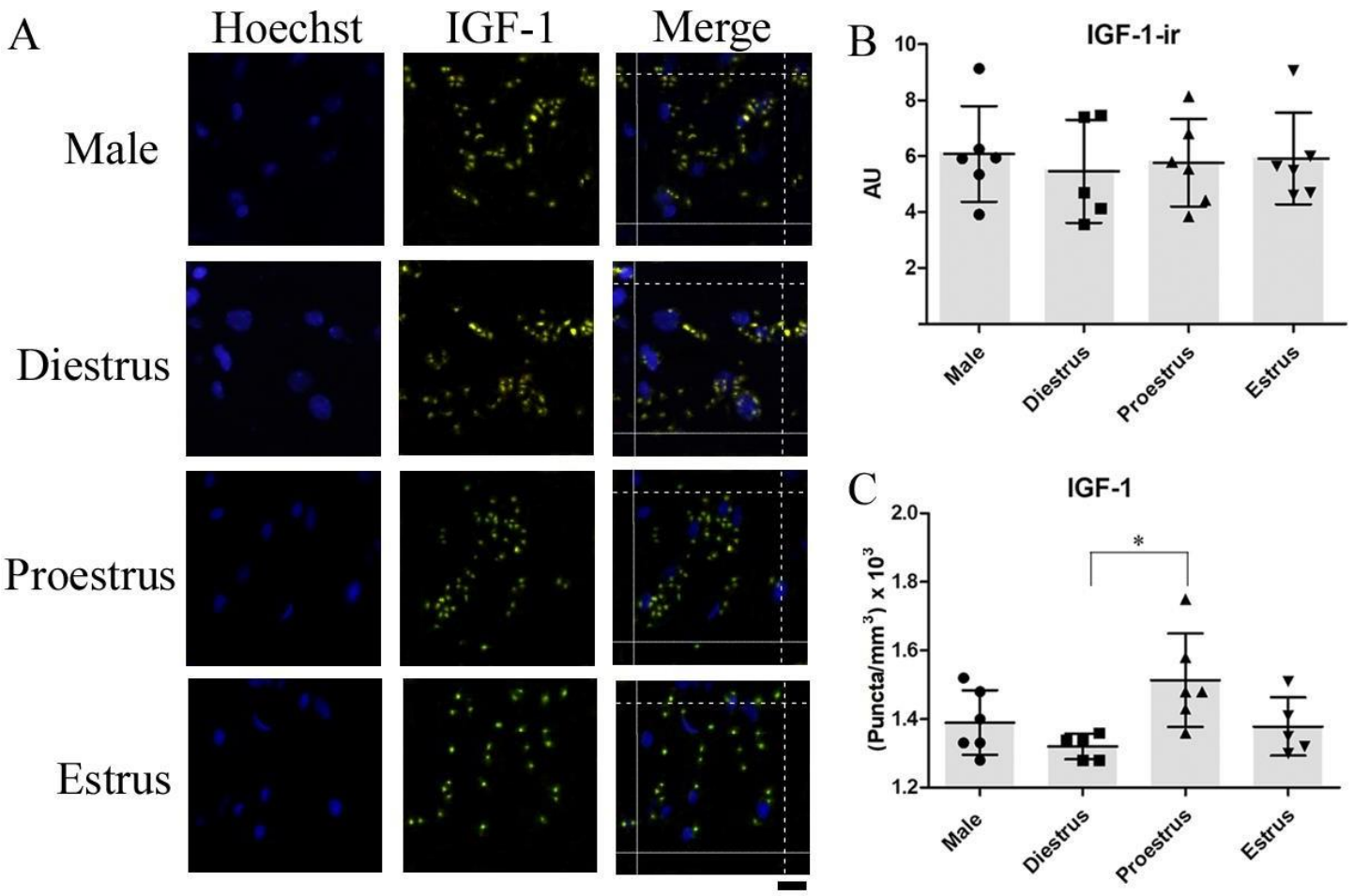

A
Hoechst
IGF-1
Merge
Male
Diestrus
Proestrus
Estrus
B
IGF-1-ir
AU
10
8
6
4
2
Male
Diestrus
Proestrus
Estrus
C
IGF-1
(Puncta/mm3) x 10-3
2.0
1.8
1.6
1.4
1.2
*
Male
Diestrus
Proestrus
Estrus

Figure 5

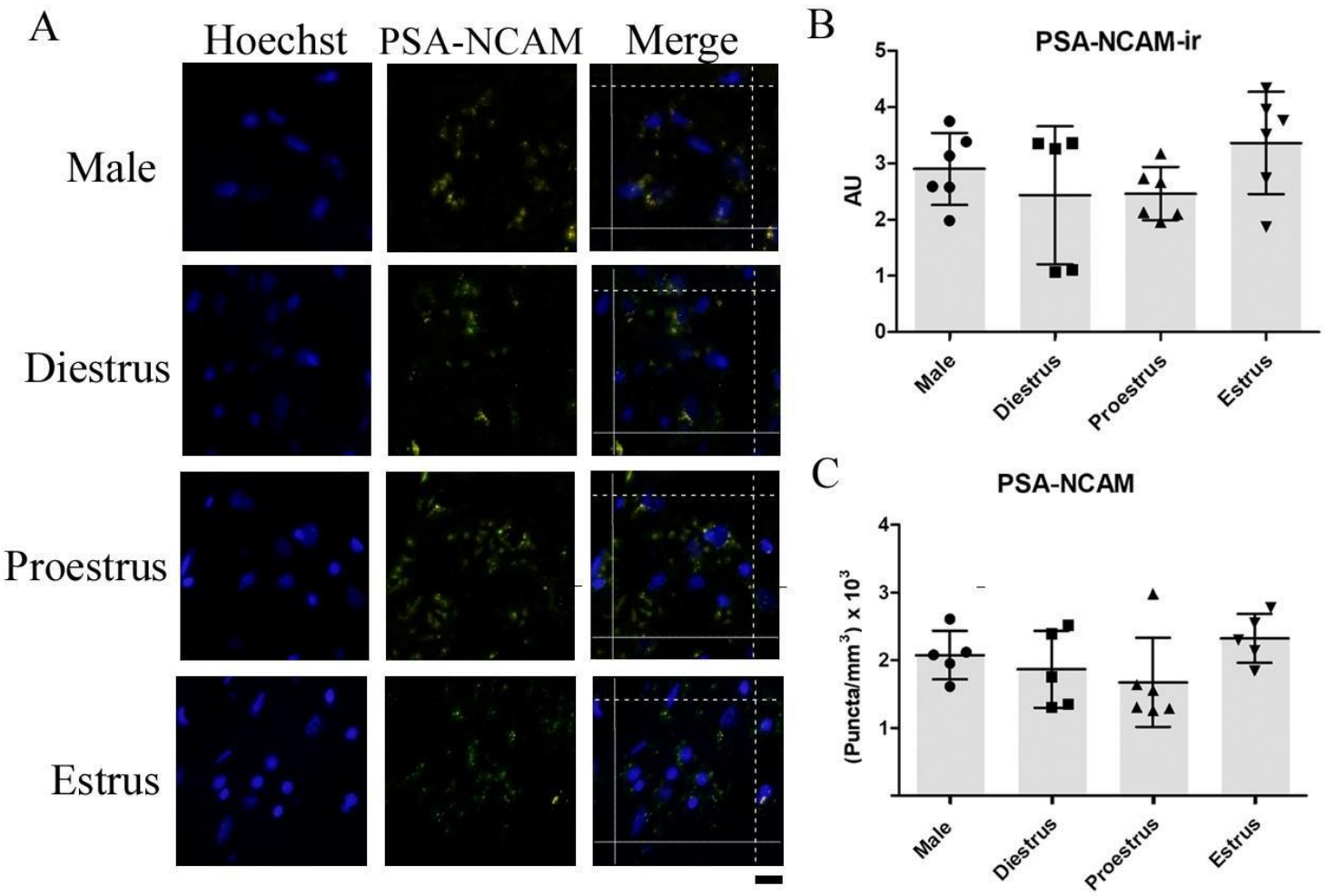
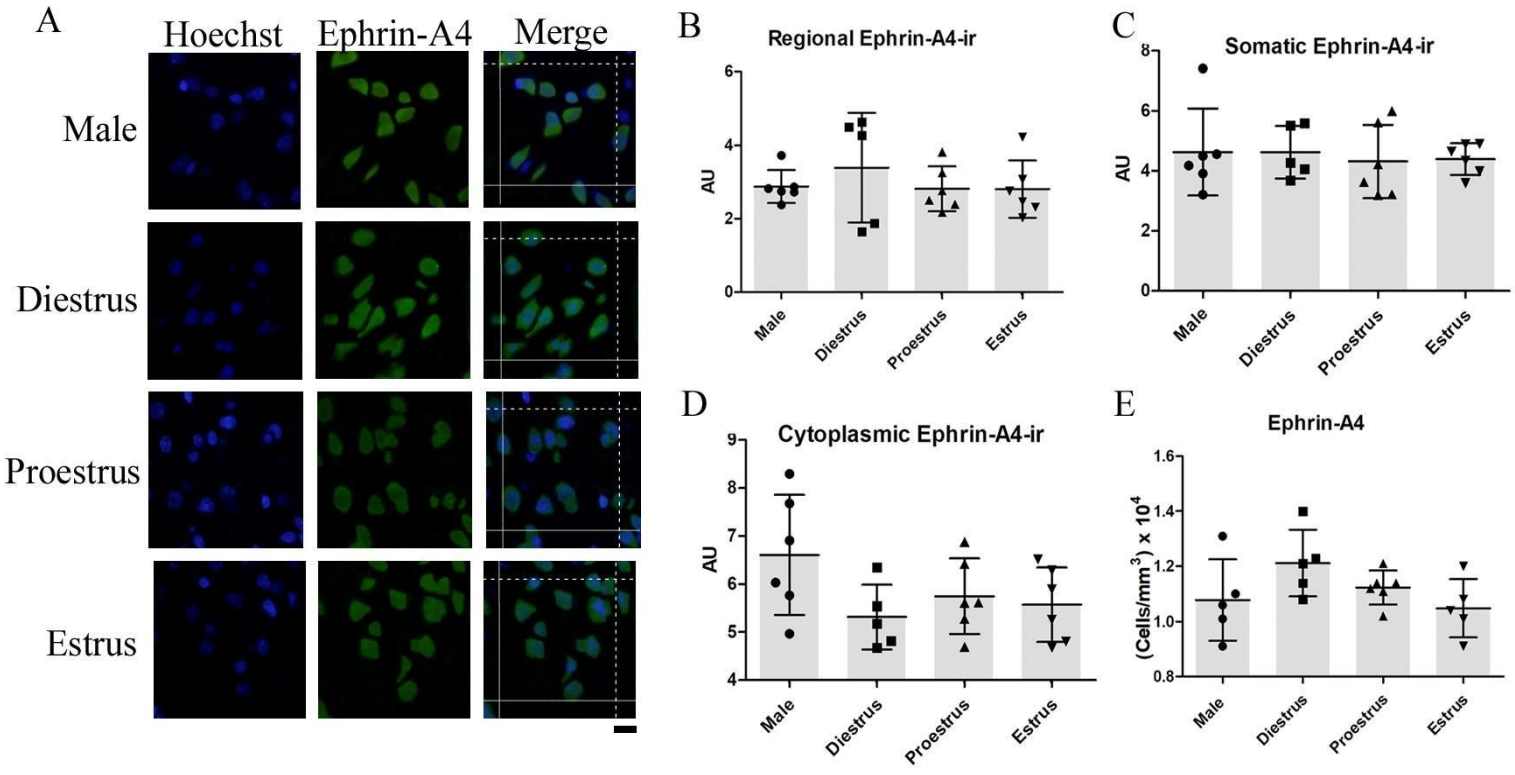

A
Hoechst
PSA-NCAM
Merge
Male
Diestrus
Proestrus
Estrus
B
PSA-NCAM-ir
AU
0
1
2
3
4
5
Male
Diestrus
Proestrus
Estrus
C
PSA-NCAM
(Puncta/mm3) x 10^3
1
2
3
4
Male
Diestrus
Proestrus
Estrus

Figure 6


A
Hoechst
Ephrin-A4
Merge
Male
Diestrus
Proestrus
Estrus
B
Regional Ephrin-A4-ir
AU
C
Somatic Ephrin-A4-ir
AU
D
Cytoplasmic Ephrin-A4-ir
AU
E
Ephrin-A4
(Cells/mm3) x 10^4
Male
Diestrus
Proestrus
Estrus

4. DISCUSSÃO

Os presentes dados avançam os conhecimentos atuais sobre a estrutura e plasticidade da MePD de ratos e serão discutidos, adicionalmente ao já realizado em cada artigo acima, de acordo com a sua apresentação. Neste momento buscou-se evitar repetições desnecessárias na abordagem do assunto, mas acrescenter raciocínios adicionais aos novos conhecimentos obtidos.

4.1 Dimorfismo sexual em ratos pré-púberes e plasticidade em ratos pós-púberes com e sem experiência sexual

Os dados do primeiro artigo mostram que machos pré-púberes tem maior densidade de espinhos dendríticos do que fêmeas. Considerando o tipo morfológico de espinho, machos tem mais espinhos achatados/espessos do que fêmeas, e fêmeas tem mais espinhos do tipo cogumelo. A seguir, não há diferença estatisticamente significativa na densidade de espinhos quando comparados os grupos de machos pré-púberes, machos pós-púberes sem experiência sexual e macho pós-púberes com experiência sexual. No entanto, o número de espinhos do tipo fino é maior em machos pré-púberes quando comparados com machos pós-púberes com experiência sexual, e o número de espinhos do tipo cogumelo é maior em machos pós-púberes com experiência sexual quando comparado com os outros dois grupos estudados.

Adicionalmente, as análises morfométricas revelaram que: 1) em machos pré-púberes, os espinhos do tipo achatado/espesso são maiores e com maior diâmetro da cabeça e os dos tipos fino e cogumelo possuem pescoço mais largo e maior diâmetro da cabeça quando comparados com fêmeas pré-púberes; 2) em fêmeas pré-púberes, o comprimento do pescoço dos espinhos do tipo cogumelo é maior do que em machos na mesma idade; 3) machos pós-púberes sem experiência sexual tem espinhos do tipo achatado/espesso menores e os espinhos do tipo cogumelo possuem menor diâmetro do pescoço; 4) machos pós-púberes com experiência sexual possuem maior comprimento dos espinhos do tipo achatado/espesso, maior diâmetro da cabeça dos espinhos dos tipos fino

e achatado/espesso assim como maior diâmetro do pescoço dos espinhos dos tipos fino e cogumelo.

Os resultados para a densidade e a forma dos espinhos dendríticos sugerem funções específicas no circuito neural e efeitos adicionais dos esteróides sexuais para a modulação intrínseca da atividade na MePD de ratos (Rasia- Filho *et al*., 2004, 2012a, 2012b). De fato, a MePD de machos e fêmeas pré-púberes já exibem diferenças na densidade e em detalhes sutis nos componentes dos espinhos dendríticos. Essas diferenças sugerem que a MePD tem processamento e função sináptica específicos relacionados ao sexo, determinados geneticamente ou pelos efeitos dos hormônios gonadais durante o período intra-uterino e/ou ao longo das primeiras poucas semanas de desenvolvimento pós-natal (Cooke e Woolley, 2009; Cooke *et al*., 2007). Independentemente da forma, espinhos dendríticos são os principais sítios de contatos sinápticos excitatórios na MePD e formam sinapses assimétricas (Brusco *et al*., 2014). Sendo assim, diferenças na densidade de espinhos dendríticos podem refletir diferenças na densidade de sinapses excitatórias e na atividade que está sendo executada nos neurônios locais (Cooke e Woolley, 2009), proposição comprovada eletrofisiologicamente mais recentemente com animais adultos (Dalpian *et al*., 2019). Isso se associa com dados morfológicos e eletrofisiológicos da MePD que demonstram dimorfismo sexual e machos pré-púberes com aproximadamente 80% mais sinapses excitatórias que fêmeas pré-púberes (Cooke & Woolley, 2005; Cooke *et al*., 2007).

Machos pré-púberes tem mais espinhos do tipo achatados/espesso que fêmeas. Este tipo de espinho pode afetar diretamente a voltagem do dendrito uma vez que não possui pescoço para compartimentalização bioquímica e/ou elétrica (Spruston, *et a*l., 2013). É possível que informações que chegam através deste tipo de espinho possam afetar a voltagem dendrítica adjacente mais rapidamente, e vice-versa (Segal, 2010). Isso pode gerar um padrão de atividade neural na MePD diferente do de fêmeas e do que ocorre nos mesmos machos depois da puberdade. Além disso, há uma redução no número de espinhos finos e um aumento no número de espinhos de cogumelos após a experiência sexual, sugerindo um remodelamento das conexões sinápticas na MePD. Isso corrobora a possibilidade de que espinhos finos estejam relacionados a processos de

aprendizagem, evidenciando seu aspecto lábil propenso a mudanças no processamento sináptico, enquanto espinhos do tipo cogumelos estão relacionados à elaboração de “memória”, mostrando maior estabilidade, grande densidade pós-sináptica com receptores glutamatérgicos e respostas sinápticas mais intensas (Bourne e Harris, 2007; Segal, 2010).

Ou seja, embora não haja diferença estatisticamente significante na densidade de espinhos dendríticos entre os grupos de machos pré-púberes e pós-púberes com e sem experiência sexual, há uma mudança na estrutura dendrítica evidenciada pelos tipos de espinhos e pelos achados morfométricos. Neste sentido, é muito plausível que o tipo de espinho esteja relacionado com o processamento e plasticidade sinápticos na MePD importante para o comportamento sexual em machos. As análises morfométricas demonstram que os espinhos tiveram sua forma alterada e pelo menos cada uma de suas medidas aumentadas no grupo de machos com experiência sexual. O comprimento e a largura dos espinhos alteram suas propriedades biofísicas, como ocorre no comprimento e diâmetro do pescoço dos espinhos finos que impõem resistência local e compartimentalização dos processos bioquímicos na cabeça do espinho (Spruston *et al*., 2013; Yuste, 2013). A geometria do pescoço do espinho é determinante na sinalização de Ca^{2+} (Yuste, 2013). Pescoços maiores permitem maior efluxo de Ca^{2+} para o tronco dendrítico, enquanto pescoços menores aumentam a concentração de cálcio no espinho (Yuste, 2013; Segal, 2010). O volume da cabeça está diretamente relacionado ao número de vesículas pré-sinápticas, à extensão da densidade pós-sináptica e à presença e quantidade de receptores de glutamato de tipos AMPA e/ou NMDA (Yuste, 2013). Na MePD, machos com experiência sexual apresentaram valores maiores de diâmetros de cabeça e pescoço de espinhos finos, maior diâmetro do pescoço de espinhos cogumelos e maior comprimento do espinho e diâmetro da cabeça de espinhos achatados/espesso em comparação com animais pré-púberes ou pós-púberes sem experiência sexual. Esses dados sugerem que os contatos sinápticos são mais eficazes para afetar a voltagem neuronal na MePD de ratos que passaram por experiência sexual do que ratos pré-púberes ou em ratos sem experiência sexual. Esses resultados indicam a importância da plasticidade sináptica na MePD como área importante na organização central

dos comportamentos sociais, seu dimorfismo sexual, a transformação estrutural ao longo do processo ontogenético da puberdade e após a aquisição da experiência sexual, revelando mudanças no número, tipo e forma dos espinhos dendríticos locais.

4.2 Complexidade da modulação sináptica na MePD de ratos

A MePD de ratos possui uma organização sináptica que envolve contatos definidos e uma plasticidade que permite modular adicionalmente comportamentos sociais (Rasia-Filho *et al*., 2012a; Zancan *et al*., 2015, 2017, 2018). Apesar de sinapses químicas serem as prevalentes, foram reportadas discretas sinapses elétricas entre células gliais na MePD em um único estudo prévio (Brusco *et al*., 2014). Com o intuito de descrever melhor esses achados, estudou-se a ultraestrura da MePD para identificar junções comunicantes e a existência de conexinas componentes inequívocas dessas junções comunicant*es* indicativas de acoplamento elétrico celular. As imagens de microscopia eletrônica revelaram a existência de imagens características de aposição entre as membranas celulares com espaço restrito entre si. Apesar de muito escassas, as junções comunicantes foram identificadas entre dendritos. Em contraste, a presença e distribuição de Cx36, Cx43 e Cx45 foi ampla e ocorreram isoladas ou formando agrupamentos de *puncta*. Esses achados, em conjunto, revelam a possibilidade de acoplamento elétrico funcional na MePD associadamente com as sinapses químicas já bem descritas (Rasia-Filho *et al*., 2012b).

No núcleo medial amigdaloide humano, a possível presença de junções comunicantes foi documentada por microscopia eletrônica de transmissão (Dal'Oglio *et al*., 2013). Verdadeiramente, a investigação de junções comunicantes na MePD é laboriosa provavelmente porque essas junções são raras e têm distribuição restrita na MePD (Brusco *et al*., 2014). No entanto, a documentação de junções comunicantes é absolutamente relevante para acrescentar novos conhecimentos sobre a transmissão de informação na MePD de diferentes espécies, e, especialmente em ratos, no que diz respeito à sincronização das informações olfativas/ vomeronasais em machos referentes a

fêmeas sexualmente receptivas e para a emissão de comportamentos sexuais (Petrulis, 2013; Rasia-Filho *et al*., 2012b). É importante considerar que a MePD é região particularmente sensível à ação dos hormônios gonadais no SNC dada a grande concentração de receptores para testosterona, receptores de tipo α e β para estradiol e receptores para progesterona em níveis comparáveis aos de núcleos hipotalâmicos (Simerly *et al.*, 1990; Gréco *et al.*, 1996; Shughrue *et al.*, 1997; Gréco *et al.*, 2001, 2003; De Vries e Simerly, 2002; Gréco *et al.*, 2003). A ação dos esteróides gonadais se relaciona diretamente com diferenças estruturais e neuroquímicas entre machos e fêmeas nessa área (Rasia-Filho *et al.*, 2004; Cooke e Woolley, 2005; Rasia-Filho *et al.*, 2012a,b). Resultados experimentais indicam que a MePD forma um circuito sensível aos hormônios gonadais e que, por suas eferências, conecta-se a vários núcleos hipotalâmicos que integram informações olfativas e regulam a atividade neuroendócrina para modular a ocorrência do comportamento sexual de machos e fêmeas (Wood e Newman, 1995; Guillamon e Segovia, 1997; Dong *et al.*, 2001; Petrovich et al., 2001; Choi *et al.*, 2005). De fato, a MePD recebe informação direta e indireta proveniente das vias olfatória e vomeronasal e projeta eferências para a AVPV para organizar temporalmente a secreção neuroendócrina de GnRH para a ocorrência de ovulação (De Vries e Simerly, 2002) e, guardadas as particularidades de cada sexo, para os núcleos pré-óptico medial, pré-mamilar ventral e a parte ventrolateral do VMH para modular o comportamento reprodutivo masculino e feminino (Canteras et al., 1995; Newman, 2002; Meredith e Westberry, 2004; Choi *et al.*, 2005; Cavalcante *et al.*, 2006). Os dados sobre a presença de junções comunicantes e a possibilidade de ocorrer acoplamentos elétricos, embora restritos, mas maior quantidade de conexinas, com chance de formação de hemicanais e suas funções, sugere que novos experimentos sejam necessários para descobrir as implicações desses dados morfológicos com a função complexa exercida pela MePD no comportamento reprodutivo.

Com relação às sinapses químicas, machos e fêmeas diferem na densidade de espinhos dendríticos na MePD, maior nos machos do que em fêmeas em proestro ou estro (Rasia-Filho et al., 2014). Outras variações na estrutura sináptica ocorrem em proestro quanto ao número e forma de espinhos

somáticos e nos contatos sinápticos feitos diretamente nos troncos dendríticos nessa fase específica do ciclo estral (Brusco *et al*., 2014; Zancan *et al*., 2015; Dalpian *et al*., 2019). Tal plasticidade dos espinhos dendríticos poderia estar relacionada com a expressão e a ação locais de fatores neutróficos, tais como o BDNF, IGF-1, PSA-NCAM e Efrina A4. Nossa hipótese inicial era de que os níveis de expressão desses fatores tivessem relação com a densidade de espinhos dendríticos (Orefice *et al*., 2013; Rattiner et al., 2005) e modulassem eventos subcelulares mediado por hormônios gonadais (especialmente de estradiol) na MePD (Zhou *et al*., 2005; revisado em Rasia-Filho *et al*., 2012b). De fato, como fator de crescimento, o BDNF é capaz de induzir aumento no número de espinhos dendríticos no hipocampo e no córtex pré-frontal, por exemplo, trabalhando de forma sinérgica e dependente dos níveis de estrógeno (Luine e Frankfurt, 2013). Já a ovariectomia de camundongos fêmeas adultas reduz a densidade de espinhos dendríticos, promove a ocorrência de formas aberrantes de espinhos em neurônios da região CA1 do hipocampo, com menor quantidade dos espinhos com formato do tipo cogumelo e maior daqueles com aspecto fino ou achatado, efeito revertido pela administração sistêmica de estradiol e elevação local da expressão de BDNF (Li *et al*., 2004, Li *et al*., 2012).

Da mesma forma, a regulação da expressão de substâncias relacionadas com a adesão celular afeta o remodelamento da forma neuronal e a plasticidade sináptica (Varea *et al*., 2007; Guirardo *et al*., 2012). É o caso da PSA-NCAM expressa por interneurônios maduros (Varea *et al*., 2005) e intensamente encontrada, por técnica imunohistoquímica, na MePD de ratos adultos jovens (Varea *et al*., 2009; Job e Cooke, 2015). Pela sua propriedade de anti-adesão, a PSA-NCAM permite que sejam formados novos contatos sinápticos e tal atividade se relaciona diretamente com os níveis locais de sinaptofisina (Varea *et al*., 2007, 2009). Ou seja, quanto maiores os níveis de PSA-NCAM, maior é a expressão de sinaptofisina (Varea *et al*., 2007), e a possível ocorrência de novas sinapses (Daly *et al*., 2000; Jang *et al*., 2009).

O IGF-1 tem efeito trófico e plástico muito bem documentado no Arc (Fernandez-Galaz *et al*., 1999). Há variação da imunorreatividade para IGF-1 durante o ciclo estral, com fêmeas na tarde do proestro e manhã de estro apresentando maior imunorreatividade do que fêmeas na manhã do proestro,

diestro e metaestro (Fernandez-Galaz et al., 1999). O número de contatos sinápticos no soma neuronal de ratas na manhã do proestro e estro é maior quando comparadas ao grupo que recebeu antagonista ao receptor de IGF-1, e a diminuição dos contatos neste grupo é acompanhado por um “enovelamento” glial sem afetar o número basal de sinapses ou a forma dos contatos sinápticos (Fernandez-Galaz *et al*., 1999). Estes dados indicam que o receptor para IGF-1 está envolvido no remodelamento fásico das sinapses no Arc ao longo do ciclo estral de ratas (Fernandez-Galaz et al., 1997; Fernandez-Galaz *et al*., 1999).

Já os receptores para Efrina-A4 são conhecidos como reguladores das vias de sinalização intracelular envolvidas no remodelamento da actina do citoesqueleto neuronal e, não é surpresa, então, que sua atividade seja importante para formação e manutenção dos espinhos dendríticos (revisado por Murai e Pasquale, 2004). Em experimento com secções de hipocampo, Efrina A4 foi identificada nos espinhos de neurônios piramidais e com ação na modulação da forma desses elementos dendríticos (Murai e Pasquale, 2004). Não está claro como isso acontece, mas acredita-se que a GTPase Rho esteja envolvida na sinalização celular que propicia a estabilidade dos espinhos (Shamah *et al*., 2001), evitando formas aberrantes (Murai e Pasquale, 2004).

Os resultados do terceiro artigo mostraram que não há diferença estatisticamente significativa entre machos e fêmeas em diestro, proesto ou estro no nível de expressão gênica e proteica de BDNF, IGF-1, PSA-NCAM e Efrina A4 na MePD. Como ocorre diferença na quantidade de puncta imunorreativa para BDNF e IGF-1 entre machos e fêmeas em fases específicas do ciclo estral, tais dados indicam que a MePD é região com particularidades no efeito desses fatores na plasticidade sináptica, principalmente durante o proestro. Nossa hipótese é que, especificamente na MePD, quando BDNF e IGF-1 estão elevados durante o pico de estrógeno e progesterona em circulação, pode ocorrer mudanças morfológicas mediadas pela alteração da actina do citoesqueleto, principal componente dos espinhos dendríticos. A regulação da forma do espinho dendrítico pode utilizar múltiplas vias de sinalização intracelular que alteram a dinâmica do remodelamento da actina (Saneyoshi e Hayashi, 2012) e que, então, pode causar alteração no número, tipo e tamanho dos espinhos dendríticos (Fisher *et at*., 2000). Além da actina, podem estar

envolvidos no remodelamento dos espinhos a enzima cofilina, reguladora da polimerização da actina, e a ação de diferentes micro-RNAs (miRNA) (Hirsch *et al*., 2018).

Sobre a actina, é fato que a estrutura e a dinâmica na modificação da forma de neurônios e células gliais depende de proteínas do citoesqueleto. O padrão de expressão da actina muda em cada região do encéfalo, particularmente quando ocorrem variações nos níveis de hormônios gonadais durante o ciclo estral (Hansberg-Pastor et al., 2015). Estrógeno estimula o remodelamento da actina, modulando a forma dos espinhos dendríticos (Srivastava, 2012), de modo que as células modificam suas conexões e a atividade nos circuitos neurais (Hansberg-Pastor et al., 2015). Por exemplo, no VMH, outra área sexualmente dimórfica, há remodelamento da conectividade neuronal, com aumento na densidade de espinhos dendríticos concomitante com o aumento dos hormônios ovarianos característico do ciclo estral (Ferri e Flanagan-Cato 2012). O remodelamento dos espinhos dendríticos e a estabilidade das sinapses, requerem, por sua vez, modificações estruturais na actina (Matus 2000). A diminuição da fosforilação da cofilina está associada a um alongamento dos filamentos de actina, que promove o desenvolvimento de espinhos dendríticos (Bernstein and Bamburg, 2010). Tais ações serão detalhadas a seguir porque representam raciocínios que podem ser feitos para compreender os efeitos dos hormônios gonadais e dos fatores neurotróficos na MePD de ratos. Esse é um tema complexo aberto para novos experimentos, ainda mais porque será preciso correlacionar a maior densidade de espinhos dendríticos em machos e a retração do número de espinhos dendríticos no proestro com o aumento dos espinhos somáticos e dos contatos feitos diretamente nos troncos dendríticos em fêmeas nessa mesma fase do ciclo ovulatório.

De fato, os mecanismos relacionados com o remodelamento do citoesqueleto não estão completamente elucidados. O rearranjo do citoesqueleto neuronal é estimulado pela ativação dos receptores para hormônios gonadais (especialmente estrógeno e progesterona; Hansberg-Pastor *et al*., 2015). Isso pode induzir a ativação de vias de sinalização intracelular, como as mediadas por MAPKs (proteína quinase ativada por mitógeno), PI3K/Akt (fosfoinositida 3-

quinase/ proteína quinase) e PKC (proteína quinase C), regular vias de sinalização de segundos-mensageiros e modular a ação de receptores para neurotransmissores por meio de outros mecanismos de ação de forma específica em cada área encefálica (Hansberg-Pastor *et al*., 2015 e referências adicionais nesse artigo). É o caso dos membros da família de GTPases (por exemplo, RhoA e Rac1), as quais regulam a atividade de diversas proteínas associadas ao citoesqueleto, como as MAPs (proteínas associadas ao citoesqueleto) e as ABPs (proteínas de ligação à actina, Gonzalez-Billault *et al*., 2012; Hansberg-Pastor *et al*., 2015). As GTPases, assim denominadas pela afinidade e ligação de nucleotídeos de guanina, ciclam de um estado inativo, ligado a GDP (difosfato de guanosina) a outro ativo, ligado a GTP (trifosfato de guanosina). Quando em sua conformação ativa, diferentes moléculas efetoras são capazes de se ligar a essas proteínas e desencadear as respostas celulares (Chazeau e Giannone, 2016). Já a MAP2, por exemplo, aumenta no hipocampo de ratas ovariectomizadas entre 24 a 48 horas após tratamento com estrógeno, progesterona ou ambos hormônios (Reyna-Neyra *et al*., 2002). Surpreendentemente, não há aumento no mRNA de MAP2, sugerindo que seu envolvimento em mudanças estruturais induzidas por estrógeno e progesterona deva ser regulado por ações pós-transcricionais (Gonzáles-Arena *et al*., 2014; Hansberg-Pastor et al., 2015).

Assim, a formação e manutenção dos espinhos dendríticos requerem atividade coordenada de moléculas de sinalização e componentes estruturais intracelulares (Yasuda, 2017; Nakahata e Yasuda, 2018). Por isso, maior atenção tem sido dada para a actina como principal componente do citoesqueleto dos espinhos dendríticos, GTPases e a função sináptica (Tada e Sheng, 2006; Woolfrey e Srivastava, 2016; Hedrick e Yasuda, 2017). Proteínas de citoesqueleto, actina e tubulina, são consideradas morfogênicas para espinhos dendríticos e moduladoras do estabelecimento e função sinápticos (Woolfrey e Srivastava, 2016; Hedrick *et al*., 2016; Nakahata e Yasuda, 2018). Considerando a posição dessas proteínas na via de sinalização intracelular, elas podem estar organizadas em grupos funcionais hierárquicos que seguem: proteínas ligantes à actina, pequenas GTPases e pequenas GTPases reguladoras da morfologia celular e efetoras (estimulam o reprimem a atividade

das proteínas; Penzes *et al*., 2008). De maneira geral, a sinalização envolvida na morfogênese dos espinhos dendríticos se inicia a partir da atividade sináptica, através do influxo de $Ca^{2+,}$ e que é transmitida para GEFs (fatores de troca do nucleotídeo guanina) ou GAPs (proteínas ativadoras de GTPases) que controlam a atividade das pequenas GTPases (Woolfrey e Srivastava, 2016; Chazeau e Giannone, 2016). Dessa forma, quando as pequenas GTPases se ligam ao GTP, que está na conformação ativa, conseguem se unir às moléculas efetoras que estão a seguir na via de sinalização, exercendo seu importante papel na conversão de sinais extracelulares em vias de sinalização intracelular (Ye e Thomas, 2010). Essas pequenas GTPases regulam a plasticidade estrutural, relacionando sinais extracelulares para modulação dos espinhos dendríticos, novamente com impacto especial na dinâmica da actina que é a principal proteína determinante da morfologia dessas especializações celulares (Hedrick e Yasuda, 2017).

A regulação da quantidade e agrupamento da actina está intimamente ligada às mudanças estruturais e a organização dos espinhos dendríticos, o sistema de sinalização de ABPs, a atividade e a plasticidade sinápticas (Nakahata and Yasuda, 2018). Sinapses excitatórias induzem elevação do Ca^{2+} intracelular via receptores NMDA ou canais de cálcio dependentes de voltagem no espinho dendrítico (Sabatini et al., 2002; Chazeau e Giannone, 2016). A elevação do Ca^{2+} nos espinhos desencadeia vias de sinalização para plasticidade sináptica ativando subsequentemente quinases e fosfatases como CaMKII e calcineurina (Fujii *et al*. 2013, Chang *et al*., 2017). A partir disso, a ativação de CaMKII é retransmitida para diversas moléculas de sinalização incluindo as GTPases, responsáveis pela organização do citoesqueleto de actina (Yasuda, 2017), conforme a Figura 2. As famílias de proteinas intracelulares Rho, Ras e Cdc42 são as mais estudadas e intimamente relacionadas com o remodelamento da actina no citoesqueleto e que organiza os espinhos estruturalmente (Tada e Sheng, 2005; Woolfrey e Srivastava, 2016, Hedrick e Yasuda, 2017). Enquanto RhoA inibe o desenvolvimento de espinhos dendríticos, Rac promove crescimento e/ou estabilidade desses elementos (Tada e Sheng, 2005). A ativação de Rac1 estimula a polimerização da actina e estabiliza os espinhos dendríticos pela ativação de efetores subsequentes na via

de sinalização intracelular, como a quinase ativada por p-21 (PAK21), LIMK1, e a proteína cofilina de ligação à actina (Zhang *et al*., 2005; Saneyoshi e Hayashi, 2012). No hipocampo, por exemplo, a ligação do estrógeno ao seu receptor β estimula a pequena GTPase RhoA que, por sua vez, ativa a via quinase RhoA (ROCK) resultando em fosforilação na quinase LIMK a qual, então, fosforila a cofilina, resultando em um aumento da actina nos espinhos dendríticos e uma reestruturação da forma destes espinhos e, consequentemente, uma reorganização da atividade sináptica (Kramar *et al*., 2009; Saneyoshi e Hayashi, 2012). O bloqueio farmacológio de PACK e ROCK cessa a alteração estrutural de volume e crescimento inicial do espinho, respectivamente, ambas ações associadas com a inibição da LTP (do inglês, l*ong-term potentiation*, Murakoshi *et al*., 2011). Em neurônios corticais expostos ao 17β-estradiol há um rápido aumento na atividade da Ras que é compatível com o aumento na densidade de espinhos dendríticos, confirmando que o estrógeno é um potente regulador da atividade de pequenas GTPases e da dinâmica da actina neuronal (Srivastava *et al*., 2008). Essas evidências sugerem que as pequenas GTPases cooperam para mudanças na densidade e morfologia dos espinhos dendríticos, embora não esteja ainda claro como os neurônios integram estas informações oriundas de mecanismos complexos que impactam na função celular.

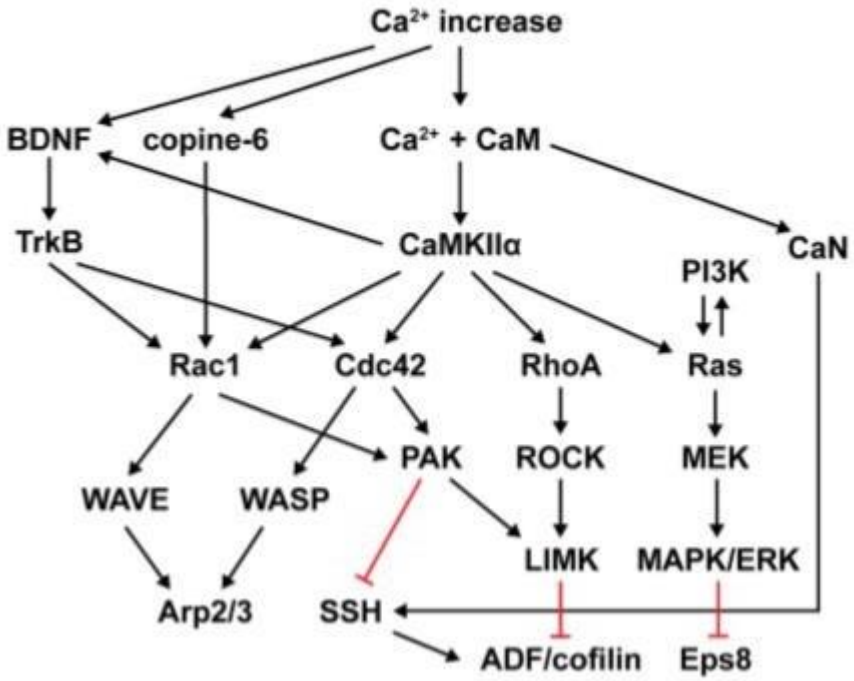


Figura 2: **Sinalização intracelular envolvendo a plasticidade dos espinhos**. Vias de sinalização que controlam as ABPs em espinhos dendríticos. Adaptado de Nakahata and Yasuda (2018). BDNF, Fator Neurotrófico Derivado do Encéfalo; CaM, calmodulina; TrkB, receptor tropomiosina quinase; CaMKIIα, proteína quinase dependente de cálcio/calmodulina de cadeia alfa tipo II; CaN; Rac1, do inglês *Ras-related C3 toxin substrate 1*; Cdc42, do inglês *cell division control protein 42 homolg* ; RhoA, do inglês *Ras homolg gene Family member A*; Ras do inglês Rat Sarcoma vírus ; PI3K, Fosfatidilinositol 3-quinase; WAVE proteína homóloga à verprolina; WASP proteína da Síndrome de Wiscott-Aldrich; PAK proteína quinase ativada por p21; ROCK, do inglês *Rho-associated coiled-coil containing protein kinase* ; MEK, proteína quinase ativada por mitógeno ; LIMK, *domínio K da proteína quinase LIM*; MAPK/ERK, proteína quinase ativada por mitógeno/quinase controlada por sinal extracelular; Arp2/3, proteína relacionada à actina; SSH, do inglês *slingshot*; ADF/Cofilin, *fator de despolimerização da actina/cofilina* ; Eps8, *substrato quinase do receptor do factor de crescimento epidérmico 8*.

Em breve resumo, o remodelamento dendrítico envolve, como elemento preponderante, as modificações estruturais da actina (Matus, 2000, Fisher *et al*.,

2000). Os mecanismos detalhados pelos quais os efetores da família Rho (incluindo ROCK e PACK) regulam a dinâmica da actina ainda não estão bem esclarecidos (Saneyoshi e Hayashi, 2012). A cofilina recebe atenção porque é a enzima responsável pela clivagem da actina, que é inativada por fosforilação (Saneyoshi e Hayashi, 2012). A “fosfo-desativação” da cofilina está associada ao alongamento dos filamentos de actina e o desenvolvimento dos espinhos dendríticos (Bernstein e Bamburg, 2010). A atividade da cofilina é complexa e é considerada resultado da integração de múltiplas vias de sinalização, incluindo ROCK, MEK, PAK, PKA, e CaMKIV, que regulam a LIMK (Saneyoshi e Hayashi, 2012). Praticamente nada se sabe sobre todo esse mecanismo de ação ou modulações de atividade na MePD de ratos machos e fêmeas relacionadas com níveis hormonais diferentes entre os sexos e ao longo das diferentes fases do ciclo estral.

Por fim, na atividade pós-transcricional existem miRNA (micro RNAs) que regulam a expressão de diferentes proteínas relacionadas com a morfogênese de espinhos dendríticos em diversas áreas encefálicas, incluindo o complexo amigdaliano (Siegel *et al*., 2009; Olsen *et al*., 2009). Os mRNA se apresentam com sequências curtas de 21-25 nucleotídeos e com papel importante no controle translacional das sinapses, visto que muitos foram identificados como reguladores de proteínas relacionadas à actina e à plasticidade sináptica (Nakahata and Yasuda, 2018). As ações desses miRNAs são partes adicionais e complexas da plasticidade sináptica, uma vez que podem permanecer inibidos até que os neurônios sejam expostos a um estímulo, como por exemplo fatores neurotróficos, liberação de neurotransmissores ou hormônios gonadais (Schratt *et al*., 2006; Hirsch *et al*., 2018). A expressão de alguns miRNAs envolvidos no remodelamento do citoesqueleto e na plasticidade sináptica dos neurônios varia entre machos e fêmeas ao longo do ciclo estral. Por exemplo, miR138-5p é abundantemente expresso em machos quando comparados com fêmeas ao longo do ciclo estral, enquanto miR132-3p mostrou baixos níveis em machos quando comparados com fêmeas em proestro (Hirsch et al., 2018). Esses resultados sugerem que tais miRNA podem modular os efeitos dos hormônios gonadais nos espinhos dedríticos na MePD de animais adultos (Hirsch *et al*., 2018). Dessa forma, os miRNA regulam a tradução local de moléculas-chave de

sinalização em subcompartimentos dendríticos e, em conjunto com as GTPases, medeiam a regulação o citoesqueleto de actina dos espinhos dendríticos e sua plasticidade estrutural e funcional (Nakahata and Yasuda, 2018). Esse capítulo do conhecimento sobre a MePD abre-se agora para novas possibilidades de estudo.

Por fim, técnicas de imageamento mais modernas especializadas na monitorização do comportamento molecular de um único espinho têm mostrado que cada via pode ser diferentemente regulada em cada espinho de cada domínio (Nakahata e Yasuda, 2018; Colgan e Yasuda, 2014; Sambandan et al., 2017). O padrão espacial da atividade de sinalização se expande de um espinho até a área dendrítica adjacente, regulando diferentes eventos celulares em cada escala espacial (Nakahata e Yasuda, 2018). Devido à complexidade morfológica dos neurônios, a dinâmica espaço-temporal da sinalização desempenha um papel particularmente importante na plasticidade neuronal. Embora as medições quantitativas de vias de sinalização mais detalhadas sirvam para a compreensão do sistema de sinalização global, será necessário criar estruturas teóricas que possam integrar a dinâmica espaço-temporal de muitas vias de sinalização diferentes para elucidar diversas relações entre a estrutura e a função de cada neurônio em cada área nervosa (Brown et al., 2011). Esse raciocínio vale para a MePD em ambas condições *in vitro* e *in vivo* e devem fornecer próximos passos no conhecimento de sua organização funcional em machos e fêmeas.

5. CONCLUSÃO

- Há diferenças no número, tipo e forma de espinhos dendríticos na MePD de machos e fêmeas pré-púberes; e mudança no tipo e na forma de espinhos dendríticos na MePD devido à experiência sexual em machos pós-púberes. Estes resultados confirmam a relação da MePD com a reprodução e a ação dos hormônios gonadais, o refinamento funcional dependente da experiência que ocorre neste subnúcleo, além de servir para a integração e codificação sináptica de todas as etapas do comportamento sexual.

- Há poucas junções comunicantes na MePD de ratos adultos e expressão evidente de conexinas 36, 43 e 45 na MePD de ratos adultos. A contribuição do acoplamento celular elétrico na MePD auxiliaria na atividade sequencial neuronal sincronizada para machos identificarem pistas olfativas/vomeronasais socialmente relevantes de fêmeas receptivas para a ocorrência da intromissão, ejaculação, tempo do período pós- ejaculatório e saciedade sexual.

- Há maior quantidade de *puncta* imunorreativa para BDNF na MePD em fêmeas em proestro do que em machos. E ainda, há maior quantidade de *puncta* imunorreativa para IGF-1 na MePD em fêmeas em proestro do que em diestro. A notável plasticidade estrutural e sináptica na MePD de ratos adultos, em machos e em fêmeas ao longo do ciclo estral indica que este subnúcleo possui particularidades especialmente durante o proestro.

6. PERSPECTIVAS

- A função dos espinhos dendríticos poderá ser estudada por registros eletrofisiológicos *in vitro* e *in vivo* empregando-se técnica optogenética, bem como por registros eletrofisiológicos da atividade neuronal relacionada com o tipo de espinho estimulado a cada instante.

- A mesma abordagem eletrofisiológica deverá ser empregada para registro de acoplamento elétrico entre neurônios na MePD para testar o funcionamento das junções comunicantes ou a possibilidade, por técnica de passagem de substâncias fluorescentes de baixo peso molecular entre células acopladas por conexinas (identificadas por co-marcação).

- As GTPases compreendem um grande grupo de proteínas intracelulares sinalizadoras que medeiam diversas respostas fisiológicas como a organização do citoesqueleto celular. Propomo-nos a, futuramente, investigar o possível papel da GTPase RhoA em vias de sinalização responsáveis pela motilidade do citoesqueleto de forma a documentar se há variação entre machos e fêmeas ao longo do ciclo estral, empregrando-se a técnica de PCR.

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8.ANEXOS

I

CEUA –COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

1) PROTOCOLO Nº: 163/15

2) DATA DO PARECER:14/10/2015 Parecer 314/15

3) TÍTULO DO PROJETO:

Estudo sobre a morfologia e plasticidade sináptica de espinhos dendríticos da amigdala medial: efeito da puberdade e de experiência sexual em ratos.

4) PESQUISADOR RESPONSÁVEL:

Alberto Rasia Filho

5) RESUMO DO PROJETO:

Estudo das possíveis diferenças na densidade e na forma dos espinhos dendríticos de neurônios localizados na MePD de ratos e relacioná-los com a puberdade e a ocorrência de experiência sexual. Serão estudados 04 grupos de ratos pré-púberes; pós-púberes sem experiência sexual e pós-púberes com experiência sexual; Espera-se obter dados sobre a densidade e frequência de ocorrência de espinhos dendríticos. A hipótese inicial é de que a ocorrência da puberdade aumente a densidade de espinhos e a experiência sexual venha a gerar espinhos dendríticos com morfologia sugestiva de maior estabilidade estrutural e funcional.

6) OBJETIVOS DO PROJETO:

Estudar as possíveis diferenças na densidade e na forma dos espinhos dendríticos de neurônios localizados no MePD de ratos e relacioná-los com a puberdade e a ocorrência de experiência sexual

7) FINALIDADE DO PROJETO: [x] Ensino [ ] 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:	**Número Amostral:**
Ratos Wistar machos 3 meses	24
Ratos Wistar machos 30 dias	08
Ratos Wistar fêmeas	32

Redução Amostral: [ ] Sim [x] Não

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:

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

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

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

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

11) CRONOGRAMA DE UTILIZAÇÃO DE ANIMAIS

Data	Espécie	Sexo	Quantidade

12) RECOMENDAÇÃO:

[x] Aprovado

[ ] Com Pendência

[ ] Não aprovado

Data de início____/____/_____ Data de Término____/____/_____

Comentários gerais sobre o projeto:

Realizado os ajustes pertinentes. Aprovado.

II

CEUA –COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

1) PROTOCOLO Nº: 162/15 parecer 310/15

2) DATA DO PARECER: 19/08/2015

3) TÍTULO DO PROJETO:

Estudo sobre a ocorrência de sinapses elétricas e efeito do dimorfismo sexual e da variação cíclica de hormônios ovarianos na expressão de fatores neurotróficos moduladores da plasticidade sináptica na amígdala medial póstero-dorsal de ratos.

4) PESQUISADOR RESPONSÁVEL:

Alberto A. Rasia Filho

5) RESUMO DO PROJETO:

Estudar por microscopia eletrônica e imunohistoquímica a amígadal medial de ratos procurando identificar a presença de sinapses elétrica e comprovando pela presença de marcadores imunohistoquímicos que serão comparados entre machos e fêmeas em diferentes fases do ciclo estral.

6) OBJETIVOS DO PROJETO:

Geral: Estabelecer conhecimentos inéditos a respeito da morfologia e da plasticidade sináptica dos neurônios da MePD de ratos machos e fêmeas ao longo do ciclo estral.

Específicos:

1) Estudar a ultraestrutura local procurando identificar a presença de “gap junctions” através da microscopia eletrônica e determinar a expressão da conexina-43 por imunohistoquímica em ratos adultos.
2) Estudar o possível dimorfismo sexual e a variação ao longo do ciclo estral (fêmeas em diestro, proestro e estro) na expressão local de BDNF, IGF-1, PSA, NCAM e Efrina-A4 por técnica imunohistoquímica e densitometria óptica em ratos adultos.

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:** 55

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): [ ] Adequada [ ] Inadequada

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

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

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

Faltou informar no formulário e descrever adequadamente no projeto.

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

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

Não foi indicado o método de eutanásia aplicado nas fêmeas não utilizadas.

Local de Realização (Biotério/Labotarório):

Laboratório de Fisiologia

Outra instituição. Qual?

11) CRONOGRAMA DE UTILIZAÇÃO DE ANIMAIS

Data	Espécie	Sexo	Quantidade

12) RECOMENDAÇÃO:

[x] Aprovado

[ ] Com Pendência

[ ] Não aprovado

Data de início 01/08/2015 Data de Término 01/06/2017

III

CEUA –COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

1) PROTOCOLO Nº: 227/18 Parecer 564/18

2) DATA DO PARECER: 09/052018

3) TÍTULO DO PROJETO:

Estudo Sobre a Variação Cíclica dos Hormônios Ovarianos na Expressão Gênica de Fatores Neurotrópicos Moduladores da Plasticidade Sináptica na Amígdala Medial Póstero-dorsal de ratos

4) PESQUISADOR RESPONSÁVEL:

Alberto A Rasia Filho

5) RESUMO DO PROJETO:

A amígdala medial póstero-dorsal (MePD) é uma estrutura cerebral sexualmente dimórfica que depende dos níveis de hormônios gonadais circulantes. Com relação às sinpses químicas na MePD em ratos,e stá descrito o dimorfismo sexual e efeito da variação cíclica dos hormônios ovarianos na densidade de espinhos dendritos somáticos locais. O objetivo do projeto proposto é estudar se a diferença entre machos e fêmeas e a variação da densidade de espinhos na MePD tem influência na expressão g~enica de BDNF, IGF-1, PSA-NCAM e Efrina-A4. Para essa análise será empregada a técnica de PCR em tempo real. A escolha dessa técnica se deve ao fato de não terem sido observadas diferenças nesses marcadores por meio da técnica de imunofluorescência. A

hipótese dos autores é a de que variações na expressão gênica seja muito sutis e possam ser detectadas por meio da técnica de PCR em tempo real.

6) OBJETIVOS DO PROJETO:

Analisar a expressão gênica dos fatores neurotrópicos BDNF, IGF-1, PSA-NCAM e Efrina-A em machos e fêmeas ao longo do ciclo estral (diestro, proestro e estro)

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: Rato heterogênico **Número Amostral:** 40

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:

Não utilizar lidocaína como analgésico, pois o uso comum é como anestésico.

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

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

Eutanásia dos animais (se aplicável): [X] Adequada [ ] Inadequada

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

Local de Realização (Biotério/Labotarório):

Outra instituição. Qual?

UFCSPA Laboratório de Fisiologia

11) CRONOGRAMA DE UTILIZAÇÃO DE ANIMAIS

Data	Espécie	Sexo	Quantidade
	Rato heterogênico	M/F	40

12) RECOMENDAÇÃO: As pendências deverão ser respondidas em uma carta, indicando as páginas do projeto que foram alteradas (nova versão), assinadas pelo pesquisador responsável.

[X] Aprovado

[ ] Com Pendência

[ ] Não aprovado