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Estudo dos espinhos dendríticos na amígdala medial póstero-dorsal de ratos: morfologia e conectividade

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SUMÁRIO

LISTA DE ABREVIATURAS

ACe	Núcleo central da amígdala
AMe	Núcleo medial da amígdala
MeAD	Núcleo medial da amígdala, subdivisão ântero-dorsal
MeAV	Núcleo medial da amígdala, subdivisão ântero-ventral
MePD	Núcleo medial da amígdala, subdivisão póstero-dorsal
MePDi	Parte intermediária da amígdala medial póstero-dorsal
MePDl	Parte lateral da amígdala medial póstero-dorsal
MePDm	Parte medial da amígdala medial póstero-dorsal
AMPA	α-amino-3-hidroxi-5metil-isoxazoiproprionato
AVPV	Núcleo periventricular ântero-ventral hipotalâmico
BLA	Núcleo basolateral da amígdala
DiI	Perclorato de 1,1'-Dioctadecil-3,3,Y,3'-tetrametilindocarboeianina
GABA	Acido γ-aminobutírico
$GABA_A$	Receptor tipo A para GABA
GluR	Receptores glutamatérgicos
GluR1	Subunidade 1 do receptor glutamatérgico do tipo AMPA
GluR1/2	Subunidades 1 e 2 do receptor glutamatérgico do tipo AMPA
GluR1-4	Subunidades 1,2, 3 e 4 do receptor glutamatérgico do tipo AMPA
Lhx	Fatores de transcrição da família homeodomínio LIM
NMDA	N-metil-*D*-aspartato
NMDAR	Receptor glutamatérgico do tipo NMDA
GluN1	Subunidade GluN1 do receptor glutamatérgico do tipo NMDA

PMv	Núcleo pré-mamilar ventral hipotalâmico
ET	Estria terminal
TO	Trato óptico

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RESUMO

Introdução: O subnúcleo póstero-dorsal da amígdala medial (MePD) modula comportamentos sociais e fatores de transcrição LIM (Lhx) participam da diferenciação de neurônios locais. Espinhos dendríticos são especializações pós-sinápticas, mas detalhes sobre sua morfologia em fêmeas (comparativamente com machos), em diferentes subpopulações neuronais e a ocorrência de receptores glutamatérgicos e GABAérgico nesses espinhos são conhecimentos ainda inéditos para o MePD.

Objetivos: 1) Estudar a morfologia dos espinhos dendríticos de neurônios do MePD em ratas ao longo do ciclo estral, 2) Estudar a morfologia dos espinhos dendríticos das subpopulações neuronais Lhx6, Lhx5 e Lhx9 e, 3) Estudar a presença e distribuição dos receptores AMPA (subunidade GluR1-4), NMDA (subunidade GluN1) e $GABA_A$ em espinhos dendríticos do MePD de ratos.

Material e Métodos: Estudaram-se ratos Wistar adultos mantidos sob condições padrão de biotério e cuidados éticos. Os experimentos envolveram secções coronais do MePD e reconstrução tridimensional das imagens dos espinhos dendríticos visualizados com fluorescência pela carbocianina DiI associada com imunomarcações sob microscopia confocal.

Resultados: A densidade de espinhos dendríticos das fêmeas, respectivamente em diestro, proestro e estro, foi (média±desvio padrão): 0,9±0,1; 0,6±0,2; e, 0,6±0,1 espinhos/µm dendríticos. A imunomarcação para Lhx6, Lhx5 e Lhx9 ficou restrita ao corpo neuronal e espinhos dendríticos dos tipos fino e espessos/achatados foram os mais abundantes nesses neurônios (80% do total), variando entre 0,4-2,3 espinhos/µm, sem diferença estatística

entre as diferentes subpopulações neuronais locais. Imunomarcações para os receptores testados ocorreram nos ramos dendríticos proximais e distais e em diferentes espinhos. Observamos a presença espinhos multisinápticos, ou seja, a co-localização desses receptores sugere que os espinhos recebem mais de uma sinapse ao mesmo tempo. **Conclusões:** Esses resultados complementam os obtidos pela técnica de Golgi e microscopia eletrônica ao revelar características importantes dos espinhos dendríticos e da composição celular do MePD de ratos adultos, além de proporcionar uma nova visão sobre a complexidade da organização sináptica deste subnúcleo.

ABSTRACT

Introduction: The rat posterodorsal medial amygdala (MePD) modulates social behaviors and LIM homeobox transcription factors (Lhx) are involved in the differentiation of local cells. Dendritic spines are post-synaptic specializations, but details about their morphological differences in females (compared to males), presence in different neuronal subpopulations and expression of GABAergic and glutamatergic receptors are currently unkown for the MePD.

Objectives: 1) To describe the morphology of MePD dendritic spines in females along the estrous cycle, 2) To describe the morphology of dendritic spines in the different Lhx-expressing neuronal subpopulations, and 3) the presence and distribution of AMPA (GluR1-4 subunits), NMDA (GluN1 subunit) and $GABA_A$ receptors on each different type of dendritic spine in the MePD of male rats.

Materials and Methods: Adult Wistar rats were housed under standard laboratory conditions and ethical care. Data were gathered from coronal sections of the MePD and three-dimensional reconstructions of dendritic spines were visualized by DiI dye fluorescence and immunolabeling procedures under confocal microscopy.

Results: Females showed a spine density (mean±standard deviation) of 0.9±0.1; 0.6±0.2; and 0.6±0.1 spines/dendritic µm in diestrus, proestrus and estrus, respectively. Immunostaining for Lhx6, Lhx5 Lhx9 was restricted to the neuronal cell bodies, thin and stubby/wide spines were the most abundant types (~80%) and showed 0.4-2.3 spines/dendritic µm, but no statistically significant difference in their occurrence was found among these cell subpopulations. Immunolabeling for glutamatergic and GABAergic receptors was found in

proximal and distal dendritic branches as well as in different dendritic spines. The colocalization of receptors suggests that spines receive both excitatory and inhibitory synapses in the MePD.

Conclusions: Results agree with previous Golgi method and electron microscopy data and revealed relevant features of dendritic spines and the cellular composition of the adult rat MePD. They also provide new insights into the complexity of the synaptic organization of this subnucleus.

1. INTRODUÇÃO

1.1 Amígdala

A amígdala (complexo amigdalóide ou, preferentemente, 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 e cols., 1995; Canteras e cols., 1995; Everitt, 1995; Swanson e Petrovich, 1998; Brusco, 2012). Embora historicamente tenha sido considerada como uma estrutura unitária, a amígdala, no entanto, não é nem homogênea anatomicamente e nem funcionalmente (Swanson e Petrovich, 1998) além de se estender além de seus limites anatômicos (Johnston, 1923). Atualmente sabe-se que a amígdala é uma estrutura que compreende núcleos e subnúcleos que formam uma complexa rede estrutural inter-relacionada e multifuncional, uma vez que está envolvida na modulação de diversos comportamentos e ajustes homeostáticos de diversas variáveis fisiológicas (Alheid e cols., 1995; Everitt,1995; Swanson e Petrovich, 1998; Rasia-Filho e cols., 2000; de Olmos e cols., 2004; Rasia-Filho e cols., 2009; Brusco, 2012).

Estudos mais recentes sobre a divisão da amígdala de ratos apresentam-na dividida em quatro regiões, segundo a sua citoarquitetura, hodologia e funcionalidade, a saber: 1) amígdala “expandida”, denominada assim por se estender além de seus limites anatômicos, sendo formada pelos núcleos medial (AMe) e central (ACe); 2) amígdala com características corticais, subdividida em porção basolateral (BLA) e em porções que se ligam às vias olfativas e vomeronasal; 3) área de transição, localizada entre a porção

ventral dos núcleos da base e a amígdala “expandida”; e 4) núcleos ainda não classificados, constituídos por um grande grupo de células dispersas na substância branca e no interior do núcleo próprio da estria terminal (ET; Alheid e cols., 1995; Canteras e cols., 1995; Heimer e cols., 1997; de Olmos e cols., 2004; Quagliotto, 2006; Figura 1).

1.2 Núcleo Medial da Amígdala

Um dos núcleos que tem recebido muita atenção nos últimos anos é a AMe (Newman, 1999; Gréco e cols., 2003; Zhou e cols., 2005; Lehman e Erskine, 2005; de Castilhos e cols., 2006; Hermel e cols., 2006a; Bennur e cols., 2007; Cooke e cols., 2007; Brusco e cols., 2010; Rasia-Filho e cols., 2012a,b). A AMe é um dos componentes da denominada “amígdala expandida” e um dos núcleos superficiais amigdalianos (Alheid e cols., 1995; de Olmos e cols., 2004). É formado por uma coluna proeminente de células que surgem em justaposição à superfície lateral do trato óptico (TO) e, mais caudalmente, em posição ventral em relação à ET (de Olmos e cols., 2004). A AMe encontra-se em posição medial e posterior ao núcleo do trato olfatório e, como limite posterior, está aproximadamente onde surgem as porções temporais dos ventrículos cerebrais (de Olmos e cols., 1985; 2004; Canteras e cols., 1995; Alheid e cols., 1995).

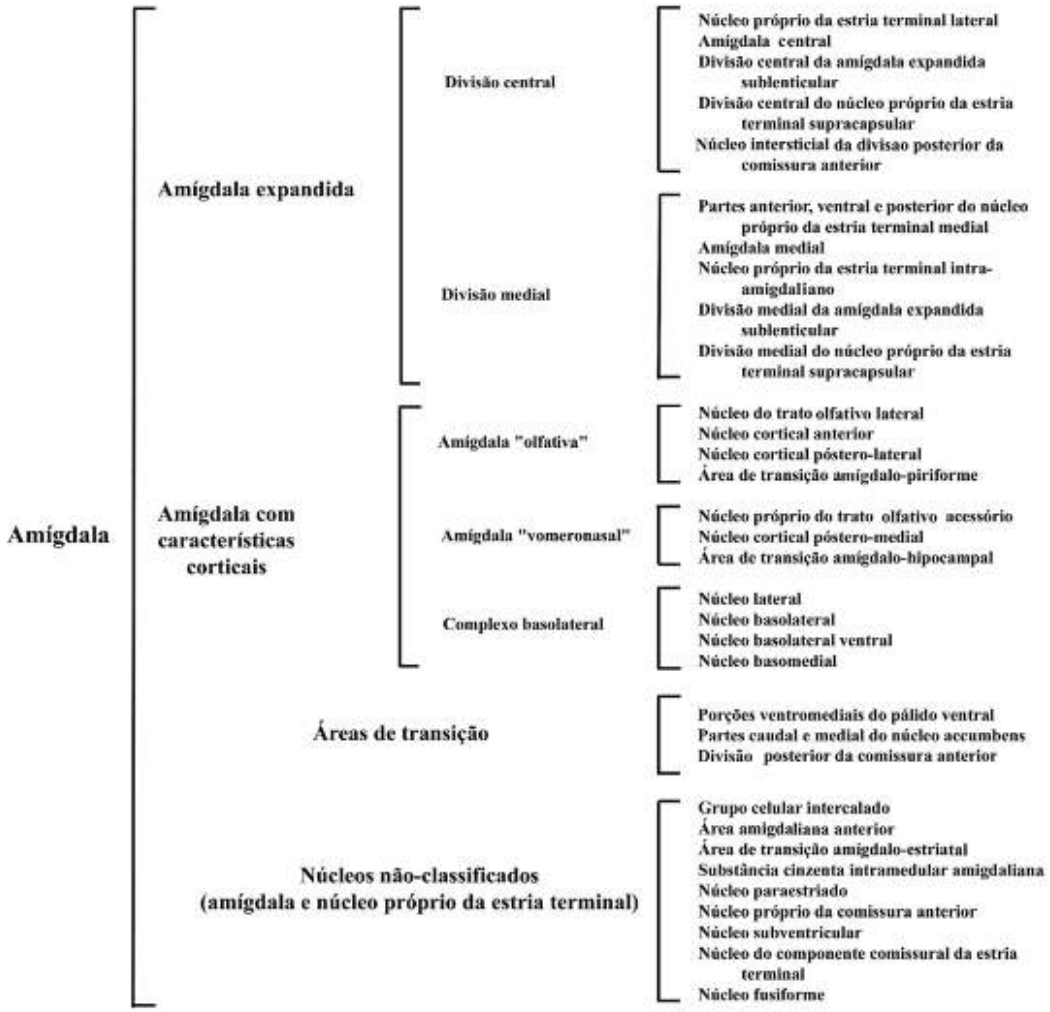

Figura 1: Classificação dos núcleos que compõe a amígdala do rato com suas subdivisões anatômicas e seus componentes principais, baseado em Alheid e cols. (1995), modificado por Rasia-Filho e cols. (2000) e como apresentado em Marcuzzo (2006).

O rato tem sido o modelo experimental mais empregado nos estudos da citoarquitetura, conexões, funções e plasticidade neuroglial dessa área complexa (como revisado em Rasia-Filho e cols., 2009). Não obstante, a AMe do camundongo tem sido utilizada para estudos citogenéticos (García-López e cols., 2008; Carney et al., 2010; Bupesch et al., 2011). Nestes, a AMe é formada por células oriundas do pálio ventral, da porção caudoventral da eminência ganglionar medial (MGEcv), da porção comissural da área pré-óptica e da região paraventricular hipotalâmica formando como que um "mosaico" de subpopulações celulares locais (García-López e cols., 2008; Bupesh e cols., 2011).

Ademais, a AMe é subdividida nos subnúcleos ântero-dorsal (MeAD), ântero-ventral (MeAV), póstero-dorsal (MePD) e póstero-ventral (MePV) o que veio a ser ratificado posteriormente por outros autores (Alheid e cols., 1995; Canteras e cols., 1995; Newman, 1999; de Olmos e cols., 2004; Dall'Oglio et al., 2008a,b; Figura 2). Nos quatro subnúcleos da AMe de ratos há praticamente uma homogeneidade quanto aos tipos morfológicos de neurônios que podem ser observados pela técnica de Golgi tanto em machos quanto em fêmeas (Rasia-Filho e cols., 1999, 2004; de Castilhos e cols., 2006). Trata-se de neurônios multipolares de tamanho pequeno (corpos celulares com 8-10 µm de diâmetro) a médio (corpos celulares com 10-15 µm de diâmetro; McDonald, 1992; Rasia-Filho e cols., 1999; Niimi e cols., 2012). Seus corpos celulares podem ser ovais, arredondados ou fusiformes (Alheid e cols., 1995; McDonald, 1992; Rasia-Filho e cols., 1999). Pelo aspecto dendrítico, esses são neurônios multipolares (Ramón y Cajal, 1995) do tipo "bitufted" ("bipenachados", como tentativa de aproximação do termo à língua portuguesa) caracterizados por

apresentarem dois ramos dendríticos primários, e do tipo estrelado, com três ou mais ramos dendríticos primários (Rasia-Filho e cols., 1999; Marcuzzo e cols., 2007; Dall'Oglio e cols., 2008a).

As propriedades eletrofisiológicas dos neurônios da AMe foram estudados por técnicas de "current-clamp" e "voltage-clamp" (modo "whole-cell") em secções do cérebro de camundongos (Niimi e cols., 2012). Os neurônios foram classificados, baseado no padrão de atividade em resposta a despolarização mediada por pulsos de corrente, em três tipos: neurônios com disparos de potenciais de ação regulares (tipo I), neurônios que reduzem a frequência de potenciais de ação mediante corrente despolarizante (tipo II) e neurônios com adaptação completa e param de disparar potencial de ação (tipo III). O neurônio tipo I foi o mais comum (56%), seguido pelos tipos III (12%) e II (3%; Niimi e cols., 2012).

O MePD será objeto de estudo deste trabalho e descrito com maiores detalhes. Localiza-se adjacente ao TO e ventralmente à ET na parte mais posterior e dorsal da MeA (Figura 3). Em cortes histológicos coronais corados pela técnica de Nissl, aparece com a forma de um triângulo alongado, com uma base ventral que se estende no terço caudal da AMe (Alheid e cols., 1995; de Olmos e cols., 2004). O MePD apresenta como característica citoarquitetônica uma região de células densas que se estendem superficialmente e profundamente, separadas por uma região intermediária de células esparsas (de Olmos e cols., 1985). Com isso organiza-se em colunas orientadas paralelamente à superfície lateral do subnúcleo, as quais podem ser subdivididas em três regiões: medial, intermediária e lateral (Alheid e cols., 1995; Coolen e cols., 1997; de Olmos e cols., 2004). A coluna superficial ou

medial (MePDm) é formada por células densamente agrupadas, de pequeno à médio tamanho; a coluna mais lateral (MePDl) é constituída de células de tamanho médio densamente compactadas, e a terceira coluna possui células de tamanho médio, que se organizam de maneira a constituir uma coluna intermediária (MePDi) entre as colunas MePDm e MePDl (de Olmos e cols., 2004; Figura 4).

O MePD possui aferências que advêm de diferentes regiões do encéfalo (McDonald, 1998; Pitkanen,2000) entre as aferências mais estudadas, estão as hipotalâmicas (da área hipotalâmica anterior, áreas pré-ópticas medial e lateral, núcleo arqueado, núcleos dorsomedial, posterior, lateral, prá-mamilar ventral, supra-óptico, tuberal e ventromedial), as do córtex cerebral (da área pré-límbica, córtex entorrinal, infalímbico e perirrinal dorsal), as da área septal e as aferências talâmicas (do núcleo medial, parafascilular e posterior, por exemplo), as do tronco encefálico (núcleo dorsal da rafe e núcleo parabraquial) e as da via olfativa (do córtex piriforme, bulbo olfativo acessório e núcleo endopiriforme). Ademais, existem também as aferências intra-amigdalianas onde destacam-se as da área de transição amígdalo-hipocampal e dos núcleos basal e acessório e as dos núcleos corticais anterior, posterior, lateral e medial (McDonald, 1998). A atuação de diferentes substâncias como os esteróides sexuais, por exemplo, pode alterar as regiões de aferências com o MePD e, desta forma, modificar sítios sinápticos importantes como os espinhos dendríticos (Brusco, 2012).

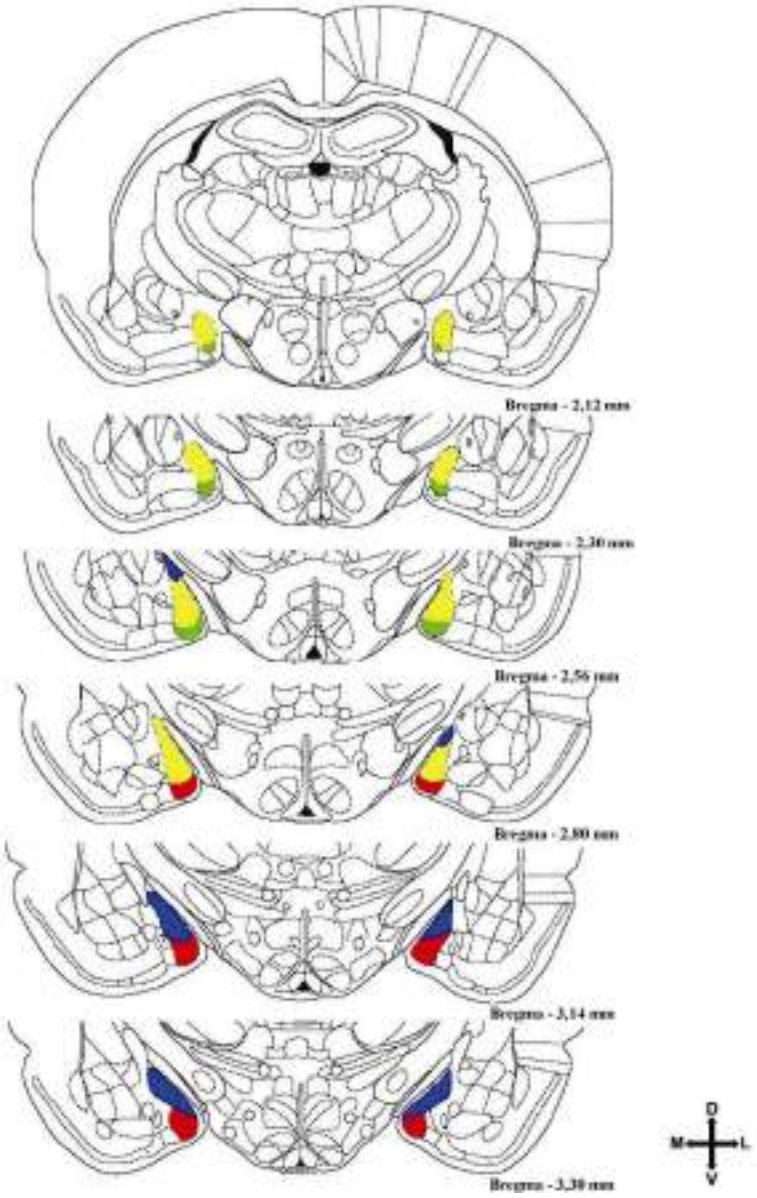

Figura 2: Representação esquemática de cortes coronais do encéfalo do rato onde se pode observar os quatro subnúcleos do AMe: MeAD (em amarelo), MeAV (em verde), MePD (em azul) e MePV (em vermelho). Os valores em mm colocados no lado direito das imagens referem-se à distância posterior ao bregma. As 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).

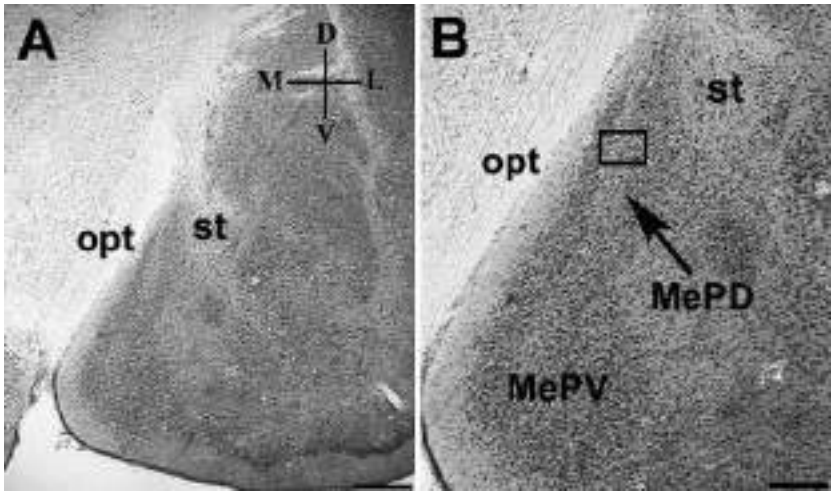

Figura 3: Fotomicrografia do MePD de rato. Opt, trato óptico; st, estria terminal; MePD, amígdala medial póstero-drosal; MePV, amígdala medial póstero-ventral; D, dorsal; V, ventral; M, medial; L, lateral. Escala = 500 µm (A) e 250 µm (B), como apresentado em Hermel (2006b).

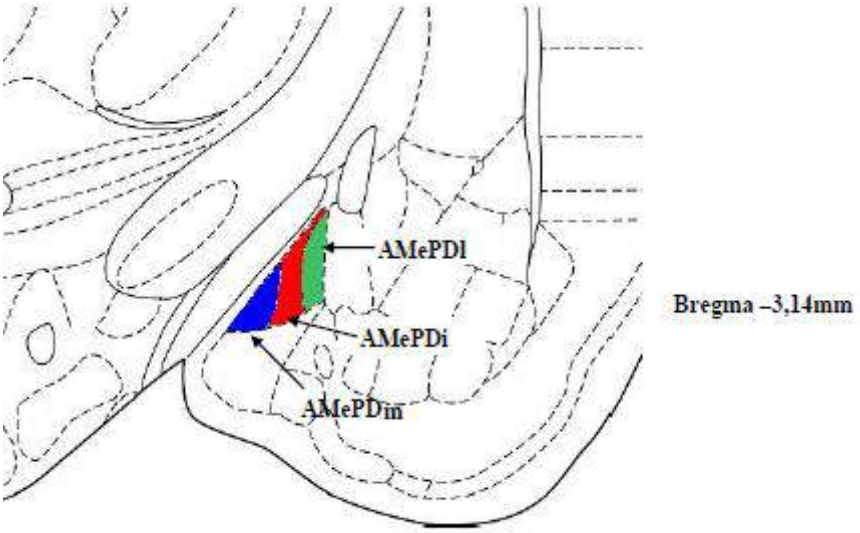

Figura 4: Esquema representativo de corte coronal do encéfalo do rato onde se observa as subdivisões do MePD. Adaptado de Paxinos e Watson (1998), correspondente à figura 32 do atlas mencionado, conforme apresentado em Forti (2005).

Em relação às eferências do MePD, as mais estudadas e significativas são as para os núcleos hipotalâmicos periventricular ântero-ventral (AVPV) e pré-mamilar ventral (PMv), as corticais (principalmente para a área entorrinal lateral, área de transição pós-piriforme, área CA1 hipocampal e subículo), as do tronco encefálico (para a área tegmental dorsal e substância cinzenta periaqueductal) e para outras regiões, como, por exemplo, para o núcleo próprio da ET (parte ântero-dorsal e posterior principal). Existem também as eferências intra-amigdalianas onde, dentre outras, destacam-se aquelas para os núcleos central, cortical póstero-lateral e póstero-medial (Canteras e cols., 1995; Petrovich e cols., 2001).

Os subnúcleos da AMe, e principalmente o MePD, modulam de forma relevante atividades relacionadas com a percepção de informações olfativas, vomeronasais e genitosensoriais (Guillamón e Segovia, 1997; Pfaus e Heeb, 1997; Dielenberg e McGregor, 2001; Meredith e Westberry, 2004; Pro-Sistiaga e cols., 2007), afetam a ocorrência de comportamentos reprodutivos de machos e fêmeas, como os comportamentos sexual e maternal (Fleming e cols., 1980; Rasia-Filho e cols., 1991; Coolen e cols., 1997; Newman, 1999; Sheehan e cols., 2001; de Castilhos e cols., 2006), os comportamentos agressivo e defensivo (Newman, 1999; Savonenko e cols., 1999; Rasia-Filho e cols., 2012a), a elaboração de respostas emocionais a estímulos em que a ansiedade e o medo estejam envolvidos (Adamec e Morgan, 1994; Duncan e cols., 1996) e participam nos ajustes cardiovasculares simpáticos e parassimpáticos relacionados à gênese e à modulação de comportamentos sociais (Quagliotto e cols., 2008; Neckel et al., 2012; Quagliotto e cols., 2012). O estudo dessas funções necessita que sejam detalhados a morfologia

neuronal local, a plasticidade dos espinhos dendríticos, os contatos sinápticos e os receptores para neurotransmissores presentes nos neurônios do MePD de ratos, o que será abordado a seguir.

1.3 Ação dos hormônios gonadais no dimorfismo sexual do MePD

A exposição do sistema nervoso a esteróides sexuais influencia uma variedade de características, tais como o tamanho e o número de neurônios, os ramos dendríticos e as conexões sinápticas, não somente no desenvolvimento, mas também em idade adulta (Gomez e Newman, 1991; Woolley e cols., 1997; De Castilhos e cols., 2008; Brusco e cols., 2008; McCarthy, 2008; De Castilhos e cols., 2010; McCarthy e Arnold, 2011; Rasia-Filho e cols., 2012a; Rasia-Filho e cols., 2012b). O dimorfismo sexual no sistema nervoso é um fenômeno onde fêmea e macho da mesma espécie diferem entre si em parâmetros morfológicos e, consequentemente, funcionais incluindo-se a secreção cíclica das gonadotrofinas e o comportamento sexual, o de agressividade, o emocional e o cognitivo, respostas a estímulos estressantes e ansiedade (Stefanova e Ovtscharoff, 2000; McCarthy, 2008).

O MePD de ratos é uma região particularmente sensível à ação dos hormônios gonadais dada a alta expressão de receptores para esses esteróides e diferenças estruturais e neuroquímicas entre os sexos (Nishizuka e Arai, 1981; Rasia-Filho e cols., 2004; Cooke e Woolley, 2005; Rasia-Filho e cols., 2012a,b). De fato, o MePD apresenta uma alta concentração de receptores para testosterona e receptores de tipo α e β para estradiol, além de receptores para progesterona (Simerly e cols., 1990; Gréco e cols., 1996;

Shughrue e cols., 1997; Gréco e cols., 2001,2003; De Vries e Simerly, 2002). A aplicação de cristais de testosterona ou de estradiol no MePD é capaz de aumentar o comportamento sexual de hamsters e ratos machos castrados, respectivamente (Rasia-Filho e cols., 1991; Wood e Newman, 1995; Newman, 2002). Em ratos, o MePD está relacionado com a ocorrência de atividade copulatória e de ejaculação (Coolen e cols., 1996; De Castilhos e cols., 2006) ou na percepção da estimulação vaginocervical (Coopersmith e cols., 1996; Pfaus e Hebb, 1997; Lehmann e Erskine, 2005; Lehmann e cols., 2005). Além disso, neurônios que expressam receptores para hormônios gonadais fazem-no também para c-fos após a percepção de estímulo olfativo e comportamento sexual (Gréco e cols., 1996 e 2003; Coolen e cols., 1997). Vários resultados experimentais indicam que o MePD forma um circuito sensível aos hormônios gonadais e que, por suas eferências, conecta-se a vários núcleos hipotalâmicos que integram informações olfativas e a regulação da atividade neuroendócrina para modular a ocorrência do comportamento sexual de machos e fêmeas (Wood e Newman, 1995; Guillamon e Segovia, 1997; Dong e cols., 2001; Petrovich e cols., 2001; Choi e cols., 2005). De fato, o MePD recebe informação direta e indireta proveniente das vias olfatória e vomeronasal e projeta eferências para os núcleos hipotalâmicos periventricular ântero-ventral (AVPV) para alterar a secreção neuroendócrina de GnRH (De Vries e Simerly, 2002) e para os núcleos pré-óptico medial, pré-mamilar ventral e a parte ventrolateral do núcleo ventromedial para modular o comportamento reprodutivo (Canteras e cols., 1995; Guillamon e Segovia, 1997; Petrovich e cols., 2001; Newman, 2002; Meredith e Westberry, 2004; Choi e cols., 2005; Cavalcante e cols., 2006). Muitas dessas conexões são recíprocas e fazem

sinapse ao longo de sua projeções em subregiões do núcleo intersticial da estria terminal (Dong e cols., 2001; Choi e cols., 2005).

O volume do MePD de ratos machos é maior, aproximadamente 85%, do que nas fêmeas (Hines e cols., 1992). E, baseado em dados publicados nos últimos anos (Rasia-Filho e cols., 1999, 2002 e 2004; Hermel e cols., 2006a; Martinez e cols., 2006; Dall'Oglio e cols., 2008a,b), a ação dos hormônios gonadais em ratos adultos podem afetar marcadamente o volume do soma neuronal e a estrutura do neurópilo do MePD (composto por ramos dendríticos, espinhos dendríticos, conexões sinápticas e células gliais) tanto em machos quanto em fêmeas. Por exemplo, embora a quantidade de ramos dendríticos seja a mesma em neurônios de machos e fêmeas em diestro, o padrão de orientação espacial dendrítico é diferente entre os sexos e provavelmente relacionado com a chegada de axônios diferente no MePD de cada um deles (Dall'oglio, 2008a).

1.4 Espinhos Dendríticos

Dendritos e espinhos são os principais sítios celulares para as funções conectivas e integrativas dos neurônios, fato igualmente evidente no MePD (Rasia-Filho e cols., 2004; Hermel e cols., 2006b; Dall'Óglio e cols., 2008b; Rasia-Filho e cols., 2009; Brusco et al., 2010) e relevantes para a formação de circuitos e da plasticidade sináptica (Alvarez e cols., 2007; Bourne e Harris, 2007; Yuste, 2011; Rasia-Filho e cols., 2012; de Vivo e cols., 2013; Hill e Zito, 2013). O padrão de desenvolvimento, forma e função dos espinhos dendríticos modifica de forma marcante a excitabilidade neuronal (Coolen e cols., 1996; Dong e cols., 2001; Segal, 2005; Genoux e cols., 2007; Segal, 2010; McKinney, 2010; Niimi e cols., 2012; Rochefort e Konnerth, 2012).

Os espinhos são pequenas protrusões neuronais mais abundantemente encontradas em ramos dendrítico, descobertos faz mais de cem anos por Ramón y Cajal (1888), heterogêneos tanto morfologicamente quanto funcionalmente (Lee e cols., 2012; Rochefort e Konnerth, 2012). Espinhos podem ser encontrados no corpo da célula neuronal e no segmento inicial do axônio de algumas células do SNC (Peters e cols., 1991; Rasia-Filho e cols., 1999, 2004). Os espinhos dendríticos são encontrados em todos os vertebrados e em alguns invertebrados, sendo que o seu tamanho varia ao longo das diferentes regiões nervosas ou dentro de uma mesma, assim como entre as espécies (Rochefort e Konnerth, 2012). Os espinhos dendríticos são unidades integradoras multifuncionais que formam compartimentos especializados pós-sinápticos com receptores de neurotransmissores para alterar as propriedades neuronais locais e para ativar seqüencias bioquímicas intracelulares (Shepherd, 1996; Nimchinsky e cols., 2002; Segal, 2005). Diferenças no comprimento do pescoço e diâmetro da cabeça dos espinhos também são evidenciadas ao longo dos ramos dendríticos do mesmo neurônio. A diferenciação dos espinhos pode ser baseada em sua morfologia geral, como o comprimento do pescoço, a forma da cabeça e o número de protusões a partir de um único pescoço. Dessa forma, os espinhos podem ser classificados como de tipo achatado/espesso, fino, com formato de cogumelo ou com outras formas menos frequentes, como com ramificações (Valverde, 1962; Peters e Kaiserman-Abramof, 1970; Woolley e McEwen, 1993; Wearne e cols., 2005; Arellano e cols., 2007; Kim e cols., 2007; Brusco e cols., 2008; Dall'Oglio e cols., 2008b; Brusco et al., 2010; Dall'Oglio e cols., 2013; Figura 5). Algumas formas intermediárias também podem ser visualizados entre as diferentes

classificações (Brusco e cols., 2010). Esta característica impressionante dos espinhos, relacionada a variedade de seus formatos e tamanhos, sugere uma possível diversidade funcional (Rochefort e Konnerth, 2012).

Os espinhos dendríticos podem ser estáveis ou podem sofrer mudanças notáveis em sua forma e número sob diferentes condições fisiológicas ou experimentais, e isso no decurso de minutos, horas ou dias (Toni e cols., 1999; Nimchinsky e cols., 2002; Kasai e cols., 2003; Marcuzzo e cols., 2007).

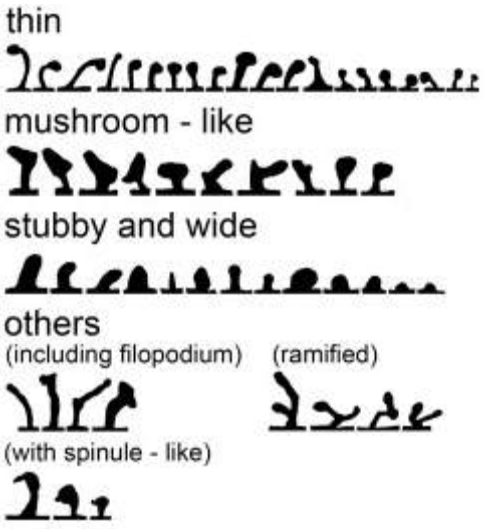

Figura 5: Desenho representativo das diferentes formas dos espinhos dendríticos do MePD de ratos adultos machos evidenciados pela fluorescência com o corante extracelular DiI e microscopia confocal, como publicado em Brusco e cols. (2010)

A maioria dos neurônios do MePD possuem espinhos dendríticos, embora outros escassos apresentem-se sem espinhos nesta região. Nesses casos, porém, algumas proeminências dendríticas são identificadas, o que coloca em dúvida a presença ou não de espinhos do tipo achatado nesses ramos irregulares (Rasia-Filho e cols., 2009). Os dendritos do MePD apresentam uma quantidade moderada de espinhos e a distribuição dos espinhos parece ser relativamente homogênea ao longo do comprimento dendrítico (Marcuzzo e cols., 2007). Não há, no entanto, diferença na densidade de espinhos nos primeiros 40 µm dendríticos nem entre as colunas celulares medial e lateral do MePD (de Castilhos e cols., 2006) nem entre o MePD dos hemisférios direito e esquerdo (Arpini et al., 21010).

Pela microscopia confocal e com imunomarcação para sinaptofisina, proteína caracteristicamente pré-sináptica, demonstrou um *punctum* característico em aposição aos ramos dendríticos e espinhos (Brusco e cols., 2010; Figura 6). Poucos espinhos não apresentavam a imunomarcação, mas, muito importante, outros pareciam ser espinhos multisinápticos, apresentando múltiplos *puncta* de sinaptofisina próximos a eles, o que sugere uma versatilidade sinaptogênica no MePD de ratos (Brusco e cols., 2010). Dados ultraestruturais revelaram a presença de espinhos multisinápticos na AMe humana (Dall`Oglio e cols., 2013).

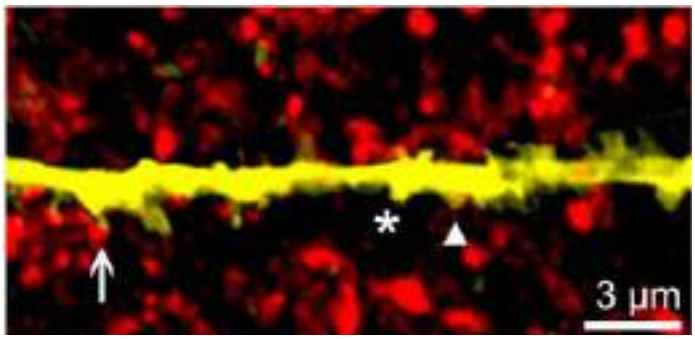

Figura 6: Ramos dendríticos e espinhos do MePD de ratos como evidenciado pela fluorescência do DiI (amarelo) associado com a imunomarcação da sinaptofisina (vermelho) reconstruída por microscopia confocal. Observe os *puncta* de sinaptofisina em aposição aos ramos dendríticos e espinhos dendríticos com suas diferentes formas (seta). Não obstante, nem todos os espinhos apareceram marcados (asterisco) e outros mostraram um agrupamento de marcação próximo a eles (ponta da seta), como publicado em Brusco e cols. (2010).

1.4.1 Plasticidade dos Espinhos Dendríticos no MePD

Várias ações relevantes dos hormônios gonadais diretamente em dendritos e espinhos já estão relatados na literatura, inclusive para a AMe de roedores (Gomez e Newman, 1991; Blaustein e cols., 1992; Lorenzo e cols., 1992; Toran-Allerand, 1995; Zehr e cols., 2006; DonCarlos e cols., 2006). O estudo dos espinhos dendríticos proximais é muito relevante, pois estes espinhos estão localizados estrategicamente para afetar a voltagem do soma neuronal, a geração do potencial de ação e as eferências para diferentes redes neurais (Rasia-Filho e cols., 2004; de Castilhos e cols., 2006, 2008; Marcuzzo e cols., 2007; Arpini e cols.2010; Rasia-Filho e cols. 2012a).

A densidade dos espinhos dendríticos (ou seja, o número de espinhos por unidade de comprimento dendrítico) nos primeiros 40 µm dos ramos dendríticos primários de neurônios do MePD, impregnados pela técnica de Golgi, está entre 2,0 a 2,9 espinhos/µm em ratos machos Wistar adultos (Rasia-Filho e cols., 2004; de Castilhos e cols., 2006; Marcuzzo e cols., 2007; de Castilhos e cols., 2008; Arpini e cols., 2010). Já a densidade dos espinhos dendríticos do MePD, em neurônios visualizados como emprego da carbocianina DiI e por microscopia confocal, foi de 1,15 ± 0,6 espinhos/µm (Brusco e cols., 2010; Rasia-Filho e cols., 2012a). As diferenças metodológicas na preparação histológica e na retração tecidual podem explicar a disparidade dos resultados da densidade dos espinhos entre as técnicas de Golgi e a que emprega DiI (Rasia-Filho e cols., 2012a).

Os neurônios multipolares, “bitufted” e estrelados, do MePD de ratos não apresentam diferença na densidade de espinhos dendríticos entre si (de Castilhos e cols., 2006; Marcuzzo e cols., 2007). Empregando-se a técnica de Golgi, foi observado que a densidade desses 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, o que sugere que a densidade de espinhos dendríticos na MePD é sexualmente dimórfica (Rasia-Filho et al., 2004). Sendo assim, é relevante considerar que a variação cíclica dos esteróides ovarianos ou sua elevação durante a gravidez modifica o número de locais pós-sinápticos de processamento de informação no MePD (Rasia-Filho e cols., 2004, 2012a,b). Ademais, a reposição hormonal afeta igualmente a densidade de espinhos dendríticos no MePD de ratas. Ou seja, a terapia substitutiva com estradiol e progesterona, uma semana depois a

castração, aumenta o número de espinhos dendríticos em relação às ratas controle não tratadas (de Castilhos e cols., 2008).

Os hormônios gonadais podem também alterar a morfologia dos neurônios do MePD de machos. A castração de ratos Sprague Dawley pré-puberes reduz o número de espinhos dendríticos sem afetar o comprimento e ramificação dendrítica de neurônios do MePD (Cooke e Woolley, 2009), enquanto a exposição de ratos Long Evans à testosterona por 4 semanas aumenta em 67% a densidade de espinhos dendríticos em ramos dendríticos distais (Cunningham e cols., 2007). Em ratos Wistar adultos, a castração reduziu a densidade de espinhos dendríticos proximais coincidindo no tempo com a redução do comportamento sexual desses animais (de Castilhos e cols., 2008).

Avançando os achados sobre a quantidade de espinhos dendríticos no MePD e sua modulação pelos hormônios gonadais em machos e fêmeas, descobriu-se que o maior percentual de espinhos dendríticos no MePD de ratos adultos apresenta formato fino (Brusco et al., 2010), como mencionado anteriormente. Não se conhecia, no entanto, qual a morfologia preferencial dos espinhos dendríticos no MePD de fêmeas ao longo do ciclo estral. Esta é uma das contribuições da presente dissertação.

1.5 Importância dos Fatores de Transcrição Lhx no desenvolvimento e na conectividade do MePD

As diferentes subdivisões da AMe estão envolvidas no controle de comportamentos sociais, dentre eles o reprodutivo, o defensivo e o agressivo (Choi et al., 2005; Rasia-Filho et al., 2009, 2012b). Essas funções são moduladas por subpopulações neuronais geneticamente distintas no MePD,

por exemplo, as quais expressam diferentes fatores de transcrição da família homeodomínio LIM (Lhx; Choi e cols., 2005). Os genes específicos de regulação para os fatores de transcrição Lhx são expressos de forma diferente ao longo do desenvolvimento das células locais do MePD (Choi e cols., 2005; García-López e cols., 2008; Bupesh e cols., 2011). Três genes da classe homeodomínio LIM, Lhx5, Lhx6 e Lhx9, são diferentemente expressos no MePD (Choi e cols., 2005). Estudos com microscopia confocal indicaram que células imunorreativas para Lhx6 constituem uma alta proporção (em torno de 80% ± 1.5%) de todos os neurônios do MePD (Zirlinger e cols., 2001; Choi e cols., 2005). Esses neurônios são particularmente originados da MGEcv formando um agrupamento celular que expressa calbindina e, provavelmente, ácido γ-amino-butírico (GABA) relacionados com o comportamento reprodutivo (Bupesh e cols., 2011). A presença do fator de transcrição Lhx5 caracteriza uma subpopulação neuronal importante derivada dos núcleos supra-óptico e paraventricular do hipotálamo (Medina e cols., 2011). Já células oriundas do pálio ventral expressam Lhx9 no MePD (García-López e cols., 2008; Bupesh e cols., 2011).

O estudo das subpopulações neuronais imunomarcadas com os fatores de transcrição Lhx6, Lhx5 e Lhx9 no MePD avança os conhecimentos sobre os genes relacionados com a circuitaria desta região, contribuindo para o esclarecimento do seu desenvolvimento e da sua organização funcional (Choi e cols., 2005). Além disso, por ser uma estrutura essencial para o controle das emoções e do comportamento social (Newman, 1999; Swanson, 2000; Phelps and LeDoux 2005), a disfunção da amígdala está associada com diversas doenças neuropsiquiátricas humanas, incluindo a epilepsia do lobo temporal

(Pitkänen e cols., 1998), o autismo (Amaral e cols., 2008), entre outras. Desta forma, o estudo do desenvolvimento desta estrutura é importante para a compreensão da fisiopatologia destas doenças e o rato pode servir como modelo animal. Os espinhos dendríticos, dada sua relevância para as sinapses, nas diferentes subpopulações neuronais que expressam esses fatores de transcrição e que compõe o MePD de ratos são igualmente estudados nesta dissertação.

1.6 Importância dos receptores glutamatérgicos e GABAérgicos na plasticidade dos espinhos dendríticos

Uma sinapse é tipicamente definida pela presença de uma zona pré-sináptica ativa contendo as vesículas sinápticas, uma fenda sináptica bem definida e uma densidade pós-sináptica (PSD) que é caracterizada por uma região elétron-densa à microscopia eletrônica (Holtmaat e Svoboda, 2009; Rochefort e Konnerth, 2012). As diferenças no tamanho da cabeça do espinho são correlacionadas com diferenças no tamanho da densidade pós-sináptica (PSD, McKinney, 2010). Baseado nisso, a forma dos espinhos pode afetar a estabilidade sináptica e a função sináptica (McKinney, 2010; Niimi e cols., 2012; Rochefort e Konnerth, 2012). Por exemplo, a formação, o remodelamento, e a eliminação de sinapses excitatórias nos espinhos dendríticos representam formas de refinamento da atividade da microcircuitaria cerebral (McKinney, 2010). Sugere-se que o formato dos espinhos possa ser regulado pelos subtipos de receptores glutamatérgicos que ocorrem por causa e para modificar certas formas de plasticidade sináptica (Matus, 2000). Sendo assim, espinhos com morfologia classificada como fina (com um pescoço mais longo e uma cabeça de pequeno volume) se mostram muito mais instáveis

quanto a seu número e forma (Lin e cols., 2004; Matus, 2005), enquanto espinhos maiores e com formato de cogumelo poderiam ser mais estáveis e capazes de atividades sinápticas mais duradouras (Nimchinsky e cols., 2002; London e Häusser, 2005). Embora ainda seja assunto em debate (Segal, 2010), acredita-se que espinhos menos estáveis apresentem preferentemente receptores glutamatérgicos de tipo NMDA e aqueles mais estáveis receptores de tipo AMPA (Nimchinsky e cols., 2002; London e Häusser, 2005; Arellano e cols., 2007; Bourne e Harris, 2007). Está descrita uma correlação estreita entre o tamanho do espinho dendrítico e o tamanho da PSD e entre o tamanho da PSD e a densidade de receptores glutamatérgicos do tipo AMPA na sinapse (Hering e Sheng, 2001; McKinney, 2010; Hanley, 2008). Então, quanto menor for a cabeça do espinho, menor é a sua PSD e menor é a densidade de receptores glutamatérgicos do tipo AMPA (McKinney, 2010). Em contraste, espinhos maiores como os do tipo cogumelo, por exemplo, tem PSDs maiores que são frequentemente de aspecto “perfurado” (Harris e Stevens, 1989; Harris e cols., 1992).

Contribuições teóricas e experimentais vêm sugerindo que, além da geometria do espinho dendrítico afetar o processamento biofísico do potencial elétrico gerado pela atividade sináptica em relação ao dendrito com o qual está conectado, há presença de receptores para neurotransmissores diferentes entre os diferentes tipos de espinhos (Segev e Rall, 1998; Nimchinsky e cols., 2002; London e Häusser, 2005). Os receptores glutamatérgicos do tipo AMPA e do tipo NMDA estão colocalizados na membrana pós-sináptica da maioria das sinapses excitatórias (Kharazia & Weinberg, 1997; Nusser, 2000). Tendo papel muito importante na formação das sinapses mais estáveis, encontram-se

os receptores glutamatérgicos de tipo AMPA, com as subunidades GluR1/2 (Tsui, 1996; Oray e cols., 2006; Zito e cols., 2009). A subunidade GLUR1 tem sido envolvida na morfogênese do espinho, com sua porção C-terminal podendo interagir diretamente com a maquinaria de sinalização intracelular para promover mudanças apropriadas no citoesqueleto (Hanley, 2008). Receptores glutamatérgicos do tipo NMDA são encontrados em neurônios tanto nas sinapses que são feitas em espinhos dendríticos como em locais extrasinápticos (Petralia e cols., 2010). A subunidade GluN1 do NMDA é essencial para a formação desse tipo de receptor (Watanabe, 1992).

O glutamato também exerce um importante papel na expansão e retração do espinho. Os receptores glutamatérgicos regulam essa propriedade por ações sobre o citoesqueleto de actina, como a indução da despolimerização da actina (Richards e cols., 2004). Reduções significativas na densidade e no comprimento dos espinhos dendríticos dos neurônios da região CA1 hipocampal foram observadas após a aplicação de antagonistas do receptor AMPA ou bloqueio da liberação do glutamato pela adição da toxina botulínica à cultura de células (McKinney, 2010). Isso poderia explicar porque a ausência da ativação do receptor AMPA, após a degeneração de aferências pré-sinápticas, inicia um processo de retração dos espinhos nas células pós-sinápticas em CA1 (McKinney, 2010). Desta forma, o glutamato liberado exerce um efeito trófico sobre os espinhos, agindo sobre os receptores AMPA, o que é essencial para sua manutenção (McKinney e cols., 1999). A ativação de receptores glutamatérgicos do tipo NMDA é capaz de induzir mudanças rápidas na morfologia dos dendritos e no recrutamento dos receptores glutamatérgicos do tipo AMPA nas sinapses dos espinhos dendríticos (Matus, 2000; Lin e cols.,

2004; McKinney, 2010). A ativação dos receptores do tipo NMDA causa um aumento rápido e transitório no tamanho de espinhos pré-existentes e, em seguida, a formação gradual de novas protrusões dendríticas e espinhos dendríticos (Lin e cols., 2004). Cada uma destas formas de plasticidade pode ter efeitos significativos na eficácia da transmissão sináptica (McKinney e cols., 1999; Fischer e cols., 2000; Bnhoeffer e Yuste, 2002; Lin e cols., 2004).

A transmissão sináptica inibitória rápida na maioria das sinapses do SNC é mediada pelos receptores GABAérgicos ionotrópicos de tipo A (Marowsky e cols., 2004; Heldt e Ressler, 2007) que são compostos por cinco subunidades a partir de um conjunto de sete famílias de subunidades (Whiting e cols., 1999; Marowsky e cols., 2004). A variedade de combinações das subunidades resulta em uma população heterogênea dos receptores $GABA_A$ (Heldt e Ressler, 2007); no entanto, a composição mais abundante consiste em duas subunidades α (1-6), duas subunidades β (1-3) e uma subunidade γ (1-3). Os números entre parênteses representam as isoformas de cada subunidade do receptor $GABA_A$ (Farrar e cols., 1999; Sieghart e cols., 1999; Whiting, 1999; Marowsky e cols., 2004; Heldt e Ressler, 2007).

O padrão de distribuição das subunidades do receptor $GABA_A$ dependente de cada região nervosa e sugere que diferentes receptores estão envolvidos em circuitos neuronais distintos e com a capacidade de auxiliar uma sinalização celular específica (Marowsky e cols., 2004). Há RNAm para a subunidade γ2 em todo complexo amigdaloide de camundongos com diferenças de expressão em cada núcleo, sendo expresso de forma moderada no AMe e no ACe e fortemente expressa na BLA (Heldt e Ressler, 2007). A imunomarcação para GABA evidenciou uma intensa marcação em todas as

estruturas da divisão medial da amígdala extendida de ratos. A marcação foi principalmente observada no soma, incluindo a porção proximal dos dendritos e axônios (Pereno e cols., 2011; Niimi e cols., 2012). A grande quantidade de neurônios contendo GABA em todos os núcleos da divisão medial da amígdala extendida sugere que a AMe tem um importante papel no controle inibitório do processamento de aferências sensoriais e das projeções para os diferentes núcleos hipotalâmicos moduladores de comportamentos sociais (Choi et al., 2005; Pereno e cols., 2011; Niimi e cols., 2012).

As sinapses GABAérgicas desempenham diferentes funções, dependentes do tempo exato de sua ativação e da sua localização subcelular (Pouille e Scanziani, 2001; Gulledge e Stuart, 2003). Receptores do tipo $GABA_A$ podem estar presentes em contatos sinápticos que se fazem principalmente no tronco dendrítico proximal e, em casos mais raros, em espinhos dendríticos (Kisvárday e cols., 1990; Peters e cols., 1991; López-Bendito e cols., 2004; Cooke e Woolley, 2005). Essas sinapses inibitórias formadas com os espinhos são estrategicamente localizadas para regular as respostas pós-sinápticas (Keller, 2002).

Imunomarcação para GABA foi demonstrada em terminal sináptico simétrico de sinapses formadas em espinhos, à microscopia eletrônica, no AMe de ratos pré-púberes (Cooke e Woolley, 2005). Não há nenhum dado até o momento para os receptores glutamatérgicos AMPA e NMDA ou para o receptor $GABA_A$ e sua relação com os espinhos dendríticos no MePD de ratos adultos. Isso importa muito no processamento da informação sináptica, na estabilidade morfofuncional do espinho e de seus contatos e, na atividade eletrofisiológica e bioquímica do dendrito adjacente (Benavides-Piccione e

cols., 2002; Nimchinsky e cols., 2002; London e Häusser, 2005; Ballesteros-Yanez e cols., 2006; Bourne e Harris, 2007). Esse tipo de dado pode direcionar esforços futuros com outras técnicas de Biologia Molecular, microscopia eletrônica e imunocitoquímica para glutamato e GABA no MePD de ratos. Todos esses dados são ainda inéditos, relevantes para a área de estudo e contribuem diretamente e com os esforços que estão sendo realizados para elucidar a morfologia e a plasticidade dos neurônios do MePD de ratos (Rasia-Filho e cols., 2004; de Castilhos e cols., 2006, 2008; Dall'Óglio e cols., 2008a; Rasia-Filho e cols., 2009, 2012a,b). Podem auxiliar também a compreender as características morfológicas e funcionais dos neurônios dessa região e o papel integrado do MePD dentro de circuitos envolvidos com a modulação de diferentes comportamentos sociais em machos. Isso é absolutamente relevante para estabelecer uma correlação com os dados sobre a densidade de espinhos dendríticos de machos obtidos pela técnica de Golgi (Rasia-Filho e cols., 2004; de Castilhos e cols., 2006, 2008; Arpini e cols., 2010; Brusco e cols., 2010). Neste sentido, a microscopia confocal e a reconstrução tridimensional de imagens servem como método adequado para estudo da presença e localização de receptores em espinhos dendríticos (Rasia-Filho e cols., 2010).

2. OBJETIVOS

2.1 Objetivo Geral

- Estabelecer conhecimentos inéditos a respeito da morfologia e da conectividade dos espinhos dendríticos do MePD de ratos machos e fêmeas, valendo-se da técnica de reconstrução tridimensional com microscopia confocal, a fim de obter dados relevantes e complementares para o entendimento de certas formas de plasticidade sináptica e da organização sináptica desta região. Tais dados devem avançar o conhecimento sobre a base celular de organização funcional dessa região amigdaliana, sendo muito relevante para os experimentos futuros que visem estudar a importância do comprometimento da amígdala medial em condições neuropatológicas.

2.2 Objetivos Específicos

Objetivo 1 - Estudo da densidade e da morfologia tridimensional dos espinhos dendríticos da amígdala medial póstero-dorsal de ratas ao longo do ciclo estral

- Estudar a densidade e a morfologia tridimensional dos espinhos dendríticos, após reconstrução de imagens de microscopia confocal, no MePD de fêmeas ao longo do ciclo estral.

- Comparar com dados prévios a respeito da morfologia dos espinhos dendríticos do MePD de machos, analisar o dimorfismo sexual nos espinhos dendríticos dessa região amigdaliana e avaliar se a secreção cíclica dos esteróides ovarianos pode influenciar a morfologia desses espinhos.

Objetivo 2 - Estudo da morfologia tridimensional dos espinhos dendríticos em neurônios Lhx5$^+$, Lhx6$^+$ e Lhx9$^+$ na amígdala medial póstero-dorsal de ratos

- Descrever a presença das subpopulações neuronais que expressam os fatores de transcrição Lhx6$^+$, Lhx5$^+$, e Lhx9$^+$ no MePD de ratos.

- Estudar a densidade e a morfologia tridimensional dos espinhos dendríticos, após reconstrução de imagens de microscopia confocal, nas subpopulações neuronais, Lhx6$^+$, Lhx5$^+$ e Lhx9$^+$ do MePD de ratos, machos e adultos.

Objetivo 3 - Estudo da morfologia tridimensional dos espinhos dendríticos e da presença e da distribuição de receptores glutamatérgicos e GABAérgico na amígdala medial póstero-dorsal de ratos

- Descrever a presença e a localização de receptores pós-sinápticos dos principais neurotransmissores excitatório e inibitório nos espinhos dendríticos do MePD de ratos.

- Estudar os pontos de imunomarcação da subunidade GluR1-4 do receptor AMPA, da subunidade GluN1 do receptor NMDA e do receptor $GABA_A$ sobre a estrutura tridimensional dos espinhos dendríticos de neurônios do MePD de ratos machos adultos pela reconstrução tridimensional de imagem obtida por microscopia confocal.

3. ARTIGOS CIENTÍFICOS

Objetivo 1 = Para contemplar este objetivo, os dados foram obtidos e publicados na revista Histology and Histopathology no ano de 2012. Este artigo contém também uma revisão atual e geral dos conhecimentos correlatos da MePD de ratos.

Objetivos 2 e 3 = Os dados relacionados com estes objetivos foram obtidos e compuseram o segundo artigo aqui apresentado, a ser submetido em breve para julgamento na revista Brain Structure and Function.

Histol Histopathol (2012) 27: 985-1011

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Histology and Histopathology

Cellular and Molecular Biology

Review

Dendritic spines of the medial amygdala: plasticity, density, shape, and subcellular modulation by sex steroids

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Summary. The medial nucleus of the amygdala (MeA) is a complex component of the "extended amygdala" in rats. Its posterodorsal subnucleus (MePD) has a remarkable expression of gonadal hormone receptors, is sexually dimorphic or affected by sex steroids, and modulates various social behaviors. Dendritic spines show remarkable changes relevant for synaptic strength and plasticity. Adult males have more spines than females, the density of dendritic spines changes in the course of hours to a few days and is lower in proestrous and estrous phases of the ovarian cycle, or is affected by both sex steroid withdrawal and hormonal replacement therapy in the MePD. Males also have more thin spines than mushroom-like or stubby/wide ones. The presence of dendritic fillopodia and axonal protusions in the MePD neuropil of adult animals reinforces the evidence for local plasticity. Estrogen affects synaptic and cellular growth and neuroprotection in the MeA by regulating the activity of the cyclic AMP response element-binding protein (CREB)-related gene products, brain-derived neurotrophic factor (BDNF), the anti-apoptotic protein B-cell lymphoma-2 (Bcl-2) and the activity-regulated cytoskeleton-related protein (Arc). These effects on signal transduction cascades can also lead to local protein synthesis and/or rearrangement of the cytoskeleton and subsequent numerical/morphological alterations in dendritic spines. Various working hypotheses are raised from these experimental data and reveal the MePD as a relevant region to study the effects of sex steroids in the rat brain.

Key words: Amygdala/cytology, CREB, Gonadal hormones, Neural pathways/axonal network, Sexual dimorphism

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Introduction

The amygdaloid complex in the basal forebrain is composed of various nuclei and subnuclei with anatomical and functional particularities (Brodal, 1981; McDonald, 1998; Pitkänen, 2000; Rasia-Filho et al., 2000; de Olmos et al., 2004). Considerable efforts have been devoted to its study in rodents (e.g., rat, mouse, and hamster), monkeys, and humans. The medial nucleus of the amygdala (MeA) is a superficial and relatively large component of the "extended amygdala" in rats (Alheid et al., 1995; Alheid, 2003; de Olmos et al., 2004). The embryological development of the mouse MeA indicates that it is a "mosaic" formed by cells coming from a caudoventral pallidal subdivision, the ventral pallium, the commissural preoptic area, and the supraopto-paraventricular domain of the hypothalamus (García-López et al., 2008; Bupesh et al., 2011). These data did not support an exclusively striatal nature for the mice MeA (Swanson and Petrovich, 1998). The rat MeA can be further subdivided according to histological, connectional, neurochemical, and functional criteria in

the anterodorsal (MeAD), anteroventral (MeAV), posterodorsal (MePD), and posteroventral (MePV) subnuclei (Alheid et al., 1995; Petrovich et al., 2001; de Olmos et al., 2004; Carrillo et al., 2007; Dall'Oglio et al., 2008a,b). In coronal sections, the MePD shows three parallel vertically-oriented columns of aggregated cells that extend from the medial to the lateral border of this subnucleus, close to the optic tract (OT) and ventrally to the stria terminalis (ST; de Olmos et al., 2004). Surrounding the external borders of the MePD and the MePV there is a cell-sparse rim initially termed the "molecular layer", but rather formed by axons coming from outside the MeA subnuclei (Scalia and Winans, 1975; Nishizuka and Arai, 1983; de Olmos et al., 2004).

Neuroanatomical and functional differences of MeA subnuclei were revealed by different methodological approaches (Coolen et al., 1997; Newman, 1999; Petrovich et al., 2001; de Olmos et al., 2004; Rasia-Filho et al., 2004; Blake and Meredith, 2011). Previous results suggested that the MeA could have an anterior chemosensory information-sensitive part and a hormonally-sensitive posterior aspect (Gomez and Newman, 1991; Malsbury and McKay, 1994; Wood and Newman, 1995; Rasia-Filho et al., 1999). However, due to its axonal projections, the MePD appeared to be the most different component within the rat MeA, even when compared to the MePV (Canteras et al., 1995). The proposition of a "ventral" MeA, made by the MeAD and the MePV, received additional commentaries questioning its actual existence in rats (Dall'Oglio et al., 2008b). Therefore, as previously stated for the whole amygdaloid complex (Brodal, 1981; Swanson and Petrovich, 1998), the MeA is neither an anatomical nor a functional unit. The MeA can have subregion-specific features and this heterogeneity can affect the interpretation of the data from the whole MeA (see parallel comments in Rosa et al., 2011). This implies that the experimental data obtained from the whole MeA have to be considered as the "resultant" of all subnuclei contributions. Thus, remarkable results could be due to a great effect consistently found in one or more than one of its subnuclei. Otherwise, it is also possible that a significant effect in one subnucleus can be masked or diminished after mixing data from other subnuclei where the actions are less intense.

The MeA subnuclei are part of brain circuits sensitive to sex steroids where local cells show plastic changes according to the level of gonadal hormones in circulation (Gréco et al., 1998, 2001, 2003; Newman, 1999; Rasia-Filho et al., 2004, 2009, 2012; de Castilhos et al., 2008). The expression of receptors for testosterone, estradiol or progesterone is different in the MeA subnuclei (Simerly et al., 1990; Shughrue et al., 1997; De Vries and Simerly, 2002). The MePD presents one of the highest concentrations of sex steroid hormones receptors in the rat brain, resembling those found in the hypothalamic nuclei that control reproduction (Simerly et al., 1990; Shughrue et al., 1997). This suggests that males and females have to be studied apart and, in adult females, the phases of the estrous cycle have to be considered as a presumed source of variability in the results. If females had the experience of motherhood, the MePD differed from that of a virgin age-matched female in various respects (Rasia-Filho et al., 2004). These methodological procedures were achieved after many trials, based on many pioneering results, and they are helping us to conduct future work. In the present review, whenever possible, attention will be given to the MePD (Figure 1) because of its known plasticity.

The posterodorsal medial amygdala

The basic morphological description of the MePD was provided from prepubertal Sprague Dawley rats (Cooke et al., 2007). In these animals, the MePD volume was greater in males in both left and right hemispheres (mean ± standard error of the mean, 0.242±0.03 and 0.258±0.026 mm^3, respectively) than females (0.203±0.01 and 0.216±0.023 mm^3, respectively), which represents a statistically significant sex difference close to 15±5 %. The neuronal density was estimated using the optical disector in Nissl-stained coronal sections. In the left and right MePD of males there were 101,744±7,686 and 110,168±7,905 cells/mm^3, whereas females had 110,306±10,223 and 107,208±6,930 cells/mm^3, respectively. By the relative neuronal density and total volume of the MePD, males had more neurons in the right, but not in the left, MePD than females. That is, for the left and right MePD, values were 24,611±3,858 and 28,550±4,525 for males and 22,411±2,934 and 23,079±2,355 for females, respectively. From electron micrograph images the neuropil of the left MePD of males, compared to females, showed significantly higher values, mainly due to the volume occupied by dendritic shafts (41% vs. 32%) and glial cells (15% vs. 13%). On the other hand, more axons filled the left MePD in females than in males (50% vs. 40%). Dendritic spines and synapses accounted for 1% each in both sexes. That is, genetic and/or early epigenetic-mediated sex differences affect the rat MePD prior to the pubertal "activational" period of gonadal hormone action.

In Sprague-Dawley rats (7 months of age) the total number of Nissl-stained cells in the whole MeA (including the surrounding "molecular layer") was estimated using the optical fractionator method in coronal sections (Chareyron et al., 2011). According to these authors, the MeA has (mean ± standard deviation) 188,742±19,688 neurons and 103,936±9,036 glia cells. No sex difference or lateralization was found in the data obtained from 2 males and 2 females.

On the other hand, Morris et al. (2008) studied specifically the MePD of young adult Long Evans rats. They used the thionin technique in coronal sections and counted cells by the optical fractionator method. The MePD volume was greater in the right hemisphere than in the left and males had higher values than females. For the left and right MePD of males the results were (mean

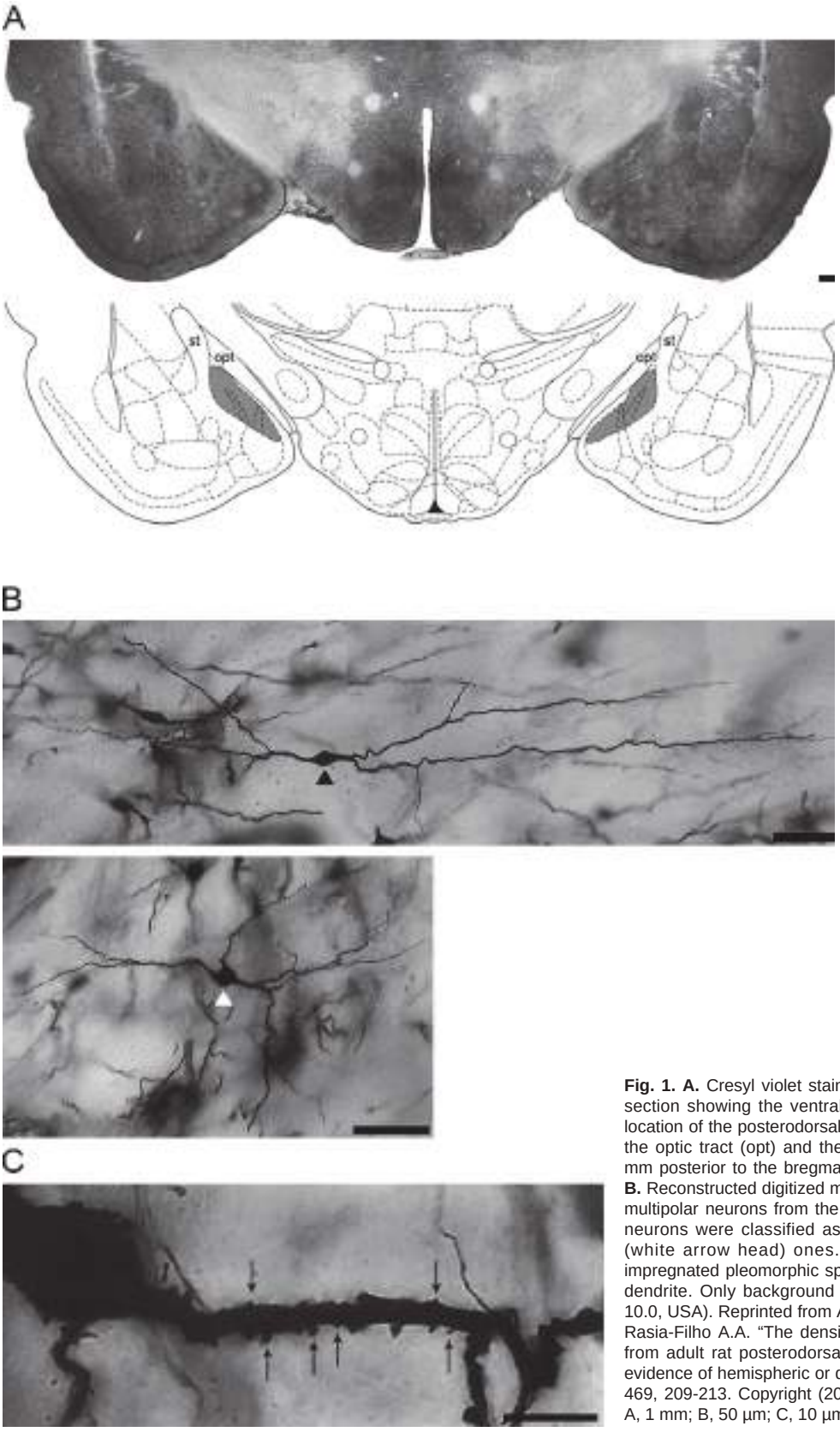

Fig. 1. A. Cresyl violet staining and schematic drawing of a coronal section showing the ventral aspect of the Wistar rat brain and the location of the posterodorsal medial amygdala (colored gray) close to the optic tract (opt) and the stria terminalis (st) approximately 3.30 mm posterior to the bregma (based on Paxinos and Watson, 1988). **B.** Reconstructed digitized microscopic image showing representative multipolar neurons from the rat MePD. Golgi-impregnated multipolar neurons were classified as bitufted (black arrow head) or stellate (white arrow head) ones. **C.** Photomicrograph showing Golgi-impregnated pleomorphic spines (arrows) protruding from a proximal dendrite. Only background contrast was adjusted (Photoshop CS3 10.0, USA). Reprinted from Arpini M., Menezes I.C., Dall'Oglio A. and Rasia-Filho A.A. "The density of Golgi-impregnated dendritic spines from adult rat posterodorsal medial amygdala neurons displays no evidence of hemispheric or dorsal/ventral differences". Neurosci. Lett. 469, 209-213. Copyright (2010) with permission from Elsevier. Bars: A, 1 mm; B, 50 µm; C, 10 µm.

± standard error of the mean) 0.29±0.01 and 0.325±0.015 mm^3, whereas females had 0.18±0.01 and 0.18±0.01, respectively (approximate data based on results presented in Figure 3A, page 855). The number of neurons was higher in the left MePD than in the right MePD and males have more neurons than females. Approximate values for the number of neurons in the left and right MePD of males were 29,000±2,500 and 26,000±2,500, and females had 21,000±2,500 and 16,000±2,000, respectively (based on data presented in Figure 4A, page 856). The number of glial cells is greater in the right than the left MePD and males have more glial cells in both hemispheres compared to females (left and right male MePD, approximately 16,000±1,000 and 24,000±2,000; in females 12,500±2,000 and 13,000±1,000, respectively; Figure 4B, page 856). That is, in the young adult rat, the left MePD is smaller in volume but contains more neurons than the right MePD, which has more glial cells. Johnson et al. (2008) confirmed these MePD volume data and found that astrocytes were more numerous on the right than in the left MePD and in males than in females. In addition, the left MePD had more complex astrocytes compared to those in the right, and the left MePD astrocytes of males had more primary branches, total number of branches, and branch points, and longer branches than females, although females had a greater astrocytic density than males.

Therefore, the rat MePD is an interesting area to study neural gonadal steroid actions. As demonstrated by the Golgi method, the neuronal population in the adult rat MePD of both sexes is comprised of multipolar cells classified as bitufted (not "bipolar", as per Ramón y Cajal's classical description; cf. Rasia-Filho et al., 1999) or stellate neurons (de Olmos et al., 1985; Gomez and Newman, 1991; McDonald, 1992; Rasia-Filho et al., 1999; Cooke et al., 2007; Marcuzzo et al., 2007; Dall'Oglio et al., 2008a). Representative images of these neurons are shown in Figure 1 (see additionally Rasia-Filho et al., 1999, 2004; Dall'Oglio et al., 2008a; de Castilhos et al., 2008). Bitufted neurons are characterized by two dendritic shafts that emerge from a rather fusiform or round soma, whereas stellate neurons have three or more primary dendrites (Rasia-Filho et al., 1999; to compare general morphology with neurochemically distinct MePD neurons, see a recent elaboration in Rasia-Filho et al., 2012). The dendritic trees are rectilinear or sinuous, branch sparingly, show preferred spatial localizations and extend over a wide range of path lengths (Alheid et al., 1985; Rasia-Filho et al., 1999; Dall'Oglio et al., 2008a). There is no clear morphological evidence for striatum-like medium spiny stellate neurons in the MePD of adult Wistar rats (for comparison, see Bennur et al., 2007; Marcuzzo et al., 2007; Dall'Oglio et al 2008a), but other way to classify neurons in the caudal MePD of rats was already reported (Akhmadeev, 2008). In mice, some neurons in the posterior part of the MeA resemble pyramidal neurons from the piriform cortex (Bian et al., 2008). In both mice and rats the local axons project with different orientations but emerge or leave the MePD via the ST, a bidirectional pathway of this subnucleus (Valverde, 1962; Cooke and Simerly, 1995; see also below).

Sex steroid actions in the MePD

The MePD neurons and glial cells in both sexes are normally affected by sex steroids, as summarized elsewhere (Rasia-Filho et al., 2009, 2012). They are also sensitive to changes in plasma levels of these hormones after experimental manipulations, such as castration and replacement therapies (Rasia-Filho et al., 2004; Cunningham et al., 2007; de Castilhos et al., 2008). By in situ hybridization, high concentrations of mRNA for androgen receptors (AR) were found in the MePD (Simerly et al., 1990). Functionally, pheromonal stimulation increased the number of AR-immunoreactive cells in MePD neurons of male hamsters (Blake and Meredith, 2011). In addition, a complete overlap was found between the immunoreactivity of the immediate early gene protein Fos, an indicator of cellular activation, and ARs in MePD neurons of males that ejaculated (Gréco et al., 1996). Local estrogen receptor (ER)-α (ER-α) increased density was associated with a single ejaculation or sexual satiety in male rats (Phillips-Farfán et al., 2007). In the MePD of female rats there were high concentrations of both ER-α and ER-ß (Simerly et al., 1990; Shughrue et al., 1997; Gréco et al., 1998, 2001, 2003). Different regional distribution of these ERs occurs in the dorsal and in the ventral parts of this subnucleus, but both ERs can also be co-localized in the same neurons (Gréco et al., 2001, 2003).

The replacement treatment with estradiol to castrated females decreased the number of MePD cells immunoreactive to ER-α and those co-expressing ER-α and ER-ß (Gréco et al., 2001). In castrated and hormone-primed female rats, Fos-ir following mating occurred in cells co-expressing ER-α or both ER-α/ER-ß in the dorsal MePD (Gréco et al., 2003). This same Fos detection occurred in cells only co-expressing ER-ß in the ventral MePD (Gréco et al., 2003). Progesterone receptors also showed complex dynamics in the female MePD along the estrous cycle or after castration (Gréco et al., 2001; Isgor et al., 2002). In addition, some MePD cells co-express ER-ß and progesterone receptors (Gréco et al., 2001). These data impose another level of complexity on the local effects of sex steroids in normal cyclic conditions or along the reorganizational period of the MePD following castration. For the latter, it is assumed that these effects can occur by two not mutually exclusive possibilities, i.e., after locally mediated/direct cellular actions, or indirectly via synaptic-induced changes mediated by other hormonally-responsive nuclei in interconnected brain circuits (Rasia-Filho et al., 1999, 2012). These propositions were already discussed by other authors (Nishizuka and Arai, 1982; Gomez and Newman, 1991; Lorenzo et al., 1992; Yoshida et al., 1994; Cooke and Woolley, 2009).

Furthermore, the rat MePD assembles coexisting subpopulations of neurons (Bupesh et al., 2011) with no obvious general morphological characteristic that identify them (Nabekura et al., 1986; Gomez and Newman, 1991; Rasia-Filho et al., 1999), but with functional particularities (Coolen et al., 1996; Gréco et al., 1998, 2003; Choi et al., 2005). Cells expressing the transcription factor Lhx6 constitute around 80% of all neurons in the MePD, as seen with confocal microscopy (Choi et al., 2005). This homogeneity for the MePD neurons would explain the low morphological variability (soma size, volume, and dendritic spine density) in male rats as described previously using the Golgi method (Rasia-Filho et al., 2004; de Castilhos et al., 2006, 2008; Arpini et al., 2010). Moreover, various morphological parameters in the rat MePD are sexually dimorphic or affected by sex steroids. Differences between males and females include: the volume of this subnucleus (Hines et al., 1992), the number of neurons and glial cells (Johnson et al., 2008; Morris et al., 2008), the neuronal somatic volume (Hermel et al., 2006a), the spatial orientation of the dendritic branches (Dall'Oglio et al., 2008a), the density of dendritic spines (Rasia-Filho et al., 2004; de Castilhos et al., 2008), the synaptic

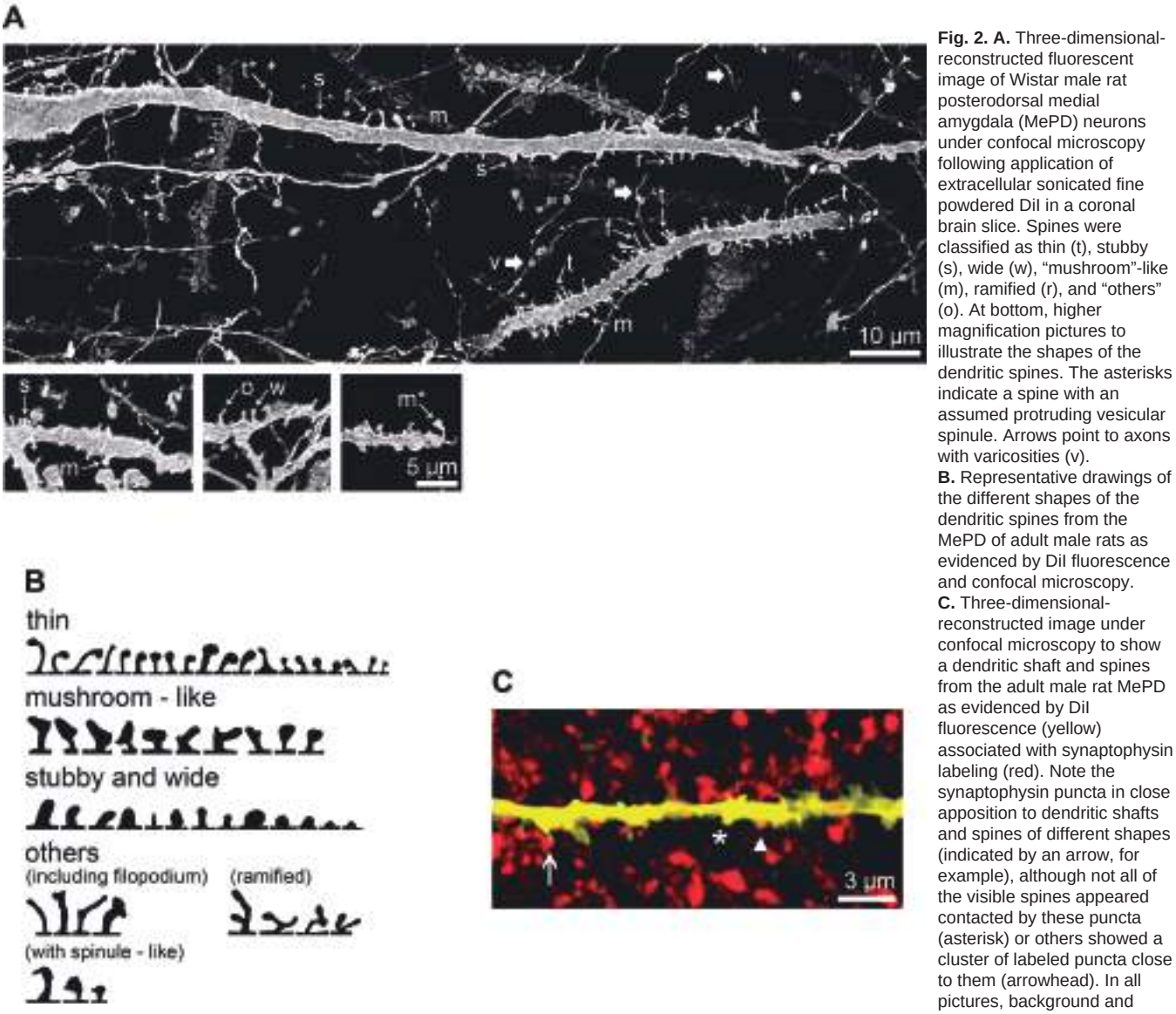

Fig. 2. A. Three-dimensional-reconstructed fluorescent image of Wistar male rat posterodorsal medial amygdala (MePD) neurons under confocal microscopy following application of extracellular sonicated fine powdered DiI in a coronal brain slice. Spines were classified as thin (t), stubby (s), wide (w), "mushroom"-like (m), ramified (r), and "others" (o). At bottom, higher magnification pictures to illustrate the shapes of the dendritic spines. The asterisks indicate a spine with an assumed protruding vesicular spinule. Arrows point to axons with varicosities (v). **B.** Representative drawings of the different shapes of the dendritic spines from the MePD of adult male rats as evidenced by DiI fluorescence and confocal microscopy. **C.** Three-dimensional-reconstructed image under confocal microscopy to show a dendritic shaft and spines from the adult male rat MePD as evidenced by DiI fluorescence (yellow) associated with synaptophysin labeling (red). Note the synaptophysin puncta in close apposition to dendritic shafts and spines of different shapes (indicated by an arrow, for example), although not all of the visible spines appeared contacted by these puncta (asterisk) or others showed a cluster of labeled puncta close to them (arrowhead). In all pictures, background and contrast were slightly adjusted using Adobe Photoshop 7.0 software (USA). Reprinted from Brusco J., Dall'Oglio A., Rocha L.B., Rossi M.A., Moreira J.E. and Rasia-Filho A.A. Descriptive findings on the morphology of dendritic spines in the rat medial amygdala. Neurosci. Lett. 483, 152-156.

connectivity (Nishizuka and Arai, 1983) and the excitatory post-synaptic current frequencies (Cooke and Woolley, 2005), the content of neuropeptides (Micevych et al., 1988; Oro et al., 1988; Malsbury and McKay, 1994; De Vries and Simerly, 2002), and astrocyte density measured by glial fibrillary acidic protein (GFAP) immunoreactivity (Rasia-Filho et al., 2002; Martinez et al., 2006; Johnson et al., 2008).

By linking the behavioral displays elicited by the MePD with gonadal hormonal effects, it became clear that sex steroids can remarkably alter the function of the MePD cells. As reviewed recently (Rasia-Filho et al., 2012), an unilateral implantation of estradiol in this subnucleus restored copulatory behavior in adult castrated male rats (Rasia-Filho et al., 1991) as in adult castrated hamsters after implantation of testosterone or estradiol, but not dihydrotestosterone, in the posterior MeA (Wood, 1996). Male mating behavior increased either after unilateral or bilateral implants of testosterone in the MePD of adult castrated hamsters, indicating redundancy, but not amplification, of the androgenic effects in this brain area (Coolen and Wood, 1999). The MePD role in the central modulation of male copulatory behavior is affected by the individual's sexual experience and involves the evaluation of the sexual receptivity of the female (De Jonge et al., 1992; Stark, 2005). This suggests that the MePD participates in a plastic circuit that changes with emotional/social processing and learning. According to Newman (1999), "These same factors, sex-steroid sensitivity and neuronal connections, are of course dynamically modulated throughout life by sexual maturation, by experience or learning, by reproductive cycles and diurnal cycles, and by disease and aging…. At the very least, these stimuli produce immediate changes in synaptic activity in the nodes of the social behavior network. In some cases the effects are long-lasting changes in the strength of synaptic connections…We will have to demonstrate mini-circuits within this network, each one independently regulating a specific aspect of a particular behavior.".

In effect, most MePD neurons are connected with the medial hypothalamic nuclei related to reproduction (Choi et al., 2005) either directly or indirectly via components of the bed nuclei of the ST (BNST; Dong et al., 2001) or the hippocampus/septum pathway (Petrovich et al., 2001). To the hypothalamus, the MePD sends projections: 1) with sparse terminals or *en passant* buttons to the anterior periventricular nucleus and the arcuate nucleus in the "neuroendocrine motor zone"; to the anterior nucleus and the dorsomedial part of the ventromedial nucleus in the medial 'behavior control column' involved with defensive display, as well to the descending division of the paraventricular nucleus related to ingestive or sympathetic/parasympathetic activities, and to the medial mammillary nucleus or the supramammilary nucleus, this one in the lateral zone; 2) moderate terminals in the ventrolateral part of the ventromedial nucleus in the medial nuclei/"behavior control column" for reproduction; and, 3) dense innervation to the anteroventral periventricular nucleus (AVPV) in the periventricular region; to the lateral part of the medial preoptic nucleus (MPOA) and the ventral premammillary nucleus (PMv) in the medial nuclei/"behavior control column" for reproduction; and, to the posterior nucleus in the lateral zone (Petrovich et al., 2001).

The relevance and functional integration for some of these connections has recently been addressed (Petrovich et al., 2001; Simerly, 2002; Choi et al., 2005; Rasia-Filho et al., 2009, 2012; Quagliotto et al., 2012). It is noteworthy that the rat MePD can affect the occurrence of emotionally-loaded social behaviors, according to Newman's proposition (Newman, 1999). Local neurochemical stimulation and inhibition support the MePD role as a node for the modulation of social behavior neural networks (see further data and comments in Rasia-Filho et al., 2012). Indeed, the MePD deals with the interpretation of the social relevance of both olfactory and vomeronasal stimuli (Meredith and Westberry, 2004; Blake and Meredith, 2011; Dhungel et al., 2011), the central processing of genitosensorial stimulation and modulation of different aspects of the sexual behavior of males (remarkably ejaculation) and females (Rasia-Filho et al., 1991, 2012; Coolen et al., 1997; Pfaus and Heeb, 1997; Coolen and Wood, 1999; Newman, 1999; de Castilhos et al., 2006 linked to Rasia-Filho and Lucion, 1996), maternal behavior (Fleming et al., 1980; Sheehan et al., 2001; Rasia-Filho et al., 2004), aggression (Halász et al., 2002; Nelson and Trainor, 2007; Rasia-Filho et al., 2012), and neuroendocrine responses to stressful stimuli (Dayas et al., 1999; Marcuzzo et al., 2007; Singewald et al., 2008). For example, the MePD projections to the hypothalamic AVPV, MPOA, and PMv are involved with pheromonal stimuli processing, neuroendocrine, behavioral, and sympathetic/parasympathetic responses (Canteras et al., 1995; Petrovich et al., 2001) with some indirect connections via GABAergic efferents from the BNST (Dong et al., 2001; Polston et al., 2004). As noted in a recent review (Rasia-Filho et al., 2009), male rat MePD activation during mating and the synaptic codification of the MePD output activity to hypothalamic areas would disinhibit brain areas involved with sexual activity (Choi et al., 2005) and modulate intromissions and ejaculation (Coolen et al., 1996; Dominguez and Hull, 2001; de Castilhos et al., 2006). Clusters of neurons medially and laterally located within the MePD are respectively involved with the occurrence of these two male activities (Coolen et al., 1996, 1997). Besides, projections to the entorhinal area and to the postpiriform transitional area would serve as other routes for pheromonal stimuli to affect the hippocampal circuitry and memory formation (Petrovich et al., 2001). From these experimental data that demonstrated multiple demands on MePD cells, it was suggested that local neurons receive "different demands from specific pathways, whose inputs are temporally and spatially integrated within neural

networks, triggering the most appropriate action according to the animal's ongoing situation" (Rasia-Filho, 2006; Rasia-Filho et al., 2012 and references therein). At the cellular level, the dendritic spines are candidates for where to look for plastic synaptic properties in these neural circuits. They are notably affected by sex steroids in adult animals.

Dendritic spine plasticity in the MePD

A new frontier in MePD neuroanatomy began when it was possible to look at individual cells within distinct circuits (Choi et al., 2005). Although dendritic spines were recognized as a cellular component for more than a century (see García-López et al., 2010), they have received much attention recently as an active research field for unraveling neuronal plastic properties. MePD neurons are basically spiny and their spines can be found emerging from cell bodies, in the initial axonal cones or, rather, from dendrites (Rasia-Filho et al., 1999; Brusco et al., 2010; Figures 1-3).

As summarized elsewhere (Rasia-Filho et al., 2010), several lines of evidence suggest that spines are complex, multifunctional, integrative units, and form specialized neuronal postsynaptic compartments with neurotransmitter receptor/ionic channels to alter local dendritic (passive and active) biophysical properties (Shepherd, 1996; Benavides-Piccione et al., 2002; Nimchinsky et al., 2002; Kasai et al., 2003; Tsay and Yuste, 2004). Therefore, the pattern of spacing and the shape of dendritic spines can alter single spine membrane potential or couple voltages among neighboring spines and/or dendritic shafts (Harris and Kater, 1994; Hayashi and Majewska, 2005; Bourne and

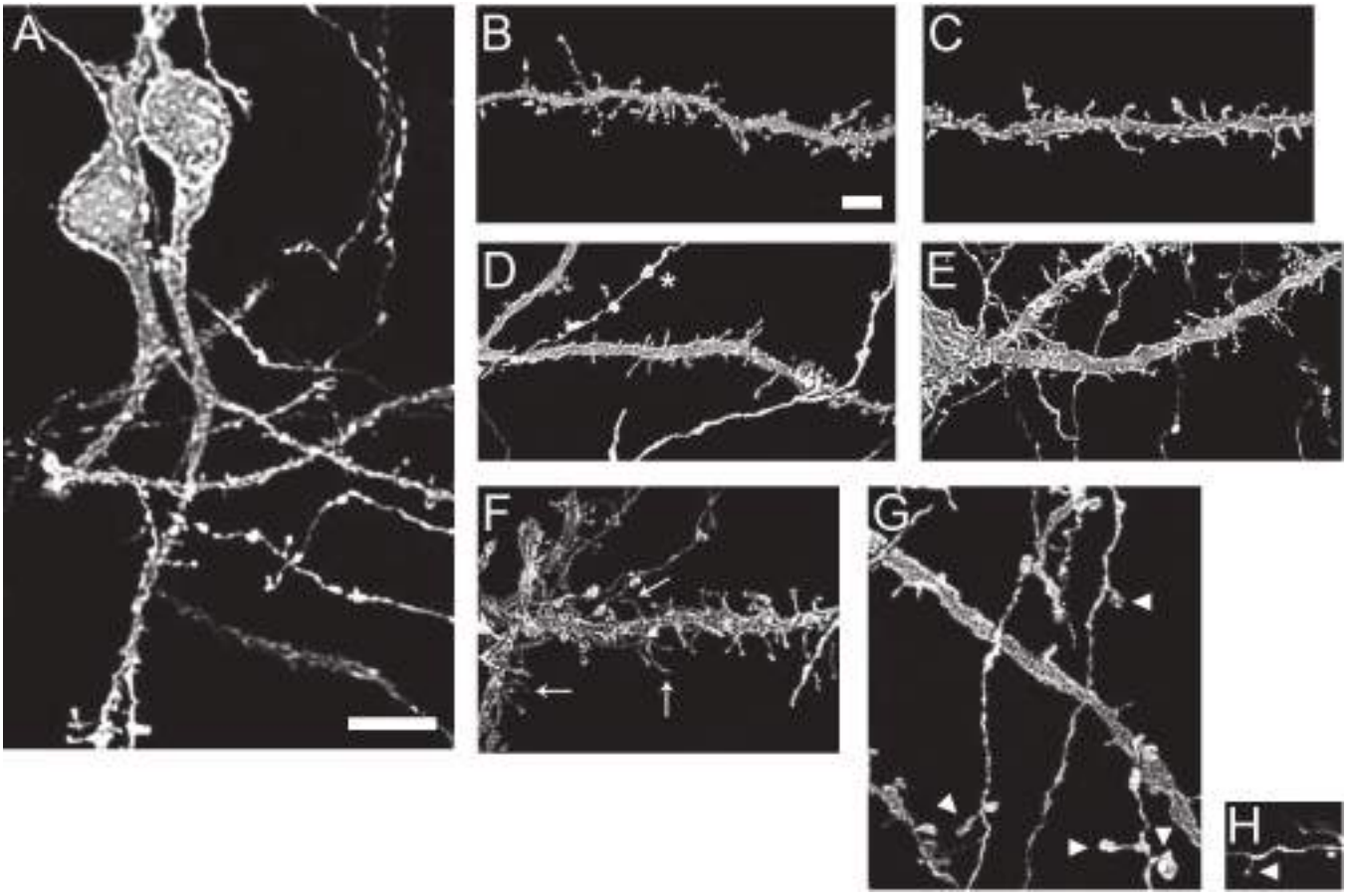

Fig. 3. A. Three-dimensional-reconstructed DiI fluorescent images of the Wistar rat posterodorsal medial amygdala (MePD; coronal brain slices approximately at 3.3 mm posterior to the bregma) neurons under confocal microscopy. **A.** Two multipolar neurons exemplifying the quality of the images obtained with this technique. Note the neuronal components and the presence of dendritic spines. **B-E.** Three-dimensional-reconstructed images at higher magnification to evidence the density and shape of dendritic spines of adult rats. Representative pleomorphic dendritic spines were obtained from the MePD of males **(B)**, females in diestrous **(C),** proestrous **(D)**, and estrous **(E)**. **F.** Note the presence of fillopodium (small arrows) among spines in the dendritic shaft, as well as axons with a regular contour and varicosities (asterisks) or **(G-H)** axonal ramifications with appendages with different shapes (arrow heads). Brightness and background contrast were slightly adjusted (Photoshop CS3 10.0, USA). Bars: A, 10 µm; B-H, 4 µm.

Harris, 2008). This activation can bring about intracellular biochemical cascades with multiple functions, such as to induce and endure long-term electrophysiological changes (Kasai et al., 2003; Segal, 2005). Spines can also serve to establish a biochemical compartmentation to deal with calcium influx, to integrate synaptic function and/or to prevent the increase of ionic concentration to a pathological level during normal synaptic transmission (Segal, 2005). Spines increase membrane surface and the receptive field for the establishment of contacts whose selectivity and functional properties determine the type of synaptically generated electric potential (Harris and Kater, 1994; Nimchinsky et al., 2002; Kasai et al., 2003; but see also Segal, 2010). In effect, few spines are non-synaptic (Arellano et al., 2007), a variable proportion is stable (Zuo et al., 2005), and others can provide new synapses related to changing neuronal inputs (Lendvai et al., 2000; Deng and Dunaevsky, 2005; see also data from Yasumatsu et al., 2008). Spines can show dynamic changes for synaptic plasticity under natural conditions (e.g., along the estrous cycle; Woolley and McEwen, 1992; Brusco et al., 2008) or pathological situations (Campbell et al., 2009). It is likely that changes in spine shape and number may be associated with various cellular processes and have an important functional implication in the synapse-specific implementation of plasticity (Hayashi and Majewska, 2005).

The number and morphology of dendritic spines have been studied using the Golgi method (Ramón y Cajal, 1909; Jones and Powell, 1969; Peters and Kaiserman-Abramof, 1970; Ramón-Moliner, 1970; Valverde, 1970; Fairén et al., 1977; Szentágothai, 1978; Woolley and McEwen, 1992; Dall'Oglio et al., 2010),

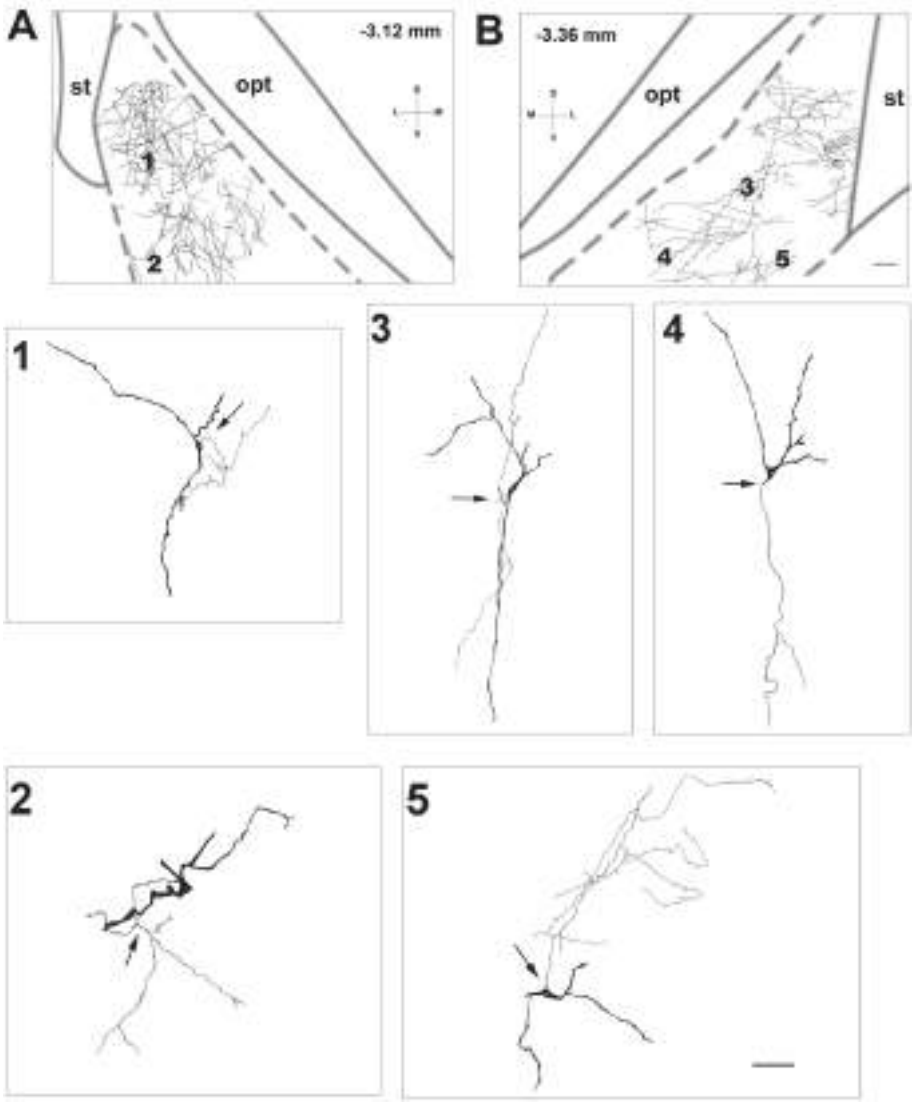

Fig. 4. A,B. Camera lucida drawings of the Golgi-impregnated axonal fibrillar network in the neuropil of the right and left posterodorsal medial amygdala (MePD), close to the optic tract (opt) and stria terminalis (st) in adult male Wistar rats. Images were 3.12 mm and 3.36 mm posterior to the bregma (according to Paxinos and Watson, 1988). Numbers inside the MePD borders represent where the neurons were found. Spatial coordinates are: D, dorsal; L, lateral; M, medial; V, ventral. (1-5) Camera lucida drawings of Golgi-impregnated neurons from the MePD of adult male rats. As a matter of correctness, spine distribution was not drawn for these spiny neurons. Arrows point to axons and their spatial distribution within the MePD. Bars: 50 µm.

intracellular microinjections of Lucifer-Yellow (Duan et al., 2003; Wearne et al., 2005), serial section reconstruction of electron microscopy images (Harris et al., 2006; Arellano et al., 2007), extracellular application of DiI, a diffusible and fluorescent carbocyanine dye, under confocal microscopy (Kim et al., 2007; Rasia-Filho et al., 2009, 2010; Brusco et al., 2010), *in vivo* two-photon imaging (Yang, et al., 2009; Fu and Zuo, 2011) and in cultured central nervous system neurons (Zagrebelsky et al., 2010; Srivastava et al., 2011a,b). Descriptive studies of this highly specialized cellular structure become even more interesting when integrated with the connectional and functional features (Freund and Somogyi, 1983; Izzo et al., 1987; Kisvárday et al., 1990; Benavides-Piccione et al., 2002; Larriva-Sahd, 2008; Lanciego and Wouterlood, 2011; Rasia-Filho et al., 2012) that are needed, for example, to co-ordinate the expression of complex behaviors and timed physiological events. Because of their strategic place to affect somatic voltage, action potential generation, and the electrical/neurochemical output coding for different networks, proximal spines of MePD neurons were counted (Rasia-Filho et al., 2004; de Castilhos et al., 2006, 2008; Marcuzzo et al., 2007; Arpini et al., 2010). Data related to these spines are described below. However, other spine locations along MePD dendrites also provided significant insights about neuronal plasticity (Gomez and Newman, 1991; Cooke and Woolley, 2005; Cunningham et al., 2007). For example, male Sprague Dawley rats castrated before puberty had a reduced dendritic spine number (31-46% lower values than control ones) along the first 70 µm of primary dendrites or along the last 70µm of terminal dendrites, without affecting overall dendritic length or branching of Lucifer Yellow-filled MePD neurons (Cooke and Woolley, 2009). This occurred together with an impaired rate of the typically juvenile rough-and-tumble playful attacks (Cooke and Woolley, 2009). As evidenced using DiI labeling and confocal microscopy, pubertal, gonadally intact Long Evans male rats exposed to testosterone proprionate for 4 weeks had a significant increase (near 67%) in spine number of MePD dendrites at least 20 µm in length and 100 µm from the cell body (Cunningham et al., 2007).

The spine density (i.e., number of spines per unit of dendritic length) in the first 40 µm of dendritic shafts of camera lucida drawing Golgi-impregnated MePD neurons ranged from approximately 2.0 to 2.9 spines/dendritic µm in adult male Wistar rats (Rasia-Filho et al., 2004; de Castilhos et al., 2006, 2008; Marcuzzo et al., 2007; Arpini et al., 2010). Using confocal microscopy and the same rat strain and sex, spine density in proximal branches was (mean ± standard deviation) 1.15±0.67 spines/dendritic µm (Brusco et al., 2010). Methodological differences in the histological preparation, mainly fixation, would account for the discrepant data. As originally depicted, pleomorphic spines were found in the adult Wistar rat MePD, either isolated or in small clusters, notably showing a *continuum* of different shapes and sizes (Rasia-Filho et al., 2004, 2009; Brusco et al., 2010). Spines were classified morphologically as thin, mushroom-like, stubby/wide, ramified, with a gemule appearance or with other transitional aspects (Peters and Kaiserman-Abramof, 1970; Fiala and Harris, 1999; Fig. 2). In the MePD of adult Wistar males, thin spines were the most abundant type (approximately 53%) followed by mushroom-like (22%) and stubby/wide spines (21%); filopodia-like protusions were also found and, together with other less frequent spine shapes, accounted for almost 3% of all sampled spines (Brusco et al., 2010). For comparison between rat brain areas, this proportion of thin spines is basically identical to that found in the stratum radiatum of hippocampal CA1 area (Fiala and Harris, 1999).

As the results in Brusco et al. (2010) indicate, "most of the MePD dendritic spines showed a thin appearance. Two interpretations could be made on spine shape and size modifications by the synaptic signal. One suggests that morphological changes are not required for all forms of synaptic plasticity (see Alvarez and Sabatini, 2007), whereas the other proposes that the diversity of dendritic spines indicates an intrinsic variability for synaptic strength and plasticity (see Arellano et al., 2007). Thin spines are usually more plastic, involve NMDA glutamate receptors, and were named as 'learning spines' (Kasai et al., 2003; Bourne and Harris, 2007). The mushroom-like spines are more stable and make synapses functionally stronger (Kasai et al., 2003; Bourne and Harris, 2007). Stubby spines have a low resistance for the flow of electrical potentials (Korkotian and Segal, 2000)." Ramified spines may enhance the surface available for synaptic contacts but the presence of filopodia is not usual in normal adult brain and might play a role in the establishment of new synapses (Fiala and Harris, 1999; Bhatt et al., 2009). Spinule-like protusions could be involved in structural remodeling and/or intercellular signaling between dendritic spines and its surrounding neuropil (Spacek and Harris, 2004).

Thus, data obtained for the adult male MePD (Brusco et al., 2010) suggest that local "spines can underlie synapses with different plastic capacities, strength, and functional consequences in adult male rats. The dendritic spine geometry would be modeled by synaptic activity to regulate the neuronal function (Hayashi and Majewska, 2005; Schmidt and Eilers, 2009). Dendritic spines can be stable in adulthood (Zuo et al., 2005; Bhatt et al., 2009) but can also show dynamic changes linked with endogenous or environmental influences (Rasia-Filho et al., 2004; Marcuzzo et al., 2007). It is likely that above this basal number, the epigenetic influences of the androgens can increase the dendritic spine density in the MePD. Furthermore, MePD dendrites with stable spines might indicate that neurons can have more steady properties in male-typical neural networks, whereas other modifiable spines would represent an intrinsic plastic capacity for local information processing and behavior modulation.

In this sense, most MePD dendritic spines receive excitatory synapses (Hermel et al., 2006b) and long-term potentiation was described in the MeA (Shindou et al., 1993), which might be related to memory formation in some parallel neural circuits (Petrovich et al., 2001). Prior experiences and associative learning involving the MePD can lead to permanent modifications, at least in the sexual performance of male rats (Stark, 2005)."

However, one must bear in mind what was critically argued by Segal (2010) about dendritic spine plasticity: "… there is an assumption that the size and number of miniature excitatory postsynaptic currents are closely correlated with, respectively, the physical size of synapses and number of spines. However, several recent observations do not conform to these generalizations, necessitating a reassessment of the model: spine dimension and synaptic responses are not always correlated. It is proposed that spines are formed and shaped by ongoing network activity, not necessarily by a 'learning' event, to the extent that, in the absence of such activity, new spines are not formed and existing ones disappear or convert into thin filopodia. In the absence of spines, neurons can still maintain synapses with afferent fibers, which can now terminate on its dendritic shaft. Shaft synapses are likely to produce larger synaptic currents than spine synapses. Following loss of their spines, neurons are less able to cope with the large synaptic inputs impinging on their dendritic shafts, and these inputs may lead to their eventual death. Thus, dendritic spines protect neurons from synaptic activity-induced rises in intracellular calcium concentrations.". These relevant ideas have to be tested in the MePD as well.

To add new data on this issue and to verify the synaptic connectivity of MePD spines, DiI was used concomitantly with the immunolabeling for synaptophysin, a pre-synaptic protein present in contact sites (Fig. 2). This approach proved to be very successful in the rat MePD under confocal microscopy (Rasia-Filho et al., 2010; Brusco et al., 2010). Most spines showed synaptophysin puncta close to its head or neck, whereas other spines had no evident labeled puncta on them or, conversely, multiple puncta appeared on one spine in the adult male rat MePD (Brusco et al., 2010). In adult male Wistar rats, ultrastructural results showed that most contacts (67.5%) were axodendritic, symmetrical and asymmetrical synaptic ones in the MePD neuropil and 92% of them appeared excitatory. Near 23% of all synapses were on dendritic spines, with no inhibitory synapses on them (Hermel et al., 2006b). Although it was suggested that dendritic spines mostly receive an asymmetrical synapse with no dense-cored vesicles (87.5% of the cases; Hermel et al., 2006b), inhibitory and multisynaptic contacts were also found on dendritic spines of the adult male MePD as evidenced in an additional electron microscopy study (Rasia-Filho et al., 2009: Brusco et al., unpublished data). The relevance of this kind of inhibitory contact on spines was hypothesized previously (Marcuzzo et al., 2007). Axospiny inhibitory synapses would account for a small proportion of contacts on MePD neurons. However, some GABAergic terminals and possible inhibitory post-synaptic currents can be dependent on spines (Kisvárday et al., 1990; López-Bendito et al., 2004; Huang et al., 2005). GABA-immunoreactivity occurred in presynaptic terminals on symmetric synapses in the MePD of prepubescent male rats (Cooke and Woolley, 2005). Although the percentage of occurrence of inhibitory spine synapses is small, they actually involve a large amount of contacts per cubic millimeter of brain tissue (Popov and Stewart, 2009). Multisynaptic spines add to the complexity of both the organization and functioning of local synapses as well (Popov et al., 2005). Therefore, the propositions regarding the associations between neurotransmission from the intra-amygdaloid or extra-amygdaloid fibre plexus to MePD dendritic spines have to include the dual possibilities of excitation and inhibition of local neurons in the course of information processing (Rasia-Filho et al., 2004, 2009; Marcuzzo et al., 2007).

Furthermore, the occurrence of "silent synapses" and the proportion of MePD spines that are genetically and functionally developed to be connectionally stable and those spines that are rather labile and plastic remains to be elucidated. One intriguing idea is that MePD dendrites with stable spines may suggest that local neurons can have some steady properties in neural networks, whereas unstable spines would represent an intrinsic plastic capacity for synaptic processing and behavior modulation; in these latter the gonadal hormones would act to modulate spine number and distribution (Rasia-Filho et al., 2009, 2012; Brusco et al., 2010).

In the MePD of adult Wistar rat brains, no statistically significant difference was found in the density of Golgi-impregnated proximal spines in bitufted or stellate neurons (de Castilhos et al., 2006), neither in the percentage of each different shape of dendritic spines in those neurons (Brusco et al., 2010), nor in the dendritic spine density in both the medial or the lateral aspects of the MePD (de Castilhos et al., 2008), which are differently involved with male sexual behavior (Coolen et al., 1996). There was also no difference in dendritic spine density in dorsally- or in ventrally-located neurons of proestrous females MePD (Arpini et al., 2010), in spite of the local heterogenic expression/distribution of ER-α and ER-ß (Gréco et al., 2001). No evidence was found for a left to right hemisphere difference in the density of MePD proximal dendritic spines when comparing data from adult males and diestrous females (Arpini et al., 2010), which contrasts with prepubertal Sprague Dawley data that showed more dendritic shafts and greater branching (Cooke et al., 2007), as well as more asymmetric excitatory synapses on dendritic spines in the left MePD neurons of males than in females (Cooke and Woolley, 2005). Apart from possible strain differences, other disparities between young and adult animals may be due

to increased levels of gonadal hormones during puberty, which may result in the rearrangement of dendrites, dendritic spines and/or other types of synapses in the MePD of both hemispheres. Indeed, there is a dendritic pruning in MeA neurons during pubertal development in male Syrian hamsters (Zehr et al., 2006) and a MePD synaptic reorganization, indicated by an increase in the number of puncta immunoreactive for vesicular glutamate transporter-2 and post-synaptic density 95, two markers of excitatory synapses, in male Siberian hamsters during puberty (Cooke, 2011).

As found originally by Rasia-Filho et al. (2004), the density of proximal dendritic spines in the MePD of adult Wistar rats is sexually dimorphic (higher in males than in proestrous, estrous or metaestrous females) and is affected by the normal fluctuations in plasma ovarian steroids along the estrous cycle (near 35% of reduction during the transition from diestrous to proestrous) or after the occurrence of motherhood (almost 24% lower in postpartum diestrous females than in age-matched virgin diestrous females). The mean spine density in the initial 40 µm of Golgi-impregnated dendritic branches studied under light microscopy ranged from approximately 2.2 spines/dendritic µm in diestrous to 1.35 spines/dendritic µm in the other phases of the estrous cycle (Rasia-Filho et al., 2004). In another set of experiments using DiI and confocal microscopy under the same methodological conditions described previously (Brusco et al., 2010), adult Wistar males had (mean ± standard deviation for proximal dendrites) 1.3±0.3 spines/dendritic µm (n=6 rats, 2.5±1.4 neurons per rat), whereas females had 0.9±0.1 spines/dendritic µm in diestrous (n=4 rats, 4.2±2.6 neurons per rat), 0.6±0.2 spines/dendritic µm in proestrous (n=4 rats, 4.2±1.5 neurons per rat), and 0.6±0.1 spines/dendritic µm in estrous (n=5 rats, 3.8±1.6 neurons per rat; Fig. 3). These data agree with the interpretation of previous Golgi results, i.e., that the number of proximal dendritic spines is different between sexes in rats (Rasia-Filho et al., 2004) and are in accordance with previous confocal data obtained in males (Brusco et al., 2010). In these newly sampled MePD neurons, diestrous females had approximately 51% of thin spines, 31% of stubby/wide, 12% of mushroom-like, and 6% belonging to the other spine shapes. Proestrous females showed near 53% of thin spines, 28% of stubby/wide, 10% of mushroom-like, and 9% belonging to the other spine shapes. Estrous females showed around 47% of thin spines, 34% of stubby/wide, 14% of mushroom-like, and 5% belonging to the other spine shapes. These novel descriptive data indicate that males have more than threefold mushroom-like spines than females along the estrous cycle. Taking into account the putative stability of this kind of spine and the relative percentages of the other shapes along the estrous phases, the present data expand the observations on cyclic synaptic plasticity in the female rat MePD (Rasia-Filho et al., 2004, 2009).

Gonadal steroid withdrawal and replacement hormonal therapies affected MePD dendritic spine density as well. Adult male castration notably reduced dendritic spine density 90 days after testes removal, leaving a lower basal value of stable spines in the MePD at this time (de Castilhos et al., 2008). This finding reinforces the proposition that androgens can affect the MePD neuropil and local synaptic organization, as previously indicated by the reduction in substance P immunoreactivity and MePD volume (Malsbury and McKay, 1994) or dendritic atrophy after castration (Gomez and Newman, 1991). Considering morphology and function, it is interesting to note that the decrease in the MePD spine density 90 days following testes removal coincides temporally with the marked postcastration reduction in ejaculatory and intromission behaviors in rats (Rasia-Filho et al., 1991; Rasia-Filho and Lucion, 1996; de Castilhos et al., 2008). It remains to be determined whether MePD spine reduction following gonadectomy is the cause or the consequence of male sexual behavior impairment.

In adult Wistar female rats, estradiol and progesterone replacement beginning 1 week after castration increased spine number to supra-physiological levels when compared to the normal number of spines during the proestrous phase (Rasia-Filho et al., 2004; de Castilhos et al., 2008). In this experiment, ovariectomized (OVX) females received one of the following treatments: (1) rats that received 2 injections of sesame oil as vehicle (0.1 mL, s.c.), 24 h apart each one, and a third injection of oil 48 h later; (2) rats that received 2 injections of estradiol benzoate (EB) (10 µg/0.1 mL, s.c.), 24 h apart each one, and a third injection of sesame oil 48 h later; and, (3) rats that received 2 injections of estradiol benzoate (10 µg/0.1 mL, s.c.), 24 h apart each one, but the third injection was of progesterone (500 µg/0.1 mL, s.c.) 48 h later (de Castilhos et al., 2008). The proximal dendritic spine density of females treated with EB and progesterone reached statistically significant values when compared to both oil-treated (near 68% more spines) or only EB-treated rats (around 42%; de Castilhos et al., 2008). The higher MePD spine density under the effects of EB and progesterone indicates a complex modulation of spine number by ovarian steroids and their receptors under different experimental or physiological conditions (compare data in Rasia-Filho et al., 2004; de Castilhos et al., 2008; see a parallel discussion in Martinez et al., 2006).

Based on dendritic morphology and spine data acquired until now, it was suggested that adult Wistar rat males and females might be receiving inputs from different spatially-oriented neural pathways (Dall'Oglio et al., 2008a). This might not occur during the pre-pubertal period in Sprague Dawley male rats submitted to gonadectomy (Cooke and Woolley, 2009). In the latter case, spine density did not show distinct changes in one particular set of inputs to the MePD, as evaluated by the general reduction in spines whatever the dendritic projection was (Cooke and Woolley, 2009). Apart from the possible differences due to the rat strain, sample size,

or methodological approach, in adult Wistar rats, MePD dendritic shafts and spines might be receiving inputs from different circuits. That is, there were more dendritic branches oriented dorsolaterally and medially in males, whereas diestrous females had predominant dorsal and ventromedial orientated dendrites (Dall'Oglio et al., 2008a). The medial dendritic orientation suggests that males are rather gathering synaptic inputs from the superficial "molecular layer", where vomeronasal information passes through (Scalia and Winans, 1975; de Olmos et al., 2004; Pro-Sistiaga et al., 2007). Males also have a higher number of dendritic shaft synapses in the medial part of the ventromedially surrounding 'molecular layer' and more spine synapses in its ventral aspect (Nishizuka and Arai, 1983). "On the other hand, morphological (dendritic spine density changes) and biochemical switches (as found for the expression of different neuropeptides) suggest that the female MePD play a relevant role in altering the course of information through interconnected circuitries during the estrous cycle (Oro et al., 1988; Fergunson et al., 2001; Polston et al., 2001; Rasia-Filho et al., 2004; Simerly, 2004). For instance, fewer dendritic spines in the MePD occur during proestrous and estrous females, coincident with a decrease in the number of synapsin reactivity, which could be associated with synaptic pruning (Rasia-Filho et al., 2004; Oberlander and Erskine, 2008). Thus, it was also hypothesized that labile dendritic spines in the MePD, which change their number along the estrous cycle, would serve to modulate phasic synaptic inputs, whereas dendritic shafts would serve to receive more stable afferences (Rasia-Filho et al., 2009). It would be interesting to know whether the synaptic inputs to dendritic spines and shafts are neurochemically different and/or whether they come from different subpopulations of input neurons…" (Rasia-Filho et al., 2012; additional comments in Rasia-Filho et al., 2009).

Another clue for elucidating the plasticity of the MePD neurons is provided by the aspect of the axonal network in the neuropil of this subnucleus. Because dendritic spines usually establish contact with one axon (Hermel et al., 2006b; but see Rasia-Filho et al., 2009; Brusco et al., 2010), the study of the features of the axonal organization in the MePD deserves further consideration. Axonal morphology of the MePD of adult male Wistar rats was studied by the "single-section" Golgi method and its variant (Gabbott and Somogyi, 1984; Izzo et al., 1987; Bolam, 1992) with light microscopy and DiI labeling on confocal microscopy. Different Golgi procedures provided separate possibilities for the evaluation of axonal features and extensions. In our hands, the Golgi procedure developed by Izzo et al. (1987) provided the best results. The spatial distribution of local axons partially resembled the one previously observed in the MePD of mice (Valverde, 1962; Fig. 4). Isolated axons in the MePD neuropil were often observed with both Golgi and DiI procedures. In these cases there were no obvious conditions to classify them as afferences or efferences because the axonal fibrillar pattern of the MePD resulted from these mixed circuits with branchings in various directions (Fig. 4). In this regard, axons might be coming from the ipsilateral MeA subnuclei or other intra-amygdaloid connections (including fibers from the intercalated nuclei or from the cortical ones) or from various extra-amygaloid connections (Nishizuka and Arai, 1983; Canteras et al., 1995; McDonald, 1998; Pitkänen, 2000; Meredith and Westberry, 2004).

In the adult male Wistar rat MePD a single Golgi-impregnated axon per neuron was found emerging from the cell body or, sometimes, from a primary dendrite. Axons had regular contours and they likely represent only the subpopulation of unmyelinated nerve fibers (Lanciego and Wouterlood, 2011). The presence of only an initial axonal cone in well-impregnated MePD neurons (Rasia-Filho et al., 1999; Dall'Oglio et al., 2008a) indicates that the Golgi reaction was impaired by the myelin sheath. The axons usually presented varicosities but the occurrence of non-classical *en passant* synapses related with these varicosities was not studied by electron microscopy until now. Axonal length was variable due to technical reasons. Local axons in coronal sections showed a tortuous course with a fibrillar aspect composed by parallel and oblique fibers in relation to the OT. Some axons were found going dorsally to the ST (Fig. 4). Supposedly, those neurons would be classified as projecting ones, but the end-target of these axons could not be determined with the present approach. Other neurons had axons directed to the medial "molecular layer" or were projected ventrally or provided an apparent innervation to its close surrounding space (Fig. 4). Some neurons appeared to have recurrent axons but their actual existence has to be confirmed. The pattern of ramification of the sampled axons was not very extensive and the number of collaterals was notably fewer than some interneurons in the rat cerebral cortex (Fig. 4). Local axonal morphology, branching and lengths did not allow the reliable classification of MePD cells as interneurons or projecting ones. This issue needs additional effort using electrophysiological recordings. As noted here, there is an interesting research field opened to new and exciting discoveries.

In addition, one morphological aspect of the MePD axons focused our attention: the presence of axonal ramifications along the axon length appearing as pleomorphic protusions from the axon membrane. They appeared as a kind of growth cone restricted to the end of the axon (Fig. 3). The appearance of each protusium under confocal microscopy was usually as a small, thin appendage extending from the parent axon. Varying shapes were observed, including a single fine prolongment that resemble a filopodium, those appearing like "spines" with a neck and a bulbous head or ramified (Fig. 3). They were not as complex as the large end bulbs of Held in the anteroventral cochlear nucleus (Lorente de Nó, 1981; Ryugo and Fekete, 1982). However, these axonal protusions appear similar to terminals found in the accessory olfactory bulb (Larriva-

Sahd, 2008) or the auditory cortex (Szentágothai, 1978), two highly plastic structures in the rat brain. It is unclear whether these axonal appendages may be in the process of forming new local connections or if they had already made mature, stable connections, adding further clues for synaptic processing in the MePD of adult animals. Interestingly, among local spines, filopodia were also observed arising from dendritic shafts of adult rats (Brusco et al., 2010). Based on these data, a working hypothesis regarding the formation and stability of axospiny synapses is as follows: 1) dendritic filiopodia grow to find an axon aiming to form a new contact, 2) an axon sends out a protusium for a new synaptic site, or 3) both phenomena may occur concomitantly (Nimchinsky et al., 2002). The spinogenesis and/or plasticity of dendritic spines may now be linked with the axonal properties in the neuropil of the adult rat MePD. Likewise, astrocytic morphology and function may be integrated in this dynamic scheme as the "third synaptic element" and for which sexually dimorphic or gonadal hormones effects were already depicted in the MePD of rats (Rasia-Filho et al., 2002, 2012; Martinez et al., 2006; Johnson et al., 2008; Morris et al., 2008).

Subcellular effects of sex steroids in the MeA

Because of its profound effects on cellular and synaptic growth, estrogen may be thought of as a trophic factor (Pfaff and Cohen, 1987). These effects may be direct or indirect via second messenger pathways, ultimately resulting in the generation of gene products (Woolley and Cohen, 2002, Scharfman and MacLusky, 2005; Srivastava et al., 2011a,b), which may elicit growth or regulatory effects, such as those for dendritic spines described above. Notably, different responses of the dendritic spines in the MePD to estrogen occur during physiological cyclic fluctuations of sex steroids or after ovariectomy and hormonal replacement, which also contrast with the region-specific effects reported in the VMH or the CA1 hippocampal field in female rats (compare data in Woolley et al. 1990; Calizo and Flanagan-Cato, 2000; Rasia-Filho et al., 2004; Brusco et al., 2008; de Castilhos et al., 2008).

Among the candidates that are likely to mediate estrogen effects on synaptic and cellular growth and neuroprotection, in general, are the cyclic AMP response element-binding protein (CREB)-related gene products, brain-derived neurotrophic factor (BDNF), the anti-apoptotic protein B-cell lymphoma-2 (Bcl-2) and the activity-regulated cytoskeleton-related protein (Arc). Furthermore, an emerging and exciting literature describes some of the rapid effects of estrogen on signal transduction cascades that lead to local protein synthesis (cf. Srivastava et al., 2011a,b for review) and/or rearrangement of the actin-based cytoskeleton (Kramár et al., 2009a,b; see also data in Sekino et al., 2007) and subsequent numerical and morphological alterations in dendritic spines.

CREB may be activated by phosphorylation at Serine-133 by various kinases, including: protein kinase A (PKA), calcium/calmodulin-dependent protein kinase IV (CaMK IV) and ribosome S6 Kinase (RSK) (cf. reviews in Lonze and Ginty, 2002; McClung and Nestler, 2008). Phosphorylated CREB (pCREB) is capable of forming homo- or heterodimers with cyclic AMP responsive modulator protein (CREM) or activating transacting factor (ATF). It can then bind to a cAMP response element (CRE), which consists of the palindromic consensus sequence TGACGTCA of promotor regions of genes (cf. reviews in Lonze and Ginty, 2002; McClung and Nestler, 2008). Along with the relevant co-activators the complex can activate transcription of CREB-related genes, such as those described above.

Previous studies (Rachman et al., 1998) indicated an ameliorative effect of estrogen in the forced swim test, a rodent model for depressive-like behavior. This finding prompted a search for molecular correlates of this action in brain areas implicated in emotional processing, including the amygdaloid nuclei and hippocampal fields. Since CREB is a target of antidepressant action (Duman et al., 1997; cf. also Carlezon et al., 2005), attention was focused on this gene transcription factor in elucidating some of the molecular mechanisms that may be involved in estrogen effects in the forced swim paradigm. Specifically, the effects of estrogen on the CREB signaling pathway, including BDNF and Bcl-2 in the whole MeA were examined. In addition to the aforementioned effects of estrogen on dendritic spines in the MeA, this brain structure was selected for study for several reasons: its relevance to reproduction (Newman, 1999; McDonald, 2003; Rasia-Filho et al., 2012); its abundance of ERs (Simerly et al., 1990; Li et al., 1997; Shughrue et al., 1997, 1998; Laflamme et al., 1998; Österlund et al., 1998; Gréco et al., 2001; Isgor et al., 2002) and its documented involvement in neuroendocrine responses to emotional stress (Dayas et al., 1999; Ebner et al., 2004; Marcuzzo et al., 2007), in which pCREB also appears to play a role (Kuipers et al., 2006). The MeA or its subdivisions have also been shown to be sensitive to steroid hormone manipulations (Nishizuka and Arai, 1981; Wood and Newman, 1995; Gréco et al., 2001; Rasia-Filho et al., 2004; de Castilhos et al., 2008), as described above, or defective androgen receptors (Morris et al., 2005). Our initial biochemical studies employed the whole amygdala because of the difficulty in dissecting out fresh subregions of this brain area. Nevertheless, this approach can provide cues for the subcellular effects of gonadal hormones in the MeA subnuclei as well.

The following section describes some of the effects of estrogen on the CREB signaling pathway, including its actions on CaMK IV, CREB, pCREB and BDNF and Bcl-2 in the MeA of OVX, estrogen-treated female rats. The central amygdala (CeA) served as a control throughout. In addition, the importance of dose and time effects of hormone treatment and a description of some preliminary findings regarding strain-dependent

differences in plasma steroid hormone levels and in pCREB levels in limbic brain areas of intact and OVX female rats are given. Some of the molecular mechanisms of dendritic spine growth in relation to signal transduction cascades and studies relating some of these cascades to sexual dimorphisms are considered. Comparisons to other brain areas, where experimental data is currently available, are needed to provide insights into MeA features and, more specifically, for further experiments aimed at the rat MePD.

Effects of long-term estrogen treatment on CRE-DNA binding, CREB, pCREB and BDNF in the amygdala and integrated areas

First, an effect of long-term estrogen treatment (10μg estradiol benzoate [EB] for 14 days) on CRE-DNA binding activity in neuroanatomical areas related to emotional processing in OVX rats using the gel-mobility shift assay on nuclear protein extracts from the amygdala, hippocampus, frontal cortex and, as a control, the cerebellum was determined (Carlstrom et al., 2001). Hormone treatment of OVX rats over the two week period resulted in an increase in CRE-DNA binding activity in the nuclear extract of amygdala of these rats compared to OVX controls, but not in extracts of hippocampus, frontal cortex, or cerebellum. Acute estrogen treatment (100 μg for one hour), on the other hand, resulted in an increase in CRE-DNA binding activity in the frontal cortex of OVX rats treated with estrogen compared to OVX controls. However, no differences were seen in the whole amygdala, hippocampus or cerebellum using this regimen. Higher CRE-DNA binding was associated with increases in levels of total and pCREB in amygdala during long-term estrogen treatment as seen by quantitative analysis of Western blots (Carlstrom et al., 2001). No differences in these parameters were seen with the acute estrogen treatment. The lack of effect of the long-term estrogen treatment in the hippocampus may be due to masking of the positive signal in the CA1 and CA3 regions, as whole hippocampus was used in these experiments.The importance of this consideration is underscored by studies showing hetereogeneity of dose and time effects of estrogen on neuron-specific neuronal protein (NeuN) and pCREB in the hippocampus of OVX rats, where EB responsiveness to different regimens varies depending on regional specificity of the hippocampus among its subregions and throughout its extent (Bakkum et al., 2011).

To further ascertain the effect of estrogen in the MeA and CeA, immunolabeling for pCREB using immunoperoxidase techniques was performed. Long-term EB treatment resulted in a significant increase in relative total immunolabeled nuclei in the MeAV; no differences were seen in the MePD or MeAD or MePV subdivisions. However, this finding does not preclude an influence of the CREB pathway in these areas. Intraamygdaloid connections or connections from other brain areas into the MePD, for example, can influence local synaptic growth and development within the MePD. Alternatively, the MePD may be sensitive to other estrogen regimens or other complex ER interactions and responses. Moreover, estrogen effects on the CREB signaling pathway in this brain structure may be subject to strain differences and/or sex differences, as discussed below. Estrogen effects on pCREB protein levels and CRE-DNA activity in the amygdala may presage CREB-binding protein (CPB) recruitment and the initiation of transcriptional activity (cf. discussion in Carlstrom et al., 2001).

The aforementioned estrogen regimen, however, did not elicit any changes in basal or cAMP-stimulated activity of protein kinase A (PKA) or in immunolabeling of the α-isoform of the catalytic subunit of PKA (PKAα-C) in the amygdala of OVX rats following EB treatment compared to OVX controls (Carlstrom et al., 2001). Therefore, an alternative path to CREB phosphorylation, i. e., CaMK IV was explored. Upon activation by calcium/calmodulin kinase kinase (CaMKK) CaMK IV can phosphorylate CREB at serine 133. The long-term estrogen regimen increased protein levels of CaMK IV in the nuclear fraction of whole amygdala and in the MeA and basomedial amygdala, but not CeA or basolateral amygdala, by quantitative Western blot analysis and immunogold labeling, respectively (Zhou et al., 2001). CaMK IV may be involved in dendritic spine growth and synaptic connectivity (Soderling, 2000) and may, therefore, mediate estrogen action on dendritic spines in the MeA.

Indeed, some of the studies on immunolabeling in the MeA of OVX rats given long-term estrogen treatment were confirmed using gold immunolabeling (Zhou et al., 2005). Estrogen treatment increased immunolabeling of CREB and pCREB in the MeA and basomedial amygdala, but not CeA or basolateral amygdala (Fig. 5). Also, long-term estrogen treatment increased gold immunolabeling and mRNA levels as seen with *in situ* polymerase chain reaction (PCR) of BDNF in the MeA and basomedial amygdala and CA1 and CA3 regions of the hippocampus, but not in any other amygdaloid or hippocampal regions examined (Zhou et al., 2005)

Dose and time effects of estrogen on the CREB signaling pathway, pCREB and Bcl-2 in the MeA and on behavior

The aforementioned data suggested a neuro-protective effect of estrogen in the MeA. However, estrogen treatment to OVX females led to opposite results on the density of MePD dendritic spines than seen with normal ovarian steroids fluctuations (Rasia-Filho et al., 2004; de Castilhos et al., 2008). That is, a reduced number of spines is found in the MePD concomitant with the physiological elevation of estradiol and progesterone during the proestrous phase, whereas supraphysiological steroid substitutive therapy to OVX females promoted an abnormal increase of spine counts

(compare Rasia-Filho et al., 2004; de Castilhos et al., 2008).Because hormonal regimens and time courses for hormone replacement therapies vary significantly, it is important to address the manner in which different regimens affect the CREB pathway. This issue is underscored by the emergence of low-dose hormone therapies, which have been developed with the assumption that the associated risks are reduced for women (cf. van de Weijer et al., 2007). Also, some animal studies report contradictory findings regarding the effectiveness of different estrogen regimens. Factors that may influence the efficacy of hormone treatments on the brain and/or behaviors include: regimen (Wise et al., 2001; Sohrabji and Lewis, 2006; Walf and Frye, 2006); dose, manner of delivery [continuous or repeated injections at specific intervals, length of treatment; acute versus chronic treatment; cf. Gibbs, 2000)]; length of time of ovariectomy (Singh et al.,1995; Cavus and Duman, 2003; Tanapat et al., 2005; Caruso et al., 2010; Lagunas et al., 2010); age of animals (Diz-Chaves et al., 2012) and/or hormone deprivation (Daniel et al., 2006)

We traditionally used the 10 µg for 14 days estrogen regimen based on previously published data (Rachman et al., 1998; Carlstrom et al., 2001; Zhou et al., 2005) so as to optimize the detection of hormonal action on cellular responses and relate our findings to previously published biochemical, molecular, and immunocytochemical data on estrogen effects on the CREB pathway, including pCREB (Gu et al., 1996; Zhou et al., 1996; Ábrahám et al., 2003; Szegö et al., 2006; cf. review in Rønnekleiv et al., 2007). However, to address some of the aforementioned issues, the effects of EB on neuronal numbers (using neuron-specific protein [NeuN] immunolabeling) and brain region volume in the MeA and CeA using stereology were determined (Fan et al., 2008a). Ovariectomized rats were injected with vehicle for 14 days; 2.5 µg EB for 4 or 14 days; or 10 µg EB for 14 days. NeuN-labeled neuronal number may be related to neuronal survival and upregulation of CREB signaling, therefore the effect of these regimens on levels of pCREB labeling in the MeA and CeA was also tested. The 2.5 µg EB for 14 days regimen increased the mean number of NeuN-labeled neurons and pCREB-labeled cells in the MeA compared to vehicle or 2.5 µg EB for 4 days, indicating that the effect was time-dependent (Fan et al., 2008a). There was also an increase in volume of the MeA with 2.5 µg EB for 14 days compared to vehicle or 2.5 µg EB for 4 days. No differences in these parameters were seen in CeA. These data indicate a neuroanatomical heterogeneity of a time effect of EB on cells expressing NeuN and pCREB in the MeA versus CeA. The time-related increase in pCREB immunolabeling may be due to an increase in the number of cells expressing pCREB or an upregulation of pCREB cells already expressing pCREB. Also, Zhou et al. (2001) showed an estrogen-induced increase in levels of the upstream regulator of CREB, CaMK IV and a decrease in levels of calcineurin (Zhou et al., 2004), a negative regulator of pCREB, in the MeA, but not CeA, of OVX rats with the 10 µg EB for 14 days regimen. Funabashi et al. (1995) have demonstrated a negative effect of proestrous or estrogen on calcineurin mRNA levels in the hypothalamic ventromedial nucleus (VMN), and Sharrow et al. (2002) have also shown a negative effect of proestrous or estrogen on protein levels and activity of calcineurin in the hippocampus.

The EB-induced increase in the mean numbers of NeuN-immunolabled neurons may be due to an increase in neurogenesis, an increase in neurotrophic and/or survival factors, such as BDNF and Bcl-2, respectively, and/or a decrease in neuronal death. Relevant to the MeA, Fowler et al. (2005) noted estrogen effects on neurogenesis in the adult posterior MeA of meadow voles. Carillo et al. (2007) have demonstrated an increase in neuron number in the MeAV in estrous female rats compared to diestrous rats. There was no

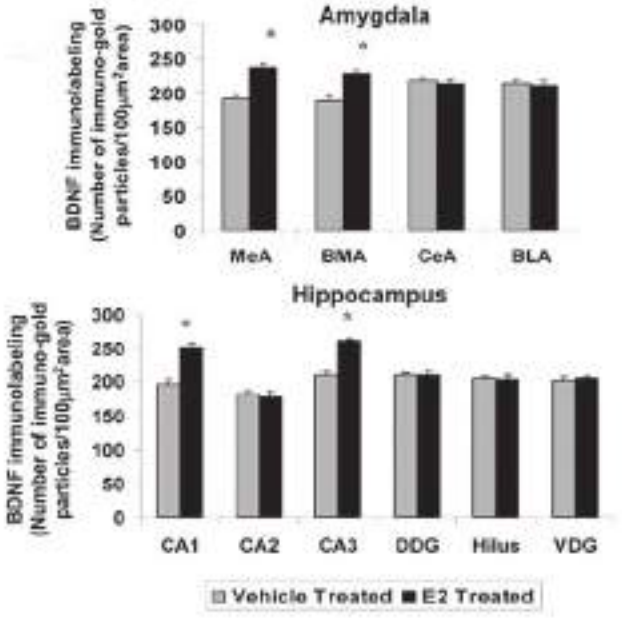

Fig. 5. Quantitation of BDNF immunogold labeling (number of immunogold particles per 100 µm^2 area) shows that estrogen treatment significantly increases levels of BDNF immunolabeling in specific subdivisions of the amygdala (top) and, for comparison, in the hippocampus (bottom). A significant increase in immunogold labeling is seen in the medial (MeA, $p<0.01$) and basomedial (BMA, $p<0.01$), but not central (CeA) or basolateral (BLA) amygdala of the estrogen-treated group (n=6) compared to the ovariectomized (OVX) control animals (n=6). A significant increase in levels of BDNF immunolabeling is seen in the CA1 ($p<0.001$) and CA3 ($p<0.001$) regions of the hippocampus, but not in the CA2, dorsal dentate gyrus (DDG), hilus, or ventral dentate gyrus (VDG) of the estrogen-treated group (n=6) compared to the OVX control (n=6) animals. Values are the mean ± S.E.M. and are represented as the mean OD/pixels of area. * Significantly different from vehicle-treated rats. Reprinted with kind permission from Neuroendocrinology; Zhou J., Zhang H., Cohen R. S. and Pandey S.C. (2005). Effects of estrogen treatment on expression of brain-derived neurotrophic factor and cAMP response element-binding protein expression and phosphorylation in rat amygdaloid and hippocampal structures. Neuroendocrinology 5, 294-310. Copyright Karger.

increase in bromodeoxyuridine or GFAP immunoreactivity, suggesting that an increase in the number of glial cells did not contribute to the increase in cell number. Also, because of the variability in cell number between estrous and diestrous rats, there was a transient sex difference between males and diestrous females.

An EB-induced increase in volume in the MeA, but not CeA, was seen with both of the 14 day EB treatments. Other studies demonstrated sex differences in the volume of unilateral MeA, with the nucleus of the adult male being larger than that seen in females; the difference was evident at postnatal (PN) day 21 (Mizukami et al., 1983). Estrogen increased the volume from PN days 1 to 30 compared to the non-treated female, whereas similar administration of estrogen did not affect the lateral nucleus in a comparable way (Mizukami et al., 1983). After day PN 30, the volume in estrogen-treated females was similar to that seen in the males. Other studies document the maintenance of MePD volume by estradiol following castration of males (Cooke et al., 2003). In the present study, differences in volume among EB regimens may be due to estrogen effects on cell survival and/or an effect of ovariectomy on apoptosis or other forms of degeneration. Several lines of evidence indicate that estrogen maintains soma size (Cooke et al., 2003; Cooke and Woolley, 2005) and synaptic structures in the MeA (Ebner et al., 2004; Cooke and Woolley, 2005; Cooke et al., 2007) and the role of estrogen in neuroprotection is well-documented (cf. reviews in Garcia-Segura et al., 2001; Scott et al., 2012). In this regard, the EB-induced volume changes may be due to a region-specific effect of estrogen on BDNF (Zhou et al., 2005) or Bcl-2 (Fan et al., 2008b) in the MeA, but not CeA.

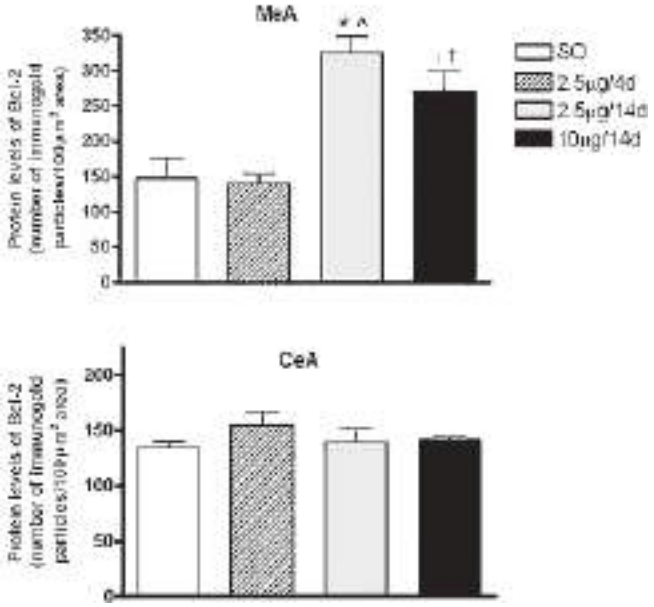

Fig. 6. Quantitation of Bcl-2 immunogold labeling (number of immunogold particles per 100 µm^2 area) in the rat medial amygdala (MeA, top) and central amygdala (CeA, bottom). There was a significant difference among treatment groups in MeA ($F_{3,22}$=15.4, p<0.001). Significant increases in immunolabeling were seen in the 2.5µg estradiol benzoate (EB) for 14 days (2.5 µg/14 days) vs. sesame oil (SO, p<0.001) and 2.5µg EB for 4 days (2.5 µg/4 days, p<0.001) groups and 10 µg estradiol benzoate (EB) for 14 days (10 µg/14 days) vs. SO (p<0.01) and 2.5 µg/4 days (p<0.01) groups. None of the groups displayed differences in gold immunolabeling in the CeA. Values are the mean ± SEM. *p<0.001; !p<0.01 compared with SO; ^p<0.001; †p<0.01 compared with 2.5 µg/4 days. Adapted from Fan L., Pandey S.C. and Cohen R.S. (2008). Estrogen affects levels of Bcl-2 protein and mRNA in medial amygdala of ovariectomized rats. J. Neurosci. Res. 86(16), 3655-3664. Copyright John Wiley and Sons.

The survival factor Bcl-2 is a CREB-related gene product and is implicated in mediating some of estrogen's action on neuroprotection. Therefore, the effects of estrogen on levels of Bcl-2 gold immunolabeling in the MeA and CeA of OVX rats treated with the abovementioned estrogen regimens were determined (Fan et al., 2008b). The 2.5 µg and 10 µg EB for 14 days regimens increased levels of Bcl-2 gold immunolabeling compared with vehicle and 2.5 µg EB for 4 days in MeA, but not CeA (Fig. 6). In addition, Bcl-2 mRNA levels in vehicle and 2.5 µg EB for 14 day groups were determined. There was a significant increase in Bcl-2 mRNA levels in MeA, but not CeA, of EB-treated OVX rats compared with vehicle controls.

Disparate effects of estrogen within the MeA may be due to estrogen effects on classical ERs or membrane ERs, which appear to mediate intracellular signaling pathways and are, also, implicated in cell proliferation, neuroprotection, and growth and survival (Gingerich et al., 2010; Srivastava et al., 2011a). Membrane ERs include: mER-Gαq, the orphan G-protein-coupled receptor, GPR30/GPER1, and the plasma membrane-associated, putative ER-X (cf. Mermelstein and Micevych, 2008 for review). In terms of sex differences, Mermelstein and colleagues have shown that activation of ER-α leads to mGluR1a signaling and phosphorylation of CREB via phospholipase C regulation of MAPK, whereas stimulation of ERα or ERß resulted in mGluR2/3 signaling, with a concomitant decrease in L-type channel-mediated phosphorylation of CREB in cultured female hippocampal pyramidal neurons (Boulware et al., 2005). These bi-directional effects of estrogen were sex-specific (Boulware et al., 2005); that is, age-matched cultures from males did not display an estradiol-induced increase in MAPK-dependent CREB phosphorylation or the estradiol-mediated decrease in CREB phosphorylation following L-type channel activation. The two pathways were dependent on calveolin proteins. Also, cultures from male rats did not display alterations in CRE-dependent transcription following estradiol treatment (Boulware et al., 2007; Mermelstein and Micevych, 2008). These effects appear to be mediated via membrane-localized receptors that stimulated group I and group II metabotropic mGluR signaling (Boulware et al., 2005). The importance of ER subtypes in mediating rapid estrogen effects on signal transduction pathways and subsequent growth and alterations to dendritic spine

morphology has been recently reviewed by Srivastava et al. (2011a,b; see also discussion below).

Implications for the estrogen effects on signaling pathways in the synaptic growth, development, and generation of sexual dimorphisms in the brain

Estrogen's effects on the CREB pathway and CREB-related gene products are likely to effect the survival of neurons and specific circuits and, consequently, estrogen-related behaviors. Growth effects of estrogen on cells and synapses are well-documented (Woolley and Cohen, 2002; Scharfmn and Maclusky, 2005; Srivastava et al., 2011a,b). Some of these effects may be mediated by increased BDNF (cf. Zhou et al., 2005) or survival factors, such as Bcl-2. The trophic effects of estrogen acting via genomic or non-genomic pathways may increase the threshold for stress-related damage, for example (see discussion below).

As mentioned, estrogen has profound effects on dendritic spines in the MeA subnuclei (e.g., Rasia-Filho et al., 2004; de Castilhos et al., 2008), as well as in other brain areas, where some of these actions appear to be mediated by pCREB (Murphy and Segal, 1997; Zhou et al., 2005). For example, estrogen treatment of cultured hippocampal neurons increased dendritic spine density, and this effect was blocked by prior treatment with antisense oligonucleotides against CREB mRNA (Murphy and Segal, 1997).

Another CREB-related gene product relevant to dendritic spine development is Arc. Flanagan-Cato et al. (2006) demonstrated the induction of Arc expression in the ventrolateral subdivision of the VMN by mating, and that the induction was consistent regardless of previous sexual experience. In these studies, there was a reduction in dendritic spines in previously mated animals. Spine density was measured five days following mating, so that the effect of mating is long lasting. However, the paradoxical result of an increased Arc induction with decreased dendritic spine density may be explained by temporal changes in spine density missed after the five day period. Also, the observed changes in spine density may have occurred in neurons other than those displaying Arc induction and/or that Arc induction is not causally related to the later action on spine density (Flangan-Cato et al., 2006). Chamniansawat and Chongthammakun (2010) demonstrated that estrogen rapidly increases the expression of Arc through non-genomic phosphoinositide-3 kinase (PI-3K)-, mitogen-activated protein kinase (MAPK)-, and ER-dependent pathways in SH-SY5Y cells.

In addition to its effect on dendritic spines, estrogen also appears to have an effect on the structure of the postsynaptic density (PSD), the dense area behind the postsynaptic membrane. Early morphological studies showed changes in the length and curvature of PSDs with the long-term regimen of estrogen (10 µg EB for 14 days) in the midbrain central gray of OVX rats (Chung et al., 1998). Also, there was an increase in the number of perforated PSDs, a putative sign of increased synaptic plasticity. Estrogen appears to regulate the expression of the PSD scaffolding protein PSD-95 in the hippocampus. Akama and McEwen (2003) demonstrated that estrogen induced a rapid increase in PSD-95 new protein synthesis in NG108-15 neurons and that this new protein synthesis depends upon Akt (protein kinase B), an intermediate in signal transduction involved in the initiation of protein translation. Waters et al. (2009) showed that ERα- and ERß-specific agonists regulate the expression of (PSD-95) in the stratum radiatum of the hippocampus. Srivastava et al. (2010) also showed that ERß activity increases dendritic spine density and PSD-95 accumulation in membrane regions of synapses.

Recently, studies have implicated the coupling of membrane-associated ERs, such as those associated with dendritic spines, with intracellular signaling pathways in mediating the rapid effects of the neurosteroid estrogen on synaptic proteins, connectivity, and synaptic function in pyramidal neurons (cf. Srivastava et al., 2011a,b for review). The studies indicate that this neurosteroid employs particular signal transduction cascades in disparate brain areas. These characteristics of synapses may form the basis for fine-tuning of neural circuitry and may contribute to differences in circuitry that, in turn, are translated to differences in learned behaviors in males and females, for example. In the model proposed by Srivastava et al. (2011a,b) estrogen affects local protein synthesis in dendritic spines by reducing translational repression with the consequent upregulation of synaptic proteins, such as PSD-95 and GluA1 which in turn may alter dendritic spine structure and the facilitation of long-term potentiation (Srivastava et al., 2011a,b). Rapid effects of estrogen may also involve the subsynaptic cytoskeleton, of which actin is a major constituent. Kramár et al. (2009a,b) have presented a scheme detailing the putative substrates for the rapid effects of estrogen on synaptic function in the adult hippocampus, involving the signaling cascade RhoA, ROCK and LIM-K, which can inactivate cofilin, a blocker of actin filament assembly. In this way estrogen can mediate synaptic structural plasticity and function (Kramár et al., 2009a). Other cascades appear to be triggered by the binding of 17-estradiol to ER-α, which induces phosphorylation of the moesin and WAVE-1 cascade, in turn leading to actin remodeling and actin branching, respectively (Sanchez and Simoncini, 2010). These types of signaling pathways may be involved in the generation of sexual dimorphism of dendritic spines and/or other dimorphic brain structures and is a subject for further investigation in the MePD.

Sexually differentiated intracellular signaling pathways have been implicated in mediation of sex-specific responses to estrogen in the brain (Gillies and McArthur, 2010; cf. also McCarthy et al, 2002; Simerly, 2002; Auger, 2003). Sex differences in CREB phosphorylation were observed in neonatal rat brain (Auger et al., 2001; McCarthy et al., 2002; Auger, 2003). Specifically, male rat pups displayed more pCREB-

immunoreactive positive cells than females in the sexually dimorphic areas of the MPOA, the VMN, the arcuate nucleus, and also in the CA1 region of the hippocampus; similar differences were not seen in two thalamic nuclei, which display little to no gonadal steroid hormone receptors (Auger et al., 2001). No sex differences were seen in the total number of CREB immunoreactive cells. Furthermore, males and testosterone-treated females displayed more pCREB immunolabeling in the VMN compared to female controls. No differences were seen in pCREB immunolabeling in any other of the areas examined. Auger et al. (2001) suggest that some of the effects of testosterone may be mediated by pathways associated with CREB phosphorylation.

Furthermore, upon phosphorylation of CREB on serine 133, coregulatory proteins may be recruited that assist in the transcription of CREB-related gene products. One of these proteins is CBP, which can also function as a nuclear receptor coactivator by interacting with steroid receptor co-activator-1 (Molenda et al., 2002), thereby enhancing steroid receptor action (Shibata et al., 1997) (cf. also Auger, 2003). CBP is also found in the amygdala (Stromberg et al., 1999). Estradiol treatment of neuronal hippocampal cultures increases the expression of CBP within 24 hours (Murphy and Segal, 1997), a time course that is consistent with the time course for estradiol-induced increases in dendritic spines. Auger et al. (2002) showed sex differences in CBP expression in neonatal rat brain, with males expressing higher levels of CBP within the VMN, MPOA, and arcuate nucleus. Infusion of antisense oligodeoxynuleotides to CBP into the hypothalamus of neonatal rats interfered with the defeminizing, but not masculinizing, effects of testosterone, suggesting that CBP expression in the developing rat brain is sexually dimorphic (Auger et al., 2002).

Auger et al. (2001; cf. McCarthy et al., 2002 and Auger, 2003, for reviews) also reported that changes from excitatory versus inhibitory GABA represent a pivotal point in steroid-mediated sexual differentiation of the brain. There appears to be an heterogeneity in responses to GABA with regard to excitatory or inhibitory on pathways that impact CREB phosphorylation depending on sex and region of the developing brain (Auger, 2003). That is, depending upon sex and brain area GABA can be excitatory or inhibitory on signal cascades associated with CREB phosphorylation. Whereas GABA is primarily inhibitory in the adult brain, GABA has excitatory effects during development (Cherubini et al., 1991). The timing of the shift from depolarizing to hyperpolarizing is brain-region specific; for example, it is relatively early in the sexually dimorphic hypothalamus (McCarthy et al., 2002). Elevated testosterone levels are aromatized to estradiol, which is responsible for many of the features of the masculine brain. Relative to the present studies, GABA-mediated sex differences in CREB phosphorylation may lead to differences in the transcription of CREB-related gene products, such as BDNF (Berninger et al., 1995; cf. also Auger, 2003, for references). Neurotrophic factors may then impact other processes, including synaptogenesis, which differentiates male and female brains (cf. McCarthy et al., 2002; Auger, 2003). However, the disparate effect of estrogen on CREB signaling cascades in male and female brains is not restricted to the neonatal period. For example, ÁAhám and Herbison (2005) noted major sex differences in rapid, non-genomic effects of estrogen on pCREB immunoreactivity in adult, gonadectomized mouse brain.

In addition, Garcia-Segura and colleagues have demonstated the importance of astrocytes in mediating estrogen effects on synaptic plasticity in the brain (Garcia-Segura et al., 1999; cf. also Chowen et al., 2000; Garcia-Segura and McCarthy, 2004). McCarthy and colleagues have presented studies underscoring the importance of immature astrocytes, which are responsive to estradiol and play a role in the establishment of sex differences in synaptic patterns in the arcuate nucleus. Arcuate astrocytes appear more complex, with an increased number of primary, secondary and tertiary processes (Mong et al., 2002), a phenomenon mediated by estrogen upregulation of glutamic decarboxylase (GAD), thereby increasing the synthesis of GABA (Davis et al., 1996). The increased structural changes in astrocytes are inversely correlated with dendritic spine development, which also exhibit increased density on neurons of female rat pups compared to those seen on neuons of males (Mong et al., 2001). The MePD shows differences in glial number and complexity in male and female rats (Rasia-Filho et al., 2002; Martinez et al., 2006; Johnson et al., 2008; Morris et al., 2008). There is an increase in GFAP immunoreactivity in proestrous females concomitant with a decrease in dendritic spines in the MePD (Rasia-Filho et al., 2004; Martinez et al., 2006). It is therefore of interest to investigate the involvement of local glial cells and GABA in cellular and synaptic organization of the MePD, a line of research that remains open to further contributions (see parallel comments in Perea et al., 2009; Faissner et al., 2010; Halassa and Haydon, 2010).

Other studies of sexual differences in behavior as they relate to levels of CREB, pCREB and BDNF, come from the laboratory of Lin et al. (2009), who examined levels of these molecules in stress-related areas of the brain (for CREB and pCREB levels: CA1, CA2 and CA3 regions of the hippocampus, paraventricular nucleus of the thalamus, amygdala, anterior cingulate area, dorsal part and infralimbic area of the prefrontal cortex; for BDNF levels: the dentate gyrus and prelimbic area of the prefrontal cortex) of male and female rats following stress recovery. Stress resulted in decreased levels of pCREB in male CA1, CA2 and CA3 regions, paraventricular nucleus, amygdala and dorsal part of the prefrontal cortex and CREB levels in CA2, but these molecular alterations were not seen in females.

The aforementioned data on estrogen-induced

changes in levels of components of the CREB signaling pathway suggest that these may be translated into behavior alterations. The percent of estrogen-induced increases in protein levels of CREB, pCREB, BDNF and mRNA levels of BDNF in the MeA displays a range of approximately 24 to 30%. Changes in pCREB levels of this magnitude either in alcohol-withdrawn rats following chronic alcohol administration or pharmacological manipulations of CREB phosphorylation in the CeA resulted in alterations in their behavior in the elevated plus maze, a test for anxiety-like behavior (Pandey et al., 2003). That is, the rats displayed a decrease in pCREB and a concommittant reduction in open arm activity in the elevated plus maze. Similarly, a reduction in protein levels of BDNF in the amygdala and hippocampal structures of CREB haplodeficient mice resulted in displays of depressive- and anxiety-like behaviors (Pandey et al., 2004). These data open new experimental possibilities to link subcellular, morphological and functional effects on the MeA subnuclei modulation of social behaviors. Experiments should involve females in addition to males, as usually used (see Rasia-Filho et al., 2012).

In effect, studies focusing on sex differences in signal transduction pathways provide fruitful avenues for unraveling the molecular and cellular bases for sexual dimorphisms in brain structure, such as those seen in dendritic spines and neuronal densities described above. These investigations may provide insight into the mechanisms that underlie differences in behavior between males and females in animal studies and in the clinical arena. Because of its well-documented role in synaptogenesis, dendritic spine formation and neuroprotection, the CREB signaling pathway appears be one of the likely candidates that generate some of these differences.

Strain differences in estrogen effects on CREB and pCREB

Finally, data from other laboratories indicate strain-dependent differences in response to some behavioral paradigms in rats (cf. Einat, 2007 for review; cf. also O'Mahony et al., 2011). To determine if plasma steroid hormone and limbic brain pCREB levels are different between strains in intact and OVX rats, estrogen, testosterone, progesterone, adreno-corticotrophic hormone (ACTH), and corticosterone levels were measured using radioimmuinoassays (RIAs) in Sprague Dawley and Wistar rats. Phosphorylated CREB levels were determined using immunogold labeling and densitometric image analysis in intact Sprague Dawley and Wistar rats. In intact rats, there was a significant increase in plasma estrogen, ACTH, and corticosterone levels in Sprague Dawley compared to Wistar rats; no differences were seen in plasma progesterone or testosterone levels between the strains (Hanbury, Pandey and Cohen, unpublished observations). In OVX rats, there were no significant differences in plasma estrogen, progesterone, testosterone or corticosterone between the strains (Hanbury, Fan, Pandey and Cohen, unpublished observations). There were significant increases in pCREB levels in intact Sprague Dawley compared to Wistar rats in the CA1, CA3 and dentate gyrus of the hippocampus, the MeA and CeA, or shell and core of the nucleus accumbens (Hanbury, Fan, Pandey and Cohen, unpublished observations). These data suggest that plasma steroid hormone and limbic pCREB levels may predispose different rat strains to disparate behaviors (cf. Einat, 2007 for review; cf. also O'Mahony et al., 2011). Moreover, these data reinforce observations that apparently discrepant results can be obtained in different rat strains even when using similar methodologies. This appeared to occur and was commented for some MePD morphological findings in the last years (e.g., Hermel et al., 2006b).

Conclusions

The adult rat MePD is a relevant area for investigating profound effects of sex steroids in the rat brain. Various morphological findings support its sexual dimorphisms and effects of sex steroids in adulthood. Alterations in dendritic spine number and morphology are examples of hormonal influences on synaptic structure and differ in males and females, the phases of the estrous cycle, or the effects supra-physiological hormonal replacement therapies. The shape of dendritic spines, the presence and aspect of dendritic fillopodium and axonal protusium in the MePD neuropil of adult animals are relevant for synaptic strength and reinforce the evidence for local cellular plasticity. Subcellular effects of estrogen in the MeA include the transcription of CREB-related gene products, such as, BDNF, Bcl-2, and Arc, which in turn affect synaptic and cellular growth and neuroprotection. Hormonal actions on various signal transduction cascades, and local protein synthesis may affect the neuronal and dendritic spine cytoskeleton and function. Various working hypotheses are raised from these experimental data. Taken together, they provide additional and exciting insights about the modulatory actions of gonadal hormones in a rat forebrain area and in integrated brain circuits relevant for reproduction and other social behaviors.

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References

Ábrahám I.M., Han S.K., Todman M.G., Korach K.S. and Herbison A.E. (2003). Estrogen receptor beta mediates rapid estrogen actions on

gonadotropin-releasing hormone neurons in vivo. J. Neurosci. 13, 5771-5777.

Ábrahám I.M. and Herbison A.E. (2005). Major sex differences in non-genomic estrogen actions on intracellular signaling in mouse brain in vivo. Neuroscience 131, 945-951.

Akama K.T. and McEwen B.S. (2003). Estrogen stimulates postsynaptic density-95 rapid protein synthesis via the Akt/protein kinase B pathway. J. Neurosci. 23, 2333-2339.

Akhmadeev A.V. (2008). Cytoarchitectonics, neuronal organization, and the effects of gender on the dendroarchitectonics of neurons in the posterior medial nucleus of the amygdaloid body in rats. Neurosci. Behav. Physiol. 38, 901-905.

Alheid G.F. (2003). Extended amygdala and basal forebrain. Ann. NY Acad. Sci. 985, 185-205.

Alheid G. F., de Olmos J. S. and Beltramino C. A. (1995). Amygdala and extended amygdala. In: The rat nervous system. Paxinos G. (ed). Academic Press. San Diego. pp 495-578.

Alvarez V.A. and Sabatini B.L. (2007). Anatomical and physiological plasticity of dendritic spines. Annu. Rev. Neurosci. 30, 79-97.

Arellano J.I., Espinosa A., Fairén A., Yuste R. and DeFelipe J. (2007). Non-synaptic dendritic spines in neocortex. Neuroscience 145, 464-469.

Arpini M., Menezes I.C., Dall'Oglio A. and Rasia-Filho A.A. (2010). The density of Golgi-impregnated dendritic spines from adult rat posterodorsal medial amygdala neurons displays no evidence of hemispheric or dorsal/ventral differences. Neurosci. Lett. 469, 209-213.

Auger A.P. (2003). Sex differences in the developing brain: crossroads in the phosphorylation of cAMP response element binding protein. J. Neuroendocrinol. 15, 622-627.

Auger A.P., Perrot-Sinal T.S. and McCarthy M.M. (2001). Excitatory versus inhibitory GABA as a divergence point in steroid-mediated sexual differentiation of the brain. Proc. Natl. Acad. Sci. USA 98, 8059-8064.

Auger A.P., Perrot-Sinal T.S., Auger C.J., Ekas L.A., Tetel M.J. and McCarthy M.M. (2002). Expression of the nuclear receptor coactivator, cAMP response element-binding protein, is sexually dimorphic and modulates sexual differentiation of neonatal rat brain. Endocrinology 143, 3009-3016.

Bakkum B.W., Fan L., Pandey S.C. and Cohen R.S. (2011). Heterogeneity of dose and time effects of estrogen on neuron-specific neuronal protein and phosphorylated cAMP response element-binding protein in the hippocampus of ovariectomized rats. J. Neurosci. Res. 89, 883-897.

Bhatt D.H., Zhang S. and Gan W-B. (2009). Dendritic spine dynamics. Annu. Rev. Physiol. 71, 261-282.

Benavides-Piccione R., Ballesteros-Yáñez I., DeFelipe J. and Yuste R. (2002). Cortical area and species differences in dendritic spine morphology. J. Neurocytol. 31, 337-346.

Bennur S., Shankaranarayana Rao B.S., Pawlak R., Strickland S., McEwen B.S. and Chattarji S. (2007). Stress-induced spine loss in the medial amygdala is mediated by tissue-plasminogen activator. Neuroscience 144, 8-16.

Berninger B., Marty S., Zafra F., da Penha Berzaghi M., Thoenen H. and Lindholm D. (1995). GABAergic stimulation switches from enhancing to repressing BDNF expression in rat hippocampal neurons during maturation in vitro. Development 121, 2327-2335.

Bian X., Yanagawa Y., Chen W.R. and Luo M. (2008). Cortical-like functional organization of the pheromone-processing circuits in the medial amygdala. J. Neurophysiol. 99, 77-86.

Blake C.B. and Meredith M. (2011). Change in number and activation of androgen receptor-immunoreactive cells in the medial amygdala in response to chemosensory input. Neuroscience 190, 228-238.

Bolam J.P. (1992). Experimental neuroanatomy: a practical approach. Oxford University Press. New York. pp 296.

Boulware M.I., Weick J.P., Becklund B.R., Kuo S.P., Groth RD. and Mermelstein P.G. (2005). Estradiol activates group I and II metabotropic glutamate receptor signaling, leading to opposing influences on cAMP response-element binding protein. J. Neurosci. 25, 5066-5078.

Boulware M.I., Kordasiewicz H. and Mermelstein P.G. (2007). Calveolin proteins are essential for distinct effects of membrane estrogen receptors in neurons. J. Neurosci. 27, 9941-9950.

Bourne J.N. and Harris K.M. (2007). Do thin spines learn to be mushroom spines that remember? Curr. Op. Neurobiol. 17, 381-386.

Bourne J.N. and Harris K.M. (2008). Balancing structure and function at hippocampal dendritic spines. Annu. Rev. Neurosci. 31, 47-67.

Brodal A. (1981). Neurological anatomy. Oxford University Press, New York.

Brusco J., Wittmann R., de Azevedo M.S., Lucion A.B., Franci C.R., Giovenardi M. and Rasia-Filho A.A. (2008). Plasma hormonal profiles and dendritic spine density and morphology in the hippocampal CA1 stratum radiatum, evidenced by light microscopy, of virgin and postpartum female rats. Neurosci. Lett. 438, 346-350.

Brusco J., Dall'Oglio A., Rocha L.B., Rossi M.A., Moreira J.E. and Rasia-Filho A.A. (2010). Descriptive findings on the morphology of dendritic spines in the rat medial amygdala. Neurosci. Lett. 483, 152-156.

Bupesh M., Legaz I., Abellán A. and Medina L. (2011). Multiple telencephalic and extratelencephalic embryonic domains contribute neurons to the medial extended amygdala. J. Comp. Neurol. 519, 1505-1525.

Calizo L.H. and Flanagan-Cato L.M. (2000). Estrogen selectively regulates spine density within the dendritic arbor of rat ventromedial hypothalamic neurons. J. Neurosci. 20, 1589-1596.

Campbell J.N., Kurz J.E. and Churn S.B. (2009). Pathological remodeling of dendritic spines. In: Dendritic spines: Biochemistry, modeling and properties. Baylog L.R. (ed). Nova Science Publishers. Hauppauge. pp 45-65.

Canteras N.S., Simerly R.B. and Swanson L.W. (1995). Organization of projections from the medial nucleus of the amygdala: a PHAL study in the rat. J. Comp. Neurol. 360, 213-245.

Carillo B., Pinos H., Guillamon A., Panzica G. and Collado P. (2007). Morphometrical and neurochemical changes in the anteroventral subdivision of the rat medial amygdala during estrous cycle. Brain Res. 1150, 83-93.

Carlezon W.A. Jr, Duman R.S. and Nestler E.J. (2005). The many faces of CREB. Trends Neurosci. 28, 436-445.

Carlstrom L., Ke Z.J., Unnerstall J.R., Cohen R.S. and Pandey S.C. (2001). Estrogen modulation of the cAMP response element-binding protein pathway. Effects of long-term and acute treatments. Neuroendocrinololgy 4, 227-243.

Caruso D., Pesaresi M., Maschi O., Giatti S., Garcia-Segura L.M. and Melcangi R.C. (2010). Effect of short-and long-term gonadectomy on neuroactive steroid levels in the central and peripheral nervous system of male and female rats. J. Neuroendocrinol. 22,1137-1147.

Cavus I. and Duman R.S. (2003). Influence of estradiol, stress, and 5-HT2A agonist treatment on brain-derived neurotrophic factor

expression in female rats. Biol. Psychiatry 54, 59-69.

Chamniansawat S. and Chongthammakun S. (2010). Genomic and non-genomic actions of estrogen on synaptic plasticity in SH-SY5Y cells. Neurosci Lett. 470, 49-54.

Chareyron L.J., Lavenex P.B., Amaral D.G. and Lavenex P. (2011). Stereological analysis of the rat and monkey amygdala. J. Comp. Neurol. 519, 3218-3239.

Cherubini E., Gaiarsa J.L., Bem-Ari Y. (1991). GABA: an excitatory transmitter in early postnatal life. Trends Neurosci. 14, 515-519.

Choi G.B., Dong H-W., Murphy A.J., Valenzuela D.M., Yancopoulos G.D., Swanson L.W. and Anderson D.J. (2005). Lhx6 delineates a pathway mediating innate reproductive behaviors from the amygdala to the hypothalamus. Neuron 46, 647-660.

Chowen J.A., Azcoitia I., Cardona-Gomez G.P. and Garcia-Segura L.M. 2000. Sex steroids and the brain: lessons from animal studies. J. Pediatr. Endocrinol. Metabol. 13, 1045-1066.

Chung S.K., Pfaff D.W. and Cohen R.S. (1998). Estrogen-induced alterations in synaptic morphology in the midbrain central gray. Exp. Brain. Res. 69, 522-30.

Cooke B.M. (2011). Synaptic reorganization of the medial amygdala during puberty. J. Neuroendocrinol. 23, 65-73.

Cooke B.M. and Simerly R.B. (2005). Ontogeny of bidirectional connections between the medial nucleus of the amygdala and the principal bed nucleus of the stria terminalis in the rat. J. Comp Neurol. 489, 42-58.

Cooke B.M. and Woolley C.S. (2005). Sexually dimorphic synaptic organization of the medial amygdala. J. Neurosci. 25, 10759-10767.

Cooke B.M. and Woolley C.S. (2009). Effects of prepubertal gonadectomy on a male-typical behavior and excitatory synaptic transmission in the amygdala. Dev. Neurobiol. 69, 141-152.

Cooke B.M., Breedlove S.M. and Jordan C.L. (2003). Both estrogen receptors and androgen receptors contribute to testosterone-induced changes in the morphology of the medial amygdala and sexual arousal in male rats. Horm. Behav. 2, 336-246.

Cooke B.M., Stokas M.R. and Woolley C S. (2007). Morphological sex differences and laterality in the prepubertal medial amygdala. J. Comp. Neurol. 6, 904-915.

Coolen L.M. and Wood R.I. (1999). Testosterone stimulation of the medial preoptic area and medial amygdala in the control of male hamster sexual behavior: redundancy without amplification. Behav. Brain Res. 98, 143-153.

Coolen L.M., Peters H.J.P.W. and Veening J.G. (1996). Fos immunoreactivity in the rat brain following consummatory elements of sexual behavior: a sex comparison. Brain Res. 738, 67-82.

Coolen L.M., Peters H.J.P.W. and Veening J.G. (1997). Distribution of Fos immunoreactivity following mating versus anogenital investigation in the male rat brain. Neuroscience 77, 1151-1161.

Cunningham R.L., Clairborne B.J. and McGinnis, M.Y. (2007). Pubertal exposure to anabolic androgenic steroids increases spine densities on neurons in the limbic system of male rats. Neuroscience 150, 609-615.

Dall'Oglio A., Gehlen G., Achaval M. and Rasia-Filho A.A. (2008a). Dendritic branching features of posterodorsal medial amygdala neurons of adult male and female rats: further data based on the Golgi method. Neurosci. Lett. 430, 151-156.

Dall'Oglio A., Gehlen G., Achaval M. and Rasia-Filho A.A. (2008b). Dendritic branching features of Golgi-impregnated neurons from the "ventral" medial amygdala subnuclei of adult male and female rats. Neurosci Lett. 439, 287-292.

Dall'Oglio A., Ferme D., Brusco J., Moreira J.E. and Rasia-Filho A.A. (2010). The "single-section" Golgi method adapted for formalin-fixed human brain and light microscopy. J. Neurosci. Meth. 189, 51-55.

Daniel J.M., Hulst J.L. and Berbling J.L. (2006). Estradiol replacement enhances working memory in middle-aged rats when initiated immediately after ovariectomy but not after a long-term period of ovarian hormone deprivation. Endocrinology 47, 607-614.

Davis A.M., Grattan D.R., Selmanoff M.K. and McCarthy M.M. (1996). Sex differences in glutamic acid decarboxylase mRNA in neonatal rat brain: implications for sexual differentiation. Horm. Behav. 30, 538-552.

Dayas C.V., Buller K.M. and Day T.A. (1999). Neuroendocrine responses to an emotional stressor: evidence for involvement of the medial but not the central amygdala. Eur. J. Neurosci. 11, 2312-2322.

de Castilhos J., Marcuzzo S., Forti C.D., Frey RM., Stein D., Achaval M. and Rasia-Filho A.A. (2006). Further studies on the rat posterodorsal medial amygdala: dendritic spine density and effect of 8-OH-DPAT microinjection on male sexual behavior. Brain Res. Bull. 69, 131-139.

de Castilhos J., Forti C.D., Achaval M. and Rasia-Filho A.A. (2008). Dendritic spine density of posterodorsal medial amygdala neurons can be affected by gonadectomy and sex steroid manipulations in adult rats: a Golgi study. Brain Res. 1240, 73-81.

de Olmos J.S., Alheid G.F. and Beltramino C.A. (1985). Amygdala. In: The rat nervous system. Paxinos G. (ed). Academic Press. Sydney. pp 223-334.

de Olmos J.S., Beltramino C.A. and Alheid G. (2004). Amygdala and extended amygdala of the rat: a cytoarchitectonical, fibroarchitectonical, and chemoarchitectonical survey. In: The rat nervous system. Paxinos G. (ed). Elsevier Academic Press. London. pp 509-603.

De Jonge FH., Oldenburger WP., Louwerse AL. and Van de Poll N.E. (1992). Changes in male copulatory behavior after sexual exciting stimuli: effects of medial amygdala lesions. Physiol. Behav. 52, 327-332.

De Vries G.J. and Simerly R.B. (2002). Anatomy, development, and function of sexually dimorphic neural circuits in the mammalian brain. In: Hormones, brain and behavior. Pfaff D.W., Arnold A.P., Etgen A.M., Fahrbach S.E. and Rubin R.T. (eds). Academic Press. San Diego. pp 137-191.

Deng J. and Dunaevsky A. (2005). Dynamics of dendritic spines and their afferent terminals: spines are more motile than presynaptic boutons. Dev. Biol. 277, 366-377.

Dhungel S., Urakawa S., Kondo Y. and Sakuma Y. (2011). Olfactory preference in the male rat depends on multiple chemosensory inputs converging on the preoptic area. Horm. Behav. 59, 193-199.

Diz-Chaves Y., Kwiatkowska-Naqvi A., Von Hülst H., Pernia O., Carrero P. and Garcia-Segura L.M. (2012). Behabioral effects of estradiol therapy in ovariectomized rats depend on the age when the treatment is initiated. Exp. Gerontol. 47, 93-99.

Dominguez J.M. and Hull E.M. (2001). Stimulation of the medial amygdala enhances medial preoptic dopamine release: Implications for male rat sexual behavior. Brain Res. 917, 225-229.

Dong H.-W., Petrovich G. and Swanson L.W. (2001). Topography of projections from amygdala to bed nuclei of the stria terminalis. Brain Res. Rev. 38, 192-246.

Duan H., Wearne S.L., Rocher A.B., Macedo A., Morrison J.H. and Hof P.R. (2003). Age-related dendritic and spine changes in

corticocortically projecting neurons in macaque monkeys. Cerebral Cortex 13, 950–961.

Duman R.S., Heninger G.R. and Nestler E.J. (1997). A molecular and cellular theory of depression. Arch. Gen. Psychiatry 54, 597-606.

Ebner K., Rupniak N M., Saria A. and Singewald N. (2004). Substance P in the medial amygdala: emotional stress-sensitive release and modulation of anxiety-related behavior in rats. Proc. Natl. Acad. Sci. USA 101, 4280-4285.

Einat H. (2007). Different behaviors and different strains: potential new ways to model bipolar disorder. Neurosci. Biobehav. Rev. 31, 850-857.

Fairén A., Peters A. and Saldanha J. (1977). A new procedure for examining Golgi impregnated neurons by light and electron microscopy. J. Neurocytol. 6, 311-337.

Faissner A., Pyka M., Geissler M., Sobik T., Frishknecht R., Gundelfinger E.D. and Seidenbecher C. (2010). Contributions of astrocytes to synapse formation and maturation- Potential functions of the perisynaptic extracellular matrix. Brain Res. Rev. 63, 26-38.

Fan L., Hanbury R., Pandey S.C. and Cohen R.S. (2008a). Dose and time effects of estrogen on expression of neuron-specific protein and cyclic AMP response element-binding protein and brain region volume in the medial amygdala of ovariectomized rats. Neuroendocrinology 88, 111-126.

Fan L., Pandey S.C. and Cohen R.S. (2008b). Estrogen affects levels of Bcl-2 protein and mRNA in medial amygdala of ovariectomized rats. J. Neurosci. Res. 86, 3655-3664.

Fergunson J.N., Aldag J.M., Insel T.R. and Young L.J. (2001). Oxytocin in the medial amygdala is essential for social recognition in the mouse. J. Neurosci. 21, 8278-8285.

Fiala J.C. and Harris K.M. (1999). Dendrite structure. In: Dendrites. Stuart G., Sprutson N. and Häusser M. (eds). Oxford University Press. New York. pp 1-34.

Flanagan-Cato L.M., Calizo L.H., Griffin G.D., Lee B.J. and Whisner S.Y. (2006). Sexual behaviour induces the expression of activity-regulated cytoskeletal protein and modifies neuronal morphology in the female rat ventromedial hypothalamus. J. Neuroendocrinol. 18, 857-64.

Fleming A.S., Vaccarino F. and Luebke C. (1980). Amygdaloid inhibition of maternal behavior in the nulliparous female rat. Physiol. Behav. 25, 731-743.

Fowler C.D., Johnson F. and Wang Z. (2005). Estrogen regulation of cell proliferation and distribution of estrogen receptor-alpha in the brains of adult female prarie and meadow voles. J. Comp. Neurol. 2, 166-179.

Freund T.F. and Somogyi P. (1983). The Section-Golgi impregnation procedure. 1. Description of the method and its combination with histochemistry after intracellular ionophoresis or retrograde transport of horseradish peroxidase. Neuroscience 9, 463-474.

Fu M. and Zuo Y. (2011). Experience-dependent structural plasticity in the cortex. Trends Neurosci. 34, 177-187.

Funabashi T., Brooks P.J., Kleopoulos S.P., Gandison L., Mobbs C.V. and Pfaff D.W. (1995). Changes in preproenkephalin messenger RNA levels in the rat ventromedial hypothalamus during the estrous cycle. Brain Res. Mol. Bain Res. 1, 129-134.

Gabbott P.L. and Somogyi J. (1984). The 'single' section Golgi-impregnation procedure: methodological description. J Neurosci. Methods 11, 221-230.

García-López M., Abellán A., Legaz I., Rubenstein J.L.R., Puelles L. and Medina L. (2008). Histogenetic compartments of the mouse centromedial and extended amygdala base on gene expression patterns during development. J. Comp. Neurol. 506, 46-74.

García-López P., García-Marin V. and Freire M. (2010). Dendritic spines and development: towards a unifying model of spinogenesis. A present day review of Cajal's histological slides and drawings. Neural Plasticity, 2010, 1-29.

Garcia-Segura, L.M. and Mccarthy M.M. (2004). Mini-review: role of glia in neuroendocrine function. Endocrinology 145, 1082-1086.

Garcia-Segura L.M., Naftolin F., Hutchison J.B., Azcoitia I. and Chowen J.A. (1999). Role of astroglia in estrogen regulation.of synaptic plasticity and brain repair. J. Neurobiol. 40, 574-584.

Garcia-Segura L.M., Azcoitia I. and DonCarlos L.L. (2001). Neuroprotection by estradiol. Prog. Neurobiol. 63, 29-60.

Gibbs R.B. (2000). Effects of gonadal hormone replacement on measures of basal forebrain cholinergic function. Neuroscience 101, 931-938.

Gillies G.E. and McArthur S. (2000). Estrogen actions in the brain and the basis for differential action in men and women: a case for sex-specific medicines. Neuroscience 101, 931-938.

Gingerich S., Kim G.L., Chalmers J.A., Koletar M.M., Wang X., Wang Y. and Belsham D.D. (2010). Estrogen receptor alpha and G-protein coupled receptor 30 mediate the neuroprotective effects of 17,-estradiol in novel murine hippocampal cell models. Neuroscience 170, 54-66.

Gomez D.M. and Newman S.W. (1991). Medial nucleus of the amygdala in the adult Syrian hamster: a quantitative Golgi analysis of gonadal hormonal regulation of neuronal morphology. Anat. Rec. 231, 498-509.

Gréco B., Edwards D.A., Michael R.P. and Clancy A.N. (1996). Androgen receptor immunoreactivity and mating-induced Fos expression in forebrain and midbrain structures in the male rat. Neuroscience 75, 161-171.

Gréco B., Edwards D.A., Michael R.P. and Clancy N.A. (1998) Androgen receptors and estrogen receptors are colocalized in male rat hypothalamic and limbic neurons that express Fos immunoreactivity induced by mating. Neuroendocrinology 67, 18-28.

Gréco B., Allegretto E.A., Tetel M.J. and Blaustein J.D. (2001). Coexpression of ER beta with ER alpha and progestin receptor proteins in the female rat forebrain: effects of estradiol treatment. Endocrinology 142, 5172-5181.

Gréco B., Blasberg M.E., Kosinski E.C. and Blaustein J.D. (2003). Response of ER·-IR and ER,-IR cells in the forebrain of female rats to mating stimuli. Horm. Behav. 43, 444-453.

Gu G., Rojo A.A., Zee M.C., Yu J. and Simerly R.B. (1996). Hormonal regulation of CREB phosphorylation in the anteroventral periventricular nucleus. J. Neurosci. 16, 3035-3044.

Halassa M.M. and Haydon P.G. (2010). Integrated brain circuits: astrocytic networks modulate neuronal activity and behavior. Annu. Rev. Physiol. 72, 335-355.

Halász J., Liposits Z., Kruk M.R. and Haller J. (2002). Neural background of glucocorticoid dysfunction-induced abnormal aggression in rats: involvement of fear- and stress-related structures. Eur. J. Neurosci. 15, 561-569.

Harris K.M. and Kater S.B. (1994). Dendritic spines: Cellular specializations imparting both stability and flexibility to synaptic function. Ann. Rev. Neurosci. 17, 341-371.

Harris K.M., Perry E., Bourne J., Feinberg M., Ostroff L. and Hulburt J. (2006). Uniform serial sectioning for transmission electron microscopy. J. Neurosci. 26, 12101-12103.

Hayashi Y. and Majewska A.K. (2005). Dendritic spine geometry: functional implication and regulation. Neuron 46, 529-532.

Hermel E.E., Ilha J., Xavier L.L., Rasia-Filho A.A. and Achaval M. (2006a). Influence of sex and estrous cycle, but not laterality, on the neuronal somatic volume of the posterodorsal medial amygdala of rats. Neurosci. Lett. 405, 153-158.

Hermel E.E., Faccioni-Heuser M.C., Marcuzzo S., Rasia-Filho A.A. and Achaval M. (2006b). Ultrastructural features of neurons and synaptic contacts in the posterodorsal medial amygdala of adult male rats. J. Anat. 208, 565-575.

Hines M., Allen L.S. and Gorski R.A. (1992). Sex differences in subregions of the medial nucleus of the amygdala and the bed nucleus of the stria terminalis of the rat. Brain Res. 579, 321-326.

Huang C.S., Shi S.H., Ule J., Ruggiu M., Barker L.A., Darnell R.B., Jan Y.N. and Jan L.Y. (2005). Common molecular pathways mediate long-term potentiation of synaptic excitation and slow synaptic inhibition. Cell 123, 105-118.

Humeau Y., Herry C., Kemp N., Shaban H., Fourcaudot E., Bissière S. and Lüthi A. (2005). Dendritic spine heterogeneity determines afferent-specific Hebbian plasticity in the amygdala. Neuron 45, 119-131.

Isgor C., Huang G., Akil H. and Watson S.J. (2002). Correlation of estrogen ß-receptor messenger RNA with endogenous levels of plasma estradiol and progesterone in the female rat hypothalamus, the bed nucleus of stria terminalis and the medial amygdala. Molecul. Brain Res.106, 30-41.

Izzo P.N., Graybiel A.M. and Bolam J.P. (1987). Characterization of substance P- and [Met]enkephalin-immunoreactive neurons in the caudate nucleus of cat and ferret by a single section Golgi procedure. Neuroscience 20, 577-587.

Johnson R.T., Breedlove S.M. and Jordan C.L. (2008). Sex differences and laterality in astrocyte number and complexity in the adult rat medial amygdala. J. Comp. Neurol. 511, 599-609.

Jones E. G. and Powell T.P.S. (1969). Morphological variation in the dendritic spines of the neocortex. J. Cell Sci. 5, 509-529.

Kasai H., Matsuzaki M., Noguchi J., Yasumatsu N. and Nakahara H. (2003). Structure-stability-function relationships of dendritic spines. Trends Neurosci. 26, 360-368.

Kim B.G., Dai H-N., McAtee M., Vicini S. and Bregman B.S. (2007). Labeling of dendritic spines with the carbocyanine dye DiI for confocal microscopic imaging in lightly fixed cortical slices. J. Neurosci. Methods 162, 237-243.

Kisvárday Z.F., Gulyas A., Beroukas D., North J.B., Chubb I.W. and Somogyi P. (1990). Synapses, axonal and dendritic patterns of GABA-immunoreactive neurons in human cerebral cortex. Brain 113, 793-812.

Korkotian E. and Segal M. (2000). Structure-function relations in dendritic spines: is size important? Hippocampus 10, 587-595.

Kramár E.A., Chen L.Y., Brandon N.J., Rex C.S., Liu F., Gall C.M. and Lynch G. (2009a). Cytoskeletal changes underlie estrogen's acute effects on synaptic transmission and plasticity. J. Neurosci. 29, 12982-12993.

Kramár E.A., Chen L.Y., Rex C.S., Gall C.M. and Lynch G. (2009b). Estrogen's place in the family of synaptic modulators. Mol. Cell Pharmacol. 1, 258-262.

Kuipers S.D., Trentani A., Westenbroek C., Bramham C R., Korf J., Kema I.P. and Ter Horst G.J. (2006). Unique patterns of FOS, phospho-CREB and BrdU immunoreactivity in the female rat brain following chronic stress and citalopram treatment. Neuropharmacology 50, 428-440.

Laflamme N., Nappi R.E., Drolet G., Labrie C. and Rivest S. (1998). Expression and neuropeptidergic characterization of estrogen receptors (ERalpha and ERbeta) throughout the rat brain: anatomical evidence of distinct roles of each subtype. J. Neurobiol. 36, 357-378.

Lagunas N., Calmarza-Font I., Diz-Chaves Y. and Garcia-Segura L.M. (2010). Long-tern ovariectomy enhances anxiety d depressive-like behaviors in mice submitted to unpredictable stress. Horm. Behav. 58, 786-791.

Larriva-Sahd J. (2008). The accessory olfactory bulb in the adult rat: a cytological study of its cell types, neuropil, neuronal modules, and interactions with the main olfactory system. J. Comp. Neurol. 510, 309-350.

Lanciego J.L. and Wounterlood F.G. (2011). A half century of experimental neuroanatomical tracing. J. Chem. Neuroanat. 42, 157-183.

Lendvai B., Stern E.A., Chen B. and Svoboda K. (2000). Experience-dependent plasticity of dendritic spines in the developing rat barrel cortex in vivo. Nature 404, 876-881.

Li X., Schwartz P.E. and Rissman E.F. (1997). Distribution of estrogen receptor-beta-like immunoreactivity in rat forebrain. Neuroendocrinology 66, 63-67.

Lin Y., Ter Horst G.J., Wichmann R., Bakker P., Liu A., Li X. and Westenbroek C. (2009). Sex differences in the effects of acute and chronic stress and recovery after long-term stress on stress-related brain regions of rats. Cereb. Cortex 19, 1978-1989.

Lonze B.E. and Ginty D.D. (2002). Function and regulation of CREB family transcription factors in the nervous system. Neuron 35, 605-623.

López-Bendito, G., Shigemoto, R., Kulik, A., Vida, I., Fairén A. and Luján R. (2004). Distribution of metabotropic GABA receptor subunits GABAB1a/b and GABAB2 in the rat hippocampus during prenatal and postnatal development. Hippocampus 14, 836-848.

Lorente de Nó R. (1981). The primary acoustic nuclei. Raven Press. New York. pp 177.

Lorenzo A., Diaz H., Carrer H. and Caceres A. (1992). Amygdala neurons in vitro: neurite growth and effects of estradiol. J. Neurosci. Res. 33, 418-435.

Malsbury C.W. and McKay K. (1994). Neurotrophic effects of testosterone on the medial nucleus of the amygdala in adult male rats. J. Neuroendocrinol. 6, 57-69.

Marcuzzo S., Dall'Oglio A., Ribeiro M.F., Achaval, M. and Rasia-Filho A.A. (2007). Dendritic spines in the posterodorsal medial amygdala after restraint stress and ageing in rats. Neurosci. Lett. 424, 16-21.

Martinez F.G., Hermel E.E., Xavier L.L., Viola G.G., Riboldi J., Rasia-Filho A.A. and Achaval M. (2006). Gonadal hormone regulation of glial fibrillary acidic protein immunoreactivity in the medial amygdala subnuclei across the estrous cycle and in castrated and treated female rats. Brain Res. 1108, 117-126.

McCarthy M.M., Auger A.P. and Perrot-Sinal T.S. (2002). Getting excited about GABA and sex differences in the brain. Trends Neurosci. 25, 307-312.

McClung C.A and Nestler E.J. (2008). Neuroplasticity mediated by altered gene expression. Neuropsychopharmacology 33, 3-17.

McDonald A.J. (1992). Cell types and intrinsic connections of the amygdala. In: The amygdala: neurobiological aspects of emotion, memory, and mental dysfunction. Aggleton J.P. (ed). Wiley-Liss. New York. pp 67-96.

McDonald A.J. (1998). Cortical pathways to the mammalian amygdala. Prog. Neurobiol. 55, 257-332.

McDonald A.J. (2003). Is there an amygdala and how far does it extend? An anatomical perspective. Ann. NY Acad. Sci. 985, 1-21.

Meredith M. and Westberry J.M. (2004). Distinctive responses in the medial amygdala to same-species and different-species pheromones. J. Neurosci. 24, 5719-5725.

Mermelstein P.G. and Micevych P.E. (2008). Nervous system physiology regulated by membrane estrogen receptors. Rev. Neurosci. 19, 423-424.

Micevych P.E., Matt D.W. and Go V.L.W. (1988). Concentrations of cholecystokinin, substance P, and bombesin in discrete regions of male and female rat brain: sex differences and estrogen effects. Exp. Neurol. 100, 416-425.

Mizukami S., Nishizuka M. and Arai Y. (1983). Sexual difference in nuclear volume and its ontogeny in the rat amygdala. Exp. Neurol.. 79, 569-575.

Molenda H.A., Griffin A.L., Auger A.P., McCarthy M.M. and Tetel M.J. (2002). Nuclear receptor coactivators modulate hormone-dependent gene expression in brain and female reproductive behavior in rats. Endocrinology 143, 436-444.

Mong J.A. and McCarthy M.M. (2002). Ontogeny of sexually dimorphic astrocytes in the neonatal rat arcuate. Dev. Brain Res. 139, 151-158.

Mong J.A., Nunez J.L. and McCarthy M.M. (2002). GABA mediates steroid-induced astrocyte differentiation in the neonatal rat hypothalamus. J. Neuroendocrinol. 14, 1-16.

Mong J.A., Roberts R.C., Kelly J.J. and McCarthy MM. (2001). Gonadal steroids reduce the density of axospinous synapses in the developing rat arcuate nucleus: an electron microscopy analysis. J. Comp. Neurol. 432, 259-267.

Morris J.A., Jordan C L., Dugger B N. and Breedlove S.M. (2005). Partial demasculinization of several brain regions in adult male (XY) rats with a dysfunctional androgen receptor gene. J. Comp. Neurol. 487, 217-226.

Morris J.A., Jordan C.L. and Breedlove S.M. (2008). Sexual dimorphism in neuronal number of the posterodorsal medial amygdala is independent of circulating androgens and regional volume in adult rats. J. Comp. Neurol. 506, 851-859.

Murphy D.D. and Segal M. (1997). Morphological plasticity of dendritic spines in central neurons is mediated by activation of cAMP response element binding protein. Proc. Natl. Acad. Sci. USA 94, 1482-1487.

Nabekura J., Oomura Y., Minami T., Mizuno Y. and Fukuda A. (1986). Mechanism of the rapid effect of 17α-estradiol on medial amygdala neurons. Science 233, 226-228.

Nelson R.J. and Trainor B.C. (2007). Neural mechanisms of aggression. Nature Rev. 8, 536-546.

Newman S.W. (1999). The medial extended amygdala in male reproductive behavior. A node in the mammalian social behavior network. Ann. NY Acad. Sci. 877, 242-57.

Nimchinsky E.A., Sabatini B.L. and Svoboda K. (2002). Structure and function of dendritic spines. Annu. Rev. Physiol. 64, 313-353.

Nishizuka M. and Arai Y. (1981). Organizational action of estrogen on synaptic pattern in the amygdala: implications for sexual differentiation of the brain. Brain Res. 2, 422-426.

Nishizuka M. and Arai Y. (1982). Synapse formation in response to estrogen in the medial amygdala developing in the eye. Proc. Natl. Acad. Sci. USA 79, 7024-7026.

Nishizuka M. and Arai Y. (1983). Male-female differences in the intra-amygdaloid input to the medial amygdala. Exp. Brain Res. 52, 328-332.

O'Mahony C.M., Clarke G., Gibney S., Dinan T.G. and Cryan J.F. (2011). Strain differences in the neurochemical response to chronic restraint stress in the rat: relevance to depression. Pharmacol Biochem. Behav. 97, 690-699.

Oberlander J.G. and Erskine M.S. (2008). Receipt of vaginal-cervical stimulation modifies synapsin content in limbic areas of the female rat. Neuroscience 153, 581-593.

Oro A.E., Simerly R.B. and Swanson L.W. (1988). Estrous cycle variations in levels of cholecystokinin immunoreactivity within cells of three interconnected sexually dimorphic forebrain nuclei. Neuroendocrinology 47, 225-235.

Österlund M., Kuiper G.G., Gustafsson J. and Hurd Y.L. (1998). Differential distribution and regulation of estrogen receptor-α and -ßmRNA within the female rat brain. Molecul. Brain Res. 54, 175-180.

Paxinos G. and Watson C. (1998). The rat brain in stereotaxic coordinates. 4th ed. Academic Press. San Diego.

Pandey S.C., Roy A. and Zhang H. (2003). The decreased phosphorylation of cyclic adenosine monophosphate (cAMP) response element binding (CREB) protein in the central amygdala acts as a molecular substrate for anxiety related to ethanol withdrawal in rats. Alcohol Clin. Exp. Res. 27, 396-409.

Pandey S.C., Roy A., Zhang H. and Xu T. (2004). Partial deletion of the cAMP response element-binding protein gene promotes alcohol-drinking behaviors. J. Neurosci. 24, 5022-5030.

Perea G., Navarrete M. and Araque A. (2009). Tripartite synapses: astrocytes process and control synaptic formation. Trends Neurosci. 32, 421-431.

Peters A. and Kaiserman-Abramof I.R. (1970). The small pyramidal neuron of the rat cerebral cortex. The perikaryon, dendrites and spines. Am. J. Anat. 127, 321-356.

Petrovich G.D., Canteras N.S. and Swanson L.W. (2001). Combinatorial amygdalar inputs to hippocampal domains and hypothalamic behavior systems. Brain Res. Rev. 38, 247-289.

Pfaff D.W. and Cohen R.S. (1987). Estrogen acting on hypothalamic neurons may have trophic effects on those neurons and the cells on which they synapse. In: Endocrinology and physiology of reproduction. Leung P.C.K., Armstrong D.T., Ruf K B., Moger W.H. and Friesen H.G. (eds). Plenum Press. New York. pp 1-11.

Pfaus J.G. and Heeb M.M. (1997). Implications of immediate-early gene induction in the brain following sexual stimulation of female and male rodents. Brain Res. Bull. 44, 397-407.

Phillips-Farfán B.V., Lemus A. E. and Fernández-Guasti E. (2007). Increased estrogen receptor alpha immunoreactivity in the forebrain of sexually satiated rats. Horm. Behav. 51, 328-334

Pitkänen A. (2000). Connectivity of the rat amygdaloid complex. In: The amygdala: a functional analysis. Aggleton J.P. (ed). Oxford University Press. Oxford. pp 31-115.

Polston E.K., Heitz M., Barnes W., Cardamone K. and Erskine M.S. (2001). NMDA-mediated activation of the medial amygdala initiates a downstream neuroendocrine memory responsible for pseudopregnancy in the female rat. J. Neurosci. 21, 4104-4110.

Polston E.K., Gu G. and Simerly R.B. (2004). Neurons in the principal nucleus of the bed nuclei of the stria terminalis provide a sexually dimorphic GABAergic input to the anteroventral periventricular nucleus of the hypothalamus. Neuroscience 123, 793-803.

Popov V.I. and Stewart M.G. (2009). Complexity of contacts between synaptic boutons and dendritic spines in adult rat hippocampus: Three-dimensional reconstructions from serial ultrathin sections in vivo. Synapse 63, 369-377.

Popov V.I., Deev A.A., Klimenko O.A., Kraev I.V., Kuz'minykh S.B., Medvedev N.I., Patrushev I.V., Popov R.V., Rogachevskii V.V., Khutsiyan S.S., Stewart M.G. and Fesenko E.E. (2005). Three-dimensional reconstruction of synapses and dendritic spines in the rat and ground squirrel hippocampus: new structural-functional paradigms for synaptic function. Neurosci. Behav. Physiol. 35, 333-341.

Pro-Sistiaga P., Mohedano-Moriano A., Ubeda-Bañon I., Arroio-Jimenez M.D.M., Marcos P., Artacho-Pérula E., Crespo C., Insausti R. and Martinez-Marcos A. (2007). Convergence of olfactory and vomeronasal projections in the rat basal telencephalon. J. Comp. Neurol. 504, 346-362.

Quagliotto E., Casali K.R., Dal Lago P. and Rasia-Filho A.A. (2012). Neurotransmitter and neuropeptidergic modulation of cardiovascular responses evoked by the posterodorsal medial amygdala of adult male rats. In: Amygdala: Structure, functions and disorders. Yilmazer-Hanke D. (ed). Nova Science Publishers. Hauppauge (in press).

Rachman I.M., Unnerstall J.R., Pfaff D.W. and Cohen R.S. (1998). Estrogen alters behavior and forebrain c-fos expression in ovareictomized ats subject to the forced swim test. Proc. Natl. Acad. Sci. U.S.A. 95, 13941-13946.

Ramón y Cajal S. (1909). Histologie du Système Nerveux de l'Homme et des Vertebrés. Maloine. Paris. pp 986.

Ramón-Moliner E. (1970). The Golgi-Cox technique. In: Contemporary Research Methods in Neuroanatomy. Nauta W.J.H. and Ebbesson S.O.E. (eds.). Springer-Verlag. Berlin. pp 32-55.

Rasia-Filho A.A. (2006). Is there anything "autonomous" in the nervous system? Adv. Physiol. Educ. 30, 9-12.

Rasia-Filho A.A. and Lucion A.B. (1996). Effects of 8-OH-DPAT on sexual behavior of male rats castrated at different ages. Horm. Behav. 30: 251-258.

Rasia-Filho A.A., Peres T.M.S., Cubilla-Gutierrez F.H. and Lucion A.B. (1991). Effect of estradiol implanted in the corticomedial amygdala on the sexual behavior of castrated male rats. Braz. J. Med. Biol. Res. 24, 1041-1049.

Rasia-Filho A.A., Londero R.G. and Achaval M. (1999). Effects of gonadal hormones on the morphology of neurons from the medial amygdaloid nucleus of rats. Brain Res. Bull. 48, 173-183.

Rasia-Filho A.A., Londero R.G. and Achaval M. (2000). On some functional activities of the amygdala: an overview. J. Psychiatry Neurosci. 25: 14-23.

Rasia-Filho A.A., Xavier L.L., Santos P., Gehlen P. and Achaval M. (2002). Glial fibrillary acidic protein immunodetection and immunoreactivity in the anterior and in the posterior medial amygdala of male and female rats. Brain Res. Bull. 58, 67-75.

Rasia-Filho A.A., Fabian C., Rigoti K.M. and Achaval M. (2004). Influence of sex, estrous cycle and motherhood on dendritic spine density in the rat medial amygdala revealed by the Golgi method. Neuroscience 126, 839-847.

Rasia-Filho A.A., Brusco J. and Moreira J.E. (2009). Spine plasticity in the rat medial amygdala. In: Dendritic spines: Biochemistry, modeling and properties. Baylog L.R. (ed). Nova Science Publishers. Hauppauge. pp 67-90.

Rasia-Filho A.A., Brusco J., Rocha L.B. and Moreira J.E. (2010). Dendritic spines observed by extracellular DiI dye and immunolabeling under confocal microscopy. Nature Protocols/Protocol Exchange. DOI: 10.1038/nprot.2010.153.

Rasia-Filho A.A., Haas D., de Oliveira A.P., de Castilhos J., Frey R., Stein D., Lazzari V.M., Back F., Pires G.N., Pavesi E., Winkelmann-Duarte E.C. and Giovenardi M. (2012). Morphological and functional features of the sex steroid-responsive posterodorsal medial amygdala of adult rats. Mini Rev. Med. Chem. (in press).

Rønnekleiv O.K., Malyala A. and Kelly M.J. (2007). Membrane-initiated signaling of estrogen in the brain. Semin. Reprod. Med. 3, 165-177.

Rosa C.B., Goularte J.F., Trindade N.A., de Oliveira A.P. and Rasia-Filho A.A. (2011). Glutamate microinjected in the posterodorsal medial amydala induces subtle increase in the consumption of a three-choice macronutrient self-selection diet in male rats. Anat. Rec. 294, 1226-1232.

Ryugo D.K. and Fekete D.M. (1982). Morphology of primary axosomatic endings in the anteroventral cochlear nucleus of the cat: a study of the endbulbs of held. J. Comp. Neurol. 210, 239-257.

Sanchez A.M. and Simoncini T. (2010). Extra-nuclear signaling of ERalpha to the actin cytoskeleton in the central nervous system. Steroids 75, 528-532.

Scalia F. and Winans S.S. (1975). The differential projections of the olfactory bulb and accessory olfactory bulb in mammals. J. Comp. Neurol. 161, 31-55.

Scharfman H.E. and MacLusky N.J. (2005). Similarities bewteen actions of estrogen and BDNF in the hippocampus: coincidence or clue? Trends Neurosci. 28, 79-85.

Scott E., Zhang Q. G., Wang R. Vadlamudi R. and Brann D. (2012). Estrogen neuroprtoection and the critical period hypothesis. Front. Neuroendocrinol. 33, 85-104.

Segal M. (2005). Dendritic spines and long-term plasticity. Nature Rev. Neurosci. 6, 277-284.

Segal M. (2010). Dendritic spines, synaptic plasticity and neuronal survival: activity shapes dendritic spines to enhance neuronal viability. Eur. J. Neurosci. 31, 2178-2184.

Sekino Y., Kojima N. and Shirao T. (2007). Role of actin cytoskeleton in dendritic spine morphogenesis. Neurochem. Internat. 51, 92-104.

Schmidt H. and Eilers J. (2009). Spine neck geometry determines spino-dendritic crosstalk in the presence of mobile endogenous calcium binding proteins. J. Comput. Neurosci. 27, 229-243.

Sharrow K.M., Kumar A. and Foster T. C. (2002). Calcineurin as a potential contributor in estradiol regulation of hippocampal synaptic function. Neuroscience 1, 89-97.

Sheehan T.P., Paul M., Amaral E., Numan M.J. and Numan M. (2001). Evidence that the medial amygdala projects to the anterior/ventromedial hypothalamic nuclei to inhibit maternal behavior in rats. Neuroscience 106, 341-356.

Shepherd G.M. (1996). The dendritic spine: a multifunctional integrative unit. J. Neurophysiol. 75, 2197-2210.

Shibata H., Spencer T.E., Oñate S.A., Jenster G., Tsai S.Y., Tsai M.J. and O'Malley B.W. (1997). Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. Recent Prog. Horm. Res. 52, 141-164.

Shindou T., Watanabe S., Yamamoto K. and Nakanishi H. (1993). NMDA receptor dependent formation of long-term potentiation in the rat medial amygdala neuron in an in vitro slice preparation. Brain Res. Bull. 31, 667-672.

Shughrue P.J., Lane M.V. and Merchenthaler I. (1997). Comparative distribution of estrogen receptor-α and ßmRNA in the rat central

nervous system. J. Comp. Neurol. 388, 507-525.

Shughrue P J., Scrimo P.J. and Merchenthaler I. (1998). Evidence of the colocalization of estrogen receptor-{beta} mRNA and estrogen receptor-alpha immunoreactivity in neurons of the rat forebrain. Endocrinology 39, 5267-5270.

Simerly R.B. (2002). Wired for reproduction: organization and development of sexually dimorphic circuits in the mammalian forebrain. Annu. Rev. Neurosci. 25, 507-36.

Simerly, R.B. (2004). Anatomical substrates of hypothalamic integration. In: The rat nervous system. Paxinos G. (ed). Academic Press. San Diego. pp 335-368.

Simerly R.B., Chang C., Muramatsu M. and Swanson L.W. (1990). Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. J. Comp. Neurol. 294, 76-95.

Singewald N., Chicchi G.G., Thurner C.C., Tsao K.L., Spetea M., Schmidhammer H., Sreepathi H.K., Ferraguti F., Singewald G.M. and Ebner K. (2008). Modulation of basal and stress-induced amygdaloid substance P release by the potent and selective NK1 receptor antagonist L-822429. J. Neurochem. 106, 2476-2488.

Singh M., Meyer E. and Simpkins J. (1995). The effect of ovariectomy and estradiol replacement on brain-derived neurotrophic factor messenger ribonucleic acid expression in cortical and hippocampal brain regions of female Sprague-Dawley rats. Endocrinology 136, 2320-2324.

Soderling T.R. (2000). CaM-kinases: modulators of synaptic plasticity. Curr. Opin. Neurobiol. 3, 375-80.

Sohrabji F. and Lewis D.K. (2006). Estrogen-BDNF interactions: implications for neurodegenerative diseases. Front. Neuroendocrinol. 27, 404-14.

Spacek J. and Harris K.M. (2004).Trans-endocytosis via spinules in adult rat hippocampus. J. Neurosci. 24, 4233-4241.

Srivastava D.P., Woolfrey K.M., Liu .F, Brandon N.J. and Penzes P. (2010). Estrogen receptor ß activity modulates synaptic signaling and structure. J. Neurosci. 30, 13454-13460.

Srivastava, D.P., Waters E.M., Mermelstein P.G., Kramár E.A., Shors T.J. and Liu F. (2011a). Rapid estrogen signaling in the brain: implications for the fine-tuning of neuronal circuitry. J. Neurosci. 31, 16056-16063.

Srivastava D.P., Woolfrey K.M. and Penzes P. (2011b). Analysis of dendritic spine morphology in cultured CNS neurons. J. Vis. Exp. 13, 53.

Stark C.H. (2005). Behavioral effects of stimulation of the medial amygdala in the male rat are modified by prior sexual experience. J. Gen. Psychol. 132, 207-224.

Strömberg H., Svensson S.P. and Hermanson O. (1999). Distribution of CREB-binding protein immunoreactivity in the adult rat brain. Brain Res. 818, 510-514.

Swanson L.W. and Petrovich G.D. (1998). What is the amygdala? Trends Neurosci. 21, 323-331.

Szegö E.M., Barabas K., Balog J., Szilagyi N. Korach K.S., Juhasz G. and Ábrahám I.M. (2006). Estrogen induces estrogen receptor-alpha-dependent cAMP response element-binding protein phosphorylation via mitogen activated protein kinase pathway in basal forebrain cholinergic neurons in vivo. J. Neurosci. 15, 4104-4110.

Szentagothai J. (1978). The neuron network of the cerebral cortex: a functional interpretation. Proc. R. Soc. Lond. B 201, 219-248.

Tanapat P. Hastings N.B. and Gould E. (2005). Ovarian steroids influence cell proliferation in the dentate gyrus of the adult female rat in a dose- and time-dependent manner. J. Comp. Neurol. 481, 252-265.

Tsay D. and Yuste R. (2004). On the electrical function of dendritic spines. Trends Neurosci. 27, 77-83.

Valverde F. (1962). Intrinsic organization of the amygdaloid complex. A Golgi study in the mouse. Trab. Inst. Cajal Invest. Biol. 54, 291-314.

Valverde F. (1970). The Golgi method: a tool for comparative structural analyses. In: Contemporary research methods in neuroanatomy. Nauta W.J.H. and Ebbesson S.O.E. (eds). Springer-Verlag. New York. pp 11-31.

van de Weijer P.H., Mattsson L.A. and Ylikorkala O. (2007). Benefits and risks of long-term low-dose oral continuous combined hormone therapy. Maturitas 56, 231-248.

Walf A.A .and Frye C.A. (2006). A review and update of mechanisms of estrogen in the hippocampus and amygdala for anxiety and depression behavior. Neuropsychopharmacology 31, 1097-1111.

Waters E.M., Mitterling K., Spencer J.L., Mazid S., McEwen B.S. and Milner T.A. (2009). Estrogen receptor alpha and beta specific agonists regulate expression of synaptic proteins in rat hippocampus. Brain Res. 1290, 1-11.

Wearne S.L., Rodriguez A., Ehlenberger D.B., Rocher A.B., Henderson S.C. and Hof P.R. (2005). New techniques for imaging, digitization and analysis of three-dimensioanl neural morphology on multiple scales. Neuroscience 136, 661-680.

Wise P.M., Dubal D.B., Wilson M.E., Rau S.W. and Liu Y (2001). Estrogens: trophic and protective factors in the adult brain. Front. Neuroendocrinol. 22, 33-66.

Wood R. (1996). Estradiol, but not dihydrotestosterone, in the medial amygdala facilitates male hamster sex behavior. Physiol. Behav. 59, 833-841.

Wood R.I. and Newman S.W. (1995). The medial amygdaloid nucleus and medial preoptic area mediate steroidal control of sexual behavior in the male Syrian hamster. Horm. Behav. 29, 338-353.

Woolley C.S. and McEwen B.S. (1992). Estradiol mediates fluctuations in hippocampal synapse density during the estrous cycle in the adult rat. J. Neurosci. 12, 2549-2554.

Woolley C.S. and Cohen, R.S. (2002). Sex steroids and neuronal growth in adulthood. In: Hormones, brains and behavior. Pfaff D.W. (ed). Academic Press. New York. pp 717-777.

Woolley C.S., Gould E., Frankfurt M. and McEwen B.S. (1990). Naturally occurring fluctuation in dendritic spine density on adult hippocampal pyramidal neurons. J. Neurosci. 10, 4035-4039.

Yang G., Pan F. and Gan W.Bi. (2009). Stably maintained dendritic spines are associated with lifelong memories. Nature 462, 920-924.

Yasumatsu N., Matsuzaki M., Miyazaki T., Noguchi J. and Kasai H. (2008). Principles of long-term dynamics of dendritic spines. J. Neurosci. 28, 13592-13608.

Yoshida M., Suga S. and Sakuma Y. (1994). Estrogen reduces the excitability of the female rat medial amygdala afferents from the medial preoptic area but not those from the lateral septum. Exp. Brain Res. 101, 1-7.

Zagrebelsky M., Schweigreiter R., Bandtlow C.E., Schwab M.E. and Korte M. (2010). Nogo-A stabilizes the architecture of hippocampal neurons. J. Neurosci. 30, 13220-13234.

Zehr J.L., Todd B.J., Schulz K.M., McCarthy M.M. and Sisk C.L. (2006). Dendritic pruning of the medial amygdala during pubertal development of the male Syrian hamster. J. Neurobiol. 66, 578-590.

Zhou Y., Watters J.J. and Dorsa D.M. (1996). Estrogen rapidly induces

the phosphorylation of the cAMP response element binding protein in rat brain. Endocrinology 5, 2163-2166.

Zhou J. Cohen R.S. and Pandey S.C. (2001). Estrogen affects the expression of Ca^{2+} /calmodulin-dependent protein kinase IV in amygdala. Neuroreport 15, 2437-2440.

Zhou J., Pandey S.C. and and Cohen R.S. (2004). Estrogen decreases levels of calcineurin in rat amygdala and hippocampus. Neuroreport 15, 2437-2440.

Zhou J., Zhang H., Cohen R.S. and Pandey S.C. (2005). Effects of estrogen treatment on expression of brain-derived neurotrophic factor and cAMP response element-binding protein expression and phosphorylation in rat amygdaloid and hippocampal structures. Neuroendocrinology 5, 294-310.

Zuo Y., Lin A., Chang P and Gan W-B. (2005). Development of long-term dendritic spine stability in diverse regions of cerebral cortex. Neuron 46, 181-189.

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Original Research

Histogenetically distinct neuronal subpopulations, dendritic spines, and synaptic diversity in the adult rat medial amygdala

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Abstract

The posterodorsal medial amygdala (MePD) has different histogenetic origins, modulates social behaviors, and displays notable plasticity in rats. Dendritic spines represent specialized integrative cellular elements that modulate and enforce synaptic inputs. Here, we describe the shape and density of dendritic spines in the main subpopulations of MePD neurons, and the presence of glutamatergic and GABAergic receptors on both proximal and distal dendritic shafts and dendritic spines. DiI dye evidenced dendritic spines in neurons that specifically express the LIM homeobox transcription factors Lhx6, Lhx5, and Lhx9 under confocal microscopy. The most abundant spine types were thin and stubby/wide spines (~80%), and the proximal dendritic spine density varied from 0.4 to 2.3 spines/dendritic µm with no statistical difference among distinct Lhx-expressing neurons. AMPA (subunit GluR1-4), NMDA (subunit GluN1R1), and $GABA_A$ receptors immunolabeling were found on both dendritic shafts and dendritic spines. AMPA receptors were predominant on mushroom and stubby/wide spines, whereas NMDA receptors were found mostly on thin spines. $GABA_A$ receptors were located on thin or stubby/wide spines of proximal dendrites and more frequently on thin spines on distal branches. Multisynaptic spines with excitatory and inhibitory receptors were also found in the MePD. These new data evidence relevant features on the cellular composition and the complex connectivity of dendritic spines in the adult rat MePD.

Key words: Extended amygdala, Lhx transcription factor, spine shape, GLUR1-4 receptor subunit, NMDA GluN1 receptor subunit, $GABA_A$ receptors.

Introduction

The posterodorsal medial amygdala (MePD) is a component of the "extended amygdala" in the rat basal forebrain (Alheid et al., 1995; Dong et al., 2001; de Olmos et al., 2004; Dall'Oglio et al., 2008a,b) and a nodal point for emotionally-loaded stress responses (Dayas et al., 1999) and the modulation of social/reproductive behavior neural networks (Newman, 1999; Polston et al., 2004; Choi et al., 2005; Rasia-Filho et al., 2012a,b). The MePD responds to species-specific socially relevant olfactory/pheromonal (Meredith and Westberry, 2004; Pereno et al., 2011) and genitosensorial stimuli (Pfaus and Heeb, 1997), has a notable local neuroplasticity related to a high expression of sex steroid receptors (Simerly et al., 1990; Gréco et al., 2001, 2003; Rasia-Filho et al., 2004; Zehr et al., 2006; Phillips-Farfán et al., 2007; Fan et al., 2008a,b; Blake and Meredith, 2011), and amends timely hypothalamic neuroendocrine secretion (Simerly, 2004).

The discovery of the embrionic origin of MePD cells and circuitry formation in rodents provided an exciting venue for local and integrated functional neuroanatomy. The MePD is a "mosaic" composed by distinct precursor cells and assembles coexisting histogenetically (Zirlinger et al., 2001; Choi et al., 2005; García-López et al., 2008; Bupesh et al., 2011) and phenotypically different (Coolen et al., 1996, 1997; Bian et al., 2008; Carney et al., 2010; Niimi et al., 2012; Bian, 2013) subpopulations of multipolar bitufted and stellate neurons (Alheid et al., 1995; Rasia-Filho et al., 1999, 2012a,b). Specific regulatory genes for LIM homeobox (Lhx) transcription factors are expressed along the development of local cells (Choi et al., 2005; García-López et al., 2008; Bupesh et al., 2011). Confocal immunofluorescent microscopy revealed that Lhx6-immunoreactive cells constitute around 80% of all neurons in the MePD (Choi et al., 2005). These neurons

derive from the subpallial, caudoventral medial ganglionic eminence, are GABAergic expressing calbindin cells (García-López et al., 2008), and reach three interconnected hypothalamic nuclei implicated in reproductive behaviors: the medial preoptic nucleus, the ventrolateral part of the ventromedial hypothalamic nucleus and the ventral premammillary nucleus (Choi et al., 2005). Comparatively, little is known about other Lhxs immunoreactive cells in the MePD. Lhx5- and Lhx9-immunoreactive cells derive from the ventral pallium and the supraoptoparaventricular hypothalamic domain (Bupesh et al., 2011). Both Lhx5- and Lhx9-immunoreacitve neurons might be glutamatergic, although Lhx5 cells would also express calbindin, vasopressin, and oxytocin (Bupesh et al., 2011).

The study of dendritic spine shape and density on these histogenetically different Lhx-expressing neuronal subpopulations in the rat MePD can illuminate the structural basis of neural circuit function and processing of complex sensorial stimuli and social behavior display. Dendritic spines are mostly associated with excitatory connections and their heterogeneous shape and number can impose functional differences in localized biochemical signaling/compartmentalization and in the synaptic organization, strength, stability or plasticity (Hayashi and Majewska, 2005; Bourne and Harris, 2007; Harms and Dunaevsky, 2007; Kasai et al., 2010; Chen and Sabatini, 2012). In randomly sampled Golgi-impregnated MePD neurons, the density of dendritic spines is sexually dimorphic (males higher than females), vary along the estrous cycle (Rasia-Filho et al., 2004), and have a marked reduction that coincides temporally with the impairment of the sexual behavior in adult castrated males (de Castilhos et al., 2008) or a reduced rough-and-tumble playful attacks in prepubertal castrated rats (Cooke and Woolley, 2009). Furthermore, in the adult rat MePD, both glutamate and GABA modulate activities such as sexual behavior (facilitating ejaculation and reducing copulatory latency, respectively; Rasia-Filho et al., 2012b) and a selective central control of the

sympathetic/parasympathetic cardiovascular reflex responses (Neckel et al., 2012). In male rats, NMDA-mediated activation of the medial amygdala induces long-term potentiation (Shindou et al., 1993) and, in females, promotes a downstream neuroendocrine memory responsible for pseudopregnancy (Polston et al., 2001), indicating that local glutamatergic innervation can promote sexually dimorphic functions. The occurrence of glutamatergic and GABAergic receptors on dendritic shafts and spines of MePD neurons is still unknown.

The aims of this study were twofold: (1) to provide the first description of the morphology and density of dendritic spines in the different immunolabeled Lhx-expressing subpopulations of neurons in the adult rat MePD, and (2) to depict the presence and distribution of glutamatergic (AMPA and NMDA) and GABAergic ($GABA_A$) receptors on proximal and distal dendritic shafts as well as on different types of dendritic spines on MePD neurons under confocal microscopy.

Materials and Methods

Animals

Adult male Wistar rats (3–4 months old, N = 26) were housed in groups with free access to food and water in standard laboratory conditions, with room temperature kept at 22 ^{o}C in a 12 h light-dark cycle. Experimental procedures were performed in accordance with the international laws for the ethical care, the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85–23, reviewed 1985, USA) and were approved by local Ethics Committees (UFCSPA, protocol 033-10 and FMRP/USP protocol 174-11).

Experimental Procedures

All animals were anesthetized with ketamine and xylazine (80 mg/kg and 10 mg/kg, i.p., respectively) and underwent transcardiac perfusion with 200 ml of 0.9% NaCl followed by 300 ml of 1.5% paraformaldehyde in 0.1 M phosphate buffer solution, pH 7.4 (PBS; Kim et al., 2007; Rasia-Filho et al., 2010), initially very fast and progressively slowing to keep the fixative running for over 15 min (Tao-Cheng et al., 2007). The brains were maintained in the same fixative solution for 1 h at room temperature (RT) and were sectioned at 80 µm-thick slices in PBS using a vibratome (Leica, Germany; Brusco et al., 2010). The MePD was found 3.0–3.30 mm posterior to the bregma, delimited laterally to the optic tract and ventrally to the stria terminalis (ST; Paxinos and Watson, 1998; de Olmos et al., 2004; Figure 1).

Lhx6, Lhx5, and Lhx9 Immunofluorescent Labeling

To study the presence of different subpopulations of Lhx-expressing neurons and their spine features, we utilized the following goat polyclonal primary antibodies against: 1) Lhx6 (N-19) to detect N-terminus of Lhx6 of human origin, 2) Lhx5 (C20) to label C-terminal of Lhx5 of human origin, and 3) Lhx9 (N-20) to detect N-terminus of Lhx9 of human origin. Antibodies were purchased from Santa Cruz Biotechnology Inc. (USA) and were diluted 1:100. Primary antibodies were labeled with anti-goat IgG Alexa 647 (1:200; Invitrogen, USA). The specificity of each antibody was verified by Western blot (according to Kurien et al., 2011). I.e., we performed additional Western blot analysis following the basic procedures described previously (Alegria-Schaffer et al., 2009) and the specific bands obtained from these control experiments are shown in Figure 2. In addition, the immunolabeling patterns for the distinct Lhx neurons were identical to those reported in a previous referential report (Choi et al., 2005).

Coronal brain slices (80 µm-thick) containing the MePD were obtained using a vibratome (Leica VT 1000S, Leica Microsystems GmbH, Germany). The sections were washed with 0.02 M Tris Buffer Solution (TBS), and immersed in 0.2% Tween in TBS (TBST) during 6 h at 4ºC. The slices were blocked with 2% bovine serum albumine (BSA) in TBST for 2 h, incubated with primary antibodies diluted in 0.5% BSA in TBST overnight at 4°C, and secondary antibodies for 2h at 4ºC. Every experiment included controls with the omission of primary antibodies (data not shown), besides the other above-mentioned control procedures.

After antibodies incubation, sonicated fine powdered carbocyanine dye DiI (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate; Molecular Probes, Invitrogen, USA) was placed over the ST for 24 h at 4°C (Rasia-Filho et al., 2010). Sections were washed in distilled water, mounted with Fluoromount (EMS, USA), and visualized under confocal microscopy, as described below. Each rat provided one brain slice for each Lhx immunoreaction tested. Both hemispheres were used. Due to technical restrictions and the inherent difficulties for obtaining acceptable multi-labeled fluorescent neurons in each brain section, no quantitative comparisons were done for the right and left MePD. For the aims of the present work, it is worth noting that previous experimental data did not evidence hemispheric lateralization for the MePD dendritic spine density in adult rats (Arpini et al., 2010).

Laser wave length of the DiI fluorescence did not overlay that one of the Alexa linked with the Lhx antibodies. Data were obtained from a total of 12, 14, and 12 different Lhx6, Lhx5 and Lhx9 neurons from 5, 8, and 9 rats [mean ± standard deviation (SD), 2.4 ± 0.5, 1.3 ± 0.5, 1.7 ± 0.7 neurons per rat], respectively. Including criteria were: 1) neurons must be located within the MePD, avoiding its ultimate borders; 2) fluorescence for both Lhx antibody and DiI must be stable and basically at the same focal plane; 3)

dendrites must be brightly fluorescent and not “tangled” with neighborhood prolongments; and, 4) spines must be clearly visbible and identifiable. Dendritic branches and spines were imaged along the first 50 µm from the soma (Rasia-Filho et al., 2004; Brusco et al., 2010; Figures 3-5). It has to be commented that the numbers generated from our confocal microscopy immunofluorescent neurons reflect the spines observed along proximal dendrites and might under-represent the actual values for entire cells (e.g., as commented in Woolley and McEwen, 1994). Nevertheless, from Golgi-impregnation studies, dendritic spines appear to have a homogeneous distribution up to more than a hundred µm away from the cell body (de Castilhos et al., 2006) and analyses of spine density have been made from data obtained along 40 µm of proximal dendrites (Rasia-Filho et al., 2004; de Castilhos et al., 2006, 2008; Marcuzzo et al., 2007; Brusco et al., 2010; Rasia-Filho et al., 2012a).

GLUR1-4, GluN1, and $GABA_A$ Immunofluorescent Labeling

To study the localization of excitatory and inhibitory receptors on MePD neurons we used: 1) rabbit polyclonal antibody against GluR1-4 subunits to label AMPA receptor (1:50), 2) mouse monoclonal antibody against the N-terminus of the NMDA receptor 1 (GluN1) subunit to detect this obligatory subunit of functional NMDA receptors (1:100), and 3) guinea pig polyclonal antibody against $GABA_A$ receptor γ2 subunit (1:300). All primary antibodies were purchased from Synaptic Systems (Germany). We also tested the specificity of each antibody by Western blot (following Kurien et al., 2011; Alegria-Schaffer et al., 2009) and the specific bands are shown in Figure 2. The final immunolabeling patterns that we obtained quite agree with previous images found in referential descriptions (Sanes and Jessell, 2000, 2013).

The primary antibodies were labeled with anti-rabbit IgG Alexa 594 (1:200), anti-mouse IgG Alexa 405 (1:200), and anti-guinea pig IgG Alexa 647 (1:400) for 2h at 4ºC,

respectively. Secondary antibodies were purchased from Invitrogen (USA). Due to the critical possibility of “merged” laser wavelengths and misleading results, data for these glutamatergic and GABAergic receptors were obtained from MePD neurons without the use of additional fluorescent antibodies.

Brains were sectioned and intially processed as abovementioned. The sections were incubated with primary antibodies against receptors GLUR1-4, GluN1, and $GABA_A$ diluted in 0.5% BSA and TBST overnight at 4°C. Each section was incubated with the primary antibodies for the 3 receptors tested here at the same time. These primary antibodies were washed in 0.5% BSA, labeled with IgG conjugated with their respective Alexas diluted in 0.5% BSA and TBST for 2 h at 4°C, and washed in TBS. Controls were incubated in the same way ommitting the primary antibodies (data not shown).

DiI was applied on the surface of the ST as well. One dendritic branch/neuron was imaged on 8 different neurons in 4 rats. The acquisition of multiple labeling was done sequentially with appropriate band pass filters to avoid cross-talk between fluorochromes under confocal microscopy.

Confocal Microscopy

This method was described in details elsewhere (Rasia-Filho et al., 2010). Images of each neuron were obtained from a confocal microscope (SP-5 AOBS, Leica Microsystems GmbH, Germany) with a plan-apochromat 40×/1.25 oil-immersion lens. The Z-stack acquisition was done at 0.33 µm step size. Lhx subpopulations and DiI were imaged using Helium/Neon laser 647 (red, 660-800nm) and 543 (green, 555-590nm), respectively. For imaging the GluR1-4, GluN1, and $GABA_A$-γ2 receptors components, laser wavelenghts were 594 (orange), 405 (blue), and 647 (red), respectively.

All images were obtained with a resolution of 2048 x 2048 pixels per frame with 4 times zooming, avoiding over and undersaturated pixels, which generated a voxel size

approximately 55 x 55 x 300 nm. The images of each neuron, dendrites and spines were three-dimensionally (3D) reconstructed using the LAS AF software (Leica Microsystems, Germany). Each spine studied was observed along the “z” axis to evaluate its morphology. Dendritic spines of the Lhx-expressing subpopulations of neurons were classified according to their shape as thin, stubby/wide, mushroom or “others”, which included ramified spines (based on Peters and Kaiserman-Abramof, 1970; Bourne and Harris, 2007; Brusco et al., 2010). Intermediate types were included in one of these cathegories according to the most evident aspects of their head and neck. Spine density was calculated as the number of spines per micrometer of 3D-measured proximal dendritic segments ranging 23 to 45 µm in all subpopulations.

Proximal and distal dendrites, respectively recognized by their origin from the cell body or by their characteristic tapered diameter, were evaluated for the presence and specific location of imunolabeling for the 3 receptors tested here (Table 2, Figure 6). Post-synaptic receptors and DiI labeled spines were considered to be in contact when their pixels were at the same focal plane with no pixel background in between, or if there was an overlap between the DiI yellow pixels and the blue, magenta and/or red puncta of the receptors labeling in at least one focal plane (Deng and Dunaevsky, 2005; Brusco et al. 2010). According to this rule, direct visual observations of each spine in each neuron was done to determine the presence of the studied receptors on the different spine types and on dendritic shafts. Representative results are shown below. We were interested in probe occurrence and found empirically that receptors immunolabeled puncta co-occurred in different proportions in dendritic spines and shafts, varying within and between structures. Then, we did not test the colocalization of receptors by the Pearson’s linear correlation coefficient to avoid poor measures and misleading conclusions (see relevant and critical methodological comments in Dunn et al., 2011).

In all pictures, only background and contrast were adjusted using Adobe Photoshop 8.0 software (USA).

Statistical Analysis

The number of each dendritic spine morphology of the 3 Lhx-expressing subpopulations of neurons was submitted to a two-way analysis of variance (ANOVA) test. Data regarding the density of dendritic spines of these subpopulations were submitted to a square root transformation, and after being tested for normality (Kolmogorov-Smirnov test) and homocedasticity (Bartlett test), were submitted to an one-way ANOVA test. The statistical level of significance was set as $p \leq 0.05$.

Results

The immunofluorescence for the 3 LIM homeobox transcription factors (Lhx6, Lhx5, and Lhx9) was restricted to the neuronal cell body and nucleus. MePD neurons had the cell body and variable lengths of DiI labeled dendrites showing usually numerous dendritic spines. The coincidence of fluorescence for a specific Lhx in the nucleus together with the DiI labeling of the neuronal cell body, dendrites, and spines, guided the selection of cells to be further studied in each subpopulation. The number of sampled neurons was compatible with previous results of MePD cell subtypes (Carney et al., 2010; Bian, 2013).

Lhx 6-, Lhx5-, and Lhx9-expressing neurons in the MePD

Representative Lhx6-immunoreactive neurons in the MePD are shown in Figure 3. These were basically multipolar neurons with round, ovoid or fusiform cell bodies and few primary dendrites (frequently only two dendritic shafts) and sparingly branched trees

(Figure 3A-D). Spines in clusters or isolated were found on cell bodies and dendrites. These spines showed a continuum of shapes and sizes (Figure 3E-F). Axons were not identified in these cells.

Representative Lhx5-immunoreactive neurons are shown in Figure 4. These were also multipolar cells (Figure 4A-D), but some usually presented thin ramified dendritic branches (Figure 4B-C). There were pleomorphic spines with an apparent similar distribution as in the Lhx6 subpopulation (Figure 4E-F).

Representative Lhx9-immunoreactive neurons are shown in Figure 5. They were also multipolar neurons (Figure 5A-C), but with some large fusiform or angular cell bodies (Figure 5A-B), and small neurons with various radiating dendritic branches arising from a round cell body (Figure 5D). Pleomorphic spines were also observed in these neurons (Figure 5E-F).

Dendritic spine categories and density in Lhx neuronal subpopulations

The number of dendritic spines on each Lhx-expressing neurons are shown in Table 1. Thin and stubby/wide spines constitute the highest proportion of all spines in the LhX neuronal subpopulations (85-78%), whereas mushroom spines and other morphologies accounted for fewer values. On the other hand, the total number of filopodia was variable, but notably very low, in the 3 different neuronal subpopulations of the MePD. I.e., median values (and interquartile ranges) were 0.5 (0/2), 1 (0/2), and 0 (0/1) filopodia on the studied dendrites of Lhx6-, Lhx5-, and Lhx9-expressing neurons, respectively.

The different origins/Lhx transcription factors expressed by the MePD neurons did not affect the morphology or number of proximal dendritic spines of these neurons. There was no predominance of any spine type in those neuronal subpopulations [$F(6, 140)= 0.51$; $P= 0.80$] nor subpopulations have a statistically significant difference in the density

of proximal spines [F(2,140)= 0.55; P= 0.58]. However, there were significantly more thin and stubby/wide spines over mushroom and other spine types [F(3,140)= 91.35; P < 0.001], indicating that the spine type influenced the total spine number in these different neuronal subpopulations.

Mean values for the density of proximal dendritic spines of Lhx6, Lhx5, and Lhx9 expressing neurons are shown in Table 1. There was an evident variability in the values for dendritic spine density of the MePD Lhx neuronal subpopulations (Table 1, untransformed data). This could be attributed to an overlap of “low density spiny neurons” (arbitrarily considered as a neuron with a spine density below 1 spine/dendritic µm) and “more densily spiny neurons” (with more than 1 spine/dendritic µm; see parallel data in DiFia et al., 1976; Feldman, 1984) in each subpopulation. In this regard, Lhx6-immunoreactive neurons had a spine density ranging from 0.6 to 2.2 spines/dendritic µm. Lhx5-immunoreactive neurons had a spine density ranging from 0.7 to 1.9 spines/dendritic µm. In these both subpopulations, approximately 60% of the sampled cells could be classified as densily spiny neurons. Lhx9-immunoreactive neurons had a spine density ranging from 0.4 to 2.3 spines/dendritic µm, but only 2/12 (17%) neurons had 0.8 $\pm$ 0.2 spine/dendritic µm whereas 10/12 (83%) neurons had higher values of 1.6 $\pm$ 0.5 spines/dendritic µm. Nevertheless, no statistically significant difference was found for the dendritic spine density among subpopulations in the MePD [F (2,34) = 1.72; P = 0.18].

Glutamatergic and GABAergic receptors on dendritic shafts and spines

Immunofluorescent labeling for AMPA, NMDA, and $GABA_A$ receptors were found in proximal and distal dendritic branches and in different dendritic spines in the MePD. Data are shown in Table 2 and Figure 6.

Immunoreactive puncta for glutamatergic and GABAergic receptors occurred directly on dendritic shafts along both proximal (Figure 6A, C, E) and distal (Figure 6B, D, G, H) branches. Colocalization of these receptors on adjacent segments of the same dendritic shaft was usual (Figure 6I-K, merged images in L). There was an apparent similar presence of AMPA receptors in proximal and distal dendritic shafts (Figure 6, compare A and B). The same was true for NMDA (Figure 6, compare C and D) and $GABA_A$ receptors (Figure 6, compare E, G, H).

The immunoreactive puncta for these receptors were found on differently shaped spines along proximal and distal dendrites in the MePD (Table 2). That is, dendritic spines exhibited excitatory as well as inhibitory receptors. AMPA receptors were predominant on mushroom and stubby/wide spines, whereas NMDA receptors were mostly observed on thin spines. Intestingly, $GABA_A$ receptors were found on either thin or stubby/wide spines of proximal dendrites, whereas, at distal branches, they were basically on thin spines (Table 2). The receptors were rather found on the spine head and, less frequently, on the spine neck, with the exception of $GABA_A$ receptors that were often found on spine necks at distal dendrites.

Putative multisynaptic spines were identified in the MePD. Double-labeling for AMPA and NMDA receptos was detected on stubby/wide and mushroom spines (e.g., Figure 6 L); for AMPA and $GABA_A$ receptors on all spine types (e.g., Figure 6 N), and for NMDA and $GABA_A$ receptors on thin and stubby/wide spines (e.g., Figure 6 L). These 3 receptors were found concomitantly on stubby/wide, thin, and mushroom spines at proximal and distal dendritic branches (Table 2).

Finally, no filopodium appeared labeled for any of the receptors tested here (Figure 6 O).

Discussion

The present data provides new knowledge about the cellular composition and on the spines and synaptic complexities in the rat MePD. Four results are available indicating that 1) three histogenetically distinct Lhx6-, Lhx5-, and Lhx9-expressing neurons are present in the MePD of adult rats, 2) these different local subpopulations display a similar percentage of dendritic spine shapes and density; 3) glutamatergic (AMPA and NMDA) receptors and $GABA_A$ receptors are located along proximal and distal dendritic shafts as well as on spines of MePD neurons, and 4) dendritic spines have excitatory, inhibitory and multisynaptic contacts with colocalization of these receptors to form diverse synaptic units.

Different Lhx-expressing subpopulations in the MePD

Our results from adult rats are in accordance with the reported presence of LIM homeodomain transcription factors in subpopulations of MePD neurons of mouse embryos (Bupesh et al., 2011) and adult mouse brain (Choi et al., 2005). However, a difference was evidenced. In adult mice, Lhx5-expressing cells were specifically observed within the anterior medial amygdala and the Lhx9 ones were found in the posteroventral medial amygdala, but not in the MePD (Choi et al., 2005). Here, we observed the expression of the Lhx 6, 5, and 9 in the adult rat MePD neurons. This can indicate a species-specific difference between the MePD histogenetic components of mice and rats with likely additional functional implications for the MePD of rats. In mice, Lhx6-expressing neurons were related with reproductive behavior whereas both Lhx5- and Lhx9-immunolabeled cells were involved with defensive behavior (Choi et al., 2005). In rats, the MePD has a clear modulatory role on male sexual behavior (Harris and Sachs, 1975; Rasia-Filho et al., 1991; Newman, 1999; de Castilhos et al., 2006; Rasia-

Filho et al., 2012b). On the other hand, ibotenic acid lesion of the rat MePD did not block anxiety-like behavior tested in the elevated plus maze or the innate fear expression of rats faced to a live caged cat, although microinjection of somatostatin in the MePD dramatically reduced rat aggressive display in a resident-intruder paradigm (Rasia-Filho et al., 2012b). Other experimental data can add to this scenario and advance the relevant proposition of the rat MePD as a nodal point for social behavior neural network (Newman, 1999; Bian, 2013; additional comments in Rasia-Filho et al., 2012b and references therein).

We propose that further scholarly debate can reconcile the nomenclature for the different neurons in the MePD based on histogenetic markers, general morphology, membrane intrinsic electrophysiological properties, diverse expression patterns (e.g., for classical neurotransmitters, neuropeptides, binding proteins and/or enzymes), connectional and functional features. Formerly, no general aspect allowed the reliable identification of responsive or not responsive cells to gonadal hormone actions in MePD neurons of rats (Nabekura et al., 1986; Rasia-Filho et al., 1999) or hamsters (Gomez and Newman, 1991). Nevertheless, as demonstrated by the Golgi method, the diversity of the neuronal population in the adult rat MePD of both sexes is basically comprised of multipolar cells classified as bitufted (suggested to be not “bipolar”, as per Ramón y Cajal’s classical description, 1909) and stellate neurons (Alheid et al., 1995; Gomez and Newman, 1991; McDonald, 1992; Rasia-Filho et al., 1999; Cooke et al., 2007; Dall’Oglio et al., 2008a; Arpini et al., 2010; Brusco et al., 2010; Rasia-Filho et al., 2012a,b; Bian, 2013). Unlike the basolateral and cortical amygdaloid nuclei, the cell types of the rat central and medial amygdala do not resemble those of the cerebral cortex (McDonald, 1992;1998). Additional characterization is also warranted to typify intrinsic or projecting neurons in the MePD of rats. For example, to reveal whether the small, spiny and more

ramified stellate neuron expressing Lhx9 is a class of local interneuron (Figure 4D) and to what extent Lhx6-and Lhx5-expressing cells are basically projecting neurons, as described in mice (Choi et al., 2005). The former possibility is relevant since one third of all medial amygdala (MeA) synapses are of local origin (Nishizuka and Arai, 1983). At this moment, the extent of DiI labeling and the number of fluorescent channels that can be captured simultaneously under confocal microscopy (using fluorophores with non-overlapping emission spectras to avoid "cross talk" of the DiI dye, the 3 Lhx fluorescent antibodies and the 3 fluorescent receptors or even other neurotransmitters markers) did not allow us to provide additional analysis regarding the phenotype of the Lhx-expressing neurons such as glutamate vs GABAergic neurons or interneurons vs projecting neurons.

Furthermore, species differences should also be beared in mind since, compared to mice, there is no clear morphological evidence for striatum-like medium spiny stellate neurons in the MePD of adult Wistar rats [see Bennur et al. (2007) - mouse; compare Marcuzzo et al. (2007) - rat; Dall'Oglio et al. (2008a) - rat; additional relevant data in Bian et al. (2008) – mouse]. In mice, some neurons in the posterior part of the MeA resemble pyramidal neurons from the piriform cortex (Bian et al., 2008), although Niimi et al. (2012) found principal MeA spiny neurons typically projecting at least two dendrites. They also found no obvious morphological differences among neurons of different electrophysiological firing patterns (i.e., type I - regular spiking neurons, 56% of the recorded cells; type II – adapting neurons, 3%; and type III - fully accomodating neurons, 12%; Niimi et al., 2012). Recently, Bian (2013), using whole-cell patch clamp recordings in GFP expressing mice to study the GABAergic neurons in the MePD, systematically found that they were not homogenous and could be divided into three subtypes based on electrophysiological, morphological, and connectivity properties: the bitufted and stellate projecting and intrinsic GABAergic neurons.The detailed neuronal morphological analysis

of these GABAergic neurons revealed two types of projection neurons and a third type of smooth-dendrite interneurons. Carney et al. (2010) reported 3 types of MePD neurons identified by their developmental histogenetic characterization and whole-cell patch-clamp recordings also in mice. Basically, types I and II neurons were both immunoreactive for the inhibitory markers neuronal nitric oxide synthase and Forkhead box transcription factor FoxP2 whereas type III neurons were immunonegative for them. At a first glance, it could be suggested that classes I and III neurons correspond to Golgi-impregnated bitufted neurons whereas class II neurons resemble Golgi-impregnated stellate ones [see Figure 8 in Carney et al. (2010) and compare to those in Dall'Oglio et al. (2008a) and Arpini et al. (2010)]. These data reinforce the propositon for an uniform nomenclature of the MePD cells bringing together their diverse relevant features.

Dendritic spines in the MePD

The complex modulation of the dendritic spine density in the MePD was previously evidenced under different experimental conditions (sex differences, estrous cycle and motherhood effects – Rasia-Filho et al., 2004; stressful stimulus on possibly inhibitory spines – Marcuzzo et al., 2007; prepubertal and adult gonadal steroids withdrawal and/or hormonal therapy – Cunningham et al., 2007; de Castilhos et al., 2008; Cooke and Woolley, 2009). Mean dendritic spine densities found here are in accordance with a previous descriptive report of randomly sampled left MePD neurons under the same DiI dye methodological approach and confocal microscopy (i.e., 1.15 ± 0.67 spines/dendritic µm; Brusco et al., 2010). This similarity would be attributed to the high proportion of Lhx6-expressing neurons that normally compose the MePD (~ 80%; Choi et al., 2005).

The spine structure-function coupling and the impact of different spine shapes on synaptic stability and plasticity have been frequently reexamined (Nimchinsky et al., 2002;

Deng and Dunaevsky, 2005; Arellano et al., 2007; Bourne and Harris, 2007; Kasai et al., 2010; García-López et al., 2010; Segal, 2010; Lee et al., 2012). In adult hippocampus and neocortex, approximately 65% of spines are thin, 25% are mushroom shaped and 10% have “immature” shapes represented by stubby, multisynaptic, filopodial or branched spines (as described by Bourne and Harris, 2007). Here, thin spines were the most abundant type (~ 44%) on proximal dendrites of MePD neurons. If these thin spines are functionally and morphologically more labile than mushroom ones (Bourne and Harris, 2007), the three Lhx neuronal subpopulations in the MePD might have a similar inherent plasticity to deal with synaptic inputs. Mushroom spines were ~14% and would represent more stable, long-lasting synaptic arrangements for these neurons. Interestingly, proximal stubby spines accounted for ~37% in the present sample and their shape would represent a synaptic site with fewer biochemical and electrical isolation from the parent dendrite. All of these proximal spines are located strategically close to the cell body and would adjoin relevant afferent synaptic information to affect the neuronal firing output of the MePD neurons (Dall’Oglio et al., 2008a).

We also assume that “the morphological heterogeneity of spines even for a local small portion of the dendrite is consistent with the idea that synapse strength is regulated locally, at the level of a single spine” (Arellano et al., 2007). Then, proximal dendritic spines shape and density can contribute to the synaptic processing in each of these histogenetically diverse Lhx-expressing subpopulations of MePD neurons. However, more evident functional differences among these neurons would reside in other cellular phenotype. Electrophysiological recordings (Nuriya et al., 2006; Ivenshitz and Segal, 2010; Bian, 2013), multi-photon laser-scanning microscopic studies (Lee et al., 2012), and the combination of tract-tracing methods and intracellular dye injections (Lanciego

and Wounterlood, 2011) are highly desirable to test these functional hypotheses for the dendritic spines in the adult rat MePD.

Excitatory and inhibitory receptors on MePD dendritic shafts and spines

There is a notable presence of immunolabeled puncta for glutamatergic and GABAergic receptors on dendritic shafts and spines with possible functional implications for the MePD. Dendritic shaft synapses can be significantly efficient and reliable to produce postsynaptic potentials (Ivenshitz and Segal, 2010), and both dendritic shafts and spines can show different densities of glutamatergic receptors. For example, thin spines can have a structural flexibility, be formed or disappear relatively rapidly according to the incoming synaptic activity, and anchor more NMDA receptors whereas mushroom spines have larger postsynaptic densities, contain more AMPA receptors, regulate calcium levels locally, and can have polyribosomes for local synthesis of proteins (reviewed in Kasai et al., 2003; Bourne and Harris, 2007). Although most spine contacts are indeed excitatory, it must not be dismissed the presence of GABAergic terminals and receptors relevant for fine information processing and a possible source of postsynaptic variability (Keller, 2002; Klausberger and Somogyi, 2008; Pereno et al., 2011). In addition, structural dynamics and receptor trafficking in dendritic spines are crucial for synaptic plasticity (Kasai et al., 2010). Glutamate and GABA receptors have been shown to move rapidly around the neuron membrane modifying the number and composition of receptors that are available to respond to released neurotransmitters (Collingridge et al., 2004; Lin et al., 2004). These changes can be quickly determined by a local intracellular pool of fast recycling receptors available at synapses that are regulated by proteins and signaling mechanisms that associate or interact with receptor subunits, contributing to the synaptic plasticity (Collingridge et al., 2004).

Excitatory and inhibitory synapses on the spine head or neck were already found in the adult rat MePD studied in 3D reconstructions of electron microscopy serial-sections (Brusco, 2012). Here, we found spines of different shapes with glutamatergic and GABAergic receptors. In effect, AMPA receptors were usually found on mushroom and stubby/wide spines whereas NMDA receptors occurred on thin spines, either in proximal or in distal dendritic branches in the rat MePD. $GABA_A$ receptors had other distribution. Although they appeared to be similarly present on proximal and distal dendrites, $GABA_A$ receptors were found on thin and stubby/wide spines of proximal dendrites, but mainly on thin spines at distal branches. This would indicate a different impact of proximal and distal inhibitory synapses on MePD neurons.

Interestingly, behaviorally-relevant afferences to the MePD involve distinctly coded glutamatergic and GABAergic inputs. Male rats have dendrites radiating and extending preferently to the surrounding “molecular layer” close to the MePD (Dall’Oglio et al., 2008a), a cell-sparse rim that contains fibers from the main and accessory olfactory bulbs (Scalia and Winans, 1975; Pro-Sistiaga et al., 2007). AMPA-mediated, more than NMDA, monosynaptic excitatory postsynaptic currents can be evoked from the accessory olfactory bub afferents coming from ventral pathways (Niimi et al., 2012). Pheromonal socially-relevant processed information are sent to the rat MePD via GABAergic fibers from the intercallated amygdaloid nuclei (Meredith and Westberry, 2004). Likewise, a strong colocalization of Fos with GABA, calretinin, and calbindin was observed in the vomeronasal system-medial extended amygdala and indicates the important role of inhibitory control of the incoming pheromonal and olfactory sensorial inputs on the rat MePD neuronal excitability (Pereno et al., 2011). The functional consequence of this local synaptic modulation involves direct and indirect GABAergic projections to specific hypothalamic nuclei (Choi et al., 2005), which can inhibit or disinhibit circuitries involved

with the display of reproductive behaviors (Rasia-Filho et al., 2012a,b). The MePD GABAergic neurons can also establish bidirectional connections with neighboring amygdaloid nuclei in mice (Bian, 2013). This panorama might correspond to the majority of Lhx6-expressing cells within the MePD. Besides, neurons in the mice MePD, whose histogenetic identity remains to be determined, can receive direct excitatory input from upstream sensory areas and inhibitory inputs from local GABAergic neurons and, then, project glutamatergic fibers to the hypothalamic ventromedial nucleus (Bian et al., 2008).

Immunolabeling for single or multiple receptors were found on dendritic spines. The existence of multisynaptic spines in the MePD was suggested by the presence of various puncta of synaptophysin, a pre-synaptic protein, upon the same spine (Brusco et al., 2010). Multisynaptic spines are complex postsynaptic elements (Popov and Stewart, 2009) and, in the MePD, can deal with one excitatory and another inibitory receptor. It is not currently known whether different axon terminals contact AMPA and NMDA receptors on the same spine or the detailed involvement of these multisynaptic spines on the function of MePD neurons. Finally, no filopodia beared AMPA, NMDA or $GABA_A$ receptors, which might suggest that these protusions lack synapses with major excitatory and inhibitory neurotransmitters. Non-synaptic filopodia were previously observed in ultrastructural studies of the adult rat MePD (Brusco, 2012).

In conclusion, the present data evidenced relevant features on the cellular composition and the complexity of dendritic shafts and spines in the adult rat MePD. The presence and distribution of glutamatergic and GABAergic synaptic receptors on dendrites can be useful to understand the selection of cell assemblies, synaptic temporal dynamics, and neural circuits oscillations (Klausberger and Somogyi, 2008). They can also be useful in directing future studies of the MePD connectivity and function not only under normal circunstances, for sensorial processing of pheromones or other social cues,

sexual behavior modulation, stress responses, memory and learning, but also for comparisons in pathological conditions that compromise this forebrain area.

Legends

Table 1 – Percentage of different dendritic spine type and density (values are mean ± standard deviation) of three different Lhx-expressing neuronal subpopulations in the posterodorsal medial amygdala of adult male rats.

Table 2 – Number and distribution of glutamatergic (AMPA GLUR1-4 and NMDA GluN1) and GABAergic ($GABA_A$ γ2 subcomponent) receptors on the head (H) and neck (N) of differently shaped spines from proximal and distal dendritic branches. Neurons were sampled from the posterodorsal medial amygdala of adult male rats and studied using combined immunofluorescent labeling and DiI dye under confocal microscopy. Stubby/wide spines characteristically did not display an apparent neck, then indicated by "-" to mean that absence. Colocalization refers to the presence of more than one of these receptors on the head or on the head and neck of the same spine in each studied type. More details about colocalization are presented in the text.

Figure 1 – **(Left)** Cresyl violet staining of a coronal brain section shows the ventral location of the posterodorsal medial amygdala in the adult male rat brain (MePD, 3.30 mm posterior to the bregma). **(Right)** Schematic diagram of a matched coronal brain section adapted from the atlas of Paxinos and Watson (1998). opt: optic tract, st: stria terminalis. Spatial coordinates: D, dorsal; V, ventral; M, medial; L, lateral. Scale bar = 800 µm. Reprinted from Brusco J, Dall'Oglio A, Rocha LB, Rossi MA, Moreira JE, and Rasia-Filho A.A. "Descriptive findings on the morphology of dendritic spines in the rat medial amygdala". Neuroscience Letters 483, 152-156, 2010. Copyright with permission from Elsevier (no. 3097760021389).

Figure 2 – **(A)** Immunoreactivity to antibodies against the Lhx6, Lhx5, and Lhx9 transcription factors and **(B)** for receptors AMPA GLUR1-4, NMDA GluN1, and $GABA_A$ γ2 subcomponent in the cerebral cortex of adult male rats as revealed by the Western blot technique.

Figure 3. (A-F) Representative examples of immunolabeled Lhx6-expressing neurons (cell nucleus in blue, turned green due to the sobreposition with the DiI dye color) in the posterodorsal medial amydala of adult male rats. Images are three-dimensional reconstructions with combined use of DiI dye fluorescence (yellow) to reveal details of neuronal morphology under confocal microscopy. Spiny neurons have cell bodies with round (**A**, 2/12 sampled cells), ovoid (**B** and **C**, 2/12 and 4/12 cells, respectively) or fusiform (**D**, 4/12 cells) shapes, few primary dendrites, and sparse proximal branching points. (**E**, details at higher magnification in **F**) Three-dimensional reconstructed images to demonstrate the density and shape of dendritic spines commonly found on these Lhx6 neurons. A continuum of pleomorphic dendritic spines (arrows) were observed in this neuronal subpopulation. Spines were classified as thin (t), stubby/wide (s), "mushroom"-like (m), and "others" (o). Scale bar = 5 µm.

Figure 4. (A-F) Representative examples of immunolabeled Lhx5-expressing neurons (cell nucleus in red) in the posterodorsal medial amydala of adult male rats. Images are three-dimensional reconstructions with combined use of DiI dye fluorescence (yellow) to reveal details of neuronal morphology under confocal microscopy. Spiny neurons have cell bodies with triangular (**A**, 4/14 sampled cells), fusiform (**B** and **C**, 5/14 and 2/14 cells, respectively) or multiangular (**D**, 3/14 cells) shapes, few primary dendrites (except in **D**), and sparse proximal branching points. (**E,** details at higher magnification in **F**) Three-

dimensional reconstructed images to demonstrate the density and shape of dendritic spines commonly found on these Lhx6 neurons. A continuum of pleomorphic dendritic spines (arrows) were observed in this neuronal subpopulation. Spines were classified as thin (t), stubby/wide (s), “mushroom”-like (m), and “others” (o). Scale bar = 5 μm.

Figure 5. (A-F) Representative examples of immunolabeled Lhx9-expressing neurons (cell nucleus in magenta) in the posterodorsal medial amydala of adult male rats. Images are three-dimensional reconstructions with combined use of DiI dye fluorescence (yellow) to reveal details of neuronal morphology under confocal microscopy. Spiny neurons have cell bodies with fusiform (**A** and **B**, 5/12 and 2/12 sampled cells, respectively), triangular (**C**, 2/12 cells) or elongated (**D**, 3/12 cells) shapes, few primary dendrites, and sparse proximal branching points (except in **B**). (**E**, details at higher magnification in **F**) Three-dimensional reconstructed images to demonstrate the density and shape of dendritic spines commonly found on these Lhx6 neurons. A continuum of pleomorphic dendritic spines (arrows) were observed in this neuronal subpopulation. Spines were classified as thin (t), stubby/wide (s), “mushroom”-like (m), and “others” (o). Scale bar = 5 μm.

Figure 6. Representative examples of three-dimensional reconstructed images combining DiI dye (yellow) to evidence spiny dendrites and the presence of immunofluorescent labeling puncta for (**A**, **B**) AMPA GLUR1-4 (red) and (**C**, **D**) NMDA GluN1 (blue) glutamaterigic receptors and the (**E**-**H**) $GABA_A$ γ2 subcomponent receptor (magenta) under confocal microscopy. These are neurons from the posterodorsal medial amygdala of adult male rats. Receptors on close contact to dendritic shafts and spines were found in both proximal (**A**, **C**, **E**) and distal (**B**, **D**, **G** and **H**) branches. Note the presence of the three immunolabeled receptors puncta on differently shaped spines. (**F**) Higher magnification image illustrates immunoreactive colored puncta for the $GABA_A$ receptor on the spine head and on the spine neck of thin spines (arrows). (**I-K**) Three-dimensional reconstructed image of the same DiI dye fluorescent dendrite demonstrates the coexistence of imunolabeling for AMPA (**I**), NMDA (**J**), and $GABA_A$ receptors (**K**) on the same branch. These three images (**I-K**) were merged in (**L**). Note the presence of immunoreactive puncta for the glutamatergic and GABAergic receptors in close apposition to dendritic shafts and on spines of different shapes. In **L**, examples of colocalization of AMPA and NMDA receptors or NMDA and $GABA_A$ receptors on spines. (**M**) Colocalization of AMPA and $GABA_A$ labeling on the same reconstructed DiI dye immunofluorescent dendritic branch. Dendritic spines (asterisks) and dendritic shaft (arrowhead) appeared contacted by immunoreactive puncta for both receptors. At higher magnification (**N**), AMPA and $GABA_A$ receptors are colocalized on the same dendritic spine (arrows). (**O**) Reconstructed DiI dye immunofluorescent dendritic branch showing a protuding filopodium without overlapping immunolabeled puncta for glutamatergic or GABAergic receptors on the same focal plane. Scale Bar = 2 μm.

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Conflict of Interest

The authors declare no actual or potential conflict of interest.

References

Alegria-Schaffer A, Lodge A, Vattem K (2009) Performing and optimizing Western blots with an emphasis on chemiluminescent detection. Methods Enzymol 463:573-599

Alheid GF, de Olmos JS, Beltramino CA (1995) Amygdala and extended amygdala. In: The Rat Nervous System. Paxinos G (ed). Academic Press, San Diego, pp 495–578

Arellano JI, Benavides-Piccione R, Defelipe J, Yuste R (2007) Ultrastructure of dendritic spines: correlation between synaptic and spine morphologies. Front Neurosci 1:131-143

Arpini M, Menezes IC, Dall'Oglio A, Rasia-Filho AA (2010) The density of Golgi-impregnated dendritic spines from adult rat posterodorsal medial amygdala neurons displays no evidence of hemispheric or dorsal/ventral differences. Neurosci Lett 469:209–213

Bennur S, Shankaranarayana Rao BS, Pawlak R, Strickland S, McEwen BS, Chattarji S (2007) Stress-induced spine loss in the medial amygdala is mediated by tissue-plasminogen activator. Neuroscience 144:8–16

Bian X (2013) Physiological and morphological characterization of GABAergic neurons in the medial amygdala. Brain Res, http://dx.doi.org/10.1016/j.brainres.2013.03.012

Bian X, Yanagawa Y, Chen WR, Luo M (2008) Cortical-like functional organization of the pheromone-processing circuits in the medial amygdala. J Neurophysiol 99: 77-86

Blake CB, Meredith M (2011) Change in number and activation of androgen receptor-immunoreactive cells in the medial amygdala in response to chemosensory input. Neuroscience 190:228-238

Bourne JN, Harris KM (2007) Do thin spines learn to be mushroom spines that remember? Curr Op Neurobiol 17:381-386

Brusco J (2012) Amígdala medial de ratos ao longo do ciclo estral: espinhos dendríticos, ultraestrutura sináptica, expressão gênica e lateralidade. Ph.D Thesis, University of São Paulo (FMRP/USP), Brazil.

Brusco J, Dall'Oglio A, Rocha LB, Rossi MA, Moreira JE, Rasia-Filho AA (2010) Descriptive findings on the morphology of dendritic spines in the rat medial amygdala. Neurosci Lett 483:152-156

Bupesh M, Legaz I, Abellán A, Medina L (2011) Multiple telencephalic and extratelencephalic embryonic domains contribute neurons to the medial extended amygdala. J Comp Neurol 519:1505-1525

Carney RS, Mangin JM, Hayes L, Mansfield K, Sousa VH, Fishell G, Machold RP, Ahn S, Gallo V, Corbin JG (2010) Sonic hedgehog expressing and responding cells generate neuronal diversity in the medial amygdala. Neural Dev 27:5-14

Chen Y, Sabatini BL (2012) Signaling in dendritic spines and spine microdomains. Curr Opin Neurobiol 22(3):389-96

Choi GB, Dong H-W, Murphy AJ, Valenzuela DM, Yancopoulos GD, Swanson LW, Anderson DJ (2005). Lhx6 delineates a pathway mediating innate reproductive behaviors from the amygdala to the hypothalamus. Neuron 46:647-660

Collingridge GL, Issac JTR, Wang YT (2004) Receptor trafficking and synaptic plasticity. Nature Rev Neurosci 5: 952-962

Cooke BM, Stokas MR, Woolley CS (2007) Morphological sex differences and laterality in the prepubertal medial amygdala. J Comp Neurol 501:904-15

Cooke BM, Woolley CS (2009) Effects of prepubertal gonadectomy on a male-typical behavior and excitatory synaptic transmission in the amygdala. Develop Neurobiol 69:141-152

Coolen LM, Peters HJ, Veening JG (1996) Fos immunoreactivity in the rat brain following consummatory elements of sexual behavior: a sex comparison. Brain Res 738:67-82

Coolen LM, Peters HJPW, Veening JG (1997) Distribution of Fos immunoreactivity following mating versus anogenital investigation in the male rat brain. Neuroscience 77:1151–1161

Cunningham RL, Clairborne BJ, McGinnis MY (2007) Pubertal exposure to anabolic androgenic steroids increases spine densities on neurons in the limbic system of male rats. Neuroscience 150:609-615

Dall'Oglio A, Gehlen G, Achaval M, Rasia-Filho AA (2008a) Dendritic branching features of posterodorsal medial amygdala neurons of adult male and female rats: further data based on the Golgi method. Neurosci Lett 430:151-156

Dall'Oglio A, Gehlen G, Achaval M, Rasia-Filho AA (2008b) Dendritic branching features of Golgi-impregnated neurons from the "ventral" medial amygdala subnuclei of adult male and female rats. Neurosci Lett 439: 287-292

Dayas CV, Buller KM, Day TA (1999) Neuroendocrine responses to an emotional stressor: evidence for involvement of the medial but not the central amygdala. Eur J Neurosci 11:2312-22

DiFiglia M, Pasik P, Pasik T (1976) A Golgi study of neuronal types in the neostriatum of monkeys. Brain Res 114:245-56

de Castilhos J, Forti CD, Achaval M, Rasia-Filho AA (2008) Dendritic spine density of posterodorsal medial amygdala neurons can be affected by gonadectomy and sex steroid manipulations in adult rats: a Golgi study. Brain Res 1240:73-81

de Castilhos J, Marcuzzo S, Forti CD, Frey RM, Stein D, Achaval M, Rasia-Filho AA (2006) Further studies on the rat posterodorsal medial amygdala: dendritic spine density and effect of 8-OH-DPAT microinjection on male sexual behavior. Brain Res Bull 69:131-139

de Olmos JS, Beltramino CA, Alheid G (2004) Amygdala and extended amygdala of the rat: a cytoarchitectonical, fibroarchitectonical, and chemoarchitectonical survey. In: The Rat Nervous System. Paxinos G (ed). Elsevier Academic Press. London, pp 509-603

Deng J, Dunaevsky A (2005) Dynamics of dendritic spines and their afferent terminals: spines are more motile than presynaptic boutons. Develop Biol 277:366-377

Dong H-W, Petrovich G, Swanson LW (2001) Topography of projections from amygdala to bed nuclei of the stria terminalis. Brain Res Rev 38:192–246

Dunn KW, Kamocka MM, McDonald JH (2011) A practical guide to evaluating colocalization in biological microscopy. Am J Physiol Cell Physiol 300:C723-C742

Fan L, Hanbury R, Pandey SC, Cohen RS (2008a) Dose and time effects of estrogen on expression of neuron-specific protein and cyclic AMP response element-binding protein and brain region volume in the medial amygdala of ovariectomized rats. Neuroendocrinology 88:111-126

Fan L, Pandey SC, Cohen RS (2008b) Estrogen affects levels of Bcl-2 protein and mRNA in medial amygdala of ovariectomized rats. J Neurosci Res 86:3655-3664

Feldman ML (1984) Morphology of the neocortical pyramidal neurons. In: Cerebral Cortex. Jones EG, Peters A (eds). Plenum Press. New York, vol. 2, pp 123-200

García-López M, Abellán A, Legaz I, Rubenstein JLR, Puelles L, Medina L (2008) Histogenetic compartments of the mouse centromedial and extended amygdala base on gene expression patterns during development. J Comp Neurol 506:46-74

García-López P, García-Marin V, Freire M (2010) Dendritic spines and development: towards a unifying model of spinogenesis. A present day review of Cajal's histological slides and drawings. Neural Plasticity. doi: 10.1155/2010/769207

Gomez DM, Newman SW (1991) Medial nucleus of the amygdala in the adult Syrian hamster: a quantitative Golgi analysis of gonadal hormonal regulation of neuronal morphology. Anat Rec 231:498–509

Gréco B, Allegretto EA, Tetel MJ, Blaustein JD (2001) Coexpression of ER beta with ER alpha and progestin receptor proteins in the female rat forebrain: effects of estradiol treatment. Endocrinology 142:5172-5181

Gréco B, Blasberg ME, Kosinski EC, Blaustein JD (2003) Response of ERα-IR and ERβ-IR cells in the forebrain of female rats to mating stimuli. Horm Behav 43:444–453

Harms KJ, Dunaevsky A (2007) Dendritic spine plasticity: looking beyond development. Brain Res 12:1184:65-71

Hayashi Y, Majewska AK (2005) Dendritic spine geometry: functional implication and regulation. Neuron 46:529-532

Harris VS, Sachs BD (1975) Copulatory behavior in male rats following amygdaloid lesions. Brain Res 86:514-8

Ivenshitz M, Segal M (2010) Neuronal density determines network connectivity and spontaneous activity in cultured hippocampus. J Neurophysiol 104:1052-60

Kasai H, Matsuzaki M, Noguchi J, Yasumatsu N, Nakahara H (2003) Structure-stability-function relationships of dendritic spines. Trends Neurosci 26:360-368

Kasai H, Fukuda M, Watanabe S, Hayashi-Takagi A, Noguchi J (2010) Structural dynamics of dendritic spines in memory and cognition. Trends Neurosci 33:121-9

Keller A (2002) Use-dependent inhibition of dendritic spines. Trends Neurosci 25:541-3

Kim BG, Dai HN, McAtee M, Vicini S, Bregman BS (2007) Labeling of dendritic spines with the carbocyanine dye DiI for confocal microscopic imaging in lightly fixed cortical slices. J Neurosci Methods 162:237-43

Klausberger T, Somogyi P (2008) Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. Science 321:53-7

Kurien BT, Dorri Y, Dillon S, Dsouza A, Scofield RH (2011) An overview of Western blotting for determining antibody specificities for immunohistochemistry. Methods Mol Biol 717:55-67

Lanciego JL, Wounterlood FG (2011) A half century of experimental neuroanatomical tracing. J Chem Neuroanat 42:157-183

Lee KF, Soares C, Béïque JC (2012) Examining form and function of dendritic spines. Neural Plast. doi: 10.1155/2012/704103

Lin H, Huganir R, Liao D (2004) Temporal dynamics of NMDA receptor-induced changes in spine morphology and AMPA receptor recruitment to spines. Biochem Biophys Res Communic 316: 501-511

Marcuzzo S, Dall'Oglio A, Ribeiro MF, Achaval M, Rasia-Filho AA (2007) Dendritic spines in the posterodorsal medial amygdala after restraint stress and ageing in rats. Neurosci Lett 424:16-21

McDonald AJ (1992) Cell types and intrinsic connections of the amygdala. In: The amygdala: neurobiological aspects of emotion, memory, and mental dysfunction. Aggleton JP (ed). Wiley-Liss. NewYork, pp 67–96

McDonald AJ (1998) Cortical pathways to the mammalian amygdala. Prog Neurobiol 55: 257-332

Meredith M, Westberry JM (2004) Distinctive responses in the medial amygdala to same-species and different-species pheromones. J Neurosci 24:5719–5725

Nabekura J, Oomura Y, Minami T, Mizuno Y, Fukuda A (1986) Mechanism of the rapid effect of 17α-estradiol on medial amygdala neurons. Science 233:226-228

Neckel H, Quagliotto E, Casali KR, Montano N, Dal Lago P, Rasia-Filho AA (2012) Glutamate and GABA in the medial amygdala induce selective central sympathetic/parasympathetic cardiovascular responses. Can J Physiol Pharmacol 90:525-36

Newman SW (1999) The medial extended amygdala in male reproductive behavior. A node in the mammalian social behavior network. Ann N Y Acad Sci 877:242-57

Niimi K, Horie S, Yokosuka M, Kawakami-Mori F, Tanaka K, Fukayama H, Sahara Y (2012) Heterogeneous electrophysiological and morphological properties of neurons in the mouse medialamygdala in vitro. Brain Res 1480:41-52

Nimchinsky EA, Sabatini BL, Svoboda K (2002) Structure and function of dendritic spines. Annu Rev Physiol 64:313-353

Nishizuka M, Arai Y (1983) Intrinsic connections in the medial amygdala as revealed by complete deafferentation. Neurosci Lett 35:247-51

Nuriya M, Jiang J, Nemet B, Eisenthal KB, Yuste R (2006) Imaging membrane potential in dendritic spines. Proc Natl Acad Sci U S A 103:786-90

Paxinos G, Watson C (1998) The Rat Brain in Stereotaxic Coordinates. Academic Press, San Diego

Pereno GL, Balaszczuk V, Beltramino CA (2011) Detection of conspecific pheromones elicits fos expression in GABA and calcium-binding cells of the rat vomeronasal system-medial extended amygdala. J Physiol Biochem 67:71-85

Peters A, Kaiserman-Abramof IR (1970) The small pyramidal neuron of the rat cerebral cortex. The perikaryon, dendrites and spines. Am J Anat 127:321-356

Pfaus JG, Heeb MM (1997) Implications of immediate-early gene induction in the brain following sexual stimulation of female and male rodents. Brain Res Bull 44:397-407

Phillips-Farfán BV, Lemus AE, Fernández-Guasti E (2007) Increased estrogen receptor alpha immunoreactivity in the forebrain of sexually satiated rats. Horm Behav 51:328–334

Polston EK, Heitz M, Barnes W, Cardamone K, Erskine MS (2001) NMDA-mediated activation of the medial amygdala initiates a downstream neuroendocrine memory responsible for pseudopregnancy in the female rat. J Neurosci 21:4104-4110

Polston EK, Gu G, Simerly RB (2004) Neurons in the principal nucleus of the bed nuclei of the stria terminalis provide a sexually dimorphic GABAergic input to the anteroventral periventricular nucleus of the hypothalamus. Neuroscience 123:793-803

Popov VI, Stewart MG (2009) Complexity of contacts between synaptic boutons and dendritic spines in adult rat hippocampus: Three-dimensional reconstructions from serial ultrathin sections in vivo. Synapse 63:369–377

Pro-Sistiaga P, Mohedano-Moriano A, Ubeda-Bañon I, Arroio-Jimenez MDM, Marcos P, Artacho-Pérula E, Crespo C, Insausti R, Martinez-Marcos A (2007) Convergence of olfactory and vomeronasal projections in the rat basal telencephalon. J Comp Neurol 504:346-362

Ramón y Cajal S (1909) Histologie du Système Nerveux de l'Homme et des Vertebrés. Maloine, Paris

Rasia-Filho AA, Brusco J, Rocha LB, Moreira JE (2010) Dendritic spines observed by extracellular DiI dye and immunolabeling under confocal microscopy. Nature Protocols/Protocol Exchange. doi: 10.1038/nprot.2010.153

Rasia-Filho AA, Fabian C, Rigoti KM, Achaval M (2004) Influence of sex, estrous cycle and motherhood on dendritic spine density in the rat medial amygdala revealed by the Golgi method. Neuroscience 126:839-847

Rasia-Filho AA, Dalpian F, Menezes IC, Brusco J, Moreira JE, Cohen RS (2012a) Dendritic spines of the medial amygdala: plasticity, density, shape, and subcellular modulation by sex steroids. Histol Histopathol 27:985-1011

Rasia-Filho AA, Haas D, de Oliveira AP, de Castilhos J, Frey R, Stein D, Lazzari VM, Back F, Pires GN, Pavesi E, Winkelmann-Duarte EC, Giovenardi M (2012b) Morphological and functional features of the sex steroid-responsive posterodorsal medial amygdala of adult rats. Mini Rev Med Chem 12:1090-106

Rasia-Filho A.A., Londero R.G. and Achaval M. (1999). Effects of gonadal hormones on the morphology of neurons from the medial amygdaloid nucleus of rats. Brain Res. Bull. 48, 173-183

Rasia-Filho AA, Peres TMS, Cubilla-Gutierrez FH, Lucion AB (1991) Effect of estradiol implanted in the corticomedial amygdala on the sexual behavior of castrated male rats. Braz J Med Biol Res 24:1041–1049

Sanes JR, Jessell TM (2000) The formation and regeneration of synapses. In: Kandel ER, Schwartz JH, Jessell TM (ed) Principles of Neural Science, 4rd edn. McGraw Hill, New York, pp. 1087-1114

Sanes JR, Jessell TM (2013) The formation and regeneration of synapses. In: Kandel ER, Schwartz JH, Jessell TM, Siegelbaum SA, Hudspeth AJ (ed) Principles of Neural Science, 5rd edn.McGraw Hill, New York, 1233-1258

Scalia F, Winans SS (1975) The differential projections of the olfactory bulb and accessory olfactory bulb in mammals. J Comp Neurol 161:31-55

Segal M (2010) Dendritic spines, synaptic plasticity and neuronal survival: activity shapes dendritic spines to enhance neuronal viability. Eur J Neurosci 31:2178-2184

Shindou T, Watanabe S, Yamamoto K, Nakanishi H (1993) NMDA receptor dependent formation of long-term potentiation in the rat medial amygdala neuron in an in vitro slice preparation. Brain Res Bull 31:667–672

Simerly, RB (2004) Anatomical substrates of hypothalamic integration. In: Paxinos G (ed) The Rat Nervous System, 3rd edn. Academic Press, San Diego, pp 335–368

Simerly RB, Chang C, Muramatsu M, Swanson LW (1990) Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. J Comp Neurol 294:76-95

Tao-Cheng JH, Gallant PE, Brightman MW, Dosemeci A, Reese TS (2007) Structural changes at synapses after delayed perfusion fixation in different regions of the mouse brain. J Comp Neurol 501:731-40

Woolley CS, McEwen BS (1994) Estradiol regulates hippocampal dendritic spine density via an N-methyl-D-aspartate receptor-dependent mechanism. J Neurosci 14:7680-7687

Zehr JL, Todd BJ, Schulz KM, McCarthy MM, Sisk CL (2006) Dendritic pruning of the medial amygdala during pubertal development of the male Syrian hamster. J Neurobiol 66:578–590

Zirlinger M, Kreiman G, Anderson DJ (2001) Amygdala-enriched genes identified by microarray technology are restricted to specific amygdaloid subnuclei. Proc Natl Acad Sci U S A 98(9):5270-5

Original Research

Histogenetically distinct neuronal subpopulations, dendritic spines, and synaptic diversity in the adult rat medial amygdala

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Abstract

The posterodorsal medial amygdala (MePD) has different histogenetic origins, modulates social behaviors, and displays notable plasticity in rats. Dendritic spines represent specialized integrative cellular elements that modulate and enforce synaptic inputs. Here, we describe the shape and density of dendritic spines in the main subpopulations of MePD neurons, and the presence of glutamatergic and GABAergic receptors on both proximal and distal dendritic shafts and dendritic spines. DiI dye evidenced dendritic spines in neurons that specifically express the LIM homeobox transcription factors Lhx6, Lhx5, and Lhx9 under confocal microscopy. The most abundant spine types were thin and stubby/wide spines (~80%), and the proximal dendritic spine density varied from 0.4 to 2.3 spines/dendritic µm with no statistical difference among distinct Lhx-expressing neurons. AMPA (subunit GluR1-4), NMDA (subunit GluN1R1), and $GABA_A$ receptors immunolabeling were found on both dendritic shafts and dendritic spines. AMPA receptors were predominant on mushroom and stubby/wide spines, whereas NMDA receptors were found mostly on thin spines. $GABA_A$ receptors were located on thin or stubby/wide spines of proximal dendrites and more frequently on thin spines on distal branches. Multisynaptic spines with excitatory and inhibitory receptors were also found in the MePD. These new data evidence relevant features on the cellular composition and the complex connectivity of dendritic spines in the adult rat MePD.

Key words: Extended amygdala, Lhx transcription factor, spine shape, GLUR1-4 receptor subunit, NMDA GluN1 receptor subunit, $GABA_A$ receptors.

Introduction

The posterodorsal medial amygdala (MePD) is a component of the "extended amygdala" in the rat basal forebrain (Alheid et al., 1995; Dong et al., 2001; de Olmos et al., 2004; Dall'Oglio et al., 2008a,b) and a nodal point for emotionally-loaded stress responses (Dayas et al., 1999) and the modulation of social/reproductive behavior neural networks (Newman, 1999; Polston et al., 2004; Choi et al., 2005; Rasia-Filho et al., 2012a,b). The MePD responds to species-specific socially relevant olfactory/pheromonal (Meredith and Westberry, 2004; Pereno et al., 2011) and genitosensorial stimuli (Pfaus and Heeb, 1997), has a notable local neuroplasticity related to a high expression of sex steroid receptors (Simerly et al., 1990; Gréco et al., 2001, 2003; Rasia-Filho et al., 2004; Zehr et al., 2006; Phillips-Farfán et al., 2007; Fan et al., 2008a,b; Blake and Meredith, 2011), and amends timely hypothalamic neuroendocrine secretion (Simerly, 2004).

The discovery of the embrionic origin of MePD cells and circuitry formation in rodents provided an exciting venue for local and integrated functional neuroanatomy. The MePD is a "mosaic" composed by distinct precursor cells and assembles coexisting histogenetically (Zirlinger et al., 2001; Choi et al., 2005; García-López et al., 2008; Bupesh et al., 2011) and phenotypically different (Coolen et al., 1996, 1997; Bian et al., 2008; Carney et al., 2010; Niimi et al., 2012; Bian, 2013) subpopulations of multipolar bitufted and stellate neurons (Alheid et al., 1995; Rasia-Filho et al., 1999, 2012a,b). Specific regulatory genes for LIM homeobox (Lhx) transcription factors are expressed along the development of local cells (Choi et al., 2005; García-López et al., 2008; Bupesh et al., 2011). Confocal immunofluorescent microscopy revealed that Lhx6-immunoreactive cells constitute around 80% of all neurons in the MePD (Choi et al., 2005). These neurons

derive from the subpallial, caudoventral medial ganglionic eminence, are GABAergic expressing calbindin cells (García-López et al., 2008), and reach three interconnected hypothalamic nuclei implicated in reproductive behaviors: the medial preoptic nucleus, the ventrolateral part of the ventromedial hypothalamic nucleus and the ventral premammillary nucleus (Choi et al., 2005). Comparatively, little is known about other Lhxs immunoreactive cells in the MePD. Lhx5- and Lhx9-immunoreactive cells derive from the ventral pallium and the supraoptoparaventricular hypothalamic domain (Bupesh et al., 2011). Both Lhx5- and Lhx9-immunoreacitve neurons might be glutamatergic, although Lhx5 cells would also express calbindin, vasopressin, and oxytocin (Bupesh et al., 2011).

The study of dendritic spine shape and density on these histogenetically different Lhx-expressing neuronal subpopulations in the rat MePD can illuminate the structural basis of neural circuit function and processing of complex sensorial stimuli and social behavior display. Dendritic spines are mostly associated with excitatory connections and their heterogeneous shape and number can impose functional differences in localized biochemical signaling/compartmentalization and in the synaptic organization, strength, stability or plasticity (Hayashi and Majewska, 2005; Bourne and Harris, 2007; Harms and Dunaevsky, 2007; Kasai et al., 2010; Chen and Sabatini, 2012). In randomly sampled Golgi-impregnated MePD neurons, the density of dendritic spines is sexually dimorphic (males higher than females), vary along the estrous cycle (Rasia-Filho et al., 2004), and have a marked reduction that coincides temporally with the impairment of the sexual behavior in adult castrated males (de Castilhos et al., 2008) or a reduced rough-and-tumble playful attacks in prepubertal castrated rats (Cooke and Woolley, 2009). Furthermore, in the adult rat MePD, both glutamate and GABA modulate activities such as sexual behavior (facilitating ejaculation and reducing copulatory latency, respectively; Rasia-Filho et al., 2012b) and a selective central control of the

sympathetic/parasympathetic cardiovascular reflex responses (Neckel et al., 2012). In male rats, NMDA-mediated activation of the medial amygdala induces long-term potentiation (Shindou et al., 1993) and, in females, promotes a downstream neuroendocrine memory responsible for pseudopregnancy (Polston et al., 2001), indicating that local glutamatergic innervation can promote sexually dimorphic functions. The occurrence of glutamatergic and GABAergic receptors on dendritic shafts and spines of MePD neurons is still unknown.

The aims of this study were twofold: (1) to provide the first description of the morphology and density of dendritic spines in the different immunolabeled Lhx-expressing subpopulations of neurons in the adult rat MePD, and (2) to depict the presence and distribution of glutamatergic (AMPA and NMDA) and GABAergic ($GABA_A$) receptors on proximal and distal dendritic shafts as well as on different types of dendritic spines on MePD neurons under confocal microscopy.

Materials and Methods

Animals

Adult male Wistar rats (3–4 months old, N = 26) were housed in groups with free access to food and water in standard laboratory conditions, with room temperature kept at 22 ^{o}C in a 12 h light-dark cycle. Experimental procedures were performed in accordance with the international laws for the ethical care, the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85–23, reviewed 1985, USA) and were approved by local Ethics Committees (UFCSPA, protocol 033-10 and FMRP/USP protocol 174-11).

Experimental Procedures

All animals were anesthetized with ketamine and xylazine (80 mg/kg and 10 mg/kg, i.p., respectively) and underwent transcardiac perfusion with 200 ml of 0.9% NaCl followed by 300 ml of 1.5% paraformaldehyde in 0.1 M phosphate buffer solution, pH 7.4 (PBS; Kim et al., 2007; Rasia-Filho et al., 2010), initially very fast and progressively slowing to keep the fixative running for over 15 min (Tao-Cheng et al., 2007). The brains were maintained in the same fixative solution for 1 h at room temperature (RT) and were sectioned at 80 µm-thick slices in PBS using a vibratome (Leica, Germany; Brusco et al., 2010). The MePD was found 3.0–3.30 mm posterior to the bregma, delimited laterally to the optic tract and ventrally to the stria terminalis (ST; Paxinos and Watson, 1998; de Olmos et al., 2004; Figure 1).

Lhx6, Lhx5, and Lhx9 Immunofluorescent Labeling

To study the presence of different subpopulations of Lhx-expressing neurons and their spine features, we utilized the following goat polyclonal primary antibodies against: 1) Lhx6 (N-19) to detect N-terminus of Lhx6 of human origin, 2) Lhx5 (C20) to label C-terminal of Lhx5 of human origin, and 3) Lhx9 (N-20) to detect N-terminus of Lhx9 of human origin. Antibodies were purchased from Santa Cruz Biotechnology Inc. (USA) and were diluted 1:100. Primary antibodies were labeled with anti-goat IgG Alexa 647 (1:200; Invitrogen, USA). The specificity of each antibody was verified by Western blot (according to Kurien et al., 2011). I.e., we performed additional Western blot analysis following the basic procedures described previously (Alegria-Schaffer et al., 2009) and the specific bands obtained from these control experiments are shown in Figure 2. In addition, the immunolabeling patterns for the distinct Lhx neurons were identical to those reported in a previous referential report (Choi et al., 2005).

Coronal brain slices (80 µm-thick) containing the MePD were obtained using a vibratome (Leica VT 1000S, Leica Microsystems GmbH, Germany). The sections were washed with 0.02 M Tris Buffer Solution (TBS), and immersed in 0.2% Tween in TBS (TBST) during 6 h at 4ºC. The slices were blocked with 2% bovine serum albumine (BSA) in TBST for 2 h, incubated with primary antibodies diluted in 0.5% BSA in TBST overnight at 4°C, and secondary antibodies for 2h at 4ºC. Every experiment included controls with the omission of primary antibodies (data not shown), besides the other above-mentioned control procedures.

After antibodies incubation, sonicated fine powdered carbocyanine dye DiI (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate; Molecular Probes, Invitrogen, USA) was placed over the ST for 24 h at 4°C (Rasia-Filho et al., 2010). Sections were washed in distilled water, mounted with Fluoromount (EMS, USA), and visualized under confocal microscopy, as described below. Each rat provided one brain slice for each Lhx immunoreaction tested. Both hemispheres were used. Due to technical restrictions and the inherent difficulties for obtaining acceptable multi-labeled fluorescent neurons in each brain section, no quantitative comparisons were done for the right and left MePD. For the aims of the present work, it is worth noting that previous experimental data did not evidence hemispheric lateralization for the MePD dendritic spine density in adult rats (Arpini et al., 2010).

Laser wave length of the DiI fluorescence did not overlay that one of the Alexa linked with the Lhx antibodies. Data were obtained from a total of 12, 14, and 12 different Lhx6, Lhx5 and Lhx9 neurons from 5, 8, and 9 rats [mean ± standard deviation (SD), 2.4 ± 0.5, 1.3 ± 0.5, 1.7 ± 0.7 neurons per rat], respectively. Including criteria were: 1) neurons must be located within the MePD, avoiding its ultimate borders; 2) fluorescence for both Lhx antibody and DiI must be stable and basically at the same focal plane; 3)

dendrites must be brightly fluorescent and not “tangled” with neighborhood prolongments; and, 4) spines must be clearly visbible and identifiable. Dendritic branches and spines were imaged along the first 50 µm from the soma (Rasia-Filho et al., 2004; Brusco et al., 2010; Figures 3-5). It has to be commented that the numbers generated from our confocal microscopy immunofluorescent neurons reflect the spines observed along proximal dendrites and might under-represent the actual values for entire cells (e.g., as commented in Woolley and McEwen, 1994). Nevertheless, from Golgi-impregnation studies, dendritic spines appear to have a homogeneous distribution up to more than a hundred µm away from the cell body (de Castilhos et al., 2006) and analyses of spine density have been made from data obtained along 40 µm of proximal dendrites (Rasia-Filho et al., 2004; de Castilhos et al., 2006, 2008; Marcuzzo et al., 2007; Brusco et al., 2010; Rasia-Filho et al., 2012a).

GLUR1-4, GluN1, and $GABA_A$ Immunofluorescent Labeling

To study the localization of excitatory and inhibitory receptors on MePD neurons we used: 1) rabbit polyclonal antibody against GluR1-4 subunits to label AMPA receptor (1:50), 2) mouse monoclonal antibody against the N-terminus of the NMDA receptor 1 (GluN1) subunit to detect this obligatory subunit of functional NMDA receptors (1:100), and 3) guinea pig polyclonal antibody against $GABA_A$ receptor γ2 subunit (1:300). All primary antibodies were purchased from Synaptic Systems (Germany). We also tested the specificity of each antibody by Western blot (following Kurien et al., 2011; Alegria-Schaffer et al., 2009) and the specific bands are shown in Figure 2. The final immunolabeling patterns that we obtained quite agree with previous images found in referential descriptions (Sanes and Jessell, 2000, 2013).

The primary antibodies were labeled with anti-rabbit IgG Alexa 594 (1:200), anti-mouse IgG Alexa 405 (1:200), and anti-guinea pig IgG Alexa 647 (1:400) for 2h at 4ºC,

respectively. Secondary antibodies were purchased from Invitrogen (USA). Due to the critical possibility of “merged” laser wavelengths and misleading results, data for these glutamatergic and GABAergic receptors were obtained from MePD neurons without the use of additional fluorescent antibodies.

Brains were sectioned and intially processed as abovementioned. The sections were incubated with primary antibodies against receptors GLUR1-4, GluN1, and $GABA_A$ diluted in 0.5% BSA and TBST overnight at 4°C. Each section was incubated with the primary antibodies for the 3 receptors tested here at the same time. These primary antibodies were washed in 0.5% BSA, labeled with IgG conjugated with their respective Alexas diluted in 0.5% BSA and TBST for 2 h at 4°C, and washed in TBS. Controls were incubated in the same way ommitting the primary antibodies (data not shown).

DiI was applied on the surface of the ST as well. One dendritic branch/neuron was imaged on 8 different neurons in 4 rats. The acquisition of multiple labeling was done sequentially with appropriate band pass filters to avoid cross-talk between fluorochromes under confocal microscopy.

Confocal Microscopy

This method was described in details elsewhere (Rasia-Filho et al., 2010). Images of each neuron were obtained from a confocal microscope (SP-5 AOBS, Leica Microsystems GmbH, Germany) with a plan-apochromat 40×/1.25 oil-immersion lens. The Z-stack acquisition was done at 0.33 µm step size. Lhx subpopulations and DiI were imaged using Helium/Neon laser 647 (red, 660-800nm) and 543 (green, 555-590nm), respectively. For imaging the GluR1-4, GluN1, and $GABA_A$-γ2 receptors components, laser wavelenghts were 594 (orange), 405 (blue), and 647 (red), respectively.

All images were obtained with a resolution of 2048 x 2048 pixels per frame with 4 times zooming, avoiding over and undersaturated pixels, which generated a voxel size

approximately 55 x 55 x 300 nm. The images of each neuron, dendrites and spines were three-dimensionally (3D) reconstructed using the LAS AF software (Leica Microsystems, Germany). Each spine studied was observed along the “z” axis to evaluate its morphology. Dendritic spines of the Lhx-expressing subpopulations of neurons were classified according to their shape as thin, stubby/wide, mushroom or “others”, which included ramified spines (based on Peters and Kaiserman-Abramof, 1970; Bourne and Harris, 2007; Brusco et al., 2010). Intermediate types were included in one of these cathegories according to the most evident aspects of their head and neck. Spine density was calculated as the number of spines per micrometer of 3D-measured proximal dendritic segments ranging 23 to 45 µm in all subpopulations.

Proximal and distal dendrites, respectively recognized by their origin from the cell body or by their characteristic tapered diameter, were evaluated for the presence and specific location of imunolabeling for the 3 receptors tested here (Table 2, Figure 6). Post-synaptic receptors and DiI labeled spines were considered to be in contact when their pixels were at the same focal plane with no pixel background in between, or if there was an overlap between the DiI yellow pixels and the blue, magenta and/or red puncta of the receptors labeling in at least one focal plane (Deng and Dunaevsky, 2005; Brusco et al. 2010). According to this rule, direct visual observations of each spine in each neuron was done to determine the presence of the studied receptors on the different spine types and on dendritic shafts. Representative results are shown below. We were interested in probe occurrence and found empirically that receptors immunolabeled puncta co-occurred in different proportions in dendritic spines and shafts, varying within and between structures. Then, we did not test the colocalization of receptors by the Pearson’s linear correlation coefficient to avoid poor measures and misleading conclusions (see relevant and critical methodological comments in Dunn et al., 2011).

In all pictures, only background and contrast were adjusted using Adobe Photoshop 8.0 software (USA).

Statistical Analysis

The number of each dendritic spine morphology of the 3 Lhx-expressing subpopulations of neurons was submitted to a two-way analysis of variance (ANOVA) test. Data regarding the density of dendritic spines of these subpopulations were submitted to a square root transformation, and after being tested for normality (Kolmogorov-Smirnov test) and homocedasticity (Bartlett test), were submitted to an one-way ANOVA test. The statistical level of significance was set as $p \leq 0.05$.

Results

The immunofluorescence for the 3 LIM homeobox transcription factors (Lhx6, Lhx5, and Lhx9) was restricted to the neuronal cell body and nucleus. MePD neurons had the cell body and variable lengths of DiI labeled dendrites showing usually numerous dendritic spines. The coincidence of fluorescence for a specific Lhx in the nucleus together with the DiI labeling of the neuronal cell body, dendrites, and spines, guided the selection of cells to be further studied in each subpopulation. The number of sampled neurons was compatible with previous results of MePD cell subtypes (Carney et al., 2010; Bian, 2013).

Lhx 6-, Lhx5-, and Lhx9-expressing neurons in the MePD

Representative Lhx6-immunoreactive neurons in the MePD are shown in Figure 3. These were basically multipolar neurons with round, ovoid or fusiform cell bodies and few primary dendrites (frequently only two dendritic shafts) and sparingly branched trees

(Figure 3A-D). Spines in clusters or isolated were found on cell bodies and dendrites. These spines showed a continuum of shapes and sizes (Figure 3E-F). Axons were not identified in these cells.

Representative Lhx5-immunoreactive neurons are shown in Figure 4. These were also multipolar cells (Figure 4A-D), but some usually presented thin ramified dendritic branches (Figure 4B-C). There were pleomorphic spines with an apparent similar distribution as in the Lhx6 subpopulation (Figure 4E-F).

Representative Lhx9-immunoreactive neurons are shown in Figure 5. They were also multipolar neurons (Figure 5A-C), but with some large fusiform or angular cell bodies (Figure 5A-B), and small neurons with various radiating dendritic branches arising from a round cell body (Figure 5D). Pleomorphic spines were also observed in these neurons (Figure 5E-F).

Dendritic spine categories and density in Lhx neuronal subpopulations

The number of dendritic spines on each Lhx-expressing neurons are shown in Table 1. Thin and stubby/wide spines constitute the highest proportion of all spines in the LhX neuronal subpopulations (85-78%), whereas mushroom spines and other morphologies accounted for fewer values. On the other hand, the total number of filopodia was variable, but notably very low, in the 3 different neuronal subpopulations of the MePD. I.e., median values (and interquartile ranges) were 0.5 (0/2), 1 (0/2), and 0 (0/1) filopodia on the studied dendrites of Lhx6-, Lhx5-, and Lhx9-expressing neurons, respectively.

The different origins/Lhx transcription factors expressed by the MePD neurons did not affect the morphology or number of proximal dendritic spines of these neurons. There was no predominance of any spine type in those neuronal subpopulations [$F(6, 140)= 0.51$; $P= 0.80$] nor subpopulations have a statistically significant difference in the density

of proximal spines [F(2,140)= 0.55; P= 0.58]. However, there were significantly more thin and stubby/wide spines over mushroom and other spine types [F(3,140)= 91.35; P < 0.001], indicating that the spine type influenced the total spine number in these different neuronal subpopulations.

Mean values for the density of proximal dendritic spines of Lhx6, Lhx5, and Lhx9 expressing neurons are shown in Table 1. There was an evident variability in the values for dendritic spine density of the MePD Lhx neuronal subpopulations (Table 1, untransformed data). This could be attributed to an overlap of “low density spiny neurons” (arbitrarily considered as a neuron with a spine density below 1 spine/dendritic µm) and “more densily spiny neurons” (with more than 1 spine/dendritic µm; see parallel data in DiFia et al., 1976; Feldman, 1984) in each subpopulation. In this regard, Lhx6-immunoreactive neurons had a spine density ranging from 0.6 to 2.2 spines/dendritic µm. Lhx5-immunoreactive neurons had a spine density ranging from 0.7 to 1.9 spines/dendritic µm. In these both subpopulations, approximately 60% of the sampled cells could be classified as densily spiny neurons. Lhx9-immunoreactive neurons had a spine density ranging from 0.4 to 2.3 spines/dendritic µm, but only 2/12 (17%) neurons had 0.8 $\pm$ 0.2 spine/dendritic µm whereas 10/12 (83%) neurons had higher values of 1.6 $\pm$ 0.5 spines/dendritic µm. Nevertheless, no statistically significant difference was found for the dendritic spine density among subpopulations in the MePD [F (2,34) = 1.72; P = 0.18].

Glutamatergic and GABAergic receptors on dendritic shafts and spines

Immunofluorescent labeling for AMPA, NMDA, and $GABA_A$ receptors were found in proximal and distal dendritic branches and in different dendritic spines in the MePD. Data are shown in Table 2 and Figure 6.

Immunoreactive puncta for glutamatergic and GABAergic receptors occurred directly on dendritic shafts along both proximal (Figure 6A, C, E) and distal (Figure 6B, D, G, H) branches. Colocalization of these receptors on adjacent segments of the same dendritic shaft was usual (Figure 6I-K, merged images in L). There was an apparent similar presence of AMPA receptors in proximal and distal dendritic shafts (Figure 6, compare A and B). The same was true for NMDA (Figure 6, compare C and D) and $GABA_A$ receptors (Figure 6, compare E, G, H).

The immunoreactive puncta for these receptors were found on differently shaped spines along proximal and distal dendrites in the MePD (Table 2). That is, dendritic spines exhibited excitatory as well as inhibitory receptors. AMPA receptors were predominant on mushroom and stubby/wide spines, whereas NMDA receptors were mostly observed on thin spines. Intestingly, $GABA_A$ receptors were found on either thin or stubby/wide spines of proximal dendrites, whereas, at distal branches, they were basically on thin spines (Table 2). The receptors were rather found on the spine head and, less frequently, on the spine neck, with the exception of $GABA_A$ receptors that were often found on spine necks at distal dendrites.

Putative multisynaptic spines were identified in the MePD. Double-labeling for AMPA and NMDA receptos was detected on stubby/wide and mushroom spines (e.g., Figure 6 L); for AMPA and $GABA_A$ receptors on all spine types (e.g., Figure 6 N), and for NMDA and $GABA_A$ receptors on thin and stubby/wide spines (e.g., Figure 6 L). These 3 receptors were found concomitantly on stubby/wide, thin, and mushroom spines at proximal and distal dendritic branches (Table 2).

Finally, no filopodium appeared labeled for any of the receptors tested here (Figure 6 O).

Discussion

The present data provides new knowledge about the cellular composition and on the spines and synaptic complexities in the rat MePD. Four results are available indicating that 1) three histogenetically distinct Lhx6-, Lhx5-, and Lhx9-expressing neurons are present in the MePD of adult rats, 2) these different local subpopulations display a similar percentage of dendritic spine shapes and density; 3) glutamatergic (AMPA and NMDA) receptors and $GABA_A$ receptors are located along proximal and distal dendritic shafts as well as on spines of MePD neurons, and 4) dendritic spines have excitatory, inhibitory and multisynaptic contacts with colocalization of these receptors to form diverse synaptic units.

Different Lhx-expressing subpopulations in the MePD

Our results from adult rats are in accordance with the reported presence of LIM homeodomain transcription factors in subpopulations of MePD neurons of mouse embryos (Bupesh et al., 2011) and adult mouse brain (Choi et al., 2005). However, a difference was evidenced. In adult mice, Lhx5-expressing cells were specifically observed within the anterior medial amygdala and the Lhx9 ones were found in the posteroventral medial amygdala, but not in the MePD (Choi et al., 2005). Here, we observed the expression of the Lhx 6, 5, and 9 in the adult rat MePD neurons. This can indicate a species-specific difference between the MePD histogenetic components of mice and rats with likely additional functional implications for the MePD of rats. In mice, Lhx6-expressing neurons were related with reproductive behavior whereas both Lhx5- and Lhx9-immunolabeled cells were involved with defensive behavior (Choi et al., 2005). In rats, the MePD has a clear modulatory role on male sexual behavior (Harris and Sachs, 1975; Rasia-Filho et al., 1991; Newman, 1999; de Castilhos et al., 2006; Rasia-

Filho et al., 2012b). On the other hand, ibotenic acid lesion of the rat MePD did not block anxiety-like behavior tested in the elevated plus maze or the innate fear expression of rats faced to a live caged cat, although microinjection of somatostatin in the MePD dramatically reduced rat aggressive display in a resident-intruder paradigm (Rasia-Filho et al., 2012b). Other experimental data can add to this scenario and advance the relevant proposition of the rat MePD as a nodal point for social behavior neural network (Newman, 1999; Bian, 2013; additional comments in Rasia-Filho et al., 2012b and references therein).

We propose that further scholarly debate can reconcile the nomenclature for the different neurons in the MePD based on histogenetic markers, general morphology, membrane intrinsic electrophysiological properties, diverse expression patterns (e.g., for classical neurotransmitters, neuropeptides, binding proteins and/or enzymes), connectional and functional features. Formerly, no general aspect allowed the reliable identification of responsive or not responsive cells to gonadal hormone actions in MePD neurons of rats (Nabekura et al., 1986; Rasia-Filho et al., 1999) or hamsters (Gomez and Newman, 1991). Nevertheless, as demonstrated by the Golgi method, the diversity of the neuronal population in the adult rat MePD of both sexes is basically comprised of multipolar cells classified as bitufted (suggested to be not “bipolar”, as per Ramón y Cajal’s classical description, 1909) and stellate neurons (Alheid et al., 1995; Gomez and Newman, 1991; McDonald, 1992; Rasia-Filho et al., 1999; Cooke et al., 2007; Dall’Oglio et al., 2008a; Arpini et al., 2010; Brusco et al., 2010; Rasia-Filho et al., 2012a,b; Bian, 2013). Unlike the basolateral and cortical amygdaloid nuclei, the cell types of the rat central and medial amygdala do not resemble those of the cerebral cortex (McDonald, 1992;1998). Additional characterization is also warranted to typify intrinsic or projecting neurons in the MePD of rats. For example, to reveal whether the small, spiny and more

ramified stellate neuron expressing Lhx9 is a class of local interneuron (Figure 4D) and to what extent Lhx6-and Lhx5-expressing cells are basically projecting neurons, as described in mice (Choi et al., 2005). The former possibility is relevant since one third of all medial amygdala (MeA) synapses are of local origin (Nishizuka and Arai, 1983). At this moment, the extent of DiI labeling and the number of fluorescent channels that can be captured simultaneously under confocal microscopy (using fluorophores with non-overlapping emission spectras to avoid "cross talk" of the DiI dye, the 3 Lhx fluorescent antibodies and the 3 fluorescent receptors or even other neurotransmitters markers) did not allow us to provide additional analysis regarding the phenotype of the Lhx-expressing neurons such as glutamate vs GABAergic neurons or interneurons vs projecting neurons.

Furthermore, species differences should also be beared in mind since, compared to mice, there is no clear morphological evidence for striatum-like medium spiny stellate neurons in the MePD of adult Wistar rats [see Bennur et al. (2007) - mouse; compare Marcuzzo et al. (2007) - rat; Dall'Oglio et al. (2008a) - rat; additional relevant data in Bian et al. (2008) – mouse]. In mice, some neurons in the posterior part of the MeA resemble pyramidal neurons from the piriform cortex (Bian et al., 2008), although Niimi et al. (2012) found principal MeA spiny neurons typically projecting at least two dendrites. They also found no obvious morphological differences among neurons of different electrophysiological firing patterns (i.e., type I - regular spiking neurons, 56% of the recorded cells; type II – adapting neurons, 3%; and type III - fully accomodating neurons, 12%; Niimi et al., 2012). Recently, Bian (2013), using whole-cell patch clamp recordings in GFP expressing mice to study the GABAergic neurons in the MePD, systematically found that they were not homogenous and could be divided into three subtypes based on electrophysiological, morphological, and connectivity properties: the bitufted and stellate projecting and intrinsic GABAergic neurons.The detailed neuronal morphological analysis

of these GABAergic neurons revealed two types of projection neurons and a third type of smooth-dendrite interneurons. Carney et al. (2010) reported 3 types of MePD neurons identified by their developmental histogenetic characterization and whole-cell patch-clamp recordings also in mice. Basically, types I and II neurons were both immunoreactive for the inhibitory markers neuronal nitric oxide synthase and Forkhead box transcription factor FoxP2 whereas type III neurons were immunonegative for them. At a first glance, it could be suggested that classes I and III neurons correspond to Golgi-impregnated bitufted neurons whereas class II neurons resemble Golgi-impregnated stellate ones [see Figure 8 in Carney et al. (2010) and compare to those in Dall'Oglio et al. (2008a) and Arpini et al. (2010)]. These data reinforce the propositon for an uniform nomenclature of the MePD cells bringing together their diverse relevant features.

Dendritic spines in the MePD

The complex modulation of the dendritic spine density in the MePD was previously evidenced under different experimental conditions (sex differences, estrous cycle and motherhood effects – Rasia-Filho et al., 2004; stressful stimulus on possibly inhibitory spines – Marcuzzo et al., 2007; prepubertal and adult gonadal steroids withdrawal and/or hormonal therapy – Cunningham et al., 2007; de Castilhos et al., 2008; Cooke and Woolley, 2009). Mean dendritic spine densities found here are in accordance with a previous descriptive report of randomly sampled left MePD neurons under the same DiI dye methodological approach and confocal microscopy (i.e., 1.15 ± 0.67 spines/dendritic µm; Brusco et al., 2010). This similarity would be attributed to the high proportion of Lhx6-expressing neurons that normally compose the MePD (~ 80%; Choi et al., 2005).

The spine structure-function coupling and the impact of different spine shapes on synaptic stability and plasticity have been frequently reexamined (Nimchinsky et al., 2002;

Deng and Dunaevsky, 2005; Arellano et al., 2007; Bourne and Harris, 2007; Kasai et al., 2010; García-López et al., 2010; Segal, 2010; Lee et al., 2012). In adult hippocampus and neocortex, approximately 65% of spines are thin, 25% are mushroom shaped and 10% have “immature” shapes represented by stubby, multisynaptic, filopodial or branched spines (as described by Bourne and Harris, 2007). Here, thin spines were the most abundant type (~ 44%) on proximal dendrites of MePD neurons. If these thin spines are functionally and morphologically more labile than mushroom ones (Bourne and Harris, 2007), the three Lhx neuronal subpopulations in the MePD might have a similar inherent plasticity to deal with synaptic inputs. Mushroom spines were ~14% and would represent more stable, long-lasting synaptic arrangements for these neurons. Interestingly, proximal stubby spines accounted for ~37% in the present sample and their shape would represent a synaptic site with fewer biochemical and electrical isolation from the parent dendrite. All of these proximal spines are located strategically close to the cell body and would adjoin relevant afferent synaptic information to affect the neuronal firing output of the MePD neurons (Dall’Oglio et al., 2008a).

We also assume that “the morphological heterogeneity of spines even for a local small portion of the dendrite is consistent with the idea that synapse strength is regulated locally, at the level of a single spine” (Arellano et al., 2007). Then, proximal dendritic spines shape and density can contribute to the synaptic processing in each of these histogenetically diverse Lhx-expressing subpopulations of MePD neurons. However, more evident functional differences among these neurons would reside in other cellular phenotype. Electrophysiological recordings (Nuriya et al., 2006; Ivenshitz and Segal, 2010; Bian, 2013), multi-photon laser-scanning microscopic studies (Lee et al., 2012), and the combination of tract-tracing methods and intracellular dye injections (Lanciego

and Wounterlood, 2011) are highly desirable to test these functional hypotheses for the dendritic spines in the adult rat MePD.

Excitatory and inhibitory receptors on MePD dendritic shafts and spines

There is a notable presence of immunolabeled puncta for glutamatergic and GABAergic receptors on dendritic shafts and spines with possible functional implications for the MePD. Dendritic shaft synapses can be significantly efficient and reliable to produce postsynaptic potentials (Ivenshitz and Segal, 2010), and both dendritic shafts and spines can show different densities of glutamatergic receptors. For example, thin spines can have a structural flexibility, be formed or disappear relatively rapidly according to the incoming synaptic activity, and anchor more NMDA receptors whereas mushroom spines have larger postsynaptic densities, contain more AMPA receptors, regulate calcium levels locally, and can have polyribosomes for local synthesis of proteins (reviewed in Kasai et al., 2003; Bourne and Harris, 2007). Although most spine contacts are indeed excitatory, it must not be dismissed the presence of GABAergic terminals and receptors relevant for fine information processing and a possible source of postsynaptic variability (Keller, 2002; Klausberger and Somogyi, 2008; Pereno et al., 2011). In addition, structural dynamics and receptor trafficking in dendritic spines are crucial for synaptic plasticity (Kasai et al., 2010). Glutamate and GABA receptors have been shown to move rapidly around the neuron membrane modifying the number and composition of receptors that are available to respond to released neurotransmitters (Collingridge et al., 2004; Lin et al., 2004). These changes can be quickly determined by a local intracellular pool of fast recycling receptors available at synapses that are regulated by proteins and signaling mechanisms that associate or interact with receptor subunits, contributing to the synaptic plasticity (Collingridge et al., 2004).

Excitatory and inhibitory synapses on the spine head or neck were already found in the adult rat MePD studied in 3D reconstructions of electron microscopy serial-sections (Brusco, 2012). Here, we found spines of different shapes with glutamatergic and GABAergic receptors. In effect, AMPA receptors were usually found on mushroom and stubby/wide spines whereas NMDA receptors occurred on thin spines, either in proximal or in distal dendritic branches in the rat MePD. $GABA_A$ receptors had other distribution. Although they appeared to be similarly present on proximal and distal dendrites, $GABA_A$ receptors were found on thin and stubby/wide spines of proximal dendrites, but mainly on thin spines at distal branches. This would indicate a different impact of proximal and distal inhibitory synapses on MePD neurons.

Interestingly, behaviorally-relevant afferences to the MePD involve distinctly coded glutamatergic and GABAergic inputs. Male rats have dendrites radiating and extending preferently to the surrounding “molecular layer” close to the MePD (Dall’Oglio et al., 2008a), a cell-sparse rim that contains fibers from the main and accessory olfactory bulbs (Scalia and Winans, 1975; Pro-Sistiaga et al., 2007). AMPA-mediated, more than NMDA, monosynaptic excitatory postsynaptic currents can be evoked from the accessory olfactory bub afferents coming from ventral pathways (Niimi et al., 2012). Pheromonal socially-relevant processed information are sent to the rat MePD via GABAergic fibers from the intercallated amygdaloid nuclei (Meredith and Westberry, 2004). Likewise, a strong colocalization of Fos with GABA, calretinin, and calbindin was observed in the vomeronasal system-medial extended amygdala and indicates the important role of inhibitory control of the incoming pheromonal and olfactory sensorial inputs on the rat MePD neuronal excitability (Pereno et al., 2011). The functional consequence of this local synaptic modulation involves direct and indirect GABAergic projections to specific hypothalamic nuclei (Choi et al., 2005), which can inhibit or disinhibit circuitries involved

with the display of reproductive behaviors (Rasia-Filho et al., 2012a,b). The MePD GABAergic neurons can also establish bidirectional connections with neighboring amygdaloid nuclei in mice (Bian, 2013). This panorama might correspond to the majority of Lhx6-expressing cells within the MePD. Besides, neurons in the mice MePD, whose histogenetic identity remains to be determined, can receive direct excitatory input from upstream sensory areas and inhibitory inputs from local GABAergic neurons and, then, project glutamatergic fibers to the hypothalamic ventromedial nucleus (Bian et al., 2008).

Immunolabeling for single or multiple receptors were found on dendritic spines. The existence of multisynaptic spines in the MePD was suggested by the presence of various puncta of synaptophysin, a pre-synaptic protein, upon the same spine (Brusco et al., 2010). Multisynaptic spines are complex postsynaptic elements (Popov and Stewart, 2009) and, in the MePD, can deal with one excitatory and another inibitory receptor. It is not currently known whether different axon terminals contact AMPA and NMDA receptors on the same spine or the detailed involvement of these multisynaptic spines on the function of MePD neurons. Finally, no filopodia beared AMPA, NMDA or $GABA_A$ receptors, which might suggest that these protusions lack synapses with major excitatory and inhibitory neurotransmitters. Non-synaptic filopodia were previously observed in ultrastructural studies of the adult rat MePD (Brusco, 2012).

In conclusion, the present data evidenced relevant features on the cellular composition and the complexity of dendritic shafts and spines in the adult rat MePD. The presence and distribution of glutamatergic and GABAergic synaptic receptors on dendrites can be useful to understand the selection of cell assemblies, synaptic temporal dynamics, and neural circuits oscillations (Klausberger and Somogyi, 2008). They can also be useful in directing future studies of the MePD connectivity and function not only under normal circunstances, for sensorial processing of pheromones or other social cues,

sexual behavior modulation, stress responses, memory and learning, but also for comparisons in pathological conditions that compromise this forebrain area.

Legends

Table 1 – Percentage of different dendritic spine type and density (values are mean ± standard deviation) of three different Lhx-expressing neuronal subpopulations in the posterodorsal medial amygdala of adult male rats.

Table 2 – Number and distribution of glutamatergic (AMPA GLUR1-4 and NMDA GluN1) and GABAergic ($GABA_A$ γ2 subcomponent) receptors on the head (H) and neck (N) of differently shaped spines from proximal and distal dendritic branches. Neurons were sampled from the posterodorsal medial amygdala of adult male rats and studied using combined immunofluorescent labeling and DiI dye under confocal microscopy. Stubby/wide spines characteristically did not display an apparent neck, then indicated by "-" to mean that absence. Colocalization refers to the presence of more than one of these receptors on the head or on the head and neck of the same spine in each studied type. More details about colocalization are presented in the text.

Figure 1 – **(Left)** Cresyl violet staining of a coronal brain section shows the ventral location of the posterodorsal medial amygdala in the adult male rat brain (MePD, 3.30 mm posterior to the bregma). **(Right)** Schematic diagram of a matched coronal brain section adapted from the atlas of Paxinos and Watson (1998). opt: optic tract, st: stria terminalis. Spatial coordinates: D, dorsal; V, ventral; M, medial; L, lateral. Scale bar = 800 µm. Reprinted from Brusco J, Dall'Oglio A, Rocha LB, Rossi MA, Moreira JE, and Rasia-Filho A.A. "Descriptive findings on the morphology of dendritic spines in the rat medial amygdala". Neuroscience Letters 483, 152-156, 2010. Copyright with permission from Elsevier (no. 3097760021389).

Figure 2 – **(A)** Immunoreactivity to antibodies against the Lhx6, Lhx5, and Lhx9 transcription factors and **(B)** for receptors AMPA GLUR1-4, NMDA GluN1, and $GABA_A$ γ2 subcomponent in the cerebral cortex of adult male rats as revealed by the Western blot technique.

Figure 3. (A-F) Representative examples of immunolabeled Lhx6-expressing neurons (cell nucleus in blue, turned green due to the sobreposition with the DiI dye color) in the posterodorsal medial amydala of adult male rats. Images are three-dimensional reconstructions with combined use of DiI dye fluorescence (yellow) to reveal details of neuronal morphology under confocal microscopy. Spiny neurons have cell bodies with round (**A**, 2/12 sampled cells), ovoid (**B** and **C**, 2/12 and 4/12 cells, respectively) or fusiform (**D**, 4/12 cells) shapes, few primary dendrites, and sparse proximal branching points. (**E**, details at higher magnification in **F**) Three-dimensional reconstructed images to demonstrate the density and shape of dendritic spines commonly found on these Lhx6 neurons. A continuum of pleomorphic dendritic spines (arrows) were observed in this neuronal subpopulation. Spines were classified as thin (t), stubby/wide (s), "mushroom"-like (m), and "others" (o). Scale bar = 5 µm.

Figure 4. (A-F) Representative examples of immunolabeled Lhx5-expressing neurons (cell nucleus in red) in the posterodorsal medial amydala of adult male rats. Images are three-dimensional reconstructions with combined use of DiI dye fluorescence (yellow) to reveal details of neuronal morphology under confocal microscopy. Spiny neurons have cell bodies with triangular (**A**, 4/14 sampled cells), fusiform (**B** and **C**, 5/14 and 2/14 cells, respectively) or multiangular (**D**, 3/14 cells) shapes, few primary dendrites (except in **D**), and sparse proximal branching points. (**E,** details at higher magnification in **F**) Three-

dimensional reconstructed images to demonstrate the density and shape of dendritic spines commonly found on these Lhx6 neurons. A continuum of pleomorphic dendritic spines (arrows) were observed in this neuronal subpopulation. Spines were classified as thin (t), stubby/wide (s), “mushroom”-like (m), and “others” (o). Scale bar = 5 μm.

Figure 5. (A-F) Representative examples of immunolabeled Lhx9-expressing neurons (cell nucleus in magenta) in the posterodorsal medial amydala of adult male rats. Images are three-dimensional reconstructions with combined use of DiI dye fluorescence (yellow) to reveal details of neuronal morphology under confocal microscopy. Spiny neurons have cell bodies with fusiform (**A** and **B**, 5/12 and 2/12 sampled cells, respectively), triangular (**C**, 2/12 cells) or elongated (**D**, 3/12 cells) shapes, few primary dendrites, and sparse proximal branching points (except in **B**). (**E**, details at higher magnification in **F**) Three-dimensional reconstructed images to demonstrate the density and shape of dendritic spines commonly found on these Lhx6 neurons. A continuum of pleomorphic dendritic spines (arrows) were observed in this neuronal subpopulation. Spines were classified as thin (t), stubby/wide (s), “mushroom”-like (m), and “others” (o). Scale bar = 5 μm.

Figure 6. Representative examples of three-dimensional reconstructed images combining DiI dye (yellow) to evidence spiny dendrites and the presence of immunofluorescent labeling puncta for (**A**, **B**) AMPA GLUR1-4 (red) and (**C**, **D**) NMDA GluN1 (blue) glutamaterigic receptors and the (**E**-**H**) $GABA_A$ γ2 subcomponent receptor (magenta) under confocal microscopy. These are neurons from the posterodorsal medial amygdala of adult male rats. Receptors on close contact to dendritic shafts and spines were found in both proximal (**A**, **C**, **E**) and distal (**B**, **D**, **G** and **H**) branches. Note the presence of the three immunolabeled receptors puncta on differently shaped spines. (**F**) Higher magnification image illustrates immunoreactive colored puncta for the $GABA_A$ receptor on the spine head and on the spine neck of thin spines (arrows). (**I-K**) Three-dimensional reconstructed image of the same DiI dye fluorescent dendrite demonstrates the coexistence of imunolabeling for AMPA (**I**), NMDA (**J**), and $GABA_A$ receptors (**K**) on the same branch. These three images (**I-K**) were merged in (**L**). Note the presence of immunoreactive puncta for the glutamatergic and GABAergic receptors in close apposition to dendritic shafts and on spines of different shapes. In **L**, examples of colocalization of AMPA and NMDA receptors or NMDA and $GABA_A$ receptors on spines. (**M**) Colocalization of AMPA and $GABA_A$ labeling on the same reconstructed DiI dye immunofluorescent dendritic branch. Dendritic spines (asterisks) and dendritic shaft (arrowhead) appeared contacted by immunoreactive puncta for both receptors. At higher magnification (**N**), AMPA and $GABA_A$ receptors are colocalized on the same dendritic spine (arrows). (**O**) Reconstructed DiI dye immunofluorescent dendritic branch showing a protuding filopodium without overlapping immunolabeled puncta for glutamatergic or GABAergic receptors on the same focal plane. Scale Bar = 2 μm.

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Conflict of Interest

The authors declare no actual or potential conflict of interest.

References

Alegria-Schaffer A, Lodge A, Vattem K (2009) Performing and optimizing Western blots with an emphasis on chemiluminescent detection. Methods Enzymol 463:573-599

Alheid GF, de Olmos JS, Beltramino CA (1995) Amygdala and extended amygdala. In: The Rat Nervous System. Paxinos G (ed). Academic Press, San Diego, pp 495–578

Arellano JI, Benavides-Piccione R, Defelipe J, Yuste R (2007) Ultrastructure of dendritic spines: correlation between synaptic and spine morphologies. Front Neurosci 1:131-143

Arpini M, Menezes IC, Dall'Oglio A, Rasia-Filho AA (2010) The density of Golgi-impregnated dendritic spines from adult rat posterodorsal medial amygdala neurons displays no evidence of hemispheric or dorsal/ventral differences. Neurosci Lett 469:209–213

Bennur S, Shankaranarayana Rao BS, Pawlak R, Strickland S, McEwen BS, Chattarji S (2007) Stress-induced spine loss in the medial amygdala is mediated by tissue-plasminogen activator. Neuroscience 144:8–16

Bian X (2013) Physiological and morphological characterization of GABAergic neurons in the medial amygdala. Brain Res, http://dx.doi.org/10.1016/j.brainres.2013.03.012

Bian X, Yanagawa Y, Chen WR, Luo M (2008) Cortical-like functional organization of the pheromone-processing circuits in the medial amygdala. J Neurophysiol 99: 77-86

Blake CB, Meredith M (2011) Change in number and activation of androgen receptor-immunoreactive cells in the medial amygdala in response to chemosensory input. Neuroscience 190:228-238

Bourne JN, Harris KM (2007) Do thin spines learn to be mushroom spines that remember? Curr Op Neurobiol 17:381-386

Brusco J (2012) Amígdala medial de ratos ao longo do ciclo estral: espinhos dendríticos, ultraestrutura sináptica, expressão gênica e lateralidade. Ph.D Thesis, University of São Paulo (FMRP/USP), Brazil.

Brusco J, Dall'Oglio A, Rocha LB, Rossi MA, Moreira JE, Rasia-Filho AA (2010) Descriptive findings on the morphology of dendritic spines in the rat medial amygdala. Neurosci Lett 483:152-156

Bupesh M, Legaz I, Abellán A, Medina L (2011) Multiple telencephalic and extratelencephalic embryonic domains contribute neurons to the medial extended amygdala. J Comp Neurol 519:1505-1525

Carney RS, Mangin JM, Hayes L, Mansfield K, Sousa VH, Fishell G, Machold RP, Ahn S, Gallo V, Corbin JG (2010) Sonic hedgehog expressing and responding cells generate neuronal diversity in the medial amygdala. Neural Dev 27:5-14

Chen Y, Sabatini BL (2012) Signaling in dendritic spines and spine microdomains. Curr Opin Neurobiol 22(3):389-96

Choi GB, Dong H-W, Murphy AJ, Valenzuela DM, Yancopoulos GD, Swanson LW, Anderson DJ (2005). Lhx6 delineates a pathway mediating innate reproductive behaviors from the amygdala to the hypothalamus. Neuron 46:647-660

Collingridge GL, Issac JTR, Wang YT (2004) Receptor trafficking and synaptic plasticity. Nature Rev Neurosci 5: 952-962

Cooke BM, Stokas MR, Woolley CS (2007) Morphological sex differences and laterality in the prepubertal medial amygdala. J Comp Neurol 501:904-15

Cooke BM, Woolley CS (2009) Effects of prepubertal gonadectomy on a male-typical behavior and excitatory synaptic transmission in the amygdala. Develop Neurobiol 69:141-152

Coolen LM, Peters HJ, Veening JG (1996) Fos immunoreactivity in the rat brain following consummatory elements of sexual behavior: a sex comparison. Brain Res 738:67-82

Coolen LM, Peters HJPW, Veening JG (1997) Distribution of Fos immunoreactivity following mating versus anogenital investigation in the male rat brain. Neuroscience 77:1151–1161

Cunningham RL, Clairborne BJ, McGinnis MY (2007) Pubertal exposure to anabolic androgenic steroids increases spine densities on neurons in the limbic system of male rats. Neuroscience 150:609-615

Dall'Oglio A, Gehlen G, Achaval M, Rasia-Filho AA (2008a) Dendritic branching features of posterodorsal medial amygdala neurons of adult male and female rats: further data based on the Golgi method. Neurosci Lett 430:151-156

Dall'Oglio A, Gehlen G, Achaval M, Rasia-Filho AA (2008b) Dendritic branching features of Golgi-impregnated neurons from the "ventral" medial amygdala subnuclei of adult male and female rats. Neurosci Lett 439: 287-292

Dayas CV, Buller KM, Day TA (1999) Neuroendocrine responses to an emotional stressor: evidence for involvement of the medial but not the central amygdala. Eur J Neurosci 11:2312-22

DiFiglia M, Pasik P, Pasik T (1976) A Golgi study of neuronal types in the neostriatum of monkeys. Brain Res 114:245-56

de Castilhos J, Forti CD, Achaval M, Rasia-Filho AA (2008) Dendritic spine density of posterodorsal medial amygdala neurons can be affected by gonadectomy and sex steroid manipulations in adult rats: a Golgi study. Brain Res 1240:73-81

de Castilhos J, Marcuzzo S, Forti CD, Frey RM, Stein D, Achaval M, Rasia-Filho AA (2006) Further studies on the rat posterodorsal medial amygdala: dendritic spine density and effect of 8-OH-DPAT microinjection on male sexual behavior. Brain Res Bull 69:131-139

de Olmos JS, Beltramino CA, Alheid G (2004) Amygdala and extended amygdala of the rat: a cytoarchitectonical, fibroarchitectonical, and chemoarchitectonical survey. In: The Rat Nervous System. Paxinos G (ed). Elsevier Academic Press. London, pp 509-603

Deng J, Dunaevsky A (2005) Dynamics of dendritic spines and their afferent terminals: spines are more motile than presynaptic boutons. Develop Biol 277:366-377

Dong H-W, Petrovich G, Swanson LW (2001) Topography of projections from amygdala to bed nuclei of the stria terminalis. Brain Res Rev 38:192–246

Dunn KW, Kamocka MM, McDonald JH (2011) A practical guide to evaluating colocalization in biological microscopy. Am J Physiol Cell Physiol 300:C723-C742

Fan L, Hanbury R, Pandey SC, Cohen RS (2008a) Dose and time effects of estrogen on expression of neuron-specific protein and cyclic AMP response element-binding protein and brain region volume in the medial amygdala of ovariectomized rats. Neuroendocrinology 88:111-126

Fan L, Pandey SC, Cohen RS (2008b) Estrogen affects levels of Bcl-2 protein and mRNA in medial amygdala of ovariectomized rats. J Neurosci Res 86:3655-3664

Feldman ML (1984) Morphology of the neocortical pyramidal neurons. In: Cerebral Cortex. Jones EG, Peters A (eds). Plenum Press. New York, vol. 2, pp 123-200

García-López M, Abellán A, Legaz I, Rubenstein JLR, Puelles L, Medina L (2008) Histogenetic compartments of the mouse centromedial and extended amygdala base on gene expression patterns during development. J Comp Neurol 506:46-74

García-López P, García-Marin V, Freire M (2010) Dendritic spines and development: towards a unifying model of spinogenesis. A present day review of Cajal's histological slides and drawings. Neural Plasticity. doi: 10.1155/2010/769207

Gomez DM, Newman SW (1991) Medial nucleus of the amygdala in the adult Syrian hamster: a quantitative Golgi analysis of gonadal hormonal regulation of neuronal morphology. Anat Rec 231:498–509

Gréco B, Allegretto EA, Tetel MJ, Blaustein JD (2001) Coexpression of ER beta with ER alpha and progestin receptor proteins in the female rat forebrain: effects of estradiol treatment. Endocrinology 142:5172-5181

Gréco B, Blasberg ME, Kosinski EC, Blaustein JD (2003) Response of ERα-IR and ERβ-IR cells in the forebrain of female rats to mating stimuli. Horm Behav 43:444–453

Harms KJ, Dunaevsky A (2007) Dendritic spine plasticity: looking beyond development. Brain Res 12:1184:65-71

Hayashi Y, Majewska AK (2005) Dendritic spine geometry: functional implication and regulation. Neuron 46:529-532

Harris VS, Sachs BD (1975) Copulatory behavior in male rats following amygdaloid lesions. Brain Res 86:514-8

Ivenshitz M, Segal M (2010) Neuronal density determines network connectivity and spontaneous activity in cultured hippocampus. J Neurophysiol 104:1052-60

Kasai H, Matsuzaki M, Noguchi J, Yasumatsu N, Nakahara H (2003) Structure-stability-function relationships of dendritic spines. Trends Neurosci 26:360-368

Kasai H, Fukuda M, Watanabe S, Hayashi-Takagi A, Noguchi J (2010) Structural dynamics of dendritic spines in memory and cognition. Trends Neurosci 33:121-9

Keller A (2002) Use-dependent inhibition of dendritic spines. Trends Neurosci 25:541-3

Kim BG, Dai HN, McAtee M, Vicini S, Bregman BS (2007) Labeling of dendritic spines with the carbocyanine dye DiI for confocal microscopic imaging in lightly fixed cortical slices. J Neurosci Methods 162:237-43

Klausberger T, Somogyi P (2008) Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. Science 321:53-7

Kurien BT, Dorri Y, Dillon S, Dsouza A, Scofield RH (2011) An overview of Western blotting for determining antibody specificities for immunohistochemistry. Methods Mol Biol 717:55-67

Lanciego JL, Wounterlood FG (2011) A half century of experimental neuroanatomical tracing. J Chem Neuroanat 42:157-183

Lee KF, Soares C, Béïque JC (2012) Examining form and function of dendritic spines. Neural Plast. doi: 10.1155/2012/704103

Lin H, Huganir R, Liao D (2004) Temporal dynamics of NMDA receptor-induced changes in spine morphology and AMPA receptor recruitment to spines. Biochem Biophys Res Communic 316: 501-511

Marcuzzo S, Dall'Oglio A, Ribeiro MF, Achaval M, Rasia-Filho AA (2007) Dendritic spines in the posterodorsal medial amygdala after restraint stress and ageing in rats. Neurosci Lett 424:16-21

McDonald AJ (1992) Cell types and intrinsic connections of the amygdala. In: The amygdala: neurobiological aspects of emotion, memory, and mental dysfunction. Aggleton JP (ed). Wiley-Liss. NewYork, pp 67–96

McDonald AJ (1998) Cortical pathways to the mammalian amygdala. Prog Neurobiol 55: 257-332

Meredith M, Westberry JM (2004) Distinctive responses in the medial amygdala to same-species and different-species pheromones. J Neurosci 24:5719–5725

Nabekura J, Oomura Y, Minami T, Mizuno Y, Fukuda A (1986) Mechanism of the rapid effect of 17α-estradiol on medial amygdala neurons. Science 233:226-228

Neckel H, Quagliotto E, Casali KR, Montano N, Dal Lago P, Rasia-Filho AA (2012) Glutamate and GABA in the medial amygdala induce selective central sympathetic/parasympathetic cardiovascular responses. Can J Physiol Pharmacol 90:525-36

Newman SW (1999) The medial extended amygdala in male reproductive behavior. A node in the mammalian social behavior network. Ann N Y Acad Sci 877:242-57

Niimi K, Horie S, Yokosuka M, Kawakami-Mori F, Tanaka K, Fukayama H, Sahara Y (2012) Heterogeneous electrophysiological and morphological properties of neurons in the mouse medialamygdala in vitro. Brain Res 1480:41-52

Nimchinsky EA, Sabatini BL, Svoboda K (2002) Structure and function of dendritic spines. Annu Rev Physiol 64:313-353

Nishizuka M, Arai Y (1983) Intrinsic connections in the medial amygdala as revealed by complete deafferentation. Neurosci Lett 35:247-51

Nuriya M, Jiang J, Nemet B, Eisenthal KB, Yuste R (2006) Imaging membrane potential in dendritic spines. Proc Natl Acad Sci U S A 103:786-90

Paxinos G, Watson C (1998) The Rat Brain in Stereotaxic Coordinates. Academic Press, San Diego

Pereno GL, Balaszczuk V, Beltramino CA (2011) Detection of conspecific pheromones elicits fos expression in GABA and calcium-binding cells of the rat vomeronasal system-medial extended amygdala. J Physiol Biochem 67:71-85

Peters A, Kaiserman-Abramof IR (1970) The small pyramidal neuron of the rat cerebral cortex. The perikaryon, dendrites and spines. Am J Anat 127:321-356

Pfaus JG, Heeb MM (1997) Implications of immediate-early gene induction in the brain following sexual stimulation of female and male rodents. Brain Res Bull 44:397-407

Phillips-Farfán BV, Lemus AE, Fernández-Guasti E (2007) Increased estrogen receptor alpha immunoreactivity in the forebrain of sexually satiated rats. Horm Behav 51:328–334

Polston EK, Heitz M, Barnes W, Cardamone K, Erskine MS (2001) NMDA-mediated activation of the medial amygdala initiates a downstream neuroendocrine memory responsible for pseudopregnancy in the female rat. J Neurosci 21:4104-4110

Polston EK, Gu G, Simerly RB (2004) Neurons in the principal nucleus of the bed nuclei of the stria terminalis provide a sexually dimorphic GABAergic input to the anteroventral periventricular nucleus of the hypothalamus. Neuroscience 123:793-803

Popov VI, Stewart MG (2009) Complexity of contacts between synaptic boutons and dendritic spines in adult rat hippocampus: Three-dimensional reconstructions from serial ultrathin sections in vivo. Synapse 63:369–377

Pro-Sistiaga P, Mohedano-Moriano A, Ubeda-Bañon I, Arroio-Jimenez MDM, Marcos P, Artacho-Pérula E, Crespo C, Insausti R, Martinez-Marcos A (2007) Convergence of olfactory and vomeronasal projections in the rat basal telencephalon. J Comp Neurol 504:346-362

Ramón y Cajal S (1909) Histologie du Système Nerveux de l'Homme et des Vertebrés. Maloine, Paris

Rasia-Filho AA, Brusco J, Rocha LB, Moreira JE (2010) Dendritic spines observed by extracellular DiI dye and immunolabeling under confocal microscopy. Nature Protocols/Protocol Exchange. doi: 10.1038/nprot.2010.153

Rasia-Filho AA, Fabian C, Rigoti KM, Achaval M (2004) Influence of sex, estrous cycle and motherhood on dendritic spine density in the rat medial amygdala revealed by the Golgi method. Neuroscience 126:839-847

Rasia-Filho AA, Dalpian F, Menezes IC, Brusco J, Moreira JE, Cohen RS (2012a) Dendritic spines of the medial amygdala: plasticity, density, shape, and subcellular modulation by sex steroids. Histol Histopathol 27:985-1011

Rasia-Filho AA, Haas D, de Oliveira AP, de Castilhos J, Frey R, Stein D, Lazzari VM, Back F, Pires GN, Pavesi E, Winkelmann-Duarte EC, Giovenardi M (2012b) Morphological and functional features of the sex steroid-responsive posterodorsal medial amygdala of adult rats. Mini Rev Med Chem 12:1090-106

Rasia-Filho A.A., Londero R.G. and Achaval M. (1999). Effects of gonadal hormones on the morphology of neurons from the medial amygdaloid nucleus of rats. Brain Res. Bull. 48, 173-183

Rasia-Filho AA, Peres TMS, Cubilla-Gutierrez FH, Lucion AB (1991) Effect of estradiol implanted in the corticomedial amygdala on the sexual behavior of castrated male rats. Braz J Med Biol Res 24:1041–1049

Sanes JR, Jessell TM (2000) The formation and regeneration of synapses. In: Kandel ER, Schwartz JH, Jessell TM (ed) Principles of Neural Science, 4rd edn. McGraw Hill, New York, pp. 1087-1114

Sanes JR, Jessell TM (2013) The formation and regeneration of synapses. In: Kandel ER, Schwartz JH, Jessell TM, Siegelbaum SA, Hudspeth AJ (ed) Principles of Neural Science, 5rd edn.McGraw Hill, New York, 1233-1258

Scalia F, Winans SS (1975) The differential projections of the olfactory bulb and accessory olfactory bulb in mammals. J Comp Neurol 161:31-55

Segal M (2010) Dendritic spines, synaptic plasticity and neuronal survival: activity shapes dendritic spines to enhance neuronal viability. Eur J Neurosci 31:2178-2184

Shindou T, Watanabe S, Yamamoto K, Nakanishi H (1993) NMDA receptor dependent formation of long-term potentiation in the rat medial amygdala neuron in an in vitro slice preparation. Brain Res Bull 31:667–672

Simerly, RB (2004) Anatomical substrates of hypothalamic integration. In: Paxinos G (ed) The Rat Nervous System, 3rd edn. Academic Press, San Diego, pp 335–368

Simerly RB, Chang C, Muramatsu M, Swanson LW (1990) Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. J Comp Neurol 294:76-95

Tao-Cheng JH, Gallant PE, Brightman MW, Dosemeci A, Reese TS (2007) Structural changes at synapses after delayed perfusion fixation in different regions of the mouse brain. J Comp Neurol 501:731-40

Woolley CS, McEwen BS (1994) Estradiol regulates hippocampal dendritic spine density via an N-methyl-D-aspartate receptor-dependent mechanism. J Neurosci 14:7680-7687

Zehr JL, Todd BJ, Schulz KM, McCarthy MM, Sisk CL (2006) Dendritic pruning of the medial amygdala during pubertal development of the male Syrian hamster. J Neurobiol 66:578–590

Zirlinger M, Kreiman G, Anderson DJ (2001) Amygdala-enriched genes identified by microarray technology are restricted to specific amygdaloid subnuclei. Proc Natl Acad Sci U S A 98(9):5270-5

Table 1 – Dendritic spine shapes and density in each Lhx neuronal subpopulation in the posterodorsal medial amygdala of adult male rats.

Spine Shape\ (%) Transcription Factor-Expressing Neurons	Lhx 6	Lhx5	Lhx9
Thin	43	42	48
Stubby/Wide	35	40	37
Mushroom	16	14	12
Others	6	4	3
Spine Density	**1.2 ± 0.5**	**1.1 ± 0.4**	**1.5 ± 0.5**

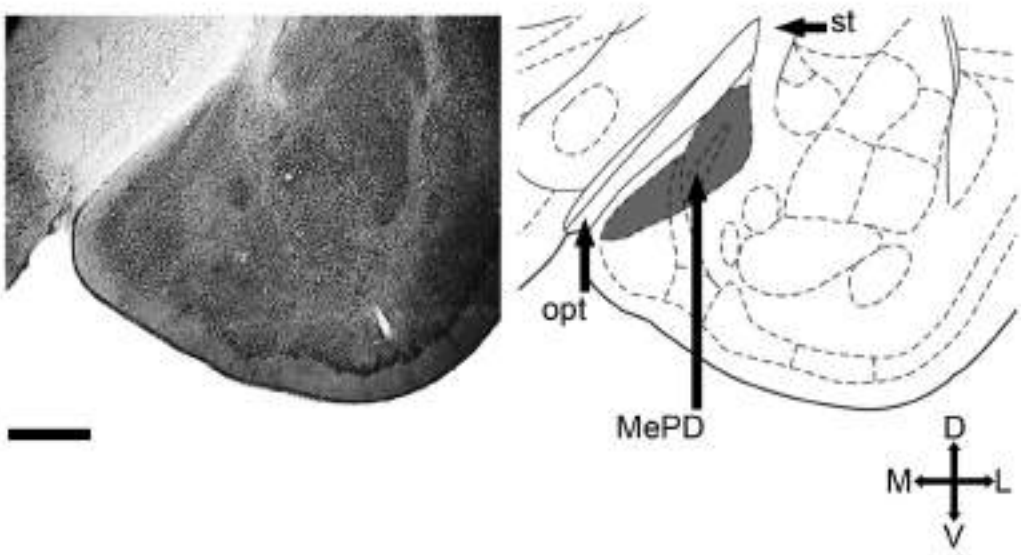
Table 2 – Glutamatergic and GABAergic receptors and contacts in different spines from proximal and distal dendritic branches in the rat posterodorsal medial amygdala.

Proximal Dendritic Branches

Spine Shape	AMPA (GLUR1-4)		GluN1		$GABA_A$		COLOCALIZATION	
	H	N	H	N	H	N	H	H + N
Thin	2	0	7	1	12	2	3	1
Stubby/ Wide	6	-	1	-	10	-	5	-
Mushroom	10	0	0	0	2	0	1	1
Others	0	0	0	0	1	0	1	1
Total	18	0	8	1	25	2	10	3

Distal Dendritic Branches

Spine Shape	AMPA (GLUR1-4)		GluN1		$GABA_A$		COLOCALIZATION	
	H	N	H	N	H	N	H	H + N
Thin	6	2	16	2	22	12	12	6
Stubby/Wide	18	-	2	-	4	-	12	-
Mushroom	9	0	0	0	0	0	9	2
Others	2	0	2	0	4	0	2	0
Total	35	2	20	2	30	12	35	8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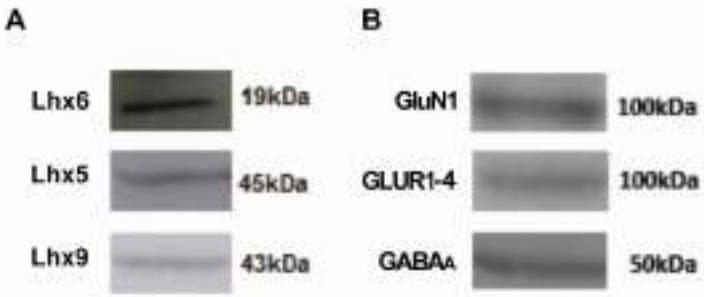
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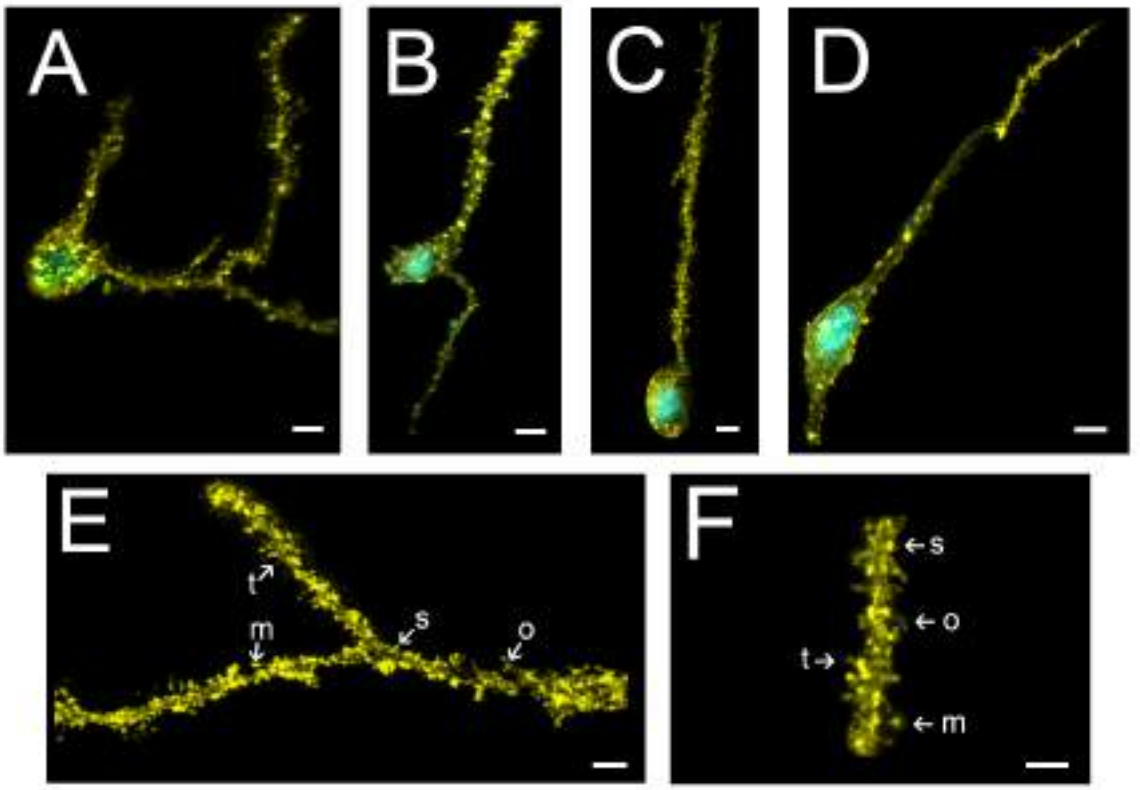
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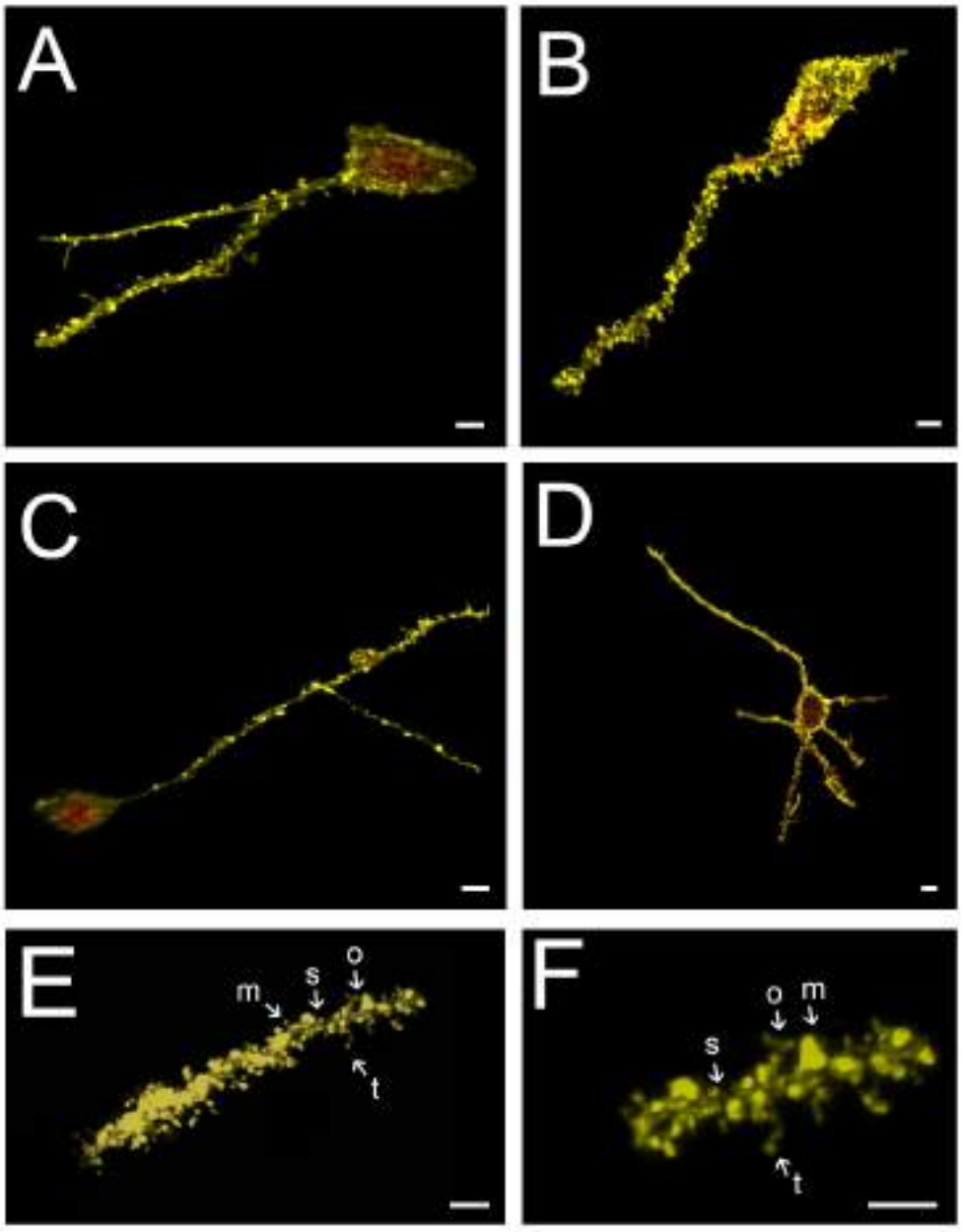
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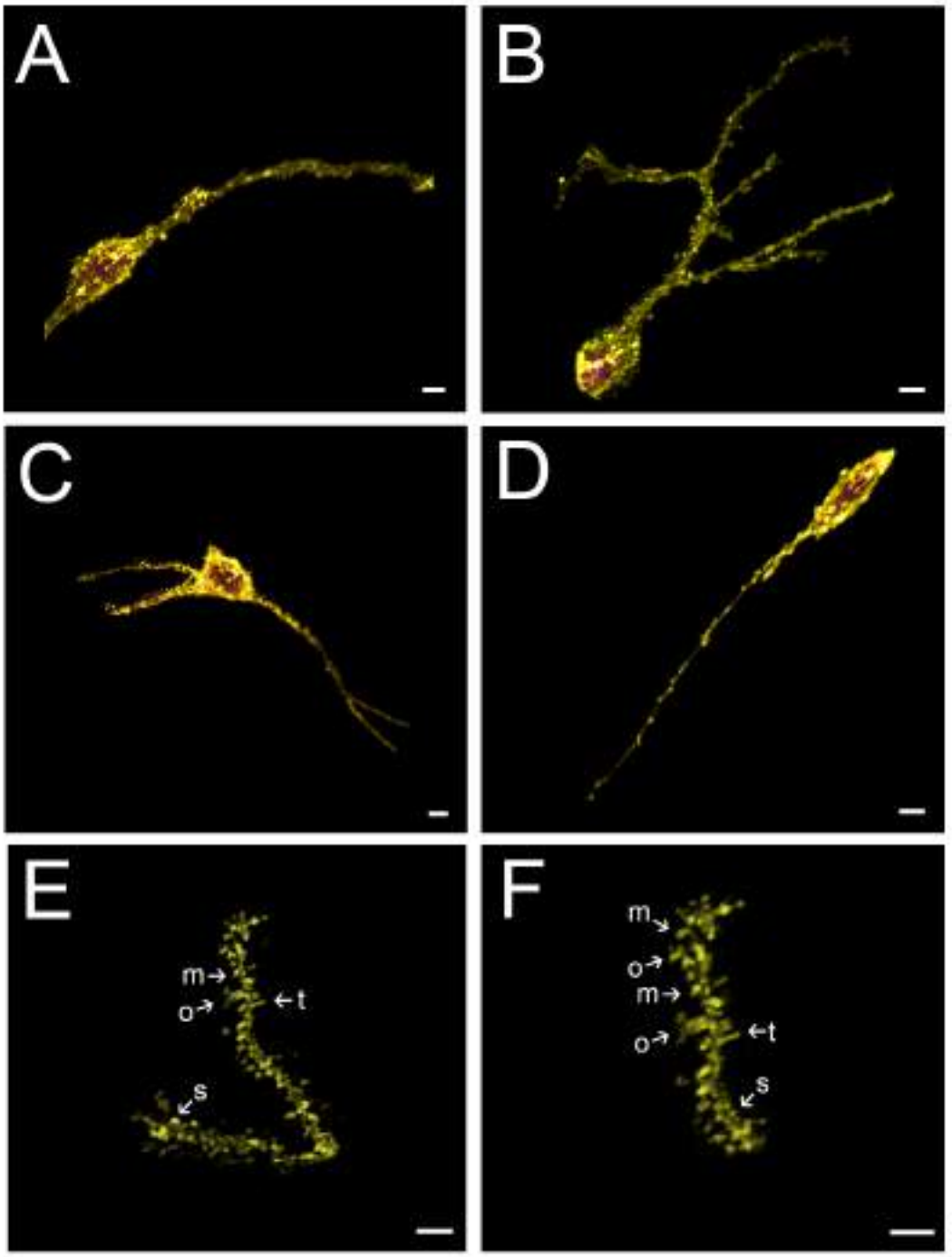
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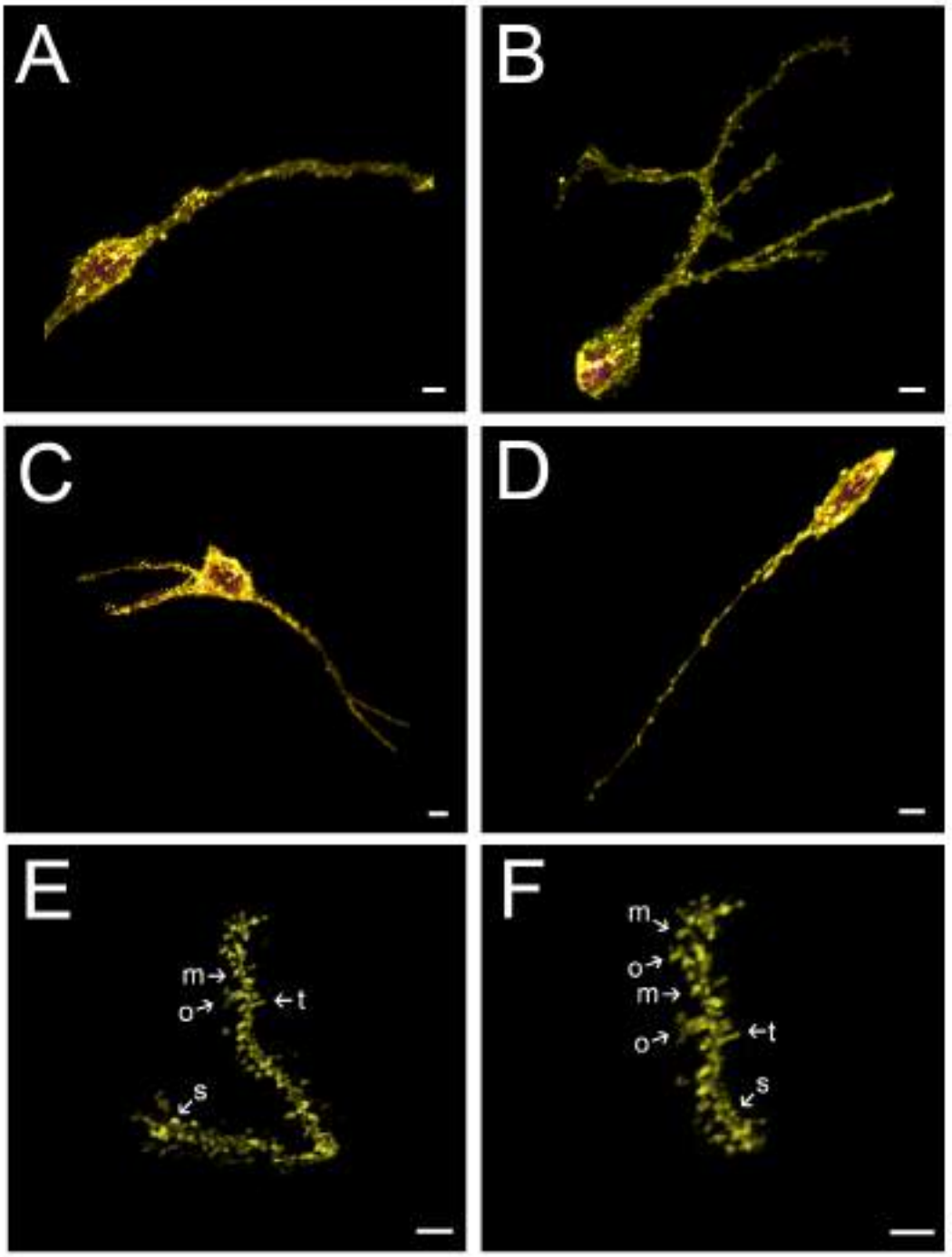
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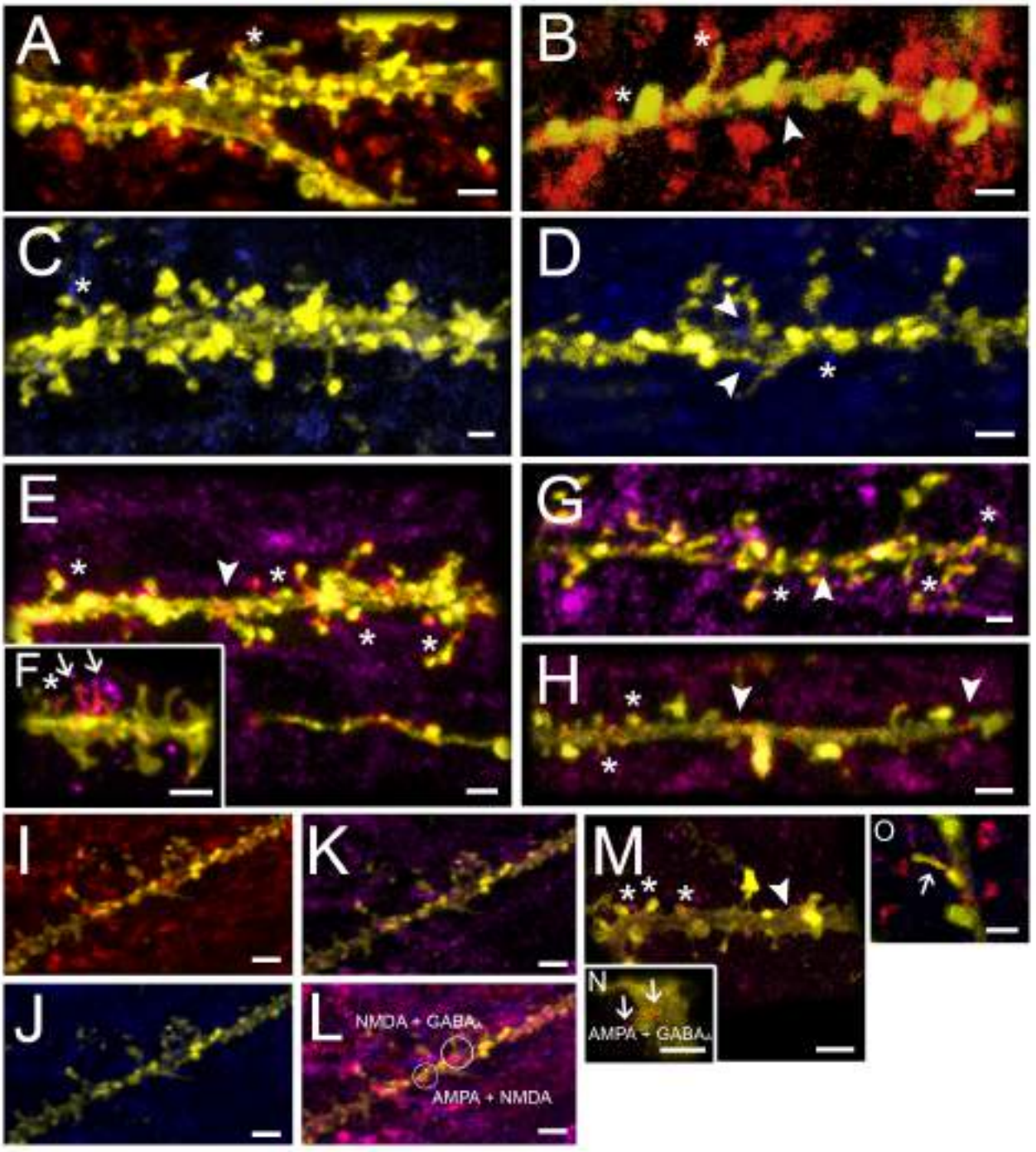
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4. CONSIDERAÇÕES FINAIS

O presente trabalho contribui de forma relevante com o avanço do conhecimento sobre características importantes dos espinhos dendríticos e da composição celular do MePD de ratos adultos. Baseado na metodologia empregada e nos resultados obtidos[1], conclui-se que:

1) O dimorfismo sexual e a secreção cíclica dos esteróides ovarianos influencia a morfologia e a densidade dos espinhos dendríticos dessa região amigdaliana.

2) Três subpopulações de neurônios histogeneticamente distintas estão presentes no MePD de ratos: neurônios $Lhx6^{+}$, $Lhx5^{+}$ e $Lhx9^{+}$.

3) As três subpopulações neuronais apresentam percentuais semelhantes de densidade e tipos morfológicos de espinhos dendríticos.

4) Receptores glutamatérgicos do tipo AMPA e NMDA e o receptor $GABA_A$ foram encontrados nos ramos dendríticos distais e proximais, assim como nos espinhos dendríticos dos neurônios do MePD.

5) Este trabalho mostra que a correlação dos espinhos com os receptores pós-sinápticos também pode indicar diferentes padrões de organização sináptica, gerando hipóteses sobre a plasticidade dos espinhos locais.

Os dados aqui encontrados avançam de forma inédita os conhecimentos sobre o MePD, somam-se com dados recentemente publicados e abrem novas linhas de pesquisa básica. Como perspectivas de trabalho, pode-se indicar:

1. Estudar os sítios extrassinápticos dos receptores glutamatérgicos do tipo NMDA. Esses sítios ainda são pouco caracterizados, mas estão usualmente concentrados em uma variedade de locais denominados sítios de contato, como os axônios, os terminais axonais e a glia, por exemplo, com fatores de adesão como as caderinas e as cateninas (Petralia e col., 2010). De acordo com a literatura, a função dos NMDAR extrassinápticos difere da função dos NMDAR localizados nas sinapses e isto depende do tipo de receptor e das proteínas associadas a estes receptores (Kim e cols., 2005; Newpher e Ehlers, 2009; Petralia e cols., 2010). Em relação aos receptores glutamatérgicos do tipo AMPA extrassinápticos, sabe-se que estes receptores são amplamente expressos no sistema nervoso central e periférico. Estes receptores estão envolvidos com um tipo de transmissão sináptica atividade dependente e as mudanças em sua funcionalidade estão relacionadas com diversas neuropatias no SNC (Kopach e cols., 2012).

2. Estudar os sítios extrassinápticos dos receptores GABAérgicos tipo A. Assim como os receptores GABAérgicos localizados nas sinapses, os receptores GABAérgicos em sítios extrassinápticos também podem alterar respostas sinápticas inibitórias importantes (Tao e cols., 2013). Estudos adicionais a respeito dos receptores extrassinápticos são necessários para elucidar a sua

funcionalidade e a sua importância no processamento de informações nos circuitos neurais.

3. Estudar a função dos espinhos dendríticos dos neurônios do MePD. Futuros experimentos devem auxiliar a desvendar, por exemplo, se eles formam sinapses “silenciosas” ou verdadeiras, quantos espinhos participam do processamento da informação sináptica e, somando-se a isso, a descrição neuroquímica mais completa da região, com seus circuitos de aferência, eferência e locais. Ademais, como perspectivas na continuação deste trabalho, a confirmação e complementação dos dados obtidos pela reconstrução tridimensional de espinhos dendríticos à microscopia eletrônica.

6. REFERÊNCIAS BIBLIOGRÁFICAS

Adamec RE, Morgan HD. The effect of kindling of different nuclei in the left and right amygdala on anxiety in the rat. Physiol. Behav. 1994 Jan;55: 1-12.

Alheid GF, de Olmos JS, Beltramino CA. Amygdala and extended amygdala. In: PAXINOS, G. The Rat Nervous System. San Diego: Academic Press; 1995. pp. 495-578.

Alvarez VA, Sabatini BL. Anatomical and physiological plasticity of dendritic spines. Annu Rev Neurosci. 2007;30:79-97.

Amaral DG, Schumann CM, Nordahl CW. Neuroanatomy of autism. Trends Neurosci. 2008 Mar;31:137–145.

Arellano JI, Benavides-Piccione R, Defelipe J, Yuste R. Ultrastructure of dendritic spines: correlation between synaptic and spine morphologies. Front Neurosci. 2007 Nov;1(1):131-43.

Arpini M, Menezes IC, Dall'Oglio A, Rasia-Filho AA. The density of Golgi-impregnated dendritic spines from adult rat posterodorsal medial amygdala neurons displays no evidence of hemispheric or dorsal/ventral differences. Neurosci Lett. 2010 Jan;469(2):209-13.

Ballesteros-Yáñez I, Benavides-Piccione R, Elston GN, Yuste R, DeFelipe J. Density and morphology of dendritic spines in mouse neocortex. Neuroscience. 2006;138(2):403-9.

Benavides-Piccione R, Ballesteros-Yáñez I, DeFelipe J, Yuste R. Cortical area and species differences in dendritic spine morphology. J Neurocytol. 2002 Mar-Jun;31(3-5):337-46.

Bennur S, Shankaranarayana Rao BS, Pawlak R, Strickland S, McEwen BS, Chattarji S. Stress-induced spine loss in the medial amygdala is mediated by tissue-plasminogen activator. Neuroscience. 2007 Jan;144(1):8-16.

Blaustein JD, Lehman MN, Turcotte JC, Greene G. Estrogen receptors in dendrites and axon terminals in the guinea pig hypothalamus. Endocrinology. 1992 Jul;131(1):281-90.

Bonhoeffer T, Yuste R. Spine motility. Phenomenology, mechanisms, and function. Neuron. 2002 Sep;35(6):1019-27.

Bourne J, Harris KM. Do thin spines learn to be mushroom spines that remember? Curr Opin Neurobiol. 2007 Jun;17(3):381-6.

Brusco J, Wittmann R, de Azevedo MS, Lucion AB, Franci CR, Giovenardi M, et al. Plasma hormonal profiles and dendritic spine density and morphology in the hippocampal CA1 stratum radiatum, evidenced by light microscopy, of virgin and postpartum female rats. Neurosci Lett. 2008 Jun;438(3):346-50.

Brusco J, Dall'Oglio A, Rocha LB, Rossi MA, Moreira JE, Rasia-Filho AA. Descriptive findings on the morphology of dendritic spines in the rat medial amygdala. Neurosci Lett. 2010 Oct;483(2):152-6.

Brusco J. Amígdala medial de ratos ao longo do ciclo estral: espinhos dendríticos, ultraestrutura sináptica, expressão gênica e lateralidade. [dissertação]. Ribeirão Preto (SP): Universidade de São Paulo; 2012.

Bupesh M, Legaz I, Abellán A, Medina L. Multiple telencephalic and extratelencephalic embryonic domains contribute neurons to the medial extended amygdala. J. Comp. Neurol. 2011 Jun;519:1505-1525.

Canteras NS, Simerly RB, Swanson LW. Organization of projections from the medial nucleus of the amygdala: a PHAL study in the rat. J Comp Neurol. 1995 Sep;360(2):213-45.

Carney RS, Mangin JM, Hayes L, Mansfield K, Sousa VH, Fishell G, Machold RP, Ahn S, Gallo V, Corbin JG. Sonic hedgehog expressing and responding cells generate neuronal diversity in the medial amygdala. Neural Dev. 2010 May;5:14.

Cavalcante JC, Sita LV, Mascaro MB, Bittencourt JC, Elias CF. Distribution of urocortin 3 neurons innervating the ventral premammillary nucleus in the rat brain. Brain Res. 2006 May;1089(1):116-25.

Choi GB, Dong HW, Murphy AJ, Valenzuela DM, Yancopoulos GD, Swanson LW, et al. Lhx6 delineates a pathway mediating innate reproductive behaviors from the amygdala to the hypothalamus. Neuron. 2005 May;46(4):647-60.

Cooke BM, Stokas MR, Woolley CS. Morphological sex differences and laterality in the prepubertal medial amygdala. J Comp Neurol. 2007 Apr;501(6):904-15.

Cooke BM, Woolley CS. Effects of prepubertal gonadectomy on a male-typical behavior and excitatory synaptic transmission in the amygdala. Dev. Neurobiol. 2009;69 (2-3): 141-52.

Cooke BM, Woolley CS. Sexually dimorphic synaptic organization of the medial amygdala. J Neurosci. 2005 Nov;25(46):10759-67.

Coolen LM, Peters HJ, Veening JG. Fos immunoreactivity in the rat brain following consummatory elements of sexual behavior: a sex comparison. Brain Res. 1996 Oct;738(1):67-82.

Coolen LM, Peters HJ, Veening JG. Distribution of Fos immunoreactivity following mating versus anogenital investigation in the male rat brain. Neuroscience. 1997 Apr;77(4):1151-61.
.

Coopersmith C, Gans SE, Rowe DW, Erskine MS. Infusions of lidocaine into the amygdala, but not the preoptic area, block pseudopregnancy in the rat. J Neuroendocrinol. 1996 Apr;8(4):259-66.

Cunningham RL, Clairborne BJ, McGinnis MY. Pubertal exposure to anabolic androgenic steroids increases spine densities on neurons in the limbic system of male rats. Neurosci. 2007 Dec;150: 609-615.

Dall'Oglio A, Gehlen G, Achaval M, Rasia-Filho AA. Dendritic branching features of posterodorsal medial amygdala neurons of adult male and female rats: further data based on the Golgi method. Neurosci Lett. 2008a Jan;430(2):151-6.

Dall'Oglio A, Gehlen G, Achaval M, Rasia-Filho AA. Dendritic branching features of Golgi-impregnated neurons from the "ventral" medial amygdala subnuclei of adult male and female rats. Neurosci Lett. 2008b Jul;439(3):287-92.

Dall'oglio A, Xavier LL, Hilbig A, Ferme D, Moreira JE, Achaval M, Rasia-Filho AA. Cellular components of the human medial amygdaloid nucleus. J Comp Neurol. 2013 Feb;521(3):589-611.

de Castilhos J, Marcuzzo S, Forti CD, Frey RM, Stein D, Achaval M, et al. Further studies on the rat posterodorsal medial amygdala: dendritic spine density and effect of 8-OH-DPAT microinjection on male sexual behavior. Brain Res Bull. 2006 Mar;69(2):131-9.

de Castilhos J, Forti CD, Achaval M, Rasia-Filho AA. Dendritic spine density of posterodorsal medial amygdala neurons can be affected by gonadectomy and sex steroid manipulations in adult rats: a Golgi study. Brain Res. 2008 Nov;1240:73-81.

de Castilhos J, Hermel EE, Rasia-Filho AA, Achaval M. Influence of substitutive ovarian steroids in the nuclear and cell body volumes of neurons in the posterodorsal medial amygdala of adult ovariectomized female rats. Neurosci Lett. 2010 Jan;469(1):19-23.

de Olmos JS, Alheid GF, Beltramino CA. Amygdala. In: Paxinos, G. The Rat Nervous System. Academic Press: Sydney; 1985. pp. 223-234.

de Olmos JS, Beltramino CA, Alheid G. Amygdala and extended amygdala of the rat: a cytoarchitectonical, fibroarchitectonical, and chemoarchitectonical survey. In: PAXINOS G. The Rat Nervous System. San Diego: Academic Press; 2004. pp. 509-603.

de Vivo L, Landi S, Panniello M, Baroncelli L, Chierzi S, Mariotti L, Spolidoro M, Pizzorusso T, Maffei L, Ratto GM. Extracellular matrix inhibits structural and functional plasticity of dendritic spines in the adult visual cortex. Nat Commun. 2013;4:1484. doi: 10.1038/ncomms2491.

de Vries GJ, Simerly RB. Anatomy, development, and function of sexually dimorphic neural circuits in the mammalian brain. In: PFAFF DW, ARNOLD AP, ETGEN AM, FAHRBACH SE, RUBIN RT. Hormones, Brain and Behavior. San Diego: Academic Press; 2002. pp. 137-191.

Dielenberg RA, McGregor IS. Defensive behavior in rats towards predatory odors: a review. Neurosci. Biobehav. Rev. 2001 Dec;25: 597-609.

DonCarlos LL, Sarkey S, Lorenz B, Azcoitia I, Garcia-Ovejero D, Huppenbauer C, et al. Novel cellular phenotypes and subcellular sites for androgen action in the forebrain. Neuroscience. 2006;138(3):801-7.

Dong HW, Petrovich GD, Swanson LW. Topography of projections from amygdala to bed nuclei of the stria terminalis. Brain Res Brain Res Rev. 2001 Dec;38(1-2):192-246.

Duncan GE, Knapp DJ, Breese, GR. Neuroanatomical characterization of Fos induction in rat behavioral models of anxiety. Brain Res. 1996 Mar;713: 79-91.

Everitt B. Limbic lobe and olfactory pathways. In: BERRY MM, BANNISTER LH, STANDRING SM. Gray's Anatomy. London: Churchill Livingstone; 1995. pp. 1115-1141.

Farrar SJ, Whiting PJ, Bonnert TP, McKernan RM. Stoichiometry of a ligand-gated ion channel determined by fluorescence energy transfer. J Biol Chem. 1999 Apr;274(15):10100-4.

Fischer M, Kaech S, Wagner U, Brinkhaus H, Matus A. Glutamate receptors regulate actin-based plasticity in dendritic spines. Nat Neurosci. 2000 Sep;3(9):887-94.

Fleming AS, Vaccarino F, Luebke C. Amygdaloid inhibition of maternal behavior in the nulliparous female rat. Physiol. Behav. 1980 Nov;25, 731–743.

Forti CD, Estudo da densidade dos espinhos dendríticos na região póstero-dorsal da amígdala medial de ratos intactos e orquiectomizados [dissertação]. Porto Alegre (RS): Universidade Federal do Rio Grande do Sul; 2005.

García-López M, Abellán A, Legaz I, Rubenstein JLR, Puelles L, Medina L. Histogenetic compartments of the mouse centromedial and extended amygdala base on gene expression patternsduring development. J. Comp. Neurol. 2008 Jan;506: 46-74.

Genoux D, Montgomery JM. Glutamate receptor plasticity at excitatory synapses in the brain. Clin. Exp. Pharmac. Physiol. 2007;34: 1058–1063.

Gomez DM, Newman SW. Medial nucleus of the amygdala in the adult Syrian hamster: a quantitative Golgi analysis of gonadal hormonal regulation of neuronal morphology. Anat Rec. 1991 Dec;231(4):498-509.

Gréco B, Edwards DA, Michael RP, Clancy AN. Androgen receptor immunoreactivity and mating-induced Fos expression in forebrain and midbrain structures in the male rat. Neuroscience. 1996 Nov;75(1):161-71.

Gréco B, Allegretto EA, Tetel MJ, Blaustein JD. Coexpression of ERβ with ERα and progestin receptor proteins in the female rat forebrain: effects of estradiol treatment. Endocrinology. 2001 Dec;142(12):5172-81.

Gréco B, Blasberg ME, Kosinski EC, Blaustein JD. Response of ERα-IR and ERβ-IR cells in the forebrain of female rats to mating stimuli. Horm Behav. 2003 Apr;43(4):444-53.

Guillamón A, Segovia S. Sex differences in the vomeronasal system. Brain Res Bull. 1997;44(4):377-82.

Gulledge AT, Stuart GJ. Excitatory actions of GABA in the cortex. Neuron. 2003 Jan;37(2):299-309.

Hanley JG. AMPA receptor trafficking pathways and links to dendritic spine morphogenesis. Cell Adh Migr. 2008 Oct-Dec;2(4):276-82.

Harris KM, Jensen FE, Tsao B. Three-dimensional structure of dendritic spines and synapses in rathippocampu at postnatal day 15 and adult ages: Implications for the maturation of synapticphysiology and long-term potentiation. Journal of Neurosci. 1992;12(7):2687-2705.

Harris KM, Stevens JK. Dendritic spines of CA 1 pyramidal cells in the rat hippocampus: serial electron microscopy with reference to their biophysical characteristics. J Neurosci. 1989 Aug;9(8):2982-97.

Heimer L, Harlan RE, Alheid GF, Garcia MM, de Olmos J. Substantia innominata: a notion which impedes clinical-anatomical correlations in neuropsychiatric disorders. Neurosci. 1997 Feb;76:957-1006.

Heldt SA, Ressler KJ. Forebrain and midbrain distribution of major benzodiazepine-sensitive GABAA receptor subunits in the adult C57 mouse as assessed with in situ hybridization. Neuroscience. 2007 Dec 5;150(2):370-85.

Hering H, Sheng M. Dendritic spines: structure, dynamics and regulation. Nat Rev Neurosci. 2001 Dec;2(12):880-8.

Hermel EE, Ilha J, Xavier LL, Rasia-Filho AA, Achaval M. Influence of sex and estrous cycle, but not laterality, on the neuronal somatic volume of the posterodorsal medial amygdala of rats. Neurosci Lett. 2006 Sep;405(1-2):153-8.

Hermel EE, Faccioni-Heuser MC, Marcuzzo S, Rasia-Filho AA, Achaval M. Ultrastructural features of neurons and synaptic contacts in the posterodorsal medial amygdala of adult male rats. J Anat. 2006b May;208(5):565-75.

Hill TC, Zito K. LTP-induced long-term stabilization of individual nascent dendritic spines. J Neurosci. 2013 Jan;33(2):678-86.

Hines M, Allen LS, Gorski RA. Sex differences in subregions of the medial nucleus of the amygdalaand the bed nucleus of the stria terminalis of the rat. Brain Res. 1992 May;579: 321-326.

Holtmaat A, Svoboda K. Experience-dependent structural synaptic plasticity in the mammalian brain. Nat Rev Neurosci. 2009 Sep;10:647-58.

Johnston JB. Further contributions to the study of the evolution of the forebrain, J Comp Neurol. 1923; 35:337–481.

Kasai H, Matsuzaki M, Noguchi J, Yasumatsu N, Nakahara H. Sructure-stability-function relationships of dendritic spines. Trends Neurosci. 2003 Jul;26: 360-368.

Keller A. Use-dependent inhibition of dendritic spines. Trends Neurosci. 2002 Nov;25(11):541-3; discussion 543-4.

Kim MJ, Dunah AW, Wang YT, Sheng M. Differential roles of NR2A- and NR2B-containing NMDA receptors in Ras-ERK signaling and AMPA receptor trafficking. Neuron. 2005 Jun 2;46(5):745-60.

Kim BG, Dai HN, McAtee M, Vicini S, Bregman BS. Labeling of dendritic spines with the carbocyanine dye DiI for confocal microscopic imaging in lightly fixed cortical slices. J Neurosci Methods. 2007 May 15;162(1-2):237-43.

Kisvárday ZF, Gulyas A, Beroukas D, North JB, Chubb IW, Somogyi P. Synapses, axonal and dendritic patterns of GABA-immunoreactive neurons in human cerebral cortex. Brain. 1990 Jun;113(Pt 3):793-812.

Kharazia VN, Weinberg RJ. Tangential synaptic distribution of NMDA and AMPA receptors in rat neocortex. Neurosci Lett. 1997 Nov 28;238(1-2):41-4.

Kopach O, Voitenko N. Extrasynaptic AMPA receptors in the dorsal horn: Evidence and functional significance. Brain Res Bull. 2012 Nov 26.

Lee KF, Soares C, Béïque JC. Examining form and function of dendritic spines. Neural Plast. 2012;2012:704103. doi: 10.1155/2012/704103.

Lehmann ML, Erskine MS. Glutamatergic stimulation of the medial amygdala induces steroid dependent c-fos expression within forebrain nuclei responsive to mating stimulation. Neuroscience. 2005 Sep;136(1):55-64.

Lehmann ML, McKellar H, Erskine MS. Coding for the initiation of pseudopregnancy by temporally patterned activation of amygdalar NMDA receptors. J Neurosci. 2005 Sep;25(38):8696-703.

Lin H, Huganir R, Liao D. Temporal dynamics of NMDA receptor-induced changes in spine morphology and AMPA receptor recruitment to spines. Biochem Biophys Res Commun. 2004 Apr;316(2):501-11.

London M, Häusser M. Dendritic computation. Annu Rev Neurosci. 2005;28:503-32.

López-Bendito G, Shigemoto R, Kulik A, Vida I, Fairén A, Luján R. Distribution of metabotropic GABA receptor subunits GABAB1a/b and GABAB2 in the rat hippocampus during prenatal and postnatal development. Hippocampus. 2004;14(7):836-48.

Lorenzo A, Díaz H, Carrer H, Cáceres A. Amygdala neurons in vitro: neurite growth and effects of estradiol. J Neurosci Res. 1992 Nov;33(3):418-35.

Marcuzzo S, Estudo sobre a densidade de espinhos dendríticos de neurônios da amígdala medial póstero-dorsal de ratos em diferentes condições experimentais [dissertação]. Porto Alegre (RS): Universidade Federal do Rio Grande do Sul; 2006.

Marcuzzo S, Dall'oglio A, Ribeiro MF, Achaval M, Rasia-Filho AA. Dendritic spines in the posterodorsal medial amygdala after restraint stress and ageing in rats. Neurosci Lett. 2007 Aug;424(1):16-21.

Marowsky A, Fritschy JM, Vogt KE. Functional mapping of GABA A receptor subtypes in the amygdala. Eur J Neurosci. 2004 Sep;20(5):1281-9.

Martinez FG, Hermel EE, Xavier LL, Viola GG, Riboldi J, Rasia-Filho AA, et al. Gonadal hormone regulation of glial fibrillary acidic protein immunoreactivity in the medial amygdala subnuclei across the estrous cycle and in castrated and treated female rats. Brain Res. 2006 Sep;1108(1):117-26.

Matus A. Actin-based plasticity in dendritic spines. Science. 2000 Oct;290(5492):754-8.

Matus A. MARCKS for maintenance in dendritic spines. Neuron. 2005 Oct;48(1):4-5.

McCarthy MM. Estradiol and the developing brain. Physiol. Rev. 2008 Jan;88:91–124,

McCarthy MM, Arnold AP. Reframing sexual differentiation of the brain. Nat Neurosci. 2011 Jun;14(6):677-83.

McDonald AJ. Cell types and intrinsic connections of the amygdale. In: AGGLETON JP. The Amygdala. New York: Wiley-Liss; 1992. pp. 67-96.

McDonald AJ. Cortical pathways to the mammalian amygdala. Prog Neurobiol. 1998 Jun;55(3):257-332.

McKinney RA, Capogna M, Dürr R, Gähwiler BH, Thompson SM. Miniature synaptic events maintain dendritic spines via AMPA receptor activation. Nat Neurosci. 1999 2,44–49.

McKinney RA. Excitatory amino acid involvement in dendritic spine formation, maintenance and remodelling. J Physiol. 2010 Jan 1;588(Pt 1):107-16.

Medina L, Bupesh M, Abellán A. Contribution of genoarchitecture to understanding forebrain evolution and development, with particular emphasis on the amygdala. Brain Behav Evol. 2011;78(3):216-36.

Meredith M, Westberry JM. Distinctive responses in the medial amygdala to same-species and different-species pheromones. J Neurosci. 2004 Jun;24(25):5719-25.

Neckel H, Quagliotto E, Casali KR, Montano N, Dal Lago P, Rasia-Filho AA. Glutamate and GABA in the medial amygdala induce selective central sympathetic/parasympathetic cardiovascular responses. Can J Physiol Pharmacol. 2012 May;90(5):525-36.

Newman SW. The medial extended amygdala in male reproductive behavior. A node in the mammalian social behavior network. Ann N Y Acad Sci. 1999 Jun;877:242-57.

Newman SW. Pheromonal signals access the medial extended amygdala: one node in a proposed social behavior network. In: PFAFF DW, ARNOLD AP, ETGEN AM, FAHRBACH SE, RUBIN RT. Hormones, Brain and Behavior. San Diego: Academic Press; 2002. pp. 17-31.

Newpher TM, Ehlers MD. Spine microdomains for postsynaptic signaling and plasticity. Trends Cell Biol. 2009 May;19(5):218-27.

Niimi K, Horie S, Yokosuka M, Kawakami-Mori F, Tanaka K, Fukayama H, Sahara Y.Heterogeneous electrophysiological and morphological properties of neurons in the mouse medialamygdala in vitro. Brain Res. 2012 Oct;1480:41-52.

Nimchinsky EA, Sabatini BL, Svoboda K. Structure and function of dendritic spines. Annu Rev Physiol. 2002;64:313-53.

Nishizuka M, Arai Y. Sexual dimorphism in synaptic organization in the amygdala and its dependence on neonatal hormone environment. Brain Res. 1981 May;212(1): 31-38.

Nusser Z. AMPA and NMDA receptors: similarities and differences in their synaptic distribution. Curr Opin Neurobiol. 2000 Jun;10(3):337-41.

Oray S, Majewska A, Sur M. Effects of synaptic activity on dendritic spine motility of developing cortical layer v pyramidal neurons. Cereb Cortex. 2006 May;16(5):730-41.

Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. San Diego: Academic Press; 1998.

Pereno GL, Balaszczuk V, Beltramino CA. Detection of conspecific pheromones elicits fos expression in GABA and calcium-binding cells of the rat vomeronasal system-medial extended amygdala. J Physiol Biochem. 2011 Mar;67(1):71-85.

Peters A, Kaiserman-Abramof IR. The small pyramidal neuron of the rat cerebral cortex. The perikaryon, dendrites and spines. Am. J. Anat. 127: 321-356, 1970.

Peters A, Palay S, Webster H. Dendrites. In: PETERS A, PALAY S, WEBSTER H. The fine structure of the nervous system. New York: Oxford University Press; 1991. pp. 70 –100.

Petralia RS, Wang YX, Hua F, Yi Z, Zhou A, Ge L, et al. Organization of NMDA receptors at extrasynaptic locations. Neuroscience. 2010 Apr 28;167(1):68-87.

Petrovich GD, Canteras NS, Swanson LW. Combinatorial amygdalar inputs to hippocampal domains and hypothalamic behavior systems. Brain Res Brain Res Rev. 2001 Dec;38(1-2):247-89.

Pfaus JG, Heeb MM. Implications of immediate-early gene induction in the brain following sexual stimulation of female and male rodents. Brain Res Bull. 1997;44(4):397-407.

Phelps EA, LeDoux JE. Contributions of the amygdala to emotion processing: from animal models to human behavior. Neuron. 2005 Oct 20;48(2):175-87.

Pitkänen A, Tuunanen J, Kälviäinen R, Partanen K, Salmenperä T. Amygdala damage in experimental and human temporal lobe epilepsy. Epilepsy Res. 1998;32: 233–253.

Pitkänen A. Connectivity of the rat amygdaloid complex. In: Aggleton, JP. The Amygdala. Oxford University-Press, p.31-115, 2000.

Pouille F, Scanziani M. Enforcement of temporal fidelity in pyramidal cells by somatic feed-forward inhibition. Science. 2001 Aug 10;293(5532):1159-63.

Pro-Sistiaga P, Mohedano-Moriano A, Ubeda-Bañon I, Arroio-Jimenez MDM, Marcos P, et al. Convergence of olfactory and vomeronasal projections in the rat basal telencephalon. J. Comp. Neurol. 2007 Oct;504: 346-362.

Quagliotto E. Efeito da Microinjeção de Histamina e Metil-Histamina no Núcleo Póstero-Dorsal da Amígdala Medial Sobre o Controle da Pressão Arterial em Ratos [dissertação]. Porto Alegre (RS): Universidade Federal do Rio Grande do Sul; 2006.

Quagliotto E, Neckel H, Riveiro DF, Casali KR, Mostarda C, et al. Histamine in the posterodorsal medial amygdala modulates cardiovascular reflex responses inawake rats. Neurosci. 2008 Dec;157(4): 709-719.

Quagliotto E, Casali KR, Dal Lago P, Rasia-Filho AA. Neurotransmitter and neuropeptidergic modulation of cardiovascular responses evoked by the posterodorsal medial amygdala of adult male rats. In: Amygdala: Structure, Functions and Disorders. (ed. Yilmazer-Hanke D). Hauppauge: Nova Science Publishers; 2012.

Ramón y Cajal S. Estructura de los centros nerviosos de las aves. Revista Trimestral de Histología Normal y Patológica. 1888 vol.1 1-10.

Ramón y Cajal S. (traduzido da edição francesa de 1909). Neurons: size and general morphology. In: Swanson N, Swanson SW, editors. Histology of the Nervous System of Man and Vertebrates. New York: Oxford University Press; 1995. pp. 46-57.

Rasia-Filho AA, Peres TM, Cubilla-Gutierrez FH, Lucion AB. Effect of estradiol implanted in the corticomedial amygdala on the sexual behavior of castrated male rats. Braz J Med Biol Res. 1991;24(10):1041-9.

Rasia-Filho AA, Londero RG, Achaval M. Effects of gonadal hormones on the morphology of neurons from the medial amygdaloid nucleus of rats. Brain Res Bull. 1999 Jan;48(2):173-83.

Rasia-Filho AA, Londero RG, Achaval M. Functional activities of the amygdala: an overview. J Psychiatry Neurosci. 2000 Jan;25(1):14-23.

Rasia-Filho AA, Xavier LL, dos Santos P, Gehlen G, Achaval M. Glial fibrillary acidic protein immunodetection and immunoreactivity in the anterior and posterior medial amygdala of male and female rats. Brain Res Bull. 2002 May;58(1):67-75.

Rasia-Filho AA, Fabian C, Rigoti KM, Achaval M. Influence of sex, estrous cycle and motherhood on dendritic spine density in the rat medial amygdala revealed by the Golgi method. Neuroscience. 2004;126(4):839-47

Rasia-filho AA, Hilbig A. Papel da amígdala e do hipocampo no transtorno do estresse pós-traumático. In: CAMINHA R. Transtornos do estresse pós-traumático. São Paulo: Casa do Psicólogo; 2005. pp. 37-53.

Rasia-filho AA, Brusco J, Moreira JE. Spine plasticity in the rat medial amygdala. In: COLUMBUS O. Dendritic Spines: Biochemistry, Modeling and Properties. New York: Nova Science Publishers; 2009.

Rasia-filho AA, Brusco J, Moreira JE, Rocha LB. Dendritic spines observed by extracellular DiI dye and immunolabeling under confocal microscopy. Nature Protocols/Network Protocols, dói: 10.1038/nprot.2010. 2010. p.153.

Rasia-Filho AA, Dalpian F, Menezes IC, Brusco J, Moreira JE, Cohen RS. Dendritic spines of the medial amygdala: plasticity, density, shape, and subcellular modulation by sex steroids. Histol Histopathol. 2012a Aug;27(8):985-1011.

Rasia-Filho AA, Haas D, de Oliveira AP, de Castilhos J, Frey R, Stein D, et al. Morphological and functional features of the sex steroid-responsive posterodorsal medial amygdala of adult rats. Mini Rev Med Chem. 2012b Oct;12(11):1090-106.

Richards DA, De Paola V, Caroni P, Gähwiler BH, McKinney RA. AMPA-receptor activation regulates the diffusion of a membrane marker in parallel with dendritic spine motility in the mouse hippocampus. J Physiol. 2004 Jul;558(Pt 2):503-12.

Rochefort NL, Konnerth A. Dendritic spines: from structure to in vivo function. EMBO Rep. 2012 Aug;13(8):699-708.

Savonenko A, Filipkowski RK, Werka T, Zielinski K, Kaczmarek L. Defensive conditioning-related functional heterogeneity among nuclei of the rat amygdala revealed by c-Fos mapping. Neurosci. 1999 Oct;94:723-733.

Segal M. Dendritic spines and long-term plasticity. Nature Rev. Neurosci. 2005;6: 277-284.

Segal M. Dendritic spines, synaptic plasticity and neuronal survival: activity shapes dendritic spines to enhance neuronal viability. Eur. J. Neurosci. 2010;31(12): 2178-2184.

Segev I, Rall W. Excitable dendrites and spines: earlier theoretical insights elucidate recent direct observations. Trends Neurosci. 1998 Nov;21(11):453-60.

Sheehan TP, Paul M, Amaral E, Numan MJ, Numan M. Evidence that the medial amygdala projectsto the anterior/ventromedial hypothalamic nuclei to inhibit maternal behavior in rats. Neurosci. 2001 Sep;106: 341–356.

Shepherd GM. The dendritic spine: a multifunctional integrative unit. J. Neurophysiol. 1996;75: 2197-2210.

Shughrue PJ, Lane MV, Merchenthaler I. Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. J Comp Neurol. 1997 Dec;388(4):507-25.

Sieghart W, Fuchs K, Tretter V, Ebert V, Jechlinger M, Höger H, Adamiker D. Structure and subunit composition of GABA(A) receptors. Neurochem Int. 1999 May;34(5):379-85.

Simerly RB, Chang C, Muramatsu M, Swanson LW. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. J Comp Neurol. 1990 Apr;294(1):76-95.

Stefanova N, Ovtscharoff W. Sexual dimorphism of the bed nucleus of the stria terminalis and the amygdala. Adv Anat Embryol Cell Biol. 2000;158:III-X, 1-78.

Swanson LW. Cerebral hemisphere regulation of motivated behavior. Brain Res. 2000 Dec;886(1-2):113-164.

Swanson LW, Petrovich GD. What is the amygdala? Trends Neurosci. 1998 Aug;21(8):323-31.

Tao W, Higgs MH, Spain WJ, Ransom CB. Postsynaptic GABAB Receptors Enhance Extrasynaptic $GABA_A$ Receptor Function in Dentate Gyrus Granule Cells. J Neurosci. 2013 Feb;33(9):3738-43.

Toni N, Buchs PA, Nikonenko I, Bron CR, Muller D. LTP promotes formation of multiple spine synapses between a single axon terminal and a dendrite. Nature. 1999;402: 421-425

Toran-Allerand D, Sohrabji F, Miranda R. Identification of a putative estrogen response element in the gene encoding BDNF. Proc Natl Acad Sci. 1995 Nov;92(24):11110-4.

Tsui LC, Scherer SW, Duvoisin RM, Kuhn R, Heng HH, Belloni E. Localization of two metabotropic glutamate receptor genes, GRM3 and GRM8, to human chromosome 7q. Genomics. 1996 Jan;31(2):230-3.

Valverde F. Intrinsic organization of the amygdaloid complex. A Golgi study in the mouse. Trab. Inst. Cajal Invest. Biol. 1962;54: 291–314.

Watanabe M, Inoue Y, Sakimura K, Mishina M. Developmental changes in distribution of NMDA receptor channel subunit mRNAs. Neuroreport. 1992 Dec;3(12):1138-40.

Wearne SL, Rodriguez A, Ehlenberger DB, Rocher AB, Henderson SC, Hof PR. New techniques for imaging, digitization and analysis of three-dimensioanl neural morphology on multiple scales. Neuroscience 2005;136: 661-680.

Whiting PJ, Bonnert TP, McKernan RM, Farrar S, Le Bourdellès B, Heavens RP, et al. Molecular and functional diversity of the expanding GABA-A receptor gene family. Ann N Y Acad Sci. 1999 Apr;868:645-53.

Wood RI, Newman SW. Hormonal influence on neurons of the mating behavior pathway in male hamsters. In: MICEVYCH, P.E. & HAMMER Jr, R.P. Neurobiological Effects of Sex Steroid Hormones. New York: Cambridge; 1995. pp. 3-39.

Woolley CS, McEwen BS. Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. J Comp Neurol. 1993 Oct;336(2):293-306.

Woolley CS, Weiland NG, McEwen BS, Schwartzkroin PA. Estradiol increases the sensitivity of hippocampal CA1 pyramidal cells to NMDA receptor-mediated synaptic input: correlation with dendritic spine density. J Neurosci. 1997 Mar;17(5):1848-59.

Yuste R. Dendritic spines and distributed circuits. Neuron. 2011 Sep;71(5):772-81.

Zehr JL, Todd BJ, Schulz KM, McCarthy MM, Sisk CL. Dendritic pruning of the medial amygdala during pubertal development of the male Syrian hamster. J Neurobiol. 2006 May;66(6):578-90.

Zhou J, Zhang H, Cohen RS, Pandey SC. Effects of estrogen treatment on expression of BDNF and CREB expression and phosphorylation in rat amygdaloid and hippocampal structures. Neuro Endocrinol Lett. 2005 Sep;81(5): 294-310.

Zirlinger M, Kreiman G, Anderson DJ. Amygdala-enriched genes identified by microarray technology are restricted to specific amygdaloid subnuclei. Proc Natl Acad Sci U S A. 2001 Apr;98(9):5270-5.

Zito K, Scheuss V, Knott G, Hill T, Svoboda K. Rapid functional maturation of nascent dendritic spines. Neuron. 2009 Jan;61(2):247-58.

7. ANEXO A

Parecer de Aprovação do Protocolo Experimental pela Comissão de Ética em Experimentação Animal (CETEA) da Faculdade de Medicina de Ribeirão Preto e pela Comissão de Ética no Uso de Animais (CEUA) da Universidade Federal de Ciências da Saúde de Porto Alegre.

UNIVERSIDADE DE SÃO PAULO
FACULDADE DE MEDICINA DE RIBEIRÃO PRETO

— Comissão de Ética em Experimentação Animal —

CERTIFICADO

Certificamos que o Protocolo para Uso de Animais em Experimentação **n° 174/2011**, sobre o projeto intitulado *"Estudo dos espinhos dendríticos na amígdala medial póstero-dorsal de ratos: morfologia e conectividade"*, sob a responsabilidade do **Professor Doutor Jorge Eduardo Moreira** está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi **APROVADO** em reunião de 30 de janeiro de 2012.

(We certify that the protocol ***n° 174/2011***, about ***"Study of dendritic spines on the rat posterodorsal medial amygdala: morphology and connectivity"***, agrees with the ETHICAL PRINCIPLES IN ANIMAL RESEARCH adopted by Brazilian College of Animal Experimentation (COBEA) and was approved by the College of Medicine of Ribeirão Preto of the University of São Paulo – Ethical Commission of Ethics in Animal Research (CETEA) in ***01/30/2012***.

Ribeirão Preto, 31 de janeiro de 2012.

Prof. Dr. Márcio Dantas
Presidente da Comissão de Ética em Experimentação Animal

CEUA –COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

1) PROTOCOLO Nº: 033/10 **Parecer 052/10**

2) DATA DO PARECER: 12/01/2011

3) TÍTULO DO PROJETO:

Estudo dos espinhos dendríticos na amígdala medial póstero-dorsal de ratos: morfologia e conectividade

4) PESQUISADOR RESPONSÁVEL:

Prof. Alberto Rasia Filho

5) RESUMO DO PROJETO:

Projeto dividido em 2 subprojetos:
1) Estudo da morfologia tridimensional dos espinhos dendríticos e da presença e distribuição de receptores glutamatérgicos e GABAérgicos na maigdala medial póstero-dorsal de ratos;
2) Estudo da densidade e da morfologia tridimensional dos espinhos dendríticos da amígdala medial póstero-dorsal de ratos ao longo do ciclo estral.

6) OBJETIVOS DO PROJETO:

Estudo em animais (ratos – machos e fêmeas) com o intuito de verificar a morfologia e a conectividade dos espinhos dendríticos do núcleo medial da amígdala através da técnica de reconstrução tridimensional em microscopia confocal.

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** [X] **D** [ ] **E** [ ]
Justifique:

Espécie: Ratos Wistar **Número Amostral:** 36

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:

Eutanásia dos animais (se aplicável): [X] Adequada [ ] Inadequada
Se achar inadequada cite abaixo as melhorias necessárias com metodologia substituta:

Local de Realização (Biotério/Labotarório): Laboratório de Fisiologia UFCSPA

Outra instituição. Qual?

11) CRONOGRAMA DE UTILIZAÇÃO DE ANIMAIS

Data	Espécie	Sexo	Quantidade
Fevereiro/2011	Ratos	machos	12
Janeiro/2012	Ratos	fêmeas	24

12) RECOMENDAÇÃO:

[X] Aprovado

[ ] Com Pendência

[ ] Não aprovado