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PROGRAMA DE PÓS-GRADUAÇÃO EM NEUROCIÊNCIAS

EFEITO DOS HORMÔNIOS GONADAIS SOBRE OS FILAMENTOS INTERMEDIÁRIOS DE ASTRÓCITOS HIPOCAMPAIS, DURANTE O DESENVOLVIMENTO E O CICLO ESTRAL: UMA ABORDAGEM IMUNOISTOQUÍMICA

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DEDICATÓRIA

Este trabalho é humildemente dedicado à Matilde.

“O ignorante afirma, o sábio duvida, o sensato reflete”

Aristóteles

AGRADECIMENTOS

À Prof. Dr. Matilde Achaval Elena, meu carinho e reconhecimento por sua confiança, apoio, amizade, exemplo de dedicação à ciência acima de tudo. E sua capacidade de nos ensinar a ensinar.

A todos, que de uma forma ou de outra contribuíram para que este trabalho se tornasse realidade.

RESUMO

O hipocampo compõe uma das estruturas do sistema límbico, suas funções são variadas, tendo seu papel na regulação do comportamento emocional e em processos relacionados com a estocagem de memórias. Apesar de ter pouco ou nenhum papel no controle da função reprodutiva, o hipocampo é ainda assim um alvo sensível aos esteróides gonadais. Filamentos intermediários, como a proteína ácida fibrilar glial (GFAP) é um eficiente marcador do citoesqueleto de astrócitos e sofrem modificações sob ação de hormônios gonadais. Os objetivos do presente estudo foram mensurar o numero de células imunorretivas à GFAP (GFAP-ir), avaliar a imunorreatividade dos marcadores para filamentos intermediários astrocitários, bem como sua morfologia pelo método de Scholl, a GFAP-ir, no estrato radiatum da região CA1 do hipocampo, de ratos machos e fêmeas durante o desenvolvimento pós-natal. Além disso, também foi investigada a GFAP-ir nas mesmas regiões de fêmeas adultas, tanto em condições fisiológicas de variação hormonal (ao longo do ciclo estral), como em situações suprafisiológicas (reposição hormonal após ovariectomia). Três experimentos foram realizados: no primeiro foram utilizados ratos machos e fêmeas durante o desenvolvimento pós-natal (n = 48), cujas idades foram: 11 dias pós-natal (PN11), 21 (PN21), 31 (PN31) e 45 (PN45). No segundo experimento, utilizou-se ratos Wistar adultos machos e fêmeas nulíparas adultas durante as fases do ciclo estral (diestro, proestro, estro e metaestro; n = 30) para revelar diferenças provocadas pelos hormônios gonadais femininos na composição astrocitária do hipocampo. No terceiro experimento utilizaram-se fêmeas adultas nulíparas submetidas à ovariectomia (OVX) (n = 18) e tratadas com substituição hormonal de benzoato de estradiol adicionado ou não à progesterona. Os dados foram comparados entre os grupos por meio de um teste de análise da variância (ANOVA) de uma via, sendo aplicado o teste

post-hoc Bonfferoni. A comparação dos dados de GFAP-ir apresentou diferenças significativas no número de células e na orientação dos prolongamentos. O número de prolongamentos centrais, aumentaram com a idade, sendo mais significativo para as fêmeas (F45xF11, $p \leq 0,001$, F45xF21, $p \leq 0,01$ e F45xF31 $p \leq 0,01$) que nos machos (M45x M21 $p \leq 0,05$). Já o número de processos laterais mostrou um aumento mais significativo com a idade nos machos (M45XM11, $p \leq 0,001$; M45XM21, $p \leq 0,001$ e M45xM31, $p \leq 0,01$) que nas fêmeas (F45xF11, $p \leq 0,01$). Havendo ainda diferenças quando analisados a orientação dos processos primários separadamente de suas ramificações. Os ratos machos apresentaram maior GFAP-ir do que as fêmeas em todas as fases do ciclo estral (diestro, proestro, estro e metaestro; $p < 0,001$), assim como apresentaram mais células que as fêmeas ($p<0,001$). Para orientação mais uma vez as diferenças ficaram restritas aos machos, tanto para os processos centrais como para os laterais ($p<0,001$ e $p<0,05$ respectivamente). Em fêmeas ovariectomizadas, injeções de estradiol provocaram aumento da GFAP-ir ($P < 0,001$), assim como houve um aumento do numero de células ($P < 0,05$) enquanto a morfologia não mostrou diferenças entre os grupos. Esses achados sugerem que a GFAP astrocitária pode ser afetada tanto por níveis fisiológicos de hormônios ovarianos quanto por manipulação hormonal destes esteróides, o que pode contribuir para a plasticidade neuro-glial relacionada a atividades locais e integradas destas áreas encefálicas em machos e fêmeas. Esses achados permitem concluir que esta influência hormonal sobre o hipocampo está presente ainda em fases iniciais do desenvolvimento do SN de ratos, apresentando particularidades morfológicas acima de tudo. Além disso, as diferenças de composição astrocitárias encontradas, ao longo do desenvolvimento, provavelmente se relacionam com a interação neurônio-glia em consonância com aspectos comportamentais e ajustes neuroendócrinos pertinentes a cada sexo, e a cada fase de diferenciação do sistema nervoso.

ABSTRACT

The hippocampus is part of the limbic system, its functions are varied, and their role in the regulation of emotional behavior and in processes related to the memories storage. Despite having little or no role in the control of reproductive function, the hippocampus still a sensitive target to gonadal steroids. Intermediate filaments such as glial fibrillary acidic protein (GFAP) is an efficient marker of the cytoskeleton of astrocytes and the action of gonadal hormones could alter this structure. The objectives of this study were to measure the number of GFAP imunorreactive cells (GFAP-ir), analysing the immunoreactivity the intermediate filaments of astrocyte, and their morphology by the Scholl's method, GFAP-ir in the stratum radiatum of the CA1 region from dorsal hippocampus of male and female rats during postnatal development. In addition, we investigated the GFAP-ir in the same regions of adult males and females, these under physiological hormonal changes (during the estrous cycle), as shown by supraphysiological (hormone replacement after ovariectomy). Three experiments were performed: the first was used male and female rats during postnatal development (n = 48), whose ages were 11 days postnatal (PN11), 21 (PN21), 31 (PN31) and 45 (PN45). In the second experiment, we used adult male Wistar rats and nulliparous adult females during the estrous cycle (diestrus, proestrus, estrus and metestrus, n= 30) to reveal differences caused by female gonadal hormones on the content of astrocytes in the hippocampus. In the third experiment, a nulliparous adult females subjected to ovariectomy (OVX) (n = 18) and treated with hormone replacement therapy of estradiol benzoate added or not with progesterone. Data were compared between groups by means of a test analysis of variance (ANOVA) one-way, and applied post-hoc Bonfferoni's test. A comparison of the GFAP-ir showed significant differences in cell number and orientation of the extensions. The

number of central extensions, increased with age, being more significant for females (F45xF11, $p \leq 0,001$, F45xF21, $p \leq 0,01$ and F45xF31 $p \leq 0,01$) than in males (M45x M21 $p \leq 0,05$). Since the number of lateral processes showed more significantly with age in males (M45XM11, $p \leq 0,001$; M45XM21, $p \leq 0,001$ and M45xM31, $p \leq 0,01$) than in females (F45xF11, $p \leq 0,01$). We also found differences when analyzed the orientation of primary processes separately from their branches. Male rats showed higher GFAP-ir than females at all stages of the estrous cycle (diestrus, proestrus, estrus and metestrus, $p < 0,001$) and had more cells than females ($p < 0,001$). About cell orientation, the differences were observed just in males, both for the core processes and to the lateral ($p < 0,001$ and $p < 0,05$ respectively). In ovariectomized females, injections of estradiol benzoate caused an increase in GFAP-ir ($P < 0,001$), as well as an increase in the number of cells ($p < 0,05$), but the morphology did not differ between groups. These findings suggest that the GFAP astrocyte can be affected both by physiological levels of ovarian hormones and on the hormonal manipulation of these steroids, which may contribute to neuro-glial plasticity related to local activities and integrated these brain areas in males and females. These findings show that hormonal influence on the hippocampus were present in early stages of development of the SN of rat, specially in the morphological characteristics. In addition, differences in astrocytic composition found throughout the development, probably were related to the neuron-glia interaction in accordance with aspects of behavioral and neuroendocrine adjustments, specific to each gender, and each stage of differentiation of the nervous system.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

AOI	Área de interesse
DO	Densidade óptica
E	Estrógeno
EB	Benzoato de estrógeno
ER	Receptor de estrógeno
FI	Filamentos intermediários
FSH	Hormônio folículo estimulante
GABA	Ácido γ-aminobutírico
GFAP	Proteína fibrilar ácida glial
GFAP-ir	GFAP Imunorreativo
GnRH	Hormônio liberador de gonadotrofinas
NMDA	N-metil-*D*-aspartato
OVX	Ovariectomia
P	Progesterona
PAP	Peroxidase anti-peroxidase
PB	Tampão fosfato
PBS	Tampão fosfato salino
PN	Dias pós-natal
REL	Retículo endoplasmático liso
RER	Retículo endoplasmático rugoso
SDN-POA	Núcleo sexualmente dimórfico – Área pré-óptica
SNC	Sistema nervoso central

SUMÁRIO

INTRODUÇÃO

1 CONSIDERAÇÕES GERAIS

O estudo das diferenças sexuais no encéfalo tem sido um dos focos mais ativos de investigação por aproximadamente 50 anos, e ironicamente, tem sido revigorado na última década pelo afluxo de mulheres cientistas. Para os endocrinologistas, não há dúvidas sobre a existência de diferenças sexuais no encéfalo, incluindo os seres humanos. As diferenças sexuais são óbvias na fisiologia reprodutiva; fêmeas ovulam em uma base periódica e regular, engravidam, tem filhos e amamentam, enquanto os machos não; exigindo que as regiões do encéfalo que regulam esses perfis diversificados sejam diferentes.

O "dimorfismo" sexual é um fenômeno comum, onde fêmeas e machos da mesma espécie diferem entre si, o que se manifesta em diversas características fisiológicas e comportamentais das espécies animais, incluindo reprodução, agressividade, emoções e cognição (STEFANOVA; OVTSCHAROFF, 2000). A reprodução sexual depende de uma variedade de comportamentos individuais e sociais, os quais diferem entre machos e fêmeas. O funcionamento do sistema hormonal gonadal tônico do macho contrasta com o caráter cíclico das fêmeas e muitas diferenças se estabelecem entre os gêneros, refletidas tanto estruturalmente como no aspecto comportamental. O comportamento e a morfologia espelham as funções endócrinas, uma vez que os hormônios têm poderosa influência sobre ambos, atuam durante períodos prolongados de tempo e podem variar em qualquer direção (MACLUSKY; NAFTOLIN, 1981; BECKER et al., 2002; PFAFF; PHILIPS; RUBIN, 2004).

Esteróides sexuais modulam o comportamento reprodutivo em uma grande variedade de espécies animais (PFAFF; PHILLIPS; RUBIN, 2004). Esta característica

filogeneticamente primitiva inclui de roedores a seres humanos. O comportamento reprodutivo, incluindo o sexual propriamente dito, o comportamento maternal, defensivo, territorial e de exploração do ambiente dependem da plasticidade das células neuronais e gliais provocadas por hormônios sexuais (McEWEN et al., 1990; WOOD; NEWMAN, 1995; GARCIA-SEGURA et al., 1999).

A ação transformadora dos hormônios soma-se às do genoma e do ambiente, para determinar o desenvolvimento de estruturas nervosas sexualmente "dimórficas" que, por sua vez, modulam a fisiologia do comportamento reprodutivo típico de machos e fêmeas (KELLY, 1991). Além disso, os hormônios são componentes críticos da interação neurônio-glia, provocando ações neuromodulatórias e neurotróficas em situações fisiológicas ou patológicas (GARCIA-SEGURA; McCARTHY, 2004). Células neurais e gliais possuem receptores para hormônios esteróides, os quais provocam efeitos genômicos e não-genômicos sobre o sistema nervoso central (McEWEN; COIRINI; SCHUMACHER, 1990; TORAN-ALERAND, 1995; FERNANDEZ-GALAZ et al., 1997; GARCIA-SEGURA et al., 1999). A ação dos hormônios sexuais sobre o SNC provoca mudanças definitivas na vida do animal, como é o caso de áreas sexualmente "dimórficas", diferentes entre machos e fêmeas em vários aspectos, como forma e arranjo celular das estruturas encefálicas (McEWEN; COIRINI; SCHUMACHER, 1990; WOOD; NEWMAN, 1995). Tais mudanças podem ocorrer durante um período crítico chamado "organizacional", quando ocorrem mudanças permanentes em estruturas sensíveis do SNC, ou sobre animais púberes ou adultos. Esses hormônios desempenham papéis "ativacionais", controlando a função reprodutiva adulta, embora haja exceções quanto a esta regra. O período organizacional ocorre no período pré-natal e, em ratos, se estende de aproximadamente 4 a 5 dias antes do nascimento até 5 a 10 dias pós-natais (KELLY, 1991).

Enquanto várias regiões cerebrais contribuem para um processo que chamamos de um modo geral de "cognição", a maior parte da atenção tem sido dada ao hipocampo, ao qual tem-se atribuído diferenças sexuais. Excelentes e provocativos estudos sobre o córtex cerebral de roedores e humanos têm recebido grande foco na pesquisa científica (WITELSON, 1991; WITELSON, GLEZER, KIGARD, 1995; MARKHAM; JURASKA, 2002; MARKHAM et al., 2003)

Muitos, se não todos, os estudos sobre diferenças sexuais na cognição se baseiam nos princípios fundamentais da diferenciação sexual: no período neonatal a testosterona nos machos é localmente aromatizada para estradiol no encéfalo, induzindo a masculinização. Endocrinologistas fizeram a suposição razoável de que a relação observada entre os níveis séricos de testosterona, correlacionada com níveis elevados de estradiol no hipotálamo na hora de nascimento (WEISZ; WARD, 1980), poderiam ser generalizados para qualquer região do cérebro que possuísse atividade da aromatase. Em neonatos, o telencéfalo apresenta valores baixos, mas detectáveis, para a atividade da aromatase (MACLUSKY et al., 1994). Essa mesma região apresenta o mais alto nível de expressão do receptor para estrógeno nos primeiros dias de vida, tanto no córtex cerebral como no hipocampo, depois diminuindo para níveis menores nos adultos (MIRANDA, SOHRABJI, TORAN-ALLERAND, 1994; O'KEEFE et al., 1990; O'KEEFE et al., 1993).

Que o estradiol participa de uma diferenciação sexual do telencéfalo em desenvolvimento para um fenótipo masculino parece evidente, e é geralmente aceito que esta é a base para uma vantagem masculina no aprendizado espacial. Esta afirmação baseia-se em pelo menos três pressupostos fundamentais: (1) os níveis de estradiol estão mais elevados durante o desenvolvimento do telencéfalo masculino em relação ao feminino (2), portanto o telencéfalo é sexualmente diferente, e (3) os machos passam a ter uma vantagem inerente na

aprendizagem espacial. Cada pressuposto é baseado em verificações independentes, tanto empiricamente, como através do conhecimento existente sobre o desenvolvimento do hipocampo e suas funções (McCARTHY, KONKLE, 2005).

1.1 DESENVOLVIMENTO E HORMÔNIOS GONADAIS

O encéfalo começa a vida como um elemento genérico, sem definição de macho ou fêmea, aguardando para ser impactado pelo perfil hormonal circulante, que será determinado pelas gônadas. A melhor exemplificação disto é o rato de laboratório, a partir do qual sabemos que em um pré-determinado ponto do tempo durante o desenvolvimento, no final da gestação, o testículo fetal libera grandes quantidades de testosterona para a circulação. Através do sangue, a testosterona ganha acesso ao cérebro, onde é localmente aromatizada para estradiol, hormônio responsável pela diferenciação do substrato neural para um fenótipo masculino (Figura 1).

Modelo clássico dos efeitos organizacionais e ativacionais dos esteróides no desenvolvimento: Parâmetros reprodutivos

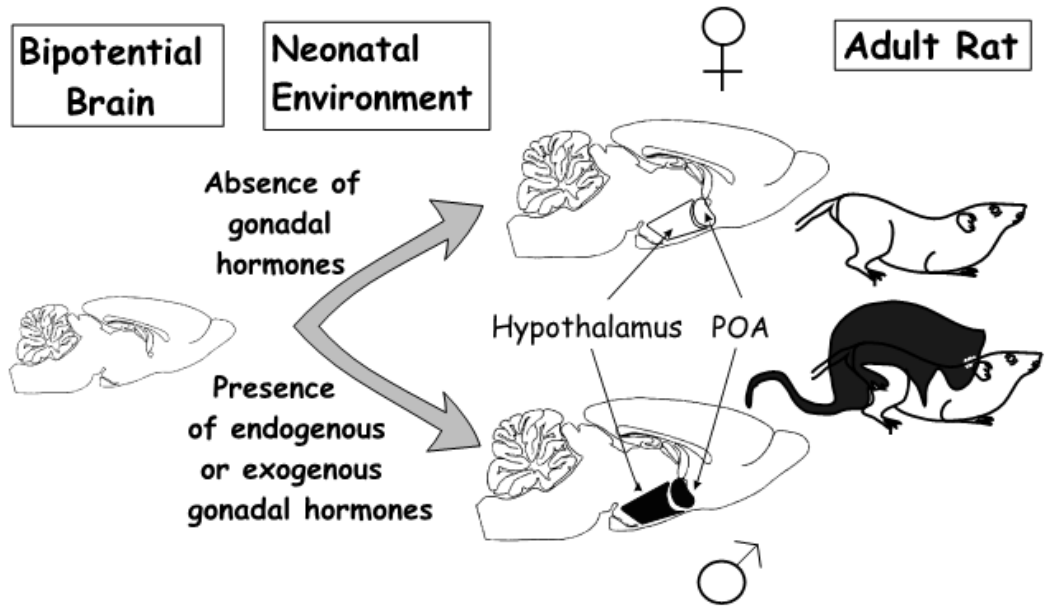


Figura 1 - Diferenciação sexual de um núcleo hipotalâmico reprodutivamente relevante. O encéfalo embrionário do rato é bipotente. O fenótipo do encéfalo de um macho adulto resulta da exposição ao estradiol (E2) no período final da gestação/neonatal, que é aromatizada a partir da testosterona (T). Administração exógena de testosterone ou estradiol em um animal fêmea geneticamente, resulta em um fenótipo masculino completo para as respostas reprodutivas do hipotálamo. Já a ausência de hormônios gonadais em ratos recém nascidos irá constituir um encéfalo feminilizado e que apresentará os reflexos das ações hormonais do adulto sobre seu comprotamento.

Fonte: McCarthy e Konkle, 2005

Testosterona e seu produto reduzido, a diidrotestosterona, também exercem efeitos diferenciando o sistema nervoso; sendo um bom exemplo o núcleo espinhal bulbocavernoso. Em outras espécies, especialmente em primatas, a maioria de diferenciação, considerada masculinização, é mediada por andrógenos em oposição a estrógenos (SMITH, 1975).

O conceito de diferenciação sexual do encéfalo foi dado em um artigo de Phoenix (1959), que postulou a hipótese organizational/ativacional da ação de hormônios esteróides sobre o encéfalo. Este quadro conceitual fora construído com base em evidências de uma

extensa variedade de espécies, mas foi empiricamente estabelecido pelo tratamento hormonal em porquinhas-da-india gestantes e da observação do comportamento da prole adulta. Colocando de forma simples, os fetos que foram expostos a andrógenos iriam se comportar como machos quando adultos se novamente expostos aos andrógenos, mas se eles não tinham a exposição ao andrógeno durante o desenvolvimento, eles não assumiam tal comportamento. Outros estudos realizados na mesma época, em ratos, observaram que os filhotes recém-nascidos do sexo feminino expostos a androgênios resultavam em adultos inférteis devido a defeitos na ovulação. Parecia bastante evidente em retrospecto, que deveria ter havido uma mudança permanente na arquitetura neural que regula o controle da secreção de gonadotrofinas e o comportamento sexual, e que logo deveria haver diferenças sexuais que ocorreriam naturalmente no encéfalo, mas naquele momento não era claro que as mudanças simplesmente não se limitavam ao ovário ou a hipófise. Uma vez que fora determinado que o encéfalo era responsável pela infertilidade induzida pelo andrógeno, uma enxurrada de estudos examinaram uma série de parâmetros químicos neurais, com relatos de diferenças sexuais na atividade da histamina acetiltransferase, parâmetros colinérgicos e serotoninérgicos, bem como da síntese e metabolismo dos esteróides, principalmente do estradiol. Surpreendentemente, pouca atenção foi dada à anatomia, embora tenha havido alguns estudos. Na castração precoce, estava a maneira encontrada para induzir mudanças na área preoptica do macho adulto (PFAFF, 1999a), que é logicamente consistente, mas a noção de diferenças sexuais encefálicas não veio à tona realmente, até o relato de um núcleo sexualmente dimórfico no cérebro de mamíferos (GORSKI, 1978) e das dramáticas diferenças sexuais no cérebro dos canários (NOTTEBOHM; ARNOLD, 1976). Estes relatos, de alto impacto, foram seguidos por uma explosão de interesses sobre os aspectos morfométricos do dimorfismo sexual, os quais continuam até hoje. Parecendo que onde quer que você olhe,

haverá uma diferença sexual; seja no tamanho de uma região, na densidade de neurônios ou no padrão de conectividade, entre outras. No entanto, permanece o questionamento: essas diferenças existem mesmo?

1.2 HORMÔNIOS GONADAIS: CICLO ESTRAL E "DIMORFISMO" SEXUAL

Hormônios são tradicionalmente definidos como compostos liberados na corrente sangüínea, principalmente pelas glândulas endócrinas, os quais agem em sítios-alvo localizados a variadas distâncias da glândula secretora (KAWATA, 1995; GREENSPAN; GARDNER, 2004, PFAFF; PHILLIPS; RUBIN, 2004, BALTHAZART; BALL, 2006). O colesterol é o precursor das cinco principais classes de hormônios esteróides: progestinas, estrogênios, androgênios, glicocorticóides, mineralocorticóides e vitamina D (KAWATA, 1995; GREENSPAN; GARDNER, 2004). Sabemos, hoje, que o colesterol pode ser um fator derivado tanto dos neurônios (ULLIAN et al., 2001) quanto dos astrócitos (HU et al., 2007).

O SNC é um tecido-alvo de hormônios gonadais, onde estes últimos promovem alterações na expressão gênica e na síntese protéica, interferindo então no fenótipo celular derivado do genoma (MURDACH e GORSKI, 1991; TORAND-ALLERAND, 1995). Esses processos ocorrem por meio da ligação dos hormônios aos receptores, preferencialmente no núcleo da célula, onde então estabelecem contato com o sítio de reconhecimento, o que provoca aumento na duplicação do DNA e, subseqüentemente, o aumento da síntese da proteína correspondente (TORAND-ALLERAND, 1995; McEWEN; ALVES, 1999; CORNIL; BALL; BALTHAZART, 2006). Essas proteínas incluem enzimas de síntese (como a tirosina hidroxilase), transportadores, receptores, proteínas de transdução de sinal

(fosfatases, kinases, proteínas acessórias, etc) ou enzimas de degradação (como monoamina oxidases), as quais modificam a neurotransmissão (McEWEN; ALVES, 1999).

Os esteróides sexuais não se restringem às ações genômicas. Eles podem agir alterando o potencial de membrana neuronal e o metabolismo de neurotransmissores, influenciando as transmissões sinápticas. Ações gênicas e não-gênicas diferem em tempo de ação: efeitos resultantes em segundos são considerados não-gênicos, enquanto efeitos gênicos demandam vários minutos ou mesmo dias (McEWEN et al., 1991; CORNIL; BALL; BALTHAZART, 2006).

Uma vez que as mudanças no ambiente neural causadas por hormônios sexuais como o estradiol são rápidas e não envolvem mecanismos genômicos, esses hormônios poderiam inclusive ser classificados como neuromoduladores ou mesmo neurotransmissores (BALTHAZART; BALL, 2006; CORNIL; BALL; BALTHAZART, 2006; WATERS et al., 2009). Considera-se ainda que o estrogênio possa ser sintetizado no encéfalo por meio da enzima aromatase, sendo uma fonte local de altas concentrações deste esteróide (CORNILL; BALL; BALTHAZART, 2006). As alterações neuroquímicas provocadas por hormônios têm repercussão na modulação comportamental e nas atividades neuroendócrinas, entre outras funções (McEWEN et al., 1990).

O eixo hipotálamo-hipófise-gonadal (HHG) é regulado primariamente pelo hormônio liberador de gonadotrofinas (GnRH) a partir do hipotálamo. O GnRH estimula a secreção de gonadotrofinas, hormônio luteinizante (LH) e folículo-estimulante (FSH) por parte da hipófise anterior, a qual, por sua vez: a) estimula o crescimento folicular e produção de estrogênio (FSH), formação do corpo lúteo e produção de progesterona (LH) no ovário da fêmea, e b) estimula a produção de testosterona (LH) e produção de espermatozóides (FSH) no testículo do macho (GREENSPAN; GARDNER, 2004, PFAFF; PHILLIPS; RUBIN,

2004). Esses hormônios esteróides, produzidos predominantemente pelas gônadas, mas com uma mínima quantidade de andrógenos sintetizados nas adrenais, atuam sobre a diferenciação do sistema nervoso por meio da ligação de receptores específicos para testosterona, estrógeno e progesterona (SIMERLY et al., 1990; KELLY, 1991; GORSKY, 1993; FITCH; DENESBERG, 1998).

Os estrogênios, importante classe de hormônios esteróides, exercem uma ampla variedade de ações em funções reprodutivas e não-reprodutivas (CORNIL; BALL; BALTHAZART, 2006) relacionando-se a processos neuroendócrinos, fisiológicos e comportamentais tanto durante o desenvolvimento quanto em animais adultos (PILGRIM; REISERT, 1992; LI; SCHWARTZ; RISSMAN, 1997). A administração experimental de estrogênios (48 h ou mais) seguida de progesterona em roedores promove o comportamento sexual típico da fêmea. Já o estrogênio em altos níveis, paralelamente à queda de progestinas, configura a combinação ótima para o comportamento maternal. O comportamento social, por sua vez, envolve outras configurações hormonais, envolvendo além de E e P, a ocitocina e, dependendo da espécie, a vasopressina (PFAFF, 1999).

Hormônios gonadais atuam também sobre células gliais, podendo modificar a morfologia e função destas células, especialmente nas áreas sabidamente “dimórficas” (GARCIA-SEGURA; McCARTHY, 2004). Diferenças sexualmente “dimórficas” já foram também detectadas na glia, uma vez que fêmeas apresentaram uma maior expressão da GFAP nos astrócitos da amígdala medial do que os ratos machos (RASIA-FILHO et al., 2002).

Por muitos anos as células gliais não foram consideradas como componentes relevantes no processo de diferenciação sexual das estruturas do SNC. Todavia, evidências recentes têm demonstrado que as células gliais são igualmente afetadas por esteróides gonadais, e ativamente envolvidas nos efeitos desses esteróides sobre a organização estrutural

do sistema nervoso (GARCIA-SEGURA et al., 1999; GARCIA-SEGURA; McCARTHY, 2004).

O ciclo sexual ou estral de ratas compõe um meio natural e repetitivo para estudar as variações dos hormônios esteróides e suas ações fisiológicas. Muito do conhecimento que há sobre o controle do ciclo ovariano de vários mamíferos que possuem ovulação espontânea é baseado em estudos sobre o controle do ciclo estral da rata. Esse ciclo tem uma duração de 4 a 5 dias, se repete durante o ano inteiro e é composto por quatro fases: diestro (55 a 57 h), proestro (12 a 14 h), estro (25 a 27 h) e metaestro (6 a 8 h). Cada uma das fases do ciclo caracteriza-se por expressar mudanças no útero e cérvix uterino, detectáveis por características do epitélio vaginal, como a presença predominante de leucócitos (diestro), de células epiteliais nucleadas (proestro), de células epiteliais cornificadas (estro), ou uma mistura homogênea de todos os tipos já citados (metaestro). As fases mudam conforme as variações nas concentrações de gonadotrofinas e, conseqüentemente, de esteróides gonadais (SMITH, FREEMAN, NEILL, 1975). A Figura 2 esquematiza o ciclo estral conforme a concentração hormonal presente no plasma da fêmea.

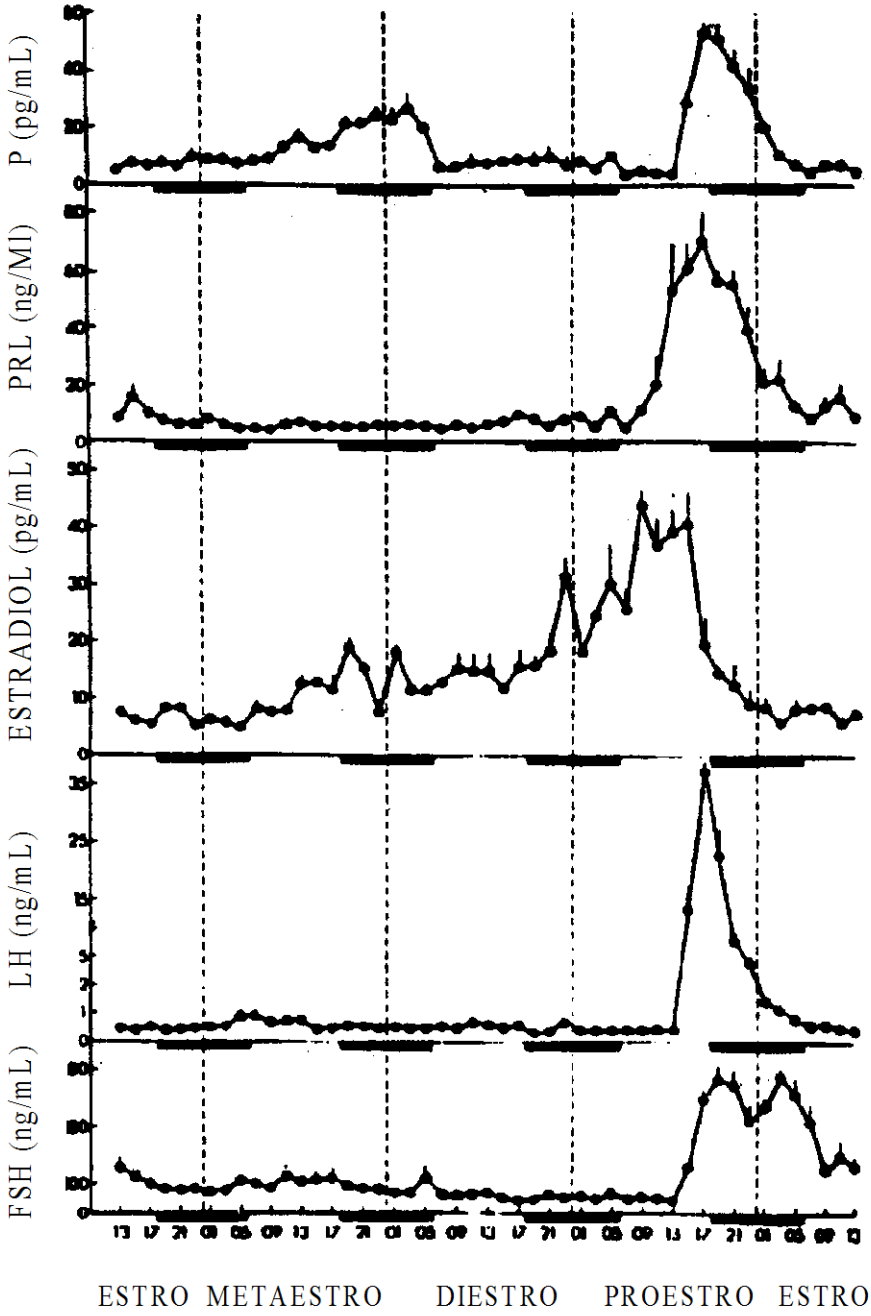


Figura 2 - Concentrações plasmáticas de progesterona (P), prolactina (PRL), estradiol, hormônio luteinizante (LH) e hormônio folículo-estimulante (FSH) obtidas em intervalos de 2 h nas quatro fases do ciclo estral da rata. O traço mais largo no eixo horizontal representa o período escuro do ciclo diário claro-escuro.

Fonte: Smith, Freeman and Neill., 1975.

Na fase do proestro o estradiol chega à sua máxima concentração, de forma a desencadear um *feedback* positivo, o qual promove a liberação do GnRH, um requisito para a ovulação (FREEMAN, 1994). Nesta fase, a presença de um rato macho e uma fêmea fértil dá início a um comportamento reprodutivo de ambos que inclui uma prévia investigação social do casal, por meio de informações sensoriais, como olfatórias e ultra-sônicas (MEISEL; LUMIA; SACHS, 1980; MO et al., 1992). A fêmea receptiva responde às lambidas realizadas pelo macho em sua genitália, com um comportamento que envolve corridas breves em direção oposta a ele, saltos e paradas rápidas, sempre posicionando os quadrantes posteriores em direção ao macho (KARPAS et al., 1983). A intromissão peniana e a ejaculação do macho na fêmea só ocorrem na ocasião do reflexo de lordose, desencadeado pela monta do macho sobre a fêmea. O circuito neural e alguns dos genes envolvidos nos mecanismos de suporte para este tipo de comportamento requerem estrogênio seguido de progesterona, a fim de que o comportamento sexual da fêmea seja sincronizado com o fenômeno da ovulação, o que apresenta o mesmo efeito combinatorial (PFAFF et al., 1994; PFAFF, 1999; PFAFF; PHILLIPS; RUBIN, 2004).

1.3 HIPOCAMPO: CONCEITO, LOCALIZAÇÃO E FUNÇÕES

O hipocampo compõe uma das estruturas do sistema límbico, e possui funções variadas, tendo seu papel na regulação do comportamento emocional inicialmente apontado por Papez em 1937. Outra função importante é sua participação no fenômeno de memórias diversas e em processos relacionados com o armazenamento de memórias (WYSS & VAN GROEN, 1992). A formação hipocampal é uma importante estrutura no aprendizado

episódico e espacial, bem como um componente de controle de uma variedade de funções vegetativas.

O sistema vomeronasal, o núcleo cortical póstero-medial da amígdala, a área pré-óptica medial e o hipotálamo ventromedial apresentam características sexualmente "dimórficas". Todas estas regiões conectam-se com o sistema límbico, o que sugere haver uma circuitaria neural exclusiva de cada sexo envolvendo essas regiões (CANTERAS; SIMERLY; SWANSON, 1995; GUILLAMÓN; SEGOVIA, 1997).

De fato, apesar de ter pouco ou nenhum papel no controle da função reprodutiva, o hipocampo é ainda assim um alvo sensível aos esteróides gonadais. Considerável atenção tem sido dada à modulação do estradiol na plasticidade sináptica em adultos (WOOLLEY et al, 1997; HU et al, 2007), e seus efeitos sobre o as funções cognitivas (KAMPE; SHERWIN, 1994; SHERWIN, 1997; DANIEL, ROBERTS, DOHANICH, 1999; DANIEL; DOHANICH, 2001; SANDSTROM; WILLIAMS, 2001; LUINE; JACOME; MACLUSKY; 2003; LI et al., 2004). Há cada vez mais interesse sobre as ações do estradiol no hipocampo adulto e durante o envelhecimento, dada a controvérsia em torno do uso da terapia de reposição hormonal em mulheres. Não há provas irrefutáveis de que o estradiol aumenta potencialmente a eficácia sináptica no hipocampo, aumentando a densidade e a funcionalidade das sinapses em espinhas dendriticas de ratas fêmeas (WOOLLEY et al., 1997; RUDICK; WOOLLEY, 2003). Os testes comportamentais destinados a correlacionar temporalmente mudanças fisiológicas com a aprendizagem espacial forneceram provas igualmente fortes quanto ao reforço da capacidade cognitiva em resposta à ação do estradiol no hipocampo (SANDSTROM; WILLIAMS, 2001).

A quantidade considerável de trabalhos sobre o hipocampo e estradiol levam para o pressuposto lógico que existem diferenças substanciais entre os sexos no hipocampo também. Na verdade, o efeito do estradiol sobre as tarefas de aprendizagem espacial em ratos e tarefas

de recordação verbal em seres humanos é frequentemente interpretada como uma diferença sexual nas habilidades cognitivas. No entanto, quando as perguntas são especialmente moldadas para saber se há diferenças sexuais no hipocampo e nas funções reguladas pelo mesmo, as evidências são muitas vezes em favor da inexistência de qualquer diferença, ou diferenças sexuais muito sutis, ou ainda de diferenças em que os dados nada mais são que a média desta variação hormonal, induzida por sua modulação, porém permanecendo dentro do intervalo da resposta do sexo masculino.

1.3.1 Organização laminar

O Corno de Amon no hipocampo possui uma organização laminar presente em todas as sub-regiões. A camada mais superficial limita-se com o ventrículo e a mais profunda com o giro denteado. Desta forma encontramos nesta ordem a camada de células ependimárias, o estrato alveus, o estrato oriens, o estrato piramidal, o estrato radial, o estrato lacunoso e o estrato molecular (RAMÓN y CAJAL, 1911; LORENTE DE NÓ, 1934), porém alguns autores falam de estrato lacunoso-molecular (AMARAL & WITTER, 2005).

A sensibilidade ao estrógeno tem sido descrita para o estrato radiatum da região CA1 do hipocampo, onde altos níveis de estradiol resultam em um incremento da densidade de espinhas dendríticas e número de sinapses (GOULD et al., 1990; WOOLLEY; MCEWEN, 1992), tamanho das espinhas e suas formas (WOOLLEY et al., 1999; LI et al., 2004), neurotransmissores (WOOLLEY et al., 1999; SCHARFMAN et al., 2003; LEDOUX et al., 2009) e a modulação do aprendizado e memória dependente do hipocampo (LUINE et al., 2003; SANDSTROM; WILLIAMS, 2004)

Enquanto as contribuições relativas dos tipos de receptor de estrogênio, β versus α, na transcrição de genes versus tradução da proteína ou degradação aguardam um estudo mais aprofundado, os relatórios sobre os efeitos diferenciais da ativação ERα e β na dinâmica intracelular de Ca^{2+}, fosforilação de moléculas sinalizadoras e neuroproteção sugerem que cada receptor tem contribuições diferenciadas para plasticidade neural normal da sinapse, e dos comportamentos dependentes do hipocampo (DAY et al., 2005; SZYMCZAK et al., 2006, ZHAO; BRINTON, 2007, FOSTER et al. 2008; WALF; FRYE, 2008; WATERS et al, 2009). Apesar de agonistas seletivos de ERα e β não completamente imitarem as ações do ERβ analisados, tanto individualmente e em ações simultâneas de ERα e β podem contribuir para os efeitos do estrogênio sobre a plasticidade do hipocampo e memória.

No SNC, células gliais participam da regulação hormonal de funções sinápticas, plasticidade sináptica, formação de mielina, cognição, sono e respostas do tecido nervoso à lesão, sendo também elementos fundamentais para o entendimento de ações hormonais no SNC e na regulação de eventos neuroendócrinos (GARCIA-SEGURA; McCARTHY, 2004). A plasticidade neuro-glial mediada pela ação de hormônios e suas repercussões funcionais, bem como o próprio ajuste neuroendócrino, são a base neural para comportamento reprodutivo e parental tão diferente estabelecido entre machos e fêmeas de uma mesma espécie (KELLY, 1991; RASIA-FILHO et al., 1991; 2002).

1.4 ASTRÓCITOS: ESTRUTURA, FUNÇÕES, INTERAÇÕES E PLASTICIDADE

As células gliais são classicamente conhecidas como células não-excitáveis do encéfalo, sendo os astrócitos as mais significantes. O termo neuroglia foi primeiramente utilizado pelo patologista Rudolf Virchow, em 1846. As células gliais foram descritas como

componentes intersticiais das paredes dos ventrículos e da medula espinhal, denominando-as "nerve-glue". Virchow, em 1846 observou que a neuroglia possui forma estrelar e é morfologicamente diferente dos neurônios, e separa o tecido nervoso dos vasos sangüíneos.

No fimdo século XIX e início do século XX, com as técnicas de coloração baseadas na impregnação metálica introduzidas pelo citologista Camillo Golgi, aliadas à microscopia óptica, foi possível obter maiores esclarecimentos sobre a morfologia da glia (CAJAL, 1995). Em seguida, Ramón y Cajal em 1913, e Del Río Hortega em 1919, produziram uma vasta literatura da microscopia óptica sobre a neuroglia, onde reconheceram duas categorias de células gliais: a macroglia e a microglia. A macroglia compreende os astrócitos e oligodendrócitos, assim como as células ependimais e suas derivadas. Estas células, como os neurônios, têm origem ectodérmica, mas diferem dos mesmos por terem somente um tipo de processo celular e por reterem a capacidade para se dividir ao longo da vida, particularmente sobre a influência de danos ao SNC (KETTENMANN; RANSOM, 2005; PETERS et al.,1991; PRIVAT et al., 1995).

Na mesma época, Penfield entre 1924 e 1932, preocupava-se em correlacionar as características das células gliais, através de impregnações metálicas, com as características nucleares e citoplasmáticas destas mesmas células incluídas em parafina e coradas com as técnicas clássicas de coloração. A microglia foi considerada por Del Río Hortega em 1932 e outros autores como originária do tecido mesodérmico e que posteriormente migra ao encéfalo neonatal.

As primeiras células progenitoras da linhagem de células macrogliais são os glioblastos provenientes das células neuroepiteliais do tubo neural (RAFF, 1989). Os glioblastos têm núcleos claros, poucas organelas, muitos ribossomos livres e não apresentam filamentos intermediários. Por sua vez, os pró-astroblastos são os precursores astrogliais. Eles

lembram os glioblastos, mas apresentam alguns grânulos de glicogênio e filamentos intermediários contendo nestina e vimentina (KALMAN; AJTAI, 2001). Já os astroblastos representam o estágio de desenvolvimento tardio, contendo mais organelas, grânulos de glicogênio, corpos densos e filamentos intermediários que contém GFAP (KETTENMANN; RANSOM, 2005; PRIVAT et al.,1995). Os astrócitos originam-se no período pré-natal das células neuroepiteliais, na zona germinal ventricular, e migram subseqüentemente para o parênquima do SNC, onde se associam com os vasos sangüíneos e contribuem para a formação da barreira hemato-encefálica (KETTENMANN; RANSOM, 2005).

São conhecidos os amplos papéis da glia na fisiologia normal do sistema nervoso central, e de sua capacidade de reação frente aos mais distintos tipos de insultos ao SNC, no intuito da proteção tecidual. Sabe-se que muitos estímulos podem ativar os astrócitos, tais como: físicos, químicos, virais e ou bacterianos (NORENBERG et al., 1994). Esta gliose reativa é caracterizada por hipertrofia ou hiperplasia das células e de seus processos astrocíticos (BIGNAMI; DAHL, 1976). Ao nível molecular, a ativação dos astrócitos esta relacionada com um aumento na expressão dos FI.

Os astrócitos, tal como as células de Schwann, por sua íntima associação com sinapses, estão estrategicamente posicionados para regular a transmissão sináptica. Esta capacidade permite a inferência de que astrócitos são elementos integrais da circuitaria e plasticidade sináptica. Além disso, devido ao fato de que a alta razão glia-neurônio é encontrada no topo da árvore filogenética no encéfalo humano, recentes demonstrações da sinalização entre astrócitos e neurônios deixam a questão se astrócitos são elementos reguladores-chave para altas funções corticais (ARAQUE; CARMIGNOTO; HAYDON, 2001).

As células astrocitárias podem ainda responder à atividade neuronal e ativamente modular a neurotransmissão por meio da liberação de transmissores gliais, tais como o glutamato, ATP e serina D (HAYDON; CARMIGNOTO, 2006). A liberação vesicular de gliotransmissores em resposta à ativação de receptores de membrana é cálcio-dependente, o que depende de proteínas *SNARE*. Entretanto, muitas questões acerca da modulação da exocitose de gliotransmissores permanecem desconhecidas (CRAVEN, 2005).

Astrócitos são considerados elementos sinalizadores dinâmicos que integram aferências neuronais, exibem excitabilidade ao cálcio e podem modular neurônios vizinhos. A atividade neuronal pode levar a uma ativação de receptores astrocíticos, considerada neurotransmissor-evocada. Elevações do cálcio astrocitário, por sua vez, provocam a liberação de transmissores químicos dos astrócitos, os quais podem causar ações modulatórias sustentadas nos neurônios vizinhos (LAMING et al., 2000; ARAQUE; CARMIGNOTO; HAYDON, 2001).

A comunicação entre neurônios e astrócitos é recíproca, de forma que o astrócito responde à atividade neuronal, mas também modula a excitabilidade dos primeiros e até a transmissão sináptica (GARCIA-SEGURA; McCARTHY, 2004). Os astrócitos parecem liberar alguns gliotransmissores, os quais atuam como moduladores da fisiologia neuronal. Evidências recentes indicam que os astrócitos possuem algumas propriedades-chave. Eles discriminam atividades de diferentes sinapses e respondem seletivamente a diferentes vias axonais (KETENMAN; RANSOM, 2005). Além disso, o sinal de Ca^{2+} astrocitário é modulado pela atividade simultânea de diferentes aferências sinápticas. Esta modulação do sinal de Ca^{2+} depende das propriedades intrínsecas do astrócito, sendo regulada bidirecionalmente pelo nível da atividade sináptica, bem como por controles da extensão

espacial do sinal de Ca^{2+} intracelular (COOPER, 1995; ARAQUE; CARMIGNOTO; HAYDON, 2001).

O modelo arquitetural das interdigitações entre astrócitos vizinhos com processos sobrepostos, baseados nos procedimentos de prata e imunorreatividade à GFAP, foi desafiado pelos resultados obtidos com o uso de microinjeções de corantes fluorescentes em astrócitos hipocampais. O arranjo ordenado destas células pode ser mostrado com a mínima sobreposição. Esta ordem determinada no período pós-natal precoce, em paralelo com os territórios vascular e neuronal, permite que cada astrócito cubra um território específico entre vasos e sinapses. Frações deste território podem, todavia, ser controladas autonomicamente por microdomínios astrocitários especializados. Estes microdomínios de finas expansões celulares, de composição estrutural e motilidade única, permitem interrelações específicas com as sinapses que as rodeiam, com os pés sugadores, de onde os sinais podem ser propagados aos pés terminais seguintes sem difundir pelo resto da célula (Figura 3).

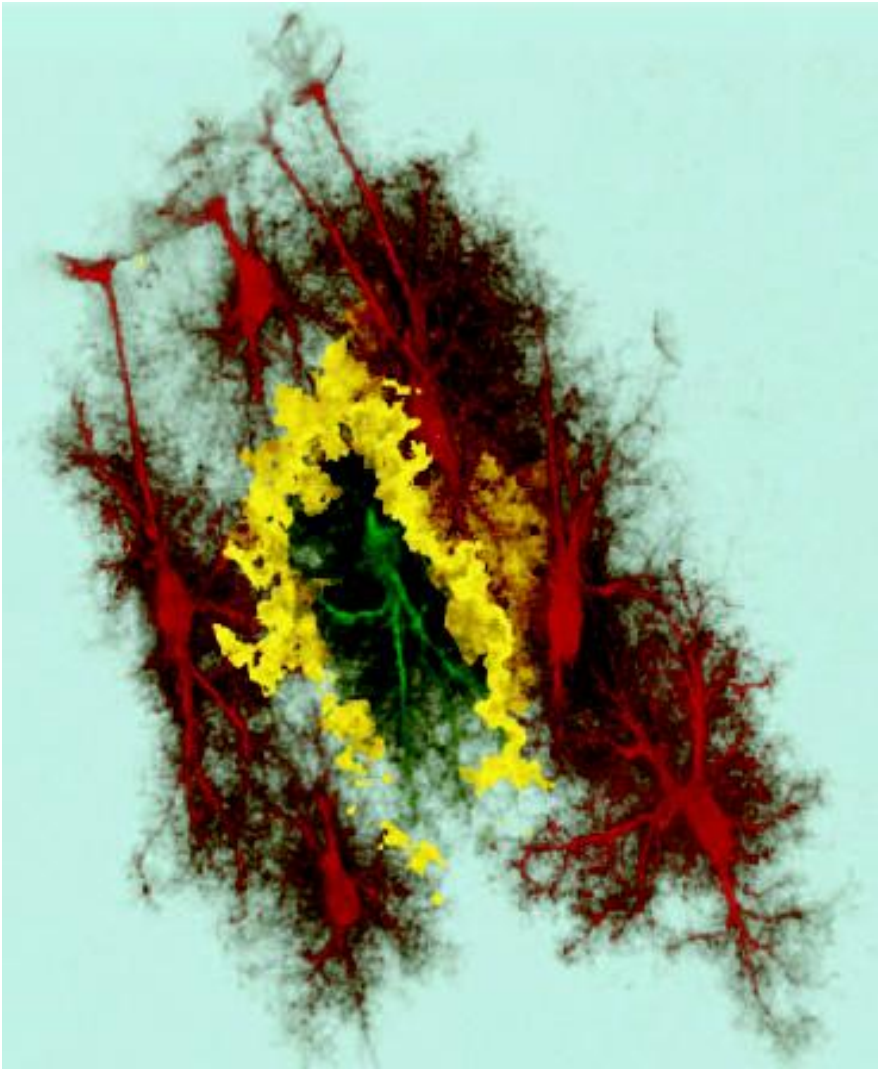


Figura 3 - Visão tridimensional da organização de astrócitos em domínios anatômicos separados. Um grupo de astrócitos protoplasmáticos corados, da camada molecular da CA1 do hipocampo foi examinado por meio de células limitantes, com diferentes corantes fluorescentes (CF; Alexa 468, CF verde e Alexa 488, CF vermelho) por microinjeção. A discreta região de interação dos finos processos terminais foi revelada pelo amarelo, primeiramente esboçando levemente como imagens (utilizando um filtro normal) e então remapeando a cor da área resultante de sobreposição para destacar o amarelo. Isto mostra onde os finos processos terminais dos astrócitos divisórios estão intimamente próximos um ao outro, apesar de não haver sobreposição de fato. Uma fronteira de cada astrócito tem uma superfície distinta que limita astrócitos circunjacentes. Os processos finos e longos que se estendem de cada célula, apresentados nesta figura, são o sifão dos processos dos astrócitos, os quais terminam em superfícies podais que se alinham ao vaso sangüíneo adjacente.

Fonte: Bushong et al., 2002

Como conseqüência de evidências sugestivas da existência da comunicação bidirecional entre astrócitos e neurônios, há uma forte indicação de papel ativo das células astrocitárias na fisiologia do sistema nervoso. Como conseqüência desta evidência, um novo conceito de fisiologia sináptica, "a sinapse de três elementos", tem sido proposto, onde a sinapse é formada por três elementos funcionais: os elementos pré e pós-sinápticos e os astrócitos circunjacentes (ARAQUE et al., 1999; PEREA; ARAQUE, 2002; 2005, 2009).

As interações neurônio-glia desempenham um importante papel em várias etapas da morfogênese do sistema nervoso, desde os primeiros estágios de neurogênese e gliogênese até estágios tardios de estabelecimento de conexões neurais. Muitos fatores de crescimento estão envolvidos na gliogênese. Além disso, fatores epigenéticos estão relacionados ao destino neural, especificação de linhagens e diferenciação celular no SNC. Dentre eles, alguns iniciam em fases muito precoces do desenvolvimento, devido a fatores difusíveis e contatos celulares encontrados no ambiente do desenvolvimento (GOMES; MARTINEZ; MOURA-NETO, 2001). A síntese e secreção do fator de crescimento transformador β1 (TGF β1) estão fortemente envolvidas no processo de diferenciação astrocitária, o que provavelmente seja mediado pelos neurônios (FREITAS et al., 2002).

Estudos em cultura de células apontam outro papel para as células gliais na formação e plasticidade de conexões sinápticas. Neurônios necessitam importar colesterol derivado da glia, via lipoproteínas para formar conexões sinápticas numerosas e eficientes (GÖRITZ et al., 2002).

A presença de receptores para esteróides hormonais não é limitada a neurônios, sendo também encontradas concentrações similares em elementos gliais (BOHN et al., 1991). Além disso, o complexo enzimático 5α-redutase-3α-hidroxiesteróide desidrogenase é também funcional nas células gliais. Conseqüentemente, hormônios sexuais como a progesterona e a

testosterona podem ser convertidos em seus respectivos metabólitos (MELCANGI et al., 1993; 1994).

As células gliais estão ativamente envolvidas nos efeitos ativacionais e organizacionais dos hormônios esteróides (GARCIA-SEGURA et al., 1999). De fato, parece haver uma participação simultânea de neurônios e células gliais no metabolismo da testosterona e talvez de outros esteróides, em diferentes populações celulares do SNC (MELCANGI et al., 1993; 2002).

O estradiol pode modular o formato celular e a distribuição de proteínas nos astrócitos em cultura, sugerindo que os astrócitos podem ser suscetíveis a esteróides sexuais durante o desenvolvimento do SNC (GARCIA-SEGURA et al., 1989). Estudos *in vivo* e *in vitro* evidenciaram diferenças nos níveis de estradiol em astrócitos e microglia de ratos em diferentes fases do ciclo estral (STONE et al., 1997), demonstrando também que a GFAP imunorreativa pode ser modificada pela manipulação dos hormônios esteróides em áreas encefálicas sexualmente dimórficas (GARCIA-SEGURA et al., 1994; KOHAMA et al., 1994; HAJÓS et al., 2000; CONEJO et al., 2003). Um estudo imunoistoquímico de GFAP no globo pálido, hipocampo e hipotálamo demonstrou a influência do estradiol sobre as células gliais (TRANQUE et al.,1987).

A castração de ratos machos provocou o aumento dos níveis de RNA mensageiro (RNAm) para GFAP no hipocampo (DAY et al., 1990; 1993), efeito que foi revertido pelo estradiol, mas não pela testosterona e dihidrotestosterona. Houve redução da GFAP-ir no hipotálamo após a castração, o que foi revertido pela testosterona ou dihidrotestosterona, mas não pelo estradiol (DAY et al., 1993).

Considera-se ainda que células gliais podem modular a atividade de neurônios que sintetizam e liberam o hormônio GnRH. Astrócitos tanto de origem cortical quanto

hipotalâmica também podem intervir na liberação do LH (MELCANGI et al.,1995). Os fatores de crescimento envolvidos nestes efeitos são o TGFα, o TGF β1, o TGF β2, o fator de crescimento fibroblástico básico (BFGF) e o fator de crescimento similar à insulina (IGF-I), o que se une ao controle de secreção de GnRH por meio de aferências neuronais e ação dos hormônios gonadais por meio de *feedback* positivo ou negativo (MELCANGI; MARTINI; GALBIATI, 2002).

Células gliais são consideradas elementos-chave do SNC por serem capazes tanto de enviar quanto de receber sinais hormonais. Embora haja um interesse investigativo evidente quanto à glia e ao dimorfismo sexual, poucas pesquisas referentes a este aspecto especificamente no hipocampo têm sido realizadas. A existência de dimorfismo sexual em algumas áreas do sistema límbico, como os núcleos amigdalianos, contribuem para diferenças comportamentais entre ratos fêmeas e machos (RASIA-FILHO et al., 1999; 2004; DE CASTILHOS et al., 2006; HERMEL et al., 2006). Todavia, investigações sobre a plasticidade glial no hipocampo por influências hormonais são necessárias, tanto em animais adultos quando durante o desenvolvimento. Tal demanda motivou a realização deste trabalho.

Hipotetiza-se aqui que o ciclo estral da rata provoque variações na GFAP-ir do estrato radiatum no hipocampo, o que também deve ocorrer com a ovariectomia seguida de reposição de estradiol adicionado ou não à progesterona. Além disso, acredita-se que haja dimorfismo sexual quanto à GFAP-ir nesta mesma região, durante o desenvolvimento pós-natal de ratos.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Estudar as diferenças relacionadas ao sexo, ao ciclo estral e à influência dos hormônios gonadais na expressão de gliofilamentos do *estrato radiatum* no hipocampo.

2.2 OBJETIVOS ESPECÍFICOS

Considerando-se a susceptibilidade dos gliofilamentos do *estrato radiatum* no hipocampo aos níveis de hormônios gonadais de roedores, estudar-se-a:

- A plasticidade astrocitária do *estrato radiatum* no hipocampo durante o desenvolvimento pós-natal de ratos machos e fêmeas utilizando-se imunoistoquímica para o marcador de filamentos intermediários de células gliais, GFAP.
- O dimorfismo sexual de ratos durante o desenvolvimento através da avaliação da relação entre a expressão dos filamentos intermediários de GFAP e as variáveis idade e sexo no *estrato radiatum* do hipocampo;
- A influência dos hormônios gonadais femininos em níveis fisiológicos sobre a expressão de GFAP no estrato radiatum do hipocampo, relacionando as fases do ciclo estral;
- A influência da castração de fêmeas ou dos hormônios estradiol e progesterona em níveis suprafisiológicos, combinados ou não, com terapia substitutiva sobre a

expressão de GFAP no *estrato radiatum* do hipocampo, relacionando aos grupos experimentais.

3 MATERIAIS E MÉTODOS

3.1 ANIMAIS

Foram utilizados ratos Wistar adultos (3 a 5 meses de idade; fêmeas nulíparas) e também filhotes da mesma espécie em diferentes idades: 11 dias (PN11), 21 dias (PN21), 31 dias (PN31) e 45 (PN45); machos e fêmeas. Todos os animais eram provenientes do biotério do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul e foram mantidos sob temperatura ambiente em torno de 22˚C, um ciclo claro-escuro de 12:12 h e com água e comida *ad libitum.*

Esforços máximos foram realizados para minimizar o número de animais estudados e seu sofrimento. Os ratos utilizados para os experimentos foram manipulados de acordo com as normas brasileiras para o uso ético de animais, além das leis internacionais de cuidados éticos e uso de animais de laboratório (Conselho Diretivo da Comunidade Européia de 24 de novembro de 1986, 86/609/EEC).

Para a realização deste estudo, foram realizados três experimentos. O primeiro analisou-se machos e fêmeas em diferentes fases do desenvolvimento pós-natal. No segundo e terceiro utilizaram–se machos e fêmeas adultas. Os procedimentos realizados para cada um dos experimentos estão descritos a seguir.

3.2 EXPERIMENTO 1: DETECÇÃO DA GFAP-IR DE MACHOS E FÊMEAS EM DIFERENTES FASES DO DESENVOLVIMENTO PÓS-NATAL

Ratas pesando entre 280-320 g foram acasaladas com machos hígidos. Fêmeas prenhas foram isoladas em um ambiente de 22°C de temperatura média e um ciclo claro-escuro 12:12 h e receberam acesso livre à comida e à água. As ratas prenhas foram conferidas regularmente para detectar o dia do nascimento de filhotes (P0). Filhotes e mães foram mantidos juntos até o dia do sacrifício, um casal de filhotes foi obtido de cada fêmea. Os ratos foram identificados e agrupados por idade ou dias pós-natais PN11, PN21, PN31 e PN45. Cada subgrupo foi composto por 6 animais de cada sexo (n=48). Todos os animais de diferentes idades foram submetidos a todos procedimentos experimentais ao mesmo tempo, incluindo perfusão, secção do encéfalo e procedimento imunoistoquímico.

3.2.1 Procedimento imunoistoquímico para GFAP

O procedimento imunoistoquímico para GFAP foi realizado conforme descrito abaixo, adequando-se soluções para o peso de cada filhote. Após a anestesia com tiopental sódico (50 mg/Kg, i.p.; Cristalia, Brasil), foi injetado heparina (500 -1000 UI Cristalia, Brasil) no ventrículo cardíaco esquerdo e perfundidos utilizando-se uma bomba peristáltica (Control Company, Brasil, 20 ml/min) com 50 - 200 mL de solução salina, seguida por 50 - 200 mL de paraformaldeído 4% (Reagen, Brasil) diluído em tampão fosfato 0,1 M e pH 7,4. Os encéfalos foram removidos, pós-fixados na mesma solução fixadora em temperatura ambiente por 4 h, crioprotegidos em uma solução de sacarose 30% em PB a a 4°C até afundarem. Os encéfalos foram então congelados em isopentano e nitrogênio líquido e seccionados coronalmente em

criostato (Leitz, Alemanha). Secções de 50 µm foram coletadas em solução salina de tampão fosfato e processadas para imunoistoquímica de GFAP seguindo o procedimento do anticorpo não marcado PAP (STERNBERGER, 1979).

Secções em livre flutuação foram pré-tratadas com metanol 10% diluído em H_2O_2 3% por 30 min e então cuidadosamente lavadas e bloqueadas com soro de cabra normal (NGS) a 3% em PBS contendo Triton X-100 0,4% (PBS-Tx; Sigma Chemical Co., USA) por 30 min. As secções foram então incubadas com anticorpo policlonal de GFAP em coelho (Sigma Chemical Co., USA) com diluição de 1:150 em NGS 3% em PBS-Tx por 48 h a 4°C em agitação contínua.

Após várias lavagens com PBS-Tx, os cortes foram incubados com imunoglobulina G (IgG) coelho anti-coelho (Sigma Chemical Co., USA) com diluição de 1:50 em PBS-Tx por 2 h a temperatura ambiente. As secções foram então lavadas novamente em PBS e incubadas em PAP para coelho (Sigma Chemical Co., USA) diluído em PBS 1:500 por 2 h a temperatura ambiente. A reação foi revelada pela incubação das secções em meio histoquímico o qual continha 3,3'-diaminobenzidina 0,06% (DAB, Sigma Chemical Co., USA) dissolvida em PBS por 10 min e na mesma solução contendo 1µL de H_2O_2 3% por mL de meio de DAB por mais 10 min. As secções foram finalmente lavadas em PBS, montadas em lâminas histológicas, desidratadas com etanol, clareadas com xilol e cobertas com Permount e lamínulas. Secções-controle foram realizadas omitindo-se o anticorpo primário, substituído por PBS.

Todos os encéfalos utilizados foram fixados e pós-fixados durante o mesmo período de tempo em soluções idênticas. Foram igualmente processados ao mesmo tempo, seguindo-se exatamente os mesmos passos histológicos, bem como incubados nos mesmos meios, pelo mesmo período de tempo. Estes procedimentos foram realizados a fim de reduzir a

possibilidade de *overreaction,* diferenças na reação do cromógeno ou alterações nos níveis de fundo nos grupos experimentais.

3.2.2 Aquisição dos dados

Os estratos hipocampais foram localizados e selecionados para a aquisição dos dados de acordo com um atlas anatômico de rato (PAXINOS; WATSON, 1998)

A intensidade de imunorreação para GFAP foi medida por meio de análise densitométrica semi-quantitativa (RASIA-FILHO, et al., 2002; XAVIER, et al., 2005). Para isso utilizou-se um microscópio Nikon Eclipse E-600 (500x, Japão) acoplado a uma câmera de alta performance Pro-Series CCD e ao *software* Image Pro Plus 4.1 (Media Cybernetics, USA). As imagens digitalizadas obtidas das áreas selecionadas foram convertidas em uma escala de cinza de 8-bit (0-255 níveis de cinza). As condições de iluminação e ampliação de imagem foram mantidas constantes durante todo o procedimento.

Neste estudo, devido às diferenças do tamanho dos encéfalos e, portanto do volume dos estratos hipocampais durante o desenvolvimento, foram utilizadas diferentes áreas de interesse (AOI). Os filhotes com idade PN11 receberam sobre cada um dos estratos hipocampais selecionados, um quadrado de 8256 μm^2, enquanto para os animais de idades PN21, PN31 e PN45 utilizaram-se dois AOIs de 8256 μm^2. A média de duas leituras de densidade óptica (DO) foi empregada para cada secção analisada. Os elementos de imagem (pixels) empregados para medida da densidade óptica foram sobrepostos sobre uma imagem de escala de cinza. Uma grade de quadros foi disposta sobre as imagens obtidas dos diferentes estratos hipocampais. Todas as AOIs foram colocadas seguindo-se um padrão constante com intervalos equidistantes na grade "máscara". Lúmen de vasos e artefatos evidentes foram evitados. Foram obtidas pelo menos 10 leituras em cada secção analisada e a média dos dados

de cada corte foi registrada. Foram utilizados ambos os lados de cada encéfalo. No mínimo 30 secções foram analisadas por estrato hipocampal de cada grupo estudado (6 cortes por animal) ao longo do desenvolvimento pós-natal. Uma área de tecido livre foi determinada e utilizada para corrigir as medidas da densidade óptica, servindo como imagem de *background.* Foi realizada a subtração de cada fundo marcado com o fundo de correção (XAVIER, et al., 2005).

A densidade óptica (DO) foi calculada usando a fórmula abaixo:

$$DO\ (x,y) = -\log\left[\frac{(INT_{(x,y)} - preto)}{(INC - preto)}\right],$$

onde: "DO(x,y)" é a densidade óptica de $pixel_{(x,y)}$, "$INT_{(x,y)}$" ou intensidade = intensidade de $pixel_{(x,y)}$, "preto" = imagem capturada na ausência de luz (5,3), e "INC" ou incidente = intensidade de luz incidente (252,4).

3.2.3 Medida da densidade celular (número de astrócitos GFAP-ir por mm^2)

Para verificar os resultados apontados pela densidade optica, o número de astrócitos foi quantificado. As imagens digitalizadas da região CA1 do hipocampo dorsal foram obtidas utilizando-se o mesmo AOI para a medida da DO, sobrepondo-o as imagens. O soma dos astrócitos GFAP-ir localizados dentro deste polígono ou os que faziam intersecção com a borda inferior e /ou lateral direita foram quantificados. Enquanto os astrócitos que tocavam a borda superior e/ou esquerda dopolígono não foram considerados. No mínimo 3 secções decada encéfalo foram analisadas (Chieco et al., 2001; Xavier et al.,2005).

3.2.4 Círculos de Scholl

Para a análise da morfologia de astrócitos, analisamos o número e distribuição radial dos processos em 15 astrócitos em cada animal (3 secções, 5 astrócitos / secção), através da adaptação da técnica dos círculos concêntricos de Sholl (DALL'OGLIO, et al., 2008, VIOLA et al, 2009). Círculos virtuais, com intervalos de 10, 15, 20, 25 e 30 µm, foram desenhados em torno de cada astrócitos, bem como foi quantificado o número de intersecções dos processos dos astrócitos com cada círculo virtual, tanto nos quadrantes laterais (direita ou esquerda) quanto nos centrais (isto é, superior ou inferior) para cada um dos astrócitos. Os processos foram contados por dois observadores em separado que desconheciam os grupos experimentais, e os resultados para ambos os observadores formaram em média para os resultados finais.

3.2.5 Análise Estatística

Os dados foram comparados quanto ao efeito de idade, sexo e região por meio de um teste da ANOVA de duas vias para medidas repetidas. As comparações múltiplas envolvendo todas as combinações dos fatores (sexo e idade e estrato hipocampal) foram realizados através dos testes *post-hoc* Bonfferoni. O nível estatístico estabelecido foi $P < 0,05$ (ZAR, 1999).

3.3 EXPERIMENTO 2: DETECÇÃO DA GFAP-IR DURANTE O CICLO ESTRAL

3.3.1 Avaliação do ciclo estral

O estudo da citologia vaginal foi realizado nas ratas por duas semanas antes do experimento para determinar a regularidade do ciclo estral. Durante este período, diariamente foi utilizada uma pipeta de tamanho adequado preenchida com solução salina. Após a coleta, o material obtido do conteúdo vaginal foi colocado sobre uma lâmina histológica e observado ao microscópio óptico para a identificação dos tipos celulares característicos de cada fase (Diestro: predominância de leucócitos; Proestro: predominância de células epiteliais nucleadas; Estro: predominância de células queratinizadas; Metaestro: presença de todos os tipos celulares apresentados nas outras fases do ciclo; (NELSON, 1995). Foram excluídas deste estudo as ratas que não apresentavam dois ciclos estrais normais consecutivos, consideradas com secreção de esteróides ovarianos irregular.

3.3.2 Procedimento imunoistoquímico para GFAP

Foram utilizadas 5 machos e 5 fêmeas em cada fase do ciclo estral (diestro, metaestro, estro e proestro) (n= 25), as quais foram sacrificados na tarde de cada fase. O procedimento imunoistoquímico para GFAP foi realizado conforme descrito no experimento 1, adequando-se soluções para o peso dos animais adultos: tiopental sódico (50 mg/Kg, i.p.; Cristalia, Brasil), 1000 UI de heparina (Cristalia, Brasil), 200 mL de solução salina, 200 mL de paraformaldeído 4 % (Reagen, Brasil) em tampão fosfato (PB) 0,1 M e pH 7,4.

Todos os demais procedimentos foram idênticos aos do experimento 1, previamente descrito.

3.3.3 Aquisição dos dados

Os estratos hipocampais também foram localizados e selecionados para a aquisição dos dados de acordo com um atlas anatômico de rato (PAXINOS; WATSON, 1998)

A intensidade de imunorreação para GFAP foi medida por meio de análise densitométrica semi-quantitativa conforme descrito para o primeiro experimento, utilizando-se a mesma metodologia usada nos animais com 45 dias.

3.3.4 Círculos de Scholl

Assim como os demais procedimentos seguiu-se exatamente o protocolo descrito pra o primeiro experimento.

3.3.5 Análise Estatística

Foram realizadas as médias dos valores para cada corte e então os dados foram submetidos à análise estatística, por meio de uma análise de variância (ANOVA) de uma via para medidas repetidas, considerando-se o sexo (macho ou fêmea) as fases do ciclo estral (diestro, proestro, estro ou metaestro) como o fator entre-sujeitos. Foi utilizado ainda o teste *post hoc* de Bonfferoni. O nível estatístico estabelecido foi $P < 0,05$ (ZAR, 1999).

3. 4 EXPERIMENTO 3: DETECÇÃO DA GFAP-IR DE FÊMEAS OVARIECTOMIZADAS E TRATADAS COM HORMÔNIOS OVARIANOS

Para o segundo experimento realizou-se ovariectomia e injeção de hormônios para detectar o efeito destes sobre a expressão de GFAP no estrato radiatum do hipocampo. Nesta etapa, objetivou-se detectar a ação da terapia substitutiva com hormônios gonadais sobre a glia hipocampal.

Os animais (30 fêmeas em idade adulta, 3 meses de idade) foram perfundidos no período da manhã e seus encéfalos processados da mesma forma como no Experimento 1.

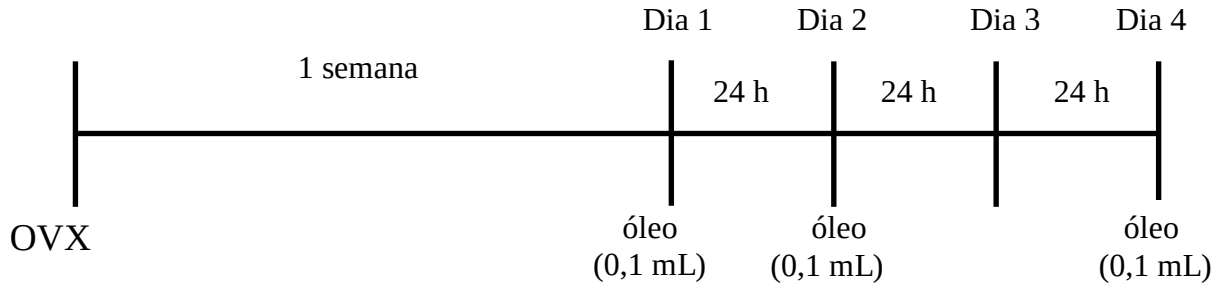
3.4.1 Procedimento cirúrgico

As fêmeas adultas foram estudadas ao longo do ciclo estral. Durante três semanas, estudos diários da citologia vaginal foram realizados sempre no mesmo período do dia (entre 8 e 10 h da manhã). As células da mucosa vaginal eram observadas em microscópio óptico para verificação da fase do ciclo em que o animal se encontrava. Desta forma, ratas com o ciclo estral irregular foram descartadas deste estudo.

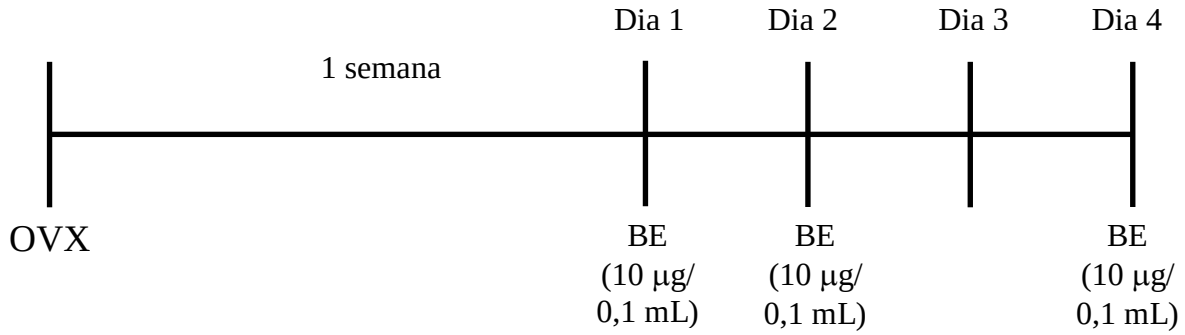
As fêmeas foram então submetidas a ovariectomia. Para tanto, os animais foram anestesiados com cloridrato de cetamina e xilazina (Vetbrands, Brasil; 80 mg/kg e 20 mg/kg, respectivamente) intramuscular (i.m.). Após assepsia e tricotomia da região lombar, as ratas foram ovariectomizadas bilateralmente, por acesso cirúrgico dorsal. Após a retirada dos ovários e a sutura por planos, os animais foram cuidadosamente devolvidos para o biotério, onde ocorreu a recuperação pós-operatória. Houve acompanhamento diário das fêmeas por

uma semana, quando se iniciou o protocolo de injeção dos hormônios. Nesta etapa, as ratas foram divididas em três grupos experimentais:

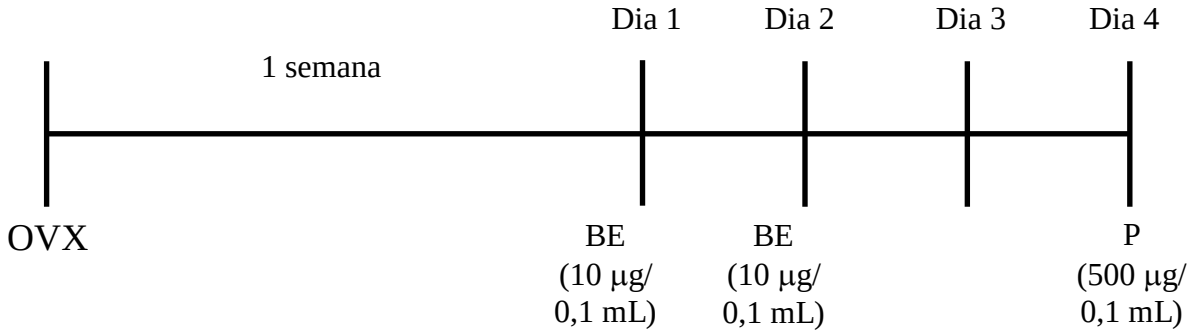
Grupo 1 (n=6): fêmeas OVX que receberam três injeções subcutâneas (s.c.) de injeção de veículo (óleo de gergelim) (V; 0,1 mL/sc), no horário da manhã (às 9 h), servindo como grupo controle. A primeira dose foi dada no primeiro dia da terapia substitutiva (dia 1), a segunda dose de óleo foi dada 24 h depois da primeira dose (dia 2), e a terceira dose foi dada 48 h após a segunda dose e 5 h antes do sacrifício, conforme o seguinte esquema:



Grupo 2 (n=6): fêmeas OVX que receberam injeção de benzoato de estradiol (BE; Sigma Chemicals Co., EUA) na dose de 10 μg diluídos em 0,1 mL de veículo (óleo de gergelim) sob o seguinte protocolo: a primeira dose de BE foi dada no primeiro dia de terapia substitutiva (dia 1), a segunda dose de BE foi dada 24 h depois da primeira dose (dia 2) e a terceira dose na dose 48 h após a segunda dose de BE e 5h antes do sacrifício (dia 4), conforme o esquema a seguir:



Grupo 3 (n=6): fêmeas OVX e que receberam 10 μg de BE e 500 μg de progesterona (P; Sigma Chemicals Co., EUA) ambos diluídos em 0,1 mL de veículo (óleo de gergelim), sob o seguinte esquema: a primeira dose de BE foi dada no primeiro dia da terapia substitutiva (dia 1), a segunda dose de BE foi dada 24 h depois da primeira dose (dia 2) e a terceira dose foi de progesterona, 48 h após a segunda dose de BE e 5 h antes do sacrifício, conforme o esquema abaixo:



Não houve controle para progesterona. Os animais foram então anestesiados e perfundidos para realização de procedimento imunoistoquímico e densitometria óptica para GFAP e o método de Scholl para seus prolongamentos. Nesta etapa, os procedimentos foram idênticos aos realizados no primeiro experimento, descrito previamente.

Logo após o sacrifício dos animais, o útero de cada rata foi cuidadosamente retirado, através de uma incisão cirúrgica na região inferior da parede abdominal. O peso do útero de cada animal foi obtido e registrado com a finalidade de comparação estatística dos dados, para então serem constatados os efeitos sistêmicos dos hormônios administrados.

3.4.2 Análise Estatística

Foram realizadas as médias dos valores para cada corte e então os dados foram submetidos à análise estatística, por meio do teste da ANOVA para medidas repetidas, considerando-se o grupo experimental (veículo, EB ou EB + P) como o fator entre-sujeitos. Foi realizado também o teste *post hoc* Bonfferoni. O nível estatístico estabelecido foi $P < 0,05$.

O peso do útero dos animais estudados foi comparado pelo teste da ANOVA de uma via e o teste *post hoc* Bonfferoni. Igualmente, o nível de significância ficou considerado como $P < 0,05$ (ZAR, 1999).

4 RESULTADOS

4.1 ARTIGO EXPERIMENTO 1 – SUBMETIDO À NEURON AND GLIA BIOLOGY

THE DISTRIBUTION OF GFAP IMMUNOREACTIVITY IN THE HIPPOCAMPUS OF MALE AND FEMALE RATS DURING POST-NATAL DEVELOPMENT

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Abstract- The aim of the present study was to describe morphological modifications in astrocytes in the dorsal hippocampus during postnatal development in female and in male rats, as assessed by glial acidic fibrillary protein immunoreactivity (GFAP-ir) using an immunohistochemical procedure. Local immunoreaction was analyzed by estimating astrocytic density (number of astrocytes/mm^2) and Sholl's method was used to identify the morphological changes in the astrocytic processes. Morphological differences in the pattern of protoplasmic astrocytic GFAP-ir were identified between genders, though, at some postnatal developmental stages, they were found to be temporary. In the female hippocampus, the astrocytes were mainly fusiform in shape, while in males they were more frequently spongiform. The data obtained on astrocytic density failed to show any age-related difference between males and females in the intensity of GFAP-ir in the area studied. In females the distribution of GFAP-ir appears to become homogenous earlier than in males, suggesting an earlier maturation in the former. These findings indicate the existence of slight differences between males and females in the pattern of hippocampal astrocytic development during the postnatal period that could be related either to genetic characteristics of the hippocampus in formation and/or to the early epigenetic actions of gonadal steroids and aromatase activity during brain development.

Keywords: Hippocampus, Neurodevelopment, Astrocytes, GFAP immunohistochemistry, Sholl's method

Running Title: HIPPOCAMPAL GFAP OF MALE AND FEMALE RATS DURING DEVELOPMENT

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Introduction

The hippocampus is a telencephalic region closely involved in a variety of critical brain functions including learning/memory and stress responses (McEwen and Sapolsky, 1995). It can be distinguished by its laminated appearance, organized synaptic inputs and clear functional significance. It contains numerous astrocytes that are located in the Ammon's horn, dentate gyrus and subiculum (Amaral and Witter, 2004). Within the context of the hippocampal development in rats, neurons have received considerable attention while glial cells have been given relatively little.

Previous studies have shown that neurogenesis in the Ammon's horn occurs in embryonic periods and extends beyond birth and continues postnatally, whereas mature astrocytes develop during postnatal life (Bayer, 1980) and that most astrocytes are generated by postnatal-day 16 (PN) (Nixdorf-Bergweiler et al.,1994). In the first two postnatal weeks there is a sharp decline in astrogliogenesis, with radial glia disappearing and being replaced by stellate astrocytes by the end of the first week. To date, the development of the stellate astrocytes in the CA1 has been best characterized through the analysis of GFAP maturation (Catalani et al., 2002). This study has established that this cytoskeletal element progressively elongates and ramifies during the first 3–4 postnatal weeks after which there is a slow decline in the volume and number of branches until stabilization occurs around 5 months of age (Catalani et al., 2002).

Astrocytes support the central nervous system during development, and contribute to the maintenance of the brain microenvironment and regulation of neural activity and plasticity (Volterra and Meldolesi, 2005; Kettenmann and Ramson, 2005). Moreover, astrocytes express neurotransmitters and hormone receptors such as β-adrenoreceptors (Shao and Sutin, 1992), serotonin (Merzak et al., 1996) glucocorticoids and estrogen receptors (Mong et al., 1999).

Since steroid hormones bind these receptors, they are critical regulators of GFAP gene expression. Findings from *in vivo* and *in vitro* experiments have shown that GFAP immunoreactivity (GFAP-ir) is modified by gonadal steroid manipulation in different sexually dimorphic brain areas (Leranth et al., 2003). The effects of gonadal steroids on GFAP are complex and may also be exerted indirectly through changes in the surrounding neurons and/or glial elements (Melcangi et al., 1998). Throughout life, gonadal steroid receptors may contribute to sexually different functions that occur in limbic regions exposed to different levels of gonadal hormones (Araque et al., 2001; Rasia-Filho et al., 2002, 2004).

Sexual dimorphism of astrocytes in the adult rat has been reported in the cerebellum, hippocampus, striatum and hypothalamus (McCarthy and Konkle, 2005). Furthermore, effects of gonadal hormones on astroglial morphology and expression of GFAP have been described in the hippocampus (Lam and Leranth, 2003) and in the hypothalamic arcuate nucleus (Mong and McCarthy, 1999) of the adult rat. However, the existing information on the functional implications of the effects of gonadal steroids on astroglia during the developmental period is scarce.

Most of the information about GFAP-ir astrocytic development comes from *in vitro* investigations (Catalani et al., 2002), while *in vivo* studies have only analyzed the development of hippocampal astrocytes (Garcia-Segura et al., 1991; Nixdorf-Bergweiler et al.,1994; Catalani et al., 2002; Conejo et al., 2003) or assessed possible developmental differences between hippocampal astrocytes in males and females. On the other hand, there is no study available on the astrocytic process orientation and branching during hippocampal development, which probable play a role in neuronal plasticity.

The aim of the our study was to describe postnatal changes in the immunoreactivity and distribution patterns of GFAP-ir astrocytes in the stratum radiatum of the hippocampal CA1

of male and female rats ranging from 11 to 45 postnatal days of age, using the GFAP immunohistochemical procedure. We also analyzed the number of astrocytes/mm2, using the astrocytic density measurement, and the quantification of processes and their branches using the modified Sholl`s method (Dall'Oglio et al., 2008; Viola et al., 2009).

Materials and methods

Animals

Female Wistar rats weighing 280-320 g, were housed and mated with adult male rats obtained from the ICBS bioterium, UFRGS. Pregnant females were isolated and allowed to give birth. Food and water was freely available and a temperature of around 22º C and a 12:12 h light-dark cycle were maintained. On postnatal days (PN) 11, 21, 31 and 45 one pair (a single male and a single female) were obtained from each litter. Each subgroup was composed of 6 animals of each gender (n=48). All animals from the different age groups were submitted to the experimental procedures at the same time (Martinez et al., 2005; Xavier et al., 2005). Rats were manipulated according to international laws for the ethical care and use of laboratory animals (European Communities Council Directive of 24 November 1986, 86/609/EEC) and to Brazilian guidelines on the ethical use of animals.

The animals were deeply anesthetized with sodium thiopental (50 mg/kg, i.p.) injected with heparin (500-1000 IU), and transcardially perfused through the left cardiac ventricle, using a peristaltic pump (10-20 mL/min) with 50-200 mL of saline solution followed by the same volume of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4; PB). The brains were removed and post-fixed in the same fixative solution at room temperature for 4 h. Afterwards, they were cryoprotected in a 30% sucrose solution in PB at 4º C until they sank,

frozen in isopentane and liquid nitrogen and then stocked at -70º C. Semi-serial coronal sections (50 µm) were obtained from each brain using a cryostat (Leitz, Digital 1702) at -20° C and collected in a saline phosphate buffer, pH 7.4 (PBS). The free-floating sections were pre-treated with 10% methanol diluted in 3% hydrogen peroxide for 30 min, carefully washed and blocked with 3% normal goat serum (NGS) in a 0.1 M phosphate buffer, 0.9% NaCl, pH 7.4 (PBS) containing 0.3% Triton X-100 (PBS-Tx) for 30 min and incubated with polyclonal GFAP antiserum raised in rabbit (1:150 Sigma Chemical Co., USA) with 3% NGS in PBS-Tx for 48 h at 4°C. After several washes in PBS-Tx, tissue sections were incubated with a secondary antibody biotinylated with peroxidase-conjugated (Amersham, UK) diluted 1:200 in PBS-Tx at room temperature for 2 h. The immunohistochemical reaction was developed by incubating the sections in a medium containing 0.06% 3,3 diaminobenzidine (DAB; Sigma Chemical Co., USA) dissolved in PBS for 10 min and then in the same solution containing 1 µM of 3% H_2O_2 per mL of DAB medium for 10 min. Then, the sections were rinsed with PBS, dehydrated with ethanol, cleared with xylene and covered with Permount and coverslips. Control sections were prepared by omitting the primary antibody and replacing it with PBS.

The brains of the animals were fixed and post-fixed for the same time in identical solutions, rigorously processed at the same time, and then incubated in the same medium for the same period of time. This precaution was taken to avoid overreaction, differences in chromogen reaction, saturation of optical density or changes in background staining levels (Martinez et al., 2005; Xavier et al., 2005).

Data acquisition

a) Measuring astrocytic density (number of GFAP-ir per mm^2)

In order to estimate the number of GFAP-ir astrocytes per mm^2, digitized images of dorsal CA1 were obtained from stratum radiatum of the dorsal hippocampus. All measurements were performed in the stratum radiatum of CA1 between coordinates bregma -1.58 mm interaural 2.22 mm and bregma -2.06 mm, interaural 1.74 mm obtained from Watson and Paxinos' Atlas (1997). Quantification took place within squares measuring 8256 μm^2 (our Region of Interest, ROI), while the obvious blood vessels and other artefacts were avoided. The soma of GFAP-ir astrocytes located inside this square or intersected by the lower and/or right edge of the square were counted. The astrocytes that were intersected by the upper and/or left edge of the square were not counted. At least 3 sections from each brain were analysed (Chieco et al., 2001; Xavier et al., 2005).

b) Quantification of astrocytic processes and their branches

To study astrocytic morphology, we analyzed the number and radial distribution of 15 astrocytes in each animal (3 sections) using an adaptation of Sholl's concentric circles technique (Dall'Oglio et al., 2008; Viola et al., 2009). Four virtual circles with radii of 10, 20, 25 and 30 μm were drawn around each astrocyte, and the number of intersections of astrocytic processes with each virtual circle was quantified in both the lateral (i.e. right/left) and central (i.e. superior/inferior) quadrants around the astrocytes (Figure 1). The same AOI and inclusion and exclusion parameters used for astrocytic density were employed when selecting the astrocytes analyzed using Sholl's method. The method was performed on a single focal plane, after first focusing on the astrocytic soma, the image was digitalized. To obtain a detailed analysis of the processes and their branches, they were identified and counted

separately. Processes and their branches were counted by two observers who were blind to the experimental groups, and the results from both observers were averaged in the final results.

Statistical analysis

The data obtained from the estimation of the astrocytic GFAP-ir density and the quantification of the processes and branches in the studied stratum radiatum, in both sexes and all ages, were compared using Analysis of Variance (ANOVA) for Repeated Measures followed by the post-hoc Bonferroni test. In all cases, the level of statistical significance was set as $p < 0.05$.

Results

General observations

Exposure of hippocampal sections of to polyclonal antibody raised against GFAP resulted in a highly selective and reproducible staining pattern. GFAP appeared in the cytoplasm of astrocytic cell bodies and their processes. A difference was seen in the morphology of GFAP-ir astrocytes from PN11 to PN45 (Figure 1). Furthermore, the fact that the astrocytic end-feet envelop the vessels indicates the degree of maturity of the GFAP expression. In older age groups (PN31 to PN45), the immunoreactivity seems to be similar in both male and female rats, with little difference in terms of process orientation (figure 1 and table 1).

Qualitative light microscopy observations

To detect any possible sexual difference in the distribution of astrocytes in the stratum radiatum of CA1, different features were analyzed, such as the immunoreactive variation during postnatal development and the distribution pattern of their processes (Figure 2). Two elements were found to be present during the hippocampal postnatal development: i) the variation in the cellular expression of the GFAP, with a lower progressive increase during all the studied postnatal days and ii) an increase in the density of the immunoreactive astrocytes that contribute, with the other structural components (neurons and vessels), to increase the space occupied within each hippocampal layer. Consequently, there is a modification to the GFAP-ir astrocytic domains (vascular and neuronal). As described below, in Table 1.

At PN11, the GFAP-ir appeared more widely distributed, because the labelled astrocytes were detected throughout the stratum radiatum extending towards the stratum lacunosum-moleculare. While the stratum radiatum grew at the same rate in males and females and the quantity of astrocytic processes was the same, those of males were found to be shorter while those of females had more branches. This "migration" in the GFAP-ir astrocytic process distribution took place earlier in females (PN21) than in males, and there was greater homogeneity of the labelled astrocytic distribution in females. In the stratum radiatum of the females at PN21, numerous astrocytes with long fine processes (always perpendicular to the pial surface) sprouted from the GFAP-ir cell bodies and appeared to be longer than those seen in males, in which there were only sparse strongly labelled cell bodies with short processes and branches, which projected laterally.

At PN31, the GFAP-ir astrocytic processes appeared to be larger, and differences were detected in relation to the distribution. However, there was no difference between the genders in terms of the astrocytic density, of GFAP-ir cells. In females, intense labelled astrocytes

exhibited scanty lateral processes or ramifications, and an evident main process perpendicular to the stratum pyramidale. In males, the ramification pattern was denser and a single main process was not always identified. At PN45 the astrocytic distribution was different in both genders, though the stellate pattern previously observed in males changed to fusiform shape and the number of lateral processes and branches was higher. Interestingly, in females, both processes and the branches retained a perpendicular distribution to the pial surface with an increment in the number of branches (Figure 2).

When the relationships between vessels and astrocytic density were compared; there was no evidence of correlation. However, an "intense" immunoreaction of the astrocytic processes was detected, especially in the more complex astrocytes, such as those forming vascular end-feet along the arbor of processes.

The estimation of astrocytic density only indicated a statistical difference within the age groups and not among the other aspects. Thus, the test showed that astrocytic density increased with age, in consequence, at PN45 astrocytic density was highest (table I). When the orientation of all processes was evaluated some differences were identified. With age, the growth rate of the processes was seen to be different between the sexes, while in females, more growth was seen in central processes with increased age ($p \leq 0.01$ and $p \leq 0.001$; Figure 2b), in males there was more lateral growth ($p \leq 0.05$ and $p \leq 0.01$; Figure 2b). Nonetheless, the results failed to show a statistically significant difference between the genders. In addition, when primary processes and their branches were separately analyzed some slight differences were found. Centrally orientated primary processes are more numerous in males than in females at 45 days ($p \leq 0.05$; Figure 2c), while the central branches are more numerous in females than in males ($p \leq 0.05$; Figure 2d). Furthermore, regarding age, there is

a notable increase in the number of central branches ($p \leq 0.01$ and $p \leq 0.001$; Figure 2d) while in males there was a greater increase in the number of lateral branches ($p \leq 0.05$; Figure 2d).

Discussion

Our results reveal transitory structural sex-related GFAP-ir differences in the CA1 of the hippocampus. The distribution of the processes are similar in males and females during post natal development (PN11 and PN21), but from PN31 onwards the GFAP-ir pattern seems to become different in both genders.

The morphology of the astrocytic population in CA1 appears more homogeneous in females than in males by PN11, even though astrogliogenesis is still present. This suggests that the pro-differentiation signals imprinted on nascent astrocytes at this age are stronger in females than males, enabling astrocytes to elaborate significant amounts of surface area very quickly in newborns (Bushong et al., 2004).

Initially, the astrocytes also produce long varicose processes without ramifications. Gradually, these processes become highly ramified, with the branches appearing first near the soma and progressing centrifugally. In addition, Sholl's method reveals that in PN45 females the astrocytic fusiform shape is predominant, with an increase in the perpendicular orientation of their branches, similar to those found in adult morphology. However, in PN45 males with more processes and branches, similarly to the immature aspect, the astrocytes retain a spongiform shape.

Astrocytes play an important role in guiding the development and maintenance of synapses (Araque et al., 1999; Pfrier, 2002). Synapse formation begins in the embryonic hippocampus, before most glial proliferation takes place. During the third and fourth postnatal weeks, there is a three- to four-fold increase in the number of synapses, which is often associated with

some degree of glial coverage (Ventura and Harris 1999). As shown by our results and those of other authors, during this same period there is an increase in the number of astrocytes, their ramifications and the length of their processes (Nixdorf-Bergweiler et al., 1994). The extension of numerous processes during these first two postnatal weeks suggests that astrocytes play an important role in influencing and participating in the initial events of synapse formation (Ullian et al., 2001).

Accordingly, their elaborate morphology enables protoplasmic astrocytes to maintain a position close to neuronal somata and dendrites, synapses, blood vessels, and the outer edge of the nervous system. As for the relationship established between neighboring protoplasmic astrocytes, recent reports have indicated that these cells prevent overlapping between their processes and thereby establish individual domains within the neuropil, creating a patchwork of almost exclusive astrocytes. The consequences of this cellular arrangement are unknown, but may represent significant territories for astrocytic action (Bushong et al., 2004).

In the literature there are discrepancies in relation to GFAP immunohistochemical findings within the CA1 area in adult rats. In contrast to the findings of the present study, Conejo et al (2003) found more GFAP-ir astrocytes in the CA1 region in females than in males; but Catalani et al (2002) failed to find such a difference. It would be logical to assume that our morphological findings, trying to show differences between males and females could be the result of the effects of gonadal steroids, because on the first post-partum day, the endogenous estradiol content in the hippocampus is higher in females than in the males, indicating the potential for *de novo* synthesis of E2 by telencephalic regions of the female brain (Amateau et al., 2004).

Sexual differentiation of the male brain requires that during critical periods of development, adequate amounts of testosterone gain access to what will ultimately become

sexually dimorphic structures (MacLusky and Naftolin, 1981). In the rat, the fetal male gonad secretes testosterone, peaking on embryonic day 18 and continuing through the first few days of life (Weisz and Ward, 1980). Whereas some functions of brain sexual differentiation are mediated by testosterone acting at the androgen receptor (McCarthy and Konkle, 2005), aromatase is thought to be involved in the regulatory effects of this androgens, via conversion to estrogens, and therefore involve the estrogen receptor (Garcia-Segura, 2008).

The functional significance of elevated estradiol in the female hippocampus and the cerebral cortex during development is unknown. Nonetheless, the hippocampus shows modest sex differences in volume, which are apparent by the first week of life, and estradiol appears to have trophic effects on hippocampal development (Hilton et al., 2003). A role for estradiol in feminization has been previously postulated based on the effects of either targeted disruption of the aromatase gene (Bakker et al., 2002) or interference with the functioning of estrogen receptors (Amateau et al., 2004). These studies placed emphasis on reproductive behavior and physiology. Cognitive functions, such as learning and memory, in which the hippocampus and cerebral cortex play prominent roles, are much less sexually dimorphic than reproductive-related responses. The observed sex differences are largely limited to acquisition of a task with no sex differences in memory capacity or steady-state performance (McCarthy and Konkle, 2005).

Perhaps, increased estradiol synthesis by the developing female telencephalon contributes to maintaining parity in cognitive function between males and females. Recent evidence from aromatase null mice indicates both males and females show deficits in hippocampal-dependent tasks (Toran-Allerand et al., 2001). Also some studies assessing the effect of the adrenal androgen dehydroepiandrosterone confirmed the role of aromatase on hippocampal synaptic plasticity because letrozole abolished the induction of CA1 spine synapses by DHEA

in females (MacLusky et al, 2004; Sasahara et al, 2007). Interestingly, testosterone and DHEA do not need to be converted into estradiol to exert an effect on spine synapses in males (Greco, 2001; Hajszan et al., 2004; MacLusky et al, 2004), suggesting a sex-specific role of brain aromatase on synaptic plasticity. These findings indicate that brain aromatase modulates synaptic plasticity in brain regions related to cognition, such as the hippocampus.

The enzyme may also influence synaptic development and plasticity in other nonreproductive regions of the central nervous system because cerebellar Purkinje cells in ArKO mice show decreased dendritic growth, and decreased formation of dendritic spines and synapses (Sasahara et al, 2007).

Other studies have discussed the influence of the female hormones on synaptic plasticity in the hippocampus through effects on the astroglia (Kohama et al., 1994; Garcia-Segura et al., 1994, 1999). It has been demonstrated that estrogen has a direct effect on astrocytes in the hippocampus and that astrocytes have estrogen receptors (Azcoitia et al., 1999; Gomes et al., 2001). A decrease in the number of astroglial processes in the hippocampus as a reaction to increased estrogen concentrations has been previously observed (Gomes et al., 1999; McCarthy and Konkle, 2005), as well as the fluctuation of astroglial volume fraction during the estrous cycle of the female rat (Klintsova et al.,1995). Complementary to the increase in the spine synapse density, with higher estrogen concentrations (Wooley and McEwen 1992), large astroglial volumes correspond to low estrogen levels, whereas small volumes correspond to high estrogen levels, in adult rats. Thus, given that astrocytes modify their morphology, so influencing the synaptic contacts in response to the hormonal fluctuations of the estrous cycle, it would be logical to expect that, during brain development, hormonal fluctuations would be needed in order to prepare the glial cells to alter their morphology upon

receiving suitable neuronal signaling. Furthermore, perhaps the morphological alterations, shown in our study may occur in response to developmental hormonal fluctuations.

While it is possible that the distinct astrocytic morphologies observed at PN45 simply reflect various stages of development along a continuum of increasing complexity, it seems quite likely that the temporal correlation between synapse maturation and spongiform ramification is more than coincidence. Previous studies demonstrate that plastic astrocytic morphological changes could also lead to modifications in hippocampal synapses through the release of mediators such as glutamate, ATP, adenosine, D-serine, and others (Volterra and Meldolesi, 2005, Fiacco et al., 2009), as well as through changes in the uptake of neurotransmitters in the synaptic cleft (Theodosis et al., 2008).

Conclusions

Based on the findings of the present study and other published papers it is possible to hypothesize that GFAP-ir astrocytes adapt their morphology to progressively cover, with their processes, the increased neuropile as a response to neurite outgrowth and branching and that this occurs in females earlier than in males. Our study has produced evidence suggesting that these morphological findings concerning astrocytic processes coincide in a similar way to the effects of hormones and enzymes on neurons and their synapses One challenging area for future studies will be to clarify the interrelationship between synaptogenesis, astrocytic processes during hippocampal development and sexual hormones. Considering the fact that a period of gradual increase and subsequent definition of astrocytic “domains of influence” are necessary for the definition of hippocampal function.

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Figure 1

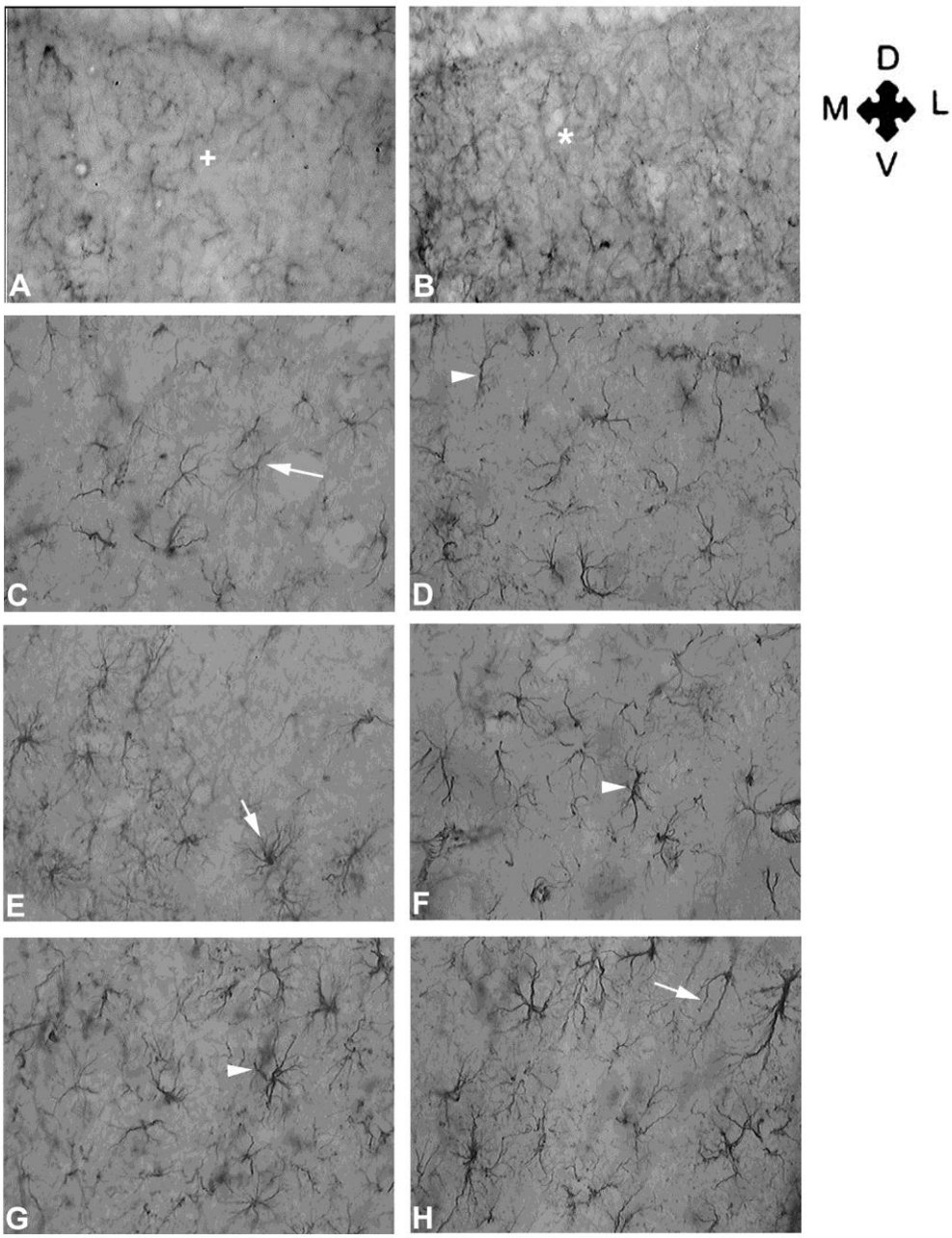
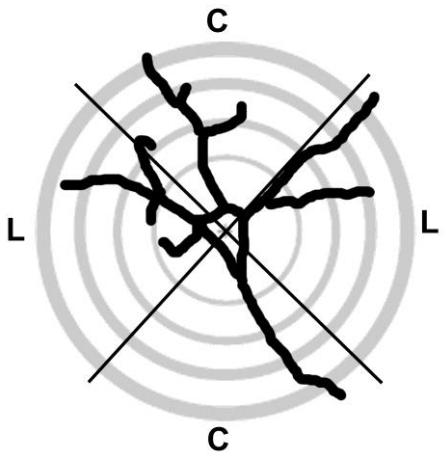


Figure 2

A-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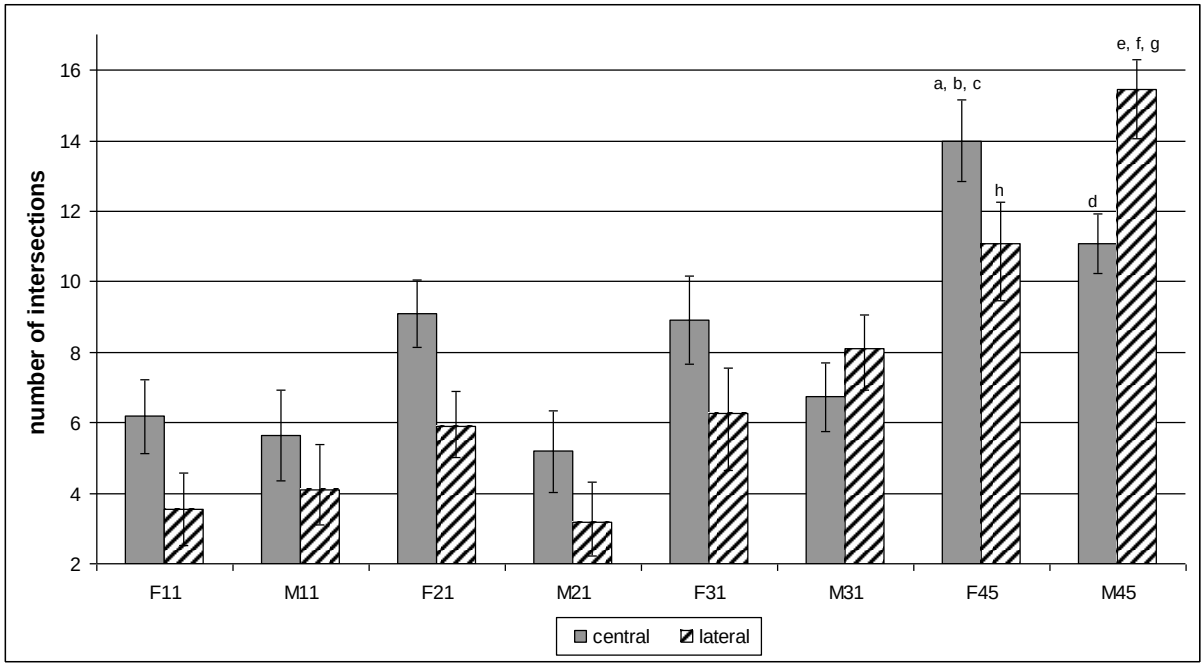
number of intersections
16
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2
a, b, c
h
d
e, f, g
F11
M11
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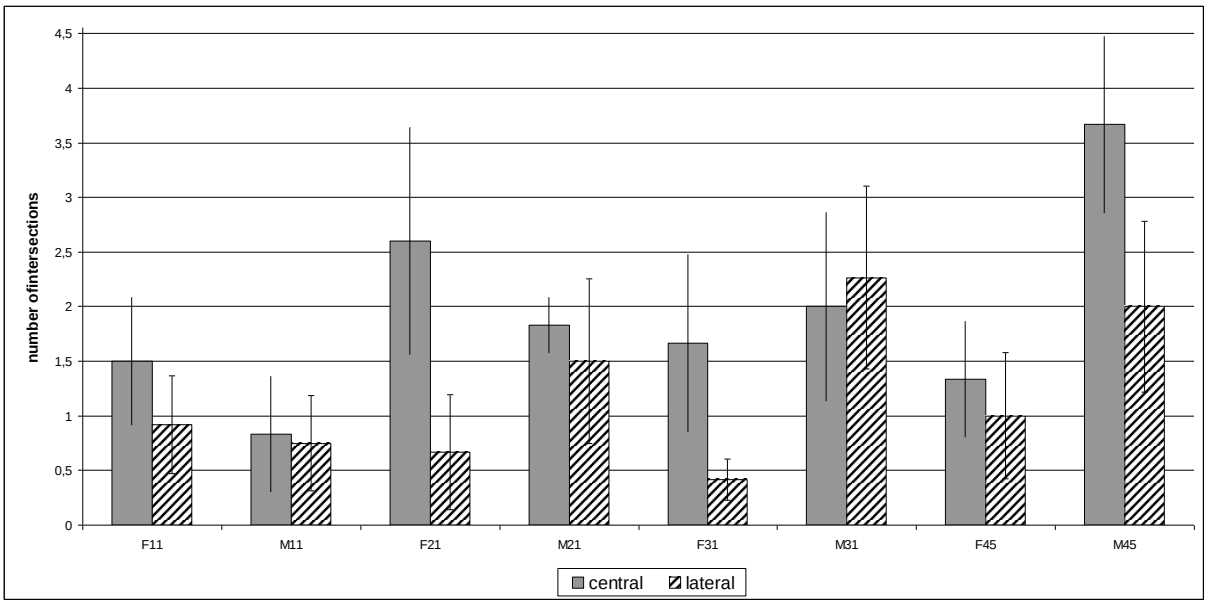
M21
F31
M31
F45
M45
central
lateral

C-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4
3,5
3
2,5
2
1,5
1
0,5
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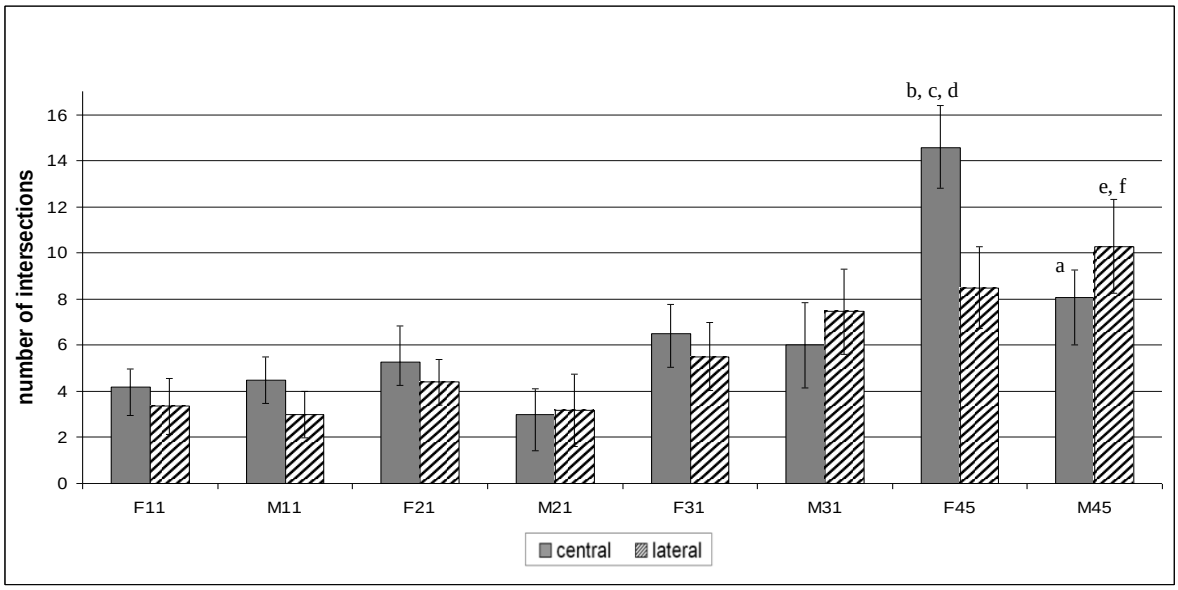
M21
F31
M31
F45
M45
central
lateral

D-


number of intersections
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14
12
10
8
6
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b, c, d
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a
F11
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M21
F31
M31
F45
M45
central
lateral

Table 1 – Characteristics observed in the analysis of stratum radiatum in relation to sex and age, during postnatal development.

Age of animal	***Nº of total processes intersections***	***Nº of cells/mm²***	***Cell orientation for pial surface***	***Cell shape***	***Immunoreactivity intensity***
F11	4,5	2998	Diagonal	Fusiform	+
M11	3,75	2912	Diagonal	Fusiform	-
F21	5,5	3340	Upright	Fusiform	++
M21	4,6	3169	Diagonal	Spongiform	+
F31	5,9	3854	Upright	Fusiform	++
M31	10,8	4197	Upright	Spongiform	++
F45	11,9	4625	Upright	Fusiform	+++
M45	14	5224	Upright	Fusiform	++

Figure legends

Figure 1: Digitized images of the stratum radiatum of hippocampal sections from male and female rats showing the CA1 region immunolabeled with GFAP antibody. (A) P11 male, there are fewer GFAP-ir astrocytes than in females (cross). (B) P11 female, only fusiform shaped GFAP-ir astrocytes were seen (asterisk). (C) P21 male, some glial processes display the spongiform pattern (arrow). (D) P21 female, there are more GFAP-ir cells, with perpendicular processes (arrow head). (E) P31 male, note the processes with diffused orientation (arrow). (F) P31 female, the glial cell process orientation is preserved (arrow head). (G) P45 male, as in females, GFAP-ir is more intense, but the distribution pattern of the processes is different (arrow). (H) P45 female, note the intensity of the GFAP-ir cells (arrow), and the length of the thin processes and branches. (A – H) 40 x.

Figure 2: Effects of sex and age on astrocytic processes and branches in the stratum radiatum of CA1 during hippocampal development.

A - Schematic representation of the Sholl's method used to establish the number of astrocytic processes and branches. Virtual circles of 10, 20, 25 and 30 μm were drawn around each astrocyte, and the number of intersections of astrocytic processes or branches with the virtual circles was quantified in the lateral (L) and central (C) quadrants around astrocytes.

B - Total number of all astrocytic processes intersecting with concentric circles in central (black bar) and lateral (striped bar) quadrants of male and female animals. The number of central processes increased with age, more significantly for female (F45xF11, a, $p \leq 0.001$, F45xF21 b, $p \leq 0.01$ and F45xF31 c, $p \leq 0.01$) than male (M45x M21d, $p \leq 0.05$). The number of lateral processes increased more significantly with age for male (M45XM11 e, $p \leq 0.001$; M45XM21 f, $p \leq 0.001$ and M45xM31 g, $p \leq 0.01$) than female (F45xF11 h, $p \leq 0.01$).

C - Total number of astrocytic primary processes intersecting with concentric circles in central (black bar) and lateral (striped bar) quadrants of male and female animals. Females showed fewer central (a, $p \leq 0.01$) processes than males, while no differences were found for lateral processes.

D - Total number of astrocytic branches intersecting with concentric circles in central (black bar) and lateral (striped bar) quadrants of male and female animals. Also regarding age, note a significant difference in central branches between the sexes (F45xM45 a, $p \leq 0.05$), note the increase in the number of central branches was greater in females (F45xF11 b $p \leq 0.01$, F45xF21 c, $p \leq 0.001$ and F45xF31 d, $p \leq 0.01$), while the increase in the number of lateral branches was greater in males (M45xM11 e, $p \leq 0.05$ and M45xM21 f, $p \leq 0.05$). All values are expressed as mean ± standard error.

4.2 ARTIGO EXPERIMENTOS 2 E 3 – A SER SUBMETIDO PARA BRAIN RESEARCH

GONADAL HORMONE REGULATION OF GLIAL FIBRILLARY ACIDIC PROTEIN IMMUNOREACTIVITY IN THE HIPPOCAMPAL STRATUM RADIATUM ACROSS THE ESTROUS CYCLE AND IN OVARIECTOMIZED FEMALE RATS

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Abstract

Sex differences in the morphology and function of neurons and glia cells from the hippocampus have been studied in several species, but it is unknown whether a sexual dimorphism exists in glial fibrillary acidic protein (GFAP) expression in the rat hippocampus. For this purpose, we analyzed GFAP immunoreactivity (GFAP-ir) in the hippocampus of adult males and females rats either across the different phases of the estrous cycle (diestrus, proestrus, estrus and metaestrus) or following ovariectomy and hormonal substitutive therapy (consisting of oil, estradiol alone, or the combination of estradiol and progesterone). In the stratum radiatum of CA1, measured by optical densitometry and astrocytic density was found that males showed a higher GFAP-ir and number of GFAP-ir cells than females across the estrous cycle ($P<0.001$ and $P<0.01$, respectively). Also, differences in astrocyte morphology were found. Fusiform cells with short processes were mainly observed in the female hippocampus during estrous cycle, whereas cells with stellate shape with numerous and long processes were identified in the hippocampus of males ($P<0.001$). In ovariectomized females, estradiol alone (EB) or estradiol plus progesterone (EB+P) increased the density of GFAP-ir cells ($P < 0.01$), but not the GFAP content in these cells or their morphology but not affected the astrocyte morphology. These findings suggest that GFAP-ir in the stratum radiatum of hippocampus can be affected either by physiological or supraphysiological levels of gonadal hormones, leading to new discovery about astrocyte morphological features in adult rats.

Keywords: stratum radiatum, hippocampus, ovarian hormones, glial plasticity, GFAP, Sholl's method, optical densitometry.

Introduction

Among others sexually dimorphic brain areas, the hippocampus is of particular interest because of its well described functions in learning, memory and stress responses, and it is a target of brain damage and aged-related diseases such as Alzheimer's (Wang et al., 2003). Several sex differences in morphology, function, and gene expression regulation have been well documented in the hippocampus (Juraska, 1991; Garcia-Segura et al., 1994; Maren et al., 1994; Madeira and Lieberman, 1995; Shors et al., 2001; Guerra-Araiza et al., 2002; Garcia-Segura and McCarthy, 2004; McCarthy and Konkle, 2005; Martinez et al., 2005), suggesting that sex steroids modulate both organizational and activational effects on this brain region. Gonadal hormones participate in the regulation of the morphology and activity of the hippocampus (Cooke and Woolley, 2005; Galea et al., 2006; Hajszan et al., 2007; Waters et al., 2008) through their effects on both neurons and glial cells, particularly astrocytes (Barreto et al., 2007; Reyna- Neyra et al., 2002; Tanapat et al., 2005).

The glial cells are involved in the developmental and activational effects promoted by sex steroids in synaptic formation and plasticity (Garcia-Segura and McCarthy, 2004; Garcia-Segura et al., 1994; Klintsova et al., 1995; Martinez et al., 2005). These findings reveal that glial cells are key targets for gonadal hormones-mediated effects in the mammalian brain. In fact, the expression of intracellular receptors for progesterone, estradiol, and androgens has been demonstrated in glial cells (Jung-Testas and Baulieu, 1998; Azcoitia et al., 1999; García-Ovejero et al., 2002; Milner et al., 2001, 2005; Tabori et al., 2005).

Ovarian hormones have widespread effects throughout the brain and modify neurons and glial cells in some specific brain areas. Estradiol (E) has been suggested as a mediator of neuronal sprouting through its effects on astrocytes (Rozovsky et al., 2002), and regulates the expression of glial fibrillary acidic protein (GFAP), an intermediate filament of astroglia and

an important cell marker of mature astrocytes, both *in vivo* and *in vitro* in the rat hypothalamus and the hippocampus (Day et al., 1993; Gomes et al., 1999; Stone et al., 1998; Garcia-Segura and McCarthy, 2004; Kettenmann and Ranson, 2005). Also progesterone (P) induces the expression of agrin, an astrocyte-derived protein involved in synapse formation (Tournell et al., 2006) while testosterone (T) regulate astrocyte number during rat hippocampal development (Conejo et al., 2005), enhances the extension of GFAP immunoreactive processes in astrocytes from hippocampal slices *in vitro* (Del Cerro et al., 1995), and modulates astrocyte reaction after brain injury (Storer and Jones, 2003; Nilsson and Pekny, 2007).

The density of GFAP immunoreactive processes in mice hippocampus fluctuates during the estrous cycle, with higher values on proestrus phase (Struble et al., 2006), when gonadal hormones are at the highest levels (Smith et al., 1975). Astrocytic volume also fluctuates in brain regions that regulate sexual and maternal behavior as well as pituitary secretions, such as the medial amygdala (Rasia-Filho et al., 2002; Martinez et al., 2005) and the ventromedial and arcuate hypothalamic nuclei (Kohama et al., 1995; Mong et al., 1999).

Although a higher number of GFAP immunoreactive cells has been found in the hippocampus of young male rats when compared with prepuberal females (unpublished results; Conejo et al., 2005), the influence of circulating gonadal hormones in physiological or supraphysiological conditions on GFAP immunoreactivity (GFAP-ir) and in the number and orientation of GFAP-positive cells in the hippocampus of adult rats have not been established. The aim of the present work was to evaluate a possible sexual difference in the number and morphology of GFAP-positive cells, GFAP-ir and GFAP-ir distribution in stratum radiatum of adult rats hippocampus. The immunohistochemical technique were used together with a

semi-quantitative analysis using optical densitometry and Sholl's method (Xavier et al., 2005; Martinez et al., 2005, Dall'Oglio et al., 2008; Viola et al., 2009).

2 Results

2.1 Qualitative light microscopic observations

GFAP-ir was clearly detected in the glial cell bodies and their processes in the stratum radiatum of hippocampus. No reaction was found when the first antibody was omitted. The GFAP-ir in the stratum radiatum displayed a visually strong labeling in males when compared to females across estrous cycle (Fig. 1), and some differences were noted in the cell morphology: in proestrus and diestrus females, GFAP-positive cells showed fusiform cell bodies with numerous and long processes; on the other hand, estrous and metaestrus females showed smaller cell bodies with shorter and fewer processes. In males were observed GFAP-positive cells with numerous and long processes resembling reactive astrocytes (Fig. 1). Data regarding to GFAP-ir in ovariectomized female rats, did not demonstrated changes in the cellular morphology (Fig. 4).

2.2 Semi-quantitative analysis

2.2.1 GFAP-ir across the estrous cycle

In the stratum radiatum of hippocampus, the optical density for GFAP-ir reached a statiscally significant difference among males and females across of the estrous cycle [$F(4,86)=9.746$; $P<0.0001$; Fig. 2A]. The Bonferroni *post hoc* test showed that males have a higher optical density for GFAP-ir when compared to diestrus ($P<0.01$), estrus ($P<0.05$), and metaestrus groups ($P<0.01$). When males and proestrus females were compared, no statistically significant difference were found ($P>0.05$). When females across estrous cycle

were compared, proestrus and estrus females have a higher optical density for GFAP-ir when compared to diestrus phase ($P<0.001$ and $P<0.05$, respectively). No statistical difference was found for the values obtained between metaestrus and all other phases of estrous cycle ($P>0.05$; Fig. 2A).

Data obtained for the number of GFAP-ir cells also reached a statistically significant difference among males and females across of the estrous cycle [$F(4,27)=7.410$; $P<0.001$; Fig. 2B]. The Bonferroni *post hoc* test showed that males have a higher number of GFAP-ir cells when compared to diestrus ($P<0.01$), proestrus ($P<0.001$), and metaestrus group ($P<0.01$). When males and estrus females were compared, no statistically significant difference were found ($P>0.05$). No statistical difference was found for the values obtained in females across the different phases of the estrous cycle ($P>0.05$; Fig. 2B).

Data regarding to astrocytic morphology [$F(9,116)=9.165$, $P<0,001$] reached a statistically significant difference among males and females across of the estrous cycle. (Fig. 3). Nevertheless the process intersections were higher in males when compared to females across the estrous cycle ($P< 0,001$, Fig. 3). This higher values was observed both in processes oriented perpendicularly to CA3-CA1 projections ("central" processes, Fig. 3, *p<0,001*), which is the usual orientation of astrocytic processes in this region. And in those oriented parallel to these fibers ("lateral" processes) were higher in males when compared to estrus ($P<0.05$) and metaestrus ($P<0.05$), but no difference were also detected to diestrus and proestrus (Fig. 3).

2.2.2 GFAP-ir in ovariectomized and treated females

In the stratum radiatum of hippocampus, the optical density for GFAP-ir reached a statically significant difference from ovariectomized females treated with V, EB alone, or EB

plus P [F(2,36)=14.42; *P*<0.0001; Fig. 5A]. Moreover, the Bonferroni *post hoc* test showed that ovariectomized females treated with EB+P have a higher optical density for GFAP-ir when compared to V and EB groups (*P*<0.001 in both cases; Fig. 5A). No statistical difference was found for the values obtained between V and EB alone treated groups (*P*>0.05; Fig. 5A).

Data obtained for the number of GFAP-ir cells [F(2,16)=5.907; *P*<0.05] also reached a statistically significant difference from ovariectomized females treated with V, EB alone, or EB plus P (Fig. 5B). The Bonferroni *post hoc* test showed that ovariectomized females treated with EB alone or EB+P have a higher number of GFAP-ir cells when compared to V (*P*<0.05). When EB ans EB+P treated females were compared, no statistically significant difference were found (*P*>0.05; Fig. 5B).

The Sholl concentric circles values, in the groups that received E or E + P, were compared to vehicle group [F(5,32)=9.929; *P*<0.001], and a statistically differences were observed only for orientation, central against lateral (*P*<0.001 in both cases; Fig. 6) within each group. But not between ovariectomized female rats, and the morphology were equivalent to that present in proestrous phase.

A significant difference was found in the weight of the uteri from ovariectomized females in the different experimental studied groups (P < 0.001). The vehicle-injected group exhibited thinner and atrophied uteri (mean ± SD, 0.20 ± 0.04 g) when compared to the E (0.47 ± 0.07 g; P < 0.001). The E group showed higher results than the E + P group (0.27 ± 0.03 g; P < 0.001). When vehicle and the E + P groups were compared, a clear trend for the latter group to present heavier uteri was found (P = 0.07).

3 Discussion

The present findings showed that GFAP-ir, in the CA1 hippocampus stratum radiatum varied across the rat estrous cycle as well as following sex differences, but were less evident for ovariectomy and hormones manipulation.

Some GFAP-ir was higher in the afternoon of the proestrus phase, when estrogen and progesterone are at their highest circulating values (Arias et al., 2009), decreasing afterwards in the estrus. Additionally, hormonal substitutive therapy provided some similarities but also differences compared with ovarian cycle effects (i.e., E alone and E + P administered to ovariectomized females small increased GFAP-ir).

Although the number of astrocytes, it is possible that the rapid modification of GFAP expression during the estrous cycle might be related to the increase in the GFAP-ir of astrocytic processes and the degree of their phosphorylation and dephosphorylation (Kettenmann and Ranson, 2005). This hypothesis is based on previous demonstrations that the number of astrocytes was not affected by estrogen in the rat globus pallidus, in the arcuate nucleus or in the hippocampal subfields CA3 and CA4 (Garcia-Segura et al., 1994; Lâm and Leranth, 2003; Tranque et al., 1987). In the arcuate nucleus, there was a probable change in astrocytic branching across the estrous cycle (Garcia-Segura et al., 1994).

Effects of sex steroids on astrocytes in the brain have been assessed with different experimental approaches. For example, it has been found an enhanced expression of GFAP in the hippocampus and hypothalamus after castration of newborn male rats (Day et al., 1990; Garcia-Segura et al., 1996), which was reverted by administration of estrogen (Day et al., 1993). Recently, Struble and col. (2006) have shown that the density of mouse hippocampal GFAP immunoreactivity processes peaked on proestrus in absence of variation of cell density.

Herein, we demonstrated by immunohistochemistry, that in intact adult female and male rats, slightly variations in GFAP-ir, as well as in the number and morphology of GFAP-positive cells. Notable differences were observed when male and female rats were compared. We observed a higher number of astrocytes of adult male rats as compared with females. Interestingly, our group (Gehlen et al, submitted 2009) found some similar values in young animals, and Conejo et al. (2005) obtained the same results but in prepuberal rats.

Astrocytes regulate neuronal development, maintenance of neuronal structures providing active molecules (Hama et al., 2004), and participate in neuronal plasticity regulating neurite outgrowth (Crone and Lee, 2002; Kanemaru et al., 2007) and synaptogenesis (Newman, 2003; Piet et al., 2004; Perea and Araque, 2005). It is conceivable by a variety of evidences that astrocytes may change their biochemical properties depending of microenvironment cues (Bushong et al, 2004). The present work support the notion that gonadal steroids from male and female rats provide different signaling conditions capable of modifying astrocyte cytoskeleton, number and morphology.

Sex hormones, particularly estrogens, produced at high levels during proestrous phase of estrous cycle, are proven to be involved in learning performance (Li et al., 2004). Moreover, it has been suggested that plastic changes in glial cells driven by gonadal steroids are involved in mechanisms of neuronal and synaptic plasticity (Reyna-Neyra et al., 2002, 2004).

Hippocampal CA1, CA3 and the hilar dentate gyrus regions posses predominantly protoplasmic astrocytes irregular in shape with short and branched processes (Privat et al., 1995) Interestingly, during the estrous cycle, astrocytes appeared with fine and longer processes and with reduced GFAP content. In contrast, in males, there were an increased number of astrocytes with GFAP immunoreactivity longer processes in male. The consequences of these differences in astrocytic processes pattern could be significant for brain

functioning, which may reveal the dynamism of their interaction with neurons (Hirrlinger et al., 2004). In fact, it has been found estradiol-mediated synaptic fluctuation during estrous cycle in adult rats in CA1 (Woolley and McEwen, 1992; Yankova et al., 2001; Cooke and Woolley, 2005).

In conclusion, the present study intended to demonstrate that astrocytic GFAP-ir in normally cycling females is slightly higher in the proestrus, accompanying the physiological fluctuations of circulating ovarian steroids. Highlighting the changes in the morphology between male and intact females. Furthermore, the stratum radiatum showed a slender enhance of GFAP-ir following injections of estradiol-benzoate alone or estradiol-benzoate and progesterone to females submitted to ovariectomy, without changes in the cell shape pattern.

4 Experimental procedures

4.1 Animals

Adult male and female Wistar rats (3–5 months old) were housed in groups with access to food and waster *ad libitum*, used; and room temperature was maintained around 22 °C in a 12:12 h light:dark cycle (lights off at 6 PM). All efforts were made to minimize the number of animals studied and their suffering. Rats were manipulated according to international laws for the ethical care and use of laboratory animals (European Communities Council Directive of 24 November 1986, 86/609/EEC) and to local guidelines on the ethical use of animals.

Vaginal smears were taken from virgin females during two weeks before the beginning of the experiment to determine the regularity of ovarian steroid secretion. Only normal cycling females were used in this study. In the first experiment, females (n=6 in each group) were sacrified in the afternoon of each phase of the estrous cycle (diestrus, proestrus, estrus, and

metaestrus). In the second experiment, virgin female rats were submitted to ovariectomy under ketamine and xylazine anesthesia (Cristalia, Brasil, 20 and 80 mg/kg i.p., respectively). After 1 week of recovery, animals were divided into three groups and submitted to hormonal therapy (n=6 in each group). The first group received two injections of sesame oil as vehicle (V; 0.1 mL, s.c.) 24 h apart, and a third injection of oil 48 h later. The second group received two injections of estradiol benzoate (EB; Sigma Chemical Co., USA; 10 μg/0.1 mL, s.c.) 24 h apart, and a third injection of EB 48 h later. Rats in the third group received two injections of EB (10 μg/0.1 mL, s.c.) as performed in the second group, but the third injection was of progesterone (P; Sigma Chemical Co., USA; 500 μg/0.1 mL, s.c) 48 h later. The action of progesterone alone was not tested here. In each of these three groups, the last injection was given 5h before sacrifice and to the beginning of the immunohistochemical study. The injection dosages and protocols were based and adapted from previously published data (Martinez et al., 2005; de Castilhos et al., 2008; 2009). Uterine weight was used to assess the systemic effects of the hormonal therapy administered to the experimental groups of ovariectomized females.

4.2 Immunohistochemical procedure

For the immunohistochemical study, all animals were deeply anesthetized with sodium thiopental (50 mg/kg, i.p.) and injected with 1000 IU of heparin. Thereafter, they were transcardially perfused through the left cardiac ventricle using a peristaltic pump (Control Company, Brasil, 20 mL/min) with 200 mL of saline solution followed by 200 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (Reagen, Brasil, pH 7.4; PB). The brains were removed and post-fixed in the same fixative solution at room temperature for 4 h, were cryoprotected in a 30% sucrose solution in PB at 4 °C until they sank and were frozen in

isopentane and liquid nitrogen. Coronal sections (50 μm) were obtained using a cryostat (Leitz, Germany). Sections were collected in phosphate buffer saline solution (PBS) and processed for GFAP immunohistochemistry following the unlabeled antibody peroxidase–antiperoxidase (PAP) procedure (Sternberger, 1979). Free-floating sections were pretreated with 10% methanol and 3% H_2O_2 for 30 min and then, carefully washed and blocked with 3% normal goat serum (NGS) in PBS containing 0.4% Triton X-100 (PBS-Tx, Sigma Chemical Co., USA) for 30 min. Then, sections were incubated with polyclonal GFAP antiserum raised in rabbit (Sigma Chemical Co., USA) diluted 1:150 in 3% NGS in PBS-Tx for 48 h at 4 °C. After being washed several times with PBS-Tx, sections were incubated in a rabbit anti-rabbit IgG (Sigma Chemical Co., USA) diluted 1:50 in PBS-Tx at room temperature for 2 h. Sections were again washed in PBS and incubated in a rabbit PAP (Sigma Chemical Co., USA) diluted 1:500 in PBS for 2 h at room temperature. The reaction was developed by incubating the sections in a histochemical medium that contained 0.06% 3,3′-diaminobenzidine (DAB, Sigma Chemical Co., USA) dissolved in PBS for 10 min and in the same solution containing 1 μL of 3% H_2O_2 per mL of DAB medium for additional 10min. Finally, the sections were rinsed in PBS, dehydrated in ethanol, cleared with xylene and covered with Permount and coverslips. Control sections were prepared omitting the primary antibody by replacing it with PBS.

The brains from males, females in the different phases of the estrous cycle and those from ovariectomized females submitted to vehicle or hormonal injections were fixed and post-fixed for the same time period and in the same solutions. Being processed together, they underwent exactly the same histological steps and were incubated in an identical medium for the same time period. These procedures were taken to reduce the possibility of overreaction,

differences in the chromogen reaction or changes in the background levels between the experimental groups of rats (Xavier et al., 2005).

4.3 Data acquisition

4.3.1 Acquiring and measuring the regional optical density

For the immunohistochemical study, stratum radiatum of the dorsal hippocampus were selected for data acquisition. All measurements were performed in the stratum radiatum of CA1 between coordinates bregma -1.58 mm interaural 2.22 mm and bregma -2.06 mm, interaural 1.74 mm obtained from Paxinos and Watson' atlas (1997). The intensity of the GFAP immunoreaction was measured by semi-quantitative densitometric analysis (Rasia-Filho et al., 2002; Xavier et al., 2005; Martinez et al, 2005) using a Nikon Eclipse E-600 microscope (500×, Japan) coupled to a Pro-Series High Performance CCD camera and to the Image Pro Plus Software 4.1 (Media Cybernetics, USA). The digitized images obtained from the selected areas were converted to an 8-bit gray scale (0–255 gray levels). All lighting conditions and magnifications were held constant. Picture elements (pixels) employed to measure optical density were obtained from squares with 1970 μm2 (our area of interest, AOI) overlaid in the gray scale image. A grid mask was placed in the images obtained from radiatum. To obtain a more representative evaluation of these regions, the first AOI was placed randomly (using a table of random numbers), all other AOIs were placed following a constant pattern with equidistant intervals in the grid mask. The obvious blood vessels and other artifacts were avoided. At least 10 readings were performed in each analyzed section and the data average of each section was recorded. Both left and right sides of each brain were used. At least, 10 sections were analyzed per hippocampus from each studied group (6 sections per animal) across the estrous cycle and in ovariectomized and treated rats. The

subtraction of background staining and the background correction were done in accordance with our previous published protocol (Xavier et al., 2005).

The optical density (OD) was calculated using the following formula:

$$OD(x,y) = -\log[INT_{(x;y)} - BL)] / (INC - BL)$$

where "OD(x,y)" is the optical density at $pixel_{(x,y)}$, "$INT_{(x,y)}$" or intensity is the intensity at $pixel_{(x,y)}$, "BL" or black is the intensity generated when no light goes through the material (5.3 in our case) and "INC" or incident is the intensity of the incident light (252.4 in our case).

4.3.2 Measuring astrocytic density (number of GFAP-ir astrocytes per mm^2)

In order to verify the results generated by optical density measurement, the number of GFAP-ir astrocytes per mm^2 was estimated. The digitized images of dorsal CA1 were obtained and the same AOI used to measure the optical density was overlaid on these regions. The soma of GFAP-ir astrocytes located inside this square or intersected by the lower and/or right edge of the square were counted. The astrocytes that were intersected by the upper and/or left edge of the square were not counted. At least 3 sections from each brain were analysed (Chieco et al., 2001; Xavier et al.,2005).

4.3.3 Quantification of astrocytic processes and their branches

For the analysis of astrocytic morphology, we analyzed the number and radial distribution of 15 astrocytes in each animal (3 sections) using an adaptation of Sholl's concentric circles technique (Dall'Oglio et al., 2008; Viola et al., 2009). Five virtual circles with radii of 10, 15, 20, 25 and 30 μm were drawn around each astrocyte, and the number of intersections of

astrocytic processes with each virtual circle was quantified in both the lateral (i.e. right/left) and central (i.e. superior/inferior) quadrants around the astrocytes. The same AOI and inclusion and exclusion parameters used for astrocytic density were employed when selecting the astrocytes analyzed using Sholl's method. The method was performed on a single focal plane, after first focusing on the astrocytic soma, the image was digitalized. To obtain a detailed analysis of the processes and their branches, they were identified and counted separately. Processes and their branches were counted by two observers who were blind to the experimental groups, and the results from both observers were averaged in the final results.

4.4 Statistical analysis

Data regarding the GFAP-ir optical densities, number of GFAP-ir cells and astrocite morphology from the stratum radiatum of the hippocampus of males and females across the estrous cycle, and in a separate test, from ovariectomized females treated with the different substitutive therapies were compared by an one-way analysis of variance (ANOVA) test. When appropriate, *post hoc* comparisons were done using the Bonferroni test. Uterine weights were compared among these last experimental groups using the same test. In all cases, the level of statistical significance was set *a priori* at $P < 0.05$.

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Legends

Figure 1. Digitized images of GFAP-ir in the stratum radiatum of CA1 dorsal hippocampus of male and female rats in different phases of the estrous cycle. Note the higher expression of the GFAP-ir in the male than proestrus and diestrus phases. GFAP-positive cells from males showed stellate bodies with long processes (arrow) while cells from females showed fusiform cell bodies with long processes (arrowhead), but estrus and metaestrus showed fewer processes (asteris). Scale bar= 20 μm.

Figure 2. (A) Optical density measurement (mean ± SEM) of GFAP immunoreactivity in the stratum radiatum of males and females across the estrous cycle. 'a' corresponds to $P<0.05$ when compared to estrus; 'b' corresponds to $P<0.01$ when compared to metaestrus; and 'c' corresponds to $P<0.001$ when compared to males and proestrus. (B) Number of GFAP-ir cells in the stratum radiatum of males and females across the estrous cycle. 'a' corresponds to $P<0.01$ when compared to diestrus and metaestrus; 'b' corresponds to $P<0.001$ when compared to proestrus.

Figure 3. Total number (mean ± SEM) of GFAP-ir astrocytic processes intersecting with concentric circles from Scholl's method. An increased number of intersections was observed for males for central processes (black bar), 'a' corresponds when compared to diestrus; 'b' to proestrus; 'c' to estrus and 'd' to proestrus (all cases $p<0,001$). And those lateral processes (striped bar) also showed higher values for males when compared estrous ('e', $p< 0.05$) and metaestrous ('f', $p< 0.05$). Images below represent gray scale images of representative astrocytes from all groups analysed. Scale bar = 10μm.

Figure 4. Digitized images of GFAP-ir in the stratum radiatum of dorsal CA1 hippocampus of ovariectomized females treated with vehicle (V), estradiol benzoate (EB) or estradiol benzoate plus progesterone (EB+P). Note the same cell shape pattern among all studied groups. Scale bar = 20 μm.

Figure 5. (A) Optical density measurement (mean ± SEM) of GFAP immunoreactivity in the CA1 stratum radiatum of ovariectomized female rats treated with vehicle (V), estradiol benzoate alone (EB), and estradiol benzoate plus progesterone (EB+P). 'a' corresponds to $P<0.001$ when compared to V and EB. (B) Number of GFAP-ir cells in the stratum radiatum of ovariectomized female rats with the same hormonal replacement. 'a' corresponds to $P<0.05$ when compared to EB and EB+P.

Figure 6. Total number (mean ± SEM) of GFAP-ir astrocytic processes intersecting with concentric circles from Scholl's method of ovariectomized female rats treated with vehicle (V), estradiol benzoate alone (EB), and estradiol benzoate plus progesterone (EB+P). There were differences within all analysed groups, in central processes (black bar), but not among lateral processes (striped bar). 'a' corresponds to $P<0.001$ when compared central to lateral for V animals, 'b' corresponds to $P<0.001$ to EB animals, and 'c' corresponds to $P<0.001$ to EB+P animals. Images below represent gray scale images of representative astrocytes from ovariectomized and hormonal treated females. Scale = 10μm

Figures

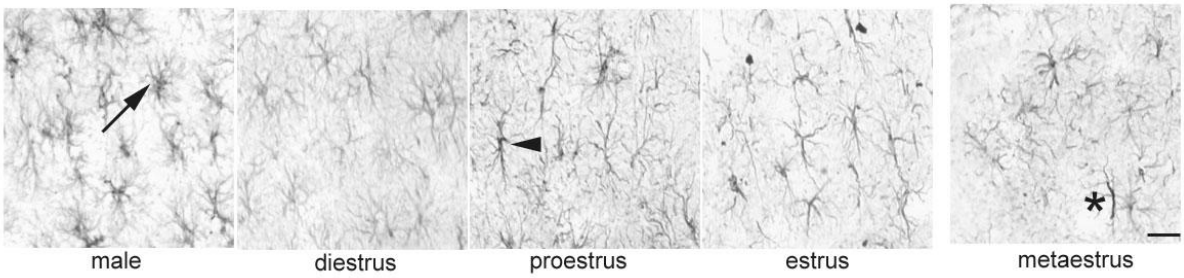


Figure 1

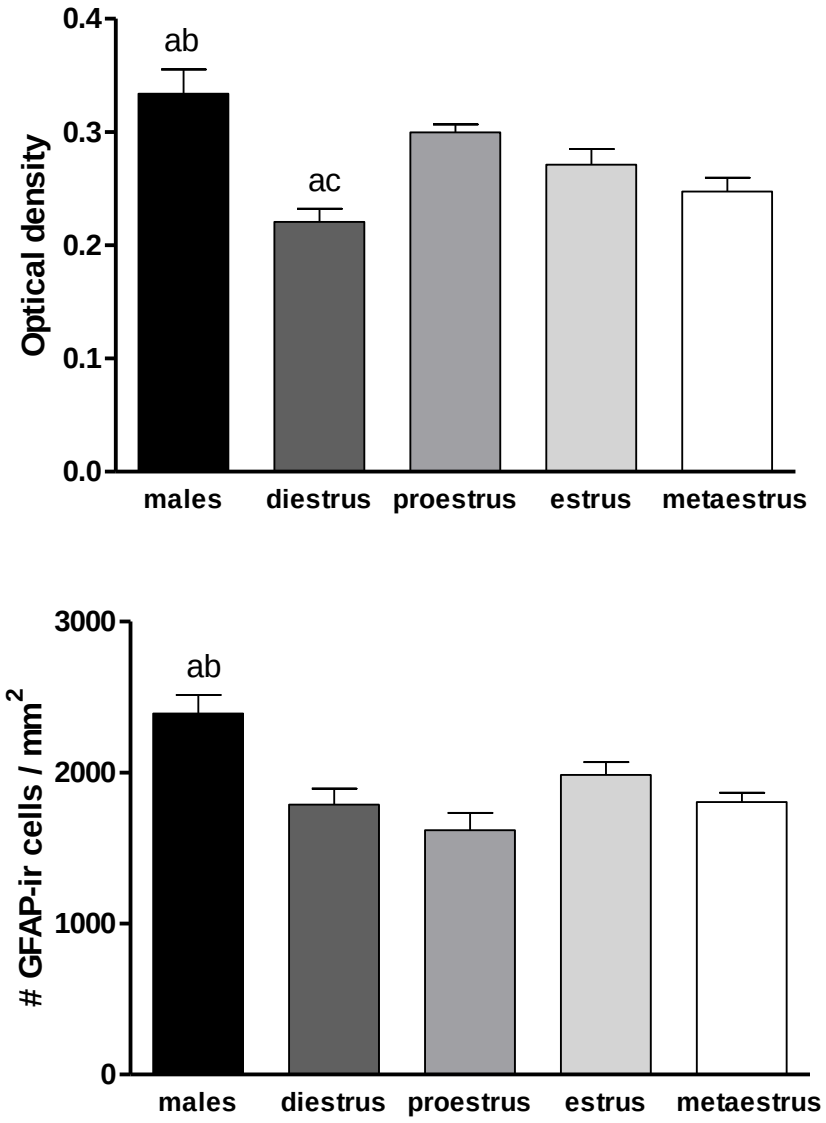


Figure 2

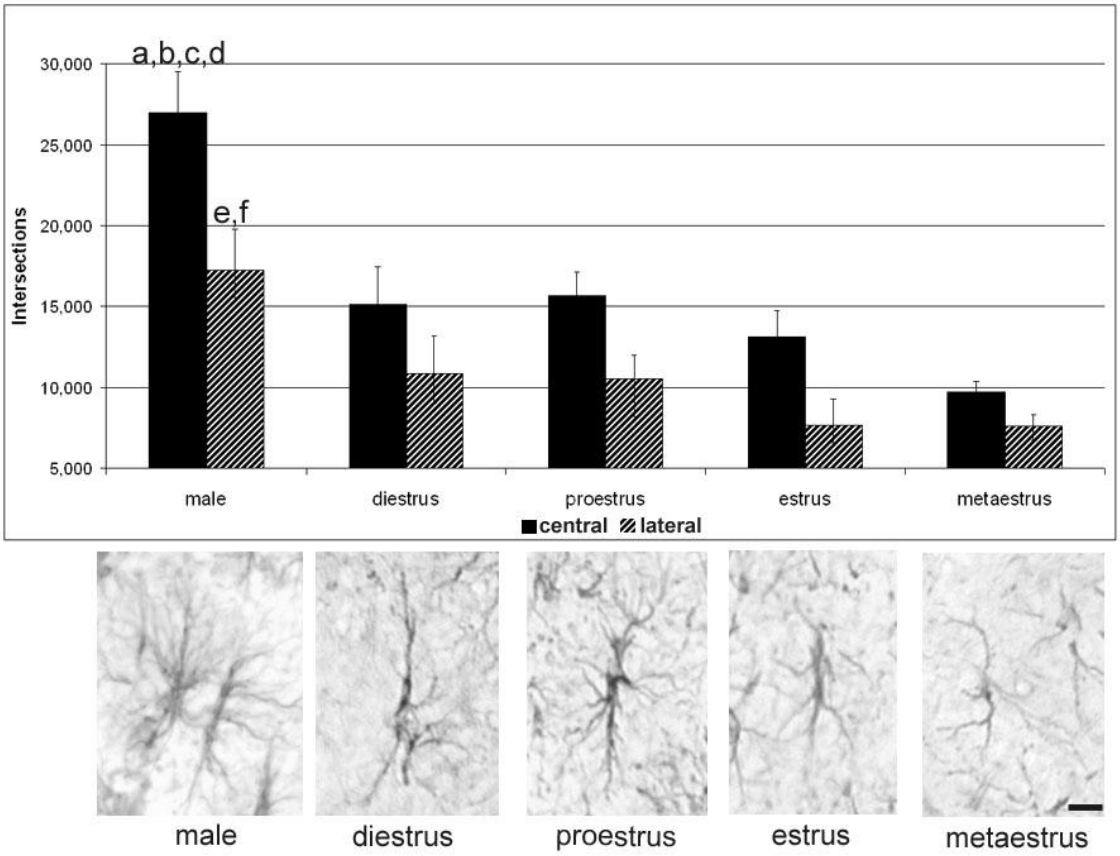


Figure 3

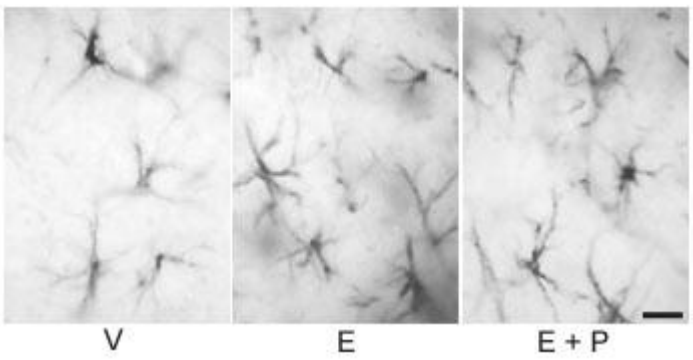
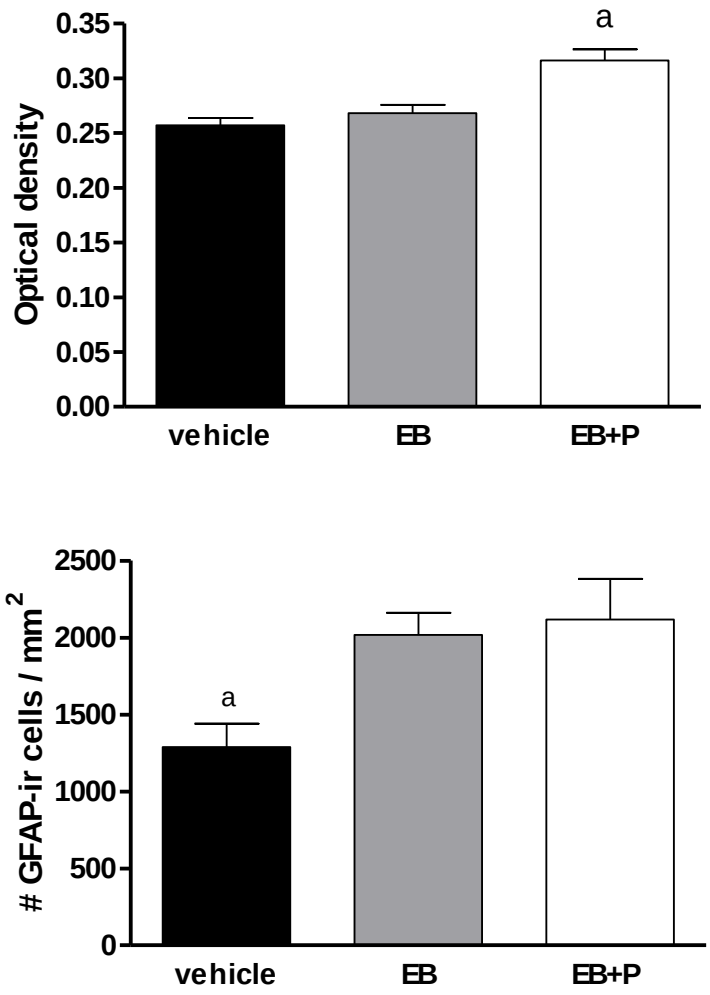


Figure 4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EB+P

Figure 5

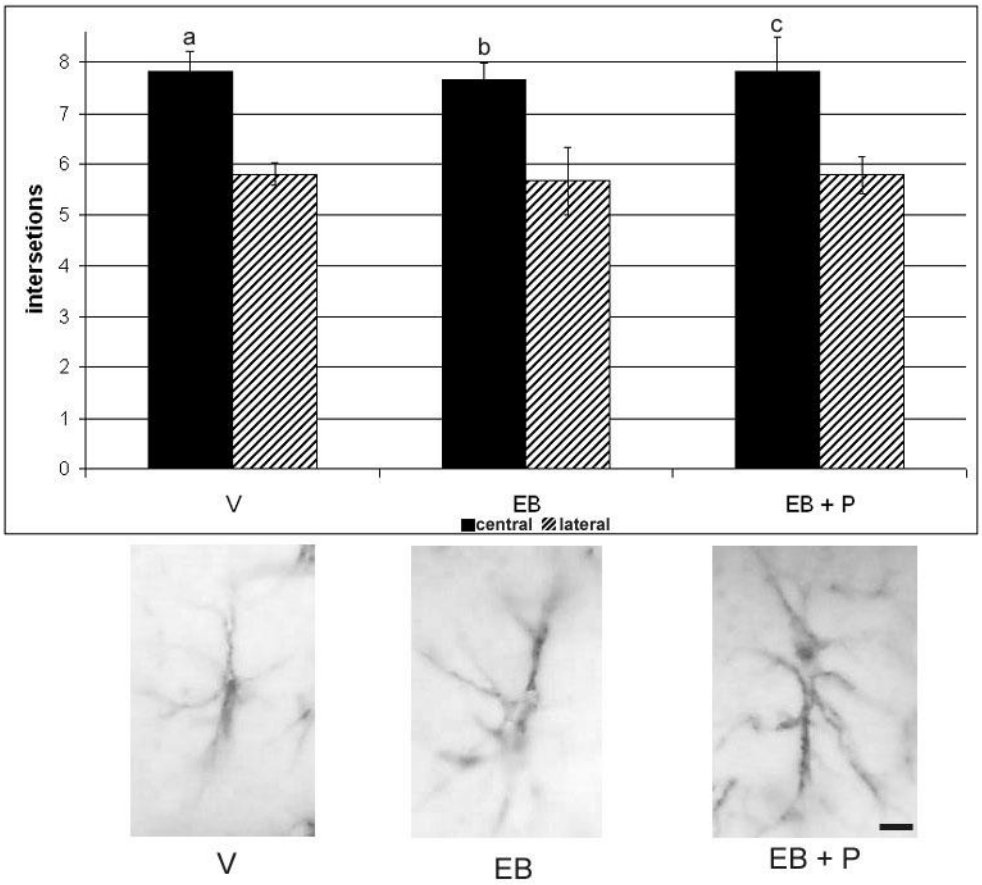
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Figure 6

5 DISCUSSÃO

5.1 DIMORFISMO VERSUS DIFERENÇAS

Através dos dados obtidos neste trabalho e da análise de outros trabalhos da literatura com modelos animais, proponho que há necessidade de um esclarecimento na literatura sobre o que é uma diferença sexual, que poderia significar, radical e permanentemente, diferenças entre machos e fêmeas frente a uma resposta hormonal modulada. Ainda, poderia representar um parâmetro que varia por vias conhecidas em resposta a mudanças nos hormônios circulantes em adultos.

Extensivos estudos têm fornecido uma descrição rica do tamanho relativo, neuroquímica, e conectividade de sub-núcleos dentro da área pré-óptica e no hipotálamo de uma variedade de espécies, tais como: anfíbios, répteis, aves e mamíferos, incluindo primatas superiores. A magnitude da diferença entre os sexos em diversos parâmetros morfométricos tende a ser grande, na faixa de 2 a 7 vezes, levando facilmente ao título de "dimorfismo sexual". Dimórfico significa de duas formas, e, portanto, é um bom termo, uma vez que essas diferenças são de grande amplitude e têm pouca sobreposição. Mas a terminologia permite deslizes e frequentemente leva ao erro. Como resultado, tem se observado uma tendência crescente para equacionar as "diferenças sexuais" com "dimorfismo sexual" que, simultaneamente, levam a um desvio da atenção quanto a questão mais importante, que são a magnitude e confiabilidade (McCARTHY; KONKLE, 2005).

5.1.1 Princípios mecanicistas comuns no estabelecimento das diferenças sexuais encefálicas

Algumas generalizações simples podem ser feitas em relação ao que podem ser consideradas diferenças sexuais encefálicas hormonalmente mediadas. Uma delas é que os hormônios esteróides, sejam eles os andrógenos ou os estrógenos, não parecem afetar a gênese de células em regiões do encéfalo que tende a ser dimórfico e, em vez disso, determinam se células pré-existentes irão sobreviver ou morrer. Por exemplo, o núcleo sexualmente dimórfico da área pré-óptica é de 5 a 7 vezes maior em volume nos machos adultos do que nas fêmeas (GARCIA-SEGURA, 1986). Machos recém-nascidos e fêmeas têm o mesmo número de neurônios na região destinada a se tornar a SDN, e a diferença sexual para adultos deve-se à morte de células, no sexo feminino, dentro da primeira semana de vida. Essas células podem ser resgatadas por tratamento em recém-nascidos do sexo feminino usando testosterona, ou com estradiol diretamente, mas se o tratamento for adiado de forma a passar da primeira semana, a fêmea vai ter permanentemente uma SDN menor do que o macho.

Por outro lado, no núcleo anteroventral periventricular ocorre o contrário, as células morrem no sexo masculino em resposta à ação do estradiol, resultando em um núcleo menor do que a quantidade encontrada nas fêmeas adultas (GORSKI et al, 1978). Estas diferenças entre os sexos em termos de volume são robustas e permanentemente estabelecidas durante o período sensível perinatal. Também são diretamente relevantes para a reprodução, em oposição às mudanças volumétricas em outras regiões do encéfalo que respondem aos esteróides gonadais circulantes na fase adulta.

Uma segunda generalização envolve o estabelecimento de dimorfismo sexual nas conexões sinápticas, de forma igualmente robusta quando identificados volumetricamente,

mas geralmente são menos investigadas devido aos desafios inerentes às técnicas utilizadas. A plasticidade sináptica na conectividade até o encerramento do período sensível é um parâmetro bem estabelecido das regiões do encéfalo influenciado por estímulos sensoriais externos, tais como componentes do sistema visual que recebem intervenção direta ou indireta da retina, ou porções do córtex que mapeiam os estímulos específicos das vibrissas em ratos (MORRIS; JORDAN; BREEDLOVE, 2004).

5.1.2 Diferenças sexuais morfométricas no hipocampo

Por definição pode-se supor que uma diferença sexual no tamanho e/ ou na forma do hipocampo satisfazem os critérios para que essa diferença seja forte, permanente e não sujeita à alterações por esteróides gonadais na fase adulta. Mas, surpreendentemente, isto geralmente não é o caso das diferenças sexuais na morfometria do hipocampo. Em termos de volume, os machos têm um hipocampo maior do que as fêmeas (MADEIRA; LIEBERMAN, 1995; NUNES; KOSS; JURASKA, 2000), com a região CA1 sendo 16% maior nos machos em um estudo (ISGOR; SENGELAUB, 1998) e apenas 10% maior em outro (NUNEZ; MCCARTHY, 2003). A diferença sexual é evidente dentro da primeira semana de vida, e parece ser devida à morte celular diferencial entre machos e fêmeas (NUNEZ; MCCARTHY, 2003; HILTON; NUNEZ; MCCARTHY, 2003).

No entanto, para alguns autores, como McCARTHY e KONKLE (2005), essas diferenças são sutis e inconsistentes, vendo a diferença sexual em um grupo de animais, mas não necessariamente em outro. Em ratos, os machos têm mais células granulares do giro denteado do que as fêmeas, mas somente em três das seis cepas examinadas (WIMER; WIMER, 1985). A diferença, em favor do sexo feminino, quanto ao número de processos

dendríticos nos neurônios granulares do giro denteado observados no desmame desaparece na idade adulta (JURASKA, 1989). Além disso, diferenças de sexo na morfologia dos dendritos de neurônios piramidais em CA3, assim como nas células granulares do giro foram significativamente alterados pela complexidade do ambiente de criação (JURASKA; FITCH; WASHBURNE, 1989). É importante comparar estes dados com as robustas diferenças sexuais volumétricas observadas no sub-núcleo pré-óptico, uma área importante reprodutivamente, onde as diferenças estão na faixa de 300 a 700% (SIMERLY, 2000; SIMERLY, 2002; GORSKI, 1978).

Essas observações também estão em nítido contraste com as diferenças sexuais das regiões cerebrais reprodutivamente relevantes, onde as diferenças sexuais são em sua maioria estáveis na vida adulta e parecem relativamente resistentes aos experimentos. Quantificações de toda a árvore dendrítica apical em CA1 revelaram mais ramos nos machos do que nas fêmeas, mas isso não mostrou divergências sexuais significantes, sendo eliminadas com o envelhecimento devido a uma perda de ramos dendríticos no sexo masculino (MARKHAM, 2002), equivalendo-se aos nossos resultados com as células GFAP-ir para o mesmo período. Nesse mesmo estudo não foi encontrada nenhuma diferença entre os sexos na densidade de espinhas dendríticas, em qualquer idade.

Em conjunto, estes estudos sugerem que ainda não existe consenso sobre a existência ou não de diferenças sexuais, mesmo que pequenas, na morfometria do hipocampo, e se estas pequenas diferenças que são observadas podem ter relação com as funções não esclarescidas do hipocampo. Se não há diferença entre os sexos no hipocampo, como fica a modulação hormonal? É reconhecido há algum tempo que o estradiol aumenta a densidade das espinhas dendríticas e suas sinapses excitatórias nos dendritos distais dos neurônios piramidais de CA1 de ratas fêmeas (WOOLLEY; MCEWEN, 1992). Nas análises do sexo masculino, a

gonadectomia reduz drasticamente a densidade de espinhas, em até 50%, só sendo restaurada pelo tratamento androgênico, com efeito relativamente baixo do estradiol (LERANTH; PETNEHAZY; MACLUSKY, 2003). Assim, fêmeas e machos adultos com níveis adequados de andrógenos ou estradiol, respectivamente, possuem quantidades relativamente equivalentes de aferências sináptica para os dendritos de neurônios piramidais em CA1, mas aparentemente os atingem através de estratégias celulares diferentes.

Não há uma diferença sexual, por si só, na morfometria, impossibilitando o uso do termo "dimorfismo sexual," mas parece ser uma "diferença sexual" nas estratégias hormonais utilizadas para alcançar o mesmo objetivo. Em outras palavras, machos e fêmeas exploraram seus perfis hormonais específicos, de andrógenos versus estrogênios, respectivamente, para atingir o mesmo parâmetro do aumento das sinapses nas espinhas dendríticas. Ainda é desconhecido se os parâmetros são verdadeiramente os mesmos, pois não há muito conhecimento sobre a funcionalidade dessas novas sinapses. Que machos e fêmeas atingem o mesmo fim por diferentes meios é um tema que deve começar a ser investigado de forma integrada aos demais aspectos das diferenças sexuais no hipocampo.

CONCLUSÕES E PERSPECTIVAS

A presente tese foi dividida em duas seções, as quais podemos definir como: primeira, o estudo imunoistoquímico da GFAP no *estrato radiatum* da região CA1 do hipocampo de ratos machos e fêmeas ao longo do desenvolvimento pós-natal e, segunda, estudo da GFAP-ir na mesma região em ratas fêmeas intactas e ovateriomizadas com tratamento hormonal. Ambos os estudos obtiveram resultados que levaram à confirmação da hipótese formulada.

No estudo realizado utilizando machos e fêmeas adultas intactas ao longo do ciclo estral, os resultados obtidos demonstraram o efeito significativo dos hormônios masculinos sobre a GFAP-ir. Além disso, quando comparados machos e femeas adultos, foram observadas diferenças na orientação dos prolongamentos celulares, indicando uma plasticidade que provavelmente esteja relacionada com a sinaptogênese local e, consequentemente, com as funções desempenhadas pelas sinapses na modulação do aprendizado e memória dependente do hipocampo (LUINE et al., 2003; SANDSTROM; WILLIAMS, 2001).

Nosso estudo demonstrou também que níveis suprafisiológicos de estradiol e progesterona, combinados ou não, também exerceram efeito trófico significativo sobre marcadores do citoesqueleto astrocitário, como o aumento do número de células GFAP-ir.

Hormônios são componentes críticos da interação neurônio-glia, provocando ações neuromodulatórias e neurotróficas em situações fisiológicas ou patológicas (GARCIA-SEGURA; McCARTHY, 2004). No presente estudo, os esteróides sexuais foram capazes de exercer uma ação no *estrato radiatum* do hipocampo.

O outro foco desta tese, o estudo do dimorfismo sexual ao longo do desenvolvimento pós-natal, também apresentou resultados interessantes. Enquanto machos apresentaram células mais imaturas ao longo do período estudado, as fêmeas apresentaram morfologia

característica do período adulto, já a partir da segunda quinzena de desenvolvimento. Esses achados remetem a considerar a diferença temporal de maturação da função das gônadas, uma vez que os testículos dos machos já produzem testosterona em PN1, enquanto os ovários das fêmeas ainda são quiescentes (RHODA; CORBIER; ROFFI, 1984). Entretanto, nossos dados foram ao encontro, pelo menos neste aspecto, da afirmação de que as mudanças plásticas ocorridas em fases precoces do desenvolvimento perduram na vida adulta (MONG et al., 1999; CATALANI, 2005).

O marcador astrocitário GFAP também apresentou características diferentes, sexualmente, durante o desenvolvimento. Analisando-se todas as idades em conjunto, machos apresentaram maior número de células GFAP-ir do que fêmeas, além das diferenças morfológicas entre os grupos estudados. Ressalta-se que o formato astrocitário e sua mobilidade são influenciados por mudanças na expressão e fosforilação de GFAP (LAPING et al, 1994), a qual é envolvida na manutenção de longo prazo da arquitetura encefálica, na função de barreira hemato-encefálica e na modulação de funções neuronais pelos astrócitos (BUSHONG, 2004).

Os astrócitos são também elementos sinalizadores dinâmicos que integram estímulos neuronais, exibem excitabilidade, conhecidas como ondas de Ca^{2+}, e podem modular a ação de neurônios vizinhos (ARAQUE; CARMIGNOTO; HAYDON, 2001, PEREA, NAVARRETE, ARAQUE, 2009). Embora sejam evidentes os avanços no campo do entendimento da interação neurônio-glia, muitas questões se mantêm desconhecidas, o que desafia muitos estudos acerca do dimorfismo sexual, conectividade do hipocampo e as estruturas do SNC a ele associados.

Desta forma, o presente estudo aponta para a necessidade de explorar as interações neurônio-astrócitos nos mesmos períodos do desenvolvimento, e de influências hormonais, através de marcadores funcionais para os dois tipos celulares envolvidos. Para tal, poderiam

ser utilizadas ferramentas como a microscopia confocal, para se obter as relações tridimensionais, volumétricas, destas interações celulares.

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ANEXOS

ANEXO 1

Dendritic branching features of posterodorsal medial amygdala neurons of adult male and female rats: Further data based on the Golgi method. **Neuroscience Letters**, 2008.

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Dendritic branching features of posterodorsal medial amygdala neurons of adult male and female rats: Further data based on the Golgi method

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Abstract

The posterodorsal portion of the medial amygdalar nucleus (MePD) contains receptors for gonadal hormones and modulates the function of a social behavior network in rodents. The aims of this study were: to provide further data about the morphology of Golgi-impregnated dendrites of neurons from the MePD of adult rats; and, to compare the results obtained for dendritic branching and predominant dendritic spatial distribution in the MePD of males and diestrus females. MePD neurons were classified as bitufted or stellate, their spiny dendrites showed variable lengths, divided sparingly and decreased the number of branches with the distance from the soma. Dendritic arborization levels, number of branches in each level, distribution of the dendrites around the cell body and away from it, and the preferred spatial distribution of dendritic branches were studied according to different techniques and compared between sexes. Statistically significant differences were found in the predominant dendritic spatial distribution in the MePD, males with branches more oriented medially and dorsolaterally and females with more dorsally and ventromedially ones ($p \leq 0.05$ in all cases). This result adds another clue to understand how information is processed and integrated in the MePD and within functionally dynamic sex steroids-responsive circuits relevant for reproduction in both sexes.

Keywords: Dendrites; Amygdaloid complex; Sex difference; Golgi procedure

The medial amygdalar nucleus (MeA) is considered a part of the medial division of the "extended amygdala" [11] or a ventral part of the striatum [38] and is composed of four main subnuclei: anterodorsal (MeAD), anteroventral, posterodorsal (MePD) and posteroventral (MePV) ones [4,11,13,30,33]. The MePD receives olfactory and vomeronasal afferences [18,23,25] and projects to the anteroventral periventricular nucleus to alter the secretion of gonadotrophin releasing hormone [37], and to the ventral premamillary nucleus, medial preoptic nucleus and ventrolateral part of the ventromedial nucleus to affect reproductive behaviors [5,13,30]. Part of these connections, some of them reciprocal, are relayed by some components of the bed nucleus of the stria terminalis (BST, [13]). Many of these regions are sexually dimorphic in rats [12,16,18,25].

Several evidences indicate that the MePD integrates a gonadal steroid-responsive circuit that can join chemosensorial information with hormonal signals in both males and females to modulate the display of social behaviors, such as the maternal and sexual behaviors in rats [5,10,25,35]. For example, studies employing c-fos expression showed that vaginocervical stimulation in female rats [17,31] and the occurrence of intromissions and ejaculations during a sexual encounter in male rats is able to activate the MePD [8]. Androgen receptors [12,25], both estrogen receptors α and β (ER-α, ER-β) [12,17,28] and progesterone receptors [12] are highly concentrated in the MePD. There is an overlap in some MePD neurons that express c-fos following mating and also present receptors for sex steroids [17]. Based on these experimental data, it is not surprising that sex steroids can affect the MePD dendritic spine density [33], synaptic connectivity [26,27], content of neuropeptides [12], presence of delta opioid receptors [39] and binding of α-bungarotoxin in dendrites [2]. Electrophysiological differences between MePD neurons of male and females could be found even in prepubertal rats [7]. Because some of these findings involve the MePD neuropil,

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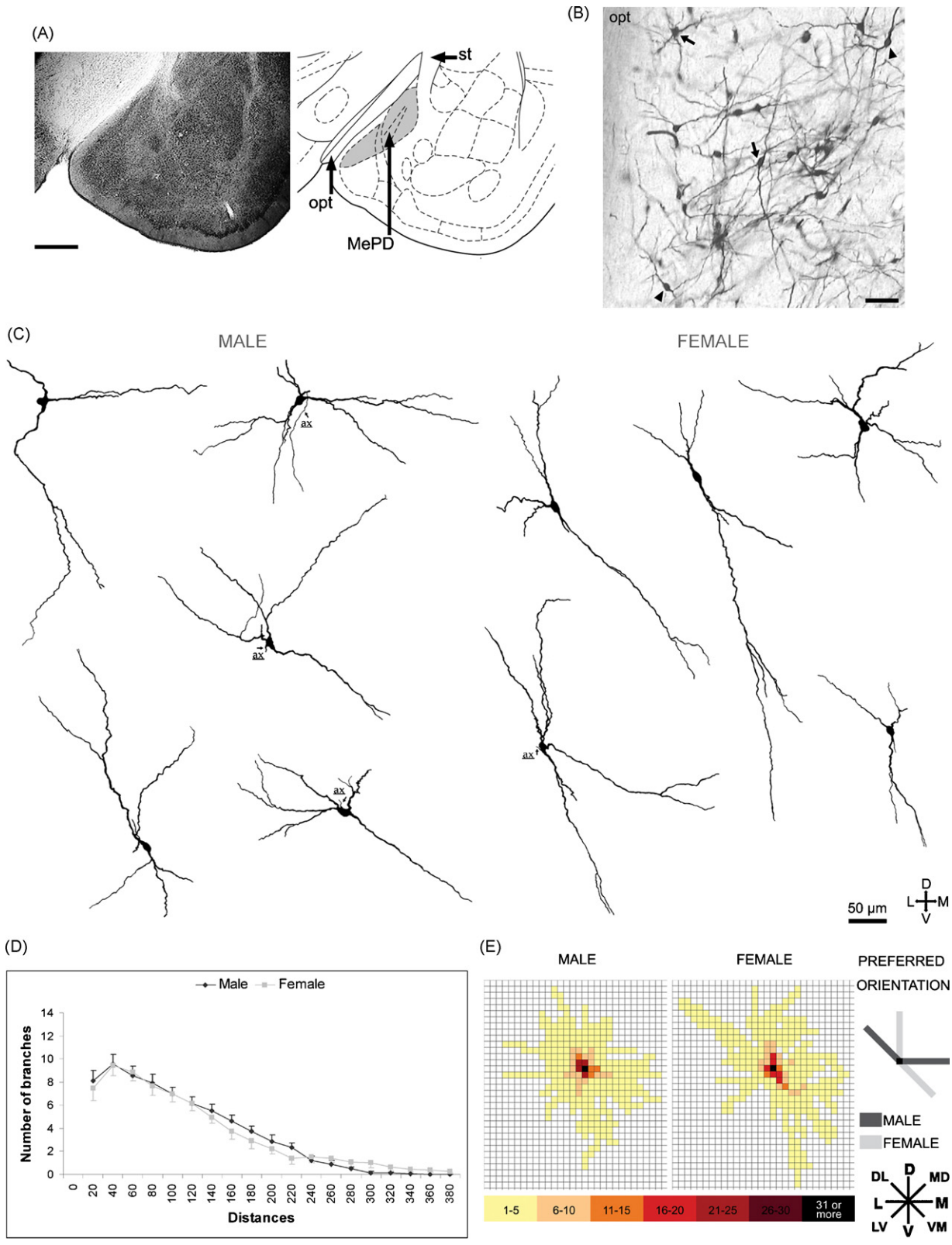

Fig. 1. (A) Left cresyl violet staining of a coronal brain section exemplifying the location of the rat posterodorsal medial amygdala (MePD) from where part of the present data was obtained (in this case, 3.30 mm posterior to the bregma). At right, schematic diagram of a matched coronal brain section showing the MePD, adapted from [29]. Scale bar = 800 μm. (B) Reconstructed digitized microscopic image showing the cellular organization of the MePD. Golgi-impregnated neurons showing bitufted (arrows) or stellate (arrows heads) appearances. Only background contrast was adjusted. (C) Camera lucida drawings of MePD Golgi-impregnated neurons of adult male (left) and female (right) rats. For a matter of correctness, spine distribution was not drawn for these spiny neurons. When visible, "ax" refers to an axon. OT, optic tract; ST, stria terminalis. Scale bar = 50 μm in (B) and (C). (D) Total number and distribution of dendritic branches along increasing distances from the soma, as revealed by the use of the Sholl's concentric circles technique. No statistical difference was found between sexes. (E) Spatial orientation of MePD

dendrites can be one of the targets for epigenetic gonadal hormones actions. Indeed, puberty was related with dendritic pruning [41] and adult castration decreased mean highest dendritic branching in the posterior MeA of male hamsters [15].

The complexity of dendritic morphology reflects the number of connections made by a neuron that, together with passive and active electrical properties, are relevant for space-temporal synaptic integration and its firing pattern [19,36]. Several evidences have shown how much dendritic branching, arborization extent and local topology contribute to the ongoing neuronal processing of information [14,19,32,36]. Usually, more elaborated inputs require the development of more complex dendrites and neuronal receptive field. Revealing if dendrites in a region have a preferential orientation can also indicate the potentiality of a neuron to make contacts with axons from a large number of cells or numerous connections with just a few of them in a parallel distribution [32]. These are important reasons to detail the general dendritic morphology of MePD neurons. In this regard, the Golgi method can provide representative samples of neuronal types within a brain area and continues to be a relevant research tool for revealing cellular morphology [9,10,22,33].

The aims of the present report were: (a) to further detail the dendritic morphology of neurons from the MePD of adult male and female rats, as revealed by the Golgi impregnation procedure; (b) to describe, quantify and compare relevant dendritic branching features, such as the number and levels of branching and dendritic spatial distribution in an attempt to identify possible sex differences in these parameters. These data have not been reported before for adult rats.

Wistar rats of both sexes (3–6 months of age) were housed in groups, with water and food *ad libitum* and room temperature around 22 °C in a 12 h light:dark cycle (lights off at 5 p.m.). Vaginal smears were taken from normally cycling virgin females, which were sacrificed in the afternoon of the diestrus phase to avoid variations in the results due to different levels of circulating ovarian steroids. All efforts were made to minimize the number of animals studied and rats were manipulated according to international laws for the ethical care and use of laboratory animals (European Communities Council Directive of 24 November 1986, 86/609/EEC).

The "single-section" Golgi method and the data acquisition procedure followed the same methodology published in details elsewhere [10,33]. Briefly, rats were deeply anesthetized with sodium thiopental (50 mg/kg, i.p.), brains were fixed with 4% paraformaldehyde and 1.5% picric acid in 0.1 M phosphate buffer (pH 7.4), sectioned coronally (200 μm thick) using a vibratome (Leica, Germany) and impregnated in 1.5% silver nitrate following 3% potassium dichromate (Merck, Germany).

The location of the MePD was based on previous descriptions [10,11] and compared with those from an atlas [29], taking the direct apposition to the lateral side of the optic tract (OT) and the dorsal position of the stria terminalis (ST) as anatomical references (Fig. 1A). The sections containing the MePD were approximately 3.0–3.30 mm posterior to the bregma [29]. Due to technical reasons, both sides of the brain were used (as employed in [10,15]).

To be selected for further analysis, neurons had to possess the following characteristics after histological procedure: (a) have neuronal cell bodies undoubtedly located within the boundaries of the MePD and relatively distant from all of its outer limits; (b) be located near the middle of the section; (c) be relatively isolated from neighboring impregnated cells to avoid "tangled" dendrites; (d) have well-impregnated dendrites with defined borders; (e) neurons must have the majority of their dendrites tapering towards their ends; and, (f) to be considered as a tapering branch, the apparent dendritic diameter at the end should be at least 50% narrower than its initial portion and should tend to be a final segment. In the neurons studied, there was no statistical difference between sexes in the number of "cut-off" branches, i.e., those dendrites whose impregnation stopped suddenly or that were sectioned due to the histological procedure. Values for them were around 30% in both sexes $\{F(1,35) = 0.002; p = 0.967$; also seen in [1]$\}$.

The first neurons that fulfilled the aforementioned including criteria were traced (400×) using a camera lucida coupled to an optic microscope (Olympus BX-41, Japan). All the selected dendritic branches were drawn in the three dimensions (including "z") and converted to a two-dimensional ("x" and "y") final image (Fig. 1C). Neurons had their dendrites initially evaluated for their number of branches and divisions. Afterwards, all drawings were scanned and measured, to complement the qualitative description (see below), using an image analysis system (Image Pro Plus 4.1., Media Cybernetics, USA).

The total number of neurons studied were 17 in males and 19 in females (from $n = 6$ and 7 rats, mean ± S.D. = 2.8 ± 1.8 and 2.7 ± 1.3 cells per rat, respectively). For each neuron, morphological analyses included the following parameters: (a) number of dendritic branches in each arborization level determined by the order of centrifugal appearance of primary dendrites from the soma; (b) number of branching points, i.e., total number of dendritic ramifications; (c) radial distribution of dendrites in relation to the distance from the center of the cell body, by the use of Sholl's concentric circles technique, and the intersection of the dendritic branching field with each virtual circle with 20 μm of radius; and, (d) the predominant spatial distribution of branches (in medial, mediodorsal, dorsal, dorsolateral, lateral, lateroventral, ventral, and ventromedial coordinates) using the overlaid square (20 μm each side) technique. That is, every selected neuron (only in this case, $n = 16$ neurons for males and females) had its cell body set at the center of the figure, the spatial location of all dendrites was assessed and the overall number

neurons dendritic branches of male and female rats. Neuronal cell body was set at the center of the figure. The different colors represent the quantity of branches in each spatial locations studied, ranging from 1 to 5 (yellow) to 31 or more branches (black). On the right, schematic representation (large lines) of the statistically significant differences found in the preferential orientation of dendritic branches in males (dark gray) and females (light gray). Spatial coordinates correspond to: D, dorsal; DL, dorsolateral; L, lateral; LV, lateroventral; M, medial; MD, mediodorsal; V, ventral; VM, ventromedial (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

of dendritic branches that radiated in each square was directly counted. As occurs with other techniques, morphological results from these Golgi-impregnated neurons probably represent conservative estimates of the changes produced by the action of sex steroids and likely under-represent the actual values for entire cells [15,33]. Notwithstanding, all experimental procedures and all measurements were rigorously the same for both sexes.

For the statistical analysis, the dendritic branches within each level of arborization were grouped as total numbers of primary, secondary, tertiary, and quaternary branches. Due to differences in the amount of studied neurons, total values were divided by the number of cells from which data were gathered in each sex. Then, it was calculated the relative values for the number of dendrites within each arborization level and the number of branching points for each studied neuron. It was assumed that pooling data from the rats of each studied area, theoretically, might provide a more powerful test for differences between groups (critical comments and recommendations in [40]). After a square root transformation, data were compared between sexes using the multivariate analyses of variance test.

The distribution of dendritic branches in relation to the distance from the neuronal soma was compared between males and females using an analysis of variance test for repeated measures. Mean numbers were used for each length interval and comparisons were done up to the greatest radial distance in which tests could be reliably carried out. Afterwards, based on the results of matrices equality, the Mauchly's test for sphericity and the Levene's test for the heterogeneity of variances, it was employed the Roy's largest root multivariate test.

For males and females, the number of dendritic branches in a preferred spatial distribution were compared using the χ^2 test employing a contingency table of 2×8 (i.e., 2 sexes and 8 subdivisions of spatial coordinates) followed by the residual analysis test. In all cases, the statistically significant level was set at 5%.

On the basis of the number of primary dendrites, multipolar neurons throughout the MePD of males and females were classified as: (a) bitufted neurons, which have two primary dendrites that give off successive branches, providing the dendritic arborization a "tufted" appearance, although branchpoints and branching appearance were typically not profuse; (b) stellate neurons, which have three or more primary dendrites that distort the soma into irregular shapes, some of them resembling (but apparently only in this parameter) a pyramidal-like form (according to [10,33]). It was unusual to find more than six primary dendrites in these cells (Fig. 1B and C). The appearance of bitufted and stellate neurons also permits their inclusion, with a slight adaptation, in the classification of "radiate cells" [32]. Usually, bitufted neurons presented cylindrical and biconical radiations and stellate neurons tended to have spherical and partially spherical radiations (Fig. 1C, [14]). Many of these neurons showed dendrites with coronal orientations, some with also a rostral to caudal distribution or with an oblique orientation throughout the section thickness.

Most dendrites in the MePD appeared to be moderately spiny, generally branched sparingly and had few branchpoints. In the "molecular layer", dendrites emanated from cell bodies that were observed in the adjacent lateral region. Dendritic shafts were basically rectilinear and extended in different directions. Perpendicular, oblique or parallel orientations towards the OT were frequently observed (Fig. 1B). As a rule, the lengths of dendrites were heterogeneous, extending over a wide range from the soma (Fig. 1B and C). From the sample studied, primary and secondary branches had very different path lengths, ranging from around 2 to 250 μm. Tertiary branches could be as long as 530 μm, as one of the highest values observed. Quaternary branches commonly represented the highest order of dendritic ramification and further orders were less frequently seen. It was usual to find branches running along higher distances and, some of them, reaching adjacent areas such as the MePV and the amygdaloid intercalated nuclei.

There were no statistically significant differences between sexes in the relative number of dendrites of primary [mean ± S.E.M. for males and females, respectively, 3.0 ± 0.2 vs. 2.7 ± 0.2; $F(1,35) = 0.939$, $p = 0.339$], secondary [3.5 ± 0.3 vs. 3.5 ± 0.4; $F(1,35) = 0.017$, $p = 0.896$], tertiary [1.5 ± 0.4 vs. 2.7 ± 0.6; $F(1,35) = 2.845$, $p = 0.101$] or quaternary levels per neuron [0.6 ± 0.3 vs. 0.8 ± 0.3; $F(1,35) = 0.466$, $p = 0.499$]. There was no sex difference in the number of branching points [mean ± S.E.M., 2.9 ± 0.5 and 3.6 ± 0.6 in males and females, respectively; $F(1,35) = 0.587$; $p = 0.499$]. The comparison of the distribution of dendritic branches between males and females showed that there was a significant difference in the number of branches related with the increase in the distance away from the soma (their number decreasing with the distance, $p < 0.001$), but there was no interaction between sex and distance ($p = 0.963$), i.e., the differences observed among the different concentric circles were the same for both sexes (Fig. 1D). Finally, a statistical difference was found in the distribution of dendritic branches in the MePD. In males, more dendritic branches were oriented dorsolaterally ($p < 0.02$) and medially ($p = 0.05$), whereas in females they were seen with predominant dorsal ($p < 0.01$) and ventromedial ($p < 0.01$) orientations (Fig. 1E).

Neurons in the MePD resembled those found in the medial subdivision of the central nucleus of the amygdala and in connected parts of the BST, but are simpler than those found in other amygdaloid areas not belonging to the "extended amygdala", such as the basolateral nucleus, for example [22]. Being treated as one sole group, bitufted and stellate neurons present a simpler morphological appearance that does not permit to undoubtedly classify MePD neurons as similar to those striatal medium-spiny ones found in rats (see a parallel discussion in [21,24]). Our qualitative descriptions are in accordance and expand previous observations that studied different MeA subnuclei altogether in this and other species [15,22]. The quantitative data on the dendritic trees and their spatial arrangement contribute with new morphological information and an additional sex difference in the MePD of adult male and female rats.

Until the moment, no general morphological characteristic allows the reliable differentiation of sex steroid responding neurons from non-responding ones in the MePD, neither is currently known their relative percentages nor the total number of neurons in the adult rat MePD. More stellate than bitufted neurons composed our final data, but their proportions in the sample

studied were identical in both sexes (around 78% of stellate ones; two-tailed Fisher's exact test, $p > 0.5$). These selected neurons might not be numerically representative of the total number of MePD neurons; nevertheless, it is our empirical impression that neuronal general morphology is relatively homogeneous in the MePD and these cells illustrate those reliable found within it in both sexes [10,33]. Other forms of sorting subpopulations of neurons were not tested here but are highly encouraged, including those linking MePD local neurons with their specific connections and neurochemical features [5,34].

Recently, Cooke et al. [6] showed that biocitin-injected neurons of prepubertal Sprague Dawley male rats have dendrites in the left MePD with a higher length when compared to age-matched females. Radial distribution of dendrites was different in the left than in the right MePD in these young males. Future research probably will allow the comparison of sexual dimorphisms in morphological data from prepubertal and postpubertal rats of the same and different strains or in other animals, and how would these data can be the result of a proper ontogenetic process and/or due to epigenetic neural gonadal steroids actions in different ages. In the present study, dendrites showed more branches radiating near the soma and, when tapering, differences in their preferred spatial localizations within the MePD of both sexes. In this sense, new experiments are foreseen to address the dendritic ionic channel activities, synaptic transmitters and receptors, local neuro-glial dynamic interactions and mechanisms of sex steroids actions that are occurring in dendrites of the rat MePD.

Because dendrites are involved with the direction of information flow and their extent and complexity of branching affect the neuronal activity [14,19,36], some ideas merit further development. First, it is interesting to note that some MePD dendrites are oriented towards the "molecular layer", where efferent fibers from the bed nucleus of the accessory olfactory tract pass through ([11], additional references in [10]). Ultrastructural studies revealed that, following lesion of the posterior cortical amygdala, synapses on dendritic shafts of the medial "molecular layer" significantly decreased in number in males, but not in female rats [26]. Sexual dimorphism was reported for the number of dendritic shaft and spine synapses in the medial and ventral parts of the "molecular layer", male data higher than female ones [27]. These findings indicate the existence of a sexually dimorphic neuronal circuitry in the "molecular layer" that medially surrounds the MePD. More dendritic branches were found here oriented medially in males than in females. Differences in the dendritic orientation might suggest that males and females would be gathering synaptic information with a different spatial distribution.

Second, other studies using c-fos expression might also suggest the functional need for a preferential dendritic spatial distribution. Pheromonal stimuli are sent to the MePD by the intercalated amygdaloid nuclei [23], which because of their location [11,29] probably come from a lateral aspect. Furthermore, the male MePD medial column of cells is involved with the occurrence of intromission behavior, whereas the MePD lateral column is mainly involved with the neural circuit for ejaculation [8]. As these are interrelated behaviors, it is plausible to suppose that the flow of information should be processed by neurons oriented and interconnected in a rather horizontal position in the male MePD. Interestingly, males showed dendrites with medial and dorsolateral preferential orientations. By its turn, in the MePD of females, a dorsal to ventral distribution of ER-α and ER-β is involved with the perception of vaginocervical stimulation during mating behavior [17], as part of a neural circuitry controlling hypothalamic neuroendocrine secretion and reproduction [31,37]. This dorsoventral gradient in ER distribution is related to the number of cell bodies [17], but both ER-α and ER-β were found in dendrites [3] and estradiol affects MeA dendritic morphology *in vitro* as well [20]. Here, females showed a rather vertical (dorsal and ventromedial) distribution of MePD dendrites. Hormonal signaling might well be interacting with the processing of afferent information and contributing to shape MePD dendrites in both sexes, tailoring neurons to perform specific roles and process synaptic inputs in distinctive ways. Additional work is needed to confirm these working hypotheses that constitute an interesting field to join neuroanatomical connectional data with their functional implication.

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ANEXO 2

Dendritic branching features of Golgi-impregnated neurons from the "ventral" medial amygdala subnuclei of adult male and female rats. **Neuroscience Letters**, 2008.

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Dendritic branching features of Golgi-impregnated neurons from the "ventral" medial amygdala subnuclei of adult male and female rats

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ABSTRACT

The anterodorsal (MeAD) and posteroventral (MePV) subnuclei would form the proposed "ventral" division of the rat medial nucleus of the amygdala (MeA). These parts receive chemosensorial inputs, have gonadal hormone receptors and modulate hypothalamic neuroendocrine secretion and defensive/reproductive behaviors. The aims of this study were: (1) to provide further data on the morphology of Golgi-impregnated dendrites from the MeAD and the MePV of adult rats; and (2) to compare the results obtained for dendritic branching and predominant dendritic spatial distribution in both these subnuclei in males and diestrus females. Dendritic arborization levels, number of branches in each level, distribution of dendrites around the cell body and distally from it, and the preferred spatial distribution of dendritic branches were studied using different techniques and compared between sexes. MeAD and MePV multipolar neurons had spiny dendrites with sparse ramifications. The main statistically significant differences were found in the predominant dendritic spatial distribution in the MeAD (rather medially and laterally in males and ventromedially in females, $p < 0.02$) and in the MePV (rather medially and mediodorsally in males and ventrally in females, $p < 0.01$). Results suggest that synaptic information might be processed and integrated differently in the dendrites of males and females in these sex steroid-responsive MeA subnuclei. The inclusion of the MeAD and the MePV in one single "ventral" MeA division is further discussed.

The rat medial nucleus of the amygdala (MeA) is a complex part of the medial division of the "extended amygdala" [6] or part of the striatum [27], caudally and medially surrounded by a cell-sparse "molecular layer" through which pass axons from the bed nucleus of the olfactory accessory tract [22]. The MeA is composed of four main subnuclei: anterodorsal (MeAD), anteroventral (MeAV), posterodorsal (MePD) and posteroventral (MePV) [1,6,17]. Based on their efferent connections, local distribution of chemical transmitters and gonadal hormone receptors, it was proposed that the MeA would be composed by two main divisions: one "ventral", including the MeAD, the MePV and, supposedly, the MeAV; and, another dorsal, formed by the MePD [1]. At least, this latter subnucleus appears to be different in many cytoarchitectonical, hodological, neurochemical and functional criteria when compared to the other MeA subnuclei [5,14,17,20].

Local neurons in the "ventral" MeA of male and female rats can be affected by circulating sex steroids [2,20,21,25,26]. Androgen receptors are equally and markedly found in the MeAD and the MePV [26]. Estrogen receptors (ERs)-α appear to be slightly more concentrated in the MePV than in the MeAD, whereas the opposite is true for ER-β (see Fig. 1 in [25]). Both subnuclei present sexual dimorphisms or sex steroid-induced changes in neuronal morphological parameters in rats [20,21] and provide an extensive hypothalamic innervation to modulate reproductive and agonistic behaviors [1,17]. For example, the density of dendritic spines is higher in males than in females across the different phases of the estrous cycle in the MeAD and in the MePV [20], which suggests that part of the "ventral" MeA neuropil may be subject to the epigenetic action executed by gonadal hormones in adulthood.

At the cellular level, there are important reasons to detail the general dendritic morphology found in the MeAD and the MePV. Dendrites represent the main site for synapses, serve for local spatial–temporal integrations and information processing, and the complexity of dendritic arbors can address the connections made by a neuron [8,24]. Detailing dendritic and axonal networks can help to reveal whether neurons establish contacts with a large number of cells or numerous connections with just a few of them distributed in parallel. The Golgi method can be useful to reveal the neuronal morphology found in several amygdaloid nuclei of male and female rodents [5,10,14,20].

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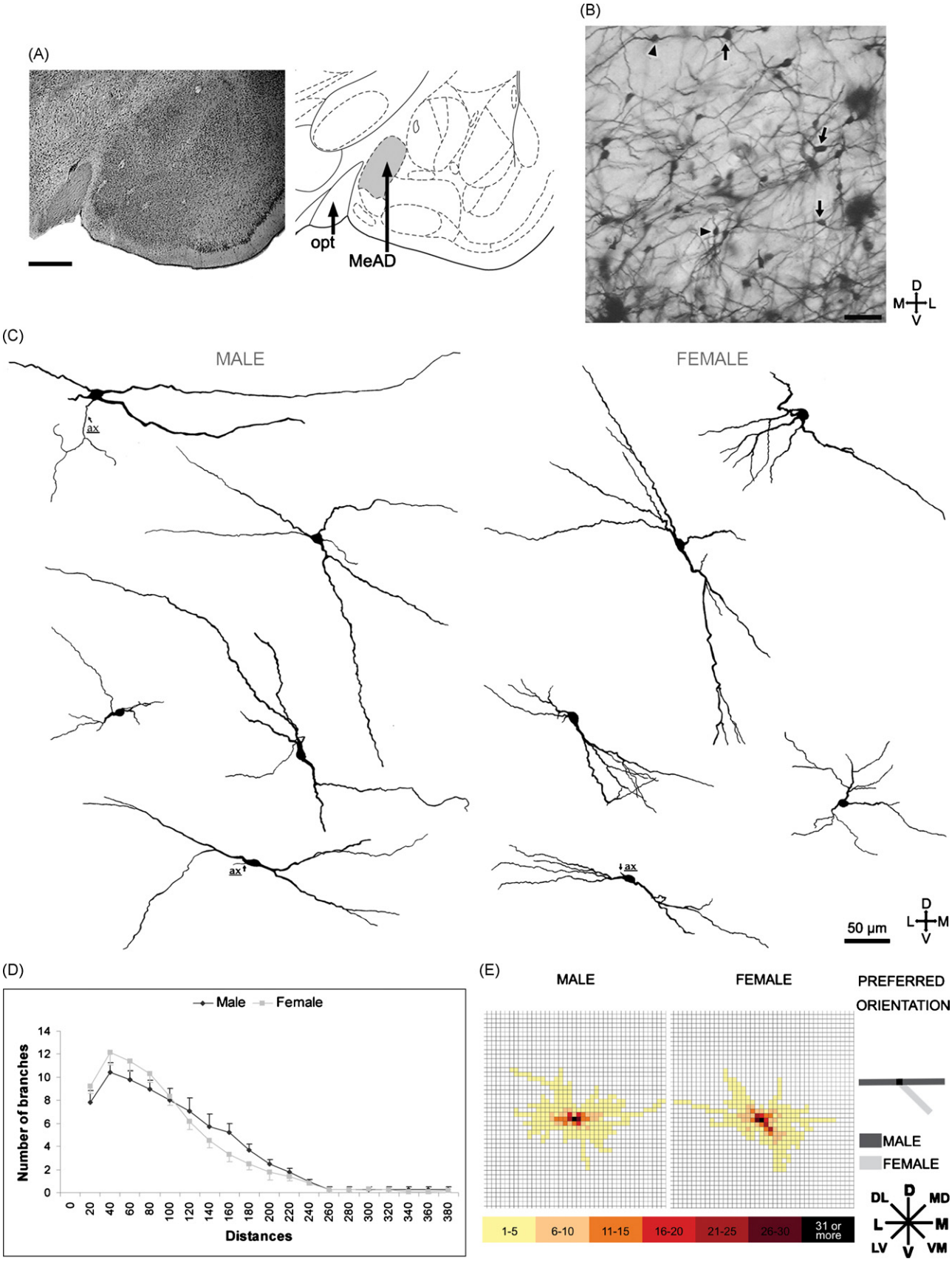
(A)
opt
MeAD
(B)
D
M
L
V
(C)
MALE
FEMALE
ax
ax
ax
50 µm
D
L
M
V
(D)
Male
Female
Number of branches
Distances
0
2
4
6
8
10
12
14
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
(E)
MALE
FEMALE
PREFERRED ORIENTATION
MALE
FEMALE
1-5
6-10
11-15
16-20
21-25
26-30
31 or more
DL
D
MD
L
M
LV
V
VM

The aims of the present study were: (1) to detail the dendritic morphology of Golgi-impregnated neurons from the MeAD and the MePV of adult male and female rats; and (2) to describe, quantify and compare dendritic branching features, such as the number and levels of branching and dendritic spatial distribution for both subnuclei, in an attempt to identify possible sex differences in these parameters. This experimental approach is identical to one recently presented in a companion paper that focused on the MePD of adult rats [5]. The inclusion of the MeAD and the MePV in one single "ventral" MeA division is further discussed at the end.

Wistar rats of both sexes (3–6 months of age) were housed in groups, with water and food *ad libitum* and room temperature around 22 °C in a 12 h light:dark cycle (lights off at 5 p.m.). Vaginal smears were taken from normally cycling virgin females, which were sacrificed in the afternoon of the diestrus phase. All efforts were made to minimize the number of animals studied and their suffering. Rats were manipulated according to international laws for the ethical care and use of laboratory animals (European Communities Council Directive of 24 November 1986, 86/609/EEC).

The "single-section" Golgi method was employed [5,20]. Briefly, rats were deeply anesthetized with thiopental (50 mg/kg, i.p.), brains were fixed with 4% paraformaldehyde and 1.5% picric acid in 0.1 M phosphate buffer solution (pH 7.4), sectioned coronally (200 μm thick) using a vibratome (Leica, Germany), and put in 3% potassium dichromate and 1.5% silver nitrate solutions (Merck, Germany). The locations of the MeAD and the MePV were based on [6] and compared with schematic representations from an atlas [16]. The rostral apposition to the lateral side of optic tract (OT) served to localize the MeAD (Fig. 1A), whereas the OT aspect, the dorsal position of the stria terminalis (ST) and the ventral border of the brain served to identify the MePV (Fig. 2A). The sections containing the MeAD and MePV were approximately 1.80–2.40 and 3.0–3.30 mm posterior to the bregma, respectively [16]. Both sides of the brain were used.

Selected neurons (1) had neuronal cell bodies undoubtedly located within the boundaries of each MeA subnuclei and relatively distant from all of their outer limits, (2) were relatively isolated, and (3) had well-impregnated dendrites with defined borders and the majority of these dendrites tapered towards their ends. To be considered as a tapering branch, the apparent dendritic diameter at the end should be at least 50% narrower than its initial portion and tending to be a final segment [5]. In the neurons studied, the quantity of "cut-off" branches per cell (i.e., those dendritic branches whose impregnation stopped suddenly or that were sectioned due to the histological procedure) was around 30% (as in [5]). There were no statistical differences between sexes in the number of "cut-off" branches in the sampled cells in the MeAD [$F(1,34) = 1.29$; $p = 0.26$] or in the MePV [$F(1,30) = 0.02$; $p = 0.87$].

The first neurons that fulfilled the aforementioned inclusion criteria were drawn (400×) using a camera lucida coupled to an optic microscope (Olympus BX-41, Japan). All the selected dendritic branches were observed in their apparent 3-dimensions and converted to a 2-dimensional ("*x*" and "*y*") final image (Figs. 1C and 2C). The total number of neurons studied in the MeAD were 14 for males and 21 for females (from $n = 8$ and 6 rats, mean ± S.D. = 1.7 ± 1.1 and 3.5 ± 1.8 cells per rat, respectively); and, in the MePV were 16 from males and 15 from females (from $n = 7$ rats in both groups, mean ± S.D. = 2.2 ± 1.1 and 2.1 ± 1.4 cells per rat, respectively). Additional methodological comments on this topic and other suggested efforts to sort local subpopulations of neurons can be found in [5].

For each neuron, morphological analysis included the following parameters: (1) number of dendritic branches in each arborization level determined by the order of centrifugal appearance of primary dendrites from the soma; (2) number of branching points, which represents the total number of dendritic ramifications; (3) radial distribution of dendrites in relation to the distance from the center of the cell body, by the use of Sholl's concentric circles technique, and the intersection of the dendritic branching field with each virtual circle (multiples of 20 μm of radius); and, (4) predominant spatial distribution of branches (in medial, mediodorsal, dorsal, dorsolateral, lateral, lateroventral, ventral, and ventromedial coordinates) using the overlaid square (20 μm each side) technique. That is, every selected neuron had its cell body set at the center of the figure, the spatial location of all dendrites was assessed and the overall number of dendritic branches that radiated in each square was directly counted [5]. It is likely that morphological results from these Golgi-impregnated neurons represent conservative estimates of the changes produced by the action of sex steroids and under-represent the actual values for entire cells (see a suggested methodological improvement in Caserta et al. [3]). Nevertheless, all experimental procedures were rigorously the same for both sexes and for each subregion.

For the statistical analysis, the dendritic branches within each level of arborization were grouped as total numbers of primary, secondary, tertiary, and quaternary branches. Due to differences in the amount of studied neurons, total values were divided by the number of cells from which data were obtained. Then, the relative values were calculated for the number of dendrites within each arborization level and the number of branching points for each studied neuron. It was assumed that pooling data from the rats of each studied area, theoretically, might provide a more powerful test for differences between groups (critical comments and recommendations in [28]). After a square root transformation, data were compared between sexes using the multivariate analyses of variance test.

For each subnucleus studied, the distribution of dendritic branches in relation to the distance from the neuronal soma was compared between males and females using an ANOVA test for repeated measures. Mean numbers were used for each length interval and comparisons were made up to the greatest radial distance in which tests could be reliably carried out. Afterwards, based on the results of matrices equality, the Mauchly's test for sphericity and the Levenes test for the heterogeneity of variances, Roy's largest root multivariate test was employed.

Fig. 1. (A) Left: cresyl violet staining of a coronal brain section exemplifying the location of the rat anterodorsal medial amygdala (MeAD) from where part of the present data was obtained (in this case, 2.30 mm posterior to the bregma). Right: schematic diagram of a matched coronal brain section showing the MeAD, adapted from [16]. Scale bar = 800 μm. (B) Reconstructed digitized microscopic image showing the cellular organization of the MeAD. Golgi-impregnated neurons with bitufted (arrows heads) or stellate (arrows) appearances. Only background contrast was adjusted. Spatial coordinates on the left are the same for (A and B). (C) Camera lucida drawings of MeAD Golgi-impregnated neurons of adult male (left) and female (right) rats. For a matter of correctness, spine distribution was not drawn for these spiny neurons. "ax" refers to an axon, when visible. OT, optic tract; ST, stria terminalis. Scale bar = 50 μm in (B and C). (D) Total number and distribution of dendritic branches over increasing distances from the soma, as revealed by the use of the Sholl's concentric circles technique. No statistical difference was found between sexes. (E) Spatial orientation of MeAD neurons dendritic branches of male and female rats. Neuronal cell body was set at the center of the figure. The different colors represent the quantity of branches in each spatial location studied, ranging from 1 to 5 (yellow) to 31 or more branches (black). On the right, schematic representation (large lines) of the statistically significant differences found for the preferential orientation of dendritic branches in males (dark gray) and females (light gray). For technical reasons, spatial coordinates were adapted in (C and E) and correspond to: D, dorsal; DL, dorsolateral; L, lateral; LV, lateroventral; M, medial; MD, mediodorsal; V, ventral; VM, ventromedial. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

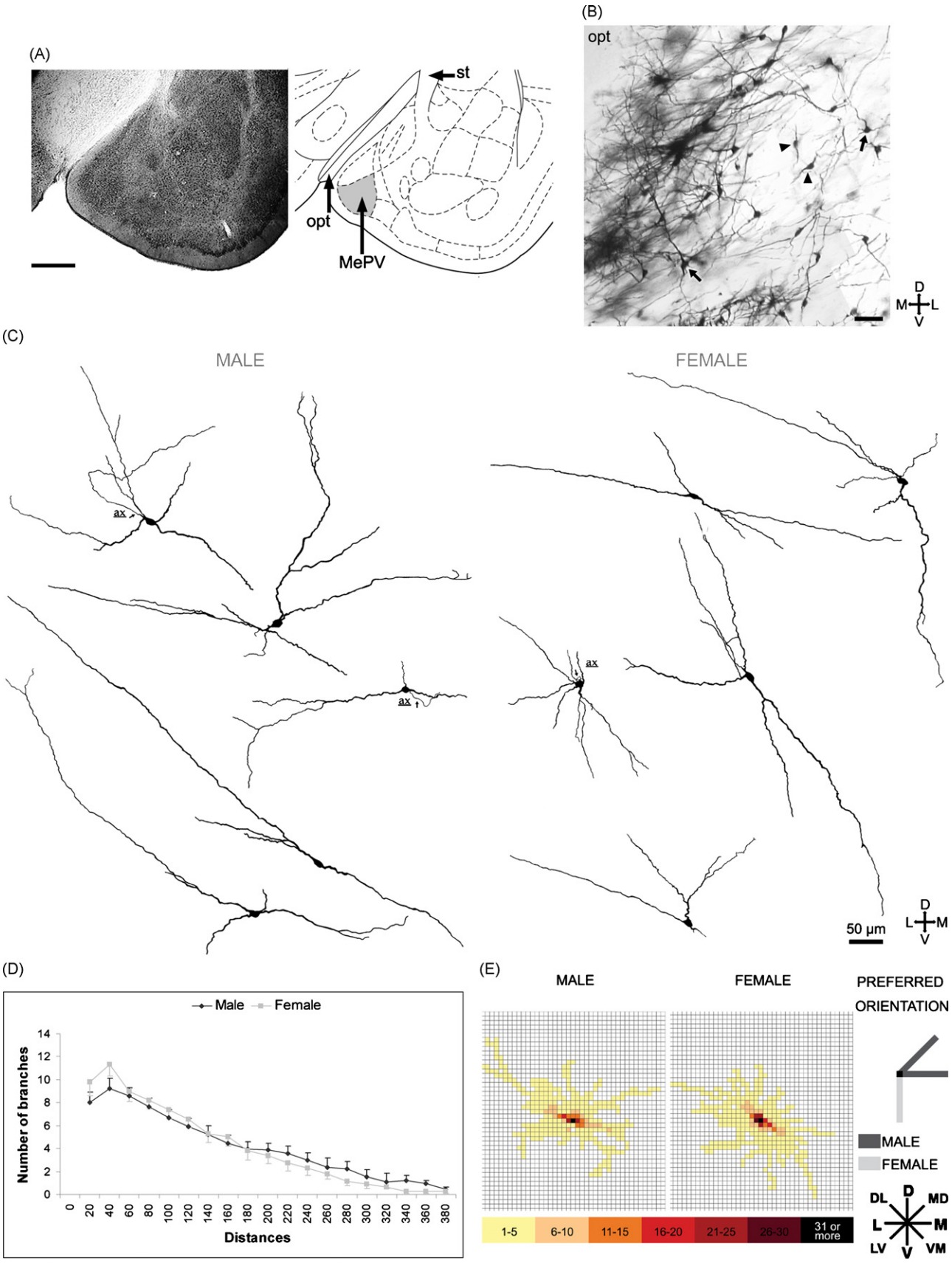

Fig. 2. (A) Left: cresyl violet staining of a coronal brain section exemplifying the location of the rat posteroventral medial amygdala (MePV) from where part of the present data was obtained (in this case, 3.30 mm posterior to the bregma). Right: schematic diagram of a matched coronal brain section showing the MePV, adapted from [16]. Scale bar = 800 μm. (B–E) Legends correspond to the same description provided in Fig. 1, but adapted for the MePV data.

Finally, males and females had the number of dendritic branches in a preferred spatial distribution compared using the χ^2 test and employing a contingency table of 2×8 (i.e., 2 sexes and 8 subdivisions of spatial coordinates) followed by the residual analysis test. In all cases, the statistically significant level was set at 5%.

Multipolar neurons in both the MeAD and the MePV of males and females had a general morphological appearance that allows their classification as bitufted (with two primary dendrites) or stellate cells (with three or more primary dendrites). Here, more stellate than bitufted neurons composed the final sample data, but their proportions were identical in both sexes and in each subregion studied (two-tailed Fisher's exact test, $p > 0.5$; data not shown).

For the MeAD of both sexes, spiny neurons had basically rectilinear dendrites with very different lengths and usually no more than fifth- or sixth-order branches (Fig. 1B and C). Some dendrites showed a parallel orientation towards the ventral part of this subnucleus or perpendicular to the dorsal position of the OT. Others crossed the limits of the MeAD and reached adjacent amygdaloid areas.

There was a sex difference in the relative number of MeAD second order dendrites [higher in females; $F(1,34) = 8.34$; $p < 0.01$], but there were no statistically significant sex differences in the values obtained for dendrites of primary [$F(1,34) = 0.02$; $p = 0.86$], tertiary [$F(1,34) = 0.14$; $p = 0.71$] or quaternary orders [$F(1,34) = 0.08$; $p = 0.77$] and neither in the number of branching points [4.0 ± 0.5 and 5.0 ± 0.5 in males and females, respectively; $F(1,34) = 1.70$; $p = 0.20$]. There was a significant difference in the number of branches regarding their distribution near the cell body (number of dendrites decreased centrifugally, $p < 0.01$), but there was no interaction between sex and distance in this parameter ($p = 0.69$; Fig. 1D). On the other hand, in the distribution of MeAD dendritic branches, males displayed significantly more medially ($p = 0.02$) and laterally ($p = 0.01$) oriented dendritic branches than females, for which dendrites were predominantly orientated ventromedially ($p < 0.01$; Fig. 1E).

In the MePV of males and females, neurons also had dendritic branches with heterogeneous lengths (Fig. 2B and C). Although these spiny dendrites were oriented in multiple directions, those located more superficially displayed a radial orientation that accompanied the ventral and the medial borders of the MePV (Fig. 2B), close to the OT and where the surrounding "molecular layer" is located. Within the inner MePV, dendrites appeared to have an oblique orientation in relation to the OT and, near the border with the MePD, they showed a more perpendicular orientation towards the OT. Sometimes dendrites projected away from the lateral and dorsal boundaries of the MePV.

There were no statistically significant differences between males and females in the relative number of primary [$F(1,30) = 0.98$; $p = 0.33$], secondary [$F(1,30) = 0.23$; $p = 0.63$], tertiary [$F(1,30) = 0.21$; $p = 0.64$] or quaternary dendritic branches [$F(1,30) = 1.11$; $p = 0.30$] and neither in the number of branching points [3.6 ± 0.7 and 3.2 ± 0.3 in males and females, respectively; $F(1,30) = 0.08$; $p = 0.76$]. There was a significant difference in the number of branches affected by the distance relative to the neuronal soma (the greater the distance, the fewer the number of branches; $p < 0.01$), although no interaction was found between sex and distance in this parameter ($p = 0.22$; Fig. 2D). Highly statistically significant differences between sexes were found in the distribution of dendritic branches in the MePV. In males, dendrites were predominantly orientated medially ($p < 0.01$) and mediodorsally ($p < 0.01$), whereas in females they were mainly positioned ventrally ($p < 0.01$; Fig. 2E).

The present general description of MeAD and MePV dendrites confirms and contributes to previous data [10,20] and resembles that of the rat MePD [5]. In the present study, differences were found in the relative number of second-order dendrites in the MeAD (more in females) and, in both subnuclei, a sex difference in the preferred dendritic spatial localization. It is interesting to note that in males and females the MePV dendrites were oriented differently towards a "molecular layer", whose ventral part showed a decrease in the number of intact synapses upon dendritic spines following lesions of the posterior cortical amygdala in male rats, but not in females [15]. Female MePV neurons had more ventrally oriented branches, which would provide dendritic shafts places for local connections. These data suggest the need for further ultrastructural and electrophysiological studies to reveal a possible gender difference in the neuronal circuitry of the "molecular layer" that medially and ventrally surrounds the MePV. The distinct preferred dendritic orientation that occurs in the MeAD also suggests a possible spatial organization for the connections in males and females. In this sense, it is speculated whether more secondary branches in female MeAD neurons might represent additional sites near the cell body to establish connections that can promptly affect firing output. Unfortunately, there is insufficient complementary data to address functional reasons for these MeAD findings. However, these results support those of a previous study [5] and suggest that dendritic sex-based differences are a common feature in the different MeA subnuclei.

Whilst the present data are not the most important to decide on the complete identity of the MeAD and the MePV, other experimental findings can be joined to suggest that these subnuclei have some particular features. For example, although dendritic spines are affected by sex steroids in both of them, the density of spines is lower in the MeAD when compared to the MePV of males or diestrus and proestrus females [20]. In addition, independently of the phase of the estrous cycle, glial fibrillary acidic protein-immunoreactivity (GFAP-ir) was higher in the MePV than in the MeAD [9]. In adult ovariectomized females, injections of estradiol alone or estradiol plus progesterone increased GFAP-ir in the MePV, but not in the MeAD [9]. These evidences indicate a distinct and subregion-specific neuron-glial responsiveness to endogenous and supra-physiological sex steroid actions within the "ventral" MeA, with the MeAD showing less marked effects. Then, another point that merits further elaboration is the proposition of the existence of a rat "ventral" MeA division.

Although there are clear and mutual connections between the MeAD and the MePV and an overlap in their innervated brain areas, afferent and efferent pathways can differ in many instances [1,17]. The MeAD, the largest component of the MeA, receives inputs from the accessory olfactory bulb and evaluates the social relevance of vomeronasal stimuli, altering the functioning of an inhibitory circuit that links the intercalated amygdaloid nuclei with the MePD [13]. The MeAD also receives fibers indirectly from the main olfactory bulb via the anterior and posterolateral cortical nucleus [1], as well from the infralimbic cortex, which can integrate olfactory information with polymodal non-olfactory inputs [11]. From the MeAD, intra-amygdalar axons are projected to several nuclei, such as the medial part and the caudal capsular part of the central nucleus, for example [1]. MeAD axons run by the ansa peduncularis (ventral amygdalofugal pathway) or via the ST [1]. Among targets that are distinctly innervated by the MeAD are parts of the striatal fundus; olfactory tubercle bulb; accessory olfactory bulb; intermediate and ventral parts of the lateral septal nucleus; undifferentiated parts of the medial preoptic area and the lateral part of the medial preoptic nucleus, the lateral hypothalamic area adjacent the cerebral peduncle, lateral regions of the arcuate nucleus, posterior hypothalamic periventricular nucleus; paraventricular nucleus, mediodorsal nucleus and rostroventral part of the nucleus reuniens in the dorsal thalamus; and, a sparse input to the ventral tegmental area and parts of the raphe nuclei [1,17]. These hodological data and other immunocytochemical and behavioral studies

indicate that the MeAD can serve as a relevant part for the general arousal or nonspecific activation of social behaviors in rodents [14]. In fact, c-fos activity in the MeAD showed a generalized and similar increase after mating, following intermale aggressive encounters in male and female hamsters or even due to tail-pinching in rats (as reviewed in [14]).

By its turn, the MePV is one of the brain areas that integrates gonadal hormone influence and pelvic viscero-sensorial information [18,23]. Directly or indirectly, MePV axons reach the periventricular zone in the anterior hypothalamic level forming a "neuroendocrine motor zone"; the subparaventricular zone of the periventricular region, which contains a "visceromotor pattern generator network" also probably related with circadian rhythms; the ventromedial nucleus (VMH) probably to regulate reproductive behavior and VMH the defensive behavior control system in the hypothalamus [1,4,7,17,21]. In this sense, c-fos activity in the MePV of female hamsters is influenced by tactile and nontactile social components of mating stimulation, the display of sexual behavior and the induction of pseudo-pregnancy [23]. Mating itself, the presence of a male during non-intromissive sexual testing or vaginocervical stimuli performed at the same time that caged males were placed near to the testing place were all able to increase c-fos in the MePV of female hamsters [23]. Moreover, the MePV is also activated by predator odors [12] and, recently, Choi et al. [4] demonstrated the existence of different subpopulations of MePV neurons related to defensive and reproductive stimuli.

Then, it is plausible to conceive that the MeAD and the MePV have to be functionally interconnected, gathering information and modulating common innervated areas, to allow the animal to perceive its internal milieu and environmental context at each moment (see a parallel comment on [19]). But, these subnuclei have some structural, connectional and functional characteristics that suggest that they do not compose a homogeneous "ventral" MeA unit; rather, they appear to be part of other larger circuits involving serial and parallel processing to execute concomitant and/or complementary behavioral activities. The aforementioned comments would add to the development of a conceptual anatomical and functional organization of these brain areas.

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