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Comportamento Sexual e Densidade de Espinhos Dendríticos na Amígdala Medial Póstero-Dorsal de Camundongos *Knockout* para o Gene da Ocitocina

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RESUMO

Os neuropeptídeos ocitocina (OT) e arginina-vasopressina (AVP) desempenham um importante papel no comportamento sexual e nos mecanismos neurohormonais em roedores. Os resultados da administração exógena de OT no comportamento sexual de machos e fêmeas são controversos. Este trabalho teve como objetivo analisar o papel da OT na modulação do comportamento sexual e na densidade de espinhos dendríticos proximais dos neurônios da amígdala medial póstero-dorsal (AMePD) de camundongos fêmeas com deleções seletivas no gene da OT (OTKO). Camundongos fêmeas (C57BL/6) foram genotipados e divididos em grupos controle (WT) e OTKO. Os experimentos foram realizados no ínicio da noite do proestro. Nossos resultados mostraram que o grupo OTKO apresentou um aumento na latência e uma diminuição na frequência, na duração e no quociente do comportamento de lordose quando comparado ao grupo WT. Além disso, o grupo OTKO apresentou uma diminuição no número de oócitos e um aumento na densidade de espinhos dendríticos proximais na AMePD quando comparado ao grupo WT. Nenhuma diferença significativa foi observada entre os grupos na concentração plasmática de AVP. Em conclusão, nossos dados sugerem que a OT modula o comportamento sexual, o número de oócitos e a densidade de espinhos dendríticos na AMePD de camundongos fêmeas.

Palavras-chave: Camundongos OTKO. Comportamento Sexual. Método de Golgi. Amígdala. Vasopressina. Ovulação.

ABSTRACT

Neuropeptides oxytocin (OT) and arginine-vasopressin (AVP) have been shown to play an important role in sexual behavior and neurohomonal mechanisms in rodentes. Results from exogenous OT administration on sexual behaviors in male and female mice are controversies. This study aimed to analyze the role of OT in the modulation of sexual behavior and density of proximal dendritic spines in the posterodorsal medial amygdala (MePD) in female mice with selective deletions of the OT gene (OTKO). Female mice C57BL/6 were genotyped and divided into control (WT) and OTKO groups. The experiments were performed in the beginning of the night of the proestrus phase. Our results showed that OTKO group increased in latency and decreased in the frequency, duration and quotient of lordosis behavior when compared to WT group. Moreover, the OTKO group decreased the number of oocytes and increased density of proximal dendritic spines in the MePD when compared to the WT group. No significant difference was observed in the plasma levels of AVP between groups. In conclusion, our data suggest that OT modulates the sexual behavior, the number of oocytes and the density of dendritic spines in the MePD of female mice.

Keywords: OTKO mice. Sexual Behavior. Golgi Method. Amygdala. Vasopressin. Ovulation.

SUMÁRIO

1. INTRODUÇÃO

A ocitocina (OT) é um nonapeptídeo membro da família das proteínas neurohipofisárias que faz parte do sistema hipotálamo-neurohipofiseal (HNH) e que está tradicionalmente relacionado ao parto e à lactação (Gimpl & Farenholz, 2001). Estruturalmente a OT assemelha-se a outro nanopeptídeo, a arginina-vasopressina (AVP), diferindo-se deste por apenas dois aminoácidos (Acher et al., 1995; Caldwell & Young, 2006).

Os genes que codificam a expressão da OT e da AVP são altamente homólogos e estão localizados no mesmo cromossomo, mas com orientação transcricional oposta (Young & Gainer, 2003). A distância entre estes genes varia de 3 a 12kb em camundongos (Hara et al., 1990), humanos (Sausville et al., 1985) e ratos (Mohr et al., 1988). Nos camundongos os genes da OT e da AVP estão localizados no cromossomo 2 e no homem no cromossomo 20 (Hara et al., 1990).

Os corpos celulares dos neurônios localizados nos núcleos hipotalâmicos paraventricular (PVN) e supraóptico (SON) produzem e liberam, respectivamente, os neuropeptídeos OT e AVP, bem como suas proteínas carreadoras, as neurofisinas (Gimpl & Fahrenholz, 2001). O SON é formado principalmente por células magnocelulares, situa-se nas bordas laterais do quiasma óptico e projeta-se maciçamente para a neuro-hipófise (Kiss & Mikkelsen, 2005).

O PVN localiza-se bilateralmente ao terceiro ventrículo onde duas populações de neurônios ocitocinérgicos estão bem destacadas, os neurônios magnocelulares e os neurônios parvocelulares. Os neurônios magnocelulares localizam-se nas porções laterais do PVN, já os neurônios parvocelulares localizam-se medialmente e constituem uma população heterogênea de neurônios destinados à realização de diversas funções (Badoer, 2001). Três zonas funcionais são descritas no PVN: a magnocelular, a mediocelular e a parvocelular (Badoer, 2001; Kiss & Mikkelsen, 2005).

1.1 Ocitocina

A zona magnocelular contém grandes neurônios secretores, que projetam seus axônios para a neuro-hipófise, formando assim o sitema HNH. Este sistema é dependente da atividade elétrica dos neurônios magnocelulares, que são ativados ou inibidos principalmente por glutamato e GABA (Oliet & Piet, 2004). Os potenciais de ação nessas células neurosecretoras desencadeiam a liberação da OT dos terminais axonais na neurohipófise para a corrente sanguínea. Um potente estímulo ocorre no final da gravidez, quando os neurônios ocitocinérgicos mudam drasticamente sua atividade elétrica de um estado quiescente para um estado altamente ativo (Poulain & Wakerley, 1982), isto ocorre devido à diminuição da concentração plasmática de progesterona (P4) e aumento de estrógeno (E2) (Bridges, 1984).

A plasticidade dos neurônios ocitocinérgicos e também dos vasopressinérgicos pode ser alterada dependendo da demanda fisiológica destas células. Isto ocorre, por exemplo, durante a desidratação, a lactação e o parto (Gimpl & Farenholz, 2001). Nesses casos os neurônios magnocelulares hipertrofiam e ocorre aumento das sinapses glutamatérgicas e GABAérgicas, bem como a diminuição da quantidade e do tamanho dos prolongamentos astrocíticos na região (Oliet & Piet, 2004). Já os neurônios do SON apresentam alterações morfológicas de acordo com a atividade desempenhada. Por exemplo, durante a lactação, o corpo celular, os dendritos e os contatos sinápticos dos neurônios ocitocinérgicos aumentam, o que ocasiona a modificação da arborização dendrítica quando comparada a ratas virgens (Cunningham et al., 2004).

A OT também é produzida, em menores quantidades, na zona parvocelular do PVN. Os neurônios desta divisão projetam-se para outras áreas do sistema nervoso central (SNC), tais como: núcleo dorsomedial do hipotálamo, núcleos talâmicos, hipocampo dorsal e ventral, córtex entorrinal, área medial e lateral do núcleo septal, amígdala, bulbo olfatório, substância nigra, locus coeruleus, núcleos da rafe, núcleo do trato solitário e núcleo motor dorsal do nervo vago (Gimpl & Farenholz, 2001; Mantella et al., 2003). Apenas 0,2% dos neurônios ocitocinérgicos enviam concomitantemente projeções para a neuro-hipófise e para outras áreas do SNC

(Figura 1) (para revisão Gimpl & Farenholz, 2001). A OT que é sintetizada pelos neurônios parvocelulares do PVN, pode ainda, ser liberada diretamente no líquor por dendritos localizados na parede do terceiro ventrículo (Dogterom et al., 1977; Leckman et al., 1994).

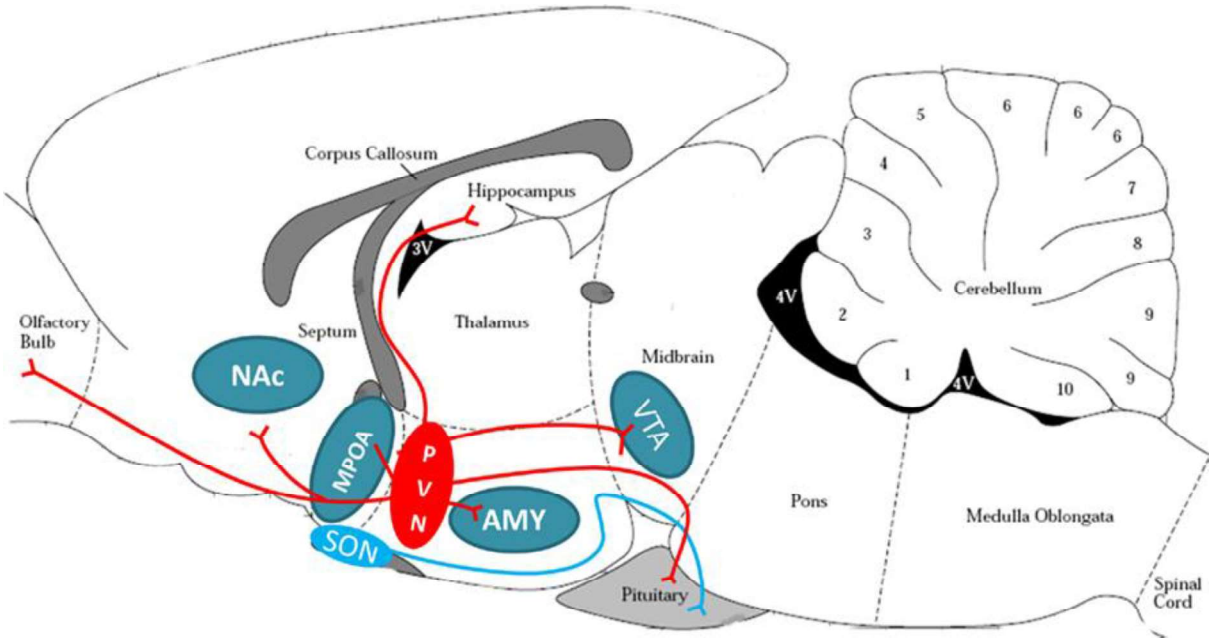

Figura 1: Projeções ocitocinérgicas no cérebro de roedores

Os neurônios magnocelulares do PVN (vermelho) e do SON (azul) enviam projeções para a hipófise posterior. Os neurônios parvocelulares do PVN projetam-se para a área tegmental ventral (VTA), núcleo accumbens (NAc), hipocampo, amígdala (AMY), intra-PVN, área pré-optica medial (MPOA), núcleo próprio da estria terminal (BNST), bulbo olfatório (OB). Figura esquemática adaptada de Paxinos & Watson (1998) (Rutherford et al., 2011).

A OT é sintetizada nos neurônios do PVN e SON a partir de uma grande molécula precursora. Esta molécula, chamada OT pré-pró-peptídeo, sofre clivagens enquanto está sendo transportada ao longo do axônio, cujos terminais localizam-se na neuro-hipófise. O produto final liberado no terminal axonal é composto pela OT e por sua molécula carreadora, a neurofisina. Existem neurofisinas associadas tanto à OT como à AVP, estas moléculas carreadoras formam uma ligação com o nanopeptídeo dentro da vesícula secretora, sendo liberadas simultaneamente. As neurofisinas também apresentam funções de empacotamento e armazenamento da OT no terminal axonal (Gimpl & Farenholz, 2001). Além da liberação axonal, sabe-se que a OT pode ser liberada centralmente pelos dendritos e pelo corpo celular, no

hipotálamo, sendo que esta liberação é independente da secreção para o sangue (Ludwig & Leng, 2006).

Os neurônios ocitocinérgicos enviam projeções para muitas regiões prosencefálicas, diencefálicas e tronco cerebral (Sofroniew, 1983), nestes locais são detectados receptores para OT (Gimpl & Farenholz, 2001). Até o momento, somente um tipo de receptor para OT é reconhecido e clonado (Caldwell et al., 2008). O receptor de ocitocina (OTR) pertence à família dos receptores hetero-triméricos acoplados à proteína G, sendo expresso por diversos tipos de células, incluindo neurônios, células ósseas, mioblastos, cardiomiócitos e células endoteliais (Gimpl & Farenholz, 2001; Zingg & Laporte, 2003).

O OTR está amplamente distribuído pelo encéfalo variando a localização de acordo com a espécie e o gênero. As diferentes expressões do OTR no encéfalo podem explicar as variações comportamentais observadas em diferentes espécies. Diferentes vias de transdução de sinais regulam a expressão do OTR e o *binding* em cada região cerebral e podem, em parte, mediar a habilidade da OT para exercer diversos efeitos comportamentais (Bale et al., 2001). Em roedores, a distribuição do OTR ocorre principalmente no bulbo e tubérculo olfatórios, no neocórtex, nos núcleos basais, no córtex piriforme, no córtex insular e perirrinal, na formação hipocampal, na amígdala central (Ace), no núcleo da base da estria terminal, no septo lateral, no núcleo accumbens, no hipotálamo ventromedial (VMH), no núcleo do trato solitário, na área tegmental ventral, no complexo mamilar, na oliva dorsal, no núcleo espinhal trigeminal, no tronco encefálico e na medula espinhal (Morris et al., 1995; Pedersen et al., 1995; Insel 1991; Veinante & Freund-Mercier, 1997).

A OT possui ações periféricas e centrais. As ações periféricas incluem a contração das células mioepiteliais que envolvem os alvéolos e ductos das glândulas mamárias e o músculo liso do endométrio, naquele estimulando a ejeção do leite e neste as contrações rítmicas do útero, que são responsáveis pela expulsão do feto (Samson & Schell, 1995). Participa também da modulação da função renal, da secreção de insulina e glucagon pelo pâncreas e da secreção de aldosterona pela glândula adrenal (Gimpl & Farenholz, 2001). No sistema cardiovascular, a OT induz a vasodilatação e diminui as contrações do miocárdio (Gimpl & Farenholz, 2001).

As ações centrais incluem sua participação no circuito ansiolítico central, na resposta supressora mediante estresse crônico, no controle do apetite pelo sal, no controle da pressão arterial e na resposta a diversos tipos de estresse em eventos hemorrágicos e ambientes novos (Carrasco & Van De Kar, 2003; Bernatova et al., 2004; Rigatto et al, 2003; Samson & Schell 1995; Michelini et al., 2003; Lang et al., 1983; Neumann, 2002). Além disso, esse neuropeptídeo é apontado como modulador dos comportamentos sociais (Pedersen, 1979; Giovenardi et al., 1998).

1.2 Vasopressina

A AVP tem sido descrita por seu papel em comportamentos agressivos, memória social e ansiedade, mas principalmente na modulação do eixo hipotálamo-hipófise-adrenal (HPA) e por agir em estruturas extrahipotalâmicas (Engelmann et al., 2006).

A síntese de AVP é realizada por dois tipos de neurônios, os neurônios parvocelulares e magnocelulares. Os neurônios magnocelulares encontram-se no PVN e SON e projetam seus axônios para a neuro-hipófise. Já os neurônios parvocelulares, encontrados na divisão parvocelular do PVN, projetam-se para a eminência mediana, sendo assim, a AVP também pode ser secretada na circulação portal. Sintetizada como um pré-pró-hormônio, que consiste no peptídeo sinalizador, no nonapeptídeo e na neurofisina associada (neurofisina II) (Caldwell et al., 2008). Os principais estímulos que induzem à liberação de AVP são o aumento da osmolalidade no sangue, a diminuição da pressão arterial e a diminuição da volemia (Treschan & Peters, 2006). Sua principal função no rim é aumentar a reabsorção de água nos ductos coletores renais, por isso também é conhecida como hormônio antidiurético (ADH). Os estímulos que induzem a sua liberação no hipotálamo são ativados através de osmorreceptores, localizados em vasos portais e mesentéricos, e barorreceptores atriais e arteriais (Petersen, 2006).

Alguns estudos sugerem que a AVP pode ser liberada em situações de estresse não osmótico, apesar de não haver um consenso, outros estímulos estressantes, tais como: imobilização, ambientes novos, nado forçado, choque, não

parecem induzir a liberação plasmática de AVP, embora induzam a liberação de OT (Wotjak et al., 1998; Laguna-abreu, 2005). Entretanto ocorre liberação de AVP intranuclearmente no PVN e SON após estresse por nado forçado, sugerindo uma dissociação da resposta central e periférica da vasopressina ao estresse (Wotjak et al., 1998). A origem da AVP liberada no PVN parece ser de origem magnocelular predominantemente (Wotjak et al., 2001).

Os receptores para AVP são acoplados à proteína G e consistem em três subtipos, V1a, V1b e V2, também conhecidos como VR1, VR2 e VR3. Os receptores V1a são encontrados em músculo liso vascular, fígado, útero e córtex adrenal, enquanto os V2 são encontrados nos ductos coletores renais. Os do tipo V1b são encontrados na adeno-hipófise onde regulam a liberação da corticotrofina (ACTH). No SNC os receptores V1a são encontrados inclusive no PVN (Dinan & Scott, 2005; Petersen, 2006). Os neurônios vasopressinérgicos expressam desde o nascimento os receptores V1a e V1b e a auto-regulação ocorre através do V1a (Ugrumov, 2002). Em roedores, foi descrita a ocorrência de receptores para AVP em várias regiões do SNC, como área septal lateral, hipocampo, área amígdalo-estriatal, núcleo da base da estria terminal e regiões do hipotálamo, esses receptores localizados nestas áreas límbicas podem mediar diversos comportamentos (Ruscio et al., 2007; Caldwell et al., 2008). Estudos em roedores demonstraram que algumas funções centrais da AVP, conjuntamente com a OT, relacionam-se com comportamentos sociais. Deste modo, a AVP parece estar relacionada com a modulação de comportamentos como a agressão entre machos, o comportamento agressivo em lactantes, o reconhecimento de odores e a preferência na escolha de parceiros (Caldwell et al., 2008).

1.3 Ocitocina, Vasopressina e Comportamento Social

Entre os mamíferos, existem espécies que vivem em diversos graus de sociabilidade, desde solitários até altamente sociais, como chimpanzés e humanos (Choleris et al., 2004). Os comportamentos sociais requerem dois ou mais animais com instinto e motivação para permanecerem próximos. Estes comportamentos podem ser considerados positivos, quando ocorrem benefícios mútuos, ou

negativos, como o comportamento agressivo. As ligações sociais podem se formar entre pais e filhotes, entre animais adultos ou entre outros membros do grupo e possui várias vantagens, entre elas a garantia da sobrevivência da espécie e a defesa contra a predação (Carter & Keverne, 2002). Durante as interações sociais consideradas como positivas (como, por exemplo, o comportamento maternal), a OT pode ser liberada tanto no SNC quanto no plasma (Uvnas-Moberg, 1998).

Em laboratório, quando ratos adultos são colocados na mesma caixa, eles interagem e apresentam comportamentos como cheirar e perseguir, podendo muitas vezes apresentarem comportamentos agressivos tais como morder, chutar e boxear (File & Hyde, 1978). Dados experimentais sugerem que as diferentes formas de interação social podem ser mediadas por sistemas neurais distintos, e que o status de familiaridade e o de isolamento do ambiente pode interferir nos comportamentos sociais (Varlinskaya & Spear, 2008). Em fêmeas, assim como ocorre com a receptividade sexual, as fases do ciclo estral podem alterar a duração dos comportamentos sociais. Ratas em proestro, por exemplo, apresentam mais tempo interagindo socialmente (Frye & Rhodes, 2008).

A OT possui importante papel nas interações sociais, em destaque o comportamento sexual, tanto de machos quanto de fêmeas (Arletti & Bertolini, 1985), o comportamento maternal (Bartels & Zeki, 2004; Consiglio & Lucion, 1996; Giovenardi et al., 1998), o agressivo maternal (Engelmann et al., 2000; Amico et al., 2004; Carter, 2005) e o *grooming* (autolimpeza) (Drago et al., 1986). Wallner e colaboradores (2006) descreveram a liberação plasmática de OT durante encontros sociais, sexuais ou não, em ratos machos e fêmeas. Engelmann e colaboradores (2000), sugerem que a OT também pode ser liberada durante a escolha e a formação de pares sexuais e, que este peptídeo associado a AVP, pode agir também influenciando comportamentos relacionados ao estresse, ao aprendizado e à memória.

O reconhecimento social, também conhecido como memória social, no qual os animais identificam e reconhecem indivíduos da mesma espécie é um pré-requisito para vários tipos de comportamentos sociais e de hierarquias dentro de um grupo (Choleris et al., 2004). A formação de pares sociais e os comportamentos

reprodutivos, também dependem da capacidade de reconhecer indivíduos familiares e não familiares dentro da mesma espécie (Ferguson et al., 2002). Em roedores, o reconhecimento social apresenta-se como um comportamento que utiliza o instinto natural de investigar indivíduos novos a partir de avaliações que envolvem o aprendizado e a memória. Além de apresentarem memória social de longo prazo, esses animais também apresentam memória social de curto prazo, reconhecendo animais apresentados por um breve período de tempo (Ferguson et al., 2002). Existem vários modelos experimentais utilizados para se testar a memória social. No modelo habituação-desabituação apresenta-se repetidamente o animal ao estímulo, que pode ser um juvenil pré-púbere e a diminuição do tempo de investigação representa que houve um reconhecimento (Bielsky & Young, 2004).

A AVP é de fundamental importância em um tipo específico de memória baseada em sinais olfatórios: a memória social. Injeções centrais e periféricas demonstram claramente que a AVP tem função importante no reconhecimento social em roedores (Ferguson et al., 2002). Como exemplo, a AVP quando injetada na área septal melhora a memória social, enquanto o antagonista injetado na mesma região prejudica o reconhecimento. Os receptores V1a e V1b relacionam-se com a memória social, entretanto, a maioria dos parâmetros comportamentais da memória social são mediados pelo V1a. O bloqueio deste tipo de receptor por antagonistas injetados centralmente impede o reconhecimento social (Bielsky & Young, 2004). Além disso, a estimulação elétrica do PVN e SON, induzindo a liberação de AVP, ocasiona melhora da memória social. Ratos Brattleboro mutantes deficientes em AVP apresentam déficits nesse tipo memória (Engelmann & Landgraf, 1994).

Em condições naturais, os ratos utilizam o comportamento de cheirar como padrão de comportamento exploratório (Kepecs et al., 2006). Além disso, os comportamentos de cheirar o corpo e a região anogenital são considerados comportamentos de investigação social (Bielsky & Young, 2004; Kepecs et al., 2006). Em roedores, o sistema olfatório principal (epitélio e bulbo olfatórios) e o sistema olfatório acessório (órgão vomeronasal e bulbo olfatório acessório) são ativados durante os encontros sociais (Ferguson et al., 2002). O sistema olfatório principal é responsável pela detecção de odores voláteis, como aqueles provenientes de alimentos, predadores e parceiros em potencial (Firestein, 2001). O

sistema olfatório acessório é utilizado para detectar odores não voláteis que influenciam nos comportamentos sexuais e agressivos, e auxiliam no reconhecimento de indivíduos da mesma espécie (Keverne, 1999).

1.4 Ocitocina, Vasopressina e Comportamento Sexual

A sobrevivência das espécies depende do seu sucesso reprodutivo. Além da coordenação dos processos fisiológicos com o meio ambiente, a reprodução necessita de vários circuitos integrados que culminam com a fertilização. Nesse processo destaca-se o papel da OT e da AVP (Debiec, 2007).

Em ratas, a receptividade sexual ocorre na fase do proestro e inclui componentes proceptivos, como a investigação dos genitais do macho, pequenas corridas e pulos dentro da caixa, vocalizações, exposição de partes do corpo e contatos físicos efêmeros (Edwards, 1970). Nestes animais, o componente mais importante do comportamento sexual é a postura de receptividade assumida pela fêmea no momento da cópula, denominada reflexo de lordose. Esta postura é caracterizada pela flexão dorsal da coluna vertebral em resposta à monta realizada pelo macho, auxiliando na intromissão peniana. Na ausência de lordose a intromissão e a ejaculação não serão possíveis, demonstrando a importância deste comportamento para o sucesso reprodutivo (Nelson, 2005). Em camundongos, as fêmeas receptivas realizam lordose quando montadas pelo macho, mas nesse momento a dorsiflexão pode não ser facilmente visualizada. Além disso, diferentemente dos ratos, os camundongos não apresentam comportamentos pré-copulatórios (Bonthuis et al., 2010).

O comportamento sexual das fêmeas varia de acordo com a fase do ciclo estral, sendo que ocorre predominantemente na noite do proestro (estro comportamental). Com duração média de doze horas, o proestro é caracterizado pela presença de muitas células epiteliais nucleadas e poucos leucócitos no muco vaginal (Nelson, 2005). O comportamento de lordose é iniciado pela presença de hormônios sexuais, E2 e P4, e ocorre em resposta à informação sensorial tátil, normalmente fornecida pela monta realizada pelo macho. Além de depender da

ação dos hormônios esteróides gonadais, o reflexo de lordose é resultante da ativição de uma circuitaria neural e, por este motivo, muitas estruturas estão envolvidas na realização desse reflexo. O hipotálamo é responsável por adicionar o componente endócrino a esse mecanismo comportamental, uma vez que os efeitos do E2 e da P4 nas propriedades eletrofisiológicas dos neurônios, a transcrição do RNA e a síntese novas proteínas e estruturas, são primariamente mediados nessa estrutura cerebral (Pfaff & Schwartz-Giblin, 1988).

Em machos e fêmeas, a OT representa um importante indutor do comportamento sexual, da excitação e do orgasmo (Carmichael et al., 1987). Em roedores, doses moderadas de OT podem facilitar a ereção peniana (Argiolas et al.,1987). No entanto, a OT é incapaz de induzir ereções sem a presença da testosterona, por isso, não ocorrem ereções penianas em animais castrados, mesmo com administração conjunta de OT (Melis et al., 1994). Além disso, injeções intracerebroventriculares ou intraperitoniais de OT reduzem a latência para a ocorrência de uma ejaculação e tempo entre os acasalamentos (Arletti et al., 1985).

Em ratas, a regulação do comportamento sexual ocorre através de interações entre o E2, a P4 e a OT (Witt, 1995). Em fêmeas ovarectomizadas e tratadas com E2 e P4, injeções intracerebroventriculares de OT podem aumentar a frequência e a duração de lordoses (Arletti et al., 1985). A frequência de lordoses também aumenta quando E2 e OT (Caldwell et al., 1986) ou P4 e OT (Gorzalka & Lester, 1987) são administrados separadamente. Essa indução do comportamento sexual feminino é mediada principalmente pela área preóptica medial (MPOA) do hipotálamo e pelo VMH (Kow & Pfaff, 1998). Por exemplo, a ocorrência do comportamento sexual feminino requer a ação do E2 no cérebro e a indução de OTR no VMH (Bale & Dorsa, 1995). De fato, o RNAm para OTR está presente desde a região rostral até a parte caudal do VMH de fêmeas, sendo que a expressão do OTR aumenta na divisão ventrolateral do VMH em resposta ao E2 e a P4 (Ostrowski, 1998).

O sistema reprodutivo feminino, em humanos e roedores, é regulado pelo eixo hipotálamo-hipófise-ovário (HPG). O principal regulador desse eixo é o hormônio liberador de gonadotrofinas (GnRH), produzido pelos neurônios da MPOA e núcleo arqueado do hipotálamo, e secretado dentro do sistema hipofisário portal. Na

hipófise, o GnRH estimula a produção do hormônio luteinizante (LH), o qual estimula o ovário a secretar E2 e P4 (Rivest et al., 1993; Ferin, 1996). Além disso, a secreção pulsátil e o pico de LH dependem da atividade dos neurônios GnRH (Kalra & Kalra, 1983). Conforme Caligioni e colaboradores (2007), nos animais em metaestro-proestro, aproximadamente 10% dos neurônios GnRH co-expressam OTR na área MPOA. Isso significa que a OT também pode interferir na secreção de LH e, portanto, na ovulação.

A expressão gonadal de OT e AVP ocorre em diversas espécies, no entanto, existem diferenças consideráveis entre elas com relação à regulação da luteólise (Russell & Leng, 1998). Em ruminantes, a OT do corpo lúteo estimula a produção de prostaglandinas pelo útero, acarretando uma liberação adicional de OT pelo corpo lúteo e a formação de um "*feed-back*" positivo que causa um aumento adicional da liberação de prostaglandina, que é o hormônio responsável pela regressão do corpo lúteo (Flint et al 1990). Nesses animais, a supressão do OTR pelo interferon τ, produzido pelo embrião, é essencial para prevenir a luteólise (Lamming et al., 1995). Em camundongos, estudos sobre o papel da OT na função gonadal indicam uma capacidade de estimular a ovulação e uma fraca expressão de OT pelas células da granulosa (Robinson & Evans 1990). Uma das metodologias utilizadas para avaliar alterações na ovulação é a contagem de oócitos, uma vez que roedores, com ciclo estral regular, apresentam um número de oócitos entre 10 e 14 a cada ciclo (Gomes et al.,1999).

Os neuropeptídeos OT e AVP exercem efeitos opostos sobre o comportamento sexual de fêmeas, uma vez que, quando administrada centralmente, a AVP inibe o comportamento sexual de fêmeas expostas a um macho sexualmente ativo (Pedersen & Boccia, 2006). Além disso, a administração central de um antagonista do V1a estimula a receptividade sexual (Caldwell et al.,2008) e a administração de um antagonista da OT causa redução nos componentes receptivos e proceptivos do comportamento sexual de fêmeas (Witt & Insel, 1991; Caldwell et al., 1992). Ambos os efeitos antagônicos estão relacionados com a MPOA, sugerindo que as interações entre OT e AVP podem contribuir para a regulação do comportamento sexual em fêmeas (Caldwell et al., 1992). Além disso, a expressão de AVP no encéfalo de machos é maior do que em fêmeas (De Vries, 2008) e está

associada aos comportamentos sociais tipicamente masculinos, como agressão e territorialidade (Donaldson & Young, 2008).

Em camundongos, a deleção do gene da OT diminuiu a transcrição do gene da AVP no PVN e no SON (Young et al., 1996; Ozaki et al., 2004) e a reposição do gene da OT restaurou a expressão do gene da AVP (Young et al., 1998). Além disso, animais com deleção do gene da OT apresentam redução da concentração plasmática basal de AVP (Lazzari et al ., 2013). Estes achados sugerem que a OT pode estar envolvida na regulação da expressão do gene da AVP e na sua liberação periférica.

1.5 Comportamento Sexual e Amígdala

A amígdala, ou complexo amigdaliano, é uma estrutura que compreende subnúcleos situados no lobo temporal, lateral ao hipotálamo e ventral ao estriado, no prosencéfalo basal de mamíferos. Em primatas, é caracterizada como uma massa ovoíde de substância cinzenta, localizada na porção terminal e rostral da formação hipocampal, tendo como limite anterior o corno temporal do ventrículo lateral (Alheid et al., 1995). Em ratos, localiza-se anteriormente ao hipocampo ventral (de Olmos et al., 2004), sendo constituída por núcleos e subnúcleos que formam uma complexa rede estrutural interrelacionada e multifuncional, que está envolvida na modulação de diversos comportamentos e ajustes vegetativos (Alheid et al., 1995; Paxinos & Watson, 1998; Rasia-Filho et al., 1999).

Estudos mais recentes sobre a divisão da amígdala de ratos apresentam-na dividida em quatros regiões, segundo a citoarquitetura, hodologia e função, que são: a amígdala “expandida”, denominada assim por se estender além de seus limites anatômicos, sendo formada pela amígdala medial (AMe) e ACe; a amígdala com características corticais, subdividida em porção basolateral e em porções que se ligam às vias olfativas e vomeronasal; a área de transição, localizada entre a porção ventral dos núcleos da base e a amígdala “expandida”; os núcleos ainda não classificados, constituídos por um grande número de células dispersas na substância branca e no interior do núcleo próprio da estria terminal (BNST) (Alheid,

1995; de Olmos et al., 2004).

A AMe é um dos subnúcleos superficiais do complexo amigdalóide, ocupando seu aspecto rostromedial, sendo formada por uma coluna proeminente de células que surgem em justaposição à superfície lateral de fibras que ascendem pelo trato óptico. Inicia medialmente e posteriormente ao núcleo do trato olfativo e ao núcleo anterior da amígdala, estendendo-se caudalmente até o surgimento da porção temporal do ventrículo lateral, em posição ventral em relação à estria terminal (Alheid et al., 1995). Na literatura a AMe tem sido dividida em subnúcleos de diversas maneiras, segundo os critérios dos autores. Pitkanen (2000) divide-a em três regiões denominadas: rostral, central (porção dorsal e ventral) e caudal. Alheid e colaboradores (1995), Paxinos e Watson (1998) e Olmos e colaboradores (2004) divdem-na nos seguintes subnúcleos: ântero-dorsal (MeAD), ântero-ventral (MeAV), póstero-dorsal (MePD) e póstero-ventral (MePV).

Vários estudos realizados na AMe de roedores têm demonstrado a presença de corpos celulares e fibras nervosas imunorreativas a diferentes neuropeptídeos, tais como: angiotensina II, Colecistoquinina (CCK), galanina, hormônio liberador de corticotrofina (CRH), GnRH, neuropeptídeo Y, neurotensina, opióides, peptídeo intestinal vasoativo (VIP), peptídeo liberador de gastrina (GRP), peptídeo relacionado ao gene da calcitonina (CGRP), polipeptídeo natiurético atrial (ANP), somatostatina, substância P, serotonina, OT e AVP (Swanson et al., 2003; Frankfurt et al., 1985; Lind et al., 1985; Gustafson et al., 1986; Oro et al., 1988; McDonald, 1989; Micevych et al., 1988; Marcos et al., 1999; De Olmos et al., 2004).

A AMe tem sido descrita como uma região envolvida na modulação de atividades endócrinas e comportamentais do animal com o seu ambiente, tais como: a percepção, a modulação e a integração das informações olfativas, vomeronasais e genitosensoriais (Guillamon & Segovia, 1997; Pfaus & Heeb, 1997; Dielenberg et al., 2001; Pro-Sistiaga et al., 2007) relacionadas com estímulos em que a ansiedade, o medo inato e o condicionado estejam envolvidos (Adamec & Morgan, 1994; Davis, 2000), no processamento de respostas emocionais, adrenocorticais e neuroendócrinas a um evento estressor (Marcuzzo et al., 2007), e uma série de comportamentos sociais, tais como o agressivo, o defensivo, o aprendizado social

(Newman, 1999; Rasia-Filho et al., 2008), o comportamento sexual, tanto em machos quanto em fêmeas, e o comportamento maternal (Fleming et al., 1980; Rasia-Filho et al., 1991; Newman, 1999; de Castilhos et al., 2006). Os subnúcleos da AMe têm diferentes funções (Canteras et al., 1995). Por exemplo, a AMePD parece influenciar as atividades neuroendócrinas e o controle dos sistemas simpático e parassimpático que o hipotálamo regula, enquanto as regiões MeAD, MeAV e MePV podem estar relacionados com a modulação hipotalâmica dos comportamentos sexual e defensivo (Canteras et al., 1995; Simerly, 2004). Especificamente, a AMePD modula alguns comportamentos, como o maternal e o sexual (Newman, 1999; de Castilhos et al., 2008).

A AMePD é separada do trato óptico por uma camada com poucos corpos celulares, a qual se torna estreita em direção rostral e dorsal até que desaparece completamente próximo da AMeAD (de Olmos et al., 2004). Essa região preferentemente deve ser considerada como local de passagem de axônios advindos do núcleo próprio da via olfativa acessória para transmissão de informação vomeronasal (de Olmos et al., 2004). Esse núcleo possui várias aferências que advêm de diferentes regiões do encéfalo. As aferências mais importantes são as hipotalâmicas (da área hipotalâmica anterior, área pré-óptica medial e lateral do núcleo arqueado, núcleos dorsomedial, hipotalâmico posterior, lateral, pré-mamilar, supraóptico, tuberal e ventromedial), as do córtex cerebral (da área pré-límbica, córtex entorrinal, infralímbico e perirrinal dorsal), as talâmicas (do núcleo medial, parafascilular, paraventricular e posterior), as do tronco encefálico (principalmente do núcleo dorsal da rafe e núcleo parabraquial), as do sistema olfativo (do córtex piriforme, bulbo olfativo acessório e núcleo endopiriforme) e de outras regiões distintas, como do núcleo da faixa diagonal de Broca, do BNST e da substância *inominata*. Além dessas, existem aferências intra-amigdaloídes, como as provenientes do córtex periamigdaloíde, núcleo basal acessório, cortical posterior e núcleo do trato olfativo lateral. Em relação às eferências da AMePD, dentre as mais significativas e estudadas, estão aquelas para os núcleos cortical porterior, o BNST (divisões ântero-dorsal e posterior principal), a substância *inominata* e alguns núcleos hipotalâmicos como o posterior, o anterior, o periventricular ântero-ventral, o pré-óptico medial e o pré-mamilar ventral (Canteras et al., 1995; Choi et al., 2005).

Acredita-se que o controle do comportamento sexual em ratos, machos e fêmeas, ocorra a partir de estímulos olfativos (Kondo & Arai et al., 1995). Em ratos, aferências quimiossensoriais do bulbo olfatório, do órgão vomeronasal e do hipocampo projetam-se para a AMe. O primeiro subnúcleo a processar a informação olfativa é a AMeAD que, via núcleos intercalados da amígdala, transfere a informação para essa área. Este núcleo, por sua vez, envia as informações olfativas para áreas motoras e neuroendócrinas do telencéfalo basal, para o tronco encefálico, bem como para a MPOA e outros núcleos hipotalâmicos (Canteras et al., 1995; Wood, 1997). Devido a estas aferências, a AMe parece ser fundamental na modulação de comportamentos que requeram a ativação quimiossensorial, como o comportamento sexual (Dominguez et al., 2001; Takahashi & Gladstone, 1988). Ratas submetidas à lesão na AMePD, quando colocadas junto a ratos, mostraram redução da ocorrência de atividade pré-copulatória (exploração olfativa) e aumento da duração da cópula. Após a cópula, elas buscavam menos frequentemente seus companheiros de acasalamento quando comparadas às ratas submetidas à lesão fictícia (Lehman & Winans, 1982). Em hamsters machos, a lesão na AMe promoveu a redução do comportamento de acasalamento e diminuição da investigação olfatória dos genitais das fêmeas. Além disso, uma lesão ampla nessa área em ratos produziu a diminuição da frequência de ejaculações, o aumento no número de intromissões e o aumento do intervalo entre as intromissões quando comparadas a animais não lesados (Meisel, 1994). Sugeriu-se, assim, que lesões na AMe causam alterações significativas nos modelos de comportamento sexual, o que reforça a sugestão de que a AMe pode ser um importante componente neural do sistema de regulação do comportamento copulatório de ratos (Takahashi & Gladstone, 1988).

É muito importante ressaltar que um dos principais papéis funcionais das projeções da AMePD para o núcleo hipotalâmico periventricular ântero-ventral (AVPV) é a regulação da liberação do GnRH, imprescindível para o ciclo reprodutivo (Simerly, 1998). Em 1999, Newman e colaboradores sugeriram que as conexões neurais e sua sensibilidade aos hormônios sexuais são dinamicamente moduladas no decorrer da vida e que essas células regulam os comportamentos sexuais em fêmeas. Neste sentido, os subnúcleos da AMe parecem estar envolvidos na organização das fases do ciclo estral, juntamente com o VMH, o AVPV, a MPOA e o núcleo arqueado. Portanto, além das conexões da AMePD com núcleos

hipotalâmicos regularem a liberação do GnRH (Simerly, 1998; Etgen et al., 1999), estas conexões podem modular a receptividade sexual em fêmeas.

1.6 Técnica de Golgi e Caracterização dos Neurônios da AMePD

Em 1873, Camilo Golgi descreveu uma técnica de impregnação celular pela prata que foi fundamental para o entendimento da estrutura do tecido nervoso e de sua organização básica. A técnica de Golgi permite a visualização da célula nervosa inteira. No entanto, apenas uma pequena porção de células nervosas (1-10%) presentes no tecido é impregnada pela prata, adquirindo uma coloração negra que contrasta com o restante do tecido em cor amarelo-parda. Ademais, nem todas as regiões do SNC de diferentes espécies impregnam-se igualmente e, conforme avança a idade do animal, torna-se muito mais difícil obter bons resultados (Ramón Y Cajal, 1909; Peters & Kaiserman-Abramof, 1970; Woolley & McEwen, 1993; Pannese, 1996; Rasia-Filho et al., 1999).

Os experimentos realizados em gatos, cachorros, camundongos e ratos evidenciaram, pela técnica de Golgi, uma morfologia neuronal relativamente simples e muito similar na AMe dessas espécies (McDonald, 1992). Os neurônios são do tipo multipolar, com corpos ovais ou fusiformes (Gomez & Newman, 1991; Rasia-Filho et al., 1999) e com tamanhos que variam de pequeno, com cerca de 8-10 micrômetros (μm) de diâmetro médio, até médio, com aproximadamente 10-15 μm nesta medida (Rasia-Filho et al., 1999). Os neurônios multipolares, característicos dessa região, são do tipo bipenachado (tradução livre para *bitufted*, do inglês) ou estrelado, conforme o número de ramificações dendríticas primárias originadas do soma celular. Sendo que os neurônios bipenachados apresentam dois ramos dendríticos primários surgindo do soma, enquanto que os estrelados possuem três ou mais ramos dendríticos primários (Rasia-Filho et al., 1999; Rasia-Filho et al., 2004).

Os dendritos podem apresentar protusões membranosas denominadas de espinhos dendríticos, que são referidos como especialização pós-sinápticas (Bradley et al., 1999) e constituem a unidade de entrada ou saída da atividade sináptica (Woolf et al., 1998). A distribuição, a forma e o tamanho dos espinhos dendríticos

estão diretamente relacionados com a função do neurônio, portanto, a determinação do número de espinhos por segmento dendrítico ou densidade por micrômetro (µm) dendrítico pode ajudar a elucidar a atividade celular local e sua plasticidade (Woolf et al., 1998). Uma característica peculiar dos espinhos dendríticos é a sua variabilidade morfológica. Este processo reflete o rearranjo rápido do citoesqueleto de actina no seu interior, o que pode levar à mudança no tamanho e no número de espinhos (Oertner & Matus, 2005; Tada & Sheng, 2006). Em geral, os espinhos dendríticos podem ser classificados de acordo com a sua morfologia com a seguinte nomenclatura: 1. Filopódio, que não apresenta uma cabeça definida, sendo fino e comprido, e acredita-se que seja a forma precursora dos espinhos; 2. Fino, o qual apresenta pescoço fino e pode não ter uma cabeça bem definida; 3. Espesso, que não apresenta um pescoço diferenciado e representa apenas uma elevação no contorno dendrítico; 4. Cogumelo, que apresenta o pescoço fino e uma cabeça grande, parecendo ser o mais estável em termos de contatos sinápticos duradouros; 5. Ramificado, no qual o pescoço pode dar origem a mais de uma cabeça (Figura 2) (Peters-Kaiserman-Abramof, 1970; Hering & Sheng, 2001; González-Burgos, 2004).

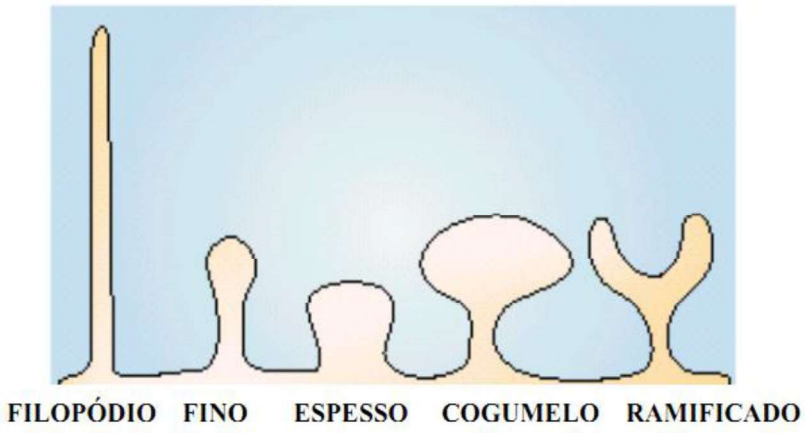

Figura 2: Morfologias dos espinhos dendríticos

Representação esquemática das diferentes morfologias dos espinhos dendríticos, como se observa à microscopia de luz, surgindo a partir de uma linha de base que representa o tronco dendrítico. Figura adaptada de Hering & Sheng (2001) e reproduzida de Marcuzzo (2006).

A arborização dendrítica da AMe é esparsa, com ramos dendríticos grossos, razoalvelmente retilíneos, longos ou de comprimento variável que se irradiam para direções variadas (Narkiewicz et al., 1978; Gomez & Newman, 1991; McDonald,

1992; Rasia-Filho et al., 1999; Rasia-Filho et al., 2004). A densidade de espinhos dendríticos nessa região varia de baixa a moderada (Narkiewicz et al., 1978; Milhouse & De Olmos, 1981; Rasia-Filho et al., 2004). Espinhos dendríticos pleomórficos são encontrados na AMeAD, na AMePD e na AMePV (Rasia-Filho et al., 2002; Rigotti, 2002). Os axônios dos subnúcleos da região posterior da AMe preferencialmente dirigem-se medialmente ao núcleo basal ou à porção principal do BNST, enquanto os da AMeAD compõem parte da ansa pediculares, também chamada de amígdalo-fungal ventral (Kamal & Kömöl, 1975; Cooke & Simerly, 2005). Pela análise ultraestrutural dos neurônios da AMePD, as sinapses axodendríticas no tronco dos dendritos são as mais frequentemente observadas e, pelo aspecto morfológico, parecem ser principalmente excitatórias (Hermel et al., 2006). Os espinhos dendríticos, quando presentes, apresentam formas variadas e encontram-se de forma aparentemente homogênea ao longo de cada dendrito, porém igualmente em alguns somas celulares e cones axonais (McDonald, 1992; Rasia-Filho et al., 1999; Rasia-Filho et al., 2004; Rigoti, 2002; Hermel et al., 2006).

Em ratos, a densidade de espinhos dendríticos proximais na AMePD é maior em machos do que em fêmeas nas fases de proestro, estro ou metaestro (Rasia-Filho et al., 2004) e ambos apresentam redução na densidade de espinhos dendríticos após remoção dos hormônios gonadais (de Castilhos et al., 2008). De fato, os hormônios gonadais influenciam no número de neurônios (Morris & Jordan, 2008), no volume celular (Hermel et al., 2006), na orientação dendrítica (Dall'Oglio et al., 2008), na densidade de espinhos dendríticos (Cooke et al., 2007; Cunningham et al., 2007; De Castilhos et al., 2008; Rasia-Filho et al., 2004), nos contatos sinápticos (Nishizuka & Arai, 1983) e no potencial excitatório pós-sináptico dessa estrutura (Cooke & Woolley, 2005). Além disso, fêmeas em proestro e estro apresentam menor densidade de espinhos dendríticos ao longo do ciclo estral. Ao mesmo tempo ocorre um decréscimo no número de sinapses na AMePD de ratas (Rasia-Filho et al., 2004; Oberlander & Erskine, 2008).

2. OBJETIVOS

2.1 Objetivo geral

Este estudo teve como objetivo avaliar o papel da OT na modulação do comportamento sexual e na densidade de espinhos dendríticos dos neurônios da AMePD em camundongos fêmeas através utilização de animais *knockout* para o gene da OT (OTKO).

2.2 Objetivos Específicos

- Analisar os efeitos do déficit de OT no comportamento sexual de camundongos fêmeas;
- Avaliar o número de oócitos presentes nos ovidutos de fêmeas OTKO;
- Quantificar as concentrações plasmáticas basais de AVP de fêmeas OTKO;
- Analisar a densidade de espinhos nos primeiros 40 µm dendríticos dos neurônios da AMePD de fêmeas OTKO na fase do proestro.

3. ARTIGO CIENTÍFICO A SER SUBMETIDO PARA PUBLICAÇÃO

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Sexual behavior and dendritic spine density of posterodorsal medial amygdala neurons in oxytocin knockout female mice

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ABSTRACT

Central oxytocin (OT) and arginine-vasopressin (AVP) have been shown to play an important role in sexual behavior and neuroendocrine secretion in rodents. Results from exogenous OT administration on sexual behaviors in male and female mice are controversial. This study aimed to analyze the role of OT in the posterodorsal medial amygdala (MePD), a forebrain area involved in pheromonal processing and reproduction, on the sexual behavior modulation and proximal dendritic spines density, , of female mice with selective deletion of the OT gene (OTKO). Female mice C57BL/6 were genotyped and divided into control (WT) and OTKO groups (n= 11 each). The experiments were performed in the beginning of the night in the proestrus phase. Our results showed that OTKO group has a notable decrease in the frequency, the duration and the quotient of lordosis behavior and a reduction in the number of oocytes. On the other hand showed a higher density of proximal dendritic spines in the MePD, when compared to the WT group. No significant difference was observed in the plasma levels of AVP between groups. Our data indicate that, without altering the occurrence of ovarian cycle phases or AVP circulating levels, OT remarkably modulates female mice sexual behavior display, ovulation, the number of dendritic spines and possibly the information processing of MePD neurons.

Keywords: Extended amygdala, OTKO mice, ovulation, reproductive behavior, vasopressin.

1. INTRODUCTION

Oxytocin (OT) and arginine-vasopressin (AVP) play a pivotal role in the regulation of social behaviors in rodents [1]. They are synthesized in the magnocellular and parvocellular neurons of the paraventricular nucleus (PVN) and in the supraoptic nucleus (SON) of the hypothalamus. Magnocellular neurons project to the neurohypophysis and release these peptides into the peripheral circulation. Parvocellular neurons project to several brain areas, including the amygdaloid nuclei, hippocampus, nucleus of the solitary tract, dorsal motor nucleus of the vagus, area postrema, hypothalamic ventromedial nucleus (VMH) and medial preoptic area (MPOA), ventral tegmental area, nucleus accumbens and bed nucleus of the stria terminalis (BnST) [2-4]. Thus, OT and AVP are likely to be key regulators of evolution and expression of different types of social systems, including the maternal care, aggression, pair bonding, sexual behavior and social memory [5-7].

The recognition of conspecifics is an initial and crucial condition for the establishment of social [8] and sexual behavior. Chemical, such as odor, scent and pheromone, are cues to mediate sexual and competitive interactions and are of major importance in individual and kin recognition as well as mate selection [9-11]. Odor signals are processed by two systems, the main olfactory and the vomeronasal pathway [12]. Both have heavy direct and indirect projections to the medial nucleus of the amygdala (MeA) [12,13], with a relatively minor input to the BnST [14]. The MeA sends massive projections to the BnST and the MPOA which, in turn, project to the lateral septum and hippocampus [13,14]. This odor investigation/recognition circuit involves two relevant neuropeptides: OT and AVP [15,16]. OT-deficient mice are not able to recognize a previously encountered, familiar conspecific during

subsequent trials [1,17], possibly due to a defect in processing of chemosensory information involving the MeA [18]. In fact, this social recognition behavior can be rescued with direct microinjection of OT into the MeA of OT knockout (OTKO) mice [17].

The MeA is composed by 4 subnuclei and, among other functions [19-21], modulate social and reproductive behaviors [22-25]. Several findings indicate that the sex steroids can alter the morphology and function of the posterodorsal medial amygdala (MePD) neurons and glial cells [26-28], making the rat MePD sexually dimorphic or modifiable by naturally occurring variations in the level of circulating ovarian steroids [23-31]. The MePD neurons from intact adult male rats have a higher density of proximal dendritic spines than females in proestrus, estrus or metaestrus, but not in diestrus [23,25]. Thus, acting locally in the MePD and/or in interconnected sex steroid sensitive regions, gonadal hormones can alter the amount of dendritic spines in the MePD, establishing and/or maintaining a higher quantity of spines in males and inducing a numerical variation across the estrous cycle in females. Dendritic spines have crucial properties for synaptic strength and plasticity and to affect neuronal activity in integrated circuits [32-35]. For example, the rat MePD is connected to hypothalamic nuclei that control reproduction [e.g., the MPOA and the anteroventral periventricular nucleus; 36,37] and modulates timely hypothalamic gonadotrophin releasing hormone (GnRH) secretion and sexual behavior display [38,39], processes olfactory/pheromonal [40,41] and vaginocervical stimuli [38], and induce long-term changes in prolactin secretion needed for pregnancy/pseudopregnancy or mnemonic events at the time of mating [42,43].

Moreover, brain OT plays an important role in the regulation of male and female sexual behavior. In male rodents, OT is implicated in the erectile function,

copulatory activity and ejaculation [44,45]. In female rats, others copulatory behavior regulation occurs through interactions between estrogen (E), progesterone (P) and OT [44]. OT induces female sexual behavior, primarily by its action in the MPOA and the VMH to control lordosis display [46,47]. In female, primed with E, the OT receptor (OTR) antisense oligodeoxynucleotides infusion into the VMH blocks female receptivity to male rats [48-50]. Furthermore, experimental evidences show that OT can regulate the GnRH cells activity, suggesting a possible modulation on luteinizing hormone (LH) peak and, consequently, the ovulation [51]. Compared to OT, AVP has been reported to exert opposite effects on female sexual behavior in rats. When administered centrally, AVP inhibits but AVP receptor (V1a) antagonists stimulates sexual receptivity [52,53]. These antagonistic effects can occur in the MPOA, suggesting that OT and AVP interaction may contribute to the regulation of sexual behavior in females [53].

Therefore, the present study aimed to analyze the role of OT in the sexual behavior, the number of oocytes and, the density of dendritic spines in the MePD of female mice. The basal plasma concentration of AVP was also measured in these animals.

2. MATERIAL AND METHODS

2.1 GENERAL METHODS

2.1.1 Animals

The mice of this study were the offspring of a backcrossed stock obtained from Dr. W. Scott Young (B6; 129S-Oxttm1Wsy/J; NIMH, USA). All animals were

littermates from heterozygous breeders (C57BL/6 mice). 34 females and 10 males, weighed 25 to 35 g between 5 and 8 months old, were raised in the animal house facility of the Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA, Brazil). Mice were housed in ventilated transparent acrylic cages (37 cm × 24 cm × 24 cm) and grouped with up to five same-sex. The room temperature was controled (22 ± 1ºC) and a 12:12 light–dark cycle (lights off at 5 pm) was adopted. Mice had free access to chow (Nuvilab, Brazil) and water.

All procedures were performed in conformity with international regulation for the care and use of laboratory animals (National Institutes of Health Publication No. 85-23, reviewed 1985, USA) as well as the Brazilian Society for Neuroscience and Behavior Guidelines. The protocols were approved by the local Ethics Committee (UFCSPA, Brazil, protocol No. 920/09).

In order to determine the regularity of the estrous cycle, vaginal smears were taken from virgin female mice during 2 weeks before the beginning of the experiment. After the occurrence of three regular estrous cycles, experiments were performed in the beginning of the night of the proestrus phase.

2.1.2 Genotyping

The colony founders were developed by Young et al. [54]. The gene was deleted by crossing a genetic construct with the WT mouse OT allele in a manner that replaced the last 2 exons. Genotyping was carried out as previously described [54]. Briefly, genomic DNA was isolated from mouse tail samples and used as template for polymerase chain reaction. The primer sequences for amplification of the WT alleles involved the forward primer 5’-CTT GGC TTA CTG GCT CTG ACCT-3’ and the reverse primer 5’-GTC AAG AGG GAG CCT AAC ACT TC-3’. To amplify

the targeted allele, an additional forward primer (NEO) was used: 5'-TGC CCC AAA GGC CTA CCC GCT TCC-3'.

After genotyping, mice were divided into WT and OTKO groups and randomly assigned in two experiments, as follow.

2.2 EXPERIMENT 1

2.2.1 Sexual Behavior

The females from the WT control group (n=11) and OTKO group (n=11) were tested with sexually experienced males. In this experiment we used sexually experienced males because they exhibited higher frequencies and shorter latencies for the behavioral components of copulation, including mounting, intromission and ejaculation [55]. The male was adapted for 10 minutes in the test apparatus. After the adaptation time, a female was placed in the same observing box and the behavioral test started. The test was performed during the dark cycle in an observation room illuminated by dim red light, and behaviors were recorded with a video camera during 15 minutes [56].

The following parameters were evaluated: latency, frequency and duration of lordosis behavior. The lordosis response was scored on a 4-point scale (0–3) as described by Hardy and DeBold [57]. For each female mouse, a lordosis quotient was calculated by dividing the number of lordosis scores of 2 or 3 by the total number of mounts x 100. The test was videotaped using a video camera and recorded using the "Observer" software (Noldus®, Holland).

2.2.2 Hormonal Measures and Counting of Oocytes

On the day after the sexual behavior test (i.e., on the estrus day and during the lightly part of the cycle, at 8 a.m.), the female mice were decapitated and trunk blood samples (n=8 for both WT and OTKO groups) and ovaries (n=11 for both WT and OTKO groups) were collected for further study.

The blood samples were placed in heparinized test tubes and centrifuged for 15 minutes at 1600g at 4°C; plasma was separated and stored at –80°C. AVP enzyme-linked immunosorbent assays (ELISA) were performed according to the manufacturer's protocol (Enzo Life Sciences, USA) using the Arg8-Vasopressin EIA kit. Briefly, 100 µL of plasma was compared to other known concentrations, an optical density reading at 405 nm with correction at 570 nm was taken and a standard curve was generated. The accepted intra-assay variability was 5.9%.

The oviducts were dissected and squashed between 2 glass slides. The number of oocytes of both oviductal ampullae was counted under an optical microscope as previously described [58].

2.3 EXPERIMENT 2

2.3.1 Histological Procedure and Data Acquisition

Females from the WT and OTKO groups (n=6 each) were anesthetized with a single intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Then, mice were submitted to transcardially perfusion with heparinized 4% paraformaldehyde and 2% picric acid diluted in 0.1 M phosphate buffer solution (pH=7.4). The brains were removed and sectioned using a vibratome (Leica, Germany). Coronal sections (150-µm thick) were received in a 3% potassium

dichromate (Merck, Germany) solution for 24 h and, afterwards, impregnated in 1.5% silver nitrate (Merck, Germany) solution for 48 h at room temperature. Sections were dehydrated, cleared with xylene, mounted on slides and covered with non-acidic synthetic balsam and coverslips [adapted from 21,31,59,61].

The sections containing the MePD corresponded to a distance of 1.46 to 1.94 mm posterior to the bregma [61]. In both hemispheres, the MePD was located laterally to the optic tract and the "molecular layer" and ventrally to the stria terminalis. Microscopical images of each brain slice were compared with the schematic drawings of an atlas [61; Figure 1A]. The criteria for neuronal selection were: (a) be undoubtedly located within the boundaries of the MePD and relatively distant from its ultimate borders; (b) be relatively isolated from neighboring impregnated cells to avoid "tangled" dendrites; (c) dendrites should have well-impregnated and defined borders; and (d) spines should be clearly distinguishable from the background [based on 21,26,59]. Because the number of impregnated neurons was variable from section to section, both sides of the brain were used [see also 59].

For each female, the first dendrites that fulfilled these aforementioned inclusion criteria had their spines drawn using a camera lucida (2000x; i.e., 100x oil-immersion objective lens and 20x ocular lens) coupled to an optic microscope (Olympus BX-41, Japan). Dendritic spines in the different microscopic focal planes were counted on proximal branches. From each female, 8 different dendrites were studied, being 1 dendrite per sampled neuron. Then, dendritic spines data were obtained from a total of 48 dendrites per experimental group. After this procedure, three-dimensional dendritic lengths were measured from the same microscopic images (400x; Olympus BX-61, Japan) and the images of the selected dendrites

were captured by a high-resolution digital camera (CCD DP72, Japan) and analyzed with the Image Pro Plus 7.0 computer software (Media Cybernetics, USA). Dendritic length sampled varied from 30-60 μm in both groups [mean ± standard deviation (SD) values of 41 + 5 μm and 39 + 7 μm for WT and OTKO mice, respectively]. Spine density was defined as the number of spines per unit length of dendritic segment [μm; 26,31,59].

2.4 STATISTICAL ANALYSIS

Parameters of female sexual behavior showed a nonparametric distribution. The latency, frequency, duration and lordosis quotient were analyzed by the Mann-Whitney test, as was the counting of oocytes in the experimental groups.

Mean values for the dendritic spine density from each mouse were calculated for further comparisons. Results fulfilled the formal requirements for the use of parametrical analyses after showing a normal distribution (Kolmogorov-Smirnov test) and equal SD. Dendritic spine density data from the two experimental groups were submitted to an unpaired two-tailed Student's t-test. The same test was used to compare the plasma concentration of AVP of WT and OTKO mice. In all cases, $P<0.05$ was considered statistically significant.

3. RESULTS

The OTKO group had a significant increase in the latency and decrease in the frequency and duration of female sexual behavior and in the lordosis quotient when

compared to WT females (Table 1).

OTKO females had a significant decrease in the number of oocytes at the oviductal ampullae (*P*=0.03) when compared to the WT group (Figure 2).

Proximal dendritic spine density showed a highly significant statistical difference between groups. Minimum to maximum ranges for the MePD spine density observed for the WT and the OTKO groups were, respectively, 1.7-1.9 and 2.2-2.5 spines/dendritic µm. The OTKO group showed consistently higher values than the WT one (*P* < 0.01; Figure 1B-D).

Finally, there was no significant difference in the plasma concentration of AVP between WT and OTKO group (mean ± standard error= 583.3±112.0 and 617.1±96.03 pg/mL, respectively; *P*=0.82).

4. DISCUSSION

To provide additional evidence for the importance of OT in physiological and behavioral processes, gene targeting has been used to eliminate certain limitations of the OT manipulations on the central nervous system [62]. This technique has own advantages and limitations, but makes a valid contribution to the complex behavioral functions of central neuropeptides such as OT. However, it is also important to keep in mind that manipulation of a single brain component is likely to co-affect multiple related systems [7].

There are currently differing views on the role of OT in the sexual behavior of male and female rodents [7,54,63-66]. Recently, we showed that sexual behavior of male mice was not affected by the lack of OT [67]. Therefore, other hormones and neurochemical mechanisms could be more critically involved in sexual behavior of

males (e.g., see 68-70). In this study, our results showed that knocking out the OT gene notably decreased the sexual behavior of female mice.

Various central effects of OT in the sexual behavior were obtained after intracerebroventricular microinjections, some showing that OT facilitates socio-sexual interactions [65,68,71]. In ovariectomized female rats under E and P replacement therapy, OT increased lordosis behavior in response to mounting attempts [44]. Lordosis is also increased when either E and OT [48] or P and OT [72] are administered concomitantly. Otherwise, the use of an OT antagonist reduced or inhibited the expression of sexual behavior in female rats [44,48,50,53].

The lordosis response, the typical expression of female receptivity, is a complex phenomenon regulated by excitatory and inhibitory neural systems in the brain [73,74]. The induction of female sexual behavior is mediated primarily by the MPOA and the VMH [47]. McCarthy et al [50] demonstrated that microinjection of antisense oligodeoxynucleotides to OTR into the VMH of females primed with E blocked female receptivity to male rats. Similarly, OT antagonist into the MPOA prior to treatment with P significantly decreased lordosis behavior and increased duration of fighting with males [75]. Our results also indicate that OT is significantly involved in female sexual behavior, and together with a previous report [76], these data show that OT is an essential modulator of lordosis behavior in mice.

Our results of oocytes quantification showed that the OTKO group had a significant decrease in the number of oocytes compared to the WT group. Several evidences show that OT modulates GnRH neurones activity and that OT constitutes the final output pathway of a neuronal network that controls the pre-ovulatory LH peak and also ovulation [51,77-80]. Additionally, the central administration of OT antiserum abolishes the proestrus LH peak [81], whereas OT induces GnRH release

from hypothalamic explants on the afternoon of proestrus [82], supporting the pivotal role for OT in GnRH release. Caligioni et al [51] demonstrated that female rats in metaestrus or proestrus had a double-labeled immunofluorescence in approximately 10% of GnRH neurones co-expressing OTRs in the MPOA, and that, few OT fibres could be found in the vicinity of these GnRH neurones. These data suggest that OT may partially control neuronal activity in a subpopulation of GnRH neurons. Moreover, our results allows us to infer that ovulation was influenced by the lack of OT, but further investigations should be conducted to clarify the OT interaction in the multifactor neural network that controls GnRH neurones during the estrous cycle. Indeed, in our study OTKO females showed normal cycles, as assessed by vaginal cytology, and could be studied in the proestrus phase.

Our results also showed that OTKO mice have a higher (~25% more) dendritic spines in the MePD than WT controls. Again, both groups were studied during proestrus, the cycle phase when there are estradiol and progesterone peak in the circulation. It is important noting that ovarian hormones and their receptors correlate with the OT and OTR expression in socially-relevant mouse brain regions [83,84]. The MePD expresses one of the highest concentrations of α and β estrogen receptors [29,30,85], local neurons also co-express progesterone receptors [29], OT and OT receptors (OTR) can be found in this subnucleus [86]. In addition, the bilateral administration of OTR antagonist into the MeA impairs social memory in adult females [87]. Then, it is highly conceivable that the female mice MePD neurons are affected by sex steroids fluctuations and local release of OT to dynamically modulate reproductive/social behaviors. OTKO mice showed a disruption of sexual receptiveness during proestrus at the same time that MePD dendritic spines density was higher. In the rat MePD, there is a significant decrease in the number of spines

when females change from diestrus to proestrus [24,26]. Considering that direct and indirect projections from the MePD to reproductive behavior-related hypothalamic areas are GABAergic, it is likely that the reduction in the MePD dendritic spines during proestrus would reduce the output inhibition for the occurrence of female sexual behavior [further elaborated in 21,31; see also relevant data in 88]. It is possible that OTKO female mice have an impaired sex steroid-mediated influence on the plastic number of MePD dendritic spines and/or an altered neural circuitry for sexual behavior, although sparing the cyclic hypothalamic GnRH neuroendocrine secretion. Other possibilities include pheromonal recognition of the conspecific male and emotional/social behavior processing by the MePD neurons of OTKO females. These are working hypotheses highlighted by the present results that deserve to be tested with further experimental approaches aiming specifically the MePD.

The involvement of OT and AVP in social recognition, as well as in numerous social behaviors, including parental and sexual behaviors [2,7] suggests a fundamental role of these hormones in the evolution of animal sociality. In contrast to OT, AVP is likely to be more important for social recognition in males than in females [89]. The expression of AVP is greater in male than in female brains across various species [62] and AVP is usually associated with male-typical social behaviors, such as male reproduction, aggression and territoriality [90]. Here, we observed that knocking out the OT gene did not change the basal AVP plasma concentration, when comparing OTKO and WT female mice, and it would be inferred that lordosis behavior was influenced only by the lack of OT.

In conclusion, our data suggest that OT modulates the sexual behavior display, decrease the number of oocytes and the density of dendritic spines in the MePD of female mice. The AVP plasma concentration was not affected in OTKO

animals.

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CONFLICT OF INTEREST

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

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LEGENDS

Table 1. Latency, frequency, duration and quotient of lordosis behavior of control (WT) and knockout (OTKO) groups (n = 11 each). The Mann-Whitney test was used to compare the experimental groups, at a significance level of P ≤ 0.05. Data are expressed as median and interquartile range.

Figure 1. (A) Schematic diagram of the ventral part of a coronal slice showing the posterodorsal medial amygdala (MePD) in the mouse forebrain and from where part of the present data was obtained (in this case, 1.70mm posterior to the bregma). Gray filled area indicates the MePD location. MePV, posteroventral medial amygdala; opt, optic tract; st, stria terminalis. Scale bar = 500 µm. Adapted from the atlas of Franklin and Paxinos (1997). **(B,C)** Digitized photomicrographs of representative Golgi-impregnated spiny proximal dendrites of posterodorsal medial amygdala neurons of **(B)** control (WT mice) and **(C)** oxytocin knockout (OTKO) adult female mice. Arrows point to pleomorphic dendritic spines. Fine adjustments in background contrast and brightness were made in Image Pro Plus 7.0 and Photoshop 7.0 softwares (USA). Scale bar = 2.5 µm. (**D**) Mean (± SD; n=6 rats in each group) of the number of spines obtained in proximal dendrites of Golgi-impregnated neurons from the MePD of WT and OTKO females. * $P < 0.001$ compared to the WT group.

Figure 2. Number of oocytes from control (WT) and OT knockout (OTKO) female mice (n = 11 each). The Mann-Whitney test was used to compare the experimental groups, at a significance level of $P \leq 0.05$. * indicates a significant difference between groups. Data are expressed as median and interquartile range.

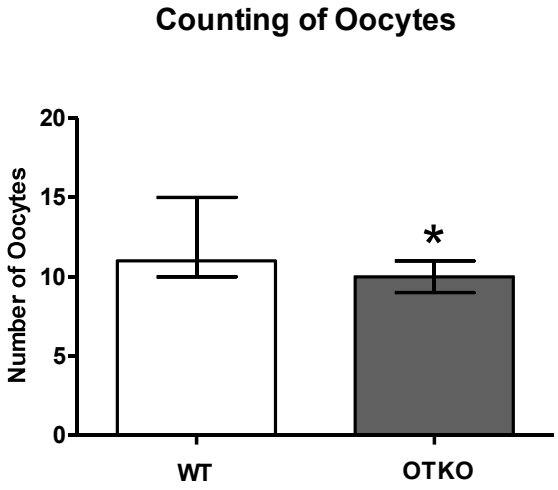
TABLE 1. Sexual Behavior of Female Mice

Parameters/Groups	WT	OTKO	*P*
Latency of Lordosis	307.0 (108.0/900.0)	900.0(900.0/900.0)	0.01
Frequency of Lordosis	1.0 (0.0/14.0)	0.0 (0.0/0.0)	0.02
Duration of Lordosis (s)	0.3 (0.00/13.9)	0.0 (0.0/0.0)	0.02
Quotient of Lordosis	0.4 (0.1/1.4)	0.0 (0.0/0.1)	0.02
Frequency of Mounts	22.0 (15.0/30.0)	11.0 (0.0/19.0)	0.07

FIGURE 1


A
st
opt
MePD
B
C
D
Dendritic Spines / µm
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
*
WT
OTKO

FIGURE 2



4. CONCLUSÕES

Com os resultados obtidos no presente estudo podemos concluir que a OT modula o comportamento sexual de camundongos fêmeas, pois fêmeas OTKO apresentam redução deste comportamento. Além disso, apresentam redução do número de oócitos e aumento da densidade de espinhos dendríticos proximais na AMePD (na fase de proestro). Outro ponto a ser destacado é que não existe diferença nas concentrações plasmáticas basais de AVP entre animais WT e OTKO.

5. PERSPECTIVAS

Pretende-se dar continuidade a este estudo realizando-se:

- Análise da densidade de espinhos nos primeiros 40 µm dendríticos dos neurônios da MePD de fêmeas OTKO nas outras fases do ciclo estral;

- Análise da densidade de espinhos nos primeiros 40 µm dendríticos dos neurônios do hipocampo e do hipotálamo de fêmeas OTKO na fase do proestro;

- Análise da capacidade de reconhecimento olfatório e memória social de camundongos machos e fêmeas com e sem déficit de OT;

- Análise da expressão gênica dos receptores de OT, AVP, estrogênio e dopamina em diferentes estruturas do SNC, de camundongos machos e fêmeas com e sem déficit de OT.

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Reference to a chapter in an edited book: [3] Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, Smith RZ, editors. Introduction to the electronic age, New York: E-Publishing Inc; 2009, p. 281–304.
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ANEXO 2: Parecer de Aprovação do Projeto pelo CEUA

Parecer Consubstanciado de Projeto de Pesquisa

Título do Projeto: Estudo do papel da ocitocina no comportamento sexual e reprodutivo de camundongos.

Pesquisador Responsável Marcia Giovenardi **Parecer** 920/09

Data da Versão 13/07/2009 | **Cadastro** 506/09 | **Data do Parecer** 13/08/2009

Grupo e Área Temática III - Projeto fora das áreas temáticas especiais

Objetivos do Projeto

- Geral: Estudar o papel da ocitocina (OT) na regulação do comportamento sexual e reprodutivo de camundongos machos e fêmeas.

Especificos: Analisar o efeito do deficit de ocitocina no comportamento sexual de camundongos fêmeas e machos;

Analisar as concentrações plasmáticas dos hormonios progesterona, estradiol, luteinizante, foliculo estimulante eprolactina, em machos e femes knockout para ocitocina;

Avaliar o número de oócitos presentes nos ovidutos das fêmeas, bem como a espermatogenese em machos de camundongos knockout para ocitocina;

Estudar o comportamento de memória social e interação social em machos com deficit de ocitocina.

Sumário do Projeto

Em mamiferos, a OT tem importante papel na reprodução de fêmeas e na modulação de diversos comportamentos como interação social, comportamento sexual, maternal, agressivo maternal. Sabe-se que diferentes vias de transdução de sinais regulam a expressão dos receptores de OT e o binding em cada região cerebral e podem, em parte, mediar a habilidade da OT em exercer seus efeitos comportamentais. A OT facilita a motivação social e o comportamento de aproximação e , também, parece ser fundamental em processos de memória social na discriminação de individuos familiares ou não.

Itens Metodológicos e Eticos	Situação
Título	Adequado
Autores	Adequados
Local de Origem na Instituição	Adequado
Projeto elaborado por patrocinador	Não
Aprovação no pais de origem	Não necessita
Local de Realização	Própria instituição
Outras instituições envolvidas	Não
Condições para realização	Adequadas

Comentários sobre os itens de identificação

Os experimentos serão realizados nos laboratórios de fisiologia e de fisiopatologia da hipertensão arterial sistêmica, UFSCPA.

Introdução	Adequada

Comentários sobre a introdução

Objetivos	Comentário

Comentários sobre os Objetivos

Pacientes e Metodos	
Delineamento	Adequado
Tamanho de amostra	Total 78 Local Lab
Cálculo do tamanho da amostra	Adequado
Participantes pertencentes a grupos especiais	Não
Seleção eqüitativa dos indivíduos participantes	Não se aplica
Critérios de inclusão e exclusão	Adequados
Relação risco- benefício	Não se aplica

Uso de placebo	Não utiliza
Período de suspensão de uso de drogas (wash out)	Não utiliza
Monitoramento da segurança e dados	Não se aplica
Avaliação dos dados	Adequada - qualitativa
Privacidade e confidencialidade	Não se aplica
Termo de Consentimento	Não se aplica
Adequação às Normas e Diretrizes	Sim

Comentários sobre os itens de Pacientes e Métodos

Cronograma	Adequado
Data de início prevista	09/09
Data de término prevista	12/11
Orçamento	Adequado
Fonte de financiamento externa	Agência de fomento

Comentários sobre o Cronograma e o Orçamento

O projeto será submetido a agências de fomento além de recursos que o pesquisador responsável já possui.

Referências Bibliográficas	Adequadas

Comentários sobre as Referências Bibliográficas

Recomendação

Aprovar

Comentários Gerais sobre o Projeto