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A ausência de ocitocina altera comportamentos sociais e o padrão de expressão gênica hipocampal e a experiência sexual influencia a morfologia dos espinhos dendríticos no hipocampo de camundongos machos

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Tese submetida ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Doutor

Orientadora: Profa. Dra. Márcia Giovenardi
Co-orientadora: Profa. Dra. Silvana de Almeida

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RESUMO

Os comportamentos sociais, essenciais para a manutenção das espécies, são modulados pelo neuropeptídeo ocitocina (OT). Importantes funções relacionadas aos comportamentos sociais tais como a motivação sexual, interação social, memória e respostas emocionais são moduladas por estruturas do sistema nervoso central (SNC), tais como bulbo olfatório (OB), hipotálamo (HPT), córtex pré-frontal (PFC) e hipocampo (HPC). OT, vasopressina (AVP), dopamina (DA) e estrogênio, bem como seus respectivos receptores (OTR, V1aR, V1bR, D2R, ERa e ERb) atuam sobre estas estruturas. Variações nos receptores e nos neurotransmissores se refletem na morfologia dos espinhos dendríticos de neurônios envolvidos e nos desfechos comportamentais observados nestes roedores. Este estudo teve como objetivo analisar o impacto do nocauteamento do gene da OT nos comportamentos sexual e de interação social; na síntese hipotalâmica de AVP e na expressão gênica dos receptores de OT, AVP, DA e estrogênio no OB, HPT, PFC e HPC. Além disso, analisar o impacto do nocauteamento do gene da OT e da experiência sexual na densidade e morfologia de espinhos dendríticos na área CA2 do HPC de camundongos machos. Camundongos C57BL/6J foram genotipados para o grupo controle (WT) e para o grupo nocaute para OT (OTKO). Os testes de comportamento sexual foram realizados com camundongos com experiência sexual compondo os grupos WT (n=13) e OTKO (n=12). Os testes de interação social utilizaram machos após uma semana de isolamento social para os grupos WT (n=8) e OTKO (n=11). Após os testes comportamentais, 6 camundongos WT e 8 OTKO foram eutanasiados e o sangue foi coletado para análise de AVP plasmática. Nossos resultados mostraram que no teste de interação social, OTKOs mostraram níveis mais baixos de comportamentos sociais, menor dominância e níveis mais elevados de comportamentos não-sociais que os WT. Na análise etológica, o grupo OTKO apresentou menor desempenho agressivo e maior investigação social que o grupo WT. Não foram observadas diferenças significativas no comportamento sexual, por outro lado, encontramos menores concentrações plasmáticas de AVP no grupo OTKO em comparação com o grupo WT. Para a análise da expressão gênica, camundongos foram genotipados e alocados no grupo WT (n=10) e no grupo OTKO (n=10) e tiveram suas estruturas encefálicas (OB, HPT, PFC e HPC) coletadas. Para a expressão gênica utilizou-se a extração de RNAm, síntese de cDNA e PCR real time como técnicas empregadas. Machos OTKO apresentaram redução significativa nos níveis de transcrição de AVP no HPT e aumento significativo de expressão gênica de ERb no PFC. No HPC, o grupo OTKO apresentou expressão gênica de OTR aumentada e expressão gênica de

D2R e V1bR diminuídas, se comparados ao grupo WT. Por fim, para a análise de densidade e morfologia de espinhos dendríticos, lâminas de encéfalo preparadas com a técnica de Golgi foram analisadas na área CA2. Quatro grupos foram utilizados: WT (n=5) e OTKO (n=6) virgens e WT (n=5) e OTKO (n=5) com experiência sexual. Os espinhos dendríticos dos primeiros 10 μm de dendritos proximais de neurônios piramidais de CA2 foram desenhados ao longo dos diferentes planos focais em "z". Para cada macho, 2-8 dendritos diferentes foram estudados com 1 dendrito por neurônio amostrado. Os 3 principais tipos de espinhos (finos, cogumelos, largos) foram identificados e contados a partir dessas amostras. Os resultados mostram que a experiência sexual reduziu a quantidade de espinhos largos na área CA2, e o grupo OTKO sexualmente experiente apresentou menor redução destes espinhos que os animais WT. Nossos principais achados nos permitem inferir que a OT é importante para o correto desfecho do comportamento social, porém que a falta dela não altera o comportamento sexual de camundongos machos. Além disso, a ausência da OT no SNC está relacionada com uma redução nas concentrações de AVP, visto que a concentração plasmática de AVP e a expressão gênica no HPT dos OTKO apresentaram-se diminuídas. Além disso, a falta de OT interfere principalmente no HPC, onde a expressão gênica de OTR, D2R e V1bR apresentaram-se alteradas. Por fim, a experiência sexual modula os espinhos dendríticos da CA2 tanto em WT quanto em OTKO, porém esta adaptação mostrou-se menos efetiva no grupo OTKO.

Palavras-chave: vasopressina, estrogênio, dopamina, córtex pré-frontal, hipotálamo.

ABSTRACT

The neuropeptide oxytocin (OT) modulates social behaviors, which are essential for species maintenance. Important functions related to social behaviors such as sexual motivation, social interaction, memory and emotional responses are modulated by structures of the central nervous system (CNS), such as olfactory bulb (OB), hypothalamus (HPT), prefrontal cortex (PFC) and hippocampus (HPC). OT, vasopressin (AVP), dopamine (DA) and estrogen, as well as their respective receptors (OTR, V1aR, V1bR, D2R, ERa and ERb) exert effects on these structures. Variations in the receptors and neurotransmitters are reflected in the morphology of dendritic spines and in the behavioral outcomes found in these rodents. This study aimed to analyze the impact of OT gene knockout on sexual and social interaction behaviors, in the hypothalamic synthesis of AVP and in the gene expression of OT, AVP, DA and estrogen receptors in OB, HPT, PFC and HPC. Also, we aimed to analyze the impact of OT gene knockout and sexual experience on the density and morphology of dendritic spines in the hippocampal area CA2 of male mice. C57BL / 6J mice were genotyped for the control group (WT) and for the OT knockout group (OTKO). Sexual behavior tests were performed on sexually experienced mice, composing the WT (n = 13) and OTKO (n = 12) groups. Social interaction tests used males after one week of social isolation for the WT (n = 8) and OTKO (n = 11) groups. After the behavioral tests, 6 WT and 8 OTKO mice were euthanized and blood was collected for plasma AVP analysis. Our results showed that in the social interaction test, OTKOs showed lower levels of social behaviors, lower dominance and higher levels of non-social behaviors than WT. In the ethological analysis, the OTKO group presented lower aggressive performance and greater social investigation than the WT group. No significant differences were observed in sexual behavior. On the other hand, we found lower plasma concentrations of AVP in the OTKO group compared to the WT group. For the analysis of gene expression, mice were genotyped and allocated to the WT (n = 10) and OTKO (n = 10) groups and their encephalic structures (OB, HPT, PFC and HPC) were collected. Gene expression was analyzed using mRNA extraction, cDNA synthesis and real time PCR as techniques employed. OTKO males showed a significant reduction in the levels of transcription of AVP in HPT and a significant increase of ERb gene expression in the PFC. In HPC, the OTKO group showed increased OTR gene expression and decreased D2R and V1bR gene expression, when compared to the WT group. Finally, for the analysis of density and morphology of dendritic spines, slices with brains sections prepared with the Golgi technique were analyzed in the CA2 area. Four groups were used: WT (n = 5) and OTKO (n =

6) virgin and WT (n = 5) and OTKO (n = 5) with sexual experience. The dendritic spines of the first 10 μm of proximal dendrites of CA2 pyramidal neurons were analyzed in light microscope and drawn. For each male, 2-8 different dendrites were studied with 1 dendrite per sampled neuron. The 3 main types of spines (fines, mushrooms, stubby/wide) were identified and counted from these samples. The results showed that the sexual experience was able to reduce the amount of stubby/wide spines in the CA2 area, and the sexually experienced OTKO group presented a smaller reduction of these spines than the WT animals. Our main findings allow us to infer that OT is important for the correct outcome of social behavior, but that lack of it does not alter the sexual behavior of male mice. In addition, the absence of OT in the CNS is related to a reduction in the concentrations of AVP, since the plasma concentration of AVP and the gene expression in the HPT of the OTKO were decreased. In addition, the lack of OT interferes mainly with HPC, where the gene expression of OTR, D2R and V1bR has been altered. Finally, the sexual experience modulates the dendritic spines of CA2 in both WT and OTKO, but this adaptation proved to be less effective in the OTKO group.

Keywords: vasopressin, estrogen, dopamine, prefrontal cortex, hypothalamus.

LISTA DE ABREVIATURAS

AVP	Vasopressina
BNST	Núcleo do leito da estrial terminal
CA	Corno de Amoon
CoA	Núcleo cortical da amigdala
D1R	Receptor de dopamina 1
D2R	Receptor de dopamina 2
DA	Dopamina
ERa	Receptor de estrógeno *alpha*
ERb	Receptor de estrógeno *beta*
HPC	Hipocampo
HPT	Hipotálamo
MeA	Núcleo medial da amigdala
MPOA	Área pré optica medial
OB	Bulbo olfatório
OT	Ocitocina
OTR	Receptor de ocitocina
OTKO	Camundongos *knockout* para o gene da ocitocina
PFC	Córtex pré-frontal
PV	Parvalbumina
PVN	Núcleo hipotalâmico paraventricular
SC	Via colateral de Schaffer
SNC	Sistema nervoso central
SON	Núcleo hipotalâmico supraóptico
V1aR	Receptor de vasopressina 1a
V1bR	Receptor de vasopressina 1b
VMHvl	Divisão ventrolateral do hipotálamo ventromedial
WT	Camundongos *wild type*

SUMÁRIO

1 REVISÃO DA LITERATURA

1.1 Introdução

A evolução molda mecanismos cognitivos e neurais, tornando-os projetados para encontrar soluções adaptativas e garantir a sobrevivência das espécies. Os padrões comportamentais de um indivíduo são oriundos da sua interação com o ambiente e com outros indivíduos (1). As respostas comportamentais ao ambiente são definidas como comportamentos não sociais (comportamento de manutenção ou de exploração), e aquelas entre indivíduos, como comportamentos sociais (2).

O reconhecimento e a interação social entre os animais são habilidades cruciais para a sobrevivência e a vida em grupo (3). As ligações sociais entre os membros de um grupo possuem várias vantagens, entre elas a de garantir a defesa da espécie contra a predação. Nos mamíferos, o comportamento social de machos e fêmeas apresenta diferentes estratégias reprodutivas: o sucesso reprodutivo nos machos é determinado por competição com outros machos para se acasalar com tantas fêmeas quanto possível. Assim, os machos são tipicamente hierárquicos, com o comportamento agressivo sendo determinante para esta condição (4). Quando dois roedores machos são colocados juntos, eles engajam-se em interações sociais, que incluem vários comportamentos, dentre eles cheirar, perseguir, morder, atacar, chutar e boxear (5). Dados experimentais sugerem que as diferentes formas de interações sociais podem ser mediadas por sistemas neurais distintos e que o status de isolamento (ou não) e a familiaridade (ou não) ao ambiente em que o teste foi realizado podem interferir nos comportamentos sociais (6).

Neste contexto dos comportamentos sociais, a ocitocina (OT) atua como um neurotransmissor/neuromodulador para modular diversas funções do sistema nervoso central (SNC), tanto em machos quanto em fêmeas (7–10).

1.2 Ocitocina

A OT é um hormônio classicamente conhecido pela sua função no parto e na lactação, funções descobertas por Dale, em 1906. Por este fato, a palavra “ocitocina” foi criada a partir da união de duas palavras gregas que significavam “nascimento rápido” (11). A OT é um nonapeptídeo hidrossolúvel que possui uma ponte cys-cys nas posições 1-6, importante para o

reconhecimento e ligação do hormônio ao receptor. Estruturalmente assemelha-se à vasopressina (AVP), diferindo-se desse por apenas dois aminoácidos (12,13). Os genes que codificam a OT e a AVP são altamente homólogos e localizados no mesmo cromossomo, mas com orientação transcricional oposta (14). A distância entre esses genes variam de 3 a 12 kb em camundongos (15), humanos (16) e ratos (17).

A OT é liberada para a circulação sanguinea e para o encéfalo. Quando este peptídeo é liberado perifericamente, sua produção ocorre nos neurônios magnocelulares do núcleo paraventricular (PVN) e do núcleo supra-óptico (SON) do hipotálamo (HPT). Os axônios de neurônios magnocelulares destes núcleos são projetados para neurohipófise, formando assim o sistema hipotálamo-neurohipofiseal (18). Já a OT liberada para o SNC é produzida, em menores quantidades, nos neurônios parvocelulares do PVN e, dependendo da espécie, no núcleo do leito da estria terminal (BNST), na área pré-óptica medial (MPOA) e amígdala lateral (14). Em roedores, os neurônios ocitocinérgicos da região parvocelular do PVN projetam-se para várias áreas cerebrais, dentre elas: núcleo hipotalâmico dorsomedial, núcleos talâmicos, hipocampo (HPC), subículo, cortex entorrinal, núcleos septais lateral e medial, amígdala, bulbo olfatório (OB), substância negra, locus ceruleus, núcleo da rafe, núcleo do trato solitário (11, 19).

Muitos estudos têm acumulado evidências de que, além das funções hormonais clássicas, a OT e a AVP desempenham papéis críticos nos comportamentos sociais em mamíferos (2,20,21). A OT possui importante papel nas interações sociais, como no comportamento maternal (22) e no agressivo maternal (7,9). Engelmann et al. (2000) sugerem que a OT também pode ser liberada durante a escolha e a formação de pares sexuais e que este peptídeo, associado à AVP, pode agir influenciando comportamentos relacionados ao estresse, ao aprendizado e a memória (23). A OT facilita a motivação social, o comportamento de aproximação e, também, parece ser fundamental em processos de memória social no que se refere à discriminação de indivíduos familiares ou não (24).

Estudos realizados com camundongos nocautes para o gene da OT (OTKO) mostraram que esta é essencial para o reconhecimento social e pode influenciar a aquisição de memória (25–27). Ferguson et al. (2001) demonstraram que animais OTKO são incapazes de reconhecer entre indivíduos conhecidos e desconhecidos em testes de habituação-desabituação, e que o tratamento de reposição de OT na amigdala medial (MeA) restaura completamente o reconhecimento social destes animais (28).

Um estudo anterior do nosso laboratório (29) analisou os comportamentos de investigação social e comportamento agressivo em camundongos machos OTKO por meio do

teste de interação social. Este estudo demonstrou que machos OTKO apresentam uma frequência aumentada de investigação oronasal e anogenital em comparação com o tipo selvagem (WT). Por outro lado, o grupo OTKO demonstrou um desempenho agressivo menor do que o grupo WT, comprovando a importância deste peptídeo para o comportamento hierárquico social. No que se refere ao comportamento sexual, machos OTKO não apresentaram alterações comportamentais e fêmeas OTKO apresentam comportamento sexual diminuído (29,30).

1.2.1 Receptor de Ocitocina

Somente um tipo de receptor para OT é reconhecido e clonado (13). Este receptor está amplamente distribuído no encéfalo, variando a localização de acordo com a espécie e o gênero. O receptor de ocitocina (OTR) pertence à família dos receptores heterotriméricos acoplados à proteína G, sendo expresso por diversos tipos de células, incluindo neurônios, células ósseas, mioblastos, cardiomiócitos e células endoteliais (19). Ele pode estar associado tanto a proteínas G do tipo Gq/11 como as proteínas Gi, e seus efeitos intracelulares dependem do tipo de acoplamento (31,32). Receptores associados à proteína Gq/11 possuem caráter excitatório e receptores associados à proteína Gi têm caráter inibitório (33).

As diferentes expressões do OTR no encéfalo podem explicar as variações comportamentais (como a monogamia ou poligamia) observadas em diferentes subespécies de roedores (34). Diferentes vias de transdução de sinais regulam a expressão do OTR e a ligação em cada região cerebral e podem, em parte, mediar a habilidade da OT para exercer diversos efeitos comportamentais (35). Em roedores, a distribuição de OTR ocorre principalmente no OB, núcleos basais, córtex piriforme, córtex insular e perirrinal, formação hipocampal, amígdala central, BNSP lateral e medial, septo lateral, núcleo accumbens e HPT ventromedial, núcleo do trato solitário e área tegmental ventral, complexo mamilar, núcleo olivar dorsal, núcleo espinal trigeminal, colículo superior do tronco encefálico e medula espinal (34,36–38).

Mitre et al (2016) quantificaram os níveis de expressão de OTR em 29 regiões cerebrais, escolhidas com base na sua importância no comportamento social. As regiões incluíram HPT, regiões neocorticais como o córtex piriforme e auditivo, amígdala, sub-regiões do HPC, córtex frontal, OB e núcleo mediano da rafe. As análises foram feitas em camundongos machos e fêmeas virgens e fêmeas mães e detectaram que as mães apresentam níveis de OTR maiores que os padrões encontrados em camundongos virgens (39).

A análise microscópica eletrônica revelou que os OTR estavam localizados em sinapses excitatórias e inibitórias (pré e pós-sinapticamente), ao longo de axônios e expressos por células gliais. O efeito predominante da modulação da OT via OTR, foi de reduzir a transmissão inibitória sem afetar diretamente a excitação. Assim, os autores sugeriram que a modulação da inibição pudesse ser um mecanismo geral pelo qual a OT pode atuar em todo o encéfalo para regular os comportamentos parentais e a cognição social (39).

A expressão dos OTR em células gliais é um dado interessante que traz outras possibilidades de modulação à liberação de OT. O fato de a glia apresentar OTRs, pode permitir que estas células detectem os níveis de OT no SNC ou no sangue, possibilitando que elas regulem a libertação de OT e de outros neurohormônios (40,41).

1.3 Neurotransmissores envolvidos na modulação da interação social e do comportamento sexual

1.3.1 Vasopressina

Além de muitos estudos relacionando comportamento social e OT, não podemos ignorar que uma série de neurotransmissores e neuropeptídeos têm sido relacionados aos elementos neurobiológicos da cognição social (42,43). Junto com a OT, o peptídeo mais estreitamente relacionado com comportamentos sociais é a AVP (44). As ações da AVP sobre o comportamento social são mediadas por dois subtipos de receptores específicos: receptor de AVP 1a (V1aR) e receptor de AVP 1b (V1bR) (45). Enquanto V1aR é expresso em várias áreas cerebrais (OB, amígdala, córtex piriforme, HPT, órgão vomeronasal, subículo do ventrículo, área tegmental ventral) (4), os V1bR de camundongos, ratos e seres humanos estão mais discretamente localizados, com proeminência em células piramidais da região CA2 do HPC e em algumas células dentro da amígdala anterior e PVN (Para uma revisão, ver (45)).

Sabe-se que os camundongos machos nocautes para V1aR apresentam uma completa interrupção no reconhecimento social, sem apresentarem prejuízos na aprendizagem não-social e em tarefas de memória. Isto sugere que as ações de AVP via V1aR são específicas no reconhecimento de odores sociais (46). Já trabalhos prévios com camundongos nocautes V1bR, relacionaram a falta de V1bR com déficit significativo de reconhecimento social e memória social, déficit de motivação para interagir com estímulos sociais e deficiências no comportamento agressivo em contextos sociais específicos (45,47).

Além do papel da AVP no comportamento social, estudos têm descrito a importância das projeções vasopressinérgicas em relação ao comportamento sexual (22). Uma das estruturas que parece estar envolvida no controle do comportamento sexual masculino é o BNST, pois os machos têm mais neurônios AVP e projeções mais densas nessa área e no núcleo amidaloide medial do que as fêmeas (48). A AVP influencia a reprodução e o comportamento do macho e está envolvida na ereção e ejaculação em várias espécies, incluindo ratos e coelhos (49); a AVP também medeia uma variedade de comportamentos sociais típicos masculinos, incluindo agressão e territorialidade em várias espécies (50).

Além disso, como a cópula libera OT e AVP, uma possibilidade é que estes neuropeptídeos estejam envolvidos no processo de formação de pares após o acasalamento (51). Estudos prévios demonstraram que a administração de AVP estimula os comportamentos associados à monogamia, tais como cuidados paternos, guarda de companheiros e uma preferência seletiva para o companheiro em ratos de pradaria. Tratamento semelhante não induz estes comportamentos em espécies não monogâmicas [para revisão, ver (50)], provavelmente pelo fato de que distribuição de receptores de AVP entre essas espécies é tão divergente quanto seu comportamento social. O simples aumento da expressão do receptor V1aR dentro do circuito de recompensa no encéfalo de ratos da montanha permite que indivíduos desta espécie não monogâmica formem uma preferência seletiva pelo seu companheiro, indicando que os padrões de V1aR influenciam o repertório sócio comportamental de uma espécie (52,53).

1.3.2 Dopamina

A existência de interação positiva entre dopamina (DA) e OT no comportamento social, em distúrbios associados como autismo, disfunção sexual, dependência e depressão tem sido mais discutida atualmente (54). Modelos animais parecem indicar a existência de circuitos cerebrais amplos e integrados onde as interações DA e OT, pelo menos em parte, medeiam comportamentos sociais. Tanto OT como DA desempenham um papel dentro do OB no reconhecimento social; no entanto, a DA, especialmente através dos receptores do tipo 2 de DA (D2R), desempenha um papel mais proeminente na consolidação da memória do que no reconhecimento em si (51).

Em ratas da pradaria o bloqueio de D2R antes do teste de preferência pelo parceiro não interfere no reconhecimento do parceiro em si, mas o bloqueio deste logo após o

acasalamento inibe a consolidação da memória de reconhecimento do parceiro (55). Em machos da pradaria, a ativação DA via D2R no núcleo acumbens é crítica para a formação de pares, já a ativação dos receptores tipo 1 (D1R) impede as preferências dos parceiros por seus pares (56).

Em um trabalho recente, Rocchetti el al. (2015) descobriram que a deleção de D2R diminui severamente o desempenho de ratos em tarefas de aprendizagem e de memória e prejudica a plasticidade sináptica na região CA1 do HPC (57). A expressão pós-sináptica D2R é restrita ao giro denteado, onde estes ativam vias de sinalização múltipla (58). Já as fibras dopaminérgicas portadoras de D2R pré-sinápticos inervaram as áreas temporais de CA1 (57), onde exercem um controle inibitório sobre a síntese e liberação de DA (59).

As conexões entre DA e OT também estão envolvidas nas interações sócio sexuais. No acasalamento, as conexões de DA e OT estão envolvidas tanto na ereção do pênis quanto nos comportamentos subsequentes relacionados com a recompensa do comportamento sexual (54). Durante a excitação sexual, a estimulação do sistema mesolímbico de DA, envolvido nos efeitos motivacionais da atividade sexual, ocorre via OT. A OT liberada na área tegmental ventral, subículo ventral e amígdala, ativa neurônios DA mesolímbicos, culminando na ativação das vias de recompensa (60). Já durante o acasalamento, a liberação de OT na amigdala, HPC e área tegmental ventral facilita o aprendizado e a memória social e estimula as projeções de recompensa dopaminérgicas mesolímbicas, que projetam-se para a núcleo acumbens e PFC. A DA no núcleo acumbens pode ser ativada para modular a ligação de pares (54).

1.3.3 Estrogênio

Além das funções clássicas descritas para o estrogênio, outro papel para este hormônio está surgindo neste contexto. A regulação estrogênica de diversos comportamentos sociais tem sido investigada em camundongos nocautes para os receptores de estrogênio alfa (ERa) e beta (ERb). Machos nocautes para ERa e ERb mostraram fenótipos comportamentais opostos quando testados para o comportamento agressivo: enquanto os nocautes ERa apresentaram menor comportamento agressivo (61), os nocautes ERb são mais agressivos do que seus respectivos controles (27). Já em testes de reconhecimento e memória, machos nocautes para o ERa mostraram reconhecimento normal, mas retenção de memória de discriminação social prejudicada. Os nocautes ERb não mostraram qualquer prejuízo no reconhecimento social em qualquer dos testes [para uma revisão, ver (42)].

Embora, Choleris et al. (2012) sugira que os receptores de estrogênio parecem facilitar as ações da OT com relação ao reconhecimento social (42), ainda não existe um concenso sobre o mecanismo exato da ação do estrogênio no comportamento social. Neste trabalho, os autores propõem que esta ação regularia genes ou sistemas de neurotransmissores que estão implicados na aprendizagem social. Estes sistemas incluem: OT, DA, peptídeos opióides, acetilcolina, fatores neurotróficos e progesterona. Um exemplo desta ação modulatória ocorre na amígdala medial, onde ERa induz a transcrição do gene de OTR e no HPT, onde a síntese de OT é modulada pela ativação de Erb (42). Assim, qualquer desbalanço em ERa ou ERb pode resultar em modificações sobre a síntese ou a ação de OT e desta forma, alterar os comportamentos sociais (62).

1.4 Circuitos neurobiológicos envolvidos na modulação da interação social e do comportamento sexual

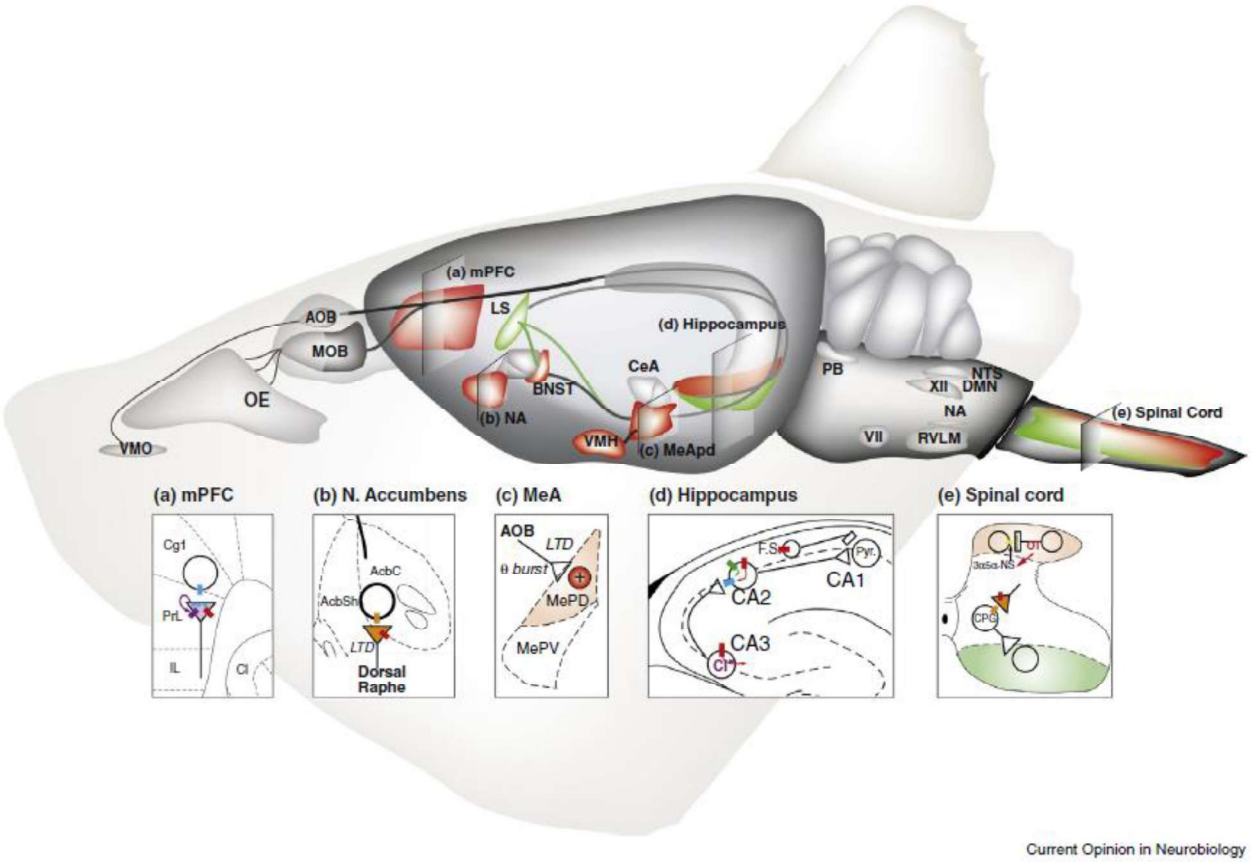

FIGURA 1 Neuromodulação dos circuitos comportamentais pela OT e AVP: Regiões expressando receptores para OT (em vermelho) e para AVP (em verde) no encéfalo de roedores e suas respectivas conexões. (a) PFC medial, mostrando a expressão de OTR

(vermelho) em neurônios que regulam a liberação de glutamato (azul). A OT atua ativando receptores pré-sinápticos canabinoides CB1 (roxo). (b, c, e) OTRs (vermelho) expressos em neurônios do núcleo accumbens, amigdala medial e medula espinal. (d) O hipocampo, que expressa os receptores de OT e AVP em diferentes níveis: na região CA3, OT afeta o potencial de equilíbrio, em CA2 onde os agonistas de OT e V1b R melhoram a transmissão sináptica glutamatérgica e na região CA1 onde OTRs afetam a transmissão excitatória em neurônios piramidais.

Fonte: http://www.sciencedirect.com/science/article/pii/S0959438814002001

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1.4.1 Bulbo Olfatório

A olfação em roedores envolve dois órgãos sensoriais: o OB principal, que está envolvido no olfato geral, e o órgão vomeronasal. Acredita-se que o reconhecimento individual envolva a detecção de feromônios pelo órgão vomeronasal, que projeta-se para o OB acessório e depois para MeA e núcleo cortical da amígdala (CoA) (63). O OB acessório, MeA e CoA são particularmente ricos em receptores de OT e são, portanto, locais candidatos para a ação da OT na formação de memória social em camundongos (4).

Dentro de uma mesma espécie, a formação de pares sociais e os comportamentos reprodutivos, dependem da capacidade de reconhecer indivíduos familiares ou não-familiares (para revisão, ver (21)). Em roedores, o OB principal e o acessório são ativados nos encontros sociais e são cruciais para este reconhecimento, sendo o OB acessório o responsável por detectar os feromônios, transmitindo a informação para iniciar um comportamento específico ou respostas endócrinas (64,65).

Ratos de pradaria servem como um excelente modelo para o estudo do comportamento sexual e das funções neuroendócrinas relacionadas (56). Quando ratos da pradaria não familiarizados se encontram, eles se envolvem em repetidas investigações olfatórias da região genital do parceiro, seguidas por períodos prolongados de grooming. Durante esse tempo, os feromônios urinários do macho, administrados ao epitélio olfatório da fêmea, ativam diretamente múltiplos processos neuroendócrinos e induzem a fêmea a seu primeiro estro. Dentro de 48 h a fêmea é sexualmente receptiva e libera seus feromônios para se engajar em repetidas interações copulatórias com o macho, que responde ao estímulo sexual realizando as montas (66).

Embora o OB não possua terminais ocitocinérgicos, ele apresenta OTR em abundância. Este desajuste de terminais com receptores é funcionalmente abordado pela

liberação neurohumoral de OT no fluido cerebrospinal, em situações como o parto e o acasalamento (4,67). Estes eventos biológicos produzem mudanças significativas na sensibilidade, eficácia sináptica e ativação neural no OB, que fazem parte do processo de aprendizagem olfativa para a familiaridade social (65,68).

1.4.2 Hipotálamo

O HPT é a região responsável por adicionar o componente endócrino aos mecanismos comportamentais, uma vez que os efeitos dos hormônios sexuais nas propriedades eletrofisiológicas dos neurônios, a transcrição do RNAm e a síntese de novas proteínas e neuropeptídios como a OT e a AVP, são primariamente mediados pelo HPT (69).

Décadas de estudos envolvendo lesão e estimulação identificaram o HPT como um centro responsável pelo controle da agressão em machos [para revisão, ver (70)]. Em gatos, bem como em roedores, este estudos comprovaram que o HPT era crítico para o comportamento de ataque (71,72). Um estudo de Lin et al. (2011) descreveu a divisão ventrolateral do HPT ventromedial (VMHvl) e o comportamento agressivo de camundongos machos (73). Por outro lado, Scott et al. (2015) demonstraram que neurônios do núcleo periventricular anteroventral do HPT que expressam tirosina hidroxilase projetam diretamente para neurônios OT no PVN e, quando estimulados, aumentam a liberação de OT na circulação (74).

Um estudo de Yang et al. (2013) identificou que o silenciamento do receptor para progesterona no VMHvl levou à redução significativa na agressão, bem como déficits específicos no comportamento sexual dos machos (75). No entanto, os machos com este silenciamento de VMHvl ainda conseguiam distinguir entre os sexos e também marcar seu território, indicando que os déficits comportamentais no acasalamento e na agressão não representavam déficits generalizados nos comportamentos sociais (75). Um estudo relacionado (76) demonstrou que a estimulação de uma população sobreposta de neurônios na VMHvl e que expressa ERa provocou manifestações sexuais dos machos em relação tanto aos camundongos fêmeas quanto aos machos, enquanto a estimulação mais forte gerou ataques direcionados a ambos os sexos.

A área cerebral mais sensível para a indução da ereção peniana pela OT é o PVN, a OT injetada nesta área é capaz de induzir a ereção peniana em doses tão baixas quanto 3 pmol (60). Quanto ao mecanismo pelo qual a OT atua no PVN para induzir essa resposta sexual, estudos sugerem que a OT ativa os seus próprios neurônios; de acordo com essa hipótese, a

interação sexual aumenta a expressão do c-*Fos* em neurônios ocitocinérgicos do PVN que se projetam para medula espinal e estão envolvidos no controle da ereção peniana (60,77). Além dos papeis já bem descritos do HPT em relação aos comportamentos agressivo e sexual, sabe-se que a infusão de OT em MPOA de ratos melhora o comportamento de reconhecimento social (3).

1.4.3 Córtex pré-frontal

Segundo Fuster (2001), o PFC não se encontra envolvido exclusivamente em processos cognitivos, pois a região órbito-frontal está relacionada com aspectos emocionais do comportamento, além do controle inibitório. Já o PFC medial é uma região envolvida na tomada de decisões, na flexibilidade comportamental, além de ser mediador potencial de inibição comportamental (78). O PFC medial contém neurônios sensíveis a OT, expressa em abundância OTRs e recebe projeções axonais de longo alcance de OT produzida no HPT (34). Na camada V do PFC medial, a OT suprime a neurotransmissão glutamatérgica em neurônios piramidais através de uma ativação pré-sináptica de receptores canabinoides CB1 (Figura 1a). Em vista das projeções do PFC para neurônios inibitórios na amigdala central, é possível que esses efeitos de OT trabalhem em conjunto com os efeitos excitatórios de OT na CeA em neurônios inibitórios (79).

Davis et al. (2010), testaram o papel do PFC medial na inibição do comportamento sexual quando associada a resultados aversivos. Os pesquisadores descobriram que as lesões do PFC medial resultam em um comportamento sexual compulsivo, independente se este foi associado com recompensa ou estímulos aversivos. Estes animais provavelmente eram incapazes de suprimir a busca de recompensa sexual em face de consequências aversivas. Estes dados sugerem um papel do PFC medial na regulação da busca compulsiva de recompensa (80).

As alterações diretas do equilíbrio excitação/inibição dentro do PFC em camundongos adultos têm um forte efeito sobre a motivação social [para uma revisão, ver (81)]. Yizhar et al. (2011) utilizaram a optogenética para manipular de forma independente a atividade de neurônios piramidais excitatórios e interneurônios inibitórios de parvalbumina (PV) do PFC durante uma tarefa de exploração social e no teste de sociabilidade. Eles descobriram que, estimulando neurônios piramidais no PFC, a exploração social foi abolida e a preferência social foi comprometida. Os efeitos de uma excitação aumentada foram melhorados ao estimular simultaneamente os interneurônios PV, mostrando que uma razão excitação/inibição

apropriada no PFC é necessária para a motivação social em camundongos (82). O aumento da razão excitação/inibição no PFC leva também a déficits no reconhecimento social, já a retenção de memórias sociais é aumentada ativando interneurônios inibitórios (83).

Um estudo recente examinou a atividade cerebral total através a expressão da *Fos* num contexto social e, descobriu que o PFC de camundongos foi ativado na interação social (84). Em camundongos, a dominância também parece estar ligada ao microcircuito no PFC. Wang et al. (2011) observaram que alterar a eficácia da transmissão sináptica no PFC provoca uma modulação bidirecional da hierarquia social (85). Especificamente, o aumento da excitabilidade aumenta a posição do camundongo dentro da hierarquia e atenuar a eficácia da transmissão sináptica diminui sua classificação. Por outro lado, a ativação optogenética do PFC medial em camundongos diminui o comportamento agressivo, enquanto silenciar esta região leva a uma escalada de agressão (86). Esse achado é interessante à luz dos achados de Wang et al., porque esses estudos juntos demonstram uma regulação contrária da agressão e da dominância pela atividade de PFC (81).

1.4.4 Hipocampo

O HPC faz parte do sistema límbico, o qual participa do processamento de respostas adequadas ao contexto em que o animal está inserido, baseado em aprendizados anteriores. Indicações químicas, tais como odor, medeiam interações sexuais e competitivas e são importantes no reconhecimento e seleção de pares (87–89). Os odores são processados por vias olfatórias e vomeronasais, que projetam-se diretas e indiretas para o MeA (67) com uma entrada relativamente menor para o BNST (44). O MeA envia grandes projeções para o BNST e MPOA, que por sua vez projetam para o septo lateral e HPC, responsável pelo processamento da memória social (30,44).

O HPC propriamente dito consiste na região de *Cornu Ammonis* (CA), denominação em latim para Corno de Ammon, que se trata de uma tira de neurônios piramidais e pelo giro dentado, que consiste em células granulares. A CA e o giro denteado estão dispostas anatomicamente numa estrutura enrolada, que constitui a formação hipocampal (90). A formação hipocampal de mamíferos é essencial para a aprendizagem e memória, exerce importantes funções relacionadas à conversão da memória de curto prazo em memória de longo prazo, na orientação espacial, no processamento do medo, na regulação da resposta ao estresse e nos comportamentos sociais (90).

Os primeiros neuroanatomistas descreveram duas áreas distintas do CA em encéfalos de roedores: a porção superior, que consistia em pequenos neurônios piramidais (regio superior de Cajal) e a porção inferior, que consistia de neurônios piramidais maiores (regio inferior de Cajal). No entanto, em 1934, Rafael Lorente de Nó notou que uma pequena área do regio inferior era suficientemente distinta em sua citoarquitetura e conectividade para justificar uma nomenclatura separada. Por essa razão, ele designou três áreas CA contendo neurónios piramidais como CA1, CA2 e CA3 (91). Lorente de Nó observou que os corpos celulares piramidais do CA2, como os do CA3, são maiores que os encontrados no CA1. No entanto, observou que os dendritos de células piramidais CA2 não possuem as excrescências espinais especializadas associadas à entrada de fibras musgosas do giro denteado, que são características dos neurônios piramidais CA3 (90).

A região CA1 do hipocampo recebe potente entrada excitatória de CA3 através da via colateral de Schaffer (SC). A ativação de axônios SC evoca um potencial pós-sináptico excitatório monossináptico sobre as células piramidais de CA1, assim como excita uma variedade de interneurônios CA1. Estes interneurônios, em seguida, entregam um potencial pós-sináptico inibitório um milissegundo atrasado, causando uma inibição direta. Deste modo, tanto o limiar de estimulação como o tempo de picos evocados nas células piramidais CA1 por ativação de SC são ditados por um balanço finamente sintonizado de entradas monossinápticas excitadoras e dissinápticas inibitórias (92,93).

No trabalho de Owen et al. (2013), demonstrou-se que a ação da OT neste contexto ocorre especificamente em interneurônios de disparo rápido e pode ser orientada para alterar a função de rede através de um ajuste fino da inibição de fundo. Através da utilização de um agonista para OTR, o aumento da atividade de interneurônios de disparo rápido não apenas suprime a ativação espontânea de células piramidais, mas também melhora a fidelidade da transmissão sináptica e agudiza o sincronismo dos picos (Figura 1d) (93). Os interneurônios de disparo rápido estimulados pela OT são fisiológica e funcionalmente distintos dos interneurônios regulares, que desempenham um papel importante na inibição e cuja produção é regulada por endocanabinoides (94). Nesta segmentação seletiva de populações de interneurônios, neuromoduladores como a OT e endocanabinóides podem ser especializados para esculpir diferentes formas de inibição (93).

Apesar de as ações da OT terem sido elucidadas em experimentos envolvendo CA1, sabe-se que a área CA2 também é rica em interneurônios inibitórios. Porém, estudos apontam que os interneurônios de CA2 podem possuir um funcionamento diferente daqueles interneurônios das áreas vizinhas CA1 e CA3 (90).

1.4.4.1 Região CA2 do hipocampo

A região CA2 foi descrita em 1934, mas pouco se soube sobre sua função até recentemente, como se esta região houvesse sido negligenciada do circuito hipocampal até então, talvez devido à sua pequena área em comparação com as outras subdivisões hipocampais (95,96). Atualmente, a área CA2 atraiu o interesse dos pesquisadores, pois em comparação com os neurônios de CA1 e CA3, os neurônios em CA2 são relativamente resistentes aos danos que surgem durante o curso de várias doenças, incluindo epilepsia, hipóxia, desordens vasulares e esquizofrenia (97,98). Além destes achados, descobriu-se que, aliado à resistência aumentada a danos, os neurônios de CA2 também apresentam resistência à plasticidade sináptica, quando comparados às outras regiões do HPC (99).

Estas peculiaridades da região CA2 intrigaram pesquisadores das mais diversas áreas e, recentemente, esta região tem sido mais estudada. Estudos anatômicos, de expressão gênica, de influências neuromoduladores, de vias de sinalização celular e de eletrofisiologia vêm demonstrando as propriedades desta área (96,100).

As principais aferências para CA2 são: neurônios vasopressinérgicos do PVN, o núcleo supramamilar, a rafe medial, o septo medial e diagonal, o córtex entorrinal, o giro denteado e CA3. Já o principal alvo das eferências da área CA2 é o CA1. Os neurônios piramidais CA2 projetam principalmente para a área *stratum oriens*, coberta pelos dendritos basais de neurônios piramidais CA1. As projeções do CA2 a CA1 tem uma vasta extensão caudal ao longo do eixo longitudinal do HPC de ratos e podem servir para integrar a informação do HPC dorsal com as das regiões mais ventrais. Além das projeções para CA1, os axônios dos neurônios piramidais CA2 ramificam-se fortemente dentro de CA2 e CA3, proporcionando assim um elevado grau de interconexão local e recursiva. Projeções de CA2 que saem do HPC incluem axônios que formam conexões recíprocas de volta aos núcleos supramamilar, septal e córtex entorrinal (para uma revisão, ver (90)) (Figura 2).

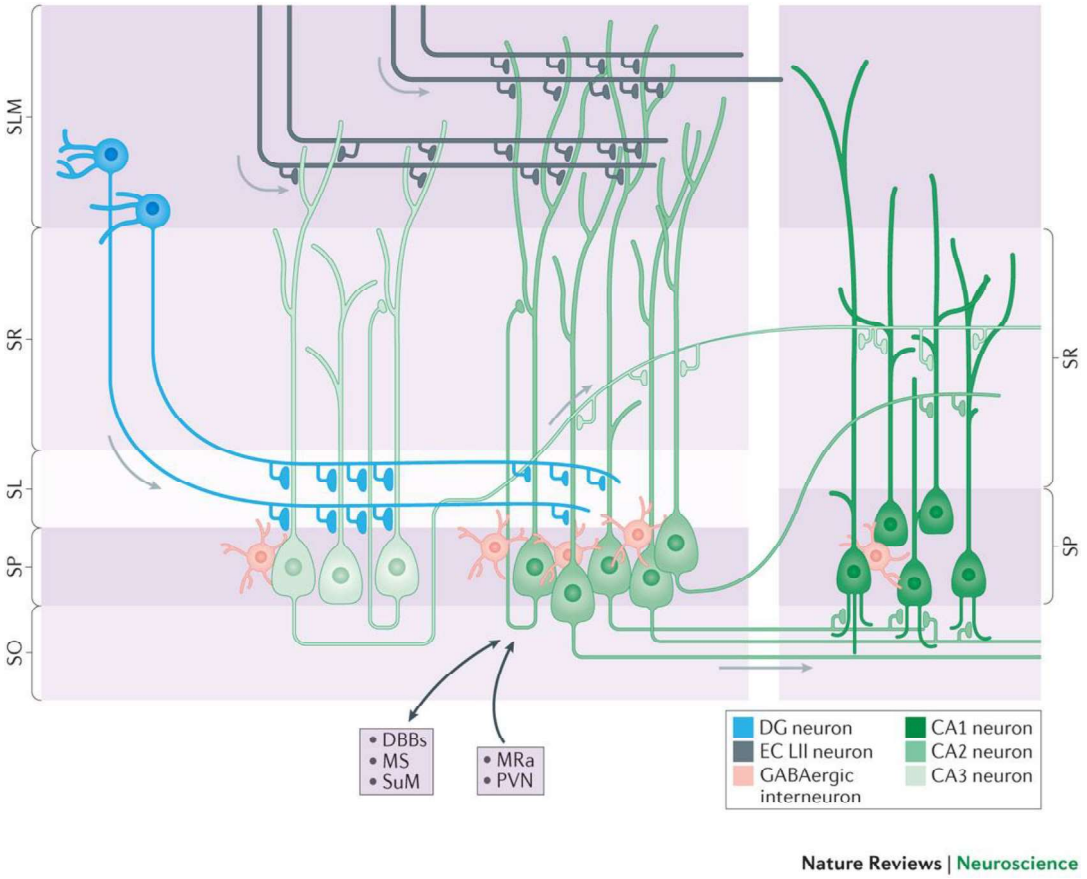

FIGURA 2 Conectividade de neurônios piramidais de CA2 dentro do circuito hipocampal de roedores: Neurônios do giro denteado (DG neuron); neurônios da camada II do córtex entorrinal (EC LII neuron); stratum oriens (SO); stratum pyramidale (SP); stratum lucidum (SL); stratum radiatum (SR); stratum lacunosum-moleculare (SLM). As entradas extra-hipocampais para CA2 (indicadas por setas pretas) incluem as de neurônios vasopressinérgicos do PVN e da rafe mediana (MRa) e conexões recíprocas com o núcleo supramammilar (SuM) septo mediano (MS) e feixe diagonal de Broca (DBBs).

Fonte: http://www.nature.com/nrn/journal/v17/n2/abs/nrn.2015.22.html
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Várias classes funcionais de proteínas são particularmente expressas em CA2. Muitas destas proteínas são preditas como tendo papéis tanto na limitação da plasticidade como na limitação dos danos dos processos patológicos (90). Entretanto, uma questão interessante é que neurônios piramidais CA2 expressam altos níveis de receptores para os neuropeptídeos envolvidos em comportamentos sociais AVP e OT (11,101), evidência sugestiva de um papel específico para CA2 neste contexto.

O trabalho de Hitti & Siegelbaum (2014) (100) comprovou que o silenciamento neuronal de CA2 resultou em um comprometimento seletivo da memória de reconhecimento social dos camundongos. Por outro lado, o silenciamento de CA2 não prejudicou outras

tarefas de memória dependentes do HPC, tais como o reconhecimento de novos objetos ou a memória espacial. Especificamente, os camundongos foram incapazes de diferenciar entre camundongos novos e familiares, o que demonstra que a transmissão sináptica a partir de neurônios piramidais CA2 é essencial para a codificação de informação social em memórias. No entanto, ainda não está claro como os neurônios CA2 integram o processamento social com outros aspectos da memória episódica, como o tempo e o espaço.

Um estudo recente de Pagani et al. (2015) propôs que AVP e OT diminuem drasticamente o limiar para a estimulação em neurônios piramidais de CA2 (Figura 1d). Segundo os autores, a AVP, via ativação de V1bR, aumenta a potencialização sináptica levando à associação de circunstâncias sociais (tais como o contexto espacial e o comportamento do outro camundongo) com odores específicos (96). Desta forma, além de participar de circuitos do HPC subjacentes ao processamento espacial, a área CA2 é crucial para a consolidação de informações socialmente relevantes na memória de longo prazo e também pode desempenhar um papel na codificação temporal (90).

1.5 Plasticidade sináptica e espinhos dendríticos

Os espinhos dendríticos são pequenas protrusões dos ramos dendríticos de vários tipos de neurônios, incluindo os neurônios piramidais do neocórtex, os neurônios medianos do estriado e as células de Purkinje do cerebelo (102). 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 (103). Espinhos dendríticos têm propriedades cruciais para a força sináptica e plasticidade e afetam a atividade neuronal em circuitos integrados (30,104).

Uma característica peculiar dos espinhos dendríticos é a sua variabilidade morfológica. Esta característica é um reflexo do rearranjo rápido dos filamentos de actina no seu interior, o que pode levar à mudança no tamanho e no número de espinhos (102). Em geral, os espinhos dendríticos podem ser classificados de acordo com a sua morfologia com a seguinte nomenclatura: filopódio, que não apresenta uma cabeça definida, sendo fino e comprido, e acredita-se que seja a forma precursora dos espinhos; fino, o qual apresenta pescoço fino e pode não ter uma cabeça bem definida; espesso, que não apresenta um pescoço diferenciado e representa apenas uma elevação no contorno dendrítico; cogumelo, que apresenta o pescoço fino e uma cabeça grande, parecendo ser o mais estável em termos de

contatos sinápticos duradouros e ramificado, no qual o pescoço pode dar origem a mais de uma cabeça (102,105,106).

Tipicamente, as cabeças de espinhos dendríticos formam sinapses excitatórias assimétricas com um axônio pré-sináptico (102). A morfologia dos espinhos dendríticos afeta a difusão e a compartimentação das proteínas associadas à membrana e a expressão dos receptores AMPA (106). Sabe-se, por exemplo, que espinhos com morfologia do tipo cogumelo apresentam mais receptores AMPA em comparação com espinhos finos (102). Sendo assim, os espinhos dendríticos mais longos (finos) têm uma menor densidade de receptores de glutamato pós-sinápticos e respondem ao glutamato com uma corrente interna menor registrada no soma do que os espinhos mais curtos. Portanto, as maiores respostas são observadas com sinapses de espinhos espessos (104).

1.6 Camundongo Nocaute para o gene da ocitocina

Os avanços da genômica, a conclusão do mapeamento do genoma humano, a busca pela cura através da fabricação de novos medicamentos resultaram no aumento de pesquisas com modelos geneticamente modificados. A técnica foi desenvolvida no final da década de 1970 em camundongos, o mamífero cujo genoma é, até hoje, o mais facilmente manipulável. Atualmente, a transgenia permite tanto a transferência de DNA exógeno para o animal, através da técnica de microinjeção pronuclear, quanto à alteração de DNA já existente no animal, através da recombinação homóloga em células-tronco embrionárias (107).

A criação dos camundongos OTKO é de responsabilidade de três grupos de pesquisadores. Nishimori et al. (1996) (108) deletaram o primeiro éxon, Young et al. (1996) (109) deletaram um segmento do segundo éxon e Gross et al. (1998) (110) deletaram os três éxons da OT. O modelo de experimentação utilizando animais nocaute permite avaliar o papel da OT em relação a diversos parâmetros de interesse do pesquisador, tais como: neuroendócrino, fisiológico, comportamental, entre outros.

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2 JUSTIFICATIVA

Muitos estudos sobre comportamento têm buscado entender os mecanismos moleculares e citológicos que regulam processos comportamentais básicos. A genética e a biologia molecular têm contribuído sobremaneira na identificação de genes associados a determinados comportamentos e como alterações genéticas ou fatores ambientais podem interferir na expressão desses genes. O modelo de experimentação utilizando animais nocautes para um determinado gene permite avaliar as alterações fisiológicas e comportamentais geradas a partir desta manipulação genética (107).

Estudos utilizando camundongos OTKO apontaram para alterações nos comportamentos sociais destes animais (25,28,60,108,111) e o desfecho comportamental descrito nestes trabalhos prévios foi associado à falta de OT. Contudo, os circuitos neurais envolvidos em um comportamento não estão associados a apenas um neuropeptídeo. Vários neurotransmissores, seus níveis de liberação e função precisam estar adequados para que, em conjunto, realizem a ativação neuronal correta para o padrão comportamental em questão. Neste contexto, uma alteração na expressão gênica pode desencadear uma série de outras alterações que contribuem para os déficits encontrados em animais nocautes. Mas quais seriam estas alterações secundárias? Em qual região do sistema nervoso elas se localizam? Quais outros neurotransmissores podem estar afetados pela falta de OT? Estas alterações poderiam estar modificando a morfologia dos neurônios envolvidos?

A partir das alterações observadas nos comportamentos sociais dos animais OTKO e das lacunas no entendimento dos mecanismos que desencadeiam estes padrões comportamentais, tornam-se necessários estudos que possam esclarecer o impacto do nocauteamento do gene da OT no SNC destes animais. Para compreender este impacto, animais OTKO devem ser estudados para os padrões comportamentais descritos e seus neurotransmissores/neuropeptídeos e receptores devem ser analisados em várias áreas envolvidas com o comportamento social. Sabendo-se qual área tem padrões de expressão mais alterados, busca-se então entender como os neurônios desta área se alteram frente a estes diferentes padrões, através da análise da densidade e morfologia de espinhos dendríticos.

3 OBJETIVOS

3.1 Objetivo geral

Este estudo teve como objetivo analisar o impacto do nocauteamento do gene da ocitocina nos comportamentos sexual e de interação social; na concentração plasmática e na expressão hipotalâmica de vasopressina e na expressão gênica dos receptores de ocitocina, vasopressina, dopamina e estrogênio em regiões encefálicas importantes aos comportamentos sociais. Além disso, analisar o impacto do nocauteamento do gene da ocitocina e da experiência sexual na densidade e morfologia de espinhos dendríticos em CA2 do hipocampo de camundongos machos.

3.2 Objetivos específicos

- Analisar os comportamentos de interação social e sexual de camundongos machos nocautes para a ocitocina (artigo 1);

- Estudar se o nocauteamento do gene da ocitocina altera concentração plasmática de vasopressina em machos nocautes para a ocitocina (artigo 1);

- Analisar a expressão do receptor de ocitocina, estrogênio α e β, dopamina, vasopressina 1a e 1b no bulbo olfatório, hipotálamo, córtex pré-frontal e hipocampo de machos nocautes para a ocitocina (artigo 2);

- Estudar se o nocauteamento do gene da ocitocina altera a síntese de vasopressina no hipotálamo de machos nocautes para a ocitocina (artigo 2);

- Analisar o impacto do nocauteamento do gene da ocitocina e da experiência sexual na densidade e morfologia de espinhos dendríticos na área CA2 do hipocampo de camundongos machos (artigo 3).

4 ARTIGOS

4.1 ARTIGO 1: Oxytocin modulates social interaction behavior but is not essential for sexual behavior in male mice

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Research report

Oxytocin modulates social interaction but is not essential for sexual behavior in male mice

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HIGHLIGHTS

- Sexual behavior is not altered in OTKO mice.
- OTKO mice are less aggressive than WT.
- OT modulates social investigation behavior of male mice.
- Non-social interactions are not altered in OTKO mice.
- OTKO mice have decreased AVP plasma concentration.

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ABSTRACT

Recently, several studies have shown different conclusions regarding the effect of oxytocin (OT) on the social behaviors of male mice. Most of these studies used exogenous OT, but currently, investigations of the neural bases of social behavior are increasingly employing gene inactivation. This study aimed to analyze the role of OT in the modulation of social behaviors (i.e., sexual and social interaction behaviors) in male mice with selective deletions of the OT gene (OTKO) and the influence of this deletion in basal vasopressin (AVP) plasma concentrations. Our results showed that in the social interaction test, OTKO mice exhibited lower levels of social behaviors and higher levels of non-social behaviors compared to the wild type (WT) group. Additionally, the OTKO group showed a decrease in the number of agonistic behaviors delivered, and consequently, their dominance score was lower than that of the WT group. In the ethological analysis, the OTKO group had a lower aggressive performance and increased social investigation than the WT group. No significant differences were observed in the sexual behavior between groups. Finally, we found lower AVP plasma concentrations in the OTKO compared with the WT group. In conclusion, our data suggest that OT modulates social investigation behavior and the aggressiveness of male mice. The decrease in AVP concentrations in the OTKO group allows us to infer that AVP is physiologically relevant to these behavioral modulations. However, sexual behaviors do not seem to be affected by the lack of OT or by a decrease in the AVP concentration.

1. Introduction

The neuropeptide oxytocin (OT) is a well-known hormone that mediates uterine contractions during labor and milk ejection during lactation in mammals [1,2]. Recently, brain OT has attracted considerable attention due to the discovery that it regulates behavioral functions. Although OT is implicated in a variety of "non-social" behaviors, it is the role of OT in various social behaviors that has received the most attention recently [3]. In fact, OT acts as a neurotransmitter/neuromodulator to regulate a diverse range of central nervous system (CNS) functions in both males and females, including emotional [4], parental [5], affiliative [6], and sexual [7] behaviors, as well as spatial and social memories [8,9].

In mammals, social groups are characterized by high levels of complexity in the type and number of social interactions [10]. The ability to recognize individuals is essential to most aspects of social behavior. For example, rodents identify each other through unique odor cues [11]. Odor cues mediate sexual and competitive

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interactions and are of major importance in both individual and kin recognition, as well as in mate detection and selection [12,13]. Therefore, the processing of social information needs to be precisely tuned for sociality to exist. This fact implies that the specific brain mechanisms associated with the recognition and interpretation of the various aspects of social information must also be precisely regulated [10].

Several studies have shown different results regarding the central effects of OT in male sexual behavior. Previous studies [14–16] have shown that OT increases socio-sexual interactions. In this context, Melis and Argiolas [17] inferred that OT plays a role in arousal and sexual motivation, following intracerebroventricular (ICV) infusion. Moreover, reproduction can be facilitated by OT effects [18] on erectile function and male sexual behavior in different species ([4,19]; for reviews, see [7]).

However, Mahalati et al. [20] showed that the ICV injection of OT produced an immediate cessation of sexual activity in male prairie voles. In the male rat, chronic ICV infusions of small amounts of OT facilitate sexual behavior, whereas high levels inhibit it [21]. Additionally, Nishimori et al. [22] showed that male mice lacking OT had no functional or reproductive deficits. Together, these findings suggest that OT may play a role in sexual satiety [22].

Together with the nonapeptide OT, arginine vasopressin (AVP) is an essential component of the hypothalamo-neurohypophysial system. Structurally, the OT molecule is very similar to AVP, differing by only two amino acids in most mammals and crosstalk at the receptor level may be physiologically relevant [18].

Great discoveries have been made as a result of studying the OT system. These discoveries also make OT a promising target for psychotherapeutic interventions and the treatment of numerous psychiatric illnesses, including anxiety disorders, social phobia, autism and postpartum depression [4]. Many studies have attempted to understand the role of the OT system with the aim of apply it therapeutically. Most of these studies were designed using exogenous OT administration. However, newer investigations of the neural and hormonal bases of social behavior and social information processing are increasingly employing gene inactivation [23,24].

The present study aimed to analyze the role of OT in the modulation of social behaviors (i.e., sexual and social interaction behaviors) in male mice with selective deletions of the OT gene (OTKO) and the influence of this deletion on basal vasopressin (AVP) plasma concentrations.

2. Material and methods

2.1. General methods

2.1.1. Animals

The animals of this study were the offspring of a backcrossed stock obtained from Dr. Scott Young's Laboratory (B6; 129S-Oxttm1Wsy/J). All subjects were littermates from heterozygous breeders. C57BL/6 mice, male ($n = 44$) and female ($n = 15$), were used. The mice weighed 25–35 g, were from 3 to 6 months old, and were raised in the animal house of the Federal University of Health Sciences of Porto Alegre (UFCSPA, Brazil). The subjects were housed in ventilated transparent acrylic cages (37 cm × 24 cm × 24 cm) with up to five same-sex mice. All subjects were maintained in a temperature-controlled room (22 ± 1 °C) on a 12:12 light–dark cycle with the lights off at 5 p.m. and free access to food (Nuvilab) and water in their home cages.

All procedures were performed in accordance with the Brazilian Society of Neuroscience and Behavior Guidelines for the care and use of laboratory animals as well as the international laws for the care of laboratory animals. The protocols were approved by the Ethics Committee of the UFCSPA.

2.1.2. Genotyping

The colony founders were developed by Young et al. [25]. The gene was deleted by crossing a genetic construct with the wild-type mouse OT allele in a manner that replaced the last 2 exons. Genotyping was carried out as previous described [25]. Briefly, genomic DNA was isolated from tail samples and used as templates for polymerase chain reactions (PCR). The primer sequences for amplification of the wild-type (WT) allele were as follows: forward primer: 5′-CTT GGC TTA CTG GCT CTG ACCT-3′ and 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 the genotype of each animal was analyzed, the groups were divided into WT and OTKO animals, and these groups were used in experiments 1 and 2.

2.2. Experiment 1

2.2.1. Ovariectomy, hormonal induction and female receptivity test

The female mice ($n = 15$) were bilaterally ovariectomized for the sexual behavior test. To perform the surgery, the females were anesthetized with a single intraperitoneal injection of a mixture of ketamine (20 mg/mL; Dopalen®, Vetbrands, Miramar, FL, USA), xylazine (4 mg/mL; Anasedan® Vetbrands) and saline solution [26]. After anesthesia, they were placed in the ventral decubitus position, and an incision was made on the dorsum immediately under the ribs, through which the ovaries were exposed and then removed. Finally, the muscle tissue and skin were sutured. After surgery, the females remained in individual boxes for approximately 2 h to recover from anesthesia, and then they were returned to their home cages.

Female sexual receptivity was induced using subcutaneous injections of estradiol benzoate (30 μg/0.1 mL of soybean oil, Sigma, St. Lois, MO) and progesterone (500 μg/0.1 mL of vegetable oil, Sigma). The hormonal induction was adapted from Carnevale and Popova [27,28]. The injections of estradiol benzoate were administered 48 h (40 μg/animal) and 24 h before the test (15 μg/animal). Progesterone was injected 4 h before the test (500 μg/animal).

To test for sexual receptivity, the females were placed in an observation box with a sexually experienced, heterozygous male. Those females who demonstrated lordosis and did not escape from male mounts were considered receptive.

2.2.2. Sexual behavioral test

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 [29]. The males from the WT ($n = 13$) and OTKO ($n = 12$) groups were tested with receptive females. Each male was given 30 min to adapt to the test apparatus. After the adaptation time, a receptive female was placed in the box, and the behavioral test started. The test was performed during the dark cycle in an observation room illuminated by a red light, and the behaviors were recorded with a video camera for 30 min. To confirm male ejaculation, each female was inspected for a vaginal plug. If the cap was not visualized, a vaginal smear was performed to verify the presence of sperm.

The following behaviors were evaluated: latency, frequency and duration of anogenital investigation of the female, mounting and intromission; latency until first ejaculation; and the duration of the first post-ejaculatory refractory period, as previously described [30,31]. The test was videotaped using a video camera and recorded using the Observer program (Noldus®, Holland).

2.3. Experiment 2

2.3.1. Social interaction test

After one week of social isolation, males from the WT ($n = 8$) and OTKO ($n = 11$) groups were tested for social interaction, as previously described [32], with modifications.

The social interaction test arena consisted of three boxes (35 cm × 35 cm × 35 cm), A, B and C, with floors covered with wood shavings. The boxes were linked two-by-two by a corridor (10 cm × 10 cm × 15 cm). The tested males (from the WT or OTKO group) were able to explore the entire space of the apparatus for 15 min to allow for habituation. When the habituated mouse was in box A, the stimulus mouse (intruder) was introduced into box C and the test started. Both mice could explore the entire apparatus. The test was performed during the dark cycle in an observation room illuminated by a red light, and behaviors were recorded with a video camera for 15 min.

The animals were tested in pairs: one male was from the WT or OTKO group, and the other was a heterozygous male mouse of approximately the same weight as the test animal. The intruder mice were maintained in social isolation for one week before testing, and each intruder was used only once. The behavioral analysis focused on the tested mouse. The behavior of the intruder was collected in relation to the behavior of the tested mouse. All of the analyzed behaviors were evaluated for latency, frequency and duration.

The behaviors were evaluated based on the ethogram by Grant and Mackintosh, modified from Clipperton Allen et al. [33]. Briefly, the social behaviors scored included the following: chasing the intruder, dominant behaviors (the resident mouse is in control), attacks delivered (dorsal/ventral bites), aggressive postures (boxing/wrestling), reciprocal attacks (the attacker cannot be identified), avoidance of the intruder, submissive behaviors (the intruder mouse is in control), attacks received, defensive upright posturing and social inactivity (sitting/lying/sleeping together). The non-social behaviors scored included the following: horizontal exploration, vertical exploration, digging, abnormal stereotypies (repeated jumps, spin turns), solitary inactivity and self-grooming. The investigative behaviors scored included the following: oronasal investigation, anogenital investigation, stretched approaches (risk assessment behavior) and approaching the intruder.

For the ethological analysis, the categories of individual behaviors were formed to assess the full spectrum of social, investigative and non-social exploration

behavior of the mice, as well as the overall levels of agonistic behavior. The total social exploration was calculated by grouping the frequency of following the intruder, dominant behaviors, attacks delivered, aggressive postures, defensive upright posturing, and inactivity together with the investigative behaviors (i.e., oronasal investigation, anogenital investigation, stretched approaches and attend to/approach the intruder). The spectrum of non-social behaviors was a combination of the frequencies of horizontal exploration, vertical exploration, digging, stereotypies, solitary inactivity and self-grooming. The dominance score was the total agonistic behavior delivered (following the intruder, dominant behaviors and attacks delivered) minus the total agonistic behavior received (avoiding the intruder, submissive behavior, attacks received and defensive upright posturing).

With this detailed view of the animal's agonistic behavior, we could calculate the "dominance score" for each pair of mice by subtracting the number of agonistic behaviors received from the number of agonistic behaviors delivered. The dominance score thus takes into account the reciprocity of dominant and submissive behaviors involved in agonistic interactions.

The evaluation was recorded using the program The Observer (Noldus®, Holland).

2.3.2. Blood samples and hormonal measurements

The day after the social interaction test during the light cycle at 3 p.m. (approximately 19 h after the behavioral test), the male mice were decapitated and blood samples were collected from the WT ($n = 6$) and OTKO groups ($n = 8$) and placed in previously heparinized test tubes. The blood samples were centrifuged for 15 min at 1600 × g at 4 °C. The plasma was extracted and stored at −80 °C.

Vasopressin enzyme-linked immunosorbent assays (ELISAs) were performed according to the manufacturer's protocol (Enzo Life Sciences, Farmingdale, New York) using the Arg8-Vasopressin EIA kit. Briefly, 100 μL of plasma were compared with known concentrations. An optical density reading at 405 nm with a correction at 570 nm was taken, and a standard curve was generated. The accepted intra-assay variability was 5.9%.

2.4. Statistical analysis

For the sexual behavioral test, the latency, frequency and duration of anogenital investigation, mounting and intromission; ejaculation latency; and post-ejaculatory refractory period duration (mean ± standard error of mean (SEM)) were analyzed using Student's t-test because all parameters were parametric. The percentage of animals that ejaculated was compared using the Chi-square test.

The first analysis of the social interaction test was performed with each behavior analyzed separately. In this step, the latency, frequency and duration of all social interaction test parameters (mean ± SEM) were analyzed using the Mann–Whitney test because they had no parametric distribution. In the behaviors measured from only a few animals from each group, the percentages of animals that developed the behavior were compared using a Chi-square test.

The second analysis of the social interaction test was performed by adding the frequencies (mean ± SEM) of social, non-social, agonistic and investigative behaviors. Taken together, these results showed a parametric distribution and were analyzed using Student's t-test. The dominance score was analyzed by adding the total number of agonistic behaviors developed minus the total number of agonistic behaviors received. This parameter also showed a parametric distribution and was analyzed using Student's t-test.

The plasma concentration of AVP (mean ± SEM) was analyzed using Student's t-test. In all cases, $p < 0.05$ was considered significant.

3. Results

3.1. Experiment 1: sexual behavior test

The results of the sexual behavior test for males that ejaculated are found in Table 1. The table shows the latency, frequency and duration of anogenital investigation, mounting and intromission; the latency to the first ejaculation; and the duration of the refractory period in both groups. No significant differences were observed in the parameters analyzed in the studied groups ($p > 0.05$). The percentage of animals that ejaculated was not different between the groups (WT = 76.9% and OTKO = 75.0%; $p = 1.00$).

3.2. Experiment 2: social interaction test: ethological analysis

Fig. 1 illustrates some of the social behaviors from the social interaction test. Fig. 1A shows that the OTKO group had a lower frequency ($p = 0.006$) and duration ($p = 0.01$) of chasing the intruder than the WT group. The groups showed no difference in the latency of this behavior ($p = 0.20$). The OTKO group had fewer attacks delivered (frequency: $p = 0.01$), and the duration of the attacks was lower ($p = 0.02$) than for the WT group. No difference in latency ($p = 0.06$) was found (Fig. 1B). Additionally, the OTKO group showed an increased latency ($p = 0.02$) and a decreased frequency ($p = 0.002$) and duration ($p = 0.002$) of aggressive postures compared to the WT group (Fig. 1C).

For the other social behaviors, no significant differences were found in reciprocal attacks (latency: $p = 0.68$; frequency: $p = 0.38$; duration: $p = 0.49$); avoidance of the intruder (latency: $p = 0.89$, frequency: $p = 0.96$, duration: $p = 0.81$); submissive behaviors (latency: $p = 0.70$, frequency: $p = 0.79$, duration: $p = 0.74$); attacks received (latency: $p = 0.79$, frequency: $p = 0.91$, duration: $p = 0.52$); or defensive upright posturing (latency: $p = 0.34$, frequency: $p = 0.34$, duration: $p = 0.34$) between the groups. None of the animals from any of the groups exhibited social inactivity behaviors.

The results of the social investigation behaviors analyzed in the social interaction test are found in Table 2. The OTKO group showed an increased frequency and duration of anogenital investigation and an increased frequency of oronasal investigation compared to the WT group. The other parameters were not significantly different between the groups (Table 2). With regard to the frequency of stretched approach behavior, 5 animals developed this behavior in the WT group, whereas only 2 animals from the OTKO group exhibited this behavior (the groups were not different as assessed using a Chi-square test for frequency, $p = 0.07$). A statistical analysis of latency and duration was not performed.

Table 1
Sexual behavior test.

	WT ($n = 10$)	OTKO ($n = 9$)
Latency		
Anogenital Investigation	7.80 ± 1.95	5.78 ± 1.56
Mount	144.80 ± 21.91	173.44 ± 23.55
Intromission	168.50 ± 28.87	204.78 ± 25.35
Ejaculation	751.90 ± 120.77	780.78 ± 166.05
Frequency		
Anogenital Investigation	22.20 ± 2.15	20.67 ± 1.82
Mount	18.90 ± 3.28	18.33 ± 4.22
Intromission	18.10 ± 3.28	16.67 ± 4.24
Duration		
Anogenital Investigation	122.30 ± 16.45	130.78 ± 9.65
Mount	217.20 ± 29.22	175.00 ± 30.09
Intromission	209.10 ± 29.11	162.89 ± 25.42
Refractory period	879.60 ± 122.69	814.45 ± 150.44

Latency, frequency and duration sexual behavior test of control (WT) and knockout (OTKO) males. The Student's t-test was used to compare the experimental groups, at a significance level of $p \leq 0.05$. The data are expressed as mean [±SEM] of the behaviors studied. The number of animals (n) is given between parentheses.

Table 2
Social investigation behaviors of the social interaction test.

	WT ($n = 8$)	OTKO ($n = 11$)	p
Latency			
Oronasal investigation	39.00 ± 19.85	14.27 ± 4.41	0.17
Anogenital investigation	158.80 ± 107.70	91.45 ± 80.89	0.12
Approaching to the intruder	48.25 ± 20.90	25.27 ± 9.04	0.28
Frequency			
Oronasal investigation	7.37 ± 1.89	19.00 ± 3.31	0.03
Anogenital investigation	5.12 ± 1.82	18.73 ± 3.75	0.02
Approaching to the intruder	31.50 ± 6.48	18.82 ± 3.09	0.16
Duration			
Oronasal investigation	10.13 ± 3.63	23.45 ± 5.64	0.14
Anogenital investigation	14.00 ± 5.71	39.45 ± 7.81	0.02
Approaching to the intruder	22.00 ± 4.32	17.82 ± 3.57	0.41

The latency, frequency and duration of social investigation behaviors in control (WT) and knockout (OTKO) groups. The Mann–Whitney test was used to compare the experimental groups at a significance level of $p \leq 0.05$. The data are expressed as the mean ± SEM of the behaviors studied. The number of animals (n) is given in parentheses.

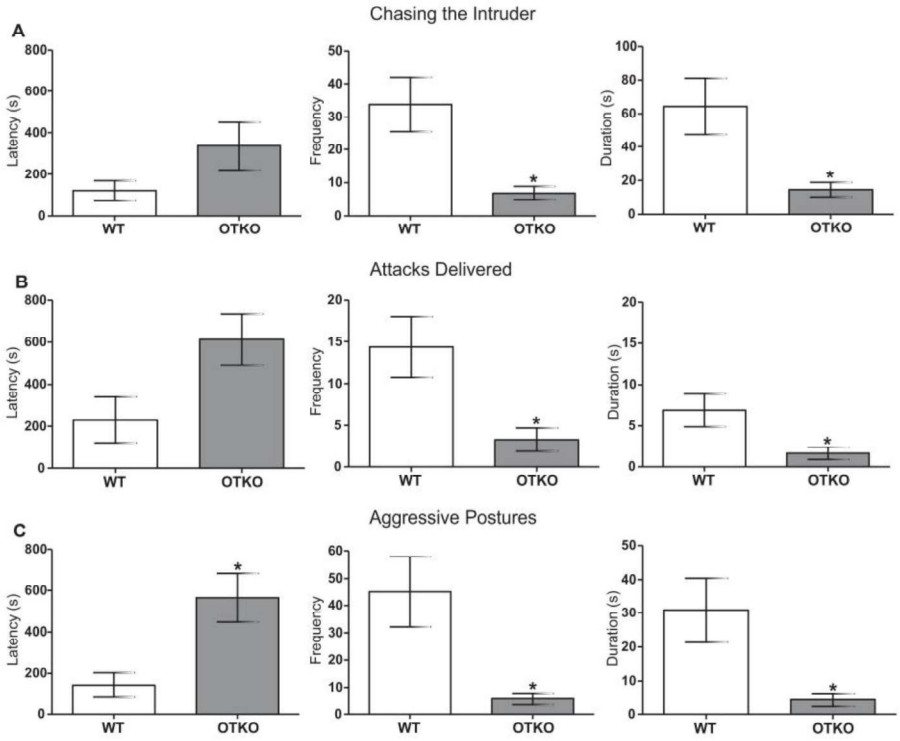

Fig. 1. Agonistic behaviors of the social interaction test: the social behaviors of the control (WT; $n = 8$) and knockout (OTKO; $n = 11$) males. (A) The latency, frequency and duration of chasing the intruder. (B) The latency, frequency and duration of attacks delivered. (C) The latency, frequency and duration of aggressive postures. The Mann–Whitney test was used to compare the experimental groups at a significance level of $p \leq 0.05$. The data are expressed as the mean ± SEM of the behaviors studied. (*) indicates significant difference between the groups.

Additionally, non-social behaviors were studied in the social interaction test. The OTKO group performed vertical exploration more frequently ($p = 0.01$) and with a longer duration ($p = 0.007$) than the WT group. The latency of this behavior was the same between groups ($p = 0.25$). The OTKO group showed a shorter latency for digging ($p = 0.02$), but the frequency ($p = 0.055$) and duration ($p = 0.07$) were the same between groups. No significant differences were observed in horizontal exploration (latency: $p = 0.09$, frequency: $p = 0.11$, duration: $p = 0.97$), solitary inactivity (latency: $p = 0.07$, frequency: $p = 0.11$, duration: $p = 0.054$) or self-grooming (latency: $p = 0.08$, frequency: $p = 0.77$, duration: $p = 0.59$). 2 animals from the OTKO group and none of the animals from the WT group showed abnormal stereotypies; therefore, further statistical analysis was not performed.

3.2.1. Social interaction test: grouped and composite behaviors

The behaviors analyzed were categorized to allow for comparisons with studies that were not analyzed using behavioral tests with ethological parameters. Fig. 2 shows that the OTKO group had a lower frequency of total social behaviors ($p = 0.02$) and a higher frequency of total non-social behaviors ($p = 0.04$) than the WT group. With regard to the delivery of agonistic behaviors, the OTKO group had a decreased frequency ($p = 0.003$), and consequently, the dominance score was lower in the OTKO group ($p = 0.03$) than in the WT group. The groups showed no differences in total social investigation ($p = 0.31$) or agonistic behaviors received ($p = 1.00$).

3.2.2. Plasma concentration of AVP

Finally, we found a significant difference in the plasma concentration of AVP (Fig. 3) between the WT and OTKO groups (WT: 120.39 ± 21.56 pg/mL and OTKO: 55.39 ± 10.95 pg/mL; $p = 0.029$).

4. Discussion

The literature presents differing views on the role of OT in the sexual behavior of rodents. Our results showed that knocking out the OT gene did not alter the sexual behavior of male mice, as the groups revealed no differences in the sexual behavioral test.

The extensive literature on the central role of OT in sexual behavior reports the use of CNS infusions. In this context, some authors [14–16] showed that OT increases socio-sexual interaction. A previous study inferred that after an ICV infusion, OT appears to be the most potent stimulator of "spontaneous" erections in rats [34]. Further, Melis and Argiolas [17,19] suggest that OT is one of several central neurotransmitters and neuropeptides that control penile erection and sexual motivation. Other researchers have observed that in male rats, chronic, low-level OT ICV infusions (1 ng) facilitate sexual behavior, whereas high-level infusions (62.5–500 ng) inhibit it [21]. Additionally, the ICV injection of OT (300 ng) in male prairie voles produced an immediate cessation in sexual activity, and the effect lasted for at least 24 h [20].

To provide additional direct evidence for the importance of OT in physiological and behavioral processes, gene targeting has been

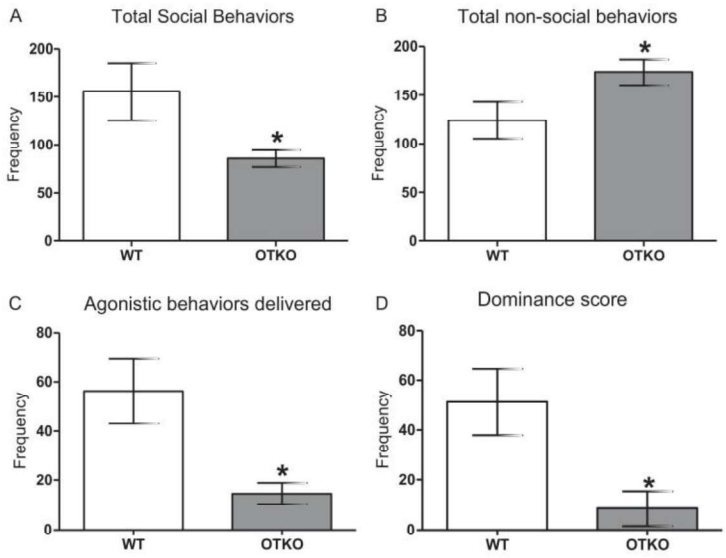

Fig. 2. Total behaviors of social interaction test: the total behaviors from the social interaction test in control (WT; $n = 8$) and knockout (OTKO; $n = 11$) males. (A) The frequency of total social behaviors. (B) The frequency of total non-social behaviors. (C) The frequency of total agonistic behaviors delivered. (D) The dominance score. Student's t-test was used to compare the experimental groups at a significance level of $p \leq 0.05$. The data are expressed as the mean ± SEM of the behaviors studied. (*) indicates a significant difference between the groups.

used to eliminate certain limitations of the OT manipulations on the CNS [35].

Previous work by Nishimori et al. [22] demonstrated that male mice lacking OT exhibit no functional or reproductive deficits. In this study, pairs of WT, heterozygous and homozygous OTKO mice were mated, and the female mice that had copulation plugs were observed until delivery. The male mice did not display reproductive deficits. The focus of our work was the sexual motivation of these animals without OT. Our results allow us to conclude that OT is not essential to the sexual behavior and motivation of male mice. Taken together with the previous report [22], these data show that OT is not an essential modulator of sexual behavior.

This result seems to contradict the extensive literature on the central role of this peptide in sexual behavior [36]. However, it is important to remember that OT is one of many neurotransmitters released in the CNS and that other neurotransmitters can influence the behaviors. Baskerville and Douglas [37] found that dopamine and OT may operate in a bidirectional manner to drive organic functions, such as penile erection and social-sexual behavior characteristics. Recent research has discovered the expression of all of the dopamine receptors in the D2 family (D2, D3 and D4) in the cell bodies of OT neurons in the paraventricular nucleus, supraoptic nucleus and medial preoptic area [38,39]. This finding provides strong neuroanatomical support to the hypothesis that dopamine and dopamine receptor agonists may directly activate OT neurons, thus compensating for the lack of OT in male mice.

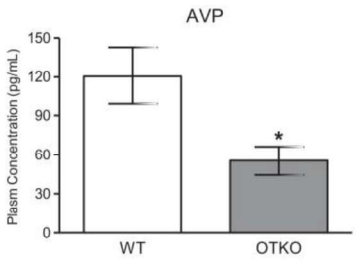

Fig. 3. Vasopressin plasma concentration: the AVP plasma concentration of control (WT; $n = 6$) and knockout (OTKO; $n = 8$) male mice. Student's t-test was used to compare the two experimental groups at a significance level of $p \leq 0.05$. (*) indicates a significant difference between the groups. The data are expressed as the mean ± SEM of the hormonal levels.

For the social interaction test, the behavioral performance of the tested mice was analyzed. For same sex interactions, particularly male–male interactions, the ordinary behaviors are often aggressive in nature and center around competition for mates and other resources, such as territory [3]. In laboratory tests of social interactions, the test conditions (light level and familiarity to the test arena) are manipulated to generate different levels of anxiety [40]. In social interaction tests, male mice will often establish and maintain dominance of a shared territory by exhibiting agonistic behaviors [33].

Classically, the literature describes the role of OT in the aggressive behavior of female rats. Previous studies [41,42] have described the role of OT in the modulation of maternal aggression. These works used techniques to reduce brain OT and showed increased aggression in lactating females. It is important to highlight that the differences observed between the aggressive behavior of females and males are normal and can be supported by well-known sex differences in social behavior that include aggression and social information processing [10].

Our results showed that OTKO mice demonstrate decreased frequency and duration in the delivery of agonistic behaviors (i.e., follow the intruder, attacks and aggressive postures) compared to the WT group. At the same time, we found no differences in submissive behaviors or in the agonistic behaviors received (i.e., reciprocal attacks, avoidance of the intruder, submissive behavior,

attacks received and defensive upright posturing) between groups. These results allow us to infer that OT stimulates the aggressiveness of these animals.

In addition to these differences in agonistic behaviors, the OTKO group showed a lower frequency of total social behaviors. Moreover, the dominance score of the OTKO group was lower than that of the WT group, which means that the OTKO males established territory dominance, but they were less aggressive than the WT group in this regard.

The evidence supporting a role for OT in male aggressive behavior is still contradictory. A previous study showed that OTKO mice are less aggressive than homozygote or heterozygote controls and show no difference in anxiety behavior in the open field test [35]. In this study, the OTKO mice were less aggressive, particularly in agonistic bouts within a neutral arena (similar to our results), but they found no differences in the frequency of attacks between groups. The duration of the test used in this work was 5 min, whereas in our test the duration was 15 min, which may have influenced in the differences found regarding the frequency of attacks between the studies.

Other studies [43,44] that used male mice from another OT knockout line reported increased aggressive behavior in the resident–intruder paradigm and decreased anxiety in the elevated plus maze. It is important to note that these differences between our study and the studies cited above may be related to methodological differences and to the type of knockout mice used. However, more studies are necessary to understand the role of the OT in the agonistic behaviors and how test conditions can influentiate in the behavioral performance of these animals.

Social recognition is essential for social interaction. Social recognition is primarily based on chemosensory cues in rodents [45]. Olfaction is clearly of primary importance because the removal of the olfactory bulbs completely blocks social recognition memory [46]. Previous studies have shown decreased performance of OTKO mice on olfactory recognition tests, even though the animals possess normal olfactory and learning abilities otherwise [12,13,47–49].

A recent study by Pobbe et al. [50] confirmed previous findings that indicating that mice that lack the ability to synthesize the oxytocin receptor display consistent deficits in social recognition and reduced levels of communication compared to their WT littermates.

Our results of social investigation showed that the OTKO group had an increased frequency of oronasal investigation and an increased frequency and duration of anogenital investigation compared to the WT group. This increase in social investigation can be caused by a social memory deficit, as has been shown in previous reports [9,49]. Another equally likely explanation is that because the OTKO mice exhibit less aggression, they exhibit increased social interactions. Our study was unable to determine whether these results are correlated. Our analysis only determined that OTKO mice are less aggressive and more investigative than WT mice under our test conditions.

Finally, the non-social behaviors evaluated in the social interaction test were higher in the OTKO than in the WT group. In the ethological view, the OTKO group showed a higher frequency and duration of vertical exploration and a higher latency for digging. In other words, the animals spent more time investigating the arena than interacting with the other male.

Additionally, the literature establishes that OT and AVP are important to the outcome of social behavior [49]. We observed that the OTKO group had decreased basal plasma concentrations of AVP compared to the WT group. This result allows us to infer that the findings concerning both aggressive and social investigation behaviors were not only influenced by the lack of OT but possibly also by the decrease in AVP. Furthermore, it is also important to consider that pharmacological studies in OTKO mice [49] have demonstrated that ICV administration of small doses of OT can fully rescue the social recognition deficit in these animals. Moreover, the effect appears specific for OT, as injections of AVP have no effect.

The AVP and OT genes are highly homologous and closely linked (tail-to-tail) in the mouse genome, as they are separated by an intragenic region of 3 kbp. Both of these genes are transcribed toward each other from opposite strands of the DNA duplex [51]. Some reported studies [25,52] are consistent with our results and discuss the effect of knocking out the OT gene on AVP gene expression. Young et al. [25] demonstrated that AVP transcript levels are decreased in the PVN and SON of OTKO mice. Ozaki et al. [52] used a different line of OTKO mice, with a deletion in a different region of the OT gene, and also demonstrated significantly lower expression of the AVP gene in PVN and SON than that that observed in WT mice. Murphy and Wells [53] argue that, the cis-effects, mediated by an enhancer in the OT transcription unit, contribute to the overall basal levels of AVP expression and are absent as a consequence of the deletion in the OTKO mice. However, Young et al. [54] replaced the OT gene in OTKO mice at a random genomic location and were able to restore normal levels of AVP gene expression, which is consistent with the existence of a feedback mechanism to reduce basal levels of AVP expression in OTKO mice [54]. These findings suggest that OT might be involved in the regulation of AVP gene expression. However, the exact molecular mechanisms underlying AVP synthesis in OTKO mice requires further investigation.

Another important point is that OTKO mice are born with already altered neuropeptide systems. This factor may have influenced their natural development patterns, thus modifying their behavioral responses in adulthood. We still have much to learn about OT and AVP systems in infants or juveniles, and the consequences of the absence of OT during development should be considered.

In conclusion, our data suggest that OT modulates social investigation behaviors and aggression in male mice. The decrease in the AVP plasma concentration in the OTKO group also suggests that the neuropeptide AVP is physiologically relevant to these behavioral modifications. However, sexual behaviors appear to be unaffected by the lack of OT or by the decrease in AVP.

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4.2 ARTIGO 2: Oxytocin gene knockout alters the gene expression of oxytocin, vasopressin 1b and dopamine 2 receptors in the hippocampus of male mice

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OXYTOCIN GENE KNOCKOUT ALTERS THE GENE EXPRESSION OF OXYTOCIN, VASOPRESSIN 1B AND DOPAMINE 2 RECEPTORS IN THE HIPPOCAMPUS OF MALE MICE

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ABSTRACT

Oxytocin (OT) and vasopressin (AVP) and a number of neurotransmitter systems play critical roles in social behaviors. In this work, we investigated the relationship between important brain areas and neurotransmitter receptors involved in social behaviors and lack of OT in OT-knockout (OTKO) male mice. We analyzed the gene expression levels of OT receptor (OTR), AVP receptors 1a and 1b (V1aR; V1bR), dopamine receptor 2 (D2R), and the estrogen receptors alpha and beta (ERa; ERb) in the hippocampus (HPC), olfactory bulb (OB), hypothalamus (HPT) and prefrontal cortex (PFC). AVP gene expression was analyzed in the HPT. We extracted RNA, synthesized cDNAs and measured gene expression with quantitative polymerase chain reaction. The absence of OT in the OTKO mice altered gene expression in the HPC; OTKOs exhibited decreased expression levels of D2R and V1bR and increased expression levels of OTR. No differences were detected in OB and HPT. Only ERb was increased in the PFC and we found reduced AVP expression levels in the HPT of the OTKOs. For the first time, we showed that OTKO male mice exhibit changes in the gene expression levels of receptors involved in other neuroendocrine systems, and these findings were more prominent in the HPC.

Key words: estrogen receptor; prefrontal cortex; hypothalamus; olfactory bulb.

1. INTRODUCTION

Recognition and social interaction between animals are crucial skills for the survival and life in groups (Choleris et al., 2009). Many studies have demonstrated that, in addition to classical hormonal functions, oxytocin (OT) and vasopressin (AVP) play critical roles in the social behaviors in mammals (Choleris et al., 2012; Guastella et al., 2011; Matsushita et al., 2010). OT and AVP are released into the brain from the parvocellular cells of the hypothalamus (HPT) (Bielsky and Young, 2004). These neuropeptides are present in many areas of the central nervous system (CNS) related to social behavior, such as the hippocampus (HPC), medial preoptic area (MPOA), nucleus accumbens (NAc), HPT, olfactory bulb (OB), fusiform area, superior temporal gyrus, amygdala, prefrontal cortex (PFC) and temporal cortex (for a review, see (Insel and Fernald, 2004)).

Several molecular and pharmacological studies have demonstrated the critical role of OT and AVP in the neuronal processing of olfactory signatures used for social discrimination and social cognition (Bielsky and Young, 2004; Ferguson et al., 2000; Winslow and Insel, 2002). In addition, a previous study (Ferguson et al., 2001) showed that OT knockout mice (OTKO) fail to recognize familiar conspecifics after repeated social exposure, and OT treatment in the medial amygdala during the initial social exposure fully restores social recognition. Additionally, in our previous study, we analyzed social investigation behaviors using a social interaction test, and we showed that OTKO male mice exhibited a decrease in social behavior and aggressive performance (Lazzari et al., 2013).

Similar to OT, the lack of AVP in male rats leads to an impairment in social recognition. This impairment was rescued by AVP administration into the septum, which provides evidence for a physiological role of AVP in this behavior (Engelmann and Landgraf, 1994). Moreover, vasopressin 1a receptor (V1aR) knockout mice show complete impairment in

social recognition, whereas vasopressin 1b receptor (V1bR) knockout mice only demonstrated partial impairment (for a review, see (Choleris et al., 2009)).

In addition to OT and AVP, other neurobiological systems also contribute to social interaction in rodents. Animal models seem to indicate the existence of broad and integrated brain circuits where interactions between dopamine (DA) and OT mediate social behaviors. Both OT and DA play a role in social recognition/memory within the OB; however, DA, especially through the D2 receptors (D2R), plays a more prominent role in the consolidation of memory rather than recognition (Insel and Young, 2001). Moreover, sex hormones are also relevant in this context. In particular, estrogens appear to facilitate social recognition by promoting the activity of OT through the specific activation of the estrogen alpha and beta receptors (ERa and ERb) (Choleris et al., 2003).

Based on the intricate relationship already described between OT and social behavior and the question of whether other neurotransmitters are associated with changes in OT expression, we investigated the gene expression levels of neurotransmitter receptors in the CNS of OTKO male mice. We selected important areas for male social behaviors and the neurotransmitters related to these behaviors to investigate the effects of the loss of OT. Specifically, this study evaluated the gene expression levels of the OT receptor (OTR), V1aR, V1bR, D2R, ERa and ERb in the HPC, OB, HPT, PFC of male mice. Additionally, we analyzed the influence of OT deletion on the AVP mRNA expression levels in the HPT.

2 MATERIAL AND METHODS

2.1 Animals

This study was conducted with the offspring from a backcrossed stock obtained from Dr. W. Scott Young (B6; 129S-Oxttm1 Wsy/J; NIMH, USA). OTKO mice were developed by gene targeting to eliminate most of the first intron and the last two exons of the OT gene,

as previously described by Young (Young III et al., 1996). All mice were littermates from heterozygous breeders. Male mice [n=4 to 10 for the control group (WT); n=4 to 10 for the OTKO group] were housed in ventilated, transparent acrylic cages (37 cm × 24 cm × 24 cm) with up to five male mice per cage. All mice had free access to water and food under controlled temperature (21 ± 1°C) and light (12:12 light-dark cycle with lights off at 5 pm) conditions.

All procedures were performed in accordance with the Brazilian Society of Neuroscience and the Guide for the Care and Use of Laboratory Animals (2011), as well as the international laws for the care of laboratory animals. The local Ethics Committee approved the protocols (UFCSPA, Brazil, protocol n°. 233/13).

2.2 Genotyping

To determine the genotypes of the mice, genomic DNA isolated from mouse tail samples was collected (Andersen and Tufik, 2015) and used as a template for polymerase chain reaction. Genotyping was carried out as previously described by our group (Lazzari et al., 2013). The primer sequences for amplification of the WT alleles involved the forward primer 5'-CTTGGCTTACTGGCTCTGACCT-3' and the reverse primer 5'-GTCAAGAGGGAGCCTAACACTTC-3'. To amplify the target allele, an additional forward primer (NEO) was used: 5'-TGCCCCAAAGGCCTACCCGCTTCC-3'. After genotyping, the mice were assigned to either the WT or OTKO groups; the brain tissue samples were collected as follows.

2.3 Brain tissue samples

The brain tissue samples were collected from male mice between 6 and 8 months old (25 to 35 g) in the morning during the light cycle in a noiseless room. The mice were decapitated, the brains were removed and the areas of interest were quickly dissected. The OB, HPC, HPT and PFC from the left hemisphere were collected with a stereomicroscope on

ice using sterile materials. The dissection of the structures was performed as previously described (Moura et al., 2014; Zimmermann-Peruzatto et al., 2016) and illustrated by Chiu et al. (Chiu et al., 2007), following the maps and guides to dissection published by Paxinos and Franklin (Paxinos and Franklin, 2012) to separate each specific brain area of interest for the present study.

2.4 Molecular Analysis

2.4.1 RNA extraction and cDNA synthesis

Total RNA was isolated from the brain samples using TRIzol reagent (Invitrogen, São Paulo, Brazil), according to the manufacturer's guidelines. Each structure was homogenized with TRIzol (1 mL), followed by the addition of chloroform (1:5, v/v), and the aqueous phase was separated by centrifugation (12,000 g, 15 min). RNA was precipitated with isopropanol for 15 min, followed by centrifugation at 12,000 g for 10 min. Then, the isopropanol was discarded, and the pellets were resuspended in 0.1% DEPC-treated water. The concentration of total RNA was determined by measuring the optical density at 260 nm, and the RNA purity was assessed based on the 260 nm/280 nm ratio (BioSpec-nano, Shimadzu).

Total RNA (patronized in 1000 ng) was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen, São Paulo, Brazil), according to the manufacturer's guidelines. RNA was first incubated with 1 µL oligo (dT) (0.5 µg/µL, Invitrogen, São Paulo, Brazil), 1 µL 10 mM dNTPs and DEPC-water for a final volume of 12 µL for 5 min at 65°C and then 1 min on ice. The following reagents were then added: 4 µL RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 2 µL 0.1 M DTT, and 1 µL RNaseOUT (40 U/µL, Invitrogen, São Paulo, Brazil). After a 2-min incubation at 37°C, 1 µL M-MLV-RT (200 U/µL, Invitrogen, São Paulo, Brazil) was added, and cDNA synthesis was performed at 50°C for 1 h. The reaction was inactivated by incubation at 70°C for 15 min.

2.4.2 Real Time PCR (qPCR)

The cDNA (1 μL) was subjected to RT-qPCR in a StepOnePlus™ thermocycler (Applied Biosystems, Foster City, CA, USA) using the SYBR® Green PCR Master Mix (Applied Biosystems, São Paulo, Brazil) for OTR, D2R, ERa and ERb. Taqman® probes (Applied Biosystems, São Paulo, Brazil) were used for V1aR and V1bR. The primers for all target genes, as well as for the reference genes [(beta-actin (ActB) and cyclophilin-A (CypA)], are provided in Table 1. The Taqman® gene expression assays used for V1aR, V1bR and beta-actin (ActB) are Mm 00444092_m 1, Mm 01700416_m 1 and Mm 00607939_s 1, respectively.

The amplification for the OTR, D2R, ERa, ERb, AVP, and reference genes [(beta-actin (ActB) and cyclophilin-A (CypA)] was carried out using 7.5 μL of SYBR Green PCR Master Mix (Applied Biosystems, São Paulo, Brazil), 0.5 μL of forward and reverse primers (0.33 μM each), and 100 ng of cDNA and nuclease-free water for a total volume of 15 μL. For the SYBR Green reactions, amplification was followed by a melting curve analysis to confirm the PCR product specificity. Taqman® probes were used for the amplification of the genes V1aR, V1bR and ActB. This protocol includes a final volume of 7.5 μl of master mix (Applied Biosystems, São Paulo, Brazil), 0.5 μL of each probe, and a cDNA concentration of 100 ng. No signals were detected in non-template controls. The experimental Ct (cycle threshold) was calculated using the algorithm enhancements provided with the equipment. The Ct value of each reaction was used to calculate the level of mRNA expression of that specific gene, after normalization to the expression of the control housekeeping genes (HKG) that were analyzed in parallel in the same reaction plate.

Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method using samples from the control group as calibrator samples (Livak and Schmittgen, 2001). All samples were analyzed

in duplicate, and the mean value of each duplicate was used for all further calculations (Moura et al., 2014; Zimmermann-Peruzatto et al., 2016).

2.5 Statistical Analysis

The data distribution was assessed by Agostino and Pearson omnibus normality test. The data were not normally distributed and were thus compared using Mann-Whitney test. The results are expressed as the mean ± SEM, and in all cases, $P<0.05$ was considered statistically significant.

3. RESULTS

The gene expression results for the HPC are shown in Figure 1. The OTKO group showed a significant increase in the OTR gene expression levels (U=11.00, P=0.028) and a significant decrease in the D2R (U=8.00, P=0.020) and V1bR (U=2.00, P=0.017) gene expression levels compared to the WT group. The gene expression levels of V1aR (U=16.00, P=0.818), ERa (U=24.00, P=0.694) and ERb (U=19.00, P=0.573) were not significantly different between the groups.

We found a significant decrease in the mRNA synthesis of AVP in the HPT of the OTKO group compared to WT (U=4.00, P=0.048), as shown in Figure 2. However, we did not find significant differences in the gene expression levels of any of the receptors evaluated in the HPT (Table 2).

In the PFC, the OTKO group exhibited increased gene expression levels of ERb compared to WT. However, there were no significant differences between the groups in the gene expression levels of the other receptors in the PFC. In addition, in the OB, we found no significant differences in the gene expression levels of any of the investigated receptors

between the WT and OTKO groups. We assessed the expression levels of V1bR in all areas, but we only detected amplification of this receptor in both groups in the HPC (Table 2).

4. DISCUSSION

The importance of OT for social behavior has already been previously demonstrated and appears to be modulating the hippocampal mechanisms related to memory retention (Choleris et al., 2003; Ferguson et al., 2000; Winslow and Insel, 2002). Here, we showed that the deletion of OT in male mice leads to changes in the gene expression of receptors involved in other neuroendocrine systems related to social behaviors, and these findings are more prominent in the HPC. Therefore, the action of OT in this area is mainly related to the action of AVP and DA, since the receptors of these neurotransmitters have been altered in OTKO animals.

The previously described (Lazzari et al., 2013) impairment in social behavior and in aggression scores in OTKO males shows the importance of OT for the correct behavioral outcome. Owen et al., 2013 described the hippocampal mechanism by which OT enhances processing of information through an increase in fast-spiking interneuron activity. This activation can improve the performance of neuronal circuitry that requires synapse specificity and millisecond precision. In the present study, the lack of OT potentially suppressed this mechanism in the OTKO group. Thus, we assume that this suppression could be associated with the alterations in the V1bR, D2R and OTR, which can be decreasing social behavior and aggression.

The increased OTR gene expression in the HPC of the OTKO group possibly indicates the existence of a compensatory mechanism. Due to the lack of OT, transcriptional and translational mechanisms may lead to an upregulation of the expression levels of the OTR

(Gimpl et al., 2001). Indeed, this result is interesting because we only found this increase in the HPC, which demonstrates the importance of OT as a modulatory neuropeptide in this area.

The interaction between the DA and OT systems is very well described; an increase in DA release to the central amygdala is involved in the regulation of the OTR gene expression levels (Bale et al., 2001; Skuse and Gallagher, 2009). In the HPC, DA is also essential for learning and memory circuits, especially through D2R activation (Rocchetti et al., 2015). Therefore, we hypothesize that the decrease in the D2R gene expression levels probably contributes to behavioral impairments observed in the OTKO animals (Ferguson et al., 2000; Winslow and Insel, 2002).

AVP is involved in social behavior by acting on the centrally expressed V1aR and V1bR (Stevenson and Caldwell, 2012). Regarding the expression of V1bR in the HPC, we found decreased gene expression levels of this receptor in the OTKO group. Previous studies using V1bR knockout mice showed a significant deficit in social recognition, social memory and aggressive behavior in specific social contexts (Pagani et al., 2015; Stevenson and Caldwell, 2012; Winslow and Insel, 2004). Interestingly, the OTKO group exhibits a reduction in the V1bR gene expression levels in the HPC, which may contribute to the behavioral changes previously demonstrated by our group (Lazzari et al., 2013). This relationship between OT and V1bR in the HPC is a novel finding that reinforces the importance of the role of neuropeptides in social behavior.

Previous studies (Ozaki et al., 2004; Young III et al., 1996) have shown that the transcription levels of the AVP gene are reduced in the PVN and SON in OTKO mice. As expected, in the present study, we found a decrease in the AVP expression levels in the HPT of the OTKO male mice. Decreased AVP has only been described in mice with exon 2 deletion of the OT gene, which is similar to the animals used in the present study; the deletion of exon 1 of the OT gene does not affect the expression of AVP (Winslow and Insel, 2002).

On the other hand, our laboratory previously demonstrated that OTKO female mice exhibit no differences in the transcription levels of AVP in the HPT (Zimmermann-Peruzatto et al., 2016). Other studies have also demonstrated lower AVP plasma concentrations in OTKO males, while the concentrations in OTKO females were not different from that in WT mice (Becker et al., 2013; Lazzari et al., 2013). These intriguing results regarding AVP gene expression in the HPT suggest that OT could modulate AVP synthesis in males but not in females, and this outcome could be due to the sexually dimorphic effects of AVP.

The formation of memory depends on the integrity of the HPC circuits but also involves a large network of cortical areas that includes the adjacent parahippocampal region and the PFC (Lee and Chirwa, 2015). We found an increase in the ERb gene expression levels in the PFC of the OTKO males. A previous study (Wide et al., 2004) in female mice found that high levels of PFC estradiol impair working memory, while low levels facilitate it. Studies with ERa and ERb knockout animals revealed that ERa in the SNC of males may facilitate social memory retention; ERb seems to play a smaller faciliatory role that may only emerge under more challenging testing conditions (for a review, see (Choleris et al., 2012)). We found no differences in the expression levels of ERa in all studied areas and only found differences in the expression levels of ERb in the PFC. These results indicate a potential relationship between ERb and the lack of OT specifically within the PFC, which may help elucidate the mechanisms underlying the behavioral modulation.

The complexity of social behavior also depends on individual recognition, and the OB plays a crucial role in the initial information processing involved in this process. Despite the importance of this area for social behavior, surprisingly the OTKO group exhibited no differences in the gene expression levels of the studied receptors in the OB. Thus, in this region, OT appears to be nonessential for the modulation of the expression of these neuroendocrine receptors.

5. CONCLUSIONS

Our data showed the relationship between OT and other neurotransmitters to relevant areas previously associated with social behavior. The findings of this study indicate the importance of OT in the HPC since there were alterations in the transcription patterns of V1bR and D2R in addition to OTR in this area. In addition, we associated the absence of OT with a reduction in the AVP gene expression levels in the HPT, which demonstrates the relationship between the functions of these peptides in the brains of male mice. These findings provide insights into the contributions of OT to deficiencies in social behavior and highlight the involvement of other neurotransmitters in this context.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicial to the impartiality of the reported research.

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Figure 1. The hippocampal relative gene expression of each studied receptor. The oxytocin receptor (OTR) gene expression levels are compared between WT (n=8) and OTKO (n=8). The dopamine receptor 2 (D2R) gene expression levels are shown in WT (n=8) and OTKO (n=7). The vasopressin receptor 1a (V1aR) gene expression levels are compare between WT (n=6) and OTKO (n=6). The vasopressin receptor 1b (V1bR) gene expression levels are shown in WT (n=5) and OTKO (n=6). The estrogen alpha receptor (ERa) gene expression levels are shown in WT (n=8) and OTKO (n=7), and the estrogen beta receptor (ERb) gene expression levels are compared between WT (n=8) and OTKO (n=6). The data are expressed as the mean [±SEM]. Mann-Whitney test (U test of Mann-Whitney). * $P<0.05$, significant difference from WT

Figure 2. The hypothalamic relative gene expression levels of vasopressin (AVP) are compared between WT (n=5) and OTKO (n=8). The data are expressed as the mean [±SEM]. Mann-Whitney test (U test of Mann-Whitney). * P<0.05

Table 1. Primers used for SYBR® Green assays

Table 2. The relative gene expression levels of the receptors in the hypothalamus (HPT), prefrontal cortex (PFC) and olfactory bulb (OB). NA = no amplification detected. The data are expressed as the mean [±SEM]. Mann-Whitney test (U test of Mann-Whitney). * $P<0.05$, significant difference between groups

Figure 1.

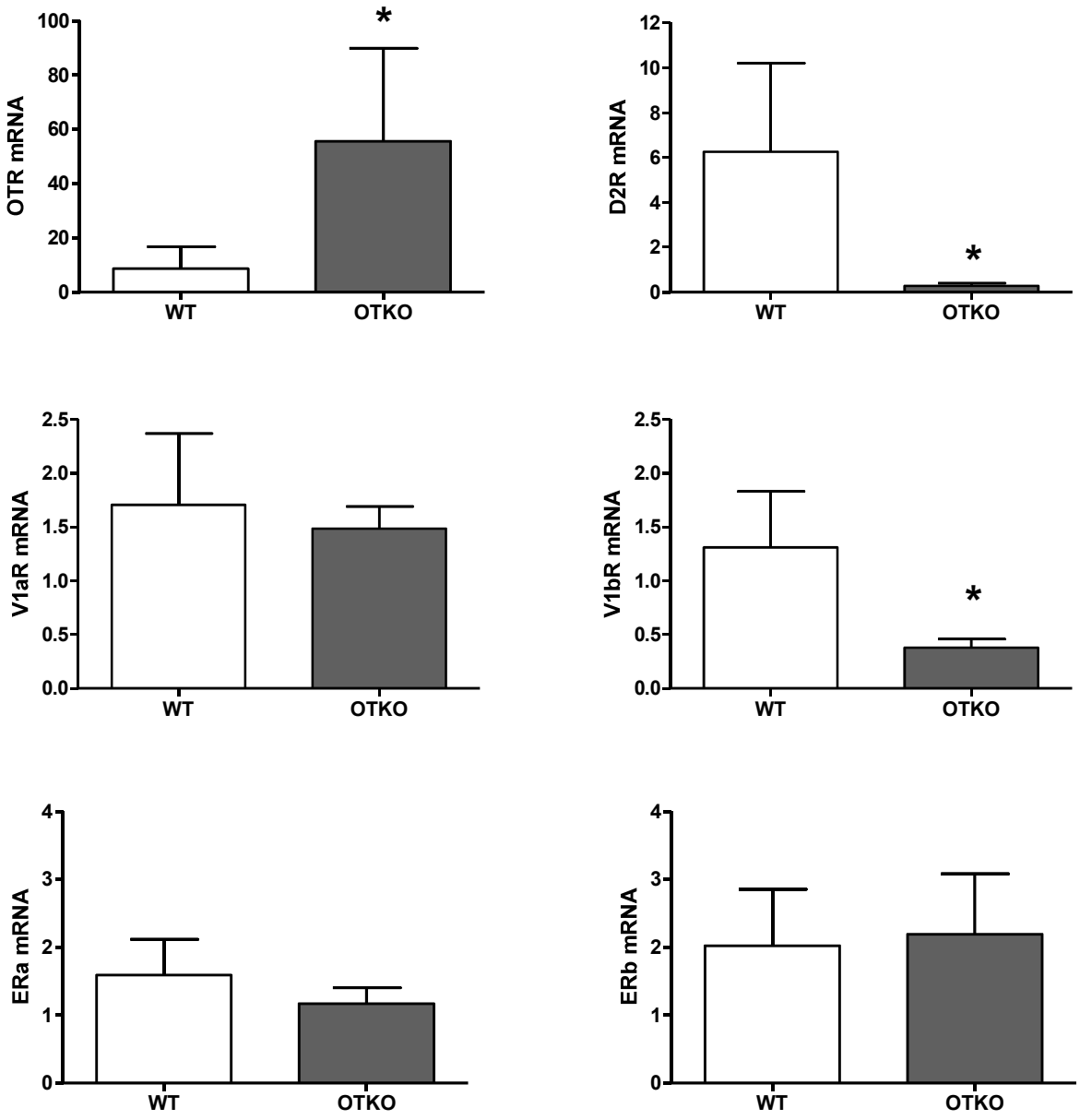

Figure 2.

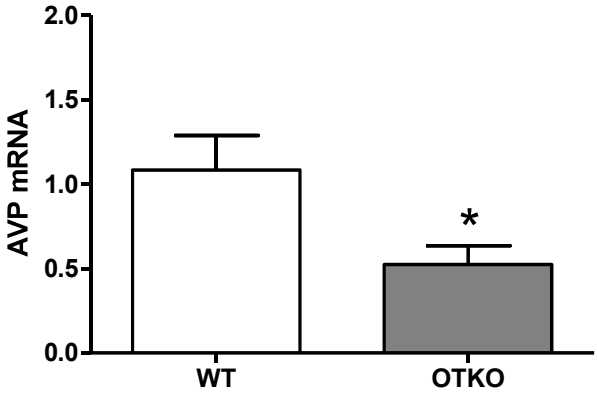

Table 1.

Target	Primer Sequencies
OTR	5’ - CGATTGCTGGGCGGTCTTCA - 3’
	5’ - CCGCCGCTGCCGTCTTGAG - 3’
D2R	5’ - CTCAGGAGCTGGAAATGGAG - 3’
	5’ - CATGCCCATTCTTTTCTGGT - 3’
ERa	5’ - CAAGAACGTTGTGCCCCTCT - 3’
	5’ - TGTAAGGAATGTGCTGAAGTGGA - 3’
ERb	5’ - GGGACATGTACCCTAGCATCG - 3’
	5’ - TGGAAAGTACAACGAGAGCCT - 3’
AVP	5’ - TCGCCATGATGCTCAACACT - 3’
	5’ - TCAGCTCCATGTCGGATGTG - 3’
ActB	5’ - TATGCCAACACAGTGCTGTCTGG - 3’
	5’ - TACTCCTGCTTGCTGATCCACAT - 3’
CypA	5’ - TATCTGCACTGGCAAGACTGAGTG - 3’
	5’ - CTTCTTGCTGGTCTTGCCATTCC - 3’

Table 2.

Area	Receptor	WT	OTKO	U	*P*
HPT		(n = 4 to 7)	(n = 8 to 9)		
	OTR	1.63 ± 0.64	1.01 ± 0.30	20.00	0.46
	V1aR	1.16 ± 0.23	1.82 ± 0.40	17.00	0.23
	V1bR	NA	NA		
	D2R	1.05 ± 0.14	0.89 ± 0.12	20.00	0.46
	ERa	1.76 ± 0.98	1.04 ± 0.20	20.00	1.00
	ERb	0.66 ± 0.19	0.60 ± 0.17	15.00	0.71
PFC		(n = 5 to 10)	(n = 5 to 9)		
	OTR	1.99 ± 0.92	0.45 ± 0.11	17.00	0.13
	V1aR	1.04 ± 0.14	1.16 ± 0.28	11.00	0.84
	V1bR	NA	NA		
	D2R	2.85 ± 1.10	2.22 ± 1.36	44.00	0.97
	ERa	1.36 ± 0.53	0.86 ± 0.14	18.00	0.73
	ERb	1.23 ± 0.30	4.73 ± 1.06	8.00	0.02*
OB		(n = 6 to 9)	(n = 5 to 7)		
	OTR	1.49 ± 0.38	1.73 ± 0.83	26.00	0.87
	V1aR	1.26 ± 0.38	0.99 ± 0.21	14.00	0.93
	V1bR	NA	NA		
	D2R	1.53 ± 0.53	1.67 ± 0.63	31.00	1.00
	ERa	2.08 ± 0.58	0.81 ± 0.38	10.00	0.17
	ERb	1.91 ± 0.73	1.07 ± 0.51	26.00	0.60

4.3 ARTIGO 3: Sexual experience induces spine-specific changes in CA2 pyramidal neurons of male mice

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SEXUAL EXPERIENCE INDUCES SPINE-SPECIFIC CHANGES IN CA2 PYRAMIDAL NEURONS OF MALE MICE

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ABSTRACT

The modulation of male sexual behavior involves contextual processing of sexual encounters and individual memories. Pyramidal neurons of hippocampal area CA2 are critical for social memory processing and highly express oxytocin (OT) receptors. OT is important to the modulation of neural circuits for social behaviors and the spine geometry can influence synaptic processing. Here, we describe the effects of sexual experience on density and shape of dendritic spines in the CA2 of male mice, with Golgi method analysis. The males were allocated into four groups: wild-type naïve (WT/Naïve), OT knockout naïve (OTKO/Naïve), WT sexually experienced (WT/SexExp) and OTKO sexually experienced (OTKO/SexExp). Even plasticity in the CA2 seems to be tightly regulated, sexual experience was able to reduce the amount of stubby/wide spines, but did not affect density or other spines in CA2. Sexually experienced OTKO had a reduction of the number of stubby/wide smaller than WT animals. Perhaps the plasticity adaptation to sexual experience occurred better in WT animals than in OTKOs. Our results show it is highly likely that the spine-specific changes induced by sexual experience alter the normal synaptic processing and excitatory responses in the CA2, which can be important to consolidate the memory of sexual experience.

Keywords: spine density, spine shape, synaptic plasticity, hippocampus, oxytocin.

Male mating behavior is managed by a complex interaction between different brain systems, which process sensory inputs, regulate reward and motivation, and integrate hormonal signals [1]. Although androgens play a major role in the regulation of sexual behavior, brain systems that integrate steroid hormone signals into behavioral outcome are modified by sexual experience. Sexual experience has long-term effects on anticipatory and consummatory male sexual behaviors [2].

The modulation of masculine sexual behavior also involves contextual processing of sexual encounters and individual memories [3]. Previous study [4] demonstrated anatomical and behavioral results that reveal pyramidal neurons of hippocampal area CA2 are critical for sociocognitive memory processing. CA2 pyramidal neurons demonstrate prominent dendritic sodium spikes and highly express OT receptors [5]. Studies showed that OT is important to the synaptic plasticity and modulation of neural circuits for social interactions [6,7]. In this context, there are several new findings on OT neuromodulation of synaptic transmission in the hippocampus (rewied in [8]). In the CA2 region, Pagani et al. [9] showed neuromodulatory effects that were mediated by V1b receptors and OT receptors by decreasing threshold for potentiation and by rendering neurons more sensitive to external stimulation.

Dendritic spines arise as small protrusions from the dendritic shaft and receive inputs from excitatory axons [10,11]. The shape of a dendritic spine depends on the synaptic demand upon it [12] and the spine geometry can influence the synaptic processing [13]. At the cellular level, dendritic spines have shapes ranging from small stubby protrusions to large mushroom-like form and are considered postsynaptic elements with critical properties for the information processing (reviewed in [11]).

The advance on understanding of the relationship between hippocampus, OT and sexual behavior modulation on synaptic plasticity was the aim of this work. Here, we describe

new data on the effects of sexual experience on density and shape of dendritic spines in the CA2 of male mice.

1. MATERIAL AND METHODS

Mice of an offspring from a backcrossed stock obtained from Dr. W. Scott Young (B6; 129S-Oxttm1Wsy/J; NIMH, USA) were used to this work. OTKO mice was developed by gene targeting as described by Young [14]. All animals were littermates from heterozygous breeders (C57BL/6 mice). Genotyping was described in details previously [15].

Adult males (n = 24) weighing 25 to 35 g, with 5 to 8 months old were housed in groups with free access to food and water. All subjects were maintained in a temperature controlled room (22 ± 1ºC) on a 12:12 light–dark cycle with the lights off at 5 p.m. The procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8^{th} edition, 2011) and the local Ethics Committee (UFCSPA, Brazil, Protocol No. 920/09 and 130/13).

At the start of the experimental phase, male mice were allocated into 4 groups: 2 groups of sexually naïve males, consisting of wild-type (WT/Naïve, n=5) and OTKO males (OTKO/Naïve, n=6), and 2 groups of sexually experienced males, consisting of WT (WT/SexExp, n=5) and OTKO males (OTKO/SexExp, n=6). For the sexual experience, each male was placed in a clean cage with two female mice along 3 weeks. The females selected were previously monitored for the regularity of estrous cycle along 2 weeks, to safeguard that males had access to females sexually receptive.

The preparation of histological samples was previously described [16]. Briefly, males were deeply anesthetized, brains were fixed, sectioned coronally (150-μm thick) and put in 3 % potassium dichromate and 1.5 % silver nitrate solutions (Merck, Germany). Sections were rinsed, dehydrated, cleared, mounted on slides and covered with synthetic balsam and

coverslips. The selected brain sections were approximately 1.46 to 1.94 mm posterior to the bregma [17].

The including criteria for the study of CA2 neurons were: (a) to be undoubtedly located within the boundaries of the intended area, as shown in Figure 1; (b) to 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 (adapted from [7]).

The first 10 μm of proximal dendrites that fulfilled these criteria had their spines drawn along the different focal planes in "z" 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). For each male, 2-8 different dendrites were studied with 1 dendrite per sampled neuron. The 3 main differently shaped spines (thin, mushroom, stubby/wide) were identified and counted from these samples. Other spine shapes (ramified or atypical) were not usually seen. 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 measured using the Image Pro Plus 7.0 computer software (Media Cybernetics, USA).

Data were submited to a square root transformation to fulfill the formal requirements of normal distribution and homocedasticity. Data (presented as mean + standard deviation, SD) were tested for the normality distribution and homocedasticity using the Kolmogorov-Smirnov test and the Bartlett test, respectively. Spine density was defined as the number of spines per unit length of dendritic segment (in μm; [18]). The overall density of dendritic spines was submitted to a two-way analysis of variance (ANOVA) test for repeated measures followed by the Bonferroni test. The number of each type of dendritic spine was submitted to a one-way ANOVA and the Tukey post hoc test for multiple comparisons. Statistical level of

significance was set as $P < 0.05$ in all cases. Statistical software was GraphPad Prism version 5.0 (GraphPad Software, USA).

2. RESULTS

Fig. 2 shows representative images of Golgi-impregnated spine neurons in the CA2 of all studied groups.

Fig. 3 shows the density of dendritic spines in the CA2 had no significant difference when compared the OTKO and WT experimental groups [$F (1, 17) = 0.10$, $P = 0.75$], the sexual experience [$F (1, 17) = 2.94$, $P = 0.10$], and the interaction of these two factors [$F(1, 17) = 0.93$, $P = 0.35$]. On the other hand, the sexual experience modified the number of the stubby/wide spines in CA2 (Fig. 4). Accordingly, males submitted to sexual experience showed marked reductions in the proximal density of stubby/wide spines [$F(3, 17) = 22.36$, $P < 0.00001$]. The Tukey post hoc comparisons showed that sexually experienced groups have a significant decrease in the proximal density of stubby/wide spines when compared to their naïve groups. WT experienced males had ~31.1% stubby/wide fewer than WT naïve group and OTKO experienced males had a reduction of ~29.4% stubby/wide density than naïve OTKOs. Moreover, OTKO sexually experienced males had higher values stubby/wide spines than WT sexually experienced ones (27.8% and 17.3%, respectively). Comparisons of mushroom spines were not different between the experimental groups [$F(3, 17) = 1.63$, $P = 0.87$] and thin spines also showed no significant differences between the experimental groups [$F(3, 17) = 1.75$, $P = 0.67$].

3. DISCUSSION

Whereas the histological features, the connectivity and the physiology of field CA3 and CA1 have been investigated by a number of studies, field CA2, possibly in view of its

small size, has been largely ignored or considered together with field CA3 [19]. However, some exciting new discoveries on the properties of CA2 neurons and their role in behavior call attention to this area recently [20]. The present findings add new data on the brain reorganization induced by sexual experience and demonstrate spine-specific changes in the CA2 of adult male mice.

For the first time, the density of proximal dendritic spines and the percentage of differently shaped spines of CA2 pyramidal neurons are described in literature. Our results agree with the current understanding that excitatory synaptic plasticity in the area CA2 is suppressed under most conditions [20]. Even plasticity in the CA2 seems to be tightly regulated, sexual experience was able to alter the amount of stubby/wide spines in the area. However, the total density dendritic spines in CA2 was not affected by sexual experience, as well as mushroom-like and thin spines shape.

The different synaptic inputs and neurotransmitter release that reach the CA2 neurons might code the complex processing of contextual memories of sexual encounters of the animal. The previous study [21] with sexual experienced male mice indicated that sexual interaction might improve the long-term recognition memory and contribute to the formation of stable recognition memory until 48 h. However, although sexual activity is able to affect neurogenesis and enhance hippocampal cell proliferation [22], its mechanism of action, and effects on learning and memory remain uncertain [21]. The general effect of sexual experience in dendritic spines were elucidated by Glasper et. al (2015) that demonstrated that mating increases dendritic spine density in the medial prefrontal cortex and the dentate gyrus, but not in the orbitofrontal cortex or CA1 region in male rats [23]. They did not study the CA2 region, but our results are complementary to their in the context where we show that CA2 region did not altered dendritic spines density.

Lines of evidences showed OT is released in the hippocampus during mating [24] and enhances synaptic plasticity [6]. Surprisingly, in our results OT does not appear to influence the density and shapes of CA2 pyramidal neurons in naïve males. Perhaps the effect of OT reported in these previous studies involves hippocampal areas other than CA2 or different effects in CA2 neuronal plasticity, such as molecular or electrical alterations. The role of OT is not clear in this context, but sexually experienced OTKO had a reduction of the number of stubby/wide spines smaller than WT animals. Perhaps the plasticity adaptation to sexual experience occurred better in animals with OT than in the ones without it. Owen et. al (2013) found a mechanism by which OT can filter signals through the hippocampus, increasing the fidelity of evoked spike transmission (EPSP-spike coupling) in the postsynaptic CA1 pyramidal neurons [25]. Maybe OTKO animals have the fidelity of neural transmission less efficient and the plasticity adaptation not occurred as properly in these animals. The lack of OT, on the other hand, do not reflected in the dendritic spine shapes or density in CA2 neurons of naïve males, indicating that OT role can be more prominent in new situations that require neuronal adaptation.

The shape of a dendritic spine depends on the specific synaptic demand upon it [12] and can influence the synaptic processing [26]. The forward and back propagation of action potentials has been shown to be sensitive to dendritic morphology [27]. Longer spines have a lower density of postsynaptic glutamate receptors, and respond to flash photolysis of caged glutamate with a smaller inward current recorded at the soma than do short ones. The largest responses are seen with stubby or shaft synapses [12]. Stubby/wide spines are considered “immature” shapes [28], 2007) and it is likely that they impose a relatively low resistance to synaptic input and fast voltage propagation to the adjacent dendrite [26]. Sexually experienced animals have less stubby/wide spines than naïve ones. This result can indicate an adaptive way to dampening the spikes and thus restrict the CA2 neural activity in these

animals. On the other hand, the density of dendritic spines did not change with sexual experience, which indicates that along with the reduction of stubby/wide spines a compensatory increase occurred in other types of spines.

In conclusion, sexual experience promoted a reduction in stubby/wide spine number, but not affected other spines or the density of dendritic spines in CA2 pyramidal neurons. It is highly likely that the spine-specific changes induced by sexual experience alter the normal synaptic processing, plasticity, and strength for excitatory responses in the CA2, which can be important to consolidate the memory of sexual experience. These data are relevant for the neuronal structural modulation made by sexual experience and the connectivity of the social behavior network in the male brain.

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LEGENDS

FIGURE 1. Coronal brain sections approximately 1.22 to 2.46 mm posterior to the bregma. Boundaries of the CA2 area (in red), in a ventral (a), intermediate (b) and dorsal (c) section.

FIGURE 2. Representative images of Golgi-impregnated spine neurons from the CA2 of naïve control (WT/naïve), naïve oxytocin knockout (OTKO/naïve), sexually experienced control (WT/SexExp) and sexually experienced oxytocin knockout (WT/SexExp) male mice. Arrows point to differently shaped proximal dendritic spines classified as thin (t), mushroom (m) or stubby/wide (s). Bar = 2 μm.

FIGURE 3. Values (mean ± standart deviation) of (A) the density of dendritic spines in the hippocampal CA2 area of wild type naïve (WT/Naïve, n = 5), oxytocin knockout naïve (OTKO/Naïve, n = 6), WT sexually experience (WT/SexExp, n = 5) and OTKO sexually experience (OTKO/SexExp, n=5) male mice. $P > 0.05$ (ANOVA test followed by Bonferroni test).

FIGURE 4. Mean values ± standart deviation of wild type naïve (WT/Naïve, n = 5), oxytocin knockout naïve (OTKO/Naïve, n = 6), WT sexually experience (WT/SexExp, n = 5) and OTKO sexually experience (OTKO/SexExp, n=5) male mice shapes of spines in 10 μm of proximal dendrites of CA2. *when compared to WT naïve group; ** when compared to OTKO naïve group; # when compared to WT sexually experienced group. $P < 0.0001$ (ANOVA test followed by Tukey test).

FIGURE 1.

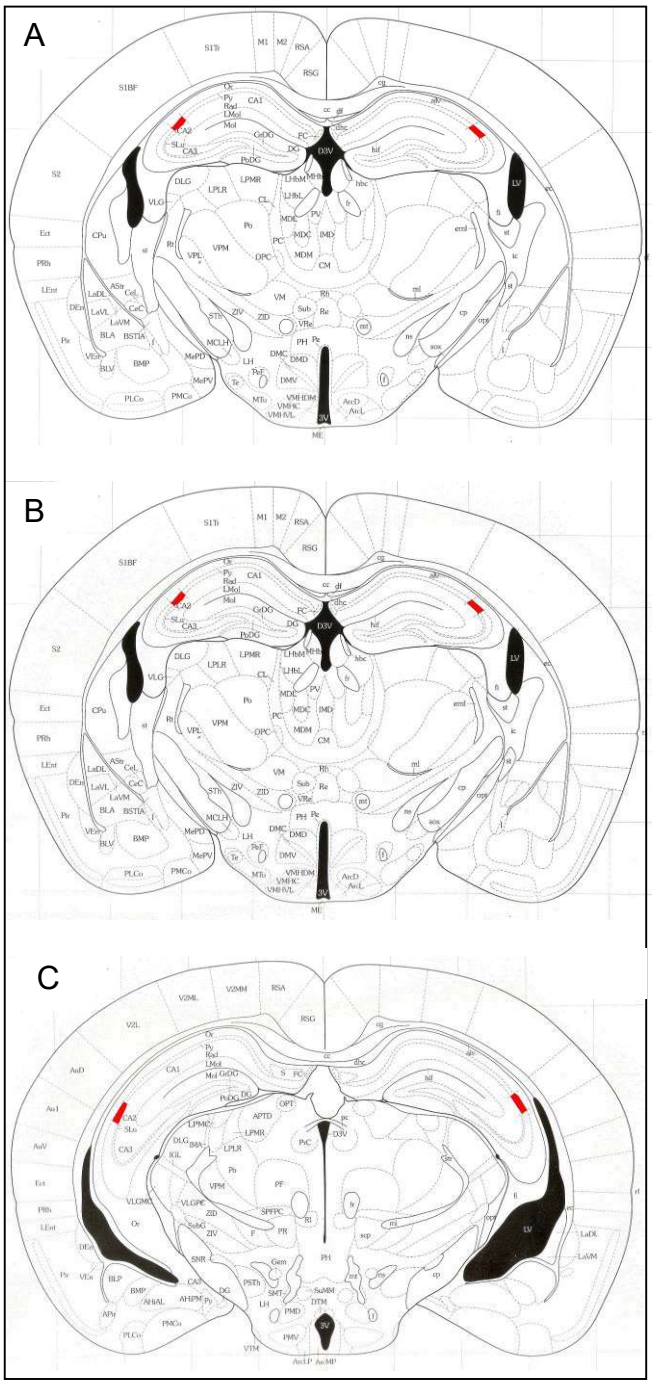


FIGURE 2.

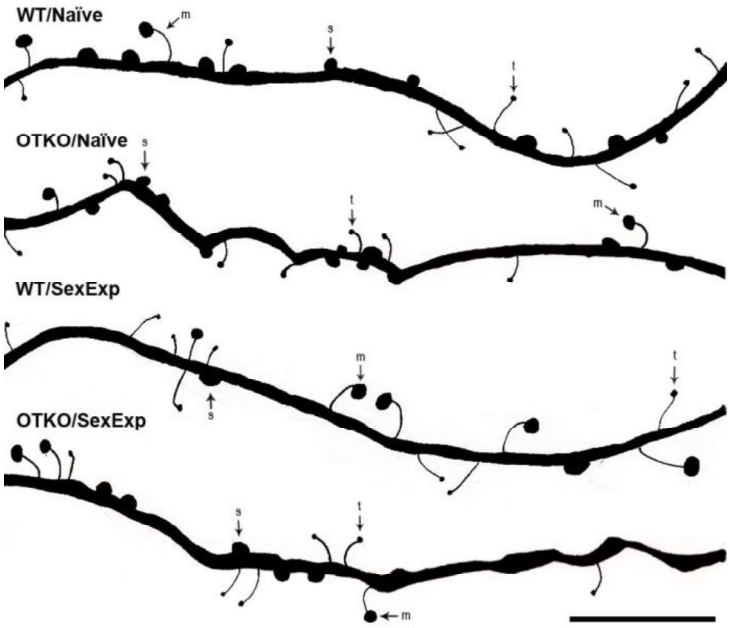


FIGURE 3.

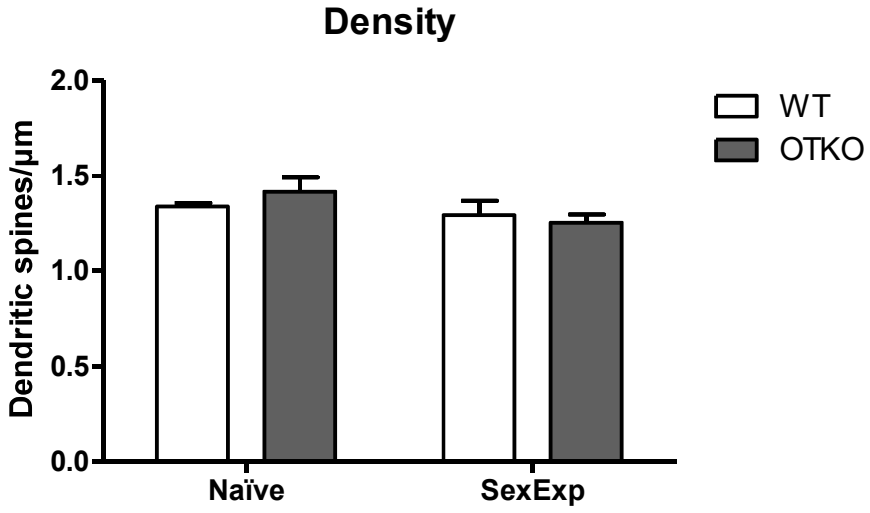
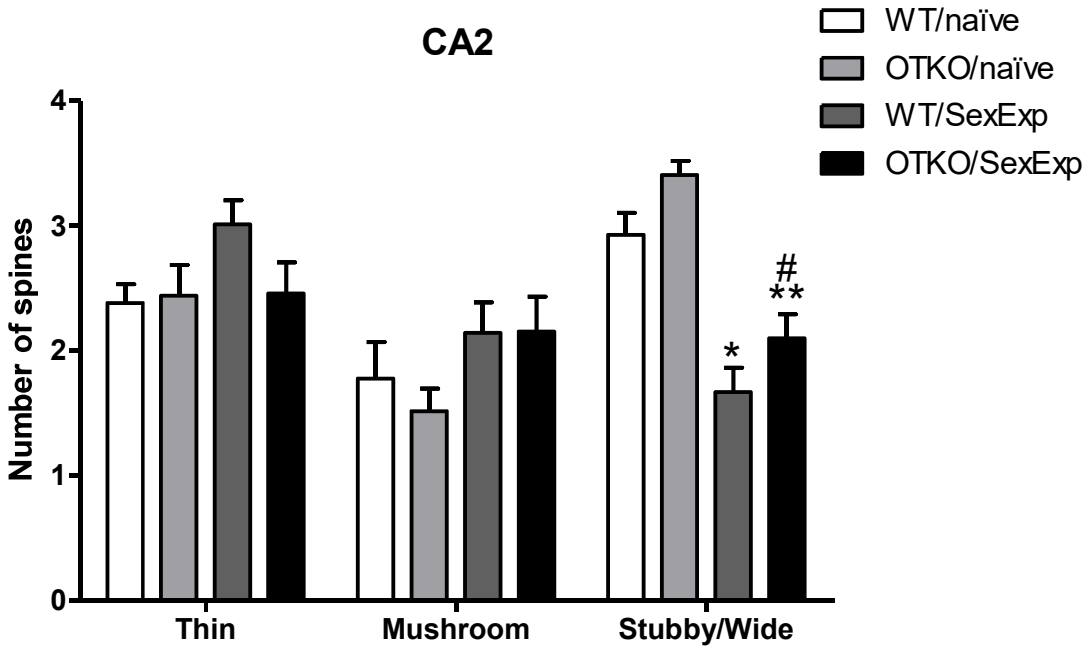


FIGURE 4.


CA2
WT/naïve
OTKO/naïve
WT/SexExp
OTKO/SexExp
Number of spines
0
1
2
3
4
Thin
Mushroom
Stubby/Wide
*
**
#

5 CONCLUSÕES E CONSIDERAÇÕES FINAIS

O papel da ocitocina nas interações sociais já é estudado há algum tempo, porém ainda se sabe pouco sobre os mecanismos de modulação deste neuropeptídeo nos padrões comportamentais. O anseio por uma explicação se baseia na possibilidade de uma ação translacional, que explique não só os padrões comportamentais de cobaias, mas também dos seres humanos e das doenças que prejudicam a sociabilidade.

Os resultados encontrados nos testes de interação social provam que a ocitocina possui papel fundamental para este correto desfecho comportamental, já que os animais nocautes para a ocitocina apresentaram menor interação social e comportamento agressivo reduzido, quando comparados aos controles. No comportamento sexual, parece que a ocitocina não possui um papel tão pronunciado em machos, pois os animais nocautes desenvolveram o comportamento normalmente e não apresentaram diferenças em relação ao grupo controle.

Os animais que foram submetidos ao teste de comportamento sexual (artigo 1), e os animais experientes utilizados para a análise dos espinhos dendríticos (artigo 3), foram alocados com fêmeas para adquirir experiência sexual, nas mesmas condições. Na análise dos espinhos dendríticos, os grupos nocaute e controle experientes foram diferentes entre si apenas em relação ao número de espinhos do tipo stubby/wide. Apesar desta diferença encontrada nos espinhos, como o padrão comportamental sexual não foi diferente entre nocautes e controles (artigo 1), podemos inferir que estes espinhos não estão influenciando no desenvolvimento do comportamento sexual. Em animais virgens, os resultados encontrados mostram que a ocitocina não tem um papel pronunciado na plasticidade neuronal da área CA2 de animais inexperientes, já que os grupos controle e nocaute apresentavam padrões de espinhos dendríticos semelhantes.

É possível que este resultado de redução de espinhos stubby/wide encontrado nos grupos experientes sexualmente esteja relacionado com a reação do animal frente à experiência social, de que forma ocorre o processamento desta experiência e não a como ele desenvolve o comportamento sexual. Neste contexto, como o grupo controle obteve uma redução de stubby/wide mais pronunciada que o grupo nocaute, parece que a ocitocina é importante para modular esta adaptação sináptica, que ocorre de forma menos eficiente no grupo nocaute.

Com os resultados encontrados nos estudos realizados, hipotetizamos que este tempo de convívio com as fêmeas, onde os animais estão livres para explorar o outro animal, cheirar,

perseguir, reconhecer o animal em questão, pode ter alterado o padrão de espinhos do tipo stubby/wide, e esta alteração poderia ser uma adaptação ao comportamento de interação social e não ao comportamento sexual em si. Porém, com as condições experimentais desenvolvidas neste trabalho, não podemos confirmar esta hipótese, para tal, seriam necessários mais estudos.

Em relação à expressão gênica do sistema nervoso de animais nocautes para a ocitocina, conseguimos concluir que a área mais afetada dentre as quatro áreas estudadas foi o hipocampo e que o neuropeptídeo mais intimamente relacionado à ocitocina foi a vasopressina. No hipocampo a ausência de ocitocina não apenas aumenta o padrão de expressão de receptores para ocitocina como também reduz dos receptores de dopamina e vasopressina 1b. Além disso, o nocauteamento da ocitocina reduziu o padrão de transcrição gênica hipotalâmica e a concentração plasmática de vasopressina. Possivelmente, estas alterações de expressão gênica contribuem para as alterações comportamentais destes animais e, portanto, não apenas a ocitocina possui um papel importante nos comportamentos sociais, mas também aos neurotransmissores a ela relacionados, como a vasopressina e a dopamina.

Este estudo contribuiu para a construção do conhecimento sobre as áreas relacionadas à modulação de comportamentos sociais e sobre o papel desempenhado pela ocitocina e outros neurotransmissores envolvidos neste contexto. Concluímos que a ocitocina não é essencial para o comportamento sexual de machos, mas possui papel importante no comportamento de interação social. Possivelmente, as alterações comportamentais encontradas em animais nocautes para a ocitocina são fruto de alterações gênicas e morfológicas no hipocampo, área pertencente ao circuito neural responsável pela modulação dos comportamentos sociais e essencial para o processamento de informações sensoriais e memórias sociais.

6 ANEXO A: Pareceres de Aprovação do CEP

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

REPÚBLICA FEDERATIVA DO BRASIL
MINISTÉRIO DA EDUCAÇÃO

UFCSPA

UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

1) PROTOCOLO Nº: 130/13 Parecer 233/13

2) DATA DO PARECER: 13/11/13

3) TÍTULO DO PROJETO:

Análise da expressão gênica de diferentes receptores de neurotransmissores no sistema nervoso central de camundongos *knockouts* para o gene da ocitocina.

4) PESQUISADOR RESPONSÁVEL:

Marcia Giovenardi

5) RESUMO DO PROJETO:

Este estudo deriva do projeto n 920/09 onde foi coletado material biológico. Será analisado as estruturas do SNC coletada no projeto anterior quanto à análise molecular extração de RNA, RT-PCR.

6) OBJETIVOS DO PROJETO:

Analisar os níveis de expressão gênica de receptores envolvidos em variações nos comportamentos sociais em camundongos machoe e fêmeas OTKO.
Correlacionar a expressão gênica dos receptores de ocitocina, vasopressina e estrógeno em diferentes estruturas do SNC.

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

8) ITENS METODOLÓGICOS E ÉTICOS DO PROJETO:

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

9) O PROJETO ESTÁ ADEQUADO À LEGISLAÇÃO VIGENTE:

REPÚBLICA FEDERATIVA DO BRASIL
MINISTÉRIO DA EDUCAÇÃO

UFCSPA

UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

10) INFORMAÇÕES RELATIVAS AOS ANIMAIS:

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

Justifique: Não se aplica será utilizado material biológico armazenado.

Espécie: **Número Amostral:**

Justifique: Não se aplica será utilizado material biológico armazenado.

Redução Amostral: [ ] Sim [x] Não

Substituição de Metodologia: [ ] Sim [x] Não

Se achar necessário, justifique e sugira uma nova metodologia:

Aprimoramento da Metodologia: [ ] Sim [x] Não

Se achar necessário, justifique e sugira aprimoramentos da metodologia:

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

Justifique: Não se aplica será utilizado material biológico armazenado.

Manipulação dos animais: [ ] Adequada [ ] Inadequada

Justifique: Não se aplica será utilizado material biológico armazenado.

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

Justifique: Não se aplica será utilizado material biológico armazenado.

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

Justifique: Não se aplica será utilizado material biológico armazenado.

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

Justifique: Não se aplica será utilizado material biológico armazenado.

Local de Realização (Biotério/Labotardrio):Laboratório de Biologia Molecular UFCSPA.

Outra Instituição. Qual?

11) CRONOGRAMA DE UTILIZAÇÃO DE ANIMAIS

Data	Espécie	Sexo	Quantidade

12) RECOMENDAÇÃO:

[x] Aprovado

[ ] Com Pendência

[ ] Não aprovado

Data de início: 2 semestre 2013 Data de Término: 2 semestre 2016

Comentários gerais sobre o projeto:

7 ANEXO B: Licenças para utilização de figuras

ELSEVIER LICENSE
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