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Ana Paula Menegolla

O PAPEL DOS RECEPTORES AMPA PERMEÁVEIS A CÁLCIO NA CONSOLIDAÇÃO E EXTINÇÃO DA MEMÓRIA

Porto Alegre
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Trabalho de conclusão de curso de graduação apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de Bacharela em Biomedicina.

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Orientador: Prof. Dr. Lucas de Oliveira Alvares

Coorientadora: Kamilla Irigaray Torquatto

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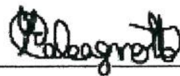

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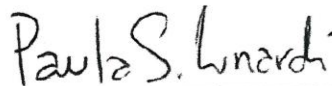

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RESUMO

O papel do receptor AMPA permeável a cálcio (CP-AMPAR) na plasticidade sináptica está relativamente bem estabelecido. Acredita-se que o CP-AMPAR seja recrutado para a sinapse durante um estado plástico da memória. Contudo, as consequências diretas de sua expressão para os processos mnemônicos ainda são pouco exploradas. Neste trabalho, investigamos a contribuição do CP-AMPAR expresso na amígdala basolateral e na região CA1 do hipocampo para a consolidação de diferentes tipos de memória, bem como para a extinção da memória aversiva. Demonstramos que o bloqueio de CP-AMPAR através da infusão de NASPM, seu antagonista seletivo, na amígdala e no hipocampo prejudicou a consolidação de memórias aversivas, ao passo que a consolidação da memória de localização de objetos, uma memória neutra, não foi afetada, e a memória espacial no labirinto aquático de Morris foi prejudicada pela infusão de NASPM no hipocampo. Além disso, a extinção de memórias aversivas não foi afetada por NASPM, porém a recuperação do medo em um contexto diferente daquele da extinção (*renewal*) foi impedida por NASPM na amígdala. Logo, a atividade de CP-AMPAR na amígdala e no hipocampo é necessária para a consolidação de memórias aversivas. Por outro lado, esse receptor não parece ser importante para memórias neutras. Ainda, ele pode não ter uma função na extinção da memória aversiva, mas é recrutado na amígdala para o *renewal*. Esses dados reforçam a ideia de que o CP-AMPAR é importante em estados da memória de alta plasticidade, como o início da consolidação de uma memória aversiva e a recuperação da memória aversiva. A valência emocional também parece influenciar a função desse receptor. Acredita-se que o tráfego de CP-AMPAR para as sinapses, evento associado à plasticidade sináptica, é o mecanismo responsável pelos efeitos comportamentais observados.

Palavras-chave: Consolidação. Extinção. Receptor AMPA permeável a cálcio. Hipocampo. Amígdala.

ABSTRACT

The role of the calcium-permeable AMPA receptor (CP-AMPAR) in synaptic plasticity is well established. CP-AMPARs are believed to be recruited to the synapse when the memory trace is in a plastic state; however, the direct implications of its expression for memory processes are less known. Here, we investigated the contribution of CP-AMPAR expressed in the basolateral amygdala (BLA) and CA1 hippocampus to consolidation of different types of memory and to fear extinction. We showed that CP-AMPAR blockade by infusion of NASPM (a selective CP-AMPAR antagonist) into the BLA and hippocampus impaired fear memory consolidation, whereas in the hippocampus it impaired spatial memory consolidation on the water maze, but not consolidation of object location memory. Furthermore, fear memory extinction was not affected by NASPM, but fear renewal was impaired by NASPM in the BLA. The activity of CP-AMPAR in the BLA and hippocampus is required for the consolidation of fear and spatial memory on the water maze; on the other hand, it is not relevant for a neutral memory. Moreover, this receptor might not be required for fear extinction, but it is recruited in the BLA during fear renewal. These findings support a role of CP-AMPAR in memory states in which plastic changes are presumably higher, such as the beginning of fear memory consolidation and fear renewal. Moreover, emotional valence seems to influence its function. CP-AMPAR trafficking to the synapses, which is associated to synaptic plasticity, is believed to be the mechanism accounting for the observed behavioral effects.

Keywords: Consolidation. Extinction. Calcium-permeable AMPA receptor. Hippocampus. Amygdala.

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LISTA DE ABREVIATURAS

AMPA: ácido α-amino-3-hidroxi-5-metil-4-isoxazol propiônico
BLA: amígdala basolateral
CA1: *cornu ammonis* 1
CAMKII: proteína cinase II dependente de Ca^{2+}/calmodulina
CREB: proteína ligadora ao elemento responsivo a AMP cíclico
CI-AMPAR: receptor AMPA impermeável a cálcio
CP-AMPAR: receptor AMPA permeável a cálcio
EC: estímulo condicionado
EI: estímulo incondicionado
LTD: depressão de longa duração
LTP: potenciação de longa duração
NASPM: 1-Naphthyl acetyl spermine trihydrochloride
NMDA: N-metil-D-aspartato (NMDA)
PKA: proteína cinase A
PKC: proteína cinase C
VGCC: canal de Ca^{2+} dependente de voltagem

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1 INTRODUÇÃO

1.1 RECEPTORES GLUTAMATÉRGICOS

O aminoácido glutamato é o principal neurotransmissor excitatório do sistema nervoso central, estimulando neurônios pós-sinápticos a partir da ligação a receptores presentes nessas células. A transmissão glutamatérgica é importante para a neurotransmissão basal, bem como para diversos tipos de plasticidade sináptica. Os receptores de glutamato são cadeias polipeptídicas transmembrana e dividem-se em duas famílias, a de receptores metabotrópicos (mGluRs) e a de ionotrópicos (iGluRs) (ROUSSEAUX, 2008).

Os receptores metabotrópicos são receptores acoplados à proteína G, proteína que medeia a função destes através da ativação de cascatas de sinalização intracelular, podendo também culminar na abertura de canais iônicos. Oito subunidades de mGluRs já foram descritas, sendo nomeadas mGluR1 a mGluR8 (ROUSSEAUX, 2008).

Já os receptores ionotrópicos são canais iônicos ativados por ligante e divididos em três classes principais, nomeadas conforme a afinidade por certos agonistas: receptores cainato, N-metil-D-aspartato (NMDA) e ácido α-amino-3-hidroxi-5-metil-4-isoxazol propiônico (AMPA). Os receptores cainato e AMPA são canais permeáveis a íons de sódio (Na^{+}) e potássio (K^{+}), ao passo que os NMDA são permeáveis também, e principalmente, a íons de cálcio (Ca^{2+}). Além dessa classificação, eles são divididos em uma diversidade de subtipos, apresentando diferentes características estruturais e funcionais. A grande diversidade de receptores glutamatérgicos se deve tanto à variedade de genes quanto a alterações pós-transcricionais (ROUSSEAUX, 2008).

1.1.1 Receptores AMPA

O receptor AMPA (AMPAR) é um subtipo de receptor de glutamato localizado na membrana pós-sináptica e se destaca na neurotransmissão excitatória rápida que ocorre no sistema nervoso central (HENLEY; WILKINSON, 2016; VERDOORN et al., 1991). Ele é um canal iônico tetramérico, ativado por voltagem, que contém diferentes combinações entre as quatro subunidades transmembrana GluA1, GluA2, GluA3 e GluA4 (DINGLEDINE et al.,

1999). Todas as subunidades possuem uma estrutura em comum: a extremidade amino-terminal é extracelular e possui um sítio de ligação ao glutamato, há três domínios transmembrana, uma porção reentrante na membrana e uma extremidade carboxi-terminal, a qual permite interação com diversas proteínas (DINGLEDINE et al., 1999). Todavia, em cada subunidade há variações que conferem propriedades biofísicas distintas ao receptor e afetam sua montagem e tráfego para as sinapses, afetando sua função.

Cerca de 80% dos AMPARs presentes nas sinapses dos neurônios piramidais da região CA1 do hipocampo são heterotetrâmeros de GluA1 e GluA2, sendo também encontrados heterômeros de GluA2 e GluA3 (LU et al., 2009). Por outro lado, a expressão de GluA4 é maior e praticamente restrita ao início do desenvolvimento, sofrendo redução acentuada ao longo da primeira semana pós-natal em ratos; em contrapartida, são observados o aumento gradativo da expressão de GluA1, GluA2 e GluA3 e a sua estabilização na terceira semana pós-natal (ZHU et al., 2000).

Portanto, a maioria dos AMPARs em um encéfalo maduro contém GluA2. Ainda, a maioria dessas subunidades está presente nas células em uma forma editada, na qual uma troca de base de adenosina para inosina no RNA mensageiro resulta em modificação de uma glutamina por uma arginina na posição 607, provocando alterações eletrostáticas no poro do receptor que impedem a passagem de íons divalentes como o Ca^{2+} (SOMMER et al., 1991). Dessa forma, o receptor contendo GluA2 editado é chamado de CI-AMPAR devido à impermeabilidade a Ca^{2+}, sendo permeável apenas a Na^{+} e K^{+}. Na presença de GluA2 não editado - uma condição rara - ou na ausência de GluA2 - característica da maioria dos AMPARs permeáveis a cálcio -, o receptor é permeável a cálcio, sendo chamado de CP-AMPAR (Figura 1). Os CI-AMPARs exibem condutância baixa e uma relação linear de corrente e voltagem. Por outro lado, os CP-AMPARs exibem retificação de corrente, sensibilidade a bloqueio por poliaminas endógenas e condutância maior em comparação aos CI-AMPARs (HESTRIN, 1993; SWANSON; KAMBOJ; CULL-CANDY, 1997).

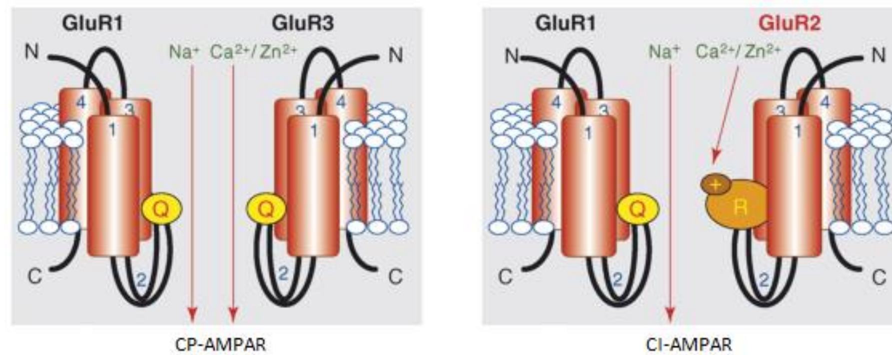

Figura 1. Composição dos AMPARs. O receptor AMPA sem a subunidade GluA2 (ou GluR2, na figura) é permeável a Na^{+} e Ca^{2+} (CP-AMPAR). Na presença de GluA2, subunidade com uma arginina (R) no poro do canal, o receptor se torna impermeável a Ca^{2+} (CI-AMPAR). Adaptado de LIU; ZUKIN (2007).

1.2 PLASTICIDADE SINÁPTICA

Plasticidade sináptica é a alteração na força das sinapses envolvida na formação das conexões neurais e considerada a base para o aprendizado e a memória. Duas formas de plasticidade sináptica são a potenciação de longa duração (LTP) e a depressão de longa duração (LTD).

A LTP é um fenômeno que permite o fortalecimento de sinapses, resultando em um aumento na comunicação entre neurônios. Ela foi identificada e caracterizada em diversos circuitos envolvendo estruturas como hipocampo, amígdala e regiões corticais. A LTP em sinapses da amígdala e do hipocampo, estruturas-chave deste projeto, possui características em comum (LYNCH, 2004).

A base para a ocorrência da LTP é um aumento na concentração de cálcio intracelular no neurônio pós-sináptico. Conforme um modelo de LTP hipocampal, da sinapse de CA3-CA1 (via colateral de Schaeffer), a despolarização e a ligação de glutamato simultâneas promovem a ativação de NMDARs, permitindo influxo de Ca^{2+} pelo canal. O Ca^{2+} é responsável pela ativação de cinases, como a proteína cinase II dependente de Ca^{2+}/calmodulina (CaMKII) e a proteína cinase C (PKC), que fosforilam a subunidade GluA1 de AMPARs, o que aumenta a condutância desses receptores e leva à incorporação de AMPARs na membrana pós-sináptica. Dessa forma, ocorre um aumento da responsividade ao glutamato (HAYASHI et al., 2000; LUSCHER;

MALENKA, 2012; MALENKA; BEAR, 2004). Além dos efeitos imediatos mediados pela LTP, há eventos a longo prazo que permitem a manutenção da alteração da força da sinapse, durante a chamada fase tardia da LTP. Nesta fase, ocorre a ativação de fatores de transcrição, levando à expressão de genes e à síntese de proteínas, incluindo a síntese de novos AMPARs que serão incorporados às sinapses (LUSCHER; MALENKA, 2012; LYNCH, 2004; MALENKA; BEAR, 2004).

Logo, A LTP promove alterações funcionais e estruturais nas sinapses, tais como aumento da sensibilidade pós-sináptica a glutamato, elevação no número e superfície de espinhos dendríticos e modificação da morfologia dos espinhos. Podem também ocorrer modificações pré-sinápticas induzidas por mensageiros retrógrados que se difundem do neurônio pós-sináptico para o pré-sináptico, estando tais alterações relacionadas ao aumento da probabilidade de liberação de neurotransmissores (LUSCHER; MALENKA, 2012).

Tipicamente, a LTP é dependente de ativação de NMDARs. No entanto, em algumas sinapses ela é induzida sem o envolvimento desses receptores. Outras formas de LTP podem envolver a ativação de canais de Ca^{2+} dependentes de voltagem (VGCCs), receptores de glutamato permeáveis a cálcio ou mGluRs (LUSCHER; MALENKA, 2012; MALENKA; BEAR, 2004).

Na LTD, um processo oposto à LTP, ocorre um enfraquecimento da conexão sináptica. Assim como a LTP, a LTD pode depender da ativação de NMDARs ou não. Baseando-se nas sinapses de CA3-CA1, a LTD dependente de NMDARs se dá com a entrada de Ca^{2+} em baixa quantidade, ao contrário da LTP, na qual se observa um aumento de Ca^{2+} intracelular de grande proporção. Com o influxo modesto de Ca^{2+}, são ativadas fosfatases, como a calcineurina. Algumas das consequências são a desfosforilação de GluA1 e a endocitose de AMPARs da membrana (BEATTIE et al., 2000; LEE et al., 1998; LUSCHER; MALENKA, 2012; MALENKA; BEAR, 2004).

1.2.1 Cálcio e plasticidade

O cálcio é um segundo mensageiro envolvido em uma ampla via de sinalização. Cascatas dependentes de Ca^{2+} envolvem a ativação de cinases que promovem a fosforilação de moléculas e a potenciação da transmissão sináptica. A importância do CP-AMPAR na plasticidade sináptica está associada à sua permeabilidade a Ca^{2+}. Apesar de a condutância do CP-AMPAR para Ca^{2+} ser inferior à do NMDAR (DINGLEDINE et al., 1999), esse receptor oferece uma forma de entrada de Ca^{2+} independente de NMDARs e VGCCs. Um dos alvos mais estudados é a subunidade GluA1 de AMPARs. Com níveis baixos de Ca^{2+}, são ativadas enzimas que desfosforilam GluA1 - a proteína fosfatase 1 e a calcineurina (LUSCHER; MALENKA, 2012; MALENKA; BEAR, 2004).

O estado de fosforilação de GluA1 é alterado com processos plásticos: a LTP está associada à fosforilação de GluA1 e inserção de AMPARs na sinapse (BARRIA et al., 1997; LEE et al., 2000), enquanto a LTD se relaciona com a desfosforilação e internalização de GluA1 (LEE et al., 2000, 1998). Ainda, foi demonstrado que estados anteriores da sinapse (em que houve anteriormente potenciação ou depressão) influenciam os pontos de fosforilação, requerendo vias diferentes para nova indução de plasticidade (LEE et al., 2000).

A entrada de cálcio leva também à regulação da expressão gênica, o que resulta em efeitos de longo prazo da plasticidade sináptica. A molécula central nesse processo é o fator de transcrição CREB (proteína ligadora ao elemento responsivo a AMP cíclico), a qual facilita a transcrição gênica (LYNCH, 2004). A síntese proteica promove mudanças sinápticas, como organização do citoesqueleto de actina de espinhos dendríticos, os quais permitem a estabilização da memória (LUSCHER; MALENKA, 2012; MALENKA; BEAR, 2004).

1.2.2 AMPARs na plasticidade sináptica

O tráfego de AMPARs para as sinapses e a mudança na composição desse receptor são processos subjacentes à formação das sinapses excitatórias e à plasticidade sináptica. A

manutenção da LTP está relacionada à inserção de AMPARs nas sinapses, e a LTD, à remoção destes (Figura 2) (BEATTIE et al., 2000; LEE et al., 1998). Dessa forma, a inserção, manutenção e remoção de determinados subtipos de AMPAR é um processo complexo e regulado.

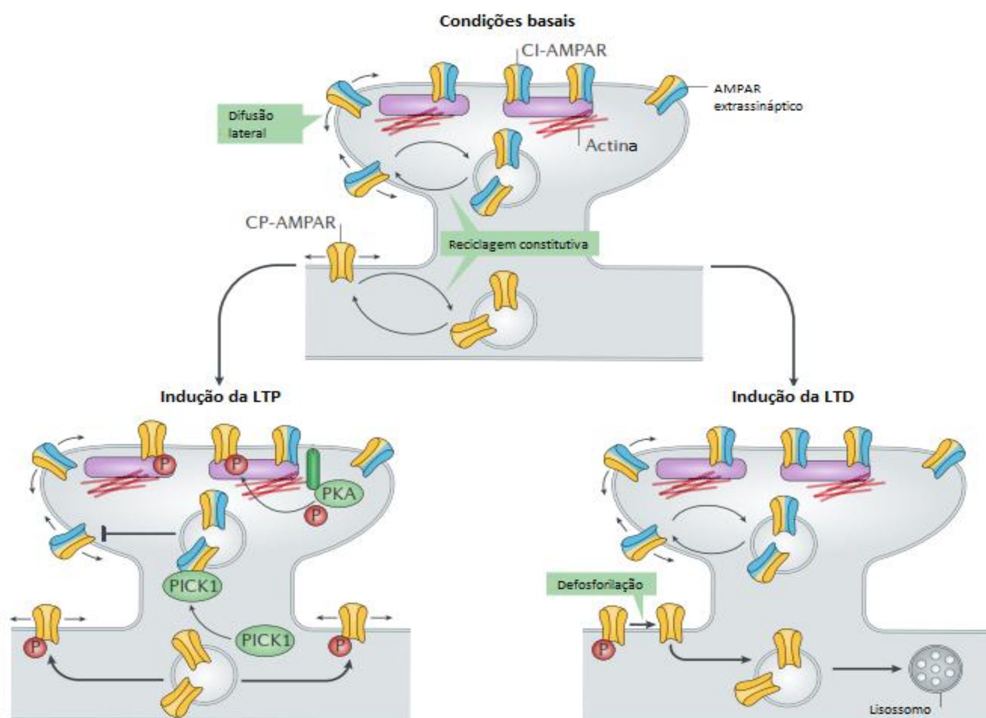

Figura 2. O tráfego de CP-AMPAR durante a plasticidade sináptica hipocampal. Em condições basais, há CI-AMPARs nas sinapses. Na indução da LTP, ocorre a fosforilação de GluA1 e o tráfego de CP-AMPARs para as sinapses. Por outro lado, a indução da LTD promove a desfosforilação de GluA1 e a internalização de CP-AMPARs, que são substituídos por CI-AMPARs nas sinapses. Adaptado de HENLEY; WILKINSON (2016).

Os CI-AMPARs são relativamente estáveis nas sinapses e participam da neurotransmissão basal. Já os CP-AMPARs são expressos de forma transitória, sendo recrutados em processos plásticos, como a LTP, e substituídos em pouco tempo por CI-AMPARs (HONG et al., 2013; PLANT et al., 2006). Diversos estudos esclarecem a relação da dinâmica de expressão de CP-AMPARs com a plasticidade sináptica, demonstrando que seu tráfego é dependente da atividade neuronal, tanto *in vitro* (BEATTIE et al., 2000; PARK et al., 2016; PLANT et al., 2006) quanto *in vivo* (CLEM; BARTH, 2006).

PLANT et al. (2006) demonstram que o CP-AMPAR tem expressão transiente na fase inicial da LTP e é importante para sua indução, e não para sua manutenção. O CP-AMPAR seria

em pouco tempo substituído por CI-AMPAR. Os CP-AMPARs recrutados na indução da LTP promovem o aumento do influxo de Ca^{2+}, estimulando a inserção de CI-AMPARs. Esse recrutamento de CP-AMPARs que antecede o de CI-AMPARs pode estar relacionado aos achados de Greger, Khatri e Ziff (2002) e Greger et al. (2003), segundo os quais GluA1 e GluA2 não editado são transportados mais rapidamente do retículo endoplasmático - onde são sintetizados - à membrana plasmática dos neurônios.

Contudo, a expressão de CP-AMPAR não é sempre necessária para a fase inicial da LTP: um estudo traz resultados contrastantes que indicam a manutenção de CI-AMPARs durante a LTP em sinapses de CA1 e a independência de CP-AMPARs nesse processo (ADESNIK; NICOLL, 2007). É possível, no entanto, que esses resultados aparentemente conflitantes sejam explicados pela existência de diferentes formas de LTP em CA1, cujas características variam conforme o protocolo de indução (PARK et al., 2016). Utilizando antagonistas de CP-AMPAR, Park et al. (2016) verificam que CP-AMPARs estão envolvidos em uma forma de LTP dependente de proteína cinase A (PKA), a qual é induzida por um protocolo com estímulos espaçados em escala de minutos. Por outro lado, não é observada a participação desses receptores com um protocolo consistindo de estímulos temporalmente muito próximos.

Além de diferenças no protocolo de indução de plasticidade, há fatores como o tipo de sinapse, o estágio do desenvolvimento do tecido e o histórico de atividade da sinapse, conforme os quais a composição e densidade de AMPARs - bem como as vias de sinalização envolvidas na indução de plasticidade ou no tráfego de AMPARs - podem variar (LEE et al., 2000; PARK et al., 2016). Todavia, devido à sua contribuição para o aumento dos níveis de cálcio no neurônio pós-sináptico, a incorporação de CP-AMPARs às sinapses é um mecanismo eficiente de potenciação da transmissão sináptica.

1.3 MEMÓRIA

A memória pode ser entendida como um conjunto de processos que contemplam aquisição, armazenamento, manipulação e evocação de informações, os quais permitem aos seres

vivos uma forma de adaptação ao ambiente, através da modificação de seus comportamentos conforme experiências (PAUSE et al., 2013).

Didaticamente, a memória de longa duração pode ser dividida em alguns processos. A aquisição de uma informação dura de segundos a poucos minutos a partir da exposição a uma experiência. Durante a consolidação, o traço de memória passa por um estado lábil, suscetível a modificações, e é progressivamente estabilizado. Este processo leva à formação da memória de longa duração. Com a evocação, a memória é desestabilizada e pode passar pelos processos de reconsolidação ou extinção (Figura 3) (DUDAI, 2004; NADER; HARDT, 2009; NADER; SCHAFE; LEDOUX, 2000b).

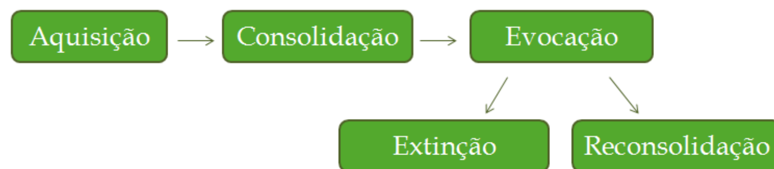

Figura 3. Processos mnemônicos. Após a aquisição de uma informação, o traço da memória passa por uma estabilização, no processo de consolidação. Quando evocada, a memória consolidada pode ser modificada, por extinção ou reconsolidação. Fonte: elaborada pelo autor.

Durante um episódio de aprendizado, informações são adquiridas e codificadas em circuitos neurais, gerando uma representação interna da experiência (DUDAI, 2004). Tais informações podem ser mantidas de forma transitória ou por mais tempo. Assim que adquirida, a representação da memória está em um estado instável, suscetível a modificações. Porém, para a formação da memória de longa duração, o traço é estabilizado ao longo do tempo e se torna mais resistente a interferências, através do processo de consolidação. Há dois níveis de consolidação: o sináptico e o sistêmico. A consolidação sináptica ocorre em minutos a horas após a aquisição e consiste em modificações de sinapses já existentes e surgimento de novas, estabilizando alterações na conectividade dos neurônios que codificam a memória. Já a consolidação sistêmica demora dias a meses para se completar e envolve a reorganização de circuitos e regiões encefálicas responsáveis pela manutenção da memória. A consolidação sináptica é mediada pela LTP e pela LTD e abrange o tráfego de receptores e síntese de novas proteínas direcionadas à sinapse (DUDAI, 2004).

As memórias após consolidadas não permanecem em um estado imutável. Elas são passíveis de modificações ou de decaimento. Todavia, para haver alteração do traço, a memória deve ser reativada e labilizada (DUDAI, 2004; NADER; HARDT, 2009). Com a evocação de uma memória, esta pode ser desestabilizada e atualizada, sendo posteriormente reestabilizada no processo de reconsolidação. A reconsolidação pode envolver a modificação da força e do conteúdo de memórias (MONFILS et al., 2009). Assim como ocorre na consolidação, foi demonstrado que a reconsolidação é um processo dependente de síntese proteica (NADER; SCHAFE; LE DOUX, 2000a).

Outra forma de alteração de expressão de memórias consolidadas é através da extinção. A extinção consiste na formação de uma nova memória que suprime a expressão de uma memória já existente, não envolvendo a modificação desta. A memória de extinção é formada a partir de um novo aprendizado, no qual certo estímulo passa a ter significado diferente daquele na primeira memória. No caso de uma memória aversiva, o estímulo condicionado (aquele que no condicionamento aversivo é associado com um estímulo aversivo e passa a desencadear resposta de medo) não está associado a um estímulo aversivo. A extinção não é um processo permanente; a resposta de medo pode voltar com o tempo (recuperação espontânea), com a apresentação do estímulo nocivo isoladamente (*reinstatement*) ou na apresentação do estímulo condicionado no contexto de aquisição da extinção (*renewal*) (DALTON et al., 2008; DUVARCI; PARE, 2014; MONFILS et al., 2009; SUZUKI, 2004).

Tanto a reconsolidação como a extinção se baseiam na apresentação do estímulo condicionado (EC) na ausência do incondicionado (EI). Um dos fatores que direcionam a ocorrência de um dos processos após a reativação da memória é o tempo de exposição ao EC: um tempo curto favorece a reconsolidação, e um longo, a extinção (SUZUKI, 2004). Os mecanismos moleculares envolvidos também são distintos (MONFILS et al., 2009; SUZUKI, 2004).

1.3.1 Memórias aversivas

A memória aversiva estudada neste trabalho é um tipo de memória episódica com valência negativa. A experiência associada a elementos negativos geralmente propicia a formação

de uma memória forte e persistente, por ter grande relevância para a sobrevivência do organismo, pois o prepara para evitar uma condição nociva (LEDOUX, 2000).

Memórias aversivas podem ser estudadas experimentalmente utilizando o condicionamento clássico, ou Pavloviano, que permite a formação eficiente de memórias duradouras. Nesse paradigma, um estímulo inicialmente neutro (EC) é pareado com um biologicamente significante, que naturalmente provoca uma resposta automática (EI), adquirindo uma valência emocional. A partir da associação entre EC (como um contexto ou tom) e EI (choque, no condicionamento aversivo), a apresentação do EC se torna suficiente para desencadear respostas defensivas a nível comportamental, autonômico e endócrino, como congelamento, maior taxa de batimentos cardíacos e liberação de hormônios (DALTON et al., 2008; LEDOUX, 2000; RODRIGUES; SCHAFE; LEDOUX, 2004), utilizadas para avaliação da memória formada. A nível comportamental, uma das respostas de medo avaliadas em roedores é o congelamento, estado no qual o animal permanece imóvel, apenas com movimentos respiratórios (BLANCHARD; BLANCHARD, 1971).

1.3.2 Memórias neutras e espaciais

Memórias neutras não apresentam valência emocional como ocorre nas memórias aversivas. Memórias que proporcionam localização própria e de objetos no espaço, com o uso de pistas externas ao organismo são neutras por não por não possuírem um componente emocional envolvido na sua constituição. Diversas tarefas foram desenvolvidas para avaliar memória espacial e de referência de roedores, dentre as quais estão a de localização de objetos e o labirinto aquático de Morris (VORHEES; WILLIAMS, 2014).

A tarefa de localização de objetos é baseada na tendência natural de roedores explorarem mais elementos em posições diferentes do ambiente do que aqueles presentes no mesmo local. Assim, a preferência por um objeto cuja localização foi alterada reflete a memória acerca do ambiente. Visto que não há estímulos que reforcem a exploração, é formada uma memória neutra (MIGUES et al., 2016).

O labirinto aquático de Morris é um teste em que, em um tanque com água, o animal deve aprender a posição de uma plataforma submersa utilizando as pistas presentes na sala e encontrá-la. Ao longo das sessões de aprendizado, o tempo para encontrar a plataforma é reduzido. Essa tarefa oferece um reforço para o aprendizado, em contraste com o teste de localização de objetos, pelo fato de o animal buscar a plataforma como motivação para escapar da água. Portanto, tem certo grau de aversividade (VORHEES; WILLIAMS, 2014).

1.3.3 Estruturas envolvidas

Diversas estruturas encefálicas participam de processos mnemônicos, porém têm envolvimento distinto conforme as propriedades da memória. A amígdala e o hipocampo são duas das estruturas envolvidas na formação, consolidação e modulação de memórias (DUVARCI; PARE, 2014; LEDOUX, 2000; LYNCH, 2004; PRESTON; EICHENBAUM, 2013).

1.3.3.1 Amígdala

A amígdala se localiza no lobo temporal e é dividida em muitos núcleos interconectados. Ela é considerada essencial para a aquisição e expressão de medo, sendo relacionada a memórias aversivas. A amígdala recebe aferências do córtex e tálamo contendo informações sensoriais, sendo a amígdala lateral o principal ponto de entrada. Especificamente pertinente ao condicionamento aversivo, as informações sobre os estímulos condicionados e incondicionados - como tons - convergem e são integrados em neurônios da amígdala lateral, cujas conexões se projetam à amígdala central. Informações espaciais ou contextuais também podem chegar à amígdala, porém a partir de projeções do hipocampo ventral, CA1 e subículo, para o núcleo basal. Ainda, também pode haver integração entre EC e EI na amígdala central, não apenas na lateral. As eferências da amígdala são muitas, tais como a substância cinzenta periaquedutal, hipotálamo, hipocampo e córtex pré-frontal (DUVARCI; PARE, 2014; LEDOUX, 2000).

Dessa forma, o condicionamento clássico é mediado por alterações no núcleo lateral da amígdala, que integra EC e EI, formando associações entre eles, as quais podem ser moduladas no núcleo basal por informações provenientes do hipocampo e córtex pré-frontal (DUVARCI; PARE, 2014; LEDOUX, 2000). Devido às aferências diretas de regiões corticais e talâmicas envolvidas no processamento auditivo, o tom é frequentemente empregado para estudo da amígdala no condicionamento aversivo (LEDOUX, 2000).

O condicionamento e, portanto, a aquisição de memórias aversivas, é mediado pela potenciação de sinapses glutamatérgicas da amígdala lateral que processam o EC, através da convergência de aferências com informação sobre EC e outras com EI (DUVARCI; PARE, 2014; LEDOUX, 2000; RODRIGUES; SCHAFE; LEDOUX, 2004). A manutenção dessas sinapses potenciadas contribui para a consolidação da memória, que tipicamente ocorre em 24 horas após o condicionamento (DUVARCI; PARE, 2014; RODRIGUES; SCHAFE; LEDOUX, 2004). A extinção de uma memória aversiva é mediada por subpopulações distintas de neurônios e circuitos dentro da amígdala e entre amígdala e córtex pré-frontal (DUVARCI; PARE, 2014). Além disso, a amígdala também está envolvida na reconsolidação (NADER; SCHAFE; LE DOUX, 2000a).

1.3.3.2 Hipocampo

O hipocampo, localizado no lobo temporal medial, é outra estrutura crucial para a formação de memórias, em especial de memórias declarativas com um componente espacial. Ele é dividido em giro denteado, CA3 (*cornu ammonis*), CA2 e CA1, e sua principal via de entrada é pelo córtex entorrinal. Seu papel em diversos tipos e aspectos da memória vem sendo extensamente estudado através de uma variedade de tarefas, desde os primeiros relatos e estudos de acordo com os quais lesões no lobo temporal medial produziam amnésia (LYNCH, 2004).

O hipocampo é uma área de convergência de vias provenientes de áreas corticais de associação multimodal, os córtices perirrinal, parahipocampal e entorrinal, formando memórias que integram a natureza dos eventos e seu contexto (PRESTON; EICHENBAUM, 2013). Em contrapartida, envia projeções de volta para essas regiões e para o córtex pré-frontal medial, formando circuitos que permitem a formação de representações e a associação de memórias

relacionadas. Algumas populações neuronais do hipocampo possuem padrões de ativação que permitem uma organização espacial e temporal de eventos, sendo ativadas conforme a localização do animal ou em tempo definidos. Acredita-se que isso permita a codificação de objetos e eventos em determinados locais, com distinção de uma sequência temporal (PRESTON; EICHENBAUM, 2013).

A reativação de conjuntos de neurônios em momentos posteriores à aquisição e sua coordenação com a atividade neocortical estão associadas à consolidação de memórias e à sua evocação (PRESTON; EICHENBAUM, 2013). A LTP observada em sinapses hipocampais após um aprendizado é considerada a base para seu envolvimento na aquisição e consolidação de memórias (LYNCH, 2004). O hipocampo também participa da reconsolidação de memórias (LEE, 2004). Apesar de não receber projeções diretas relacionadas a estímulos aversivos, o hipocampo participa do processamento de memórias aversivas através da comunicação com a amígdala (GOOSENS, 2011). No condicionamento clássico, o estímulo condicionado mais adequado para o estudo do hipocampo é o contexto, devido ao seu envolvimento com o aspecto contextual das memórias.

1.3.4 AMPARs e memória

A dinâmica de CP-AMPAR vem recebendo atenção com enfoque em processos plásticos (ADESNIK; NICOLL, 2007; PARK et al., 2016; PLANT et al., 2006). Contudo, até a atualidade, poucos estudos contextualizam a dinâmica de expressão de CP-AMPAR nos processos mnemônicos. A plasticidade sináptica é um dos fenômenos subjacentes a aprendizado e memória, e a influência dos subtipos de AMPAR para alguns processos mnemônicos já foi demonstrada (BHATTACHARYA et al., 2017; CLEM; HUGANIR, 2010; HONG et al., 2013; LOPEZ et al., 2015; MIGUES et al., 2016; RAO-RUIZ et al., 2011).

A persistência e força da memória estão associadas com a quantidade de AMPARs contendo GluA2 tanto no hipocampo (DONG et al., 2015; MIGUES et al., 2016) quanto na amígdala (MIGUES et al., 2010). Ou seja, a internalização de AMPARs resulta em prejuízos na manutenção da memória e LTP.

Há evidências de que CP-AMPARs são inseridos nas sinapses da amígdala lateral durante um aprendizado de informação aversiva, assim como ocorre inserção de CP-AMPARs *in vivo* a partir de experiências em outras regiões encefálicas (CLEM; BARTH, 2006; CONRAD et al., 2008). Foi observado um aumento de correntes excitatórias pós-sinápticas de AMPARs nas aferências do tálamo para a amígdala lateral após um condicionamento aversivo ao tom, acompanhado de maior expressão de CP-AMPARs (CLEM; HUGANIR, 2010; HONG et al., 2013; RUMPEL et al., 2005). O tempo pelo qual os CP-AMPARs persistem, no entanto, varia conforme o estudo: para Clem e Huganir (2010), a maior expressão de CP-AMPAR foi observada de 2 a 24 horas após o condicionamento, ao passo que para Hong et al. (2013) esse intervalo durou de 5 minutos a 12 horas. Apesar da diferença temporal grande, tais dados indicam inserção transitória de CP-AMPARs na amígdala lateral com o aprendizado e posterior substituição por CI-AMPARs. Ainda, sugerem que os CP-AMPARs são importantes para a consolidação das memórias aversivas, uma vez que esta ocorre após o condicionamento.

Com a evocação, as memórias podem ser labilizadas, e este processo está relacionado à dinâmica de expressão de AMPARs. Hong et al. (2013) caracterizaram alterações sinápticas na amígdala lateral após reativação da memória e concluíram que a reativação promove uma breve inserção de CP-AMPARs nessas sinapses: o aumento de CP-AMPARs foi observado em 5 minutos após a reativação, porém normalizado em 3 horas. Dentre os eventos que permitem a inserção de CP-AMPARs na reativação estão a ativação de NMDARs e a internalização de CI-AMPARs, visto que o bloqueio de NMDA e da endocitose de GluA2 impediu a labilização da memória consolidada e a inserção de CP-AMPARs. O papel dos CP-AMPARs na reconsolidação foi demonstrado a nível comportamental, sendo a reconsolidação prejudicada com o bloqueio desse receptor após a reativação, confirmando que CP-AMPARs podem desestabilizar a memória previamente consolidada ao ocorrer nova inserção nas sinapses.

No hipocampo, foi caracterizada a dinâmica de AMPARs após a reativação e constatado um processo bifásico, inicialmente com endocitose de AMPARs (1 hora após a reativação), e posterior reinserção de CI-AMPARs após o término da reconsolidação (7 horas) (BHATTACHARYA et al., 2017; RAO-RUIZ et al., 2011). Os autores não observaram aumento de CP-AMPARs em CA1 do hipocampo logo após a reativação, em contraste com o estudo de Hong et al. (2013) na amígdala lateral. Deve ser considerado o tempo pós-reativação em que o

perfil de receptores foi analisado - 1 hora em contraste com os 5 minutos de Hong et al. (2013), porém a expressão de AMPARs reduzida em 1 hora é consistente com a depressão sináptica registrada (BHATTACHARYA et al., 2017; RAO-RUIZ et al., 2011), que permitiria a reinserção de CI-AMPARs, o fortalecimento da sinapse e a reestabilização da memória após a reconsolidação. Em sinapses da amígdala e no hipocampo, a endocitose de CI-AMPARs está relacionada às alterações plásticas que permitem a alteração do conteúdo ou força da memória (CLEM; HUGANIR, 2010; RAO-RUIZ et al., 2011).

Assim como o tráfego de AMPARs é importante para o fortalecimento de sinapses excitatórias na amígdala lateral e a formação de memórias aversivas, ele também está associado à memória de extinção (DALTON et al., 2008; MIGUES et al., 2010). Um estudo demonstra uma função do CP-AMPAR mais importante para a atenuação de uma memória aversiva remota do que de uma recente, através da facilitação da memória de extinção (ZELENA et al., 2016). O bloqueio sistêmico de CP-AMPAR acelerou o aprendizado da extinção quando a extinção foi realizada 1 dia ou 28 dias após o condicionamento aversivo ao contexto, porém impediu a recuperação espontânea somente da memória remota.

No entanto, outro estudo demonstra que o CP-AMPAR pode ser relevante para a extinção a partir de uma memória recente: valendo-se do aumento de CP-AMPAR na amígdala lateral observado após o condicionamento aversivo, com pico em 24 horas, Clem e Huganir (2010) utilizaram um protocolo de reativação seguido de extinção (baseado em MONFILS et al., 2009) durante essa janela com maior expressão de CP-AMPARs, buscando atenuar uma memória aversiva durante um estado possivelmente mais lábil da memória. Desse modo, a reativação da memória 24 horas após o condicionamento permitiu redução significativa do medo na sessão de extinção, que perdurou por 7 dias, sem recuperação espontânea nem renewal. Ainda, Clem e Huganir (2010) associaram o protocolo de reconsolidação com extinção a LTD e redução da transmissão mediada por AMPARs através da internalização de CP-AMPARs na amígdala, sendo eficiente pela realização em um momento de maior expressão de CP-AMPAR. Há evidências de que CI-AMPARs também atuam na estabilização da memória de extinção, já que a inibição da sua endocitose no córtex infralímbico, área que reforça a memória de extinção, preveniu a recuperação espontânea de uma memória aversiva (MIGUES et al., 2016).

2 JUSTIFICATIVA

A atividade do CP-AMPAR é geralmente explorada em estudos *in vitro* com enfoque em plasticidade, processo que está relacionado à memória. No entanto, poucos estudos até agora abordaram a influência desses receptores diretamente na memória. Evidências sugerem que o CP-AMPAR exerce um papel importante em estados plásticos por permitir o influxo de cálcio em neurônios pós-sinápticos. Assim, hipotetizamos que esse receptor tem uma função em estados de menor estabilidade da memória, como o início da consolidação ou a formação de uma memória de extinção. Ainda, é possível que a valência emocional da memória influencie a dinâmica de AMPARs nas sinapses. Dessa forma, a relevância do presente trabalho é aumentar a compreensão dos efeitos de CP-AMPAR em determinados processos mnemônicos a nível comportamental.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar o papel do CP-AMPAR expresso na amígdala basolateral e no hipocampo na consolidação de diferentes tipos de memória e na extinção de memórias aversivas.

3.2 OBJETIVOS ESPECÍFICOS

1. Verificar se o bloqueio de CP-AMPAR na amígdala basolateral ou no hipocampo no início da consolidação sináptica de memórias aversivas prejudica a consolidação da memória.

2. Verificar se o bloqueio de CP-AMPAR no hipocampo no início da consolidação sináptica de uma memória neutra prejudica a consolidação da memória.

3. Verificar se o bloqueio de CP-AMPAR na amígdala basolateral ou no hipocampo antes da extinção de memórias aversivas prejudica a evocação da memória aversiva ou facilita a formação da memória de extinção.

4 ARTIGO CIENTÍFICO

Esse trabalho foi escrito em formato de artigo científico, seguindo as normas para submissão da revista Hippocampus. Tais normas estão presentes no Anexo A**.**

The role of calcium-permeable AMPA receptors in memory consolidation and extinction

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Abstract

The role of the calcium-permeable AMPA receptor (CP-AMPAR) in synaptic plasticity is well established. CP-AMPARs are believed to be recruited to the synapse when the memory trace is in a plastic state; however, the direct implications of its expression for memory processes are less known. Here, we investigated the contribution of CP-AMPAR expressed in the basolateral amygdala (BLA) and CA1 hippocampus to consolidation of different types of memory and to fear extinction. We showed that CP-AMPAR blockade by NASPM (a selective CP-AMPAR antagonist) infusion into the BLA and hippocampus impaired fear memory consolidation, whereas consolidation of object location memory was not affected and spatial memory consolidation on the water maze was impaired by NASPM in the hippocampus. Furthermore, fear memory extinction was not affected by NASPM, but fear renewal was impaired by NASPM in the BLA. The activity of CP-AMPAR in the BLA and hippocampus is required for the consolidation of fear and spatial memory; on the other hand, it is not as relevant for a neutral memory. Moreover, this receptor might not be required for fear extinction, but it is recruited in the BLA during fear renewal. These findings support a role of CP-AMPAR in memory states in which plastic changes are presumably higher, such as the beginning of fear memory consolidation and fear renewal. Moreover, emotional valence seems to influence its function. CP-AMPAR trafficking to the synapses, which is associated to synaptic plasticity, is believed to be the mechanism accounting for the observed behavioral effects.

Keywords

Consolidation; Extinction; Calcium-permeable AMPA receptor; Hippocampus; Amygdala.

Introduction

AMPA receptors (AMPARs) are glutamate receptors responsible for fast excitatory neurotransmission in the mammalian central nervous system (Verdoorn, Burnashev, Monyer, Seeburg, & Sakmann, 1991) and are fundamental for brain function, including cognitive processes such as learning and memory (Hong et al., 2013; Rumpel, LeDoux, Zador, & Malinow, 2005). AMPARs are typically tetrameric voltage-dependent ion channels permeable to sodium (Na^+) and potassium (K^+) due to the presence of the edited GluA2 subunit (Sommer, Köhler, Sprengel, & Seeburg, 1991). However, a less common AMPAR subtype contains non-edited GluA2 or lacks this subunit and is also permeable to calcium (Ca^{2+}). AMPAR composition determines its biophysical properties and trafficking to the synapses and, therefore, its biological function (Dingledine, Borges, Bowie, & Traynelis, 1999; Sommer et al., 1991). Around 80% of pyramidal neurons in the hippocampus contain calcium-impermeable AMPARs (CI-AMPARs) (Lu et al., 2009), and these receptors are also abundant in pyramidal neurons in the amygdala (McDonald, 1996). CI-AMPARs are known for being stable at the synapses and contributing to basal neurotransmission, whereas CP-AMPARs are transiently expressed and recruited during synaptic plasticity, a Ca^{2+}-dependent phenomenon (Hong et al., 2013; Plant et al., 2006).

Synaptic plasticity is considered the mechanism underpinning learning and memory. The memory trace for newly acquired information is initially labile, sensitive to disruption, and stabilization of the memory trace is achieved by consolidation (Dudai, 2004). Despite extensive evidence of the involvement of CP-AMPARs in synaptic plasticity in the hippocampus (Beattie et al., 2000; Park et al., 2016; Plant et al., 2006), its direct role in memory with *in vivo* studies has been less explored. Studies have revealed a transient increase in CP-AMPAR expression in lateral amygdala synapses following fear conditioning, being elevated in few minutes and persisting for hours (Clem & Huganir, 2010; Hong et al., 2013). In the course of consolidation, CP-AMPARs are replaced by CI-AMPARs, in line with memory trace stabilization (Hong et al., 2013). On the other hand, fear memory retrieval induces destabilization of the memory trace, which has been linked to a switch from CI-AMPARs to CP-AMPARs (Hong et al., 2013).

Upon retrieval, memory might be rendered labile again and be modified via different processes (Dudai, 2004; Monfils, Cowansage, Klann, & LeDoux, 2009). One such process is extinction, in which a new memory with a different meaning suppresses the original one. Fear

memory has been shown to be more easily extinguished when CP-AMPAR activity in lateral amygdala synapses is high, and extinction involves internalization of CP-AMPARs (Clem & Huganir, 2010). On the other hand, another study has shown that CP-AMPAR is more important for extinction of a remote fear memory than a recent one (Zelena et al., 2016).

In short, studies propose a role for CP-AMPAR in plasticity and that memory labilization is associated to CP-AMPAR synaptic expression, whereas memory stabilization is promoted by CI-AMPAR expression. However, there is still a lack in the understanding of the role of CP-AMPAR activity in different brain structures for memory processes, especially in the hippocampus. Furthermore, since all the *in vivo* studies focused on aversive memory tasks, whether CP-AMPAR dynamics in tasks with different requirements is similar is unknown. Therefore, this study aims to elucidate the role of CP-AMPAR expression in the amygdala and the hippocampus for fear memory consolidation and extinction, as well as to compare consolidation in different tasks.

Materials and methods

Subjects

Male adult Wistar rats from our breeding colony, aged 60-90 days and weighing 250-300g, were used. Animals were housed in plastic cages with sawdust bedding, four to five per cage, with food and water *ad libitum* under a 12 hour light-dark cycle (lights on from 07 to 19h). All experiments were performed during the lights-on phase, approved by the Ethics Committee from Universidade Federal do Rio Grande do Sul and conducted in accordance with the national animal care legislation (Brazilian Law 11794/2008).

Stereotaxic surgery

Animals underwent a stereotaxic surgery for bilateral cannula implantation in the basolateral amygdala (BLA) (in mm, from bregma: anteroposterior (AP) -0.25; latero-lateral (LL) ±0.51; dorsoventral (DV) -0.70) or the CA1 region of the dorsal hippocampus (AP -0.40; LL ±0.30; DV -0.16) (Paxinos & Watson, 2007). Rats were intraperitonially anesthetized using

ketamin (75 mg/kg) and xylazin (10 mg/kg) and given the anti-inflammatory meloxicam (0.3 mg/kg, subcutaneous) prior to the surgery. 27-gauge guide cannulae were implanted according to the coordinates, positioned 1.0 mm above the targets. Animals had a recovery period of 5 to 7 days before undergoing behavioral procedures.

Auditory fear conditioning

Animals were exposed to two conditioning chambers with stainless steel bars on the floor. Two contexts (A and B) were used, which differed by different color patterns and textures in the walls, illumination intensity and presence of vanilla scent.

First, animals were habituated to context A in 10 minute-sessions during 2 days. On day 3, they were trained: animals were placed in context B for 2 minutes, then exposed to three 30-second 5 kHz tones followed by two 1-second 0.5 mA footschocks, separated by 1-minute intervals. Animals were returned to their homecages 1 minute after the last footshock.

In experiment 1, memory consolidation was tested 24 hours after training (day 4). The test consists of placing the animals in context A for 2 minutes, then presenting three 30-second tones with 1-minute intervals. After 1 minute, animals were returned to their homecages.

In experiment 5, a memory extinction session was carried out on day 4. Animals were placed in context A for 2 minutes and were presented twenty 30-second tones with 1-minute intervals. Extinction memory was tested on day 5 in context A. To assess whether fear expression could return by exposure to the training context (renewal), animals were tested in context B on day 6.

Conditioning and testing/extinction were performed in different contexts to allow the isolation of the animal response that reflects association between shock and tone alone, excluding an influence of context. Memory was measured by quantification of freezing during each tone by an experienced observer blind to the experimental conditions. For test and renewal sessions, freezing was expressed as percentage of the total tone presentation time. For the extinction session, freezing percentage was related to tones grouped two by two to allow the generation of an extinction curve.

Contextual fear conditioning

Animals were exposed to a conditioning chamber consisting of Plexiglass walls and stainless steel bars on the floor. During the training session (day 1), animals were placed in the chamber for 3 minutes, then received two 2-second 0.7 mA footshocks separated by a 30-second interval. 30 seconds after the last footshock, they were returned to their homecages.

In experiment 2, memory consolidation was assessed 48 hours after training (day 3). Animals were placed in the conditioning context for 4 minutes and then returned to their homecages.

In experiment 6, a memory extinction session was carried out on day 3. Extinction consisted of exposing the animals to the conditioning context for 30 minutes. On day 4, memory was tested for 4 minutes in the same context.

Memory was measured by quantification of freezing and expressed as percentage of the total session time. For the extinction sessions, minutes were grouped five by five to allow the generation of an extinction curve.

Object location

In experiment 3, animals were habituated to a wooden box whose walls contained four different patterns in black and white in 5-minute sessions during 4 days. On day 5, they underwent three training sessions interspaced by 5 minutes each. During training, two identical objects were placed in two corners of the box and rats were allowed to explore them for 5 minutes. A test session was conducted on day 6, in which one of the objects was repositioned and animals could explore them for 5 minutes.

The time spent exploring each object in each session was quantified and memory was indicated by an exploration index, calculated as the ratio of the exploration time of the repositioned object to the total exploration time of both objects.

Morris water maze

In experiment 4, animals underwent training sessions for 5 days in the Morris water maze. This task was performed in a water tank inside a room with spatial cues on the walls. A platform was placed in a specific site of the tank, around 3 centimeters underwater. Each training session consisted of 6 trials, with random start positions, in which the animals had 60 seconds to swim around the tank and find the platform and then were required to remain 20 seconds on it. In case the platform was not found within 60 seconds, animals were guided by the hand of the experimenter. On day 6, a 2-minute test session was carried out with the platform removed.

The time animals took to find the platform in each trial of the training sessions (latency) was quantified and learning was assessed by comparing the average latency of the session across days. In the test, memory was measured as the time spent in the quadrant where the platform was originally located (target quadrant), time spent in the opposite quadrant and latency to cross the platform as percentage of half or the total test time, as well as the number of crossings on the platform site.

Drug infusions

1-Naphthyl acetyl spermine trihydrochloride (NASPM; Tocris), a selective calcium-permeable AMPA receptor antagonist, dissolved in 1% dimethyl sulfoxide (DMSO) in sterile isotonic saline, was used in the concentrations of 0.4 μM and 4 μM NASPM or the vehicle DMSO 1% was infused bilaterally with a microinjection pump at a rate of 20 μl/h, in a volume of 0.5 μl per hemisphere in the basolateral amygdala or 40 μl/h, 1.0 μl per hemisphere in CA1 hippocampus.

Drugs were infused at the following times: immediately after training in experiments 1 and 2; immediately after the third training session in experiment 3; immediately after each training session in experiment 4; and 20 minutes before extinction in experiments 5 and 6.

Histology

Following the behavioral procedures, animals were euthanized. Brains were removed and fixed in a 4% paraformaldehyde and 30% sucrose solution. Brains were sliced in a cryostat to

obtain sections of the BLA and HPC 50 µm thick. Cannula placement and infusion site was verified under a light microscope. Only animals with correctly positioned cannulae were included in the analyses.

Statistical analysis

Test sessions (consolidation, extinction, renewal, object location and water maze tests) were analyzed with independent Student's t test or one-way ANOVA (memory consolidation in experiment 1). The extinction sessions and the water maze learning curve were analyzed using two-way repeated measures ANOVA, followed by Bonferroni's *post hoc* test. A p-value < 0.05 was considered statistically significant.

Results

Experiment 1: NASPM impairs fear memory consolidation in the BLA

In experiment 1, we made a concentration curve to NASPM, a selective CP-AMPAR antagonist, in order to define its most effective concentration. Additionally, based on previous data (Clem & Huganir, 2010; Hong et al., 2013; Rumpel et al., 2005), we sought to confirm that fear memory consolidation was dependent on CP-AMPAR activity in the BLA, using the auditory fear conditioning paradigm. Animals were trained in context B and tested in A 24 hours later. Immediately after training, 0.4 or 4 µM NASPM or 1% DMSO was infused into the BLA (Figure 1A), and animals were tested 24 hours later. There was a significant effect of treatment on freezing levels in the consolidation test ($F_{2,16} = 10.89$; $p = 0.001$) (Figure 1B). Bonferroni's *post hoc* analysis showed that animals treated with 0.4 µM NASPM displayed lower freezing levels than controls, whereas 4 µM NASPM and control groups did not differ ($p < 0.001$). Thus, 0.4 µM NASPM hindered memory consolidation. Based on these results, 0.4 µM was the NASPM concentration chosen for the subsequent experiments.

Experiment 2: NASPM impairs fear memory consolidation in the hippocampus

In experiment 2, we assessed whether fear memory consolidation was also dependent on CP-AMPAR activity in the hippocampus, using the contextual fear conditioning paradigm. Immediately after training, 0.4 µM NASPM or 1% DMSO was infused into CA1 hippocampus (Figure 2A), and animals were tested 48 hours later. Freezing levels in the consolidation test were significantly reduced in the NASPM group compared to the control ($p < 0.05$) (Figure 2B), indicating impairment in memory consolidation.

Experiment 3: NASPM does not affect neutral memory consolidation in the hippocampus

Since our results indicated that CP-AMPAR activity is necessary both in the BLA and the hippocampus for fear memory consolidation, in experiment 3 we assessed whether neutral memory consolidation also depends on this receptor. Animals underwent the object location task, received 0.4 µM NASPM or 1% DMSO infusions into CA1 hippocampus after the last training and were tested 24 hours later (Figure 3A). Both groups explored more the object moved to a new position than the original one, as indicated by the exploration index (One Sample t test, $p < 0.05$), meaning that they learned the objects position. However, since there was no significant difference of object preference between groups ($p = 0.9191$) (Figure 3B), we conclude that consolidation was not affected by NASPM.

Experiment 4: NASPM impairs spatial memory consolidation in the hippocampus

Since CP-AMPAR activity was shown to be required for fear memory consolidation and not for neutral memory consolidation, in experiment 4 we used a task with a low level of averseness to assess spatial memory consolidation. Animals were trained in the water maze task, 0.4 µM NASPM or 1% DMSO was infused into CA1 hippocampus after each training session and memory was assessed 24 hours after the fifth training (Figure 4A). There was a significant effect of time on the latency to find the platform during the training sessions ($F_{(4,48)} = 10.41$; $p < 0.0001$) (Fig 4B), indicating that both groups decreased the latency over time and learned the task, but performance was not affected by NASPM, since there was no effect of treatment ($F_{(1,12)}$

=3.2910; $p = 0.0947$) nor interaction ($F_{(4,48)} = 0.9612$; $p = 0.4374$). In the consolidation test, the NASPM group spent less time in the target quadrant during the first minute than controls ($p < 0.05$) (Figure 4C), but when the total test time was considered, groups did not differ ($p = 0.0803$) (Figure 4D). Moreover, there was no difference in time spent in the opposite quadrant in the total test time ($p = 0.6952$) (Figure 4E), the latency to cross the platform site ($p = 0.6240$) (Figure 4F) and the number of platform crossings ($p = 0.2815$) (Figure 4G).

Experiment 5: NASPM does not affect fear memory extinction in the BLA, but prevents fear renewal

In experiment 5, we assessed whether CP-AMPAR activity in the BLA played a role in fear extinction. An extinction session was carried out 24 hours following training. 0.4 μM NASPM or 1% DMSO was infused into CA1 hippocampus 20 minutes prior to extinction (Figure 5A). Freezing levels decreased during the extinction session (time effect $F_{(9,180)} = 3.890$; $p < 0.001$) (Figure 5B), indicating memory extinction for NASPM and control groups. However, no effect of treatment was observed ($F_{(1,20)} = 1.687$; $p = 0.2087$). There was no interaction between factors (time x treatment $F_{(9,180)} = 2.136$; $p = 0.0287$). The test session confirmed extinction memory consolidation, since freezing levels remained low, without difference between groups ($p = 0.6656$) (Figure 5C). Interestingly, in the renewal session, carried out in the same context as training, which was different from the extinction one, the NASPM group expressed lower freezing levels relative to controls ($p < 0.05$) (Figure 5D), meaning that renewal (the return of fear expression) was hindered in controls. By the time spontaneous recovery was assessed, 14 days following renewal, freezing levels remained low ($p = 0.9598$) (Figure 5E).

Experiment 6: NASPM does not affect fear memory extinction in the hippocampus, but impairs fear memory retrieval.

In experiment 6, we assessed whether CP-AMPAR activity in the hippocampus played a role in fear extinction. An extinction session was carried out 24 hours following training and 0.4 μM NASPM or 1% DMSO was infused into CA1 hippocampus 20 minutes prior to extinction

(Figure 6A). There a significant effect of time on freezing levels during the extinction session ($F_{(5,100)} = 6.965$; $p < 0.0001$) (Figure 6B). However, there was no effect of treatment ($F_{(1,20)} = 1.414$; $p = 0.2483$) There was interaction between variables (time x treatment $F_{(5,100)} = 2.320$; $p < 0.05$). Bonferroni's *post hoc* analysis revealed lower freezing levels of the NASPM group during the first five minutes of extinction as compared to controls ($p < 0.05$), suggesting an impairment of fear memory retrieval (Figure 6B). The test session confirmed extinction memory consolidation, since freezing levels remained low, without difference between groups ($p = 0.3698$) (Figure 6C). By the time spontaneous recovery was assessed, 14 days following extinction, freezing levels remained low ($p = 0.7438$) (Figure 6D).

Discussion

Memory consolidation

One of the goals of experiment 1 was to test two NASPM concentrations, 0.4 and 4 μM. We found expressive freezing reduction with the former concentration, which was used in the subsequent experiments. In experiments 1 and 2, we assessed whether memory consolidation depended on CP-AMPAR activity using fear conditioning. Despite evidence that CP-AMPAR is inserted into potentiated synapses in the lateral amygdala (Clem & Huganir, 2010; Hong et al., 2013; Rumpel et al., 2005) and into the hippocampus for LTP induction (Gray, Fink, Sarinana, Vissel, & O'Dell, 2007; Park et al., 2016; Plant et al., 2006), its direct effect on memory consolidation had not been tested. We hypothesized that the initial period of consolidation would be a plastic state with crucial CP-AMPAR function. The deleterious effect of NASPM on memory consolidation in experiments 1 and 2 indicates that CP-AMPAR activity in the BLA and CA1 hippocampus was important in the beginning of consolidation, during a moment when the memory trace was still unstable, and the blockade of this receptor prevented proper stabilization of the trace. This finding is in agreement with studies that report an increase in CP-AMPAR expression in thalamus-lateral amygdala synapses after fear conditioning (Clem & Huganir, 2010; Hong et al., 2013). Nevertheless, the time window in which CP-AMPAR is detected is wide: according to electrophysiological recordings in slices, it ranged from 5 min to 12 hours (Hong et al., 2013) and from 12 to 48 hours following auditory fear conditioning (Clem &

Huganir, 2010). Differences in the fear conditioning protocols might account for this discrepancy. It was also expected to find the impairment in memory consolidation the hippocampus, as CP-AMPAR has been shown to take part in some mechanisms of hippocampal LTP induction (Park et al., 2016; Plant et al., 2006), but not all (Adesnik & Nicoll, 2007; Gray et al., 2007; Park et al., 2016). Of note, the temporal dynamics of CP-AMPAR in consolidation has not been addressed *in vivo* so far, although our results corroborate the assumption that CP-AMPAR is rapidly driven into the synapses in plastic states, such as the beginning of memory consolidation and memory retrieval. Stabilization of the trace is brought about by synaptic strengthening, related to LTP, and it is possible that NASPM perturbed plasticity by hindering CP-AMPAR contribution to LTP.

Due to our finding that fear memory consolidation was impaired by CP-AMPAR blockade, in experiment 3 we aimed at assessing consolidation of a neutral memory dependent on the hippocampus. The object location task suggests that CP-AMPAR was not necessary for consolidation of such neutral memory. Moreover, in experiment 4 we tested consolidation in the water maze, a hippocampus-dependent spatial memory task. Of all measurements, animals treated with NASPM displayed a memory deficit only in the first minute of test. The object location and the water maze task differ in that the former has no motivational components, while the latter has an averseness factor, lower than fear conditioning (cool water), and contains a reinforcement for animals to learn (the platform allows them to escape water) (Vorhees & Williams, 2014). Taken together, experiments 1 to 4 indicate that memory valence and learning conditions may influence the mechanisms underlying memory consolidation, likely by the effects of arousal and stress (Hu et al., 2007; Mumby, 2002). It has been shown that norepinephrine released by emotional stress induces serine 845 GluA1 phosphorylation in the hippocampus *in vivo*, promoting AMPAR incorporation in the post-synaptic membrane and facilitating LTP (Hu et al., 2007). Thus, besides enhancing memory formation, stress might as well influence CP-AMPAR trafficking to the synapses and account for the differences observed in our memory tasks, in line with studies that do not identify CP-AMPAR insertion in CA1 in LTP (Adesnik & Nicoll, 2007; Gray et al., 2007).

Memory extinction

Memory extinction is also associated to plasticity, since it involves, following memory retrieval, the formation of a new memory that inhibits the activation of the original trace. Previous work relating CP-AMPAR to fear memory extinction only used a protocol of reactivation followed by extinction (Clem & Huganir, 2010) or systemic CP-AMPAR antagonism (Zelena et al., 2016). In experiments 5 and 6, we aimed at elucidating the role of CP-AMPAR activity specifically in the BLA and the hippocampus in memory extinction. We hypothesized that fear memory reactivation that allows extinction would labilize the memory trace and promote CP-AMPAR synaptic insertion, which could in turn facilitate the formation of the extinction memory.

Contrary to our expectations, we did not find evidence for CP-AMPAR involvement neither in the BLA nor in the hippocampus in the acquisition and consolidation of extinction memory, as observed in the extinction curve and test. However, fear expression was reduced in the first five minutes of the extinction session in experiment 6. This suggests that CP-AMPAR is recruited in the hippocampus for memory reactivation, which occurs when the animal is exposed to a familiar stimulus (the context), and its blockade impaired retrieval. Indeed, short retrieval sessions, which are known for triggering reconsolidation (Monfils et al., 2009; Suzuki, 2004), induce CI-AMPAR endocytosis (Bhattacharya et al., 2017; Hong et al., 2013; Rao-Ruiz et al., 2011) and a transient switch from CI to CP-AMPAR (Hong et al., 2013). We assume that this switch could be also observed during extinction-mediating reactivation.

Interestingly, in the renewal test from experiment 5, freezing was lower in NASPM-treated animals than in controls. This indicates an effect of NASPM in renewal prevention. In the auditory fear extinction protocol, training and test are not performed in the same context, in order to isolate the association between tone and footshock as much as possible. However, extinction is context-dependent, and when animals are reexposed to the same context were training occurred, the context may trigger the freezing response (fear renewal). Our result indicates that CP-AMPAR activity in the BLA is important for renewal, in accordance with a mechanism proposed by Lee et al. (2013) and Park et al. (2014), according to which renewal involves increased GluA1 serine 831 phosphorylation, CP-AMPAR activity and synaptic strength in the lateral amygdala. Additionally, NASPM infused before the renewal session impaired fear renewal (Park et al.,

2014). We propose that CP-AMPAR blockade before extinction hindered the fear memory trace labilization and, even though this did not facilitate acquisition or consolidation of the extinction memory, it might have weakened the original memory and prevented the return of fear expression.

Finally, since extinction does not usually enable permanent fear reduction, we expected to observe spontaneous recovery of fear 2 weeks after extinction, but this was not corroborated by our data. 14 days might have been insufficient time for spontaneous recovery, and it is likely that memory could have been tested and recovered in 21 days (Haubrich et al., 2017).

Conclusion

Here we show that CP-AMPAR activity in the BLA is required for aversive memory consolidation. In the hippocampus, the role of CP-AMPAR likely depends on the emotional valence of the memory and other features of the task, such as reinforcement. Hippocampal CP-AMPAR is important for fear contextual memory and slightly aversive spatial memory, but not for neutral memory consolidation. Furthermore, CP-AMPAR did not play a role in formation and consolidation of extinction memory, but revealed to be important for fear retrieval in the hippocampus and renewal in the BLA. Our data support a role of CP-AMPAR in mnemonic processes that are associated to plastic changes in the brain.

Conflict of interest: the authors declare no conflict of interest.

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Legends and figures

Figure 1. NASPM infusion into the BLA impairs fear memory consolidation. (A) Protocol for testing auditory fear memory consolidation. Animals were habituated in context A (hab ctx A), trained in context B (ctx B) with tone presentations paired to 0.5 mA footshocks and tested in context A. 0.4 µM NASPM or 1% DMSO was infused into the BLA immediately after training. (B) Freezing levels in the test session. NASPM animals displayed reduced freezing compared to the controls ($p = 0.001$) (n = 6 for control, n = 6 for 0.4 µM NASPM, n = 7 for 4 µM NASPM). Data are presented as mean ± SEM. ** = $p = 0.001$.

Figure 2. NASPM infusion into the hippocampus impairs fear memory consolidation. (A) Protocol for testing contextual fear memory consolidation. Animals were trained by presenting 0.7 mA footshocks in the conditioning chamber and tested in the same context. 0.4 µM NASPM or 1% DMSO was infused into CA1 hippocampus immediately after training. (B) Freezing levels in the test session. NASPM animals displayed reduced freezing compared to the controls ($p < 0.05$) (n = 18 for control and n = 17 for 0.4 µM NASPM). Data are presented as mean ± SEM. * = $p < 0.05$.

Figure 3. NASPM infusion into the hippocampus does not affect consolidation of object location memory. (A) Protocol for testing memory consolidation in the object location task. Animals were habituated to a chamber without objects. In the training sessions, two identical objects were placed in specific positions. In the test session, one of the objects was repositioned. 0.4 µM NASPM or 1% DMSO was infused into CA1 hippocampus immediately after the last training. (B) Exploration index (exploration time for replaced object/sum of both) in the test session. NASPM and control animals explored more the object moved to a new position than the original one (One Sample t test, $p < 0.05$), but there was no difference between groups ($p = 0.9191$) (n = 8 for control and n = 8 for 0.4 µM NASPM). Data are presented as mean ± SEM.

Figure 4. NASPM infusion into the hippocampus impacts spatial memory in the water maze. (A) Protocol for testing memory consolidation in the water maze. Animals underwent five training sessions with a hidden platform under water, after each of which 0.4 µM NASPM was infused into CA1 hippocampus. In the test session, the platform was removed and spatial memory was assessed. (B) Learning curve (T1-T5 = training days 1 to 5). Both control and NASPM groups decreased the latency to

find the platform over training days (time factor $F_{(4,48)} = 10.41$; $p < 0.0001$), but no difference between groups was observed (treatment factor $F_{(1,12)} = 3.291$; $p = 0.0947$). (C) Time spent in the platform (target) quadrant relative to the first minute of the test. NASPM group spent less time in the target quadrant than controls ($p < 0.05$). There was no difference in (D) time spent in the target quadrant in the total test time ($p = 0.0803$), (E) time spent in the opposite quadrant in the total test time ($p = 0.6952$), (F) latency to cross the platform site ($p = 0.6240$) and (G) number of platform crossings ($p = 0.2815$) (n = 7 for control and n = 7 for 0.4 µM NASPM in all analyses). Data are presented as mean ± SEM.

Figure 5. NASPM infusion into the BLA does not affect fear memory extinction. (A) Protocol for testing auditory fear memory extinction. Animals were habituated in context A (hab ctx A), trained in context B (ctx B) with tone presentations paired to 0.5 mA footshocks, had extinction and test sessions in context A, renewal in the training context and spontaneous recovery in the extinction context. 0.4 µM NASPM was infused 20 into the BLA minutes prior to extinction. (B) Freezing levels during the extinction session (T_n+T_{n+1} = response to two tone presentations grouped). Freezing was reduced over time (time effect, $F_{(9,216)} = 4.2450$; $p < 0.0001$) for both groups (no effect of treatment, $F_{(1,24)} = 0.6294$; $p = 0.2087$). (C) Test session. Both groups displayed low freezing levels ($p = 0.6656$). (D) Renewal session. Freezing of the NASPM group was lower than in the control group, showing an impairment in renewal by NASPM ($p < 0.05$). (E) Spontaneous recovery. There was no spontaneous recovery of fear expression ($p = 0.9598$) (n = 11 for control and n = 11 for 0.4 µM NASPM for all analyses, except for spontaneous recovery, in which n = 10 for control). Data are presented as mean ± SEM.

Figure 6. NASPM infusion into the hippocampus does not affect fear memory extinction. (A) Protocol for testing contextual fear memory extinction. Animals were trained with 0.7 mA footshocks presented in the conditioning chamber, followed by extinction, test and spontaneous recovery sessions. 0.4 µM NASPM was infused into CA1 hippocampus 20 minutes prior to extinction. (B) Freezing levels during the extinction session (n-n+5 = responses plotted in groups of five minutes). Freezing was reduced over time (time effect, $F_{(5,100)} = 6.965$; $p < 0.0001$) for both groups (no effect of treatment, $F_{(1,20)} = 1.414$; $p = 0.2483$). The NASPM group showed lower freezing during the first five minutes of extinction relative to controls ($p < 0.05$). (C) Test session. Both groups displayed low freezing levels ($p = 0.3698$). (D) Spontaneous recovery. There was no spontaneous recovery of fear expression ($p = 0.7438$) (n = 11 for control and n = 11 for 0.4 µM NASPM for all analyses). Data are presented as mean ± SEM.

Figure 1

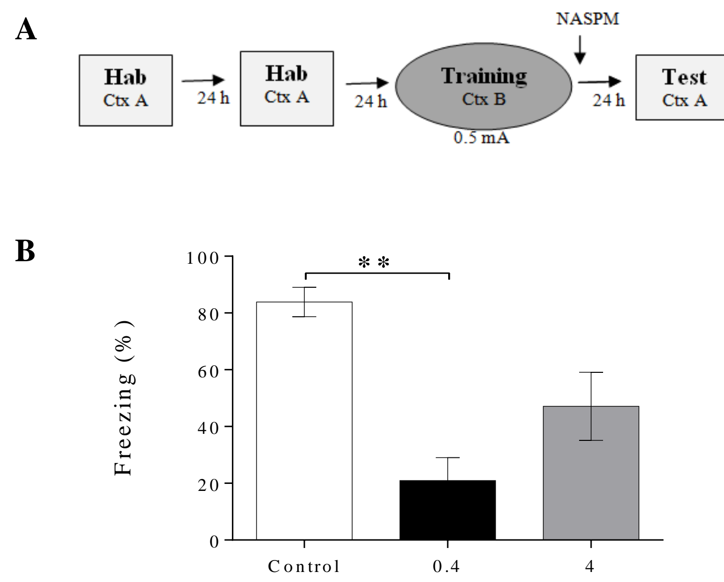


Figure 2

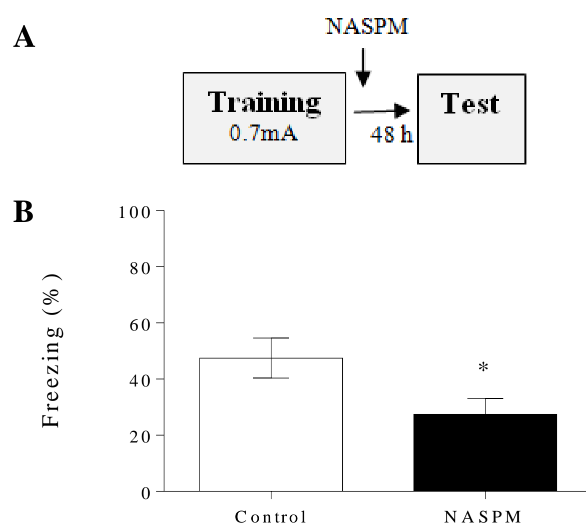
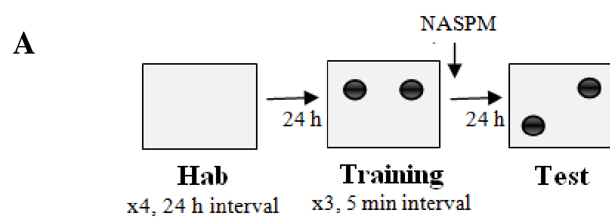


Figure 3

A



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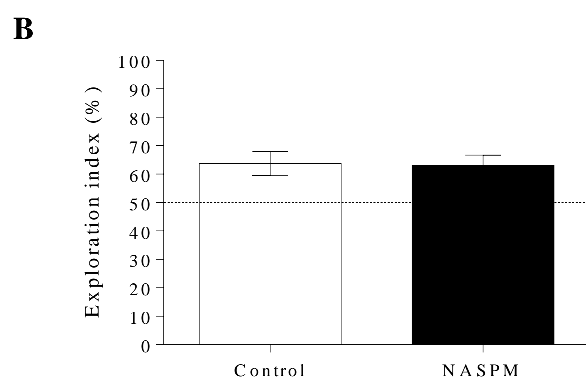


Figure 4

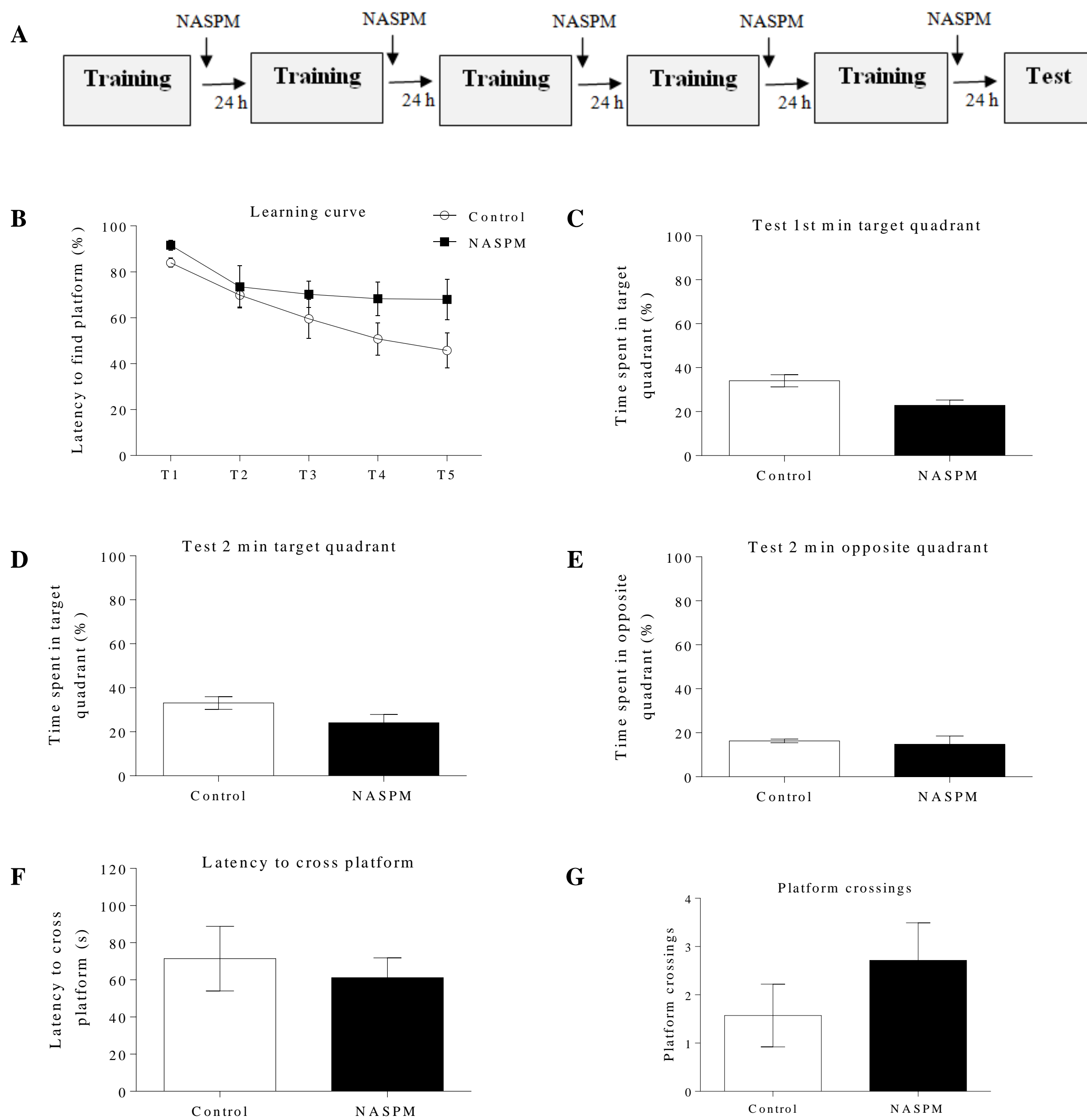
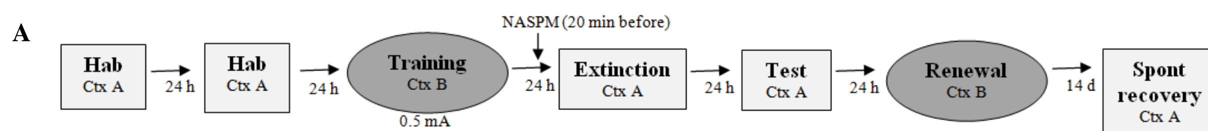
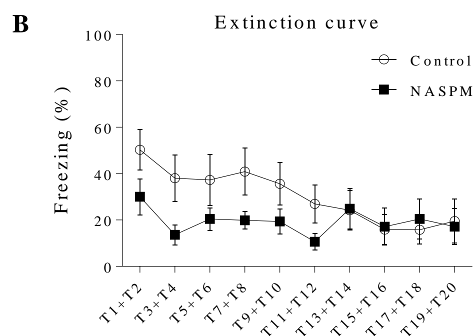
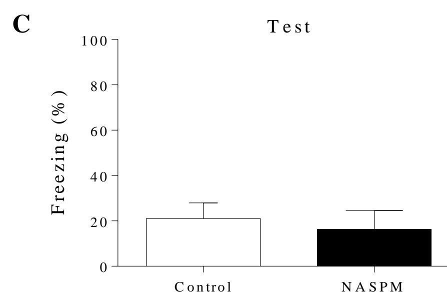
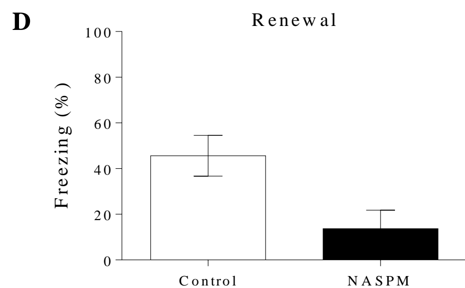


Figure 5









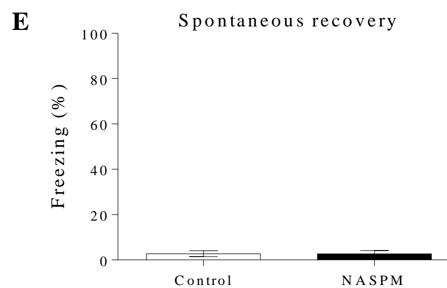
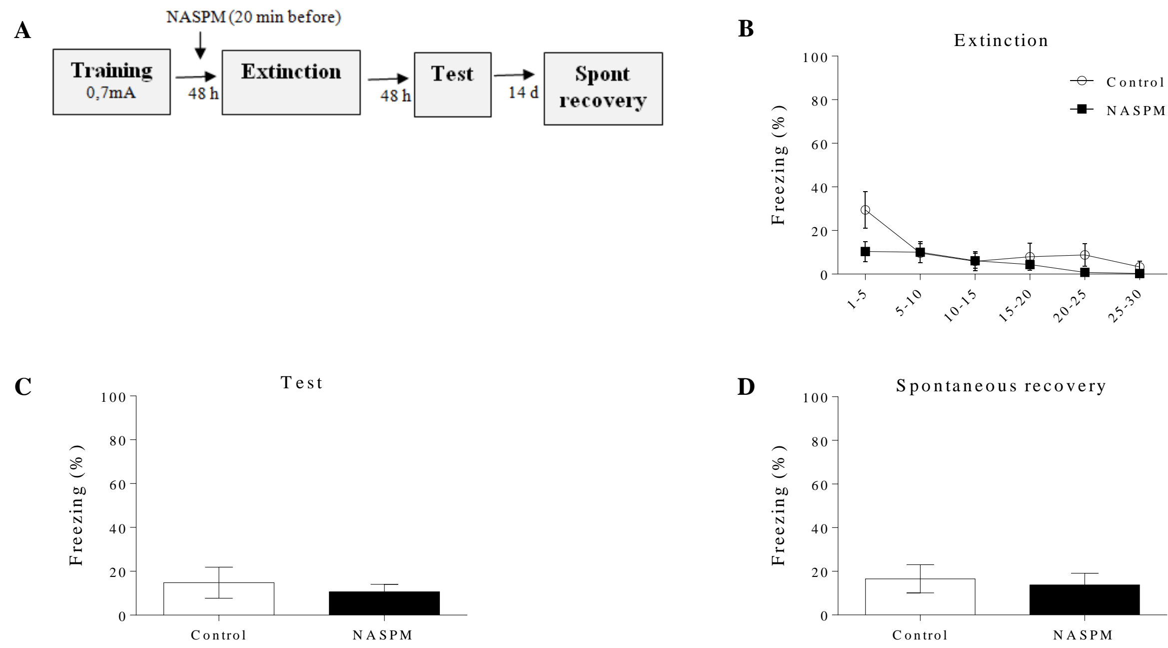


Figure 6



5 CONCLUSÕES E PERSPECTIVAS

Neste trabalho, demonstramos como o receptor AMPA permeável a cálcio (CP-AMPAR) influencia determinados processos mnemônicos a nível comportamental. Encontramos que a atividade de CP-AMPAR na amígdala basolateral é fundamental para a consolidação de memórias aversivas (condicionamento aversivo ao tom). No hipocampo, sua atividade é importante para a consolidação de memórias aversivas (condicionamento aversivo ao contexto) e para memórias espaciais com um grau baixo de aversividade (labirinto aquático de Morris), porém não para memórias neutras (localização de objetos). Assim, a atividade do CP-AMPAR parece depender da valência emocional da memória, e possivelmente de outras características da tarefa, como a presença de reforço para o aprendizado. Além disso, demonstramos que o CP-AMPAR não influencia a formação e consolidação da memória de extinção, mas é importante no hipocampo para a evocação da memória aversiva e na amígdala para o retorno da expressão de uma memória aversiva (renewal). De forma geral, esses resultados reforçam que o CP-AMPAR está envolvido em processos plásticos que são a base para a formação e modificação de memórias. Também contribuem para o entendimento do papel do CP-AMPAR diretamente em processos mnemônicos, tema ainda pouco explorado, e complementa dados já presentes na literatura com foco nos mecanismos moleculares da dinâmica de AMPARs.

Este trabalho de conclusão de curso foi desenvolvido como parte de um projeto de mestrado que visa elucidar a função do CP-AMPAR em diversos processos mnemônicos, abordando também a evocação, a reconsolidação e o esquecimento de memórias.

Nos experimentos de consolidação, buscamos explorar tipos diferentes de memória, baseados principalmente na sua valência emocional (tarefas aversivas e neutras). Todavia, é interessante verificar com maior especificidade esse fator, a fim de diferenciá-lo de outras características e exigências das tarefas. Uma das possibilidades seria realizar um condicionamento com choques mais fracos no condicionamento aversivo ao tom e ao contexto, para que fossem comparadas memórias oriundas da mesma tarefa, porém com diferentes intensidades. Ainda, poderia ser avaliado se a força da memória afeta a janela temporal de ação dos CP-AMPARs, bloqueando esse receptor em momentos diferentes da janela de consolidação sináptica e em protocolos de condicionamento de diferente intensidade.

A fim de complementar o trabalho e conferir maior consistência aos achados comportamentais, podem ser empregadas duas técnicas adicionais: o western blot e o registro eletrofisiológico. O western blot das frações sinápticas de hipocampo e amígdala permitiria quantificar as subunidades GluA1 e GluA2 e indicar a presença de CP-AMPAR (que não contém GluA2) no início da consolidação e da extinção e no renewal. Tal abordagem seria menos precisa para identificação de CP-AMPAR, visto que não há marcador específico para esse receptor (GluA1 está presente na maioria dos AMPARs, mas a ausência de GluA2 caracteriza o CP-AMPAR). Com registro eletrofisiológico das correntes excitatórias pós-sinápticas em fatias do encéfalo obtidas após procedimentos comportamentais específicos, como o início da consolidação e o renewal, seria possível avaliar o índice de retificação de corrente de AMPAR, característica dos CP-AMPARs que comprovaria mais diretamente do que por western blot sua expressão e função nas sinapses nos dados momentos.

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To ensure the highest print quality, your figures must be submitted in TIF format according to the following minimum resolutions:

- 1200 dpi (dots per inch) for black and white line art (simple bar graphs, charts, etc.)
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Forms of Manuscripts

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Please consider the following guidelines in the Resource Identification Initiative.
Hippocampus is participating in the Resource Identification Initiative, which aims to promote research resource identification, discovery, and reuse. This initiative, led by the Neuroscience Information Framework and the Oregon Health & Science University Library, provides unique identifiers for antibodies, model organisms, cell lines, and tools such as software and databases. These IDs, called Research Resource Identifiers (RRIDs), are machine-readable and can therefore be used to search for all papers in which a particular resource was used and to increase access to critical data to help researchers identify suitable reagents and tools.

As part of this pilot project, we ask authors to use RRIDs to cite the resources used in your research where applicable in the text, exactly as you would a regular citation or Genbank Accession number. For antibodies, we ask that you please include in your citation the vendor, catalogue number, and RRID in the text upon first mention in the methods section. For software tools and databases, please provide the name of the resource followed by the resource website if available, and the RRID. For model organisms, the RRID alone is sufficient.

We also ask that you please include the RRIDs in the list of keywords associated with your manuscript.

To Obtain Research Resource Identifiers (RRIDs):

1. Use the Resource Identification Portal, created by the Resource Identification Initiative Working Group.

2. Search for your research resource (please see the section titled “Search Features and Tips” for more information)

3. Click on the “Cite This” button to obtain the citation and insert the citation into your manuscript text.

If you have a resource that is not found within the Portal, we encourage you to register the resource with the appropriate resource authority. Information on how to do this is provided in the “Resource Citation Guidelines” section of the Portal.

If you experience any difficulties obtaining identifiers, please contact rii-help@scicrunch.org for assistance.

Example Citations:

Antibodies: "Wnt3 was localized using a rabbit polyclonal antibody C64F2 against Wnt3 (Cell Signaling Technology, Cat# 2721S, RRID: AB_2215411)"

Model Organisms: "Experiments were conducted in c. elegans strain SP304 (RRID:CGC_SP304)"

Cell lines: "Experiments were conducted in PC12 CLS cells (CLS Cat# 500311/p701_PC-12, RRID:CVCL_0481)"

Tools,Software, and Databases: "Image analysis was conducted with CellProfiler Image Analysis Software, V2.0 (http://www.cellprofiler.org, RRID:nif-0000-00280)"

Title Page. The Title Page should include the complete title of the paper, together with the name(s) of the author(s) and institutional affiliations (to department level); a running (abbreviated) title, not exceeding 60 characters and spaces; the number of text pages, figures, and tables; and the name, full address, telephone number, and E-mail address of the author to whom correspondence, including proofs, should be sent, and all grant information in the following format: Grant sponsor:______; Grant number:______. A list of five key words that do not occur in the title should be included for abstracting purposes. The title should represent the contents of the paper and should not include technical jargon, chemical formulas, or arbitrary abbreviations.

Abstract. The Abstract should be clearly written in 300 words or less and should succinctly state the objectives of the study, experimental design, major observations and conclusions, and their major significance. The abstract should be intelligible to neuroscientists in general and should thus be free of specialized jargon and abbreviations. References should generally not be cited in the abstract, but if they are, the complete citation should be given (e.g., Conti F et al., J Comp Neurol 1994; 343:554–565).

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Introduction. The Introduction section should provide sufficient background information to make clear the rationale and objectives of the reported studies. Extensive literature reviews are generally not necessary.

Materials and Methods. The Materials and Methods section should be concise but should adequately describe experimental procedures to allow for replication of the reported experiments. Wherever possible, references should be made to published protocols. Excessively detailed descriptions of widely used techniques or details of procedures that will not be of general interest to the reader should be avoided. Submission of a paper to *Hippocampus* implies that all animal

experimentation reported in the paper has been conducted in accordance with the guidelines laid down by the NIH (*NIH Guide for the Care and Use of Laboratory Animals*) in the USA or by the European Communities Council. When human subjects are used, adequate documentation should be included in the manuscript that the experiments were undertaken with the understanding and consent of each subject. It is assumed that with the publication of a paper in *Hippocampus* the authors will make available, whenever possible, reagents, such as antibodies, that were used in the research and are not commercially available.

Results and Discussion. In the Results section, findings should be described without discussion of their significance. Authors are encouraged to use subheadings to clarify the organization of this section. In the Discussion, authors should provide an interpretation and validation of their findings, conclusions, and their significance in relation to previously published work. Repetition of the results or extensive review of the literature should be avoided.

References

It is the responsibility of the author(s) that each reference in the text appears in the References section and that each reference listed in this section is correct and cited in the text. References should be cited in the text by author's name followed by year of publication, thus: Ben-Ari (1981) or (Ben-Ari, 1981); Squire and Zola (1983) or (Squire and Zola, 1983). In the case that there are more than two co-authors: Lopes da Silva et al. (1989) or (Lopes da Silva et al., 1989). A typical citation should follow the form: Data reported by Ben-Ari (1981) have recently been confirmed by others (Lopes da Silva et al., 1989). When more than one reference is cited, the references should be listed in chronological order. A paper that is in preparation or submitted to a journal but not yet accepted for publication should not be included in the References section; reference to a paper of this type should be cited as "unpublished observations", and the initials and surname(s) must be listed in the text for the author(s) whose unpublished experiments are cited.

In the References section, papers should be listed in alphabetical order according to the name of the first author. In the case of several references with the same first author but more than one co-author, the references should be listed in chronological order. When references are made to more than one paper by the same first author published in the same year, the postfix a, b, c, etc., should be used both in the text and in the References section; for papers published in different years, the references should be listed in chronological order. The name of the author(s) should be followed by the full title of the paper, and the complete source of the reference (abbreviations of journals should follow those used in Index Medicus), including the year of publication, volume number, and the first and last pages. The form used in the References section should follow APA reference style according to the following examples:

Journal article:
Goldstein, H. (1979). Improving police: A problem-oriented approach. *Crime & Delinquency, 3,* 236–258

Book:
Goldstein, H. (1990). Problem-oriented policing. New York, NY: McGraw-Hill. Miles, M. B., & Huberman, A. M. (1994). *Qualitative data analysis* (2nd ed.). Thousand Oaks, CA: Sage. .

Edited Book:
Gilbert, D. G., McClernon, J. F., Rabinovich, N. E., Sugai, C., Plath, L. C., Asgaard, G., ...
Botros, N. (1983). Situational crime prevention: Its theoretical basis and practical scope. In M. Tonry, & N. Morris (Eds.), *Crime and justice: An annual review of research* (Vol. 4, pp. 225–256). Chicago, IL: University of Chicago Press.

Paper Presentation:
Weiss, A., & McGarrell, E. F. (1996, November). *The impact of increased traffic enforcement on crime.* Paper presented to the Annual Meeting of the American Society of Criminology, Chicago, IL.

Symposium:
Muellbauer, J. (2007, September). Housing, credit, and consumer expenditure. In S. C. Ludvigson (Chair), *Housing and consumer behavior.* Symposium conducted at the meeting of the Federal Reserve Bank of Kansas City, Jackson Hole, WY.

Conference paper abstract retrieved online:
Liu, S. (2005, May). *Defending against business crises with the help of intelligent agent based early warning solutions.* Paper presented at the Seventh International Conference on Enterprise Information Systems, Miami, FL. Abstract retrieved from http://www.iceis.org/iceis2005/abstracts_2005.htm

Proceedings published regularly online:
Katz, I., Gabayan, K., & Aghajan, H. (2007). A multi-touch surface using multiple cameras. In J. Blanc-Talon, W. Philips, D. Popescu, & P. Scheunders (Eds.), *Lecture Notes in Computer Science: Vol. 4678. Advanced Concepts for Intelligent Vision Systems* (pp. 97–108). Berlin, Germany: Springer-Verlag. doi:10.1007/978-3-540-74607-2

Thesis/Dissertation:
Schnittker, J. (2004). *Education and the changing shape of the income gradient in health* (Unpublished doctoral dissertation or master’s thesis). Name of institution, location.

Report:
Muthen, L. K., & Muthen, B. O. (2004). *Child care and child development* (Report No. xxx). Los Angeles, CA: Publisher.

Patent:
Smith, I. M. (2011). *U. S. Patent No. 235,445.* Place: Publisher

.

Magazine:
Mathews, J., Berrett, D., & Brillman, D. (2005, May 16). Other winning equations. *Newsweek, 145*(20), 58–59.

Preparation of Illustrations. Illustrations submitted for publication should be the exact size that they will appear in print. The size of illustrations should not exceed the dimensions of the journal itself (7" x 9 3/8", or 17.8 cm x 23.8 cm). All figures, both line drawings and halftones, should be appropriately lettered and labeled. Lettering should remain at least 1/4" (6 mm) from the edges of figures to allow for trimming. The cost for printing color art is $850 per figure. The cost will be higher if the color art is submitted other than as specified above. Figures considered to be of insufficient quality for publication will be returned to the author(s) for correction. All figures must be referred to in the text and must be numbered and cited consecutively (Fig. 1, Fig. 2, etc.). Each figure should be accompanied by an explanatory legend that makes the illustration understandable without need for reference to the text.

Guidelines for cover submissions If you would like to send suggestions for artwork related to your manuscript to be considered to appear on the cover of the journal, please follow these general guidelines.

Preparation of Tables. Each table should be typed, double-spaced, as a separate doc or rtf file. The table should include an informative title and a legend that makes the table comprehensible without resorting to the text. Each column in the table should have a heading, and the columns should be formatted to be easily distinguishable by the compositor. If the table is highly complex, it should be submitted as a graphic in tiff or eps format so as to avoid introduction of errors during typesetting that would be difficult to detect in the proofreading stage. In this case, tables should be prepared using the same considerations one would apply to a line drawing illustration. All tables must be referred to in the text and must be numbered and cited consecutively (Table 1, Table 2, etc.).

Units, Symbols, and Abbreviations . For symbols of physical units, the SI system (Système International d' Unités) should be used. Abbreviations should not be used excessively in the text, and in all cases the word or words to be abbreviated should be written in full on the first occurrence, followed by the abbreviation in parentheses. The same abbreviations should be used in both the text and figures. If many abbreviations are used, including those used repeatedly in the tables or figures, they should be listed on a separate sheet, entitled Abbreviations.

Posters deposited in public access collections will not be considered as prior publication for the purposes of our acceptance at Hippocampus. However, the editors reserve the right to ask for the poster to insure that the information contained in the paper goes beyond or is different from that contained in the poster in some way and is not a duplicate publication.

Data Set Access. Authors are encouraged to make available the data sets relevant to the study and add reference to how the repository can be accessed.

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