UNIVERSIDADE FEDERAL DO PAMPA

KARINE RAMIRES LIMA

EFEITOS DA NOVIDADE NA GENERALIZAÇÃO DA MEMÓRIA AVERSIVA: UMA INVESTIGAÇÃO DA PARTICIPAÇÃO DOS SISTEMAS NORADRENÉRGICO E DOPAMINÉRGICO

TRABALHO DE CONCLUSÃO DE CURSO

URUGUAIANA
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Trabalho de Conclusão de Curso apresentado ao Curso de Farmácia da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Bacharel em Farmácia.

Orientador: Dra. Pâmela Billig Mello-Carpes

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Dedico este trabalho àqueles que nunca mediram esforços para a realização de meus objetivos; o melhor presente que a vida poderia ter me concedido: meus pais (Orpiana e Luiz).

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"Valeu a pena? Tudo vale a pena
Se a alma não é pequena.
Quem quer passar além do Bojador
Tem que passar além da dor.
Deus ao mar o perigo e o abismo deu,
Mas nele é que espelhou o céu."

Fernando Pessoa

RESUMO

A generalização da memória aversiva pode ser definida como um fenômeno no qual uma situação similar (porém distinta) a um evento aversivo prévio pode desencadear uma resposta defensiva e está relacionada a vários distúrbios psicológicos. Neste estudo, investigamos os efeitos da novidade na generalização das memórias de medo e a participação dos sistemas noradrenérgico e dopaminérgico neste processo. Para testar a memória aversiva e a generalização desta utilizamos a tarefa de memória "esquiva inibitória" (EI). No dia 1, ratos *Wistar* machos adultos foram treinados na EI. No dia 2, os animais foram expostos à uma esquiva inibitória modificada (EM) durante 3 minutos – 30 minutos antes da exposição à EM alguns animais foram expostos à uma novidade (campo aberto) durante 5 minutos. Para avaliar o envolvimento dos sistemas noradrenérgico e dopaminérgico, imediatamente antes da exposição à novidade, alguns animais receberam uma infusão intrahipocampal de timolol (antagonista β-adrenérgico), SCH23390 (antagonista D1/D5) ou veículo. No dia 3, para avaliar, respectivamente, a memória aversiva e generalização da memória aversiva metade dos animais de cada grupo foram testados na EI e outra metade na EM. Neste estudo demonstramos que a exposição à novidade evita a generalização da memória aversiva e que esse processo é dependente da ativação das vias β-adrenérgica e dopaminérgica D1/D5 hipocampal, uma vez que o bloqueio destas vias na região CA1 do hipocampo com a infusão intrahipocampal de timolol ou SCH23390, respectivamente, imediatamente antes da exposição à novidade inibe seu efeito. Além disso, observamos que a exposição à novidade eleva os níveis hipocampais de noradrenalina e dopamina, o que sugere que o aumento desses neurotransmissores pode influenciar a promoção da memória de longa duração, melhorando a memória e atenuando a generalização da memória aversiva.

Palavras-chave: generalização do medo, novidade, hipocampo, sistema noradrenérgico, sistema dopaminérgico.

ABSTRACT

The generalization of aversive memory can be defined as a phenomenon in which a similar situation (but distinct) to a previous aversive event can trigger a defensive response and is related to several psychological disorders. In this study, we investigate the effects of novelty on the generalization of fear memories and the participation of noradrenergic and dopaminergic systems in this process. In this study, we investigated the effects of novelty on the generalization of fear memories and the participation of noradrenergic and dopaminergic systems in this process. To test the aversive memory and the generalization of aversive memory, we used the "inhibitory avoidance" (IA) memory task. On day 1, adult male Wistar rats were trained in IA. On day 2, the animals were exposed to a modified inhibitory avoidance (MIA) for 3 minutes – 30 minutes before MIA exposition some animals were exposed to novelty (open field) for 5 minutes. To evaluate the involvement of noradrenergic and dopaminergic systems, immediatly before of novelty exposure, some animals received intrahippocampal infusions of timolol (β-adrenergic antagonist), SCH23390 (D1/D5 antagonist) or vehicle. On day 3, to evaluate, respectively, the aversive memory and generalization of aversive memory, half of the animals in each group were tested on IA and another half on MIA. In this study, we demonstrated that exposure to novelty avoids the generalization of aversive memory and that this process is dependent on the activation of the β-adrenergic and dopaminergic D1/D5 pathways, since the blockade of these pathways in the CA1 region of the hippocampus with the intra-hippocampal infusion of timolol or SCH23390, respectively, immediately before to exposure to novelty inhibits its effect. Moreover, we observed that exposure to novelty raises the hippocampal levels of noradrenaline and dopamine, which suggest that the increase of these neurotransmitters may influence the promotion of long-term memory (LTM), improving memory and attenuating the aversive memory generalization.

Keywords: fear generalization, novelty, hippocampus, noradrenergic system, dopaminergic system.

SUMÁRIO

APRESENTAÇÃO

O presente trabalho de conclusão de curso é composto por um estudo que buscou investigar os efeitos da novidade na generalização da memória aversiva e a participação dos sistemas noradrenérgico e dopaminérgico neste processo. Trata-se de um trabalho experimental, que foi desenvolvido após aprovação da Comissão de Ética no Uso de Animais - CEUA da UNIPAMPA (ANEXO I) e está disposto conforme o "Regulamento do Trabalho de Conclusão de Curso do Curso de Farmácia" e o "Manual de normalização de trabalhos acadêmicos: conforme normas da ABNT" da UNIPAMPA.

Este trabalho está organizado em três partes: a "Parte I" conta com a introdução, objetivos e com uma fundamentação teórica que aborda os conceitos gerais sobre a temática deste estudo; a "Parte II" inclui o desenvolvimento (materiais e métodos) e os resultados do estudo desenvolvido, e é apresentada na forma de um artigo científico, de acordo com as normas da revista *Neurobiology of Learning and Memory* (ANEXO II); por fim, a "Parte III" consiste nas principais conclusões obtidas a partir deste estudo.

PARTE I

1 INTRODUÇÃO

A memória pode ser definida como a capacidade de adquirir, consolidar e evocar informações; além disso, por ser proveniente de uma experiência, pode ser, na maioria das vezes, evocada com facilidade (IZQUIERDO, 2011). Existem diversos tipos e classificações para as memórias, a memória aversiva é uma das mais estudadas, sendo fortemente investigada através de protocolos clássicos de medo condicionado ao contexto (IZQUIERDO; FURINI; MYSKIW, 2016).

As memórias de eventos aversivos são essenciais para a sobrevivência (COSTANZI et al., 2011), pois tratam de uma habilidade cognitiva fundamental que permite lidar com a complexidade das situações do cotidiano, preparando o indivíduo à comportamentos defensivos (ONAT; BUCHEL, 2015). Entretanto, também estão frequentemente associadas à fisiopatologia de doenças como o transtorno de estresse pós-traumático (TEPT) (COSTANZI et al., 2011) e a síndrome do pânico, uma vez que podem levar a respostas exacerbadas e inapropriadas de medo (GILMARTIN; BALDERSTON; HELMSTETTER, 2014; ONAT; BUCHEL, 2015).

A generalização da memória aversiva, por sua vez, ocorre com base na semelhança entre uma situação atual e experiências aversivas anteriores. Neste caso, o indivíduo responde de uma forma defensiva a um estímulo semelhante (porém distinto) a um evento aversivo anteriormente vivenciado (ONAT; BUCHEL, 2015). Fisiologicamente, este fenômeno possui grande importância adaptativa em um ambiente em constante mudança, entretanto, essa habilidade deve ser ativamente controlada e o comportamento aversivo deve ser ressaltado somente quando necessário, pois quando este for desenfreado diferentes eventos podem ser percebidos como mais perigosos do que realmente são, levando o indivíduo a alterações do controle do medo que podem associar-se à fisiopatologia das doenças citadas anteriormente (DUNSMOOR; PAZ, 2015; ONAT; BUCHEL, 2015; HUCKLEBERRY; FERGUSON, 2016).

Apesar da generalização da memória aversiva não ser um fenômeno novo, é recente a maior atenção ao estudo de seus mecanismos neurais (JASNOW et al., 2016). A partir da literatura existente, sabe-se que o hipocampo é uma das áreas que desempenha papel fundamental nos traços de memória que envolvem informações contextuais específicas para este fenômeno (JASNOW et al., 2016). Entretanto, ainda são relativamente pouco investigadas as influências comportamentais e sensoriais sobre a generalização (HUCKLEBERRY; FERGUSON, 2016), bem como os mecanismos que possam minimizar

seus efeitos (JASNOW et al., 2016). Neste contexto se tem pensado em estratégias que possam tornar menos intensas as respostas exacerbadas de medo e atenuar a generalização, bem como buscar intervenções para o tratamento de doenças como o TEPT e seus sintomas. Desta forma, sugerimos a diferenciação de situações semelhantes, mas não iguais, como fundamental, pois embora a generalização seja importante para o nosso dia a dia, ela precisa ser comedida.

Já foi demonstrado que a exposição a uma novidade facilita a aprendizagem (MONCADA; VIOLA, 2007; MYSKIW; BENETTI; IZQUIERDO, 2013; MENEZES et al., 2015). A exposição a um ambiente novo durante uma janela de tempo crítica é capaz de induzir a síntese de proteínas relacionadas à plasticidade (PRPs) e promover a formação de uma *memória de longa duração* ou LTM (do inglês *long term memory*) a partir de um aprendizado fraco, capaz inicialmente de promover apenas a formação de uma *memória de curta duração* ou STM (do inglês *short term memory*) (MONCADA; VIOLA, 2007; MYSKIW; BENETTI; IZQUIERDO, 2013; MENEZES et al., 2015). Este fenômeno se baseia na hipótese de *marcação e captura sináptica* ou STC (do inglês *synaptic tagging and capture*), proposta por Frey e Morris (1997).

A facilitação da aprendizagem através da exposição à novidade possui grande influência neuromoduladora, provocando respostas em uma ampla variedade de áreas cerebrais (SCHOMAKER; MEETER, 2015). Sabe-se que a exposição à novos estímulos e ambientes tende a ativar regiões como *locus coerulus* (LC) e área tegmentar ventral (ATV), principais responsáveis pela liberação de noradrenalina e dopamina, respectivamente (VANKOV; HERVÉ-MINVIELLE; SARA, 1995; BUNZECK; DÜZEL, 2006; LI et al., 2003). Desta forma, o estudo da novidade tem sido relacionado à liberação destes neurotransmissores (MONCADA; BALLARINI; VIOLA, 2015).

Moncada e colaboradores (2011) estudaram o envolvimento de neurotransmissores catecolaminérgicos no processo de STC e síntese de PRPs pela exposição à novidade, e, através de suas intervenções, observaram que receptores β-adrenérgicos e dopaminérgicos D1/D5 são especificamente requeridos para induzir a síntese de PRPs. Moncada (2016) também comprovou que a ativação elétrica do LC e ATV induz a formação de LTMs, o que corrobora com os achados que relacionam a dependência dos sistemas β-adrenérgicos e dopaminérgicos para o efeito da novidade.

Além disso, em um estudo recente do nosso grupo, demonstramos que a exposição à novidade previamente a um ambiente similar a um aversivo já conhecido pelo animal evita a

generalização da memória aversiva, fenômeno este que depende da síntese proteica no hipocampo (VARGAS et al., submetido). Diante disto, estes estudos nos motivaram a investigar a participação dos sistemas noradrenérgico e dopaminérgico no efeito da novidade diante de um protocolo comportamental que induz a generalização da memória aversiva.

2 OBJETIVOS

2.1 Objetivo geral

Investigar os efeitos da novidade na generalização das memórias de medo e a participação dos sistemas noradrenérgico e dopaminérgico neste processo.

2.2 Objetivos específicos

- Confirmar se a exposição a uma novidade facilita a distinção entre dois ambientes (original e similar ao original);

- Investigar a participação do sistema noradrenérgico no efeito da novidade sobre a generalização da memória aversiva;

- Investigar a participação do sistema dopaminérgico no efeito da novidade sobre a generalização da memória aversiva;

- Verificar os níveis de noradrenalina na região CA1 do hipocampo de ratos *wistar* com e sem prévia exposição de uma novidade;

- Verificar os níveis de dopamina na região CA1 do hipocampo de ratos *wistar* com e sem prévia exposição de uma novidade.

3 FUNDAMENTAÇÃO TEÓRICA

3.1 Memória

A memória pode ser definida como a capacidade de adquirir, consolidar e evocar informações (IZQUIERDO, 2011). A formação da memória acontece basicamente por meio de três fases: aquisição, consolidação e evocação (IZQUIERDO, 2011). O aprendizado está diretamente relacionado com a aquisição de uma informação, a estabilização desta informação no cérebro se dá através da consolidação, processo fundamental para o armazenamento da informação, a lembrança, por sua vez, é quando esta informação é evocada (IZQUIERDO, 2011).

Após a evocação, a memória torna-se instável (IZQUIERDO; BELIVAQUA; CAMMAROTA, 2006) e pode seguir dois processos distintos: 1) reconsolidação, processo no qual novas informações podem ser incorporadas à memória original (FORCATO et al., 2010); ou, 2) extinção, processo caracterizado por um novo aprendizado que se sobrepõe ao aprendizado original, inibindo a evocação da memória previamente consolidada (FURINI et al., 2013).

A memória pode ter diferentes classificações que levam em conta determinados critérios, como seu tempo de duração ou sua natureza (IZQUIERDO, 2011). O tempo de duração de uma memória é variável, desta forma é comumente classificada como: memória de trabalho, quando perdura de segundos à poucos minutos; de curta duração, podendo durar minutos à poucas horas (até 6 horas); e, de longa duração, que pode permanecer durante horas, dias, meses ou anos – sua formação requer uma sequência de passos moleculares que envolve a síntese de novas proteínas (IZQUIERDO, 2011; IZQUIERDO, 2013).

Quanto à natureza, as memórias podem ser classificadas em declarativas e de procedimentos. As memórias declarativas dependem da atividade neuronal do hipocampo e estruturas relacionadas ao lobo temporal (IZQUIERDO et al., 2006) e correspondem à eventos, fatos ou conhecimentos que podem ser facilmente relatados. Estas ainda subdividem-se em memória declarativa episódica e semântica, que envolvem conceitos temporais (autobiográfico) e atemporais (conhecimentos, culturas), respectivamente (IZQUIERDO, 2011). Já as memórias de procedimentos ou não declarativas requerem a atividade do estriado e suas conexões (IZQUIERDO et al., 2006) e não são facilmente descritas por meio de palavras, compreendendo as memórias adquiridas por meio da prática e repetições (como andar de bicicleta, por exemplo) (IZQUIERDO, 2013).

Enquanto no homem a evocação das memórias pode ser medida através de relatos ou reconhecimento de pessoas, palavras, lugares ou fatos, nos animais a evocação se expressa através de mudanças comportamentais, de forma que se avalia a inibição ou o aumento de respostas naturais ou inatas, ou até mesmo a geração de novas respostas (IZQUIERDO, 1989). Por meio destas mudanças comportamentais visualizadas nos animais, ao longo dos anos pode-se melhor compreender muitos conceitos referentes ao cérebro, como a formação e o processamento da memória aversiva, uma das memórias mais estudadas, sendo fortemente investigada através de procedimentos clássicos de medo condicionado ao contexto (IZQUIERDO; FURINI; MYSKIW, 2016).

3.1.1 Memória aversiva

A memória aversiva é usualmente estudada em modelos animais através do modelo de condicionamento clássico preconizado por Ivan Pavlov (IZQUIERDO; FURINI; MYSKIW, 2016). O modelo de condicionamento para memórias aversivas (Figura 1A) consiste na apresentação de um estímulo neutro (como a caixa da esquiva inibitória, EI) juntamente com um estímulo incondicionado nocivo (como o estímulo elétrico nas patas – gera uma resposta inata de defesa), desta forma os estímulos ficam associados na memória. Após essa associação, o estímulo neutro passa a ser um estímulo condicionado, provocando uma reação aversiva, como comportamentos de congelamento ou fuga, quando o animal é re-exposto ao mesmo contexto, mesmo na ausência do estímulo incondicionado (IZQUIERDO; FURINI; MYSKIW, 2016).

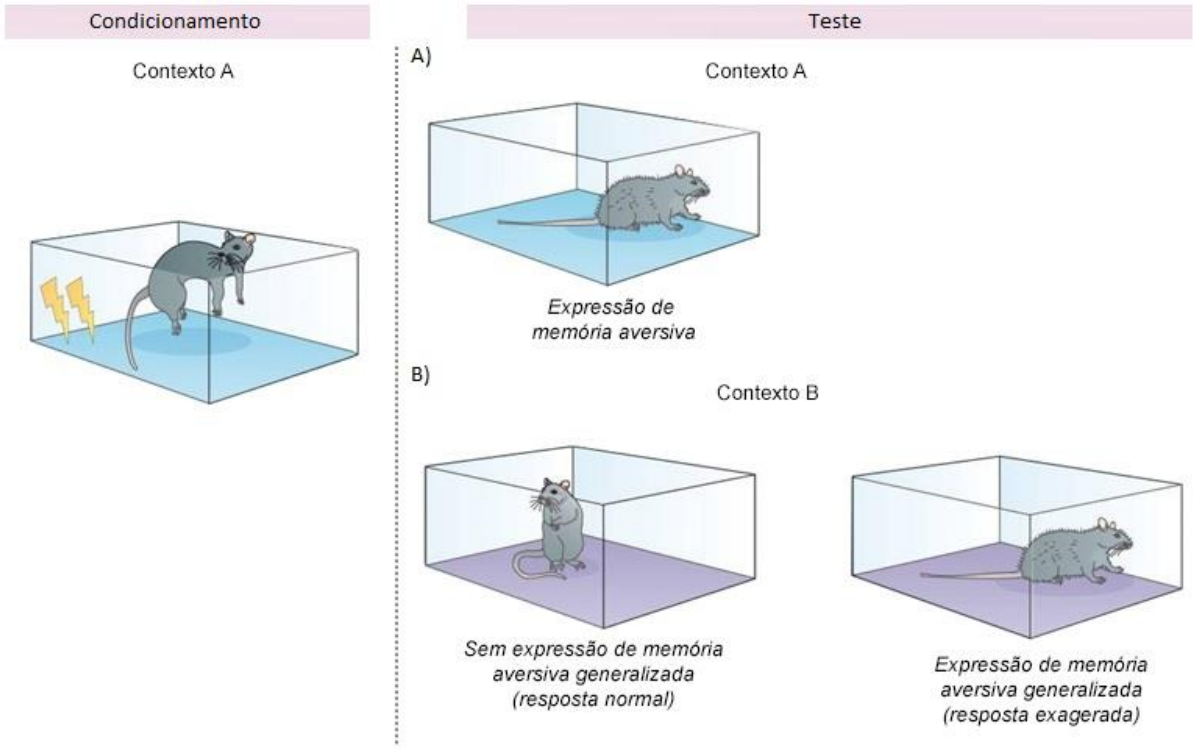

Fig. 1 - Protocolos de condicionamento da memória aversiva e memória aversiva generalizada. A) No protocolo clássico para o condicionamento da memória aversiva apresenta-se um estímulo neutro (contexto A) juntamente com um estímulo incondicionado nocivo (estímulo elétrico nas patas). Quando re-expostos ao mesmo contexto do condicionamento, mesmo sem o estímulo incondicionado, os animais tendem a evocar uma resposta aversiva. B) Para o estudo da generalização da memória aversiva, realiza-se o condicionamento da mesma forma mencionada anteriormente. Entretanto, quando testados em um contexto similar (mas não igual; contexto B) ao contexto do condicionamento, ao invés de apresentar uma resposta natural de diminuição da expressão de medo, os animais passam a apresentar uma resposta exagerada de medo, demonstrando incapacidade de diferenciar os dois ambientes.

Fonte: Dunsmoor; Paz (2015); Figura adaptada de Maren; Phan; Liberazon (2013)

Este tipo de aprendizagem representa muitas situações recorrentes com seres humanos, no qual estímulos inicialmente neutros tornam-se ameaçadores através da sua associação com outros estímulos que geram medo (IZQUIERDO; FURINI; MYSKIW, 2016). Além disso, sabe-se que quando a formação de uma memória envolve estímulos emocionais e estressores esta passa a ser armazenada de maneira mais intensa e duradoura (HAMANN, 2001; MCGAUGH, 2013).

A amígdala é uma região cerebral que está diretamente relacionada com o processamento de estímulos emocionais e estressores do meio externo e envio de informações através de projeções para outras regiões cerebrais, como o córtex pré-frontal medial e hipocampo (ASEDE et al., 2015). Desta forma, o hipocampo e a amígdala desempenham um papel crucial na consolidação da memória aversiva (MCGAUGH; IZQUIERDO, 2000), participando também do processo de evocação juntamente com o córtex pré-frontal (RISIUS et al., 2013).

O hipocampo é considerado a estrutura central para a formação de memórias episódicas (IZQUIERDO, 2011), desta forma é uma das estruturas mais estudadas para o processamento contextual relacionado a eventos aversivos (HOLLAND; BOUTON, 1999). Sua estrutura (Figura 2) configura-se sob a forma de um "C" situado na parte caudal do cérebro, e é dividida em três sub-regiões: giro denteado, subículo e o hipocampo propriamente dito, que possui ainda quatro regiões, denominadas CA1, CA2, CA3 e CA4 (YOUNGSOON; SANGYUN; JAE HYOUNG KIM, 2008; STRIEN; CAPPAERT; WITTER, 2009), dentre estas, sabe-se que a região CA1 tem real envolvimento com as funções cognitivas de aprendizado e memória, sendo a que tem funções melhores documentadas na literatura (FIORENZA et al., 2012; MELLO-CARPES; IZQUIERDO, 2013).

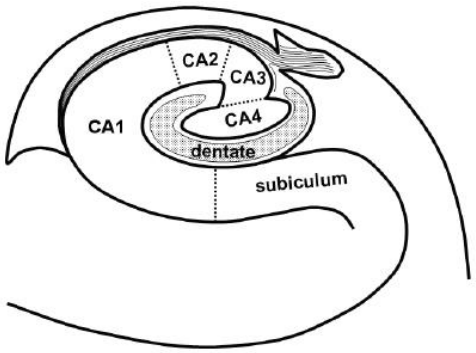

Fig. 2 - Desenho esquemático das principais regiões do hipocampo. As regiões do hipocampo correspondem basicamente ao subículo, CA1 e regiões remanescentes (incluindo CA2, CA3, CA4 e o giro denteado), respectivamente.

Fonte: YoungSoon; SangYun; Jae Hyoung Kim (2008)

Apesar de ser uma habilidade cognitiva fundamental para a sobrevivência, permitindo a detecção e prevenção do perigo (COSTANZI et al., 2011), a expressão de respostas exacerbadas e inapropriadas de medo é a base para o desenvolvimento de transtornos psiquiátricos como o TEPT (GILMARTIN; BALDERSTON; HELMSTETTER, 2014;

ONAT; BÜCHEL, 2015). O TEPT é uma condição debilitante que pode se desenvolver após um evento gravemente traumático, afetando múltiplos sistemas biológicos, tais como circuitos cerebrais, funções celulares, imunológicas, endócrinas e metabólicas, atingindo cerca de 5-10% da população, sendo duas vezes mais comum em mulheres que em homens (YEHUDA et al., 2015).

3.1.2 Generalização da memória aversiva

A generalização da memória aversiva, por sua vez, pode ser definida como um fenômeno no qual uma situação similar (porém distinta) a um evento aversivo prévio pode desencadear uma resposta defensiva (ONAT; BÜCHEL, 2015). Da mesma forma que o protocolo para o condicionamento de memórias aversivas, realiza-se inicialmente a apresentação de um estímulo neutro associado a um estímulo incondicionado nocivo (contexto A, como a EI + estímulo elétrico nas patas) (DUNSMOOR; PAZ, 2015). Para testar a generalização desta memória (Figura 1B), ou a capacidade de distinção entre dois ambientes, os animais são colocados em um contexto similar ao do condicionamento (contexto B, como uma esquiva inibitória modificada, EM) e observa-se seu comportamento (DUNSMOOR; PAZ, 2015).

Fisiologicamente, a generalização é de grande importância adaptativa em um ambiente em constante mudança, no entanto, a discriminação e especificidade de estímulos é essencial para permitir o recrutamento de comportamentos de defesa somente quando apropriado, a fim de evitar respostas exageradas ou desnecessárias (DUNSMOOR; PAZ, 2015). A generalização excessiva de estímulos pode ser considerada como simplesmente uma falha de discriminação, entretanto, pode ocorrer frente a estímulos que poderiam ser facilmente distinguidos (DUNSMOOR; MITROFF; LABAR, 2009). De acordo com Besnard & Sahay (2016) novas informações similares à memórias traumáticas prévias podem influenciar significativamente para a expressão imprópria de medo, levando a reativação destas memórias e uma consequente resposta aversiva generalizada. Alguns estudos sugerem que estas respostas comportamentais exacerbadas de medo podem estar diretamente relacionadas com a intensidade do aprendizado aversivo (GAZARINI et al., 2014; GHOSH; CHATTARJI, 2014). Segundo Pitman (1989), memórias relacionadas à um evento traumático de alta intensidade estimulam a liberação de hormônios e neuromoduladores de maneira mais intensa, o que corrobora para a potencializar seu processo de consolidação.

Do ponto de vista temporal, a generalização pode ser interpretada como um processo esperado, uma vez que com a passagem do tempo ocorre uma perda natural da precisão da memória para pistas contextuais (JASNOW et al., 2016). Assim, em modelos animais, à medida que o intervalo de retenção entre uma aprendizagem aversiva (treino em um contexto A) e o teste de generalização de memória (teste em um contexto B) aumenta, a tendência de haver níveis equivalentes de expressão de medo para ambos os contextos é maior (LYNCH et al., 2013; CULLEN et al., 2015). Em contrapartida, já foi demonstrado que a generalização do medo também ocorre em memórias recentes, descritas até um dia após o aprendizado (LYNCH et al., 2013; GAZARINI et al., 2014), o que intensifica a tese de que este fenômeno esteja associado a um processamento diferencial já durante a etapa de consolidação da memória aversiva, entretanto estes mecanismos ainda não estão bem elucidados (JASNOW et al., 2016).

Embora a generalização da memória aversiva não seja um fenômeno novo, o interesse recente em entender seus fundamentos biológicos se deve principalmente pela sua importância clínica no que diz respeito ao desenvolvimento de transtornos psiquiátricos (JASNOW et al., 2016). Expressões exageradas de comportamentos defensivos, tal como vistas na generalização, além de prejudicar a vida diária do indivíduo estão intimamente relacionadas com os transtornos relacionados ao trauma, como o TEPT (ONAT; BÜCHEL, 2015; HUCKLEBERRY; FERGUSON; DREW, 2016).

Neste contexto, protolocos de condicionamento de medo para generalização da memória aversiva, apesar de não recriarem inteiramente as condições clínicas e comportamentais de pacientes expostos a traumas (DESMEDT; MARIGHETTO; PIAZZA, 2015), são fundamentais para estudar estes transtornos e buscar intervenções que possam mitigar respostas exacerbadas de medo e atenuar a generalização. Um estudo recente do nosso laboratório (MENEZES et al., 2015) demonstrou o potencial efeito da exposição à novidade (ambiente novo) frente à extinção da memória aversiva. A partir disto, hipotetizamos que a novidade poderia também auxiliar na diferenciação de um contexto semelhante, mas não igual, sendo útil para atenuar sintomas de generalização de medo.

3.2 Exposição à novidade

A exposição à novidade pode se dar, por exemplo, pela exposição a um ambiente inicialmente desconhecido (MONCADA; VIOLA, 2007; BALLARINI et al., 2009; MONCADA et al., 2011) ou a um sabor novo (BALLARINI et al., 2009). Esta estratégia vem

sendo estudada por sua capacidade de melhorar a memória tanto em humanos como em animais (FENKER et al., 2008; SCHOMAKER; VAN BRONKHORST; MEETER, 2014; SCHOMAKER; MEETER, 2015). As principais implicações destes e outros estudos estão na atuação da novidade sobre as cascatas bioquímicas que estão associadas à formação das memórias de longo prazo (PSYRDELLIS; PAUTASSI; JUSTEL, 2016).

Os cientistas verificaram que estímulos que formariam apenas STMs podem formar LTMs diante da exposição à novidade (MONCADA et al., 2011), fenômeno que vem sendo explicado através da hipótese STC proposta por Frey e Morris (1997).

A hipótese STC (Figura 3), inicialmente testada em experimentos eletrofisiológicos realizados em fatias de hipocampo, foi proposta para descrever as mudanças sinápticas que ocorrem durante a formação da memória, e propõe que, quando uma determinada via sináptica é estimulada, dois eventos dissociáveis ocorrem: inicialmente ocorre um fenômeno conhecido como potenciação de longa duração (LTP, do inglês *long term potentiation*; principal modelo neurofisiológico e celular para a aprendizagem e formação da memória) precoce (com duração de poucas horas) e a marcação da sinapse estimulada por um *tag* – estado temporário que permite que a sinapse fique suscetível à modificações duradouras (REDONDO; MORRIS, 2011). Em paralelo, se o estímulo for forte o suficiente, haverá a síntese de PRPs que serão capturadas apenas pelas sinapses marcadas pelo *tag*. Estas PRPs, por sua vez, irão propiciar a sustentação desse estado potenciado e a formação de uma LTP tardia (com duração de dias a semanas) (BALLARINI et al., 2009; MONCADA et al., 2011; REDONDO; MORRIS, 2011). Entretanto, se o estímulo não for forte o suficiente, não haverá a síntese de PRPs e, mesmo marcada pelo *tag*, gradualmente a sinapse retorna ao seu estado basal, não potenciado e não marcado (BALLARINI et al., 2009; MONCADA et al., 2011; REDONDO; MORRIS, 2011).

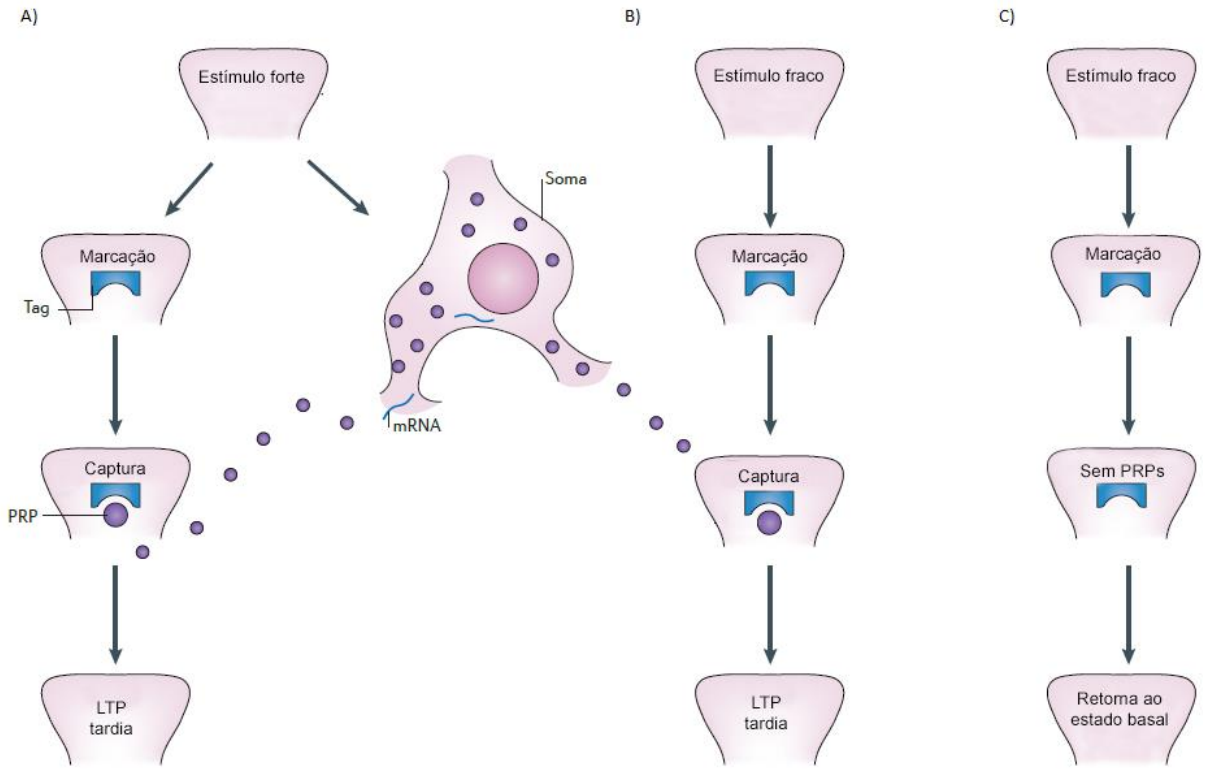

Fig. 3 - Desenho esquemático da hipótese de marcação e captura sináptica. A hipótese de marcação e captura sináptica (STC) fornece uma explicação para a especificidade sináptica e facilitação da potenciação de longa duração (LTP). A) Um estímulo forte de uma via sináptica leva a dois eventos dissociáveis: marcação local e a síntese de proteínas relacionadas à plasticidade (PRPs) difusíveis. As PRPs são então capturadas por sinapses marcadas, levando a manutenção de uma LTP tardia; B) Um estímulo fraco de uma via sináptica que tenha acesso às PRPs também conseguirá manter a LTP tardia; C) Um conjunto de sinapses fracamente estimulado sem a disponibilidade de PRPs perderá seu estado receptivo (*tag* formado) e a LTP não será sustentada, desta forma, a sinapse gradualmente retornará ao seu estado basal.

Fonte: Adaptado de Redondo e Morris (2011)

Tendo em vista a aplicação da hipótese STC para os processos de aprendizagem e memória, recentemente foi proposto um modelo semelhante que, entretanto, considera o processo de aprendizagem em um sentido comportamental mais amplo: a hipótese de *marcação comportamental* (BT, do inglês *behavioral tagging*). A principal abordagem desta hipótese é relacionar uma tarefa comportamental de aprendizagem que induz apenas uma STM com um evento comportamental forte o suficiente para induzir a síntese proteica e uma LTM (MONCADA et al., 2015; KORZ, 2018).

A exposição à novidade, por ser uma experiência forte, é capaz de induzir síntese proteica e a LTM (MONCADA; VIOLA, 2007). Esses achados foram inicialmente observados por Moncada e Viola (2007) ao expor ratos a um protocolo fraco de tarefa de EI, inicialmente capaz de induzir apenas uma STM, e associar com a exploração de uma novidade em uma janela de tempo próxima ao aprendizado/sessão de treinamento (-1h ou +1h). Posteriormente, outros estudos também evidenciaram o efeito da novidade na formação da memória. Ballarini e colaboradores (2009) observaram que tanto protocolos fracos de tarefas dependentes do hipocampo (reconhecimento espacial de objetos e condicionamento aversivo ao contexto) como independentes do hipocampo (condicionamento aversivo ao gosto), quando associados à uma novidade em uma janela de tempo crítica, tendem a formar uma LTM. Adicionalmente, nas três tarefas testadas no último estudo referido o efeito da novidade na LTM foi dependente de síntese proteica. Ainda, recentemente foi observado que exposição à novidade auxilia na extinção do medo contextual em ratos quando aplicada antes ou após de uma sessão fraca de extinção (-2, -1, -0,5 ou +1h), previamente insuficiente para inibir a memória aversiva de forma duradoura (MYSKIW; BENETTI; IZQUIERDO, 2013; MENEZES et al., 2015), o que corrobora para o seguimento de pesquisas que visam atenuar comportamentos exacerbados relacionados à memória aversiva e consequentes transtornos psiquiátricos envolvidos.

A facilitação da aprendizagem através da exposição à novidade, como relatada nos estudos mencionados, possui grande influência neuromoduladora, provocando respostas em uma ampla variedade de áreas cerebrais e estimulando vários sistemas de neurotransmissores, o que afeta, consequentemente, vários aspectos cognitivos (SCHOMAKER; MEETER, 2015). Dois neurotransmissores ganham destaque nos estudos do processamento da exposição à novidade: a noradrenalina e a dopamina (SCHOMAKER; VAN BRONKHORST; MEETER, 2014; MONCADA; BALLARINI; VIOLA, 2015). Sabe-se que a exploração de ambientes previamente desconhecidos resulta em um aumento destes neurotransmissores, no entanto os processos neurobiológicos para este fenômeno ainda não foram totalmente elucidados (SCHOMAKER; MEETER, 2015).

3.2.1 Neuromodulação e novidade: sistemas noradrenérgico e dopaminérgico

São variados os sistemas moduladores que atuam sob os processos mnemônicos (IZQUIERDO, 1989). Os sistemas noradrenérgico e dopaminérgico têm sido apontados como mediadores na melhora da memória, por atuarem significativamente na regulação da

plasticidade sináptica e, consequentemente, na formação e consolidação da memória (MONCADA et al., 2011; GAZARINI et al., 2013; MCGAUGH; ROOZENDAAL, 2009).

Estes dois neuromoduladores são liberados amplamente pelo cérebro, mais especificadamente através da ativação do LC e da ATV, que estimulam, respectivamente, a liberação de noradrenalina e dopamina em várias estruturas cerebrais, tendo eferências diretas ao hipocampo (VANKOV; HERVÉ-MINVIELLE; SARA, 1995; LISMAN; GRACE, 2005). A exposição à novos estímulos e ambientes tende a ativar estas áreas do cérebro, levando tanto à estimulação noradrenérgica quanto à dopaminérgica (VANKOV; HERVÉ-MINVIELLE; SARA, 1995; LI et al., 2003; BUNZECK; DÜZEL, 2006). Desta forma, o estudo da novidade tem sido consistentemente relacionado à liberação destes neurotransmissores (MONCADA; BALLARINI; VIOLA, 2015).

Como já se sabe, a exposição a novidade pode induzir a síntese de PRPs que, quando capturadas em locais previamente marcados, permitem a consolidação da memória e a formação da LTM (MONCADA; VIOLA, 2007). Estudos recentes demonstram que a ativação de receptores noradrenérgicos e dopaminérgicos é um importante fator para o controle da síntese destas proteínas, uma vez que desencadeiam diferentes cascatas de segundos mensageiros que, por sua vez, pode resultar em transcrição gênica e eventual processo de tradução proteica (MONCADA; BALLARINI; VIOLA, 2015; MONCADA, 2016).

Através de experimentos eletrofisiológicos já foi demonstrado que receptores β-adrenérgicos e dopaminérgicos D1/D5 são requeridos durante o processo de síntese de PRPs (O'CARROLL; MORRIS, 2004; SAJIKUMAR; FREY, 2004). Estudos posteriores avaliaram o envolvimento destes neurotransmissores a nível comportamental no processo de STC e da síntese de PRPs, confirmando os resultados eletrofisiológicos anteriormente observados (LI et al., 2003; KORZ; FREY, 2007; MONCADA et al., 2011). Moncada e colaboradores (2011) verificaram que a injeção de antagonistas β-adrenérgicos e dopaminérgicos (Propranolol e SCH23390) no hipocampo dorsal de ratos é capaz de bloquear o efeito da novidade, interferindo na promoção da LTM. Outra evidência que os mesmos autores encontraram é que, ao contrário do uso de antagonistas, agonistas β-adrenérgicos e dopaminérgicos (dobutamina e SKF 38393) por administração intraperitoneal são capazes de conduzir a promoção de LTM através de um mecanismo dependente de síntese proteica.

Em um estudo recente foi demonstrado que a ativação elétrica do LC e da ATV induz a formação de LTMs que dependem da atividade dos receptores β-adrenérgicos e

dopaminérgicos D1/D5, respectivamente (MONCADA, 2016). Moncada (2016) comprovou que estas áreas controlam a consolidação da memória e são dependentes de síntese proteica no hipocampo, através de mecanismos paralelos e complementares, uma vez que ao associar um treinamento fraco incapaz de induzir LTM com uma ativação elétrica do LC e/ou da ATV dentro de uma janela de tempo crítica houve a promoção da LTM. Ainda, outra descoberta importante é que cada uma dessas estruturas é capaz de promover a LTM mesmo quando uma destas está inativada (MONCADA, 2016). Além do mais, a co-ativação do LC e da ATV permite a formação de uma LTM "melhor" do que quando observada após a ativação de uma única área (MONCADA, 2016).

Em suma, até o momento os processos que levam à síntese de PRPs parecem depender dos sistemas noradrenérgicos e dopaminérgicos. Assim, com base nesses achados decidimos investigar a participação de ambos os sistemas no processo de modulação da generalização da memória aversiva pelo efeito da novidade.

PARTE II

NORADRENERGIC AND DOPAMINERGIC SYSTEMS INVOLVEMENT ON NOVELTY MODULATION OF AVERSIVE MEMORY GENERALIZATION

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Abstract

The generalization of aversive memory can be defined as a phenomenon in which a similar situation (but distinct) to a previous aversive event can trigger a defensive response and is related to several psychological disorders. In this study, we investigate the effects of novelty on the generalization of fear memories and the participation of noradrenergic and dopaminergic systems in this process. In this study, we investigated the effects of novelty on the generalization of fear memories and the participation of noradrenergic and dopaminergic systems in this process. To test the aversive memory and the generalization of aversive memory, we used the "inhibitory avoidance" (IA) memory task. On day 1, adult male Wistar rats were trained in IA. On day 2, the animals were exposed to a modified inhibitory avoidance (MIA) for 3 minutes – 30 minutes before MIA exposition some animals were exposed to novelty (open field) for 5 minutes. To evaluate the involvement of noradrenergic and dopaminergic systems, immediatly before of novelty exposure, some animals received

intrahippocampal infusions of timolol (β-adrenergic antagonist), SCH23390 (D1/D5 antagonist) or vehicle. On day 3, to evaluate, respectively, the aversive memory and generalization of aversive memory, half of the animals in each group were tested on IA and another half on MIA. In this study, we demonstrated that exposure to novelty avoids the generalization of aversive memory and that this process is dependent on the activation of the β-adrenergic and dopaminergic D1/D5 pathways, since the blockade of these pathways in the CA1 region of the hippocampus with the intra-hippocampal infusion of timolol or SCH23390, respectively, immediately before to exposure to novelty inhibits its effect. Moreover, we observed that exposure to novelty raises the hippocampal levels of noradrenaline and dopamine, which suggest that the increase of these neurotransmitters may influence the promotion of long-term memory (LTM), improving memory and attenuating the aversive memory generalization.

Keywords: fear generalization, novelty, hippocampus, noradrenergic system, dopaminergic system.

1 Introduction

The memories of aversive events are essential for survival (Costanzi et al., 2011), since they provide a fundamental cognitive ability that allows us to deal with the complexity of everyday situations, preparing us for defensive behavior (Onat; Büchel, 2015). However, this type of memories is frequently associated with the physiopathology of diseases such as post traumatic stress disorder (PTSD) (Costanzi et al., 2011) and panic disorder, since they can lead to exacerbated and inappropriate responses of fear (Gilmartin; Balderston; Helmstetter, 2014; Onat; Büchel, 2015).

The generalization of aversive memory occurs on the basis of the similarity between a current situation and previous aversive experiences (Onat; Büchel, 2015). In this case, the individual responds defensively to a stimulus similar to a previously experienced aversive event (Onat; Büchel, 2015). Physiologically, this phenomenon has great adaptive importance in a constantly changing environment, however, this ability must be actively controlled and the aversive behavior must be exposed only when necessary (Dunsmoor; Paz, 2015; Onat; Büchel, 2015; Huckleberry; Ferguson, 2016). When it is rampant different events can be perceived as more dangerous than they really are, leading the individual to changes in fear

control that may be associated with the physiopathology of the diseases mentioned above (Dunsmoor; Paz, 2015; Onat; Büchel, 2015; Huckleberry; Ferguson, 2016).

Although the generalization of aversive memory is not a new phenomenon, the attention to the study of its neural mechanisms is recent (Jasnow et al., 2016). From the existing literature, it is known that the hippocampus is one of the areas that plays a fundamental role in memory traces that involve contextual information specific to this phenomenon (Jasnow et al., 2016). However, the behavioral and sensory influences on generalization are still relatively seldom investigated (Huckleberry; Ferguson, 2016), as well as the mechanisms that may minimize their effects (Jasnow et al., 2016). In this context, strategies have been devised that may make the exacerbated fear responses less attentive, reducing the generalization, as well as interventions for the treatment of diseases such as PTSD and its symptoms. In this way, we suggest the differentiation of similar, but not equal, situations, as fundamental, because although generalization is important for our day to day life, it needs to be measured.

It has already been shown that exposure to novelty facilitates learning (Moncada; Viola, 2007; Myskiw; Benetti; Izquierdo, 2013; Menezes et al., 2015). The exposure to a new environment during a critical time window is capable of inducing the synthesis of plasticity-related proteins (PRPs) and promote the formation of long-term memory (LTM) from a weak learning, initially able only to promote the formation of a short-term memory (STM) (Moncada; Viola, 2007; Myskiw; Benetti; Izquierdo, 2013; Menezes et al., 2015). This phenomenon is based on the synaptic tagging and capture (STC) hypothesis proposed by Frey and Morris (1997).

The facilitation of learning through exposure to novelty has a great neuromodulatory influence, provoking responses in a wide variety of brain areas (Schomaker; Meeter, 2015). Until this moment the processes leading to the synthesis of PRPs appear to depend on adrenergic and dopaminergic systems (O'carroll; Morris, 2004; Sajikumar; Frey, 2004; Moncada et al., 2011; Moncada, 2016). It is known that the exposure to new stimuli and environments tends to activate regions such as locus coerulus (LC) and ventral tegmental area (VTA), which are responsible for the release of noradrenaline and dopamine, respectively, in diferente brain areas (Vankov; Hervé-Minvielle; Sara, 1995; Li et al., 2003; Bunzeck; Düzel, 2006).

In addition, a recent unpublished study of our group demonstrated that the exposure to a novelty previously to an environment similar to an aversive and already known by the

animal avoids the generalization of aversive memory, a phenomenon that depends on the hippocampal protein synthesis (Vargas et al., 2018). Here we confirm this novelty effect and demonstrate that the noradrenergic and the dopaminergic systems are involved in the process of modulation of generalization of aversive memory by the effect of novelty.

2 Material and methods

2.1 Animals

One hundred and eleven male Wistar rats (3 months old, 300-400g) were purchased from an registrered vivarium. They were housed four per cage and maintained under controlled light and environmental conditions (12h light/12h dark cycle at 23 ± 2°C and 50 ± 10% humidity) with food and water ad libitum. All experiments were conducted in accordance with the "Principles of Laboratory Animal Care" of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the Universidade Federal do Pampa (protocol number 001/2017).

2.2 Procedures and experimental design

The procedures were divided into three stages (Figure 1), whose protocols are described later.

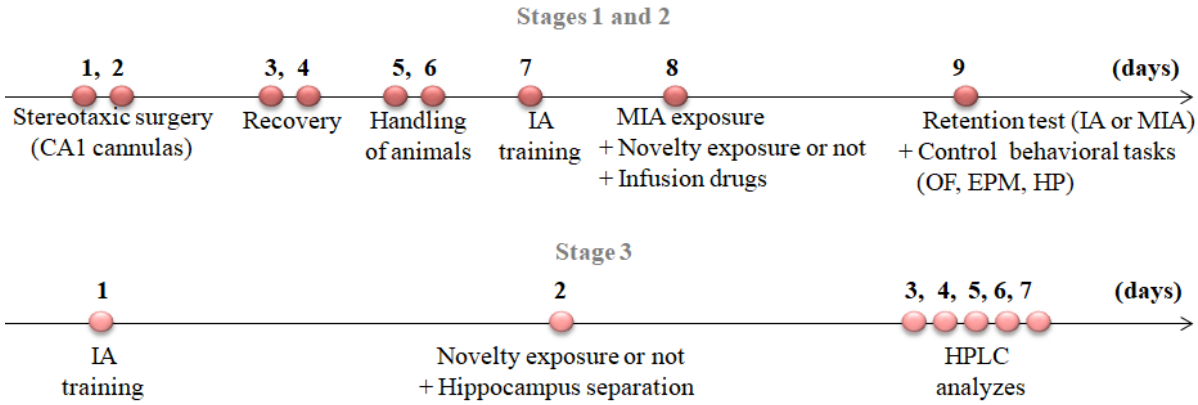

Fig. 1 Experimental design. The animals of stages 1 and 2 went through the same experimental protocols, but the experiments were carried out at different times. On days 1 and 2 the animals were submitted to stereotactic surgery for implantation of guide cannulas in the CA1 region of the hippocampus; on days 3 and 4 the animals were under recovery in their housing boxes; on days 5 and 6 animal manipulation sessions were performed; on days 7, 8 and 9 the interventions for memory test in inhibitory avoidance (IA) and modified inhibitory avoidance (MIA) were performed; finally, behavioral control tests were performed: open field

(OF), elevated plus maze (EPM) and hot plate (HP). The animals of stage 3 were used to measure levels of noradrenaline and hippocampal dopamine. On day 1 the animals were trained in IA; on day 2, 30 minutes after exposure to the novelty, or in the equivalent time, the animals were euthanized for hippocampal separation and subsequent biochemical analysis.

Stage I. Participation of the noradrenergic system in the modulation of aversive memory generalization by novelty

For this experiment, 48 animals were initially divided into three groups:

i) Control group (n = 16): animals were trained on normal inhibitory avoidance (IA) on day 1, and on day 2 they were exposed to modified inhibitory avoidance (MIA) for 3 minutes;

ii) Novelty group (n = 16): the animals were submitted to the same procedures of the group (i), but 30 minutes before to MIA exposure they were exposed to a novelty for 5 minutes and immediately before received a intrahipocampal infusion of vehicle (saline; 1μL /side);

iii) Timolol (β-adrenergic antagonist) + novelty group (n = 16): animals went through the same procedures of the group (ii) but immediately before exposure to novelty received a intrahipocampal infusion of timolol (1μg/μL; 1μL/side).

On day 3, half of the animals in each group were tested in IA and another half in MIA, totaling 6 test groups, with 8 animals each, in order to measure the step-down latency and their ability to discriminate the both environments (Figure 2).

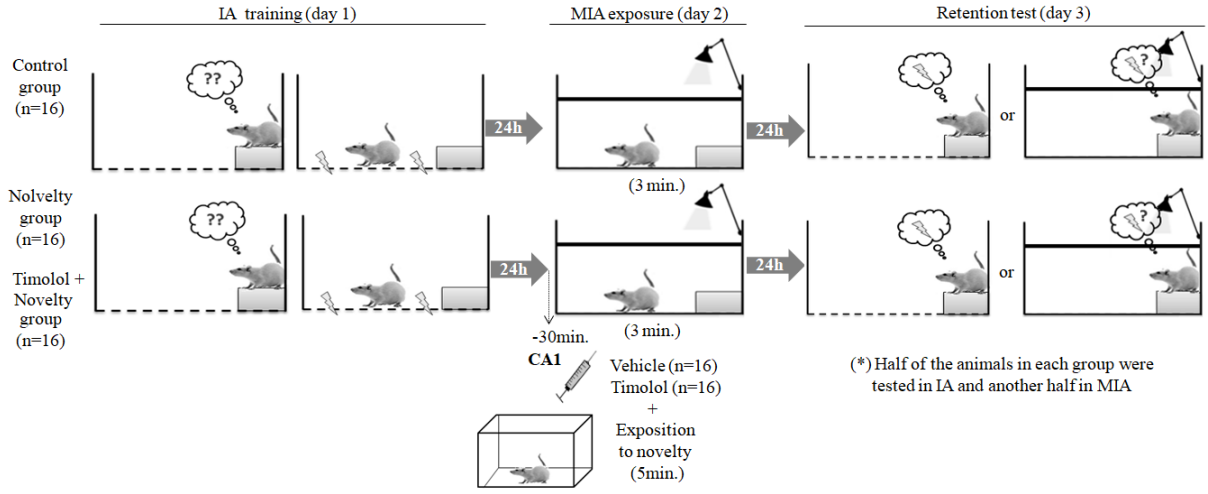

Fig. 2 Schematic drawing of behavioral experiments: investigation of the participation of noradrenergic system in the modulation of aversive memory generalization by novelty.

On day 1 all the animals were trained in IA. On day 2 the animals were exposed to MIA for 3 minutes; some animals were exposed to a novelty for 5 minutes, 30 minutes before MIA exposition, and received vehicle or timolol infusion in the CA1 region of hippocampus immediately before to the novelty exploration. On day 3, half of the animals in each group were tested on IA and another half on MIA.

Stage II. Participation of the dopaminergic system in the modulation of aversive memory generalization by novelty

For this experiment, 48 animals were initially divided into three groups:

i) Control group (n = 16): animals were trained on IA on day 1, and on day 2 they were exposed to MIA for 3 minutes;

ii) Novelty group (n = 16): the animals were submitted to the same procedures of the group (i), but 30 minutes before to MIA exposure they were exposed to a novelty for 5 minutes and immediately before received a intrahipocampal infusion of vehicle (saline; 1μL /side);

iii) SCH23390 (D1/D5 dopaminergic antagonist) + novelty group (n = 16): animals went through the same procedures of the group (ii) but immediately before exposure to novelty received a intrahipocampal infusion of SCH23390 (1μg/μL; 1μL/side).

On day 3, half of the animals in each group were tested in IA and another half in MIA, totaling 6 test groups, with 8 animals each, in order to measure the step-down latency and their ability to discriminate the both environments (Figure 3).

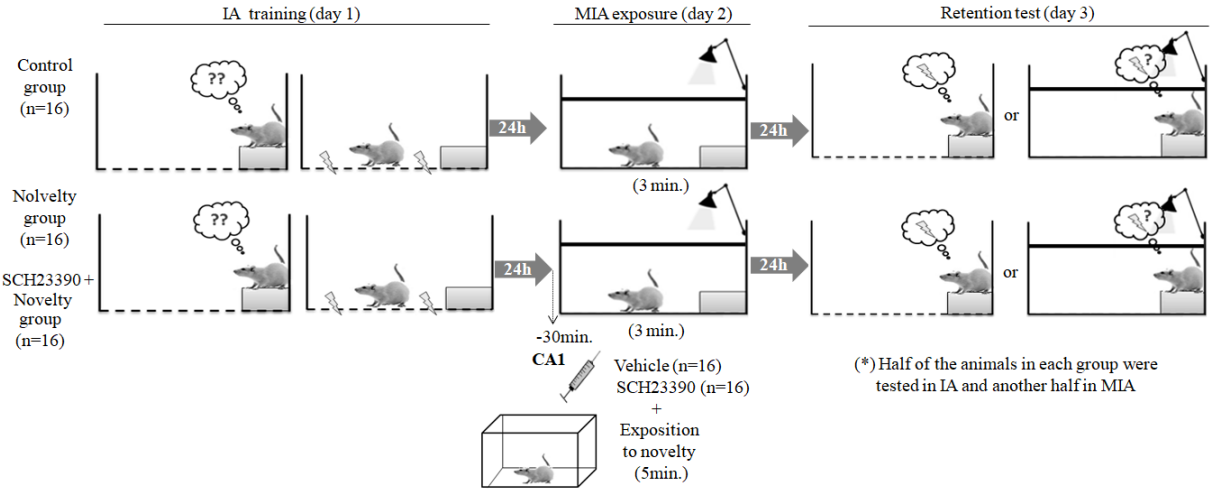

Fig. 3 Schematic drawing of behavioral experiments: investigation of the participation of dopaminergic system in the modulation of aversive memory generalization by novelty.

On day 1 all the animals were trained in IA. On day 2 the animals were exposed to MIA for 3 minutes; some animals were exposed to a novelty for 5 minutes, 30 minutes before MIA exposition, and received vehicle or SCH23390 infusion in the CA1 region of hippocampus immediately before to the novelty exploration. On day 3, half of the animals in each group were tested on IA and another half on MIA.

Stage III. Biochemical analysis of noradrenaline and dopamine levels measurement

To verify if exposure to a novelty increases the levels of noradrenaline and/or dopamine in the CA1 region of the hippocampus, fifteen animals were divided into the following groups:

i) Naive group (n = 5): wild animals not submitted to any procedure;

ii) Control group (n = 5): animals trained in IA on day 1;

iii) Novelty group (n = 5): animals submitted to the same procedure of group (ii) and exposed to a novelty for 5 minutes on day 2.

These animals were euthanized by decapitation on day 2 and the CA1 region of the dorsal hippocampus were removed for measurement of the levels of noradrenaline and dopamine by High Performance Liquid Chromatography (HPLC); the animals from group (iii) were euthanized 30 minutes after exposure to novelty (Figure 4).

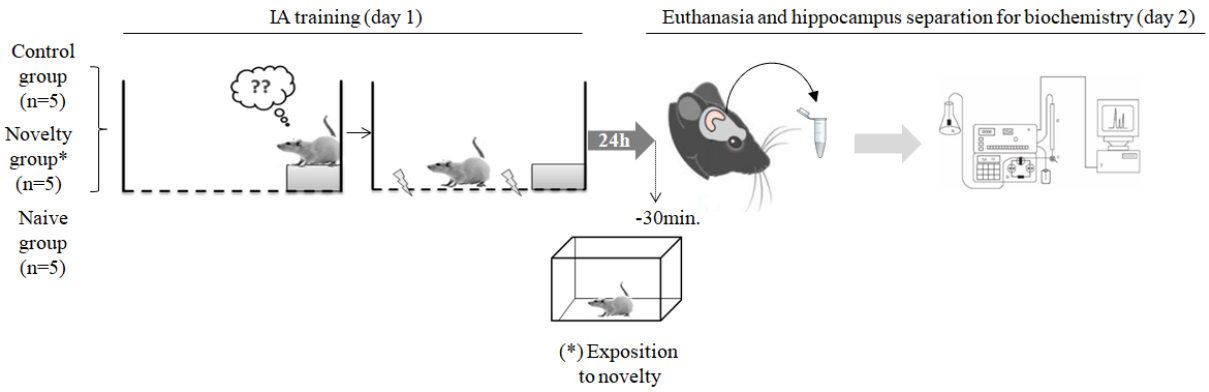

Fig. 4 Schematic drawing of behavioral experiments: biochemical analysis for the evaluation of noradrenaline and dopamine levels in the CA1 region of the hippocampus. On day 1 the animals were trained in IA. On day 2, 30 minutes after exposure to novelty, or at the equivalent time in the other groups, the animals were euthanized and their brain was dissected rapidly on ice surface to remove the hippocampus and subsequent biochemical analysis.

2.3 Experimental protocols

2.3.1 Surgery

All animals of experimental stages "*a"* and "*b"* were submitted to stereotaxic surgery (coordinates according to Paxinos and Watson, 1986), for implantation of 0.2 mm guide cannulas in the CA1 region of the dorsal hippocampus (A -4.2, L ± 3.0, V -2.0 mm). The procedure was performed with animals previously anesthetized with ketamine and xylazine (i.p., 75mg/kg and 10mg/kg, respectively). The cannulae were fixed with dental cement. Animals were allowed to recover from surgery for two days.

2.3.2 Handling of animals

In order to reduce the stress of the animals and to provide greater contact with the experimenter, two days before starting behavioral tests, the animals were subjected to two rounds of handling. During each session the animals were transported from the vivarium to the room where the experiments were to be conducted, removed from the cage and handled by the experimenter for 5 minutes.

2.3.3 Inhibitory avoidance training

The apparatus used for training in the IA task consists of a metal box (50 x 25 x 50 cm), with an acrylic made front. The floor of the cosisted of an array of parallel electridied bars with a platform box of 5.0 cm height by 7.0 cm wide placed in the left side of the box. The rats were carefully placed on the elevated platform, when they stepped down, placing their four legs on the electrified bars they received a single aversive foot shock (0.7 mA for 2s). After this event, the animais were immediately placed into their housing-boxes. This procedure results in an association of the context with the electric shock, resulting in an increase in the latency of the descent platform when a new exposure is made.

2.3.4 Modified inhibitory avoidance exposure

The MIA refers to an environment similar to the original (IA used in training), having the same characteristics previously mentioned for the IA task, and, additionally, a black floor instead of the electrifiable bars, a horizontal black stripes on the four sides of the acrylic front wall, citric aroma and a red light projected under the apparatus. During exposure to MIA (day 2) the rats were carefully placed on the elevated platform, when they stepped down, placing

their four legs on the black floor they did not receive the aversive foot shock and explored for 3 minutes the apparatus. This apparatus is used to verify the differentiation or generalization of aversive memory.

2.3.5 Novelty exposure

The novelty consists of a new environment, previously unknown by the animals, we used in this experiment the open field (OF) apparatus, a wooden box (60 x 40 x 50 cm) with the frontal wall of transparent glass. The groups exposed to novelty, 30 minutes before exposure to the MIA, were placed in the apparatus being free to explore it for 5 minutes.

2.3.6 Drugs and infusion drugs

Timolol (β-adrenergic antagonist) and SCH23390 (D1/D5 dopaminergic antagonist) were purchased from Sigma-Aldrich Brazil and individually dissolved in 0.9% saline and stored at -20°C, protected from light until use. The drugs were infused bilaterally into the CA1 region of the hippocampus (1μg/μL; 1μL/side) with the aid of micro-seringes Hamilton, according to the experimental groups specified above. The doses used were determined based on previous studies that showed the effect of each under a learning and a behavior (Menezes et al., 2015; Vargas et al., 2017). Placement of the cannulae was checked post-mortem 2-4 hours from the last behavioral test, a 4% methylene blue solution was infused (as previously described) and the dye extension 30 minutes later was taken as an indication of the presumed diffusion of the vehicle or drug previously given to each animal.

2.3.7 Retention test

The animals were tested 48 hours after the training (day 3) to measure the step-down latency in IA or MIA, to evaluate the aversive response and generalized aversive response, respectively. The animals will be carefully placed on the lateral platform of the IA or MIA, when they stepped down and places their four legs on the grids (IA) or black floor (MIA) the test was finalized and the step-down latency was recorded.

2.3.8 Control behavioral tasks

After the retention test, some behavioral control tests were performed to evaluate whether the administration of the drugs did not affect the step-down latency of the animals on this day. For this, locomotor activity and exploratory behavior were tested through the OF

task; the anxiety, through the task of the elevated plus maze (EPM) and; nociceptive response and paw sensitivity through hot plate (HP).

In order to analyze animals exploratory and locomotor activities, we used the same box used to study the effects of novelty (OF, see above). Black lines were drawn on the floor to divide it into 12 equal quadrants. Crossing and rearing, as measures for locomotor and exploratory activities, respectively, were measured over 5 minutes (Bonini et al., 2006).

To evaluate the animals anxiety state, the animals were exposed to an EPM as detailed by Pellow et al. (1985). The number of entries and the time spent into the open and closed arms were recorded over a 5 minutes session (the more anxious, the longer the length of stay and the number of entry into the closed arms).

Finally, to evaluate the nociceptive response and sensitivity of the paws of the animals, HP task was used. The procedure consists in placing the animal in an apparatus that has a heated metal plate (55 ± 0.5 ºC) and evaluating the residence time of the animal until it reacts to the thermal stimulus by lifting or licking the legs (Tita et al., 2001).

2.4 Noradrenaline and dopamine levels measurement

The levels of noradrenaline and dopamine in the homogenates prepared from the hippocampus was determined using an "Inertsil ODS-3" reverse phase column HPLC system (5µ 4.6 x 250mm, GL Sciences) with a diode array detector (as described by Menezes et al., 2015).

Thirty minutes after the exposure to novelty, or in the equivalent time, in the case of the other groups, the animals were euthanized and their brain were rapidly dissected on ice surface to remove hippocampus (Menezes et al., 2015). To prepare the samples, the samples will be homogenized with 960µL of saline (0.9% NaCl) and 40µL of HCl (25:1). The homogenization was followed by immersion in ice for 10 seconds for two consecutive times; then the samples were centrifuged at 10.000 rpm for 5 minutes at 4°C; the supernatant was filtered with a 0.22µm PTFE filter and placed in a new eppendorf; for dilution, 167µL of the sample will be added to 33µL of HCl and 800µL of saline.

2.5 Statistical analysis

For IA and MIA results, a ceiling of 300 s was imposed on step-down latencies during the retention tests (latencies equal to or higher than 300 s were counted as 300 s). So this variable did not follow a normal distribution, and data were analyzed by Kruskal–Wallis

nonparametric ANOVA. To compare the step-down latency differences between the training and test in each group, a Wilcoxon test was used IA and MIA data were expressed as mean ± SEM. The OF, EPM and HP data were analyzed using Kruskal–Wallis nonparametric ANOVA and were expressed as mean ± SD. The sample size (n, number of animals in each group) for each experiment is stated in the figure captions. In HPLC results, the data of the three groups were compared using ANOVA followed by Kruskal-Wallis test with Dunn's multiple comparisons test and were expressed as mean ± SD. The differences were considered statistically significant at $P \leq 0.05$.

3 Results

3.1 Participation of the noradrenergic system in the modulation of aversive memory generalization by novelty

3.1.1 Noradrenergic system is necessary to modulation of aversive memory generalization by novelty

Animals that were exposed to a novelty before exploring the MIA on day 2 did not present generalized aversive memory. However, the β-adrenergic receptor antagonist (timolol) injection immediately before the novelty exposure inhibited the novelty effect, suggesting that the effect of novelty on aversive memory generalization depends on the hippocampal noradrenergic system.

On the IA training day, the animals presented no significant difference in step-down latency ($H_{(3)} = 2.24$; $P = 0.3251$, Fig. 5A). In the IA retention test (day 3), there was no significant difference between the groups ($H_{(3)} = 1.51$; $P = 0.4697$, Fig. 5A). However, in the IA retention test the step-down latency was significantly higher than that presented on the training day ($P = 0.0001$ for the control group, $P = 0.0286$ for the novelty group, $P < 0.0001$ for the timolol + novelty group, Fig. 5A), indicating that all groups were able to recognize the aversive environment (they were trained and tested in the same environment).

On the MIA retention test a significant difference between the groups step-down latencies was observed ($H_{(3)} = 19.73$; $P < 0.0001$, Fig. 5B). The control group presented a high step-down latency when compared to training ($P < 0.0001$, Fig. 5B), expressing therefore, a aversive memory generalization, since there is no distinction of the environments presented in the training and in the test (they were trained and tested in similar but not equal environments). The group exposed to the novelty presented no significant difference of step-

down latencies when compared to the training (P = 0.9219, Fig. 5B), demonstrating, in this way, that the animals were able to differentiate the enviroments, avoiding the memory generalization. The intrahipocampal infusion of timolol immediately before exposure to novelty blocked the effect of novelty, and the animals presented a high step-down latency when compared to training (P = 0.0073, Fig. 5B).

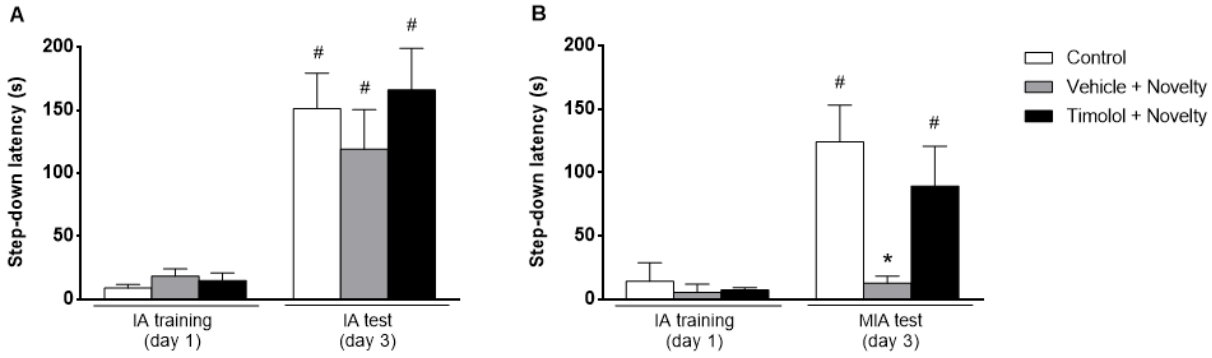

Fig. 5 Timolol infusion into the CA1 region of the hippocampus immediately before of the novelty exposition blocks the modulation effect of novelty in aversive memory generalization. On day 1 all rats were trained in IA. On day 2 all rats were submitted a MIA exploration for 3 minutes; the novelty and timolol + novelty groups were exposed to a novelty for 5 minutes 30 minutes before of the MIA exploration; timolol + novelty group received an intrahippocampal injection of timolol immediately before the novelty exposure. On day 3 half of the rats of each group were tested in IA (A) and the other half in the MIA (B). Data represent the step-down latency in IA (A) or MIA (B) test and are expressed as mean ± SEM. $^{\#}$ $P \leq 0.05$ in Wilcoxon test (training vs. test); * $P \leq 0.05$ on ANOVA followed by Kruskal-Wallis test with Dunn's multiple comparisons test; n = 8 per group.

3.1.2 Novelty exposure promotes increase of hippocampal noradrenaline levels

As shown in Fig. 6, the animals exposed to novelty presented significantly higher levels of hippocampal noradrenaline than the naive and control groups ($H_{(3)} = 23.60$; $P < 0.0001$, Fig. 6).

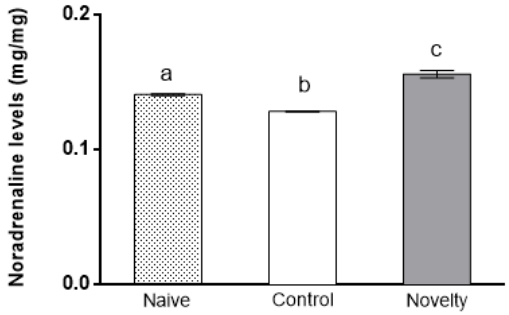

Fig. 6 Novelty exposure promotes increase of hippocampal noradrenaline levels. Rats were trained in the IA task and 24 h later were euthanized, thirty minutes after the exposure to novelty, or in the equivalent time, in the case of the other groups. The bilateral hippocampus was quickly removed and prepared as previously described for HPLC determination of noradrenaline levels. Data represent the dopamine levels in the hippocampus homogenate (mg/mg) and are expressed as mean ± SD; groups with different letters were significantly different. $P \leq 0.05$ on ANOVA followed by Kruskal-Wallis test with Dunn's multiple comparisons test; n = 5 per group, analyzed in triplicate.

3.1.3 Novelty exposure and hippocampal noradrenergic antagonist drug infusion did not impair locomotor and exploratory behaviors, anxiety and pain thresholds

Rats were exposed to OF, EPM, and HP tests after the retention test to verify whether exploratory and locomotor activity, anxiety and pain thresholds, respectively, were affected by the drug infusions or novelty exposure. As shown in Table 1, neither the drugs nor the exposure to novelty affected the evaluated parameters.

Tab. 1 Effect of the intrahippocampal infusion of vehicle or timolol and the novelty exposure on locomotor activity, anxiety and pain thresholds.

		Groups tested on IA				Groups tested on MIA			
Behavioral tasks		Control	Vehicle + Novelty	Timolol + Novelty	P value	Control	Vehicle + Novelty	Timolol + Novelty	P value
OF	Crossings, *n*	45±29.1	36.5±31.6	26.9±24.8	0.36	40.6±33.7	43±37.5	37.6±23.6	0.94
	Rearings, *n*	36.4±9.4	27.2±14.4	23.1±15.9	0.16	26.4±11.9	31.4±20.3	30.2±23.2	0.97
EPM	Entries in open arms, *n*	4.9±1.5	3.9±2.2	5.9±1.8	0.12	4.5±3.2	6.2±2.9	6±3.1	0.37
	Time in open arms, *s*	162.1±71	130±67.2	135.5±75.5	0.63	148.6±89.3	150±99.1	159.4±83.6	0.97
HP	Latency, *n*	7.9±3.2	12.2±7.8	9.1±4.8	0.30	8.2±2.5	7.6±2.7	10.9±5.4	0.50

Neither the drugs (vehicle or timolol) nor the novelty exposure affected the animals performance on the OF, EPM and HP test. Data are expressed as mean ± SD of the number of crossings and rearings (OF), the number and time spent of entries in the open arms (EPM) and latency time for the raising or licking the paws (HP). There were no differences between the groups. $P > 0.05$ on ANOVA followed by Kruskal-Wallis test; $n = 8$ per group.

3.2 Participation of the dopaminergic system in the modulation of aversive memory generalization by novelty

3.2.1 Dopaminergic system is necessary to modulation of aversive memory generalization by novelty

Animals that were exposed to a novelty before exploring the MIA on day 2 did not present generalized aversive memory. However, the D1/D5 dopaminergic receptor antagonist (SCH23390) injection immediately before the novelty exposure inhibited the novelty effect, suggesting that the effect of novelty on aversive memory generalization depends on the hippocampal dopaminergic system.

On the IA training day, the animals presented no significant difference in step-down latency ($H_{(3)} = 0.017$; $P = 0.9911$, Fig. 7A). In the IA retention test, there was no significant difference between the groups ($H_{(3)} = 5.07$; $P = 0.0791$, Fig. 7A). However, in the IA retention test the step-down latency was significantly higher than that presented on the training day ($P = 0.0006$ for the control group, $P = 0.0020$ for the novelty group, $P = 0.0005$ for the SCH23390 + novelty group, Fig. 7A), indicating that all groups were able to recognize the aversive environment (they were trained and tested in the same environment).

On the MIA retention test a significant difference between the groups step-down latencies was observed ($H_{(3)} = 20.36$; $P < 0.0001$, Fig. 7B). The control group presented a high step-down latency when compared to training ($P = 0.0023$, Fig. 7B), expressing therefore, a aversive memory generalization, since there is no distinction of the environments presented in the training and in the test (they were trained and tested in similar but not equal environments). The group exposed to the novelty presented no significant difference of step-down latencies when compared to the training ($P = 0.3464$, Fig. 7B), demonstrating, in this way, that the animals were able to differentiate the enviroments, avoiding memory generalization. The intrahipocampal infusion of SCH23390 immediately before exposure to

novelty blocked the effect of novelty and the animals presented a high step-down latency when compared to training ($P < 0.0001$, Fig. 7B).

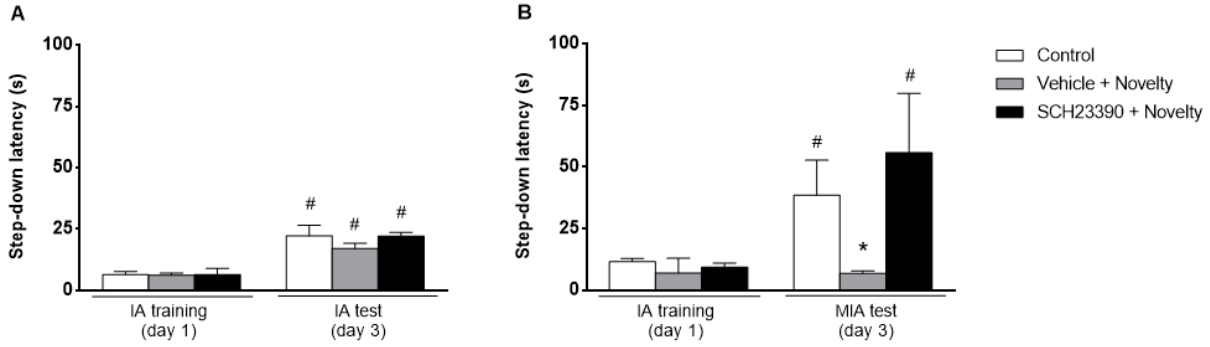

Fig. 7 SCH23390 infusion into the CA1 region of the hippocampus immediately before of the novelty exposition blocks the modulation effect of novelty in aversive memory generalization. On day 1 all rats were trained in IA. On day 2 all rats were submitted a MIA exploration for 3 minutes; the novelty and SCH23390 + novelty groups were exposed to a novelty for 5 minutes 30 minutes before of the MIA exploration; SCH23390 + novelty group received an intrahippocampal injection of SCH23390 immediately before the novelty exposure. On day 3 half of the rats of each group were tested in IA (A) and the other half in the MIA (B). Data represent the step-down latency in IA (A) or MIA (B) test and are expressed as mean ± SEM. $^{\#}$ $P \leq 0.05$ in Wilcoxon test (training vs. test); * $P \leq 0.05$ in ANOVA followed by Kruskal-Wallis test with Dunn's multiple comparisons test; n = 8 per group.

3.2.2 Novelty exposure promotes increase of hippocampal dopamine levels

As shown in Fig. 8, the animals exposed to novelty presented significantly higher levels of hippocampal dopamine than the naive and control groups ($H_{(3)} = 9.26$; $P = 0.0098$, Fig. 8).

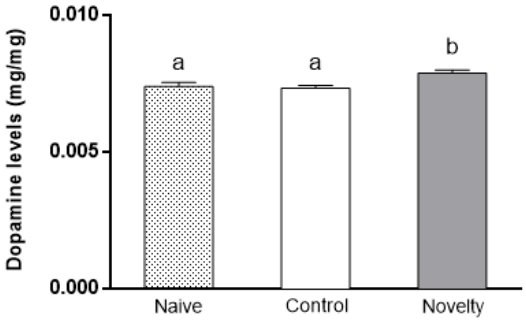

Fig. 8 Novelty exposure promotes increase of hippocampal dopamine levels. Rats were trained in the IA task and 24 h later were euthanized, thirty minutes after the exposure to novelty, or in the equivalent time, in the case of the other groups. The bilateral hippocampus was quickly removed and prepared as previously described for HPLC determination of dopamine levels. Data represent the dopamine levels in the hippocampus homogenate (mg/mg) and are expressed as mean ± SD; groups with different letters were significantly different. $P \leq 0.05$ on ANOVA followed by Kruskal-Wallis test with Dunn's multiple comparisons test; n = 5 per group, analyzed in triplicate.

3.2.3 Novelty exposure and hippocampal dopaminergic antagonist drug infusion did not impair locomotor and exploratory behaviors, anxiety and pain thresholds

Rats were exposed to OF, EPM, and HP tests after the retention test to verify whether exploratory and locomotor activity, anxiety and pain thresholds, respectively, were affected by the drug infusions or novelty. As shown in Table 2, neither the drugs nor the exposure to novelty affected the evaluated parameters.

Tab. 2 Effect of the intrahippocampal infusion of vehicle or SCH23390 and the novelty exposure on locomotor activity, anxiety and pain thresholds.

		Groups tested on IA				Groups tested on MIA			
Behavioral tasks		Control	Vehicle + Novelty	SCH23390+ Novelty	P value	Control	Vehicle + Novelty	SCH23390+ Novelty	P value
OF	Crossings, *n*	97.5±28.9	85.6±39.2	74.6±32.8	0.42	67.6±20.1	63.1±25.4	58.6±31.5	0.81
	Rearings, *n*	27.5±14.2	26.6±11.7	18.6±14	0.36	17.1±9.7	25.1±14.5	18±10.3	0.43
EPM	Entries in open arms, *n*	7.5±1.6	9.2±1.9	9.1±2.3	0.17	6.6±3.6	9±2.7	6.5±2.1	0.16
	Time in open arms, *s*	169.8±53	194.9±42.3	191.9±34	0.47	165.7±32	199.6±46	196.4±61.3	0.36
HP	Latency, *n*	3.6±1.1	4.9±1.4	4.2±1.7	0.18	4.6±1	4.4±1.2	4.4±1.6	0.94

Neither the drugs (vehicle or SCH23390) nor the novelty exposure affected the animals performance on the OF, EPM and HP test. Data are expressed as mean ± SD of the number of crossings and rearings (OF), the number and time spent of entries in the open arms (EPM) and latency time for the raising or licking the paws (HP). There were no differences between the groups. $P > 0.05$ on ANOVA followed by Kruskal-Wallis test; n = 8 per group.

4 Discussion

In this study we demonstrated that exposure to a novelty avoids the aversive memory generalization and that this process is dependent on the activation of the β-adrenergic and D1/D5 dopaminergic pathways, since the blockade of these pathways, with intra-hippocampal infusion of timolol or SCH23390, respectively, immediately before to exposure to novelty inhibits its effects.

In the test performed on IA all groups of animals were able to recognize the aversive environment. Since the training and testing for these groups were in the same environment, it is expected that they respond with an increase in the animal's aversive response (higher step-down latency). According to Izquierdo, Furini and Myskiw (2016), a conditioned stimulus, initially neutral, like the IA context, paired with an unconditioned stimulus (aversive), as an electrical stimulus, becomes associated in memory, thus establishing the conditioned stimulus as a predictive sign of the stimulus aversive, leading to defensive behaviors, such as the increase of step-down latency when the animal is re-exposed to the same context. In the MIA test, the "ideal" response would be a decrease of fear expression, since the test environment, although similar, is not not equal to that of the training. However, as seen, the animals in the control group demonstrated an aversive response in the MIA context, failing to distinguish both environments, in this way, we suggest a aversive memory generalization. In summary, we demonstrate here that the novelty modulates the aversive memory generalization, since it is able to be attenuated by the novelty exposure.

The classical conditioning model proved to be a highly effective tool to investigate the generalization of learning among species (Dunsmoor; Paz, 2015). Pavlov observed that a conditioned response was not necessarily specific to a previously conditioned stimulus, that is, it could be evoked by other similar stimuli (Dunsmoor; Paz, 2015; Jasnow et al., 2016). From this discovery is growing the interest in studying models of generalization of fear, characterized mainly by the failure to discriminate the security of the threat (Dunsmoor; Paz, 2015). In recent years, several studies have shown that both stressor and pharmacological stimuli before consolidation lead to exaggerated aversive responses to stimuli that would normally induce minimal fear responses (Adamec; Blundell; Burton, 2005; Bignante et al., 2008; Gazarini et al., 2013; 2014). However, it is known that the generalization of fear tends to occur naturally, as aversive memory becomes remote due to neural modifications (Knierim; Lee; Hargreaves, 2006; Wiltgen; Silva, 2007). These changes may involve, more precisely, a circuit that brings together cortical regions beyond the hippocampus, thalamus and amygdala

(Rozeske et al., 2015). However, excessive generalization of fear over stimuli that resemble a previous aversive event may contribute to the pathogenesis of fear and anxiety disorders (Huckleberry, Ferguson; Drew, 2016), such as specific phobias, obsessive compulsive disorder, panic disorder and PTSD (Dunsmoor; Paz, 2015). Therefore, understanding the neural and psychological mechanisms of fear generalization is fundamental (Kheirbek et al., 2012), as well as alternatives that may contribute to attenuation of symptoms (Dunsmoor; Paz, 2015).

Exposure to novelty has been widely investigated for its implications under the formation of LTMs (Moncada; Viola, 2007; Ballarini et al., 2009; Psyrdellis; Pautassi; Justel, 2016). Its action has already been observed under the aversive memory, acting in the facilitation of the extinction of the contextual fear and aversive memory in rats (Myskiw; Benetti; Izquierdo, 2013; Menezes et al., 2015) and we demonstrate in a previous work, and confirm again in these experiments, that exposure to novelty facilitates the discrimination of two similar enviroments, avoiding the aversive memory generalization (Vargas et al., 2018).

Stimulus that would form only STMs can form LTMs by novelty exposure influence (Moncada et al., 2011), a phenomenon that has been explained through the STC hypothesis proposed by Frey and Morris (1997). This hypothesis proposes that a strong stimulus can induce the synthesis of PRPs that when captured at previously marked sites, allow the consolidation of memory and the formation of LTM (Moncada; Viola, 2007; Redondo; Morris, 2011). In this study, the exposure to novelty may have acted as a strong stimulus, capable of inducing the production of PRPs, and when carried out in a time window close to a weak stimulus, in the case, the MIA exposition, was able to give PRPs for the promotion of an LTM for this context, thus, avoiding the aversive memory generalization (Figure 9). The same phenomena was observed previoulsy by our group (Vargas et al., 2018), and we showed that this novelty effect was dependent of protein synthesis.

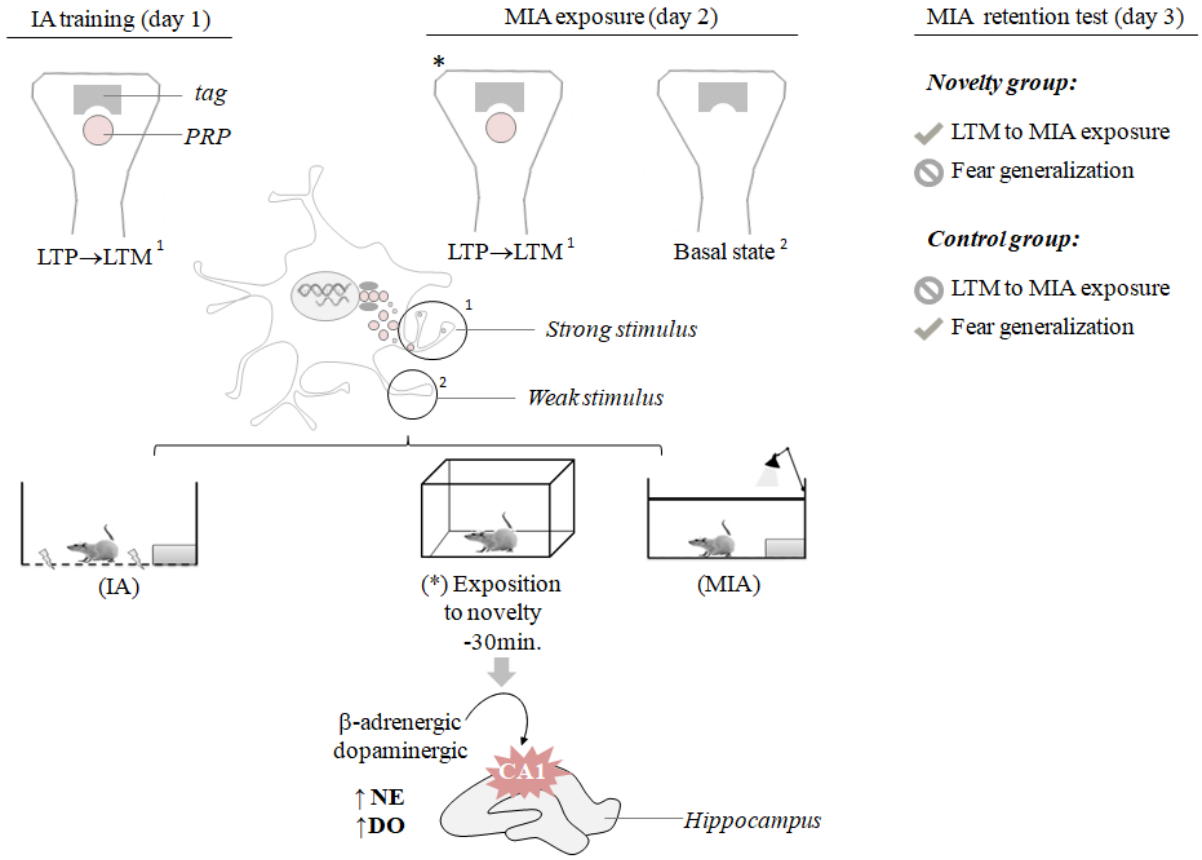

Fig. 9 Schematic drawing of STC hypothesis on the effect of novelty in the generalization of aversive memory. When a synaptic pathway is stimulated there is the marking of this synapse by a tag, if the stimulus is strong enough, there will be synthesis of PRPs, which will be captured only by previously marked synapses. The action of these proteins promotes the support of the potentiated state of the synapse and the formation of a late LTP, a fundamental phenomenon for the promotion of a LTM. Both IA training and exposition to novelty are strong stimuli capable of inducing the production of PRPs. Exposure to MIA, a weak stimulus, when associated in a time window close to a strong stimulus (such as novelty) is able to receive these PRPs which leads to the promotion of an LTP and consequent LTM. However, only the exposure to MIA is not strong enough to lead to the synthesis of PRPs and the marked synapse gradually returns to its basal state. The novelty group, in this way, tends to generate a LTM for exposure to MIA (non-aversive event), which prevents the aversive memory generalization. In contrast, the control group is not able to generate a LTM for exposure to MIA, standing out to the aversive memory and consequent aversive memory generalization. Additionally, we know now that the beneficial effect of exposure to novelty on the generalization of aversive memory depends of the activation of the β-adrenergic and dopaminergic pathways in the CA1 region of the hippocampus and; that the exposure to novelty is capable of increasing noradrenaline (NE) and dopamine (DO) levels in this region.

Recent studies have shown that the activation of noradrenergic and dopaminergic receptors is an important factor for the control of the synthesis of PRPs (Moncada; Ballarini; Viola, 2015; Moncada, 2016). Moncada et al. (2011) observed that the use of β-adrenergic and D1/D5 dopaminergic antagonists (Propranolol and SCH23390) in the dorsal hippocampus is able to block the novelty effect, interfering with the promotion of LTM. Another evidence found by the same authors is that as opposed to the use of antagonists, β-adrenergic and dopaminergic agonists (dobutamine and SKF 38393) by intraperitoneal administration are able to conduct the promotion of LTM through a mechanism dependent on protein synthesis. Additionally, Moncada (2016) showed that the LC and VTA areas, which respectively stimulate the release of noradrenaline and dopamine in various brain structures, control memory consolidation and induce the formation of LTM when electrically stimulated.

These studies are consistent with our results, since here we verified that the blockade of β-adrenergic and D1/D5 dopaminergic receptors impairs the effect of novelty, promoting the aversive memory generalization. Moreover, we have seen in our study that exposure to novelty increases the hippocampal levels of noradrenaline and dopamine, suggesting that, in fact, the increase of these neurotransmitters may influence the promotion of LTM observed in the MIA retention test. The naive group is composed of wild animals, which did not received any previous protocol (different of the control and novelty groups), in this way, hypothesized that the higher noradrenaline levels in this group when compared to the control group are related to a sympathetic nervous system response, which induces the increase of this neurotransmitter to stress situations such as the manipulation of these animals on the day of euthanasia.

It is already consolidated in the literature the role of noradrenergic and dopaminergic systems in the regulation of synaptic plasticity, corroborating to memory consolidation (Mcgaugh; Roozendaal, 2009; Moncada et al., 2011; Gazarini et al., 2013). Vankov et al. (1995) found that when rats are exposed to a new stimuli, the LC response tends to be higher. Based on these findings, more recently, Aston-Jones and Cohen (2005) have suggested that the novelty would be among a highly strong group of events capable, in fact, to causing a differential response in LC, with consequent expressive release of noradrenaline. On the other hand, the involvement of the dopaminergic system in response to novelty, according to Lisman and Grace (2005), seems to begin in the hippocampus. The authors proposed that the new stimuli are initially detected and transmited by signal via subiculum, nucleus accumbens,

and ventral pallidum to the VTA, culminating in the release of dopamine in the hippocampus and LTP expression (Lisman; Grace, 2005). However, although there are several studies that provide clear evidence that new stimuli activate these neuromodulatory systems, it is not yet clear how these systems relate to the processing of novelty influencing memory processes (Rangel-Gomez; Meeter, 2015).

According to these facts, the importance of understanding the neural mechanisms for the formation and modification of fear memories is based mainly on the search for interventions that may serve to better understand and consequently treat disorders related to fear and anxiety. Here we aim to understand the involvement of two neurotransmitter systems under the novelty and its importance to the discrimination of memory and avoid the generalization of aversive memory, which may help in future the new research for therapeutic applicability in psychiatric disorders, such as PTSD.

Authors' contributions

PBMC, DS, II and RDH defined the study design. LSV and KL performed the experiments. RR conducted the biochemical analyzes. All authors analyzed and discussed the data and read and approved the final version of the manuscript.

Acknowledgments

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Conflict of Interests

The authors declare that there are no conflicts of interests regarding the publication of this manuscript.

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PARTE III

CONCLUSÕES

Os resultados obtidos no presente trabalho de conclusão de curso permitem concluir que:

- A exposição a uma novidade facilita a distinção entre dois ambientes (original e similar ao original), evitando a generalização da memória aversiva;

- O efeito modulatório da novidade sobre a generalização da memória aversiva depende da ativação do sistema β-adrenérgico hipocampal;

- O efeito modulatório da novidade sobre a generalização da memória aversiva depende da ativação do sistema dopaminérgico hipocampal;

- A exposição à novidade aumenta os níveis de noradrenalina na região CA1 do hipocampo de ratos *Wistar* 30 minutos após este evento;

- A exposição à novidade aumenta os níveis de dopamina na região CA1 do hipocampo de ratos *Wistar* 30 minutos após este evento.

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ANEXOS

ANEXO I – Carta de aprovação do CEUA

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA
(Lei nº 11.640, de 11 de janeiro de 2008)

Pró-Reitoria de Pesquisa, Pós-Graduação e Inovação (PROPII)

CEUA

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Fone: (55)3911-0200 E-mail: ceua@unipampa.edu.br

CERTIFICADO DE APROVAÇÃO DE PROTOCOLO PARA USO DE ANIMAIS EM PESQUISA

Número de protocolo da CEUA: 001/2017

Título: Efeitos da novidade na generalização das memórias de medo: uma investigação da participação dos sistemas noradrenérgico e dopaminérgico.

Data da aprovação: 12/04/2017

Período de vigência do projeto: 12/04/2019

Pesquisador(a): Pâmela Billing Mello Carpes

Campus: Uruguaiana

Telefone: (55) 99661-2454

E-mail: pamelacarpes@unipampa.edu.br

CEUA

Comissão de Ética em Uso de Animais

Finalidade	**( ) Ensino (X) Pesquisa**
Espécie/Linhagem/Raça	Ratos Wistars
Nº de animais	192
Peso/Idade	250-300g / 120dias
Sexo	machos
Origem	Biotério da Universidade Federal do Pampa

Profª. Drª. Vanusa Manfredini
Coordenadora CEUA/UNIPAMPA

ANEXO II – Normas da revista *Neurobiology of Learning and Memory*

The Editors of *Neurobiology of Learning and Memory* will use forwarded referees' reports at their discretion. The Editors may use the reports directly to make a decision, or they may request further reviews if they feel such are necessary.

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Regardless of the file format of the original submission, at revision you must provide us with an editable file of the entire article. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier). See also the section on Electronic artwork.
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Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods
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A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

Results
Results should be clear and concise.

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This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions
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