**UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL**

**INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE**

**PROGRAMA DE PÓS-GRADUAÇÃO NEUROCIÊNCIAS**

**Bruno Popik**

# ATENUAÇÃO DE MEMÓRIAS DE MEDO ATRAVÉS DOS MECANISMOS DO DESCONDICIONAMENTO: UMA NOVA ABORDAGEM PARA ATENUAR MEMÓRIAS AVERSIVAS

**Porto Alegre**

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**BRUNO POPIK**

Tese de doutorado apresentada ao Programa de Pós-Graduação em Neurociências do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de doutor em Neurociências.

**Orientador**: Dr. Lucas de Oliveira Alvares

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***"Theory without data is fantasy, but data without theory is chaos"***

LAWLER, E. 1971

**SUMÁRIO**

**LISTA DE FIGURAS**

terminal; VA, núcleo talâmico ventral anterior; VL, núcleo talâmico ventrolateral; VM, núcleo talâmico ventromedial (adaptado de Jankowski 2013).

**LISTA DE ABREVIATURAS E SIGLAS**

| | |
|---|---|
| (Z)-3-(4-Iodophenyl) -2-mercapto-2-propenoic acid | PD150606 |
| 2-Araquidonoilglicerol | 2-AG |
| 3º ventrículo | 3V |
| Ácido-amino-3-hidroxi-5-metil-isoxazol-4-propiônico | AMPA |
| Ácido-amino-3-hidroxi-5-metil-isoxazol-4-propiônico - calcium impermeable | CI-AMPA |
| Ácido-amino-3-hidroxi-5-metil-isoxazol-4-propiônico - calcium permeable | CP-AMPA |
| Amígdala Basal | BA |
| Amígdala Basolateral | BLA |
| Amígdala Basomedial | BM |
| Amígdala Central | CeA |
| Amígdala Central Lateral | CeL |
| Amígdala Central Medial | CeM |
| Amígdala Lateral | LA |
| Anterior Cingulate Cortex | ACC |
| Cálcio | $Ca^{2+}$ |
| Canais de Cálcio Dependentes de Voltagem | VGCCs |
| Cápsula interna | IC |
| Células Intercalares dorsal | ITCd |
| Células Intercalares lateral | ITCl |
| Células Intercalares ventral | ITCv |
| Condicionamento aversivo ao contexto | CAC |
| Condicionamento aversivo ao tom | CAT |
| Conditioned stimulus | CS |
| Corno de Amon | CA1, CA2 e CA3 |
| Córtex Cingulado Anterior dorsal | ACCd |
| Córtex Dorso-Peduncular | DP |
| Estria medular do tálamo | SM |

| | |
|---|---|
| Estria terminal | ST |
| Fosfatidilinositol Bifosfato | PIP2 |
| Infra-Límbico | IL |
| Inositol 1,4,5-trifosfato | IP3 |
| Long Term Depression | LTD |
| Long Term Memory | LTM |
| Long Term Potentiation | LTP |
| N-metil-D-Aspartato | NMDA |
| Núcleo paracentral do tálamo | PC |
| Núcleo parafascicular do tálamo | PF |
| Núcleo paraventricular talâmico, parte anterior | PVA |
| Núcleo reticular talâmico | RT |
| Núcleo reuniens talâmico | RE |
| Núcleo talâmico anterodorsal | AD |
| Núcleo talâmico anteromedial | AM |
| Núcleo talâmico anteromedial, parte ventral | AMV |
| Núcleo talâmico anteroventral | AV |
| Núcleo talâmico centrolateral | CL |
| Núcleo talâmico interanteromedial | IAM |
| Núcleo talâmico laterodorsal | LD |
| Núcleo talâmico mediodorsal | MD |
| Núcleo talâmico paratenial | PT |
| Núcleo talâmico posterior lateral | LP |
| Núcleo talâmico ventral anterior | VA |
| Núcleo talâmico ventrolateral | VL |

| | |
|---|---|
| Núcleo talâmico ventromedial | VM |
| perineuronais | PNNs |
| Pré-Límbico | PL |
| Proteína Fosfolipase C | PLC |
| Short Term Memory | STM |
| Transtorno de Estresse Pós-Traumático | TEPT |
| Trato mamilotalâmico | MT |
| Unconditioned stimulus | US |

## RESUMO

As memórias aversivas estão intimamente relacionadas com os distúrbios psiquiátricos, como fobias e transtorno de estresse pós-traumático. Neste trabalho é apresentada uma nova abordagem comportamental em modelo animal, ratos *Wistar*, que atenua de forma robusta e duradoura as memórias aversivas. Esse método consiste em reescrever a memória de medo através de um gatilho, por meio de um choque fraco de 0,1mA, aplicado durante as reativações. Desta forma a memória aversiva é atenuada pelos mecanismos da reconsolidação. Os resultados sugerem que o descondicionamento é mais eficaz do que a extinção tradicional na redução das respostas de medo; além disso, tais efeitos são duradouros e resistentes a renovação, ou seja, não é dependente do contexto, retreino, reinstalação, bem como não ocorre a recuperação espontânea. Notavelmente, essa estratégia superou as condições limitantes da reconsolidação, como as memórias traumáticas remotas ou muito fortes. O mesmo efeito benéfico ocorreu tanto em machos quanto em fêmeas e demonstramos em diferentes tarefas comportamentais relacionadas a memória de medo. Ademais, foram desvendados alguns dos mecanismos celulares e moleculares atuantes no descondicionamento, tais como os canais de cálcio dependentes de voltagem do tipo L, pelos receptores CP-AMPA e NMDA-GluN2B, receptores IP3 (estoque intracelular de cálcio), pelos receptores CB1 e pela atividade das proteínas calpaínas. Por fim, foi demonstrado que o descondicionamento ocorre apenas dentro de um limite de discrepância em relação da intensidade de treino (0,3mA a 1mA) com os choques fracos aplicados durante as reativações (0,1mA), e que requer uma janela de temporal mínima de 6h entre as reativações. Os achados contribuem para uma melhor compreender de como as memórias do medo pode ser atenuada, além de atuar como uma possível estratégia de tratamento para transtornos emocionais.

## ABSTRACT

Aversive memories are closely related to psychiatric disorders such as phobias and post-traumatic stress disorder. Here, we present a novel behavioral approach in an animal model, Wistar rats, that robustly and durably attenuates aversive memories. This method consists of rewriting the fear memory through a trigger, a weak shock of 0.1mA, applied in the reactivations, in this way the aversive memory is updated by the reconsolidation mechanisms. Our results indicate that deconditioning is more effective than traditional extinction in reducing fear responses; Furthermore, such effects are long-lasting and resistant to renewal, that is, it is not context-dependent, and retraining, reinstatement, and spontaneous recovery does not occur. Notably, this strategy overcame the limiting conditions of reconsolidation, such as remote or very strong traumatic memories. The same beneficial effect occurred in both males and females and we demonstrated it in different behavioral tasks related to fear memory. Deconditioning was mediated by voltage-gated L-type calcium channels, CP-AMPA and NMDA-GluN2B receptors, IP3 receptors (intracellular calcium storage), CB1 receptors, and the activity of calpain proteins, as the blockade of these molecular markers inhibit deconditioning and consequently prevent fear memory updating. As well, the deconditioning only occurs within a discrepancy limit related to the training intensity (0.3mA to 1.5mA) with the weak footshocks applied during reactivations (0.1mA). It also requires a minimum time window of 6h between reactivations to update the fear memory, as well as it is only efficient with already consolidated memories. Our findings contribute to a better understanding of how to attenuate a fear memory, as well as act as a possible treatment strategy for emotional disorders.

**DESCONDICIONAMENTO**

# 1. INTRODUÇÃO

## 1.1 MEMÓRIA

Todos os animais estão expostos diariamente as adversidades e dinamismo do ambiente, logo, para sobreviver é fundamental que sejam capazes de se adaptarem a esse meio. Assim, torna-se primordial que armazenem as informações oriundas do ambiente e evoquem quando necessário, basicamente esse é o propósito da memória. A formação de uma memória não é um processo instantâneo, pois requer inúmeras modificações celulares e moleculares, que acarretam assim em alterações nas conexões sinápticas dos neurônios envolvidos, e que refletem no comportamento do espécime. Desta forma, para uma melhor compreensão dos fenômenos mnemônicos, é possível dividi-lo em processos, são elas: consolidação, evocação, reconsolidação e a extinção.

A consolidação ocorre imediatamente após a aquisição de informações, que decorre das experiências individuais. Durante esse período a memória ainda se encontra num estágio lábil, uma vez que é suscetível a modulações que pode ocasionar tanto o fortalecimento quanto o enfraquecimento. Após esse período, o traço da memória se torna mais resistente e insensível a modificações (Izquierdo & McGaugh, 2000; Rudy, 2015). Esse processo mnemônico é caracterizado por inúmeros mecanismos moleculares e celulares, como a dependência de síntese de proteínas, ativação dos neurônios glutamatérgicos, fatores de transcrição, reorganização de receptores de membrana e do citoesqueleto (Dudai, 2004; Dudai et al., 2015), além de, modificações nos espinhos dendríticos (Kasai et al., 2010). Segundo Casagrande e colaboradores (2018), a consolidação sináptica é dinâmica, pois vários fatores podem influenciá-la, como por exemplo, a intensidade do estímulo original (a força daquela informação) e da novidade da experiência de aprendizagem. Nesse trabalho, os autores demonstraram que um treino de alta intensidade tende a acelerar a janela temporal da consolidação, tornando-a insensível a interferências poucas horas após o aprendizado. Os autores também observaram que um treino de fraca intensidade prolonga a janela temporal da consolidação, o que resulta em um período maior de suscetibilidade a modulação da informação (Casagrande et al., 2018).

Nesse sentido, é essencial que no âmbito clínico sejam consideradas as características temporais da consolidação, uma vez que, após um evento traumático a

consolidação pode ser enfraquecida ou mesmo interrompida diante de intervenções realizadas no seu período lábil. Consequentemente, a atenuação do desenvolvimento de uma memória patológica, por exemplo, o transtorno de estresse pós-traumático (TEPT) poderia ser prevenido (Breslau, 2009; Brewin, 2011). Entretanto, o sucesso dessas intervenções fica restrito ao período no qual o processo de consolidação transcorre, limitando assim o seu efeito terapêutico.

Após o processo de consolidação sináptica, o traço mnemônico poderá persistir por meses, anos ou mesmo a vida toda, o que caracteriza a consolidação sistêmica. Nesse processo, a informação é gradualmente reorganizada em diferentes estruturas encefálicas (Kitamura et al., 2017; Squire, 2004). Inúmeros estudos demonstram que as conexões entre hipocampo e córtex, mais especificamente do córtex cingulado anterior (ACC, do inglês *anterior cingulate cortex*) sustentam a reorganização estrutural das memórias (Dudai, 2004; Frankland & Bontempi, 2005; Restivo et al., 2009). Atualmente, há duas teorias que procuram explicar o fenômeno de reorganização encefálica da consolidação, destacam-se o modelo *Standard* e a Teoria dos Múltiplos Traços.

O Modelo *Standard* da Consolidação Sistêmica prediz que, o passar do tempo ocorre uma reorganização na conectividade entre o hipocampo e o córtex, no qual as conexões hipocampo-córtex são progressivamente enfraquecidas e, as conexões córtex-córtex são fortalecidas. Sendo assim, o hipocampo seria um local temporário de armazenamento das informações e o córtex, o destino final, sustentaria tanto o armazenamento quanto a evocação dessa memória (Frankland & Bontempi, 2005).

Segundo a Teoria dos Múltiplos Traços, o circuito hipocampo-córtex interage continuamente desde a aquisição da memória até a sua evocação, porém o hipocampo sustenta as informações precisas e ricas em detalhes, principalmente contextuais (Winocur et al., 2007), enquanto que o córtex sustentaria uma memória mais genérica (Bergstrom, 2016; Pedraza et al., 2016). De Oliveira Alvares e colaboradores (2013), demonstraram que a dinâmica da consolidação sistêmica pode ser modificada por meio de reativações da memória. Com isso, a memória permanece dependente do hipocampo e consequentemente mais detalhada, ou seja, impede a consolidação sistêmica (De Oliveira Alvares et al., 2013). Em consonância com esses resultados, Jasnow e colaboradores (2016), sugerem que o processo de consolidação sistêmica permite a generalização da memória, caracterizada pela perda da precisão e dos detalhes presentes na memória original (Jasnow et al., 2017).

Em conjunto, os trabalhos apresentados permitem afirmar que a memória consiste em um processo ativo, que possibilita a adaptação dos indivíduos a um ambiente em constante mudança. Da mesma forma, a evocação da memória nos últimos anos também tem se consolidado na literatura como um processo ativo e dinâmico, pois requer síntese de proteínas (Lopez et al., 2015), tráfego de receptores glutamatérgicos (Hong et al., 2013; Torquatto et al., 2019), ativação das cascatas de proteases, bem como a reorganização de proteínas responsáveis pela reestruturação do citoesqueleto e dos espinhos dendríticos (Fernandes et al., 2022; Lunardi et al., 2018; Popik et al., 2018; Redondo et al., 2020). Portanto, quando a memória é evocada pode entrar num estado instável e passível de interferências, tanto no seu traço mnemônico original quanto acarretar na formação de uma nova memória (J. L. C. Lee, 2009; Nader & Einarsson, 2010), características dos processos de reconsolidação e extinção, respectivamente.

A capacidade de atualizar as memórias previamente consolidadas mediantes novas experiências torna-se fundamental para a sobrevivência do indivíduo. A reativação da memória possibilita que ocorra o processo da reconsolidação, esse é desencadeado pela desestabilização e reestabilização do seu traço (Nader et al., 2000). Portanto, a evocação de uma memória adquirida anteriormente pode induzir um estado lábil (desestabilização), que permite que traço original seja reescrito tanto a força quanto o seu conteúdo (De Oliveira Alvares et al., 2013; Popik et al., 2020). Sabe-se que um fator crucial para que a reconsolidação seja desencadeada é a apresentação de novas informações durante a reativação (Morris et al., 2006; Pedreira et al., 2004), ou seja, o princípio do erro de predição (*mismatch*). Define-se como a discrepância entre o que é esperado com base em experiências prévias com os eventos reais – uma relação de expectativa *vs* realidade (McNally et al., 2011; Sevenster et al., 2013).

Curiosamente o processo da reconsolidação só ganhou destaque na literatura a partir dos anos 2000 com a publicação dos achados de Nader e colaboradores (2000), entretanto, esse fenômeno foi descrito nos anos de 1968 (Mandell, n.d.). O trabalho publicado por Nader e colaboradores, demonstrou que a reativação da memória pode desencadear um período lábil e susceptível a intervenções, logo, as memórias consolidadas não são armazenadas de forma fixa e permanente, mas que pode revertê-la a um estado maleável e passível de atualização.

Desde então, a reconsolidação já foi demonstrada em diversas tarefas comportamentais que avaliam diferentes tipos de memórias, incluindo paradigmas de

memória espacial, gustativa e de medo (J. L. C. Lee, 2009; Nader & Hardt, 2009; Torquatto et al., 2019). Bem como, utilizando diferentes modelos animais, desde filos como *Nematoda* e *Mollusca* até a subclasse *Mammalia*, demonstrando assim que se trata de um processo intrínseco da memória (Anokhin et al., 2002; Chalkia et al., 2020; Dębiec & Ledoux, 2004; Eisenberg et al., 2003; Fricks-Gleason & Marshall, 2008; Jarome et al., 2015; Kim et al., 2011; Kwapis et al., 2017; J. L. C. Lee, 2009; Nader & Einarsson, 2010; Pedreira et al., 2004; Rao-Ruiz et al., 2011; Robinson & Franklin, 2010; Rose & Rankin, 2006; Sangha et al., 2003; Schiller et al., 2010; Scholl et al., 2015; Stollhoff et al., 2005; Taubenfeld et al., 2009; Tronel et al., 2005; Valjent et al., 2006; Zeng et al., 2014). Além do papel biológico da reconsolidação em flexibilizar e atualizar uma memória (Fukushima et al., 2014; Tronel et al., 2005), também pode desempenhar uma estratégia terapêutica para atenuar as memórias patológicas.

Outra consequência que a evocação da memória pode desencadear é a formação de uma memória de extinção. Essa é caracterizada pela formação de uma nova memória, ou seja, um processo ativo de aquisição de uma memória neutra que inibe o traço original de medo sem apagá-lo (Bouton ME, 2002; Furini et al., 2014; Lattal & Abel, 2001). Entretanto, a nova memória – memória de extinção – mais fraca que a memória original, e consequentemente acarreta apenas numa inibição transitória da expressão do medo, logo a memória original (de medo) eventualmente retorna. Em razão disso, duas caraterísticas são típicas desse processo, sendo elas a dependência do tempo e do contexto. Isso significa que apenas com o simples passar do tempo a memória de extinção não consegue mais inibir o traço original, assim a memória de medo volta a ser expressa, ou quando o estímulo condicionado (CS, do inglês *conditioned stimulus*) é apresentado fora do contexto seguro da extinção ou mesmo no contexto do evento aversivo/treino. Em razão disso, pode-se demonstrar que a memória original está apenas inibida e a extinção é transitória (Archbold et al., 2010; Bouton et al., 2012; Merlo et al., 2014; Monfils et al., 2009; Popik et al., 2020).

Evidências experimentais sugerem que existe de um período entre a reconsolidação e a extinção, durante a qual a memória não é sensível à intervenção tanto farmacológica quanto comportamental, fase está denominada de limbo (Bustos et al., 2009; Franzen et al., 2019; Merlo et al., 2018; Sevenster et al., 2014). Inclusive, foi demonstrado tanto em memórias neutras - apetitivas - (Flavell & Lee, 2013) quanto em memórias aversivas (Alfei et al., 2015; Merlo et al., 2014). Assim, o período em que a

memória está insensível aos agentes amnésicos representaria uma fase de transição da reconsolidação para a extinção, pois ambos os processos compartilham parcialmente uma sobreposição dos substratos neuronais (Cassini et al., 2017; Flavell & Lee, 2013; Merlo et al., 2014).

Para a compreensão desses processos mnemônicos, são utilizados modelos animais, visto que os circuitos do medo condicionado nos roedores parece ter homologias funcionais com a dos seres humanos (Milad & Quirk, 2012). Em razão disso, pode-se estudar os mecanismos da extinção da memória em roedores, bem como diferenciar dos processos subjacentes da reconsolidação. A memória de extinção é um paradigma em que o CS (contexto ou tom) é repetidamente apresentado na ausência do estímulo aversivo (choque; US, do inglês *unconditioned stimulus*), levando à redução progressiva da expressão do comportamento de medo (do inglês *freezing*). Em geral a sessão de extinção é mais longa (maior número de tons ou de tempo) em relação a sessão de reativação da reconsolidação (Merlo et al., 2014; Suzuki et al., 2004).

Os testes comportamentais permitem tanto caracterizar a memória quanto diferenciá-la da reconsolidação. Desse modo, pode-se aplicar o teste de renovação (do inglês *renewal*), quando o CS é apresentado fora do contexto de extinção, ou seja, no mesmo contexto do treino, evidenciando a nível comportamental que a memória de extinção é dependente de contexto e acarreta o retorno da memória original (Effting & Kindt, 2007); o teste de reinstalação (do inglês *reinstatement*), quando o estímulo original US é administrado inesperadamente, sem associação com o CS, serve como gatilho para o retorno da memória de medo (Gogolla et al., 2009); o teste da recuperação espontânea, ocorre com o passar do tempo, evidenciando que a memória de extinção é dependente de tempo, ou seja, a memória original acaba voltando (Schiller et al., 2010); e o teste do retreino, quando os animais são retreinados no mesmo contexto, podendo ser aplicado um US com menor intensidade, resgatando assim a memória original (Monfils et al., 2009). Esses testes comportamentais evidenciam que a memória de extinção apenas inibe momentaneamente a memória de medo, já no caso da reconsolidação a memória original é reescrita, portanto não voltaria a expressar os mesmos níveis do comportamento de medo em nenhum dos testes mencionados, consequentemente permite discernir a nível comportamental ambos os processos mnemônicos (Popik et al., 2020).

Grande parte das terapias para memórias traumáticas são baseadas na utilização dos mecanismos da memória de extinção, mesmo com as suas limitações intrínsecas.

Dentre as patologias, o transtorno de estresse pós-traumático (TEPT) destaca-se por ter uma grande variabilidade de incidência dentre as populações amostrais avaliadas. Como por exemplo, na América do Norte as taxas de prevalência podem variar de 6 a 9 % (Kessler et al., 2009; M. B. Stein et al., 1997), enquanto que no Brasil existe uma carência de estudos sobre a incidência desta patologia na população em geral (Ximenes et al., 2009). Apesar disso, a população brasileira é exposta a elevadas taxas de eventos traumáticos, favorecendo a incidência do TEPT por lesões físicas (O'Donnell et al., 2008), desastres em geral (D. J. Stein et al., 2007) e abuso sexual (Breslau, 2009). Tendo em vista que a exposição a estímulos aversivos severos pode desencadear à formação e consolidação de memórias traumáticas persistentes, torna-se fundamental desenvolver novas estratégias que atenuem essas memórias, que acarretam severos prejuízos sociais e econômicos.

Nas últimas décadas, os mecanismos da reconsolidação tornaram-se um dos principais alvos de estudo para atenuar ou mesmo eliminar uma memória traumática, pois possuem efeitos mais duradouros e eficientes. Uma das principais ferramentas empregadas nas pesquisas são os agentes farmacológicos, porém, essas intervenções possuem algumas limitações. Destaca-se o fato de que a maioria dos tratamentos que interferem com a reconsolidação são administrados diretamente nas estruturas encefálicas e tendem a ser tóxicas, logo, dificilmente poderiam ser administrados em seres humanos. Mas se a reconsolidação é tão eficiente, então por que as terapias baseadas na extinção são as mais empregadas?

Para responder esse questionamento, temos que levar em consideração as características peculiares da reconsolidação, denominadas de condições limitantes (do inglês *boundary conditions*). Destacam-se dois principais fatores que limitam esse processo, a idade da memória traumática/aversiva, pois memórias antigas tendem a serem inflexíveis e dificilmente serão modificadas. O outro fator é a intensidade do trauma/treino, da mesma forma, quanto maior a intensidade, menor a probabilidade do traço da memória se tornar suscetível a modulações (J. L. C. Lee, 2009; Nader & Hardt, 2009; Suzuki et al., 2004; Wang et al., 2009). Outro fator essencial que prediz se ocorrerá ou não o processo da reconsolidação é a apresentação de uma novidade, pois, quando apenas informações já conhecidas são apresentadas na reativação, a memória permanece estável e, consequentemente resistente a agentes amnésicos (Barrett et al., 2011; Díaz-Mataix et al., 2013; Kwapis et al., 2017, 2020; J. L. C. Lee, 2010; Von Hertzen & Giese,

2005). Entretanto, quando novas informações são apresentadas a reconsolidação pode ser iniciada e permite que o conteúdo dessa memória seja atualizado (Hupbach et al., 2007; Jarome et al., 2015; J. L. C. Lee, 2008; Sevenster et al., 2013, 2014). Logo, uma mudança de contexto (Jarome et al., 2015) ou mesmo no tempo de estímulo (Díaz-Mataix et al., 2013) já pode desencadear a atualização de memória. Essas características intrínsecas da reconsolidação acarretam nas severas limitações práticas, como por exemplo, dificulta a replicação dos achados científicos, bem como limita os tratamentos clínicos, pois grande parte dos procedimentos só iniciam muito tempo após a aquisição dessa memória traumática, ou seja, essas memórias patológicas dificilmente entrariam num estado lábil em que fossem suscetíveis a modulações (Milekic & Alberini, 2002).

## 1.2 PRINCIPAIS ESTRUTURAS ENCEFÁLICAS RELACIONADAS COM A RECONSOLIDAÇÃO E EXTINÇÃO DE UMA MEMÓRIA DE MEDO

Atualmente sabe-se que as funções cognitivas, principalmente a memória, são suportadas por uma rede de estruturas encefálicas e, não por regiões isoladas (McIntosh, 1999; Park & Friston, 2013). O comportamento dos seres vivos depende da atividade

coordenada de diversas estruturas, para a memória de medo e com destaque para os mecanismos de reconsolidação e extinção, algumas das principais estruturas encefálicas envolvidas são a amígdala, o hipocampo e córtex pré-frontal.

### *1.2.1 Amígdala*

A amígdala participa ativamente das informações aversivas (responsável pelo caráter emocional da memória), sendo crucial para o armazenamento e flexibilidade da memória de medo. Existe um sólido corpo de evidências demonstrando que a amígdala desempenha um papel fundamental na memória de medo, assim a modulação dos neurônios amigdalares modifica os parâmetros moleculares, celulares e comportamentais dos processos de flexibilização da memória de medo (Cammarota et al., 2004; Cogan et al., 2019; Díaz-Mataix et al., 2014; Hong et al., 2013; Jin et al., 2007; Kritman & Maroun, 2013; Lin et al., 2006; Mamiya et al., 2009; Mamou et al., 2006; Milton et al., 2013; Motanis & Maroun, 2012; Rehberg et al., 2010; Tronel & Alberini, 2007; Tronson et al., 2006; Tzeng et al., 2012). No caso de uma memória patológica, como o TEPT, essa estrutura está relacionada com os sintomas de hipervigilância e pelo conteúdo emocional dos eventos traumáticos vivenciados (Peri et al., 1999).

Essa estrutura está localizada profundamente no lobo temporal e recebe informações sobre o ambiente a partir do tálamo e dos córtices sensoriais (Mcdonald, 1998). Segundo Janak e Tye (2015), a amígdala é composta por grupos heterogêneos de núcleos e subnúcleos. O complexo basolateral (BLA), formado pelos grupos de células lateral (LA), basal (BA) e basomedial (BM) e o núcleo central da amígdala (CeA), constituído pela subdivisão lateral (CeL) e medial (CeM), são as principais regiões da amígdala relacionadas com o comportamento de medo. A BLA consiste principalmente de neurônios glutamatérgicos e interneurônios inibitórios, mantendo conexões com regiões como o córtex pré-frontal orbital, o hipocampo e áreas de associações sensoriais. Os neurônios do núcleo CeA são principalmente GABAérgicos, com o núcleo CeL projetando para o núcleo CeM (Janak et al., 2015). Uma bainha interligada de neurônios GABAérgicos denominada, células intercaladas também é encontrada entre o BLA e CeA, funcionando como uma importante fonte de inibição entre esses núcleos (Marowsky et al., 2005).

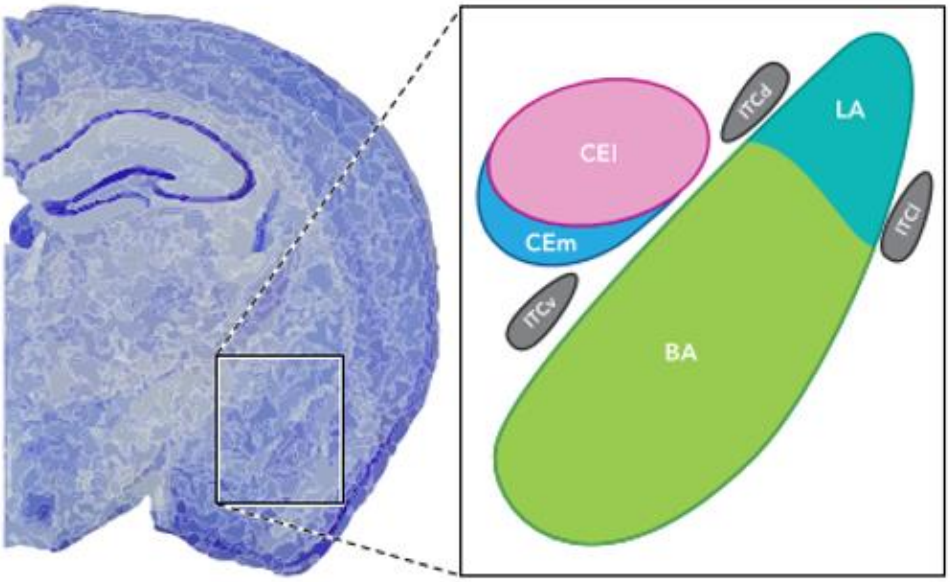


**Figura 1.** Desenho esquemático da amígdala de ratos *Wistar* em corte coronal. Representação das estruturas de interesse para a memória, o complexo basolateral (BLA) que é composto pela porção basal (BA) e lateral (LA), a núcleo central da amígdala (CeA) que é composto pelos núcleos centromedial (CeM) e centrolateral (CeL), entre a BLA e a CeA temos os grupos de células intercalares dorsal (ITCd), ventral (ITCv) e lateral (ITCl (Keifer et al., 2015).

### *1.2.2 Hipocampo*

O hipocampo é classicamente conhecido por ser uma das principais estruturas encefálicas relacionadas com a memória, exercendo um papel essencial na aquisição, consolidação e nas fases subsequentes à evocação do traço de memória (Barnes et al., 2012; Boccia et al., 2007; Bremner et al., 1995; Corcoran & Maren, 2001; de la Fuente et al., 2011; de Oliveira Alvares et al., 2008; Dolcos et al., 2004; Eichenbaum, 2000; Jobim et al., 2012; MacHado et al., 2010; Motanis & Maroun, 2012). Inclusive pacientes com TEPT apresentam tanto alterações no seu volume como também nas suas conectividades, principalmente com o córtex pré-frontal e a amígdala (Pitman et al., 2012; Shin et al., 2006).

Essa estrutura, localiza-se na porção medial do lobo temporal e faz parte do sistema límbico (O'Keefe et al., 1971). É formado por duas regiões interligadas chamadas

de giro denteado e Corno de Amon (CA), sendo esse último subdividido em outras três partes: CA1, CA2 e CA3 (Taube et al., 1990). A principal aferência para o hipocampo origina-se no córtex entorrinal e segue até os neurônios granulares da camada molecular do giro denteado, a chamada via perfurante. Os axônios desses neurônios granulares constituem as fibras musgosas, que se projetam para os neurônios piramidais da região de CA3. Por sua vez, os axônios de CA3 conectam-se também com neurônios piramidais de CA1 e CA2, a chamada via colateral de Schaffer. Em razão da reduzida área de CA2, esta é descrita em associação com CA1. Os axônios de CA1 projetam-se para o complexo subicular e então para as camadas profundas do córtex entorrinal (Brun et al., 2002; Witter & Moser, 2006). O circuito córtex entorrinal – giro denteado – CA3 – CA1 é tradicionalmente denominado "via tri-sináptica" e utiliza o glutamato como principal neurotransmissor (Witter et al., 2014).

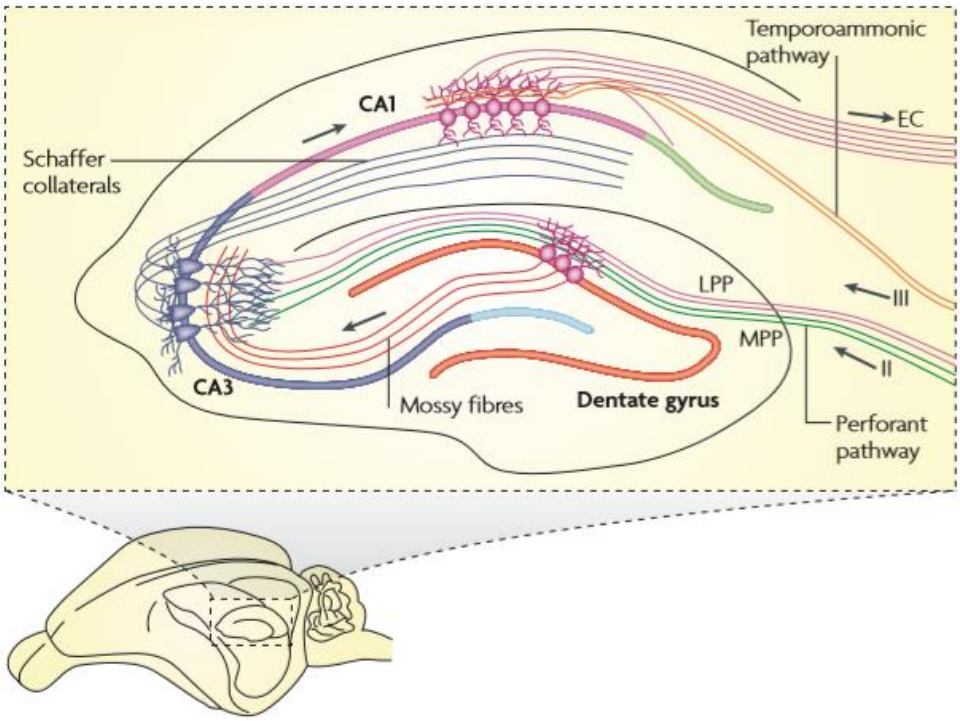


**Figura 2**. Desenho esquemático do hipocampo de ratos *Wistar* em corte coronal. Representação da formação hipocampal (giro denteado e Corno de Amon - CA, subdividido nas regiões CA1, CA2 e CA3 e das suas principais conexões). Representando a via trissináptica do hipocampo (Deng et al., 2010).

O hipocampo é uma estrutura complexa e apresenta uma dicotomia funcional e anatômica, sugere-se que o hipocampo não atua como uma estrutura unitária, mas sim

exerce papéis diferentes dependendo das regiões dorsal e ventral (M. B. Moser & Moser, 1998). Levando em consideração que os estudos anatômicos indicam que as conexões de entrada e saída do hipocampo dorsal e do hipocampo ventral são distintas (van Groen & Wyss, 1990). Bem como, a funcionalidade de cada região exerce um papel diferente nos processos cognitivos, assim a memória espacial é mais dependente da porção dorsal do hipocampo (Holt & Maren, 1999; E. Moser et al., 1993; M. B. Moser et al., 1995; Pothuizen et al., 2004; Woollett & Maguire, 2011), já as respostas ao estresse e o comportamento emocional são mais dependentes da porção ventral (Bannerman et al., 2004; Bast et al., 2001; Henke, 1990; Kjelstrup et al., 2002; Maren & Holt, 2004; Pentkowski et al., 2006; Richmond et al., 1999; Weeden et al., 2015).

### *1.2.3 Córtex Pré-Frontal*

O córtex pré-frontal está intimamente relacionado com a expressão do medo e modulação da memória de medo (Bayer & Bertoglio, 2020; Do Monte et al., 2013; Einarsson & Nader, 2012; Frankland et al., 2006; Kritman & Maroun, 2013; Mamiya et al., 2009; Stern et al., 2014). As principais regiões envolvidas na memória de medo são divididas nas porções dorsal e ventral do córtex pré-frontal medial de roedores. As quatro principais subdivisões do córtex pré-frontal medial relacionados com a memória aversiva são, na região dorsal o pré-límbico (PL) que promove a expressão do medo condicionado. Outra importante região dorsal é o córtex cingulado anterior dorsal (ACd), que também pode promover o medo. Já o córtex do infra-límbico (IL), localiza-se mais ventral que o PL, e sua função está relacionada com o fortalecimento da memória de extinção, ou seja, inibe a expressão do medo. Da mesma forma o córtex dorso-peduncular (DP), localiza-se mais ventral que o IL e também está relacionada com a inibição do medo. Assim, as regiões dorsais do córtex pré-frontal medial aumentam a expressão do medo, enquanto as regiões ventrais exercem o efeito oposto sobre o comportamento, diminuindo a expressão do medo (Milad & Quirk, 2012; Peters et al., 2010; Quirk & Mueller, 2008; Sierra-Mercado et al., 2011).

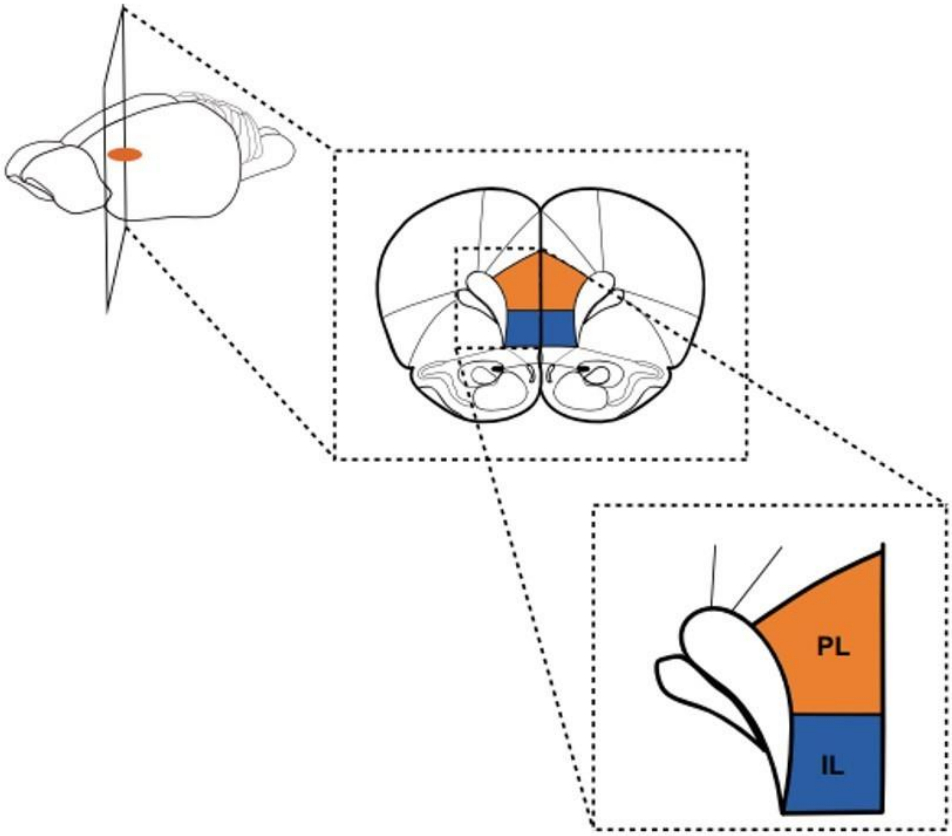


**Figura 3.** Desenho esquemático do córtex pré-frontal ventromedial de ratos *Wistar* em corte coronal. Representação das subdivisões de interesse para a memória, pré-límbico (PL) e o infralímbico (IL) (Izquierdo et al., 2016).

*1.2.4 Tálamo*

O tálamo é uma estrutura bilateral e simétrica que compreende a maior parte do diencéfalo, sendo o tálamo medial limitado pelo terceiro ventrículo. O tálamo é classicamente dividido em vários núcleos, descritos por sua localização anatômica: medial, lateral, ventral e anterior, bem como os núcleos posteriores (Wiegell et al., 2003). Em roedores, essa estrutura encefálica compreende cerca de 60 núcleos e está intimamente relacionado com a modulação das informações que recebe do meio e que serão transmitidas as outras regiões encefálicas (Cassel et al., 2021).

Desta forma, os principais núcleos talâmicos envolvidos com os processos mnemônicos são os núcleos intralaminares (ILN), as subdivisões dorsal e ventral da linha

média, que incluem os núcleos reuniens (Re) e rombóides (Rh), (Pereira de Vasconcelos & Cassel, 2015; Vertes et al., 2015). Sabe-se que os núcleos talâmicos participam da modulação das informações entre o córtex pré-frontal medial (ACC, IL, PL) e o hipocampo (CA1) (Hoover & Vertes, 2012; Varela et al., 2014). Destacam por serem estruturas-chave na consolidação de memórias espaciais e contextuais, bem como no processo de flexibilidade cognitiva (Loureiro et al., 2012; Quet et al., 2020; Pereira de Vasconcelos & Cassel, 2015; Vertes et al., 2015).

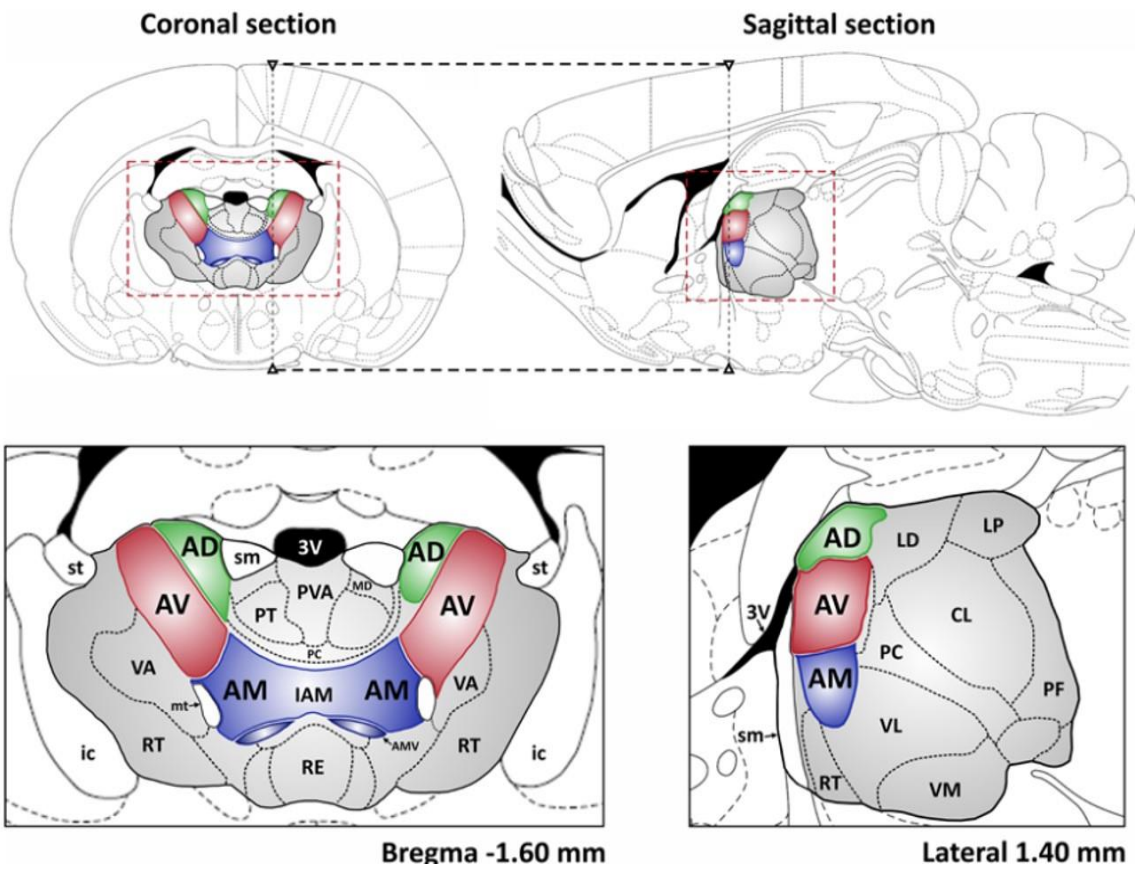


**Figura 4.** Desenho esquemático do tálamo de ratos *Wistar* em seções coronais e sagitais. Representação das estruturas de interesse para a memória, 3V, 3º ventrículo; AD, núcleo talâmico anterodorsal; AV, núcleo talâmico anteroventral; AM, núcleo talâmico anteromedial; AMV, núcleo talâmico anteromedial, parte ventral; CL, núcleo talâmico centrolateral; IAM, núcleo talâmico interanteromedial; ic, cápsula interna; LD, núcleo talâmico laterodorsal; LP, núcleo talâmico posterior lateral; MD, núcleo talâmico mediodorsal; mt, trato mamilotalâmico; PC, núcleo paracentral do tálamo; PF, núcleo parafascicular do tálamo; PT, núcleo talâmico paratenial; PVA, núcleo paraventricular

talâmico, parte anterior; RE, reúne o núcleo talâmico; RT, núcleo reticular talâmico; sm, estria medular do tálamo; st, estria terminal; VA, núcleo talâmico ventral anterior; VL, núcleo talâmico ventrolateral; VM, núcleo talâmico ventromedial (adaptado de Jankowski 2013).

## 1.3 MARCADORES MOLECULARES DA MEMÓRIA

### *1.3.1 Mecanismos moleculares de desestabilização e reestabilização da memória*

A plasticidade sináptica é a base das funções cognitivas e um correlato direto da memória, principalmente em razão da modulação na força das sinapses (Dudai, 2004). O hipocampo foi uma das primeiras estruturas encefálicas de mamíferos ao ser demonstrado as duas formas de plasticidade sináptica, a potenciação de longa duração (LTP, do inglês *Long Term Potentiation*) e a depressão de longa duração (LTD, do inglês *Long Term Depression*). Posteriormente foi apontado que ambas as formas de plasticidade sináptica estão intimamente relacionadas com a formação, persistência, manutenção e flexibilidade da memória (Fanselow & Poulos, 2005; Ledoux, 2000; G. Lynch et al., 2007; M. A. Lynch, 2004; Nabavi et al., 2014; Whitlock et al., 2006).

A indução da LTP desencadeia várias cascatas celulares e moleculares que podem ocasionar no aumento da densidade e volume dos espinhos dendríticos, bem como dos receptores ionotrópicos glutamatérgicos (Bosch et al., 2014; Kasai et al., 2010; Matsuzaki et al., 2004). Já a LTD resulta na diminuição do número desses receptores e, possivelmente, uma redução dos espinhos dendríticos (Herry et al., 2010; Mulkey & Malenka, 1992). Os espinhos dendríticos são regiões que sustentam a plasticidade e a manutenção da LTP e LTD no sistema nervoso de mamíferos, ou seja, são os *hot spots* da plasticidade (Frank et al., 2018; Maiti et al., 2015).

Sabe-se que a reativação pode acarretar a desestabilização do traço mnemônico, ou seja, um estado lábil que permite modificá-la, e consequentemente após esse período, ocorrerá a fase de reestabilização, necessária para que a memória seja atualizada e armazenada. Esses processos constituem a reconsolidação e requerem inúmeras cascatas celulares e moleculares (Kida, 2019; Nader et al., 2000).

Desta forma, compreende-se que existe uma forte correlação dos processos de desestabilização e reestabilização com a plasticidade sináptica, morfofisiologia dos espinhos dendríticos e a dinâmica do cálcio ($Ca^{2+}$). Tendo em vista que esse íon está envolvido com a ativação de inúmeras cascatas celulares e moleculares no encéfalo, ou seja, é um íon fundamental à vida. Bem como, está bem estabelecido na literatura a sua participação nos processos sinápticos que parecem ser a base da memória (Baker et al., 2013; Cavazzini et al., 2005). Por ser um íon tão crucial para inúmeros processos celulares e moleculares, a sua concentração intracelular é finamente regulada, destacando-se o papel dos receptores inositol 1,4,5-trifosfato (IP3R), um dos principais mecanismos de controle do estoque intracelular nos neurônios (Baker et al., 2013; Brini et al., 2014; Nakata & Nakamura, 2007; Raymond & Redman, 2006) . Enquanto que as principais formas de entrada de cálcio nos neurônios excitatórios são através dos canais de cálcio dependentes de voltagem (Moore & Murphy, 2020) e dos receptores glutamatérgicos (Henley & Wilkinson, 2016; Strong et al., 2014).

Existe uma organela que se destaca pela sua atuação no controle da dinâmica intracelular do $Ca^{2+}$, o retículo endoplasmático liso. Este pode se distender desde o corpo do neurônio até os espinhos dendríticos e manter a concentração basal desse íon no citoplasma extremamente baixa, exercendo, portanto, um controle das cascatas moleculares dependentes de $Ca^{2+}$ (Brini et al., 2014; Meldolesi, 2001). Desta forma, os canais IP3 abrem com a combinação da ligação do IP3, um fosfolipídio de membrana que passa a atuar como segundo mensageiro após a hidrólise do fosfatidilinositol bifosfato (PIP2) pela proteína fosfolipase C (PLC) e em resposta ao $Ca^{2+}$ (Foskett et al., 2007). Os receptores IP3 possuem três isoformas (IP3R1-3), sendo que o IP3R1 é a predominante no encéfalo, estando altamente concentrados nos espinhos dendríticos e no corpo dos neurônios (Sharp et al., 1993). Recentemente foi descoberto que esses receptores estão intrinsecamente relacionados com os períodos plásticos da memória de medo (Fernandes et al., 2022).

Já o fluxo de $Ca^{2+}$ oriundo do meio extracelular é determinado principalmente pelos canais de cálcio dependentes de voltagem (VGCCs), sendo amplamente expressos no encéfalo. Assim, nos neurônios a sua concentração intracelular está na faixa de nanomolar, enquanto a concentração extracelular está na faixa milimolar, uma diferença de mil vezes ou mais, causando uma enorme força de influxo. Este gradiente de

concentração abrupto garante um mecanismo de sinalização rápido e robusto para as inúmeras funções neuronais (Clapham, 2007).

Existem basicamente três tipos dos VGCCs, sendo que a sua nomenclatura está baseada nas suas correntes subjacentes, logo, a corrente “tipo L” é classicamente descrita como sendo grande e de longa duração, enquanto a corrente “tipo T” é minúscula e transitória, e o “tipo N” encontra-se no meio, não sendo nem L nem T (Tsien et al., 1988). Para os processos subjacentes da memória, são os canais do tipo L que se destacam, inclusive desempenham um papel crucial para sustentar a reconsolidação e extinção da memória de medo (Crestani et al., 2015; Popik et al., 2020; Suzuki et al., 2004, 2008).

Outra fonte de cálcio são os receptores ionotrópicos glutamatérgicos, inclusive também constitui o principal sistema de neurotransmissores excitatório do encéfalo. Destacando-se os receptores pós-sinápticos de N-metil-D-aspartato (NMDA) e os receptores alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiónico (AMPA). Os receptores de NMDA são uma subclasse da família de receptores de glutamato ionotrópicos e desempenham funções cruciais na plasticidade sináptica, na memória e no desenvolvimento neuronal (Wyllie et al., 2013). Esses canais são altamente permeáveis ao cálcio e são geralmente compostos por dois tipos de subunidades: o receptor de NMDA 1 da subunidade de ligação à glicina (GluN1) e as subunidades de ligação ao glutamato GluN2A-D (Laube et al., 1997). Podem apresentar diferentes combinações que consequentemente apresentam propriedades distintas nas regiões expressas (Lynch & Guttmann, 2001).

Inúmeros trabalhos apontam que as subunidades GluN2A e GluN2B atuam ativamente nos mecanismos que suportam uma memória, entretanto diferem significativamente entre si, em sua relação com a dinâmica de abertura e condutância do canal, como o tempo de permanência na membrana, pois o GluN2B é mais instável e transitório na membrana plasmática, desta forma as subunidades GluN2A estão intimamente relacionadas com a manutenção e estabilidade do traço mnemônico (Lau & Zukin, 2007; Paoletti et al., 2013; Wang et al., 2009; Yashiro & Philpot, 2008). Indicando assim, que os receptores GluN2B seriam marcadores moleculares de plasticidade no traço da memória, como o processo de desestabilização.

Já os receptores AMPA são conhecidos pela transmissão excitatória rápida no encéfalo de mamíferos (Verdoorn et al., 1991) e também sustentam os mecanismos

moleculares da memória (Henley & Wilkinson, 2016; Hong et al., 2013). Sua conformação estrutural é dada por ser um canal iônico tetramérico que contém dímeros de quatro subunidades (GluA1-4), sendo que, predominam os heterotetrâmeros compostos por dímeros GluA1 e GluA2 (K. F. H. Lee et al., 2012). A maioria dos AMPARs no encéfalo de mamíferos contém a subunidade GluA2 editada e são permeáveis ao sódio e ao potássio, mas são impermeáveis ao cálcio (CI-AMPARs; do inglês *calcium impermeable*). No entanto, existe um subtipo AMPAR menos comum sem a subunidade GluA2 ou contendo GluA2 não editada, que exibe uma alta permeabilidade ao $Ca^{2+}$ (CP-AMPAR; do inglês *calcium permeable*) (Wright & Vissel, 2012; Yelshanskaya et al., 2016). Entretanto, os CP-AMPARs são recrutados principalmente em momentos de alta plasticidade sináptica e ficam transitoriamente na membrana (Clem & Huganir, 2010; Hong et al., 2013; Plant et al., 2006). Assim, as diferentes formas de composição dos receptores AMPA estão presentes desde os processos de consolidação até a flexibilização da memória.

Uma proteína ativada pelo cálcio e que pode regular a entrada desse íon nos neurônios é denominada de calpaína. Curiosamente, os canais de cálcio dependente de voltagem do tipo L e os receptores NMDA e AMPA são substratos dessa protease, bem como o remodelamento do citoesqueleto que mantem a morfologia os espinhos dendríticos (Lu et al., 2000; Simpkins et al., 2003; Wu et al., 2004). As calpaínas atuam em inúmeras cascatas de sinalização intracelular mediadas por $Ca^{2+}$, existem duas isoformas dessa protease, μ- e m-calpaína (também designadas por calpaína-1 e calpaína-2, respectivamente), que diferem na sua sensibilidade ao cálcio para serem ativadas (Baudry & Bi, 2013, 2016). As designações μ- e m- são as abreviações de micromolar e milimolar cálcio necessário para a sua ativação, respectivamente. Ambas as isoformas desempenham um papel direto ou indiretamente nos mecanismos da consolidação, evocação e flexibilização de memórias e medo (Nagayoshi et al., 2017; Popik et al., 2018).

Além dos mecanismos citados anteriormente, existe outro sistema que se destaca pela sua atuação nos processos mnemônicos e principalmente na reconsolidação, é o sistema endocanabinóide, um neurotransmissor. Sendo caracterizado por ser um sistema complexo devido à alta mixórdia de mediadores, sobreposição com outras vias e processos metabólicos alternativos. Sabe-se que no encéfalo existe uma alta densidade dos receptores endocanabinóide 1 (CB1) e 2 (CB2), ambos receptores são acoplados à

proteína G (GPCR) que são amplamente expressos em diferentes estruturas encefálicas (Castillo et al., 2012). Em razão desse sistema complexo, é comum encontrarmos resultados antagônicos na literatura.

A modulação do sistema endocanabinóide pode contribuir para os tratamentos de transtornos psiquiátricos usando terapias baseadas na reconsolidação. Entretanto, há inúmeras evidências de dados conflitantes de como o sistema endocanabinóides modula o processo de reconsolidação, pois a atuação dos receptores endocanabinóides pode tanto favorecer quanto prejudicar a flexibilização da memória de medo (Bucherelli et al., 2006; de Oliveira Alvares et al., 2008; Lin et al., 2006; Suzuki et al., 2004, 2008).

Portanto, podemos citar alguns mecanismos moleculares responsáveis por permitir a desestabilização do traço de uma memória (mecanismo de plasticidade). Destacando para os receptores extra e intracelulares de cálcio, os receptores NMDA-GluN2B, CP-AMPA, a atividade das calpaínas e dos receptores endocanabinóides – destaque para os CB1 – (De Carvalho et al., 2014; Fernandes et al., 2022; Kida, 2019; Kim et al., 2011; Mamou et al., 2006; Merlo et al., 2015; Milton et al., 2013; Nagayoshi et al., 2017; Popik et al., 2018; Suzuki et al., 2008, 2004).

Para que a memória seja reconsolidada, é necessário a fase de reestabilização do traço mnemônico, processo pelo qual a memória desestabilizada volta a ser armazenada de forma estável após incorporar as informações atualizadas. Inúmeros estudos demonstram que o principal mecanismo molecular envolvido com a reestabilização é a síntese de proteínas, mas infelizmente existe uma carência de informações em relação aos eventos moleculares envolvidos nesse processo (Bozon et al., 2003; Duvarci et al., 2005, 2008; Gruest et al., 2004; Jiang & Schuman, 2002; J. L. C. Lee, 2008; Nader et al., 2000; Rossato et al., 2006; Takei et al., 2004).

## 2. HIPÓTESE

Há dois mecanismos clássicos que podem atenuar a expressão de uma memória de medo, a reconsolidação e a extinção. A reconsolidação é um fenômeno que permite a atualização do traço mnemônico, ou seja, o conjunto de neurônios que sustentam a memória pode ser atualizado e consequentemente pode incorporar ou retirar informações, já a extinção, é caracterizada pela formação de uma nova memória que suprime a memória original de medo, portanto, essa nova memória é mais frágil e transitória. Levando em consideração as características intrínsecas de cada uma, os mecanismos subjacentes da reconsolidação se destacam por serem um alvo perfeito para atenuar uma memória traumática. Sendo assim, levantamos a hipótese que os choques fracos, pouco aversivo à quase neutro, aplicados nas sessões de reativação funcionam como gatilho e novidade para desestabilizar o traço mnemônico, consequentemente reescrever o seu conteúdo e a sua força. Chamamos essa estratégia de descondicionamento, que consiste em atenuar de forma eficaz e duradoura uma memória do medo.

# 3. OBJETIVOS GERAIS

Avaliamos se o protocolo de descondicionamento consegue atenuar uma memória de medo em diferentes condições experimentais, bem como identificamos os principais mecanismos moleculares que sustentam o nosso protocolo.

## 3.1 OBJETIVOS ESPECÍFICOS

1. Caracterizar o protocolo de descondicionamento em diferentes tarefas comportamentais (condicionamento aversivo ao contexto – CAC –, ao tom – CAT – e *step-through)* em ratos *Wistar* machos e fêmeas;
2. Distinguir a nível comportamental os processos de reconsolidação e extinção no protocolo de descondicionamento (através *renewal*, recuperação espontânea, retreino, *reinstatement* e sessões de extinção) no CAT em ratos *Wistar* machos;
3. Avaliar se o protocolo de descondicionamento atenua a memória de medo sob as condições limitantes da reconsolidação (memória remota e treino forte) no CAT em ratos *Wistar* machos;
4. Avaliar se a inibição dos canais de cálcio voltagem dependentes do tipo L bloqueia os efeitos do descondicionamento no CAT em ratos *Wistar* machos;
5. Avaliar se a inibição dos canais intracelulares de cálcio, IP3 bloqueia os efeitos do descondicionamento no CAT em ratos *Wistar* machos;
6. Avaliar se a inibição dos receptores glutamatérgicos, CP-AMPA e NMDA-GluN2B, prejudica os efeitos do descondicionamento no CAT em ratos *Wistar* machos;
7. Verificar se a inibição dos receptores endocanabinóide - CB1 prejudica os efeitos do descondicionamento no CAT em ratos *Wistar* machos;
8. Avaliar se a inibição da atividade das calpaínas prejudica os efeitos do descondicionamento no CAT em ratos *Wistar* machos.

## COLETÂNEA DE ARTIGOS

Segue os dados produzidos durante o meu doutorado, na seguinte ordem:

1 - Artigo publicado referente ao descondicionamento;

*Shifting from fear to safety through deconditioning-update*

2 - Artigo publicado referente ao descondicionamento;

*Characterization of deconditioning-update on fear memory attenuation*

3 - Artigo publicado referente ao descondicionamento;

*Molecular mechanisms underpinning deconditioning-update in fear memory*

Anexos:

1 – Resultados obtidos referente ao projeto de amnésia infantil – dados que serão publicados;

2 – Outras ideias.

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RESEARCH ARTICLE

# Shifting from fear to safety through deconditioning-update

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**Abstract** Aversive memories are at the heart of psychiatric disorders such as phobias and post-traumatic stress disorder (PTSD). Here, we present a new behavioral approach in rats that robustly attenuates aversive memories. This method consists of 'deconditioning' animals previously trained to associate a tone with a strong footshock by replacing it with a much weaker one during memory retrieval. Our results indicate that deconditioning-update is more effective than traditional extinction in reducing fear responses; moreover, such effects are long lasting and resistant to renewal and spontaneous recovery. Remarkably, this strategy overcame important boundary conditions for memory updating, such as remote or very strong traumatic memories. The same beneficial effect was found in other types of fear-related memories. Deconditioning was mediated by L-type voltage-gated calcium channels and is consistent with computational accounts of mismatch-induced memory updating. Our results suggest that shifting from fear to safety through deconditioning-update is a promising approach to attenuate traumatic memories.

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## Introduction

Memory is a dynamic process that allows for adaptation to the demands of a continuously changing environment. The ability to update old memories in accordance with new experiences is crucial for maintaining their relevance over time. Particularly, it has been shown that after retrieval (or reactivation), memories may undergo a cycle of labilization and restabilization commonly known as reconsolidation (***Nader et al., 2000***). The labile state induced by this process can thus allow changes in memory strength and/or content (***De Oliveira Alvares et al., 2013***). This has been most extensively studied in aversive conditioning paradigms in rodents and humans and is of potential relevance to the management of psychiatric disorders involving dysfunctional memories (***Monfils and Holmes, 2018***).

Repeated exposure to a conditioned stimulus (CS) in the absence of an aversive unconditioned stimulus (US) also leads to a progressive reduction in fear responses, commonly known as extinction. However, extinction is thought not to erase the original memory; instead, it induces new learning that transiently inhibits fear expression (***Bouton, 2002***). Therefore, the fear memory typically re-emerges with the passage of time (spontaneous recovery), exposure to the US (reinstatement), or when the CS is presented independently of the extinction context (renewal) (***Rescorla and Heth, 1975***; ***Archbold et al., 2010***; ***Bouton et al., 2012***). Thus, behavioral strategies that can weaken traumatic memories and reduce memory recovery can be relevant for improving the effectiveness of extinction.

Reconsolidation has been described in several experimental paradigms and species, from invertebrates to humans, suggesting that it might be a fundamental property of memories (***Nader and Einarsson, 2010***). Beyond its biological role in memory updating, it opens a window of opportunity

as a potential therapeutic strategy to modify pathological memories. Several studies in the last decades have attempted to pharmacologically or behaviorally disrupt the reconsolidation of traumatic memories (**Beckers and Kindt, 2017**), as at least in some situations, this strategy can be less susceptible to spontaneous or induced recovery than traditional extinction (**Duvarci and Nader, 2004**; **Monfils et al., 2009**). However, most treatments that interfere with memory reconsolidation are toxic and cannot be readily administered to humans. Thus, in spite of almost two decades of research on memory reconsolidation, the evidence for the efficacy of reconsolidation-blocking treatments in clinical settings is still limited (**Monfils and Holmes, 2018**).

Research on reconsolidation-extinction boundaries suggests that the transition from one process to the other depends on the degree of mismatch between the original memory and the reactivation experience. Many studies have suggested that some degree of mismatch or prediction error is necessary for reconsolidation to occur (**Lee, 2009**; **Fernández et al., 2016**); however, if prediction error is too high, extinction may occur instead (**Suzuki et al., 2004**; **Sevenster et al., 2014**). Computational models suggest that, in models in which prediction error is low, memory updating/reconsolidation mechanisms are preferentially engaged, as the new experience is recognized as a new instance of the former one; however, as mismatch increases, the chances of it being attributed to a new latent cause (**Gershman et al., 2017**) or forming a new attractor in a neural network (**Osan et al., 2011**) increases.

This framework suggests that lowering the degree of mismatch between learning and reexposure might conceivably potentiate memory updating during extinction. This has been the rationale behind so-called retrieval-extinction procedures (**Monfils et al., 2009**; **Kredlow et al., 2016**) and has also been explored in short-term extinction protocols (**Gershman et al., 2013**). In this work, we propose a novel approach for long-term attenuation of traumatic memories that we term 'deconditioning-update'. This strategy consists in weakening fear memories by updating the aversive information, substituting the original US by a mildly aversive stimulus during the plastic state induced by reactivation.

## Results

In order to approach our hypothesis, male Wistar rats were trained in auditory fear conditioning, where they received five conditioning trial tones (CS) that co-terminated with a 0.5-mA, 1-s footshock (US). On days 3 to 6 (reactivation), animals received 3 CSs during a 400-s daily session in a different context. In the no-footshock group, CSs were presented in the absence of shock, while in the deconditioning-update group, each tone co-terminated with a 0.1-mA footshock. A third group remained in the homecage (control group). On day 7, both groups were tested in the reactivation context with 3 CSs. On day 8, animals were tested in the training context for renewal, and, on day 28, they were retested for spontaneous recovery (**Figure 1A**).

Over the course of reactivation sessions, both groups presented a decrease in freezing, but this was more marked in the deconditioning-update group (**Figure 1B**), which presented a decrease in freezing of around 70% in comparison to the no-footshock group and 80% in comparison to the homecage control group in the test session (**Figure 1C**). Animals in the deconditioning-update group also had lower freezing responses in the renewal (**Figure 1D**) and spontaneous recovery (**Figure 1E**) tests, although it should be noted that memory recovery was not observed in the no-footshock group either, perhaps due to a ceiling effect caused by insufficient extinction (for complete statistics, see **Supplementary file 1**). Pre-CS freezing varied between 37% and 69% in both groups throughout the extinction sessions (**Supplementary file 12**), suggesting some degree of generalization between both contexts.

When performing the same experiment, but with each tone co-terminating with a 0.3-mA footshock in the reactivations, no fear reduction occurred (**Figure 1—figure supplement 1**); on the contrary, the footshock group presented higher levels of freezing than the no-footshock group throughout the reactivation sessions, as well as in the test. This result is in accordance with a recent study showing that fear memory may be strengthened by reactivation with a 0.3-mA footshock (**Ferrara et al., 2019a**). It also rules out that the suppression of freezing during reactivations by deconditioning-update may be due to inhibition of delay (i.e. animals learning to freeze only at the end of the CS with extended practice). When using a single reactivation session with 0.1mA, no

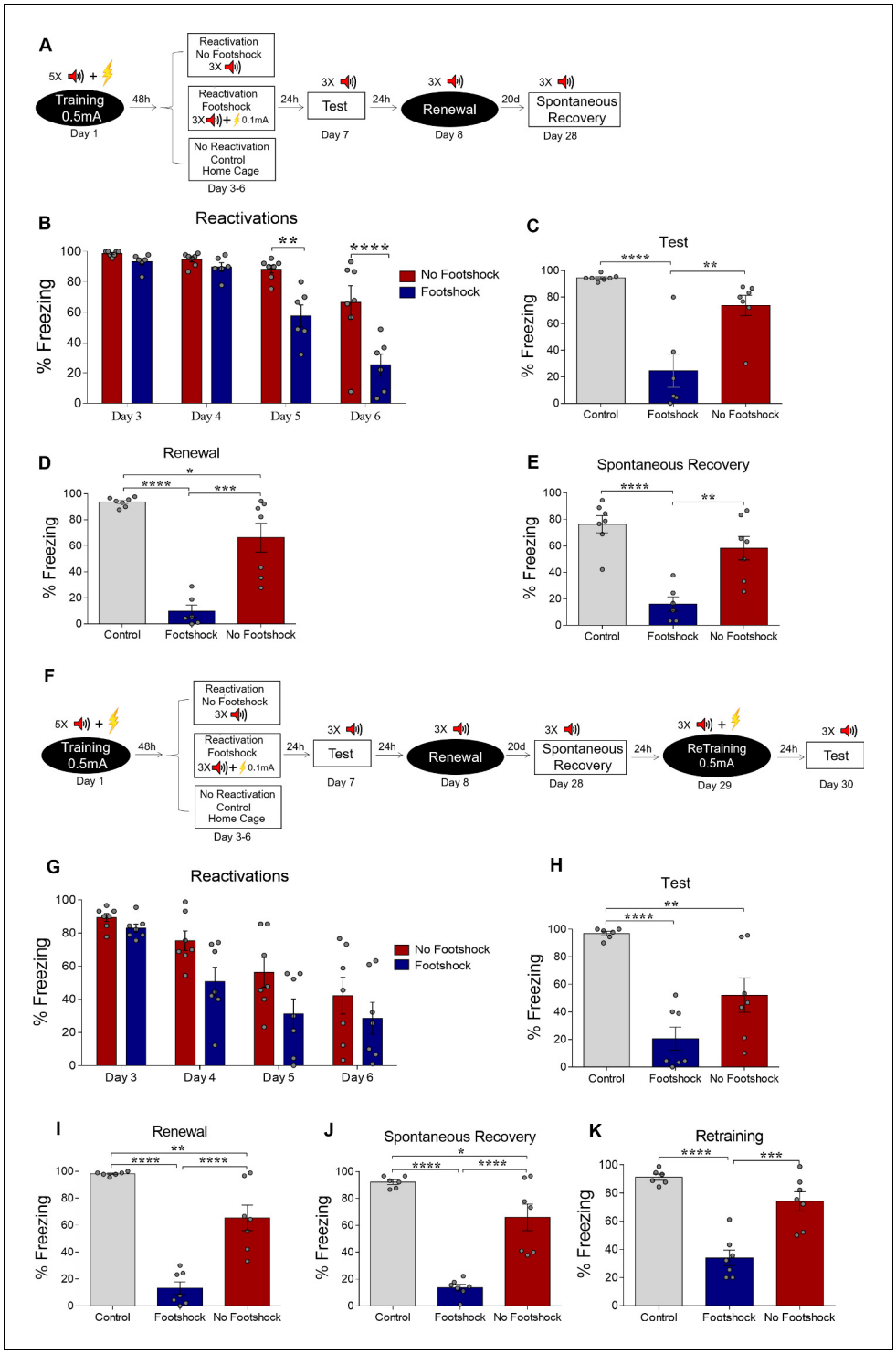


**Figure 1.** Weakening fear memory through deconditioning-update training. (**A**) Experimental design: male rats were fear-conditioned with five tone-shock pairings (context A; 5 CS + US, 0.5mA). 48 hr later, the no-footshock and footshock (deconditioning-update) groups were exposed to four daily reactivation sessions (context B). After this, animals underwent test (context B), renewal (context A) and spontaneous recovery (context B) sessions. Black circles represent context A, while white rectangles represent context B. (**B**) Freezing levels during reactivation sessions. Rats exposed to weak footshocks during reactivation sessions showed a significant reduction in freezing responses, maintained during the test (**C**), renewal (**E**) and spontaneous recovery (**D**) sessions. (**F**) Experimental design: female rats were fear-conditioned (context A; 5CS+US, 0.5mA). 48 hr later, the no-footshock and footshock groups were exposed to four daily reactivation sessions (context B). After this, all groups underwent test, renewal,

*Figure 1 continued on next page*

*Figure 1 continued*

and spontaneous recovery sessions. Animals were reconditioned (context A; 3CS+US, 0.5mA) on the next day and retested 24 hr later. (**G**) Freezing levels during memory reactivation. Rats exposed to weak footshocks showed a significant reduction in freezing responses, maintained during the test (**H**), renewal (**I**), spontaneous recovery (**J**) and retraining test (**K**) sessions. Bars represent mean ± SEM. Statistical comparisons were performed using two-way repeated-measures ANOVA followed by a Bonferroni post-hoc (reactivation sessions) or one-way ANOVA followed by Tukey post-hoc (test, renewal, spontaneous recovery, and retraining test). *p<0.05; **p<0.005; ***p<0.0005; ****p<0.0001. For full statistics, see ***Supplementary file 1***. For pre-CS freezing values, see ***Supplementary file 12***.

The online version of this article includes the following source data and figure supplement(s) for figure 1:

**Source data 1.** Raw data of ***Figure 1***.
**Figure supplement 1.** Deconditioning-update does not occur with 0.3-mA shocks.
**Figure supplement 2.** A single reactivation session does not update fear memory.
**Figure supplement 3.** Deconditioning-update is not due to US devaluation.
**Figure supplement 4.** Deconditioning-update does not occur with unpaired shocks in the reactivation sessions.

difference was found in the test in comparison to the no-footshock group (***Figure 1—figure supplement 2***), suggesting that deconditioning-update requires several sessions to take place.

One could argue that the exposure to weak footshocks could simply lead to habituation and consequent devaluation of the US, without altering the conditioned association itself (***Rescorla, 1973***; ***Rescorla and Heth, 1975***). In order to test this alternative interpretation, rats were conditioned as described above and the same 3 0.1-mA weak footshocks were given in another context without being paired with the tones (***Figure 1—figure supplement 3A***). In this case, no fear reduction was found in comparison to homecage controls. (***Figure 1—figure supplement 3B***). To further rule out the devaluation hypothesis, another set of animals was submitted to reinstatement after deconditioning, in order to test whether restoring the original footshock valence outside of the extinction context might lead to memory recovery. We found that the deconditioning group expressed a lower fear level when compared with the no-footshock group in the test even after reinstatement (***Figure 1—figure supplement 3E***), suggesting that the deconditioning-update effect is not due to US devaluation, but rather to updating of the CS-US association.

In an additional experiment, we investigated whether the stress induced by weak footshocks during reactivation could be enhancing extinction by itself. Animals underwent either the deconditioning-update protocol described in ***Figure 1A***, or a similar protocol in which both tones and footshocks were applied during the reactivation sessions, but with no pairing between them. We found that this non-contingent tone-footshock protocol did not attenuate fear expression, leading to higher freezing than deconditioning-update during reactivations and in the test (***Figure 1—figure supplement 4***).

Next, we asked whether the deconditioning-update effect would also be efficient in reducing fear in female rats. As observed in males, females from the deconditioning-update group showed lower freezing levels throughout the reactivation sessions, as well as in the test session, when compared with the control group (***Figure 1H***). The same pattern was maintained in the renewal and spontaneous recovery tests (***Figure 1I and J***, respectively). As a further way to test for persistence of the original memory, we performed a retraining session in the original context with 3 0.5-mA CS-US pairings 24 hr after the spontaneous recovery test to assess savings. The deconditioning-update group had lower freezing compared with the other groups in a subsequent test session in the extinction context, suggesting that our protocol also lowers the rate of reacquisition of an aversive memory (***Figure 1K***).

Boundary conditions such as training intensity and memory age have been reported to prevent fear memories from being modified. Protocols with high training intensity make memory less prone to interference by pharmacological agents in the reconsolidation window (***Frankland et al., 2006***; ***Suzuki et al., 2004***; ***Wang et al., 2009***), while older memories are also less susceptible to modification than recent ones (***Milekic and Alberini, 2002***; ***Frankland et al., 2006***; ***Suzuki et al., 2004***; ***Bustos et al., 2009***). Thus, our next experiments investigated whether deconditioning-update could attenuate fear expression in these cases. First, we trained the animals in the same protocol described above, but starting reactivations 40 days after conditioning (***Figure 2A***). Once more, the

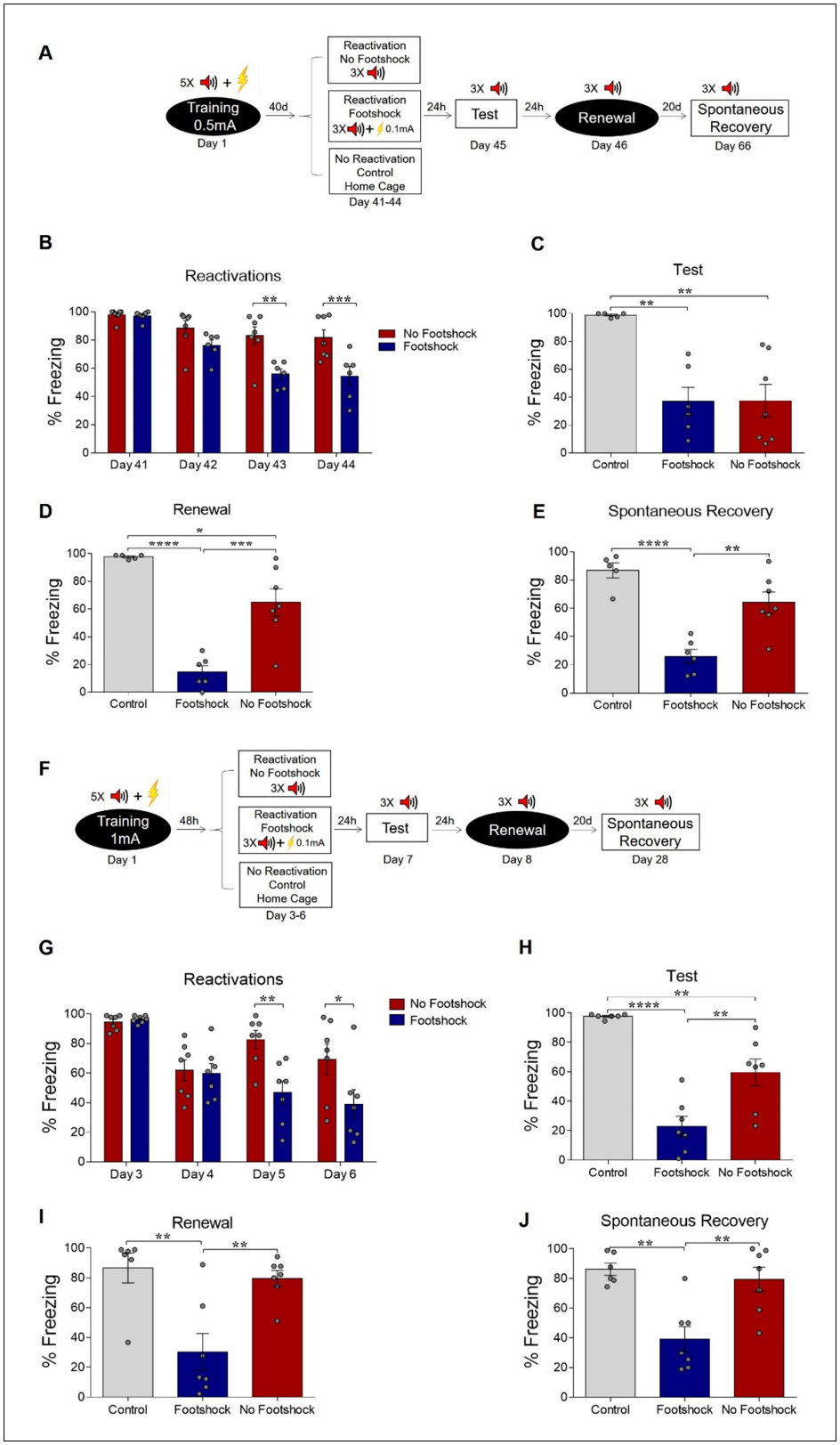


**Figure 2.** Deconditioning-update weakens both remote and strong fear memories. (**A**) Experimental design for remote memory: rats were fear-conditioned with five tone-shock pairings (context A; 5 CS + US, 0.5mA). Starting 40 days later, the no-footshock and footshock (deconditioning-update) groups were exposed to daily reactivation sessions (context B). Then, all groups underwent test (context B), renewal (context A) and spontaneous recovery

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*Figure 2 continued*

(context B) sessions. Black circles represent context A, while white rectangles represent context B. (**B**) Freezing levels during reactivation sessions. Rats exposed to weak footshocks during reactivation sessions showed similar freezing levels to no-footshock animals during the test session (**C**) and lower freezing levels at the renewal (**D**) and spontaneous recovery (**E**) ones. (**F**) Experimental design for strong training (5CS+US, 1mA). (**G**) Freezing levels during reactivation sessions. Rats exposed to weak footshocks during reactivation sessions showed a significant reduction in freezing responses that was maintained during the test (**H**), renewal (**I**) and spontaneous recovery (**J**) sessions. Bars represent mean ± SEM. Statistical comparisons are performed using two-way repeated-measures ANOVA followed by a Bonferroni post-hoc (reactivation sessions) or one-way ANOVA followed by a Tukey post-hoc (test and spontaneous recovery). *p<0.05; **p<0.005; ***p<0.0005; ****p<0.0001. For full statistics, see ***Supplementary file 2***. For pre-CS freezing, see ***Supplementary file 13***.

The online version of this article includes the following source data and figure supplement(s) for figure 2:

**Source data 1.** Raw data of ***Figure 2***.

**Figure supplement 1.** Deconditioning-update weakens strong fear memories in females.

deconditioning-update group showed lower freezing expression throughout the reactivation sessions (***Figure 2B***). 24 hr after the last reactivation, both the footshock and no-footshock groups presented a comparable decrease in freezing behavior when compared to controls (***Figure 2C***). However, in the renewal and spontaneous recovery tests, fear expression reemerged in the no-footshock group, while the deconditioning-update group maintained its low freezing levels (***Figure 2D and E***, respectively; ***Supplementary file 2***).

Next, we tested whether a stronger fear memory would constrain the deconditioning-update effect by training animals with 5 CS-US pairings using 1-mA shocks, while maintaining the rest of the protocol unchanged. In spite of the stronger shock intensity in the training session, the deconditioning-update group still presented reduced freezing levels in reactivation sessions when compared to the no-footshock group (***Figure 2G***). These lower freezing levels were maintained in the test, renewal and spontaneous recovery sessions, in which robust recovery was observed in no-footshock animals, but not in the deconditioning-update group (***Figure 2H, I and J***, respectively; ***Supplementary file 2***). Similar results were observed in females in a slightly modified protocol with three reactivation sessions (***Figure 2—figure supplement 1***). These experiments suggest that the deconditioning-update induces a plastic state, allowing the fear memory trace to be altered even in boundary conditions that usually constrain memory updating (***Pedraza et al., 2018***).

In order to investigate whether the deconditioning-update approach is effective in attenuating other types of aversive memories, we trained animals in different fear-related tasks. First, we used a contextual fear conditioning paradigm, which is known to involve a set of brain regions that is partially distinct from auditory conditioning and includes the prefrontal cortex and hippocampus (***Maren et al., 2013***). Animals were placed in the conditioning chamber for 3 min before receiving two 0.5-mA, 2 s footshocks separated by a 30 s interval. On days 3 to 6, rats were reexposed to the same context for 4 min, with those in the deconditioning-update group receiving weak footshocks (0.1mA, 2 s) 3 min after being placed in the chamber (***Figure 3A***). The deconditioning-update group had lower fear expression during reactivations (***Figure 3B***) and maintained these lower freezing levels compared with the other groups in the test (***Figure 3C***). The same pattern was observed in the spontaneous recovery test, performed 20 days later, suggesting that the decrease in freezing caused by deconditioning-update is long-lasting (***Figure 3D***; ***Supplementary file 3***).

Another set of animals underwent the step-through inhibitory avoidance task, in which training consists of applying 4 0.5-mA, 1-s footshocks with 5-s intervals when the animal enters the dark compartment of a shuttle box. During reactivations, animals were placed in the dark compartment for 30 s, with those in the deconditioning-update group receiving 2 0.1-mA shocks. In the test session, the animals were put in the light compartment, and the latency to enter the dark compartment was measured (***Figure 3E***). The deconditioning-update group showed a much shorter latency to reach the dark chamber (***Figure 3F***) and spent more time in the dark compartment over the 10-min session compared to the other groups (***Figure 3G***; ***Supplementary file 3***), suggesting that memory was more robustly updated in this group during reactivation. Taken together, these results suggest that the deconditioning-update strategy is effective in weakening distinct types of fear-related memories.

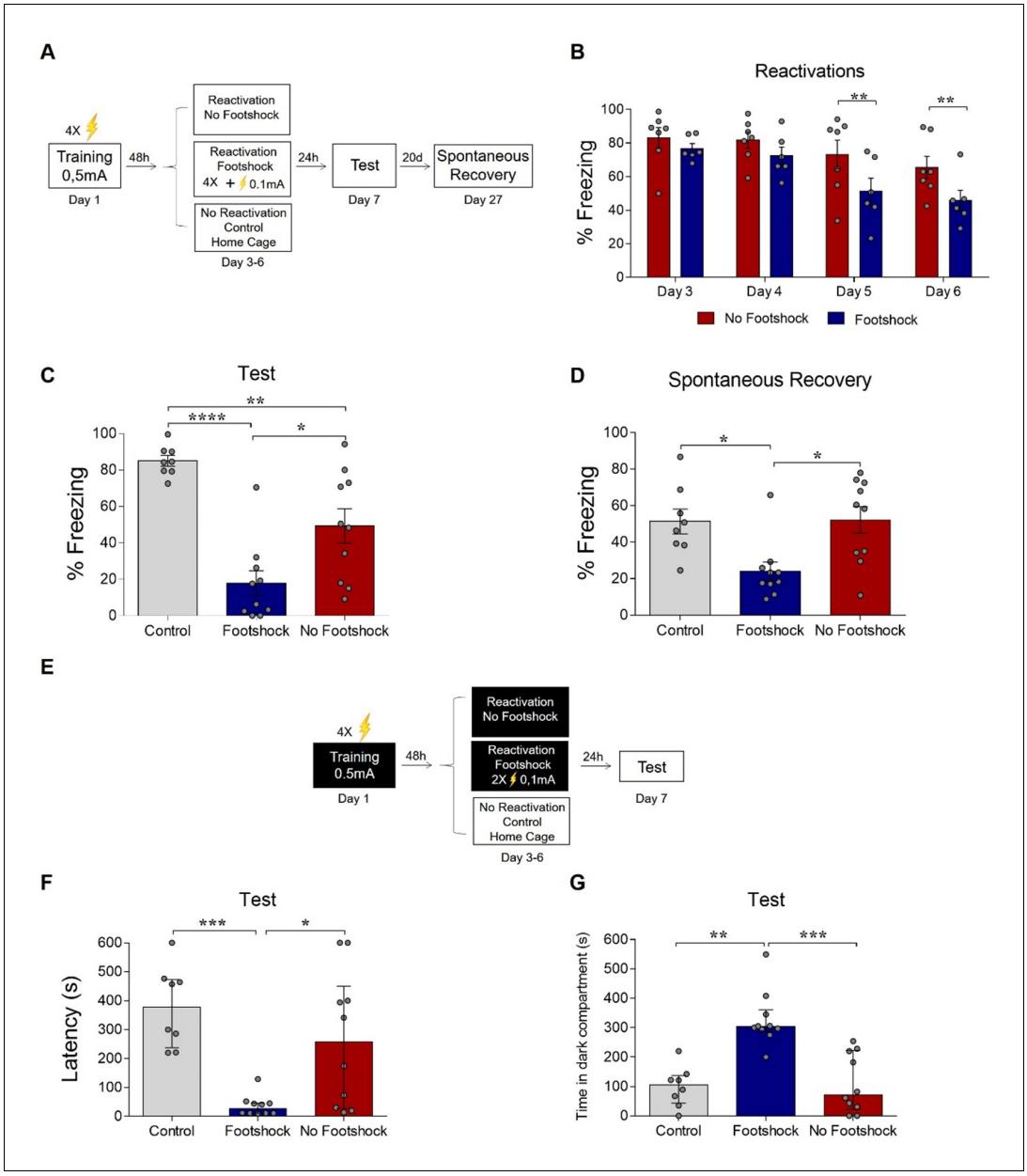


**Figure 3.** Deconditioning-update weakens fear memory in different behavioral tasks. (**A**) Experimental design in contextual fear conditioning: rats were fear-conditioned with five contextual-shock pairings (4 min context + 4 US, 0.5mA). Starting 48 hr later, the no-footshock and footshock groups were exposed to daily reactivation sessions. 24 hr after the last reactivation, all groups were tested; 20 days later, they were tested for spontaneous recovery. (**B**) Freezing levels during reactivation sessions. Rats exposed to weak footshocks during reactivation sessions showed a significant reduction in freezing responses maintained during the test (**C**) and spontaneous recovery (**D**) sessions. (**E**) Experimental design in inhibitory avoidance: rats were placed in the lighted compartment and received footshocks (4 US, 0.5mA) upon entering the dark one. Starting 48 hr later, the no-footshock and footshock groups were exposed to daily 30-s reactivation sessions in the dark compartment; 24 hr after the last reactivation, all groups were tested. Rats exposed to weak footshocks during reactivation sessions showed lower latencies to cross to the dark compartment (**F**) and spent more time in it during the test (**G**). Bars represent mean ± SEM or median with interquartile range (in F and G). Statistical comparisons for contextual fear conditioning are performed using two-way repeated-measures ANOVA followed by a Bonferroni post-hoc (reactivation sessions) or one-way ANOVA followed by a Tukey post-hoc (test, renewal, and spontaneous recovery). For inhibitory avoidance, a Kruskal-Wallis test followed by a Dunn post-hoc was performed. *p<0.05; **p<0.005; ***p<0.0005; ****p<0.0001. For full statistics, see ***Supplementary file 3***.

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To address whether the deconditioning-update effect would also be observed within a single, long-lasting extinction session, we trained animals and exposed them 48 hr later to a session containing 12 CSs, with the deconditioning-update group receiving a 0.1-mA footshock at the end of each tone. Fear reduction was limited and largely similar across groups in the extinction session and in the subsequent tests (***Figure 4—figure supplement 1***), albeit with slightly lower renewal in the deconditioning-update group, suggesting that the pairing of the CS with a weak footshock is not as effective in accelerating single-session extinction. In order to further explore this possibility, we subjected animals to a 24-tone single-session extinction protocol (***Figure 4A***). This led to robust fear reduction both within the extinction session (***Figure 4B***) and in a subsequent test (***Figure 4C***) in the no-footshock group, while freezing remained largely unchanged in the footshock group. However, fear memory reemerged in the renewal and spontaneous recovery test among no-footshock animals (***Figure 4D and E***; ***Supplementary file 4***), as typically occurs with extinction learning. These results show that the presence of a weak shock at the end of every CS not only does not enhance extinction occurring over a single behavioral session, but can actually impair it in the short-term.

Other studies have shown that activation of L-type voltage-gated $Ca^{++}$ channels (LVGCC) is necessary both for destabilizing a reactivated memory during reconsolidation (***Suzuki et al., 2008***; ***Lee and Flavell, 2014***; ***Crestani et al., 2015***; ***Haubrich et al., 2015***) and for some forms of extinction (***Cain et al., 2002***). Thus, we used the LVGCC antagonist nimodipine as a pharmacological tool to investigate whether deconditioning-update involved memory destabilization mechanisms (***Flavell et al., 2011***; ***Sierra et al., 2013***; ***Crestani et al., 2015***; ***Haubrich et al., 2015***). Animals were trained and divided into four groups: no-footshock + vehicle, no-footshock + nimodipine, deconditioning-update + vehicle and deconditioning-update + nimodipine, with nimodipine or vehicle administered systemically 30 min before each reactivation (***Figure 4F***). Nimodipine attenuated freezing decrease in both behavioral protocols, suggesting that the drug impaired both deconditioning-update and regular extinction (***Figure 4G and H***). In the renewal and spontaneous recovery sessions, high freezing levels reemerged both in the no-footshock vehicle group and in nimodipine-treated animals, while the deconditioning-update vehicle group maintained a lower freezing level (***Figure 4I and J***; ***Supplementary file 4***). This suggests that deconditioning-update is mediated by memory destabilization processes requiring $Ca^{++}$ influx through LVGCCs.

In order to address whether the effect of nimodipine treatment before reactivation sessions might be explained by state-dependency of the extinction memory in the test session, we trained a new set of animals in the same condition, except that two tests were conducted on separate days in each animal, either in the presence or absence of nimodipine treatment 30 min before. We found that animals treated with nimodipine before the test kept expressing high freezing levels, suggesting that nimodipine injection before reactivations prevented memory destabilization instead of inducing state dependency (***Figure 4—figure supplement 2***).

One explanation for our findings is that pairing the CS with a weak footshock could lead to a smaller degree of prediction error during reexposure sessions, biasing them towards memory updating as opposed to new learning. Computational models using neural networks (***Osan et al., 2011***) or Bayesian approaches (***Gershman et al., 2017***) have explored how different degrees of mismatch between stored memories and new experiences can lead to these two outcomes, suggesting lower degrees of mismatch or prediction error could lead to greater destabilization of stored memories. With this in mind, we used an adaptation of one of these models (***Osan et al., 2011***) to explore if this framework could account for our main results – that is accelerated fear reduction over multiple sessions and lower memory recovery when mismatch is reduced during reexposure. This fully connected Hopfield-like network (***Hopfield, 1984***) of 100 neurons is capable of storing patterns using Hebbian learning rules and retrieving them according to the inputs presented, which in our simulations included neurons representing tone and context information, as well as shock/non-shock information (***Figure 5A***). Additionally, the network also updates synaptic weights according to mismatch between a cue input and the retrieved network pattern (***Osan et al., 2011***).

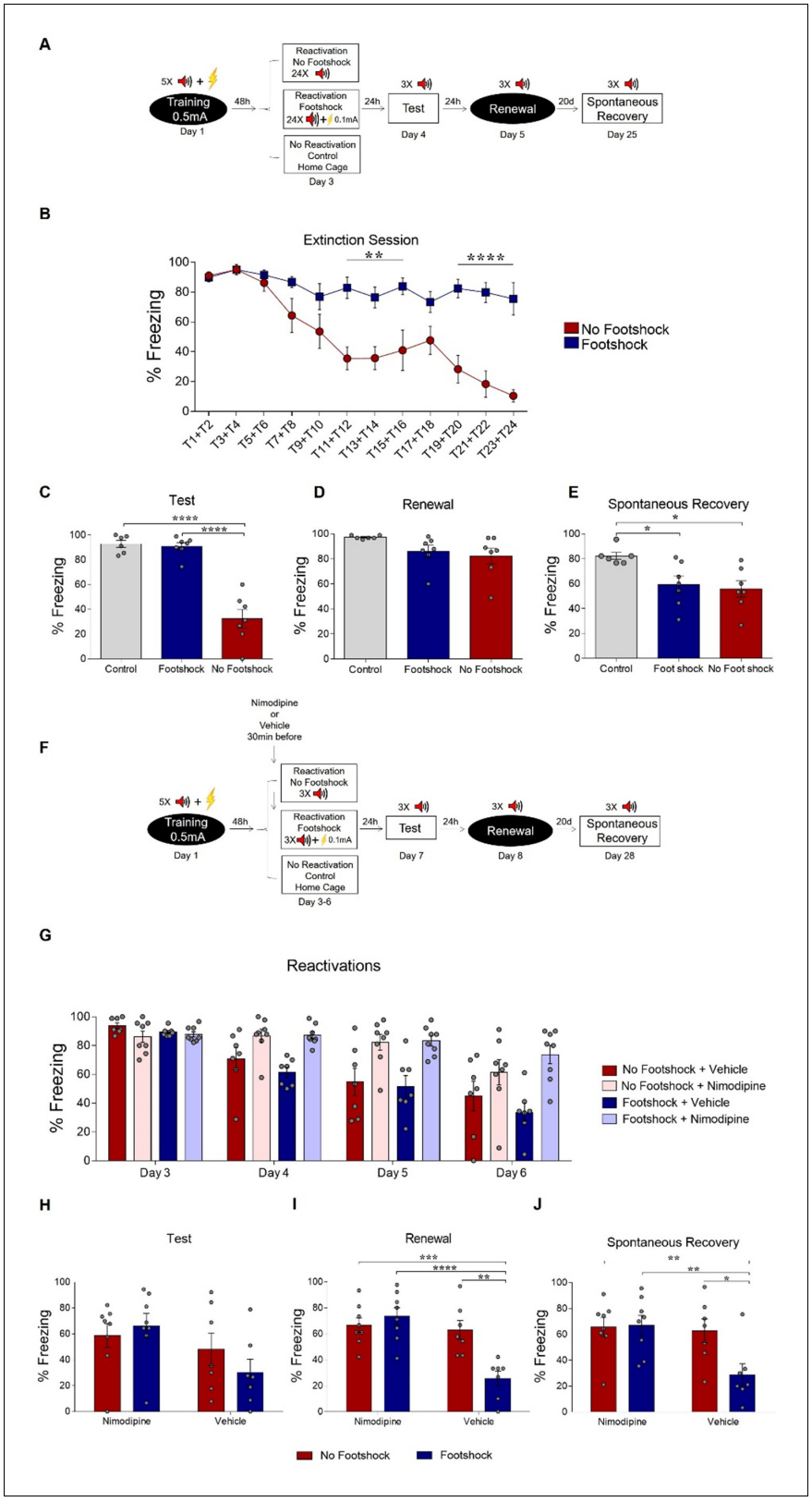


**Figure 4.** Deconditioning-update is based on memory destabilization mechanisms. (**A**) Experimental design: rats were fear-conditioned with five tone-shock pairings (context A; 5CS+US, 0.5mA). 48 hr later, the no-footshock and footshock groups underwent a single extinction session (context B, 24 CSs), followed by test (context B), renewal (context A) and spontaneous recovery (context B) sessions. (**B**) Freezing levels during extinction. Weak footshocks

*Figure 4 continued on next page*

*Figure 4 continued*

impaired extinction within the session and in the test session (**C**), but not in renewal (**D**) or spontaneous recovery (**E**). (**F**) Experimental design: rats were fear-conditioned (context A; 5CS+US, 0.5mA). 48 hr later, all animals underwent daily reactivation sessions (context B), receiving nimodipine (16 mg/kg, i.p.) or vehicle 30 min before each one. They then underwent test (context B), renewal (context A) and spontaneous recovery (context B) sessions. Nimodipine prevented freezing decrease across reactivation sessions in both groups (**G**). Freezing was similar between groups in the test session (**H**), but was lower in the vehicle-footshock group in the renewal (**I**) and spontaneous recovery (**J**) sessions. Bars represent mean ± SEM. Statistical comparisons are performed using two-way repeated-measures ANOVA followed by Bonferroni post-hoc (extinction), one-way ANOVA followed by Tukey post-hoc (test, renewal, and spontaneous recovery following extinction), three-way repeated-measures ANOVA followed by Bonferroni post-hoc (reactivation sessions with nimodipine/vehicle) and two-way ANOVA followed by Bonferroni post-hoc (test, renewal, and spontaneous recovery following nimopidine/vehicle). *p<0.05; **p<0.005; ***p<0.0005; ****p<0.0001 in between-group comparisons. For full statistics, see ***Supplementary file 4***. For pre-CS freezing, see ***Supplementary file 14***.

The online version of this article includes the following source data and figure supplement(s) for figure 4:

**Source data 1.** Raw data of ***Figure 4***.

**Figure supplement 1.** Effects of deconditioning-update in a single 12-CS extinction session.

**Figure supplement 2.** Nimodipine does not induce a state-dependent memory and does not affect open field behavior.

As shown in ***Figure 5B***, a lower degree of mismatch in the reexposure pattern, caused by weak activation of shock-related neurons (as would be expected from deconditioning-update), can indeed lead to a greater reduction in subsequent fear behavior than a 'pure extinction' pattern (sharing only the tone information with the original memory). This effect was sensitive to blockade of the mismatch-induced degradation term – the model equivalent of blocking memory destabilization mechanisms such as LVGCCs with a drug like nimodipine (***Figure 5C***). This occurs because the 'minor footshock' pattern leads to recovery of the original memory as the outcome of the initial sessions (***Figure 5D***), causing mismatch-induced updating of the weights supporting these memories and weakening of the mutual connections between shock and context neurons (***Figure 5E***). On the other hand, no-footshock extinction leads to immediate formation of a new attractor, leaving the connection weights related to the shock memory unaltered throughout the extinction process.

Because of this, deconditioning-update was sensitive to blockade of mismatch-induced degradation, while no-footshock extinction – which depends basically on new learning – was not (***Figure 5C***). Blocking the Hebbian plasticity term, on the other hand, led to blockade of no-footshock extinction, as well as reconsolidation blockade in low-mismatch conditions (***Figure 5—figure supplement 1***). Interestingly, blocking Hebbian plasticity during deconditioning-update led to greater freezing decrease after the first reactivation session, suggesting that the procedure could be modulated by pharmacological agents such as protein synthesis inhibition; on the long run, however, the same manipulation decreased extinction due to interference with new learning.

A caveat here is that, unlike in the model, nimodipine did affect regular extinction in the experimental results. This suggests that the mechanistic distinction between deconditioning-update and classic extinction is not so clear-cut, and that these two processes might share common mechanisms, as has been proposed for reconsolidation and extinction (***Almeida-Corrêa and Amaral, 2014***). Capturing these subtleties, as well as other features of the data such as distinctions between single- and multiple-session extinction, likely requires model implementations that are more complex than this simple adaptation of the classic Hopfield formulation.

## Discussion

Taken as a whole, our findings demonstrate that presenting a tone followed by a weak footshock (deconditioning-update) was more effective in reducing fear memory than presenting a tone in the absence of shock (extinction training). Attenuation of fear responses following deconditioning-update was robust, long-lasting, and less sensitive to renewal and spontaneous recovery. Remarkably, this strategy was also effective in reducing fear within boundary conditions in which memories have been described to be less sensitive to modification (e.g., very strong training protocols and remote memories). The same effect was found in other types of fear-related memory (contextual

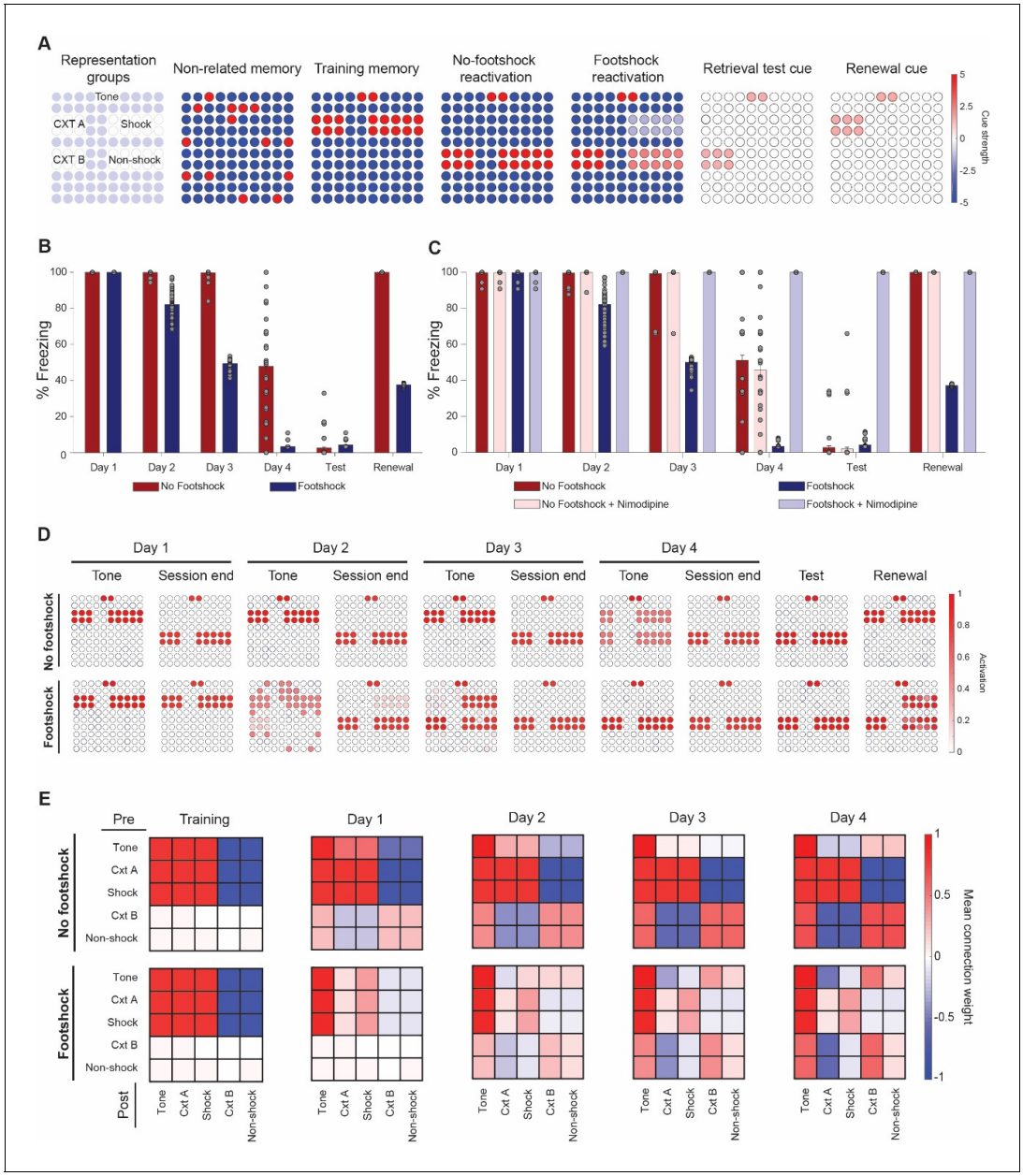


**Figure 5.** Lower mismatch accelerates fear reduction and decreases renewal in a neural network model. (**A**) Cue inputs presented to the network during training (shock memory), reexposure (with or without footshock) and test sessions (consisting of the tone and either context B (test) or A (renewal)). Color scale shows the cue received by each of the 100 neurons (**B**) Extinction over multiple sessions using the no-footshock (red bars) or footshock (blue bars) cue. Bars represent freezing, expressed as the activity ratio between shock neurons and the sum of shock and non-shock neurons in response to the test cue, at reexposure days 1 to 4. After each test, memory is updated according to the activity reached in response to the full reexposure pattern. (**C**) Effect of LVGCC blockade (i.e. setting the mismatch-induced degradation term D to 0). Removing the degradation term blocks deconditioning-update, but not regular extinction. (**D**) Network activity in retrieval tests during tone presentations (e.g. cued with the tone alone) and at the end of reexposure (e.g. cued with the full reactivation pattern), as well as on test and retrieval sessions. Lower mismatch (i.e. weak footshock) leads to retrieval of the original pattern on the first days, leading to memory updating through mismatch-induced degradation and lower retrieval on subsequent tests. (**E**) Mean synaptic weights between different neuronal groups after training and at the end of each extinction session. Heat map represents the connection from neuronal populations in the Y axis to those in the X axis in the no-footshock and footshock groups. Deconditioning-update leads to weakening of connections between context and shock neurons and of their inhibitory connections to other neurons. On no-footshock extinction, an extinction memory is formed with sparing of the shock representation.

The online version of this article includes the following figure supplement(s) for figure 5:

**Figure supplement 1.** Blockade of Hebbian plasticity impairs reconsolidation, blocks standard extinction and interacts with deconditioning-update.

fear conditioning and inhibitory avoidance tasks), and was sensitive to pharmacological blockade of LVGCCs. We suggest that the CS–US association is weakened during the deconditioning-update approach, leading to lower fear expression. Considering that (i) fear reduction is long-lasting, without spontaneous recovery, renewal and savings, (ii) weak-footshock pairings do not have the same effect in single-session extinction protocols or when stronger footshocks are applied, and (iii) this effect is prevented by LVGCC antagonism, we suggest that deconditioning-update is mediated by the memory destabilization effects commonly associated with reconsolidation.

These results are in line with predictions from a neural network model previously built to account for transitions between reconsolidation and extinction with increasing reexposure time in contextual fear conditioning (***Osan et al., 2011***). In this model, modification of existing connection weights is triggered by a protein synthesis-independent set of processes when there is mismatch between the memory pattern retrieved by the network (based on previously learned experiences) and the currently experienced sensory state, as when the prediction of a strong footshock is offset by a mild one. However, if mismatch is too extensive, as during extinction in the absence of a footshock, a new attractor is formed in the network at the first reexposure session already, preventing mismatch-induced weakening of the original memory.

Although the model is quite reductionist in its implementation, both due to the absence of realistic topology and to the non-spiking, continuous nature of neuronal activity, it is nevertheless able to account for transitions between simple retrieval, reconsolidation and extinction with increasing reexposure time (***Suzuki et al., 2004***) or number of non-reinforced CSs (***Lee et al., 2006***; ***Sevenster et al., 2014***), as assessed by the effects of pharmacological agents such as protein synthesis inhibitors, NMDA antagonists and β-blockers. The model also helps to explain why, unlike pharmacological reconsolidation blockade, deconditioning-update takes several sessions to occur, as the impact of memory labilization mechanisms on a stored memory is much more pronounced when Hebbian mechanisms are blocked concomitantly (***Osan et al., 2011***).

Although we have not tested this explicitly, our results also appear compatible with the Bayesian inference framework proposed by ***Gershman et al. (2017)***, in which the probability that an experience is attributed to a new latent cause increases in proportion to the degree of prediction error generated by previous experience. This can explain why a lower degree of mismatch, such as that caused by reactivation followed by a weak footshock, can lead to a greater probability of memory updating and decrease the recovery of fear. If this is the case, the rationale for the greater effectiveness of deconditioning-update in reducing fear might be similar to that observed in so-called retrieval extinction paradigms (***Monfils et al., 2009***; ***Kredlow et al., 2016***). In this case, the first retrieval trial, usually consisting of a single CS, is thought to induce retrieval-induced destabilization of the original memory, as the prediction error generated by this reexposure is not sufficient to form an extinction memory. Nevertheless, following this initial retrieval session with an extinction procedure leads to lower fear recovery than when extinction is performed without it, although this effect has been observed rather inconsistently across studies (***Auber et al., 2013***; ***Kredlow et al., 2016***).

Interestingly, deconditioning-update only occurred when reactivation sessions were spaced across multiple days. When a massed extinction procedure with 24 non-reinforced CSs was performed within a single extinction session, on the other hand, a mild footshock at the end of the tones actually prevented within-session extinction, and increased freezing in a test performed on the following day. This seems to reinforce the notion that computations linking the degree of prediction error with memory destabilization occur only at the end of reexposure (***Perez-Cuesta et al., 2007***; ***Osan et al., 2011***). It is also in line with the idea that within- and between-session extinction are distinct processes, with different dynamics and molecular requirements (***Plendl and Wotjak, 2010***; ***Almeida-Corrêa et al., 2015***).

An important limitation of our computational framework, however, is that time is not explicitly modeled – thus, one cannot distinguish between massed and spaced extinction protocols in order to investigate the different results found in the two settings. An interesting challenge for future theoretical models, thus, would be to study whether and how within-session extinction relates mechanistically to mismatch-induced updating and between-session extinction. In this line, it is interesting to note that the model by ***Gershman et al. (2017)*** postulates that the probability that an experience is attributed to a new latent cause increases with time between initial learning and reactivation. This could plausibly account for why fear reduction with deconditioning-update is only observed in

spaced sessions, in which new structural learning is more likely to take place in the absence of shocks (and thus prevent the original memory from updating in standard extinction conditions).

Nevertheless, in contrast to our work, ***Gershman et al. (2013)*** did find an effect of reducing mismatch at the start of a single 24-CS extinction session by pairing some of the initial tones with full-strength footshocks. That said, their effect was only observed in spontaneous recovery sessions and post-reinstatement sessions, and not within the extinction session itself. Moreover, the approach to induce lower degrees of mismatch in their experiment – gradually reducing the frequency of regular footshocks – was different from ours, in which this was achieved by providing a low-intensity shock at the end of every tone. Studying whether both approaches could be combined – by gradually decreasing footshock intensity, for example – could be an interesting topic for further investigation of the deconditioning-update effect. Another topic for future studies is whether mismatch between the training and reactivation context in auditory conditioning protocols also contributes to the deconditioning-update effect, as recently reported for reconsolidation (***Ferrara et al., 2019b***). In our study, animals showed relatively high pre-CS freezing levels, suggesting some degree of generalization between contexts, although the presence of renewal suggests that animals were also capable of differentiating them. It is thus an open question whether generalization might be important for deconditioning-update to occur.

Psychiatric disorders associated with pathological memories are prevalent, cause important social and economic burden, and approaches to translate basic knowledge of fear conditioning for potentiation of exposure therapy have met limited success so far in clinical settings (***Monfils and Holmes, 2018***). We believe that exploring the principles of deconditioning-update – that is, the notion that there is an ideal window of prediction error to potentiate reexposure effects – is a promising therapeutic avenue that could be explored in more depth in the setting of trauma-focused psychotherapy. The high efficacy, long-lasting effects, and drug-free nature of this approach make it particularly appealing for translation to human memory-related disorders, such as trauma, phobias and drug abuse.

# Materials and methods

A total of 323 male and female Wistar rats (2–3 months old, weighing between 300 and 400 g) from CREAL at the Federal University of Rio Grande do Sul (UFRGS) were used for the experiments. Only one animal was excluded (in the experiment in ***Figure 1A***) due to health conditions. They were housed in plexiglass boxes, with four animals per cage, with block randomization using the cage as subgroup to ensure that each cage contained at least one animal per experimental group. Sample sizes ranged from 6 to 10 per group across experiments, yielding statistical power estimates between 69% and 89% to detect an absolute difference of 30% in freezing time (in line with the differences in memory recovery observed in ***Monfils et al. (2009)***) with a standard deviation of 20% (our average value for test sessions in ***Figure 1***) at $\alpha = 0.05$ in a 2-tailed t test.

Animals were kept on 12/12 hr light/dark cycle under controlled temperature (21˚C ± 2), with regular chow and water available ad libitum and humidity of approximately 65%. All experiments were performed during the light cycle. All procedures followed the Brazilian ethical guidelines for animal research set by the National Council for the Control of Experimental Animal Research (CONCEA). Methods and results are reported according to the revised ARRIVE guidelines (***Percie du Sert et al., 2019***).

## Auditory fear conditioning

*Apparatus*: The conditioning chamber (context A) consisted of an illuminated plexiglass box (33 × 22 × 22 cm), with a floor grid of parallel 0.1-cm caliber stainless steel bars spaced 1 cm apart. All context chambers had the same dimensions, but context A had black walls, whereas context B was vertically striped in black and white. Context C consisted of white and brown lateral walls and a transparent front wall.

*Training session*: Rats were habituated for 2 days in context B (10 min each), and 24 hr later were placed in context A, where they received five conditioning trials consisting of a 30 s presentation of a 5-kHz, 75-dB tone (CS) that co-terminated with a 0.5-mA (or 1-mA in ***Figure 2F–H***), 1-s footshock (US) (five tone-footshock pairings). The first CS was presented 2 min into the session, with an inter-pairing interval of 1 min. One minute after the final pairing, rats were returned to their home cages.

*Reactivation sessions*: In daily sessions taking place in context B and starting 48 hr after training (or 40 days later in ***Figure 2A–E***), animals in the no-footshock group received 3 CS-only, while the footshock group (deconditioning-update) received 3 CSs that co-terminated with a 0.1-mA (or 0.3-mA in ***Figure 1—figure supplement 1***), 1-s shock. The percentage of time freezing during each tone presentation was quantified and the mean freezing percentage for the three tones was used as a measure of fear. The first CS was presented 2 min into the session, with an interpairing interval of 1 min. One minute after the final pairing, rats were returned to their home cages. Most experiments used four reactivation sessions, except for those in ***Figure 1—figure supplement 2*** (one session) and ***Figure 2—figure supplement 1*** (three sessions). In the devaluation experiment (***Figure 1—figure supplement 3A–B***), the protocol was the same, except that the 0.1-mA footshocks were presented in context C without being paired with the tone. In the paired vs. unpaired experiment (***Figure 1—figure supplement 4***), the unpaired group received uncorrelated tones and 0.1-mA footshock in a pseudorandom order. Pre-CS freezing levels were measured for the 30 s immediately preceding the first tone.

*Test session*: 24 hr after the last reactivation session, both groups were presented with 3 CSs in context B. The percentage of time freezing during each tone presentation was quantified, and the average for the three tones was used as a fear measure. The first CS was presented 2 min into the session, with an interpairing interval of 1 min. One minute after the final pairing, rats were returned to their home cages. Pre-CS freezing levels were measured for the 30 s immediately preceding the first tone.

*Renewal*: 24 hr after the test session, animals were placed in context A, where they received 3 CSs. The percentage of time freezing during each tone presentation was quantified, and the average percentage was used as a measure of fear recovery. The first CS was presented 2 min into the session, with an interpairing interval of 1 min. One minute after the final pairing, rats were returned to their home cages. Pre-CS freezing levels were measured for the 30 s immediately preceding the first tone.

*Spontaneous Recovery*: 20 days after the renewal session, animals were placed in context B and received 3 CSs. The percentage of time freezing during each tone presentation was quantified and the average was used as a measure of fear recovery. The first CS was presented 2 min into the session, with an interpairing interval of 1 min. One minute after the final pairing, rats were returned to their home cages. Pre-CS freezing levels were measured for the 30 s immediately preceding the first tone.

*Reinstatement*: 24 hr after the test session, animals were exposed in to context C for 5 s, where they received two 2-s, 0.7-mA footshocks. 24 hr later, they were tested for reinstatement in context B. Pre-CS freezing levels were measured for the 30 s immediately preceding the first tone.

*Retraining*: 24 hr after the spontaneous recovery test, rats were submitted to a training procedure (3 $\times$ 0.5 mA). They were then tested 24 hr later to assess savings. Pre-CS freezing levels were measured for the 30 s immediately preceding the first tone.

*Single-session extinction*: Animals were placed in context B 48 hr after training, where they received either 12 or 24 CSs depending on the protocol. In the no-footshock group these tones were not accompanied by the US, while the footshock group received tones that co-terminated with a 0.1-mA footshock. The first CS was presented 2 min into the session, with an interpairing interval of 1 min. One minute after the final pairing, rats were returned to their home cages. Pre-CS freezing levels were measured for the 30 s immediately preceding the first tone.

## Contextual fear conditioning

*Apparatus*: The conditioning chamber consisted of an illuminated plexiglass box (33 $\times$ 22 $\times$ 22 cm grid of parallel 0.1-cm caliber stainless steel bars spaced 1 cm apart).

*Training session*: In the training session, rats were placed in the conditioning chamber for 3 min before receiving two 2-s, 0.5-mA footshocks separated by a 30 s interval; they were kept in the conditioning context for an additional 30 s before returning to their home cage.

*Reactivation session*: 48 hr after the training session, animals were reexposed to the same conditioning chamber for 4 daily 4 min sessions. Rats from the footshock group received two pairs of 0.1-mA, 2-s shocks after 180 and 210 s, while the no-footshock group did not receive any shocks.

*Test session and spontaneous recovery*: 24 hr after the last reactivation session, animals were re-exposed to the same conditioning chamber for a 4 min test session and the percentage of time freezing was quantified. 20 days later, the procedure was repeated to assess spontaneous recovery.

## Step-through inhibitory avoidance

*Apparatus*: The apparatus consists of an automated box (Insight Ltda., Brazil) with two compartments, a dark one and a lighted one, each measuring 33 × 22×22 cm. The floor consisted of a grid of metal bars with 1 mm diameter placed 1 cm from each other.

*Training session*: Animals were placed in the lighted compartment. When they entered the dark compartment, the door was closed and the animals received 4 0.5-mA, 1-s footshocks, with intervals of 5 s between them. They were removed from the box 10 s after the last footshock.

*Reactivation sessions*: In daily sessions starting 48 hr after training, animals in the footshock and no-footshock groups were placed in the dark compartment for 30 s, with no access to the lighted compartment. The footshock group received 2 0.1-mA shocks (at 25 s and 30 s) while the no-footshock group did not receive any shocks.

*Test session*: All animals were placed in the lighted compartment and left free to explore the box. The latency to enter into the dark compartment for the first time and the time spent in each compartment were counted over a 10 min session and used as measures of memory.

## Behavioral assessment

Freezing behavior was used as a memory index in the fear conditioning tasks, being registered with a stopwatch in real time by an experienced observer that was blinded to the experimental group. Freezing was defined as total cessation of all movements except those required for respiration.

## Open field

Exploratory activity and anxiety-like behavior were assessed in the open field test in order to exclude non-specific effects of nimodipine. The apparatus consisted of a circular arena (90-cm diameter) with 50-cm high walls. The floor was subdivided into 12 quadrants and three concentric zones (periphery, intermediary and center). Animals were exposed to the apparatus for 5 min, during which the time spent on the periphery (thigmotaxis) and the number of crossings between quadrants were measured. Nimodipine (16 mg/kg) or vehicle was measured intraperitonally 30 min before the test.

## Drugs

Nimodipine (Sigma), an antagonist of the L-type voltage-gated calcium channels (LVGCCs) was dissolved in sterile isotonic saline solution with 8% dimethylsulfoxide to a concentration of 16 mg/mL. Nimodipine or its vehicle was injected intraperitoneally at a volume of 1 mL/kg (16 mg/kg) 30 min before memory reactivation sessions, test sessions or open field sessions.

## Statistical analysis

Data are expressed as mean ± SEM, always using the animal as the experimental unit. The statistical tests used and their results are detailed for every experiment in ***Supplementary file 1–11*** and in ***Figure 1—source data 1***, ***Figure 2—source data 1***, ***Figure 3—source data 1*** and ***Figure 4—source data 1***; they include two-tailed Student's *t* test; one-way, two-way or three-way analysis of variance (ANOVA), followed by Tukey's or Bonferroni's post hoc test, when necessary; and Kruskal-Wallis test, followed by Dunn's post hoc. Values of $p<0.05$ were considered statistically significant. Baseline freezing levels for all experiments are shown on ***Supplementary file 12–21***. Unit-level data for all figures are provided as ***Figure 1—source data 1***, ***Figure 2—source data 1***, ***Figure 3—source data 1*** and ***Figure 4—source data 1*** .

## Computational simulations

*Model Network*: In order to propose a mechanistic explanation for the experimental results, we used an adaptation of the attractor network model described in ***Osan et al. (2011)***. This Hopfield-like network is capable of storing and retrieving memories using Hebbian learning rules dependent on neuronal activity, which in turn depends on the inputs presented to a fully connected network of 100 neurons. In this network, the activity of each neuron *i* is determined by

$$\tau\frac{du_i}{d_t} = -u_i + \frac{1}{2}\left(1 + \tanh\left(\sum_{j=1}^{N} w_{ij}u_j + I_i\right)\right)$$

where $\tau$ is the neural time constant and $u_i$ represents the level of activation of neuron $i$ which can vary continuously from 0 to 1 – unlike in the original Hopfield continuous activity model (***Hopfield, 1984***), in which activity varies from $-1$ to 1.

As a fully connected neural network, every neuron $i$ is connected with every neuron $j$. For the learning process, the network needs to reinforce the connections between neurons that fire together, while creating inhibition when presynaptic neuron $i$ is active and postsynaptic $j$ is silent. Changes in the synaptic weight matrix $W = (w_{ij})$ are determined by the equation

$$\Delta W = -\gamma W + HLP + MID$$

where Hebbian learning plasticity (HLP) and mismatch-induced degradation are two independent learning rules (see below) and $0 \leq \gamma \leq 1$ is a time-dependent synaptic decay factor.

Learning occurs by presenting an input $I_i$ to the circuit, corresponding to sensory information provided by the environment and/or internal cues, which lead to changes in the plastic connections between neurons. The cue has a one-to-one topology to the memory network, with every neuron receiving a cue input that can be either excitatory or inhibitory. Modifications on the synaptic weight matrix follow the HLP rule, corresponding to the Hebbian formulation implemented in classic Hopfield networks and described as

$$HLP = S\left(u^T * u\right) - S\left((1-u)^T * u\right)$$

where vector $u = (u_1, u_2, \ldots, u_N)$ is the steady state of the network after input $I_i$ presentation, while S is a factor representing requirements for Hebbian plasticity, such as protein synthesis, receptor activation, intracellular signaling and other mechanisms.

When the cue input leads to the retrieval of a previously stored memory, this can cause mismatch between the cue input and the retrieved attractor if the two are not the same. This leads to concomitant activation of the MID learning rule, corresponding to a memory-updating system akin to that involved in memory destabilization during reconsolidation and defined by

$$MID = D\left(m^T * u\right)$$

where D is a factor representing requirements for memory destabilization (such as protein degradation and LVGCCs), $m = I_{norm} - u$ is the mismatch vector defined and $I_{norm}$ is a normalized cue vector. The *MID* term leads to weakening of connections responsible for the mismatch in order to update the existing memory.

## Learning, retrieval and reactivation

Non-overlapping neuron clusters in the network were chosen to represent the training or extinction contexts (six neurons each), tone (two neurons), aversive stimulus/shock (10 neurons) or safety/absence of shock (10 neurons) (***Figure 5A***). Initially, a pattern representation of a memory unrelated to fear conditioning was presented as a cue to the network. This was followed by a training pattern activating neurons representing context A, tone and shock while inhibiting the remaining ones.

Retrieval was evaluated at every training or reactivation session through presentation of a cue activating neurons representing context B and tone, with no input to the remaining neurons. This corresponds to the period in which freezing is assessed (e.g. during the tone itself, shown as 'tone' in ***Figure 5D***), and was modeled with the same cue irrespectively of the presence of shock at the end of reactivation. For the renewal test, the retrieval cue activated neurons representing context A and tone. To quantify memory retrieval, we used the mean activity of neurons representing shock and absence of shock, which was converted to a 'freezing percentage' by dividing the total activity of shock neurons by the total activity of both groups – thus, 100% freezing corresponds to full activation of shock neurons and no activation of non-shock neurons.

At the end of each reactivation session, the network underwent a new learning round with a pattern that varied according to the experimental group. To model standard extinction over multiple retrieval sessions, we activated the non-shock neuron cluster along with the neurons representing

the extinction context and tone, while inhibiting the remaining ones ('no-footshock reactivation' in ***Figure 5A***). For deconditioning-update, we assumed an intermediate representation between the learning and extinction patterns ('footshock reactivation' in ***Figure 5A***). For reconsolidation (***Figure 5—figure supplement 1***), this intermediate representation was closer to the shock pattern than to the extinction one. Synaptic weights were updated according to the activation pattern reached in response to these cues (shown as 'session end' in ***Figure 5D***) Unlike in the original model, no synaptic decay was assumed (i.e. $\gamma$ was set to 0) and learning strength (as defined by S) was assumed to be smaller during reactivation sessions in both groups due to the lower intensity of the stimuli, thus allowing extinction to occur over multiple sessions.

After each learning or reactivation session, the mean synaptic weight between each cluster of neurons (tone, contexts, shock and non-shock) was calculated by taking the average of the connections between all presynaptic neurons of a subpopulation and all postsynaptic neurons of the other subpopulation. This was used to create the synaptic weight matrix between clusters shown in ***Figure 5E***.

## Model parameters

All simulations were performed in MATLAB R2018a (Mathworks) using $N = 100$; $\tau = 1$; $\gamma = 0$; $s_0 = 1$. For training sessions, we set $S = 0.8$, while in reactivation sessions we used $S = 0.25$. $D$ was set to 0.95 for all sessions, except for reactivations using nimodipine, in which $D = 0$. Each unit $i$ during learning received an input $I_i$ varying between $-5$ and 5. In the deconditioning update group, the aversive shock cluster received an input $I_i$ of $-2.31$, while non-shock neurons received 2.31 [corresponding to t = 6 in the transformation used by ***Osan et al. (2011)*** to create intermediate patterns]. For reconsolidation, these inputs were 3.80 and $-3.80$, respectively, corresponding to t = 3 in ***Osan et al. (2011)***. In the retrieval cue, each targeted neuron had an input $I_j = 1.5$ and 0 for other neurons.

One hundred simulations of each experiment were performed, with different initial conditions determined by Gaussian noise in the initial weight matrices (with a normal distribution on [$-0.05$, 0.05]) and in the neuronal activation at the start of every session (with a normal distribution on [0, 0.1]). For each simulation, 100 retrieval trials were run in each session to determine freezing percentage. All results are displayed as the mean ± S.E.M of these 100 simulations. Matlab code to perform all simulations and generate ***Figure 5B, C and E*** and ***Figure 5—figure supplement 1*** is presented as Source Code.


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### Author contributions

Bruno Popik, Methodology, Project administration; Felippe Espinelli Amorim, Investigation, Methodology; Olavo B Amaral, Formal analysis, Supervision, Writing - original draft; Lucas De Oliveira Alvares, Conceptualization, Supervision, Funding acquisition, Writing - original draft, Writing - review and editing


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### Ethics

Animal experimentation: All procedures followed the Brazilian ethical guidelines for animal research set by the National Council for the Control of Experimental Animal Research (CONCEA) and approved by the committee on the Ethics of Animal Experiments of the UFRGS (number 34547).


### Decision letter and Author response

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## Additional files

### Supplementary files

- Source code 1. Matlab code for ***Figure 5***.
- Supplementary file 1. Weakening fear memory through deconditioning-update training.
- Supplementary file 2. Deconditioning-update approach weakens both remote and strong fear memory.
- Supplementary file 3. Deconditioning-updating weakens fear memory in different behavioral tasks.
- Supplementary file 4. Deconditioning-update is based on memory destabilization mechanisms.
- Supplementary file 5. Deconditioning-update does not occur with 0.3-mA shocks.
- Supplementary file 6. A single reactivation session does not update fear memory.
- Supplementary file 7. Deconditioning-update is not due to US devaluation.
- Supplementary file 8. Deconditioning-update does not occur with unpaired shocks in the reactivation sessions.
- Supplementary file 9. Deconditioning-update weakens strong fear memories in females.
- Supplementary file 10. Deconditioning-update does not occur in a single 12-CS extinction session.
- Supplementary file 11. Nimodipine does not affect open field behavior and does not induce a state-dependent memory.
- Supplementary file 12. Baseline (pre-CS) freezing levels for ***Figure 1***.
- Supplementary file 13. Baseline (pre-CS freezing levels for ***Figure 2***.
- Supplementary file 14. Baseline (pre-CS) freezing levels for ***Figure 4***.
- Supplementary file 15. Baseline (pre-CS) freezing levels for ***Figure 1—figure supplement 1***.
- Supplementary file 16. Baseline (pre-CS) freezing levels for ***Figure 1—figure supplement 2***.
- Supplementary file 17. Baseline (pre-CS) freezing levels for ***Figure 1—figure supplement 3***.
- Supplementary file 18. Baseline (pre-CS) freezing levels for ***Figure 1—figure supplement 4***.
- Supplementary file 19. Baseline (pre-CS) freezing levels for ***Figure 2—figure supplement 1***.
- Supplementary file 20. Baseline (pre-CS) freezing levels for ***Figure 4—figure supplement 1***.
- Supplementary file 21. Baseline (pre-CS) freezing levels for ***Figure 4—figure supplement 2***.

- Transparent reporting form

## Data availability

All data generated or analysed during this study are included in the manuscript and supporting files.

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# Figures and figure supplements

Shifting from fear to safety through deconditioning-update

**Bruno Popik *et al***

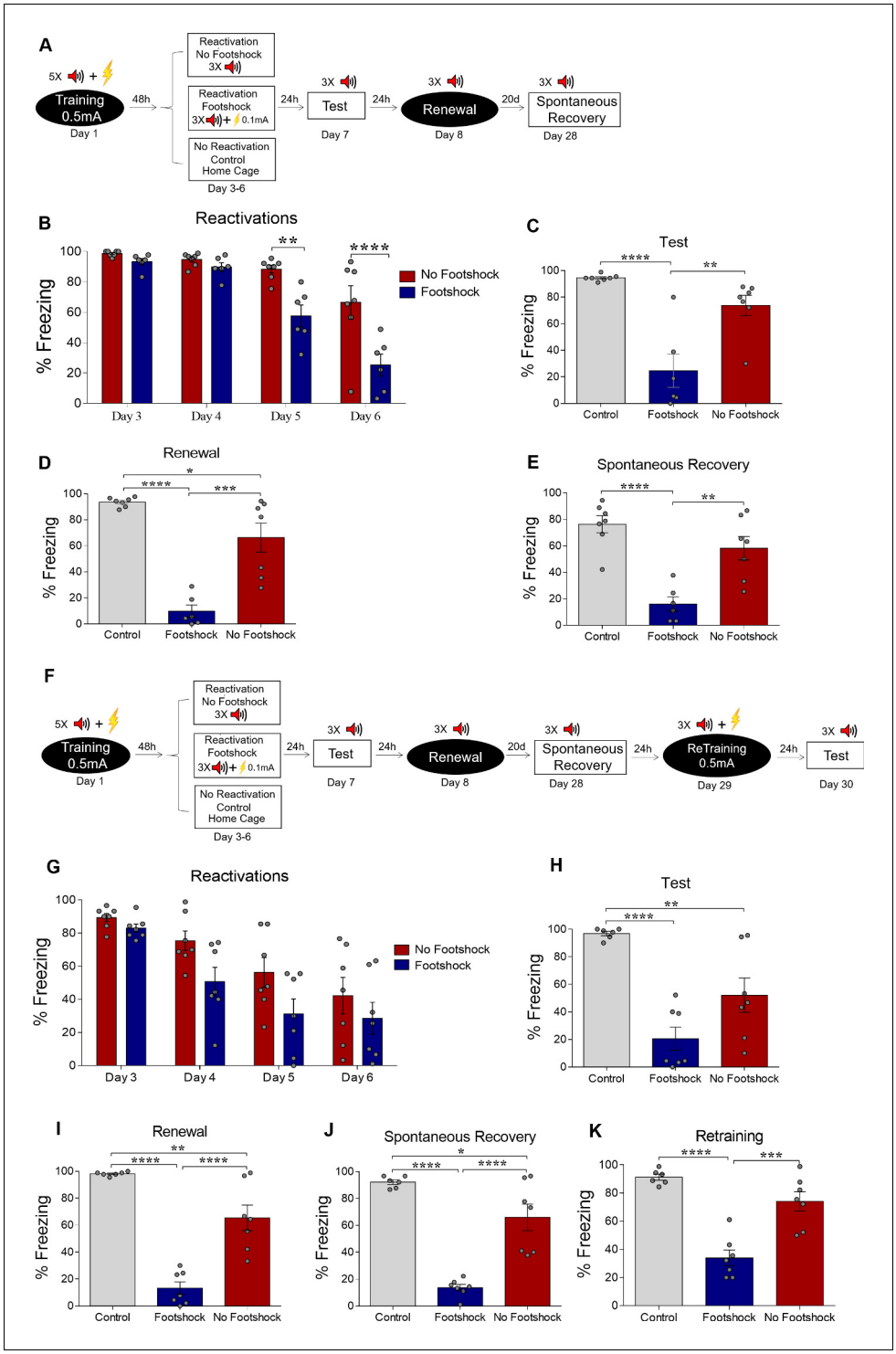


**Figure 1.** Weakening fear memory through deconditioning-update training. (**A**) Experimental design: male rats were fear-conditioned with five tone-shock pairings (context A; 5 CS + US, 0.5mA). 48 hr later, the no-footshock and footshock (deconditioning-update) groups were exposed to four daily reactivation sessions (context B). After this, animals underwent test (context B), renewal (context A) and spontaneous recovery (context B) sessions. Black circles represent context A, while white rectangles represent context B. (**B**) Freezing levels during reactivation sessions. Rats exposed to weak footshocks during reactivation sessions showed a significant reduction in freezing responses, maintained during the test (**C**), renewal (**E**) and spontaneous recovery (**D**) sessions. (**F**) Experimental design: female rats were fear-conditioned (context A; 5CS+US, 0.5mA). 48 hr later, the no-footshock and footshock

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*Figure 1 continued*

groups were exposed to four daily reactivation sessions (context B). After this, all groups underwent test, renewal, and spontaneous recovery sessions. Animals were reconditioned (context A; 3CS+US, 0.5mA) on the next day and retested 24 hr later. (**G**) Freezing levels during memory reactivation. Rats exposed to weak footshocks showed a significant reduction in freezing responses, maintained during the test (**H**), renewal (**I**), spontaneous recovery (**J**) and retraining test (**K**) sessions. Bars represent mean ± SEM. Statistical comparisons were performed using two-way repeated-measures ANOVA followed by a Bonferroni post-hoc (reactivation sessions) or one-way ANOVA followed by Tukey post-hoc (test, renewal, spontaneous recovery, and retraining test). *p<0.05; **p<0.005; ***p<0.0005; ****p<0.0001. For full statistics, see ***Supplementary file 1***. For pre-CS freezing values, see ***Supplementary file 12***.

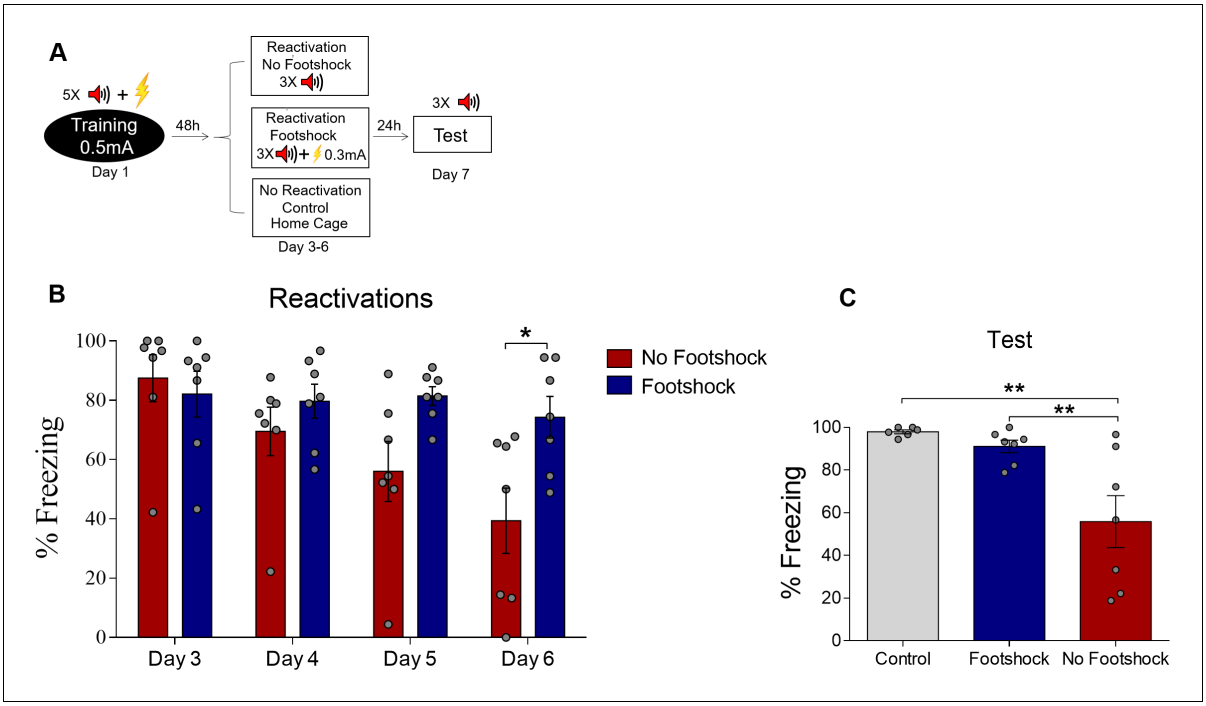


**Figure 1—figure supplement 1.** Deconditioning-update does not occur with 0.3-mA shocks. (**A**) Experimental design: rats were fear-conditioned with five tone-shock pairings (context A; 5 CS + US, 0.5mA). Starting 48 hr later, animals were exposed to four daily reactivation sessions (context B) with or without an intermediate footshock (0.3mA) at the end of tones. Subsequently, all groups underwent test sessions (context B). Black circle represents context A and white rectangles represents context B. (**B**) Freezing levels during reactivation sessions. Rats exposed to the intermediate footshock (0.3mA) showed less freezing reduction than no-footshock animals across sessions. (**C**) The no-footshock group expressed lower freezing in the test compared with footshock animals or homecage controls. Statistical comparisons are performed using two-way repeated-measures ANOVA followed by a Bonferroni post-hoc (reactivation sessions) or one-way ANOVA followed by a Tukey post-hoc (test and spontaneous recovery). Bars represent mean ± SEM. *p<0.05; **p<0.005. For full statistics, see ***Supplementary file 5***. For pre-CS freezing, see ***Supplementary file 15***.

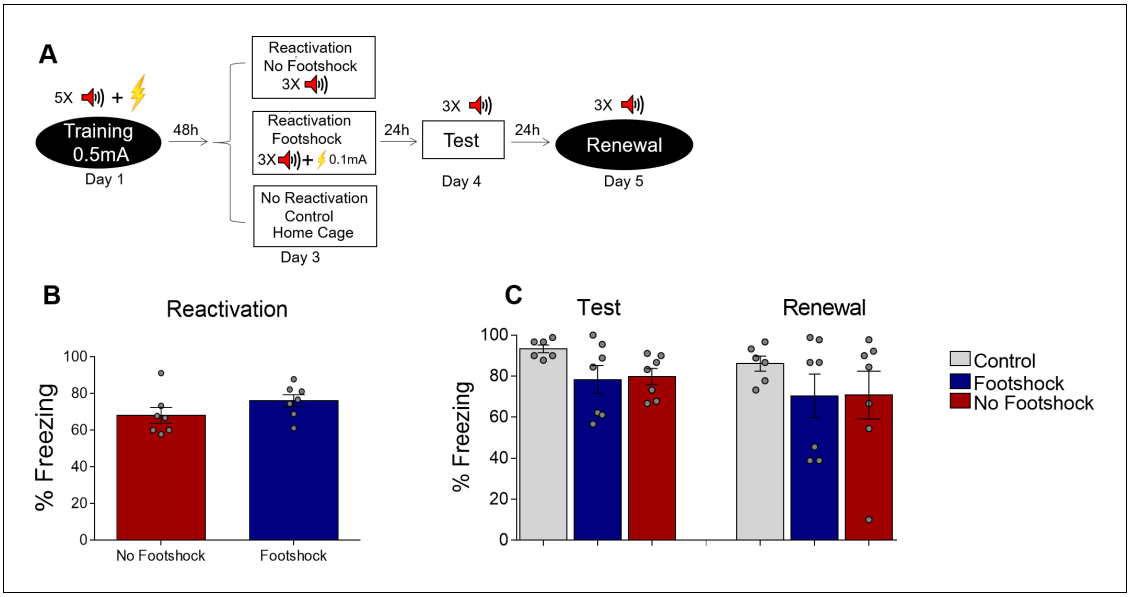


**Figure 1—figure supplement 2.** A single reactivation session does not update fear memory. (**A**) Experimental design: rats were fear-conditioned with five tone-shock pairings (context B; 5 CS + US, 0.5mA). 48 hr later, animals were exposed to a single reactivation session (context B) with or without a weak (0.1 mA) footshock. Animals then underwent test (context B), and renewal (context A) sessions. Black circles represent context A and white rectangles represents context B. There were no significant differences in freezing between groups in the reactivation (**B**), test or renewal sessions (**C**). Bars represent mean ± SEM. Statistical comparisons are performed using Student's t test (reactivation sessions) or one-way ANOVA followed by a Tukey post-hoc (test and renewal). For full statistics, see ***Supplementary file 6***. For pre-CS freezing, see ***Supplementary file 16***.

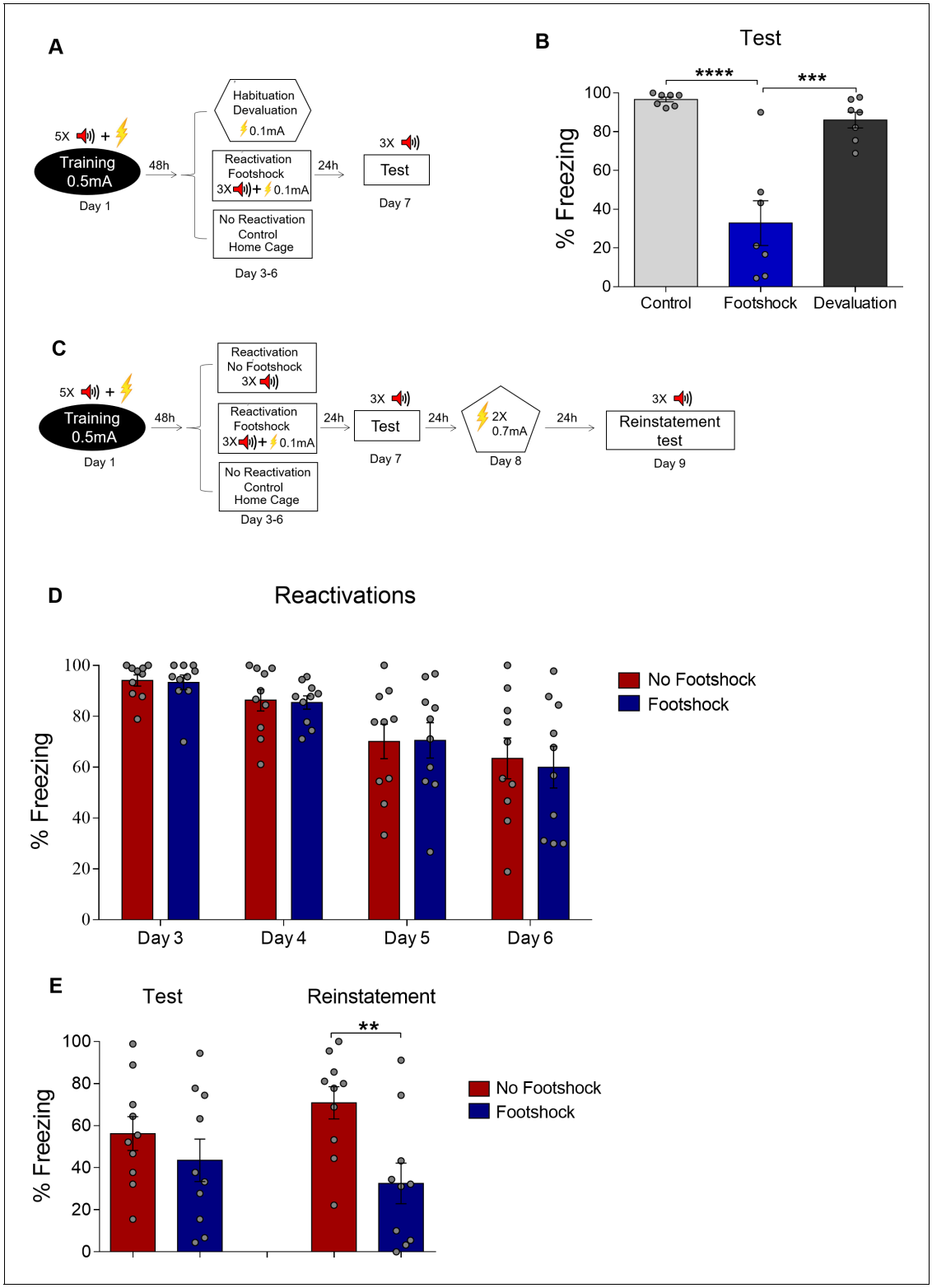


**Figure 1—figure supplement 3.** Deconditioning-update is not due to US devaluation. (**A**) Experimental design for devaluation: animals received five conditioning trial tones (CS) that co-terminated with a 0.5-mA, 1 s footshock (US) in context A. On days 3 to 6, the footshock group received 3 USs (0.1-mA footshock) at the end of the tone in context B, while the devaluation group received the same shocks in a different context (context C) without tone, and control animals remained in their home cages. Black circle represents context A, while white rectangles and hexagon represent contexts B and C, respectively. (**B**) On day 7, both groups were tested in context B with 3 CSs (tones), and the footshock group showed a decrease in freezing

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responses compared to the other two groups. (**C**) Experimental design for reinstatement: rats were fear-conditioned with five tone-shock pairings (context A; 5 CS + US, 0.5mA). Starting 48 hr later, animals were exposed to four daily reactivation sessions (context B) with or without a weak footshock (0.1 mA) at the end of tones. On day 7, both groups were tested. On the following day, animals received two 2-s non-paired footshock (reinstatement) in a different context followed by another test 24 hr later. Freezing levels were similar during reactivation (**D**) and test sessions, but rats exposed to the weak footshock during reactivation sessions showed less freezing responses after reinstatement (**E**). Bars represent mean ± SEM. Statistical comparisons are performed using two-way repeated-measures ANOVA followed by a Bonferroni post-hoc (reactivation sessions) or one-way ANOVA followed by a Tukey post-hoc (test and reinstatement sessions). *p<0.05; **p<0.005. ****p<0.0001. For full statistics, see ***Supplementary file 7***. For pre-CS freezing, see ***Supplementary file 17***.

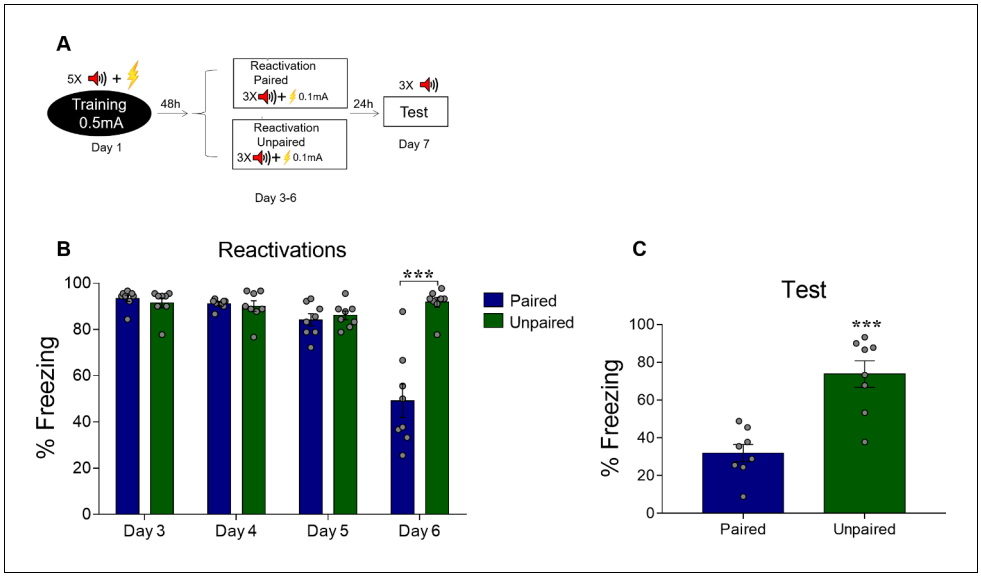


**Figure 1—figure supplement 4.** Deconditioning-update does not occur with unpaired shocks in the reactivation sessions. (**A**) Experimental design: rats were fear-conditioned with five tone-shock pairings (context A; 5 CS + US, 0.5mA). Starting 48 hr later, animals were exposed to four daily reactivation sessions (context B), where weak 0.1 mA footshocks were presented either paired (i.e. at the end of each tone) or unpaired (i.e. in pseudorandom moments during the session) with the CSs. Subsequently, all groups were tested in context B. Black circle represents context A and white rectangles represents context B. (**B**) Freezing levels during reactivation sessions. Rats exposed to unpaired footshocks showed higher freezing levels across sessions than those exposed to paired ones. (**C**) The paired CS-US group expressed lower freezing in the test compared with the unpaired group. Statistical comparisons are performed using two-way repeated-measures ANOVA followed by a Bonferroni post-hoc (reactivation sessions) or Student's t test (test session). Bars represent mean ± SEM. ***p<0.001. For full statistics, see ***Supplementary file 8***. For pre-CS freezing, see ***Supplementary file 18***.

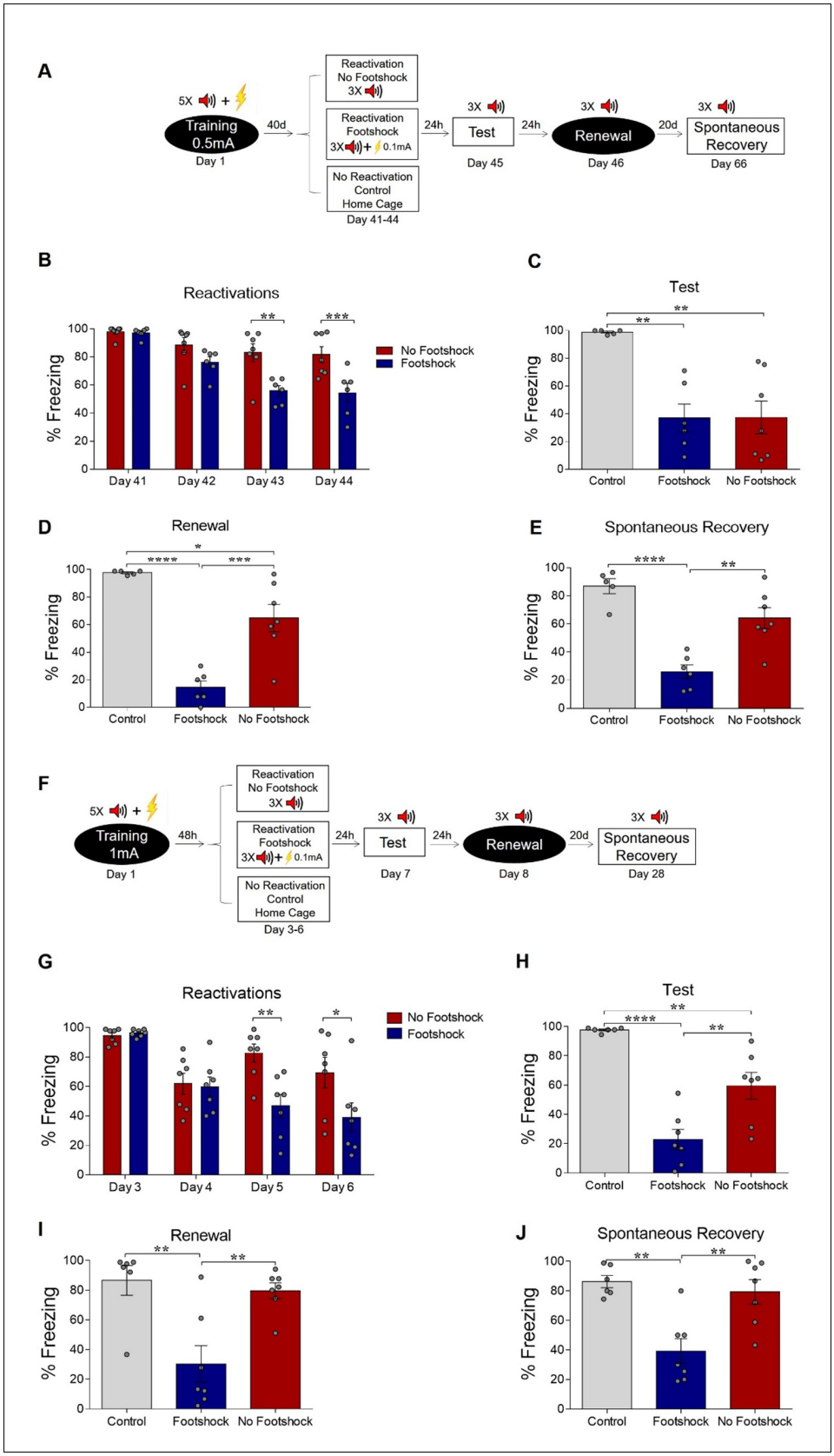


**Figure 2.** Deconditioning-update weakens both remote and strong fear memories. (**A**) Experimental design for remote memory: rats were fear-conditioned with five tone-shock pairings (context A; 5 CS + US, 0.5mA). Starting

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40 days later, the no-footshock and footshock (deconditioning-update) groups were exposed to daily reactivation sessions (context B). Then, all groups underwent test (context B), renewal (context A) and spontaneous recovery (context B) sessions. Black circles represent context A, while white rectangles represent context B. (**B**) Freezing levels during reactivation sessions. Rats exposed to weak footshocks during reactivation sessions showed similar freezing levels to no-footshock animals during the test session (**C**) and lower freezing levels at the renewal (**D**) and spontaneous recovery (**E**) ones. (**F**) Experimental design for strong training (5CS+US, 1mA). (**G**) Freezing levels during reactivation sessions. Rats exposed to weak footshocks during reactivation sessions showed a significant reduction in freezing responses that was maintained during the test (**H**), renewal (**I**) and spontaneous recovery (**J**) sessions. Bars represent mean ± SEM. Statistical comparisons are performed using two-way repeated-measures ANOVA followed by a Bonferroni post-hoc (reactivation sessions) or one-way ANOVA followed by a Tukey post-hoc (test and spontaneous recovery). *p<0.05; **p<0.005; ***p<0.0005; ****p<0.0001. For full statistics, see ***Supplementary file 2***. For pre-CS freezing, see ***Supplementary file 13***.

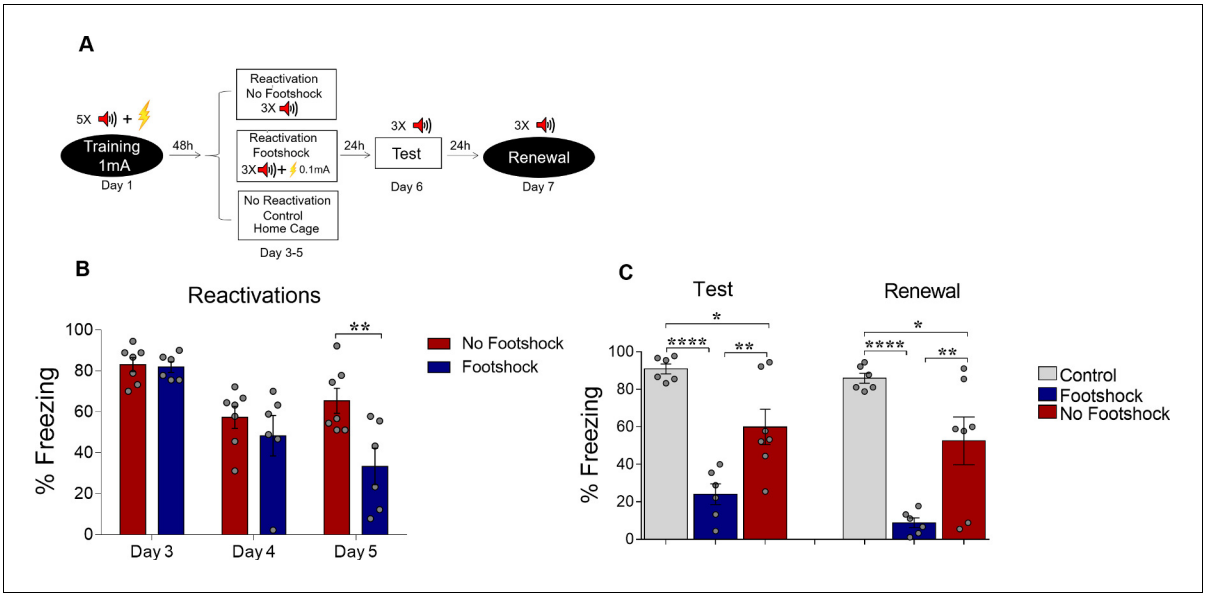


**Figure 2—figure supplement 1.** Deconditioning-update weakens strong fear memories in females. (**A**) Experimental design: rats were fear-conditioned with five tone-shock pairings (context A; 5 CS + US, 1mA). Starting 48 hr later, animals were exposed to three daily reactivation sessions (context B) with or without a weak footshock (0.1 mA) at the end of tones. Subsequently, all groups underwent test (context B) and renewal (context A) sessions. Black circles represent context A and white rectangles represent context B. (**B**) Freezing levels during reactivation sessions. Rats exposed to weak footshocks during reactivation sessions showed a decrease in freezing responses that was maintained in the test session (**C**). Bars represent mean ± SEM. Statistical comparisons are performed using two-way repeated-measures ANOVA followed by a Bonferroni post-hoc (reactivation sessions) or one-way ANOVA followed by a Tukey post-hoc (test and renewal). *p<0.05; **p<0.005. ****p<0.0001. For full statistics, see ***Supplementary file 9***. For pre-CS freezing, see ***Supplementary file 19***.

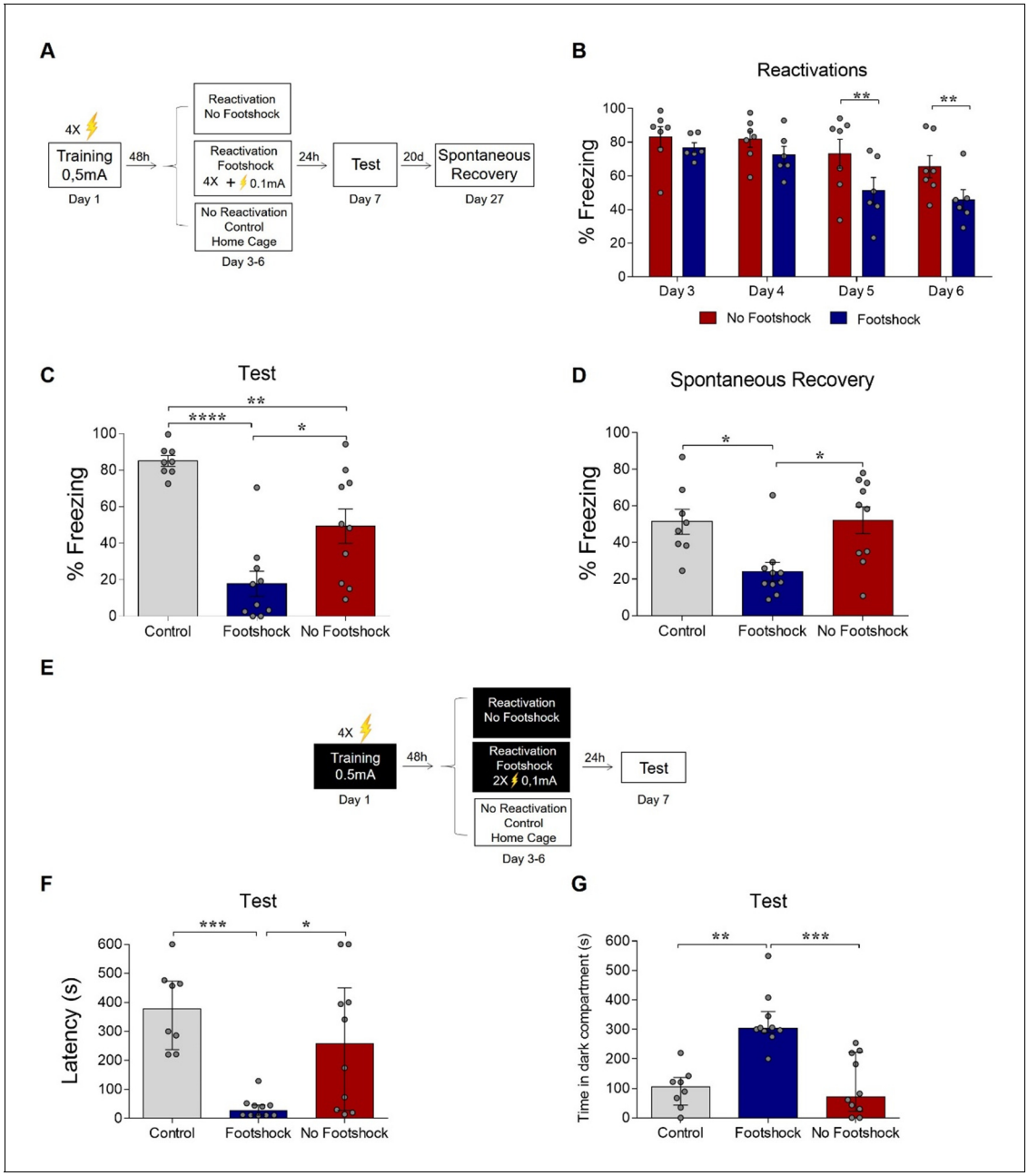


**Figure 3.** Deconditioning-update weakens fear memory in different behavioral tasks. (**A**) Experimental design in contextual fear conditioning: rats were fear-conditioned with five contextual-shock pairings (4 min context + 4 US, 0.5mA). Starting 48 hr later, the no-footshock and footshock groups were exposed to daily reactivation sessions. 24 hr after the last reactivation, all groups were tested; 20 days later, they were tested for spontaneous recovery. (**B**) Freezing levels during reactivation sessions. Rats exposed to weak footshocks during reactivation sessions showed a significant reduction in freezing responses maintained during the test (**C**) and spontaneous recovery (**D**) sessions. (**E**) Experimental design in inhibitory avoidance: rats were placed in the lighted compartment and received footshocks (4 US, 0.5mA) upon entering the dark one. Starting 48 hr later, the no-footshock and footshock groups were exposed to daily 30-s reactivation sessions in the dark compartment; 24 hr after the last reactivation, all groups were tested. Rats exposed to weak footshocks during reactivation sessions showed lower latencies to cross to the dark compartment (**F**) and spent more time in it during the test (**G**). Bars represent mean ± SEM or median with interquartile range (in F and G). Statistical comparisons for contextual fear conditioning are performed using two-way repeated-measures ANOVA followed by a Bonferroni post-hoc (reactivation sessions) or one-way ANOVA followed by a Tukey post-hoc (test, renewal, and spontaneous recovery). For inhibitory avoidance, a Kruskal-Wallis test followed by a Dunn post-hoc was performed. *p<0.05; **p<0.005; ***p<0.0005; ****p<0.0001. For full statistics, see ***Supplementary file 3***.

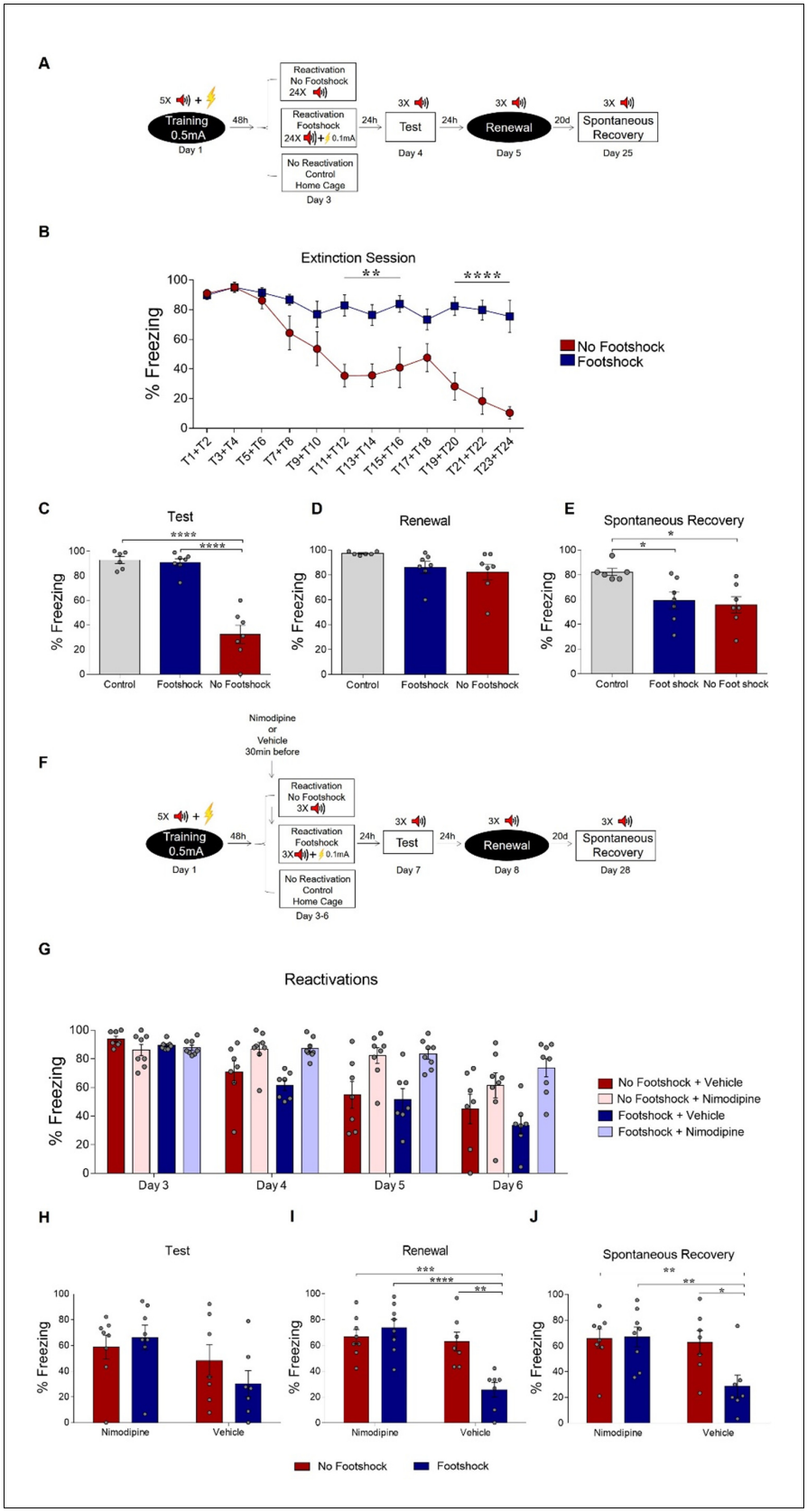


**Figure 4.** Deconditioning-update is based on memory destabilization mechanisms. (**A**) Experimental design: rats were fear-conditioned with five tone-shock pairings (context A; 5CS+US, 0.5mA). 48 hr later, the no-footshock and

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footshock groups underwent a single extinction session (context B, 24 CSs), followed by test (context B), renewal (context A) and spontaneous recovery (context B) sessions. (**B**) Freezing levels during extinction. Weak footshocks impaired extinction within the session and in the test session (**C**), but not in renewal (**D**) or spontaneous recovery (**E**). (**F**) Experimental design: rats were fear-conditioned (context A; 5CS+US, 0.5mA). 48 hr later, all animals underwent daily reactivation sessions (context B), receiving nimodipine (16 mg/kg, i.p.) or vehicle 30 min before each one. They then underwent test (context B), renewal (context A) and spontaneous recovery (context B) sessions. Nimodipine prevented freezing decrease across reactivation sessions in both groups (**G**). Freezing was similar between groups in the test session (**H**), but was lower in the vehicle-footshock group in the renewal (**I**) and spontaneous recovery (**J**) sessions. Bars represent mean ± SEM. Statistical comparisons are performed using two-way repeated-measures ANOVA followed by Bonferroni post-hoc (extinction), one-way ANOVA followed by Tukey post-hoc (test, renewal, and spontaneous recovery following extinction), three-way repeated-measures ANOVA followed by Bonferroni post-hoc (reactivation sessions with nimodipine/vehicle) and two-way ANOVA followed by Bonferroni post-hoc (test, renewal, and spontaneous recovery following nimopidine/vehicle). *p<0.05; **p<0.005; ***p<0.0005; ****p<0.0001 in between-group comparisons. For full statistics, see ***Supplementary file 4***. For pre-CS freezing, see ***Supplementary file 14***.

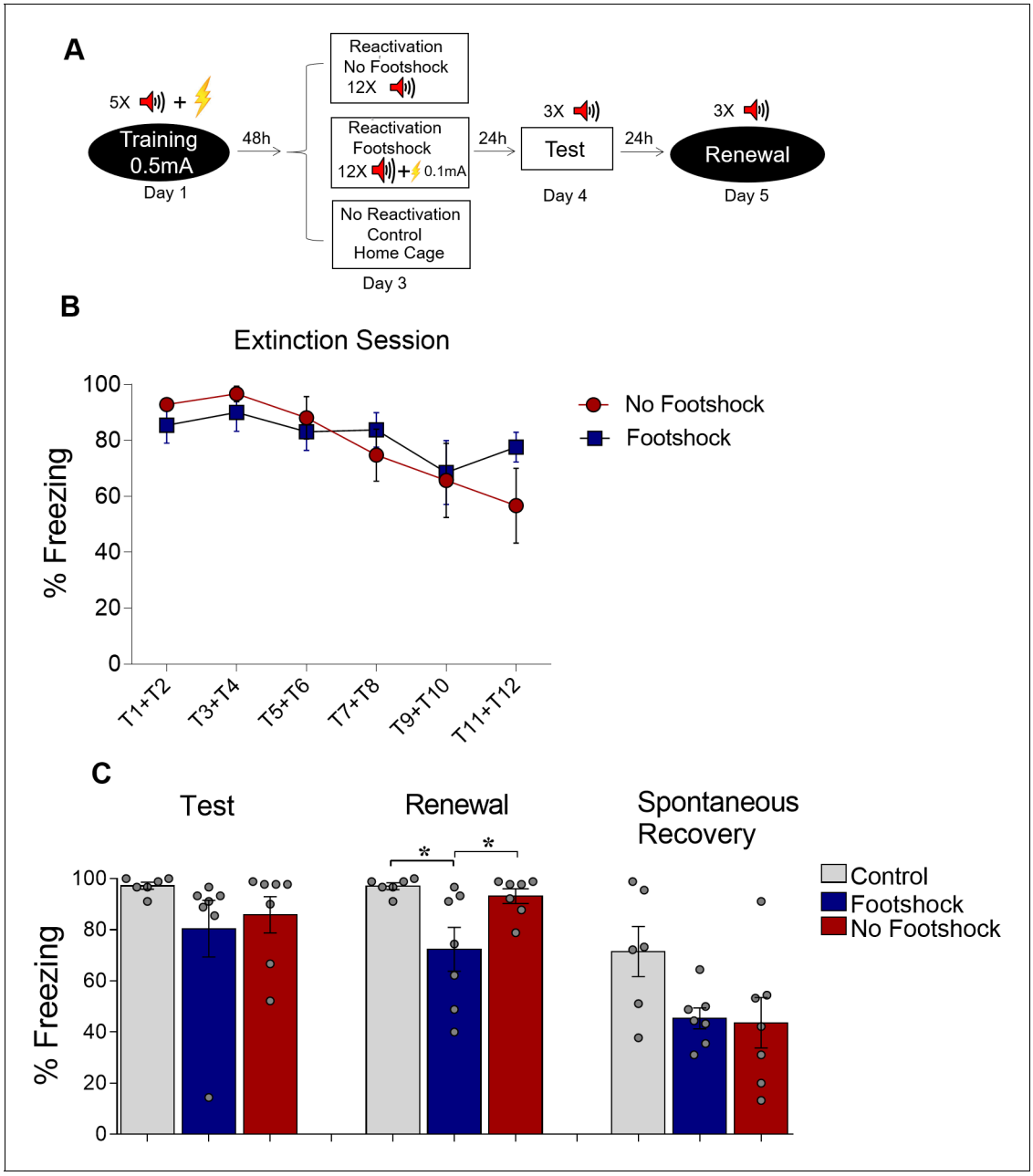


**Figure 4—figure supplement 1.** Effects of deconditioning-update in a single 12-CS extinction session. (**A**) Experimental design: rats were fear-conditioned with five tone-shock pairings (context B; 5 CS + US, 0.5mA). 48 hr later, both groups underwent a single extinction session (context A, 12 CSs) with or without a weak footshock (0.1 mA) at the end of tones. Animals then underwent test (context A), renewal (context B) and spontaneous recovery (context A) sessions. Black circles represent context A and white squares represent context B. (**B**) Freezing levels during the extinction session. No differences were found between the groups during extinction or in the test and spontaneous recovery sessions, but the deconditioning-update group showed less renewal (**C**). Bars represent mean ± SEM. Statistical comparisons are performed using two-way repeated-measures ANOVA followed by a Bonferroni post-hoc (extinction session) or one-way ANOVA followed by a Tukey post-hoc (test, renewal and spontaneous recovery). For full statistics, see ***Supplementary file 10***. For pre-CS freezing, see ***Supplementary file 20***.

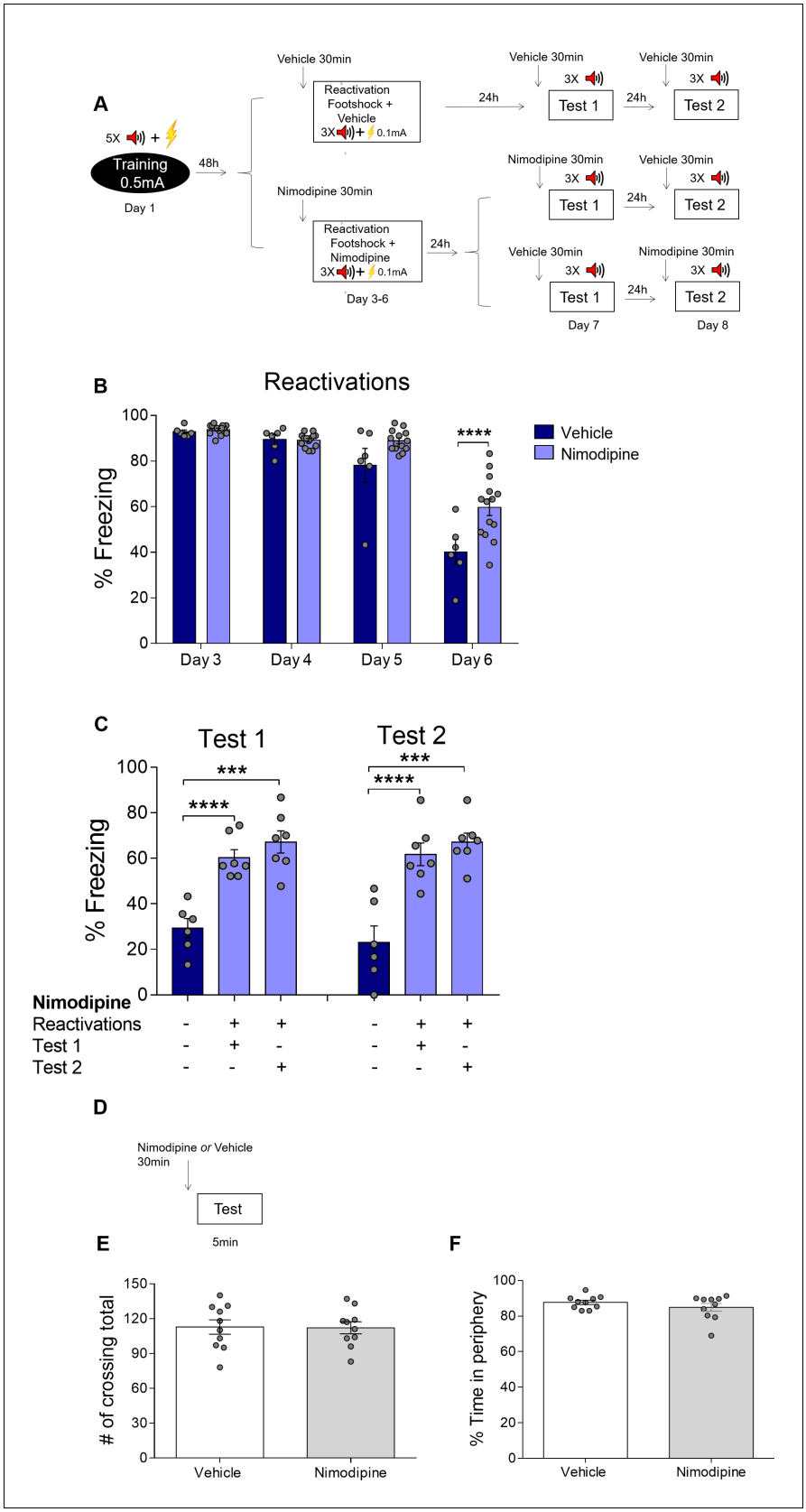


**Figure 4—figure supplement 2.** Nimodipine does not induce a state-dependent memory and does not affect open field behavior. (**A**) Experimental design: rats were fear-conditioned with five tone-shock pairings (context A;

*Figure 4—figure supplement 2 continued on next page*

*Figure 4—figure supplement 2 continued*

5CS+US, 0.5mA). 48 hr later, all animals underwent daily reactivation sessions (context B), receiving nimodipine (16 mg/kg, i.p.) or vehicle 30 min before each one. They were tested 24 hr (Test 1) and 48 hr (Test 2) later in context B. Half of the animals treated with nimodipine during reactivation received nimodipine and half received vehicle before Test 1; treatments were reversed in Test 2. Black circle represents context A, while white rectangles represent context B. (**B**) Nimodipine treatment before reactivation prevented freezing reduction across reactivation sessions. (**C**) Nimodipine injection before the test did not affect freezing expression, suggesting that it prevented deconditioning instead of inducing state-dependent extinction. Sessions in which each group received nimodipine are marked with plus signs below the graph, while those in which vehicle was given are marked with minus signs. (**D**) Experimental design of the open field task. Nimodipine administered 30 min before the test did not influence the number of crossings (**E**) or time spent in the periphery of the arena (**F**). Bars represent mean ± SEM. Statistical comparisons are performed using two-way repeated-measures ANOVA followed by a Bonferroni post-hoc (reactivation sessions), one-way ANOVA followed by a Tukey post-hoc (test sessions) or Student's t test (open field test). ****p<0.0001. For full statistics, see ***Supplementary file 11***. For pre-CS freezing, see ***Supplementary file 21***.

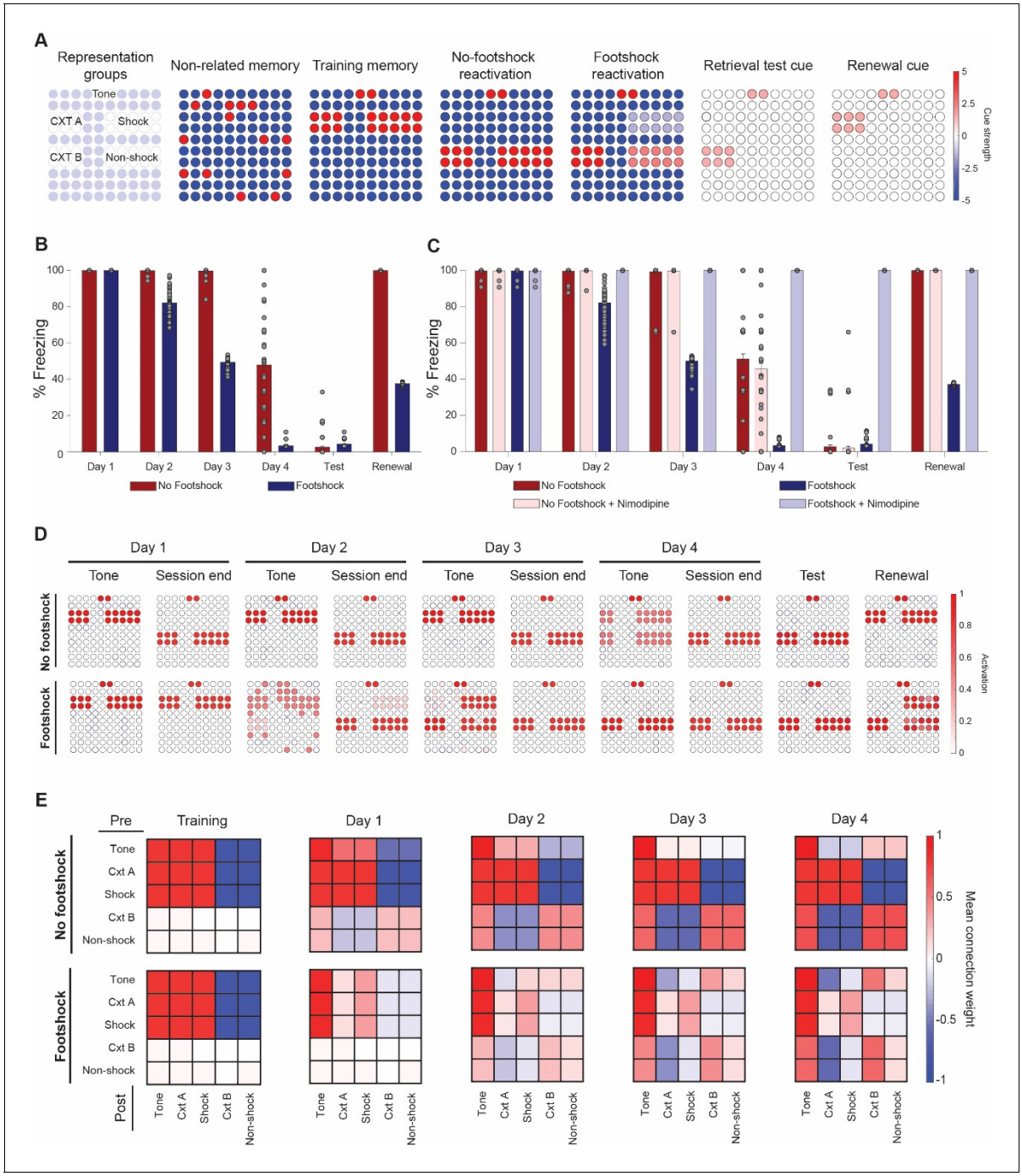


**Figure 5.** Lower mismatch accelerates fear reduction and decreases renewal in a neural network model. (**A**) Cue inputs presented to the network during training (shock memory), reexposure (with or without footshock) and test sessions (consisting of the tone and either context B (test) or A (renewal)). Color scale shows the cue received by each of the 100 neurons (**B**) Extinction over multiple sessions using the no-footshock (red bars) or footshock (blue bars) cue. Bars represent freezing, expressed as the activity ratio between shock neurons and the sum of shock and non-shock neurons in response to the test cue, at reexposure days 1 to 4. After each test, memory is updated according to the activity reached in response to the full reexposure pattern. (**C**) Effect of LVGCC blockade (i.e. setting the mismatch-induced degradation term D to 0). Removing the degradation term blocks deconditioning-update, but not regular extinction. (**D**) Network activity in retrieval tests during tone presentations (e.g. cued with the tone alone) and at the end of reexposure (e.g. cued with the full reactivation pattern), as well as on test and retrieval sessions. Lower mismatch (i.e. weak footshock) leads to retrieval of the original pattern on the first days, leading to memory updating through mismatch-induced degradation and lower retrieval on subsequent tests. (**E**) Mean synaptic weights between different neuronal groups after training and at the end of each extinction session. Heat map represents the connection from neuronal populations in the Y axis to those in the X axis in the no-footshock and footshock groups. Deconditioning-update leads to

*Figure 5 continued on next page*

*Figure 5 continued*

weakening of connections between context and shock neurons and of their inhibitory connections to other neurons. On no-footshock extinction, an extinction memory is formed with sparing of the shock representation.

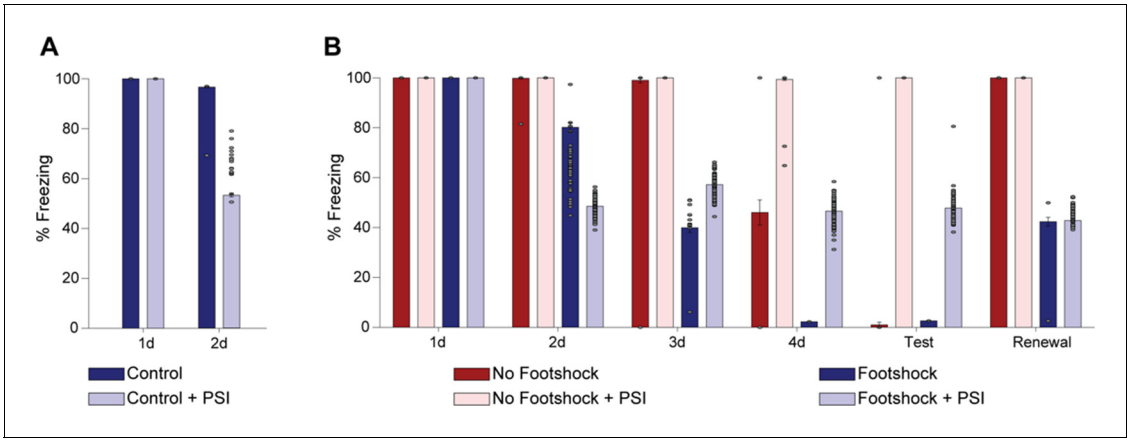


**Figure 5—figure supplement 1.** Blockade of Hebbian plasticity impairs reconsolidation, blocks standard extinction and interacts with deconditioning-update. (**A**) Effect of protein synthesis inhibition (modeled by setting the Hebbian plasticity term S to 0 in the model) during reexposure with a low degree of mismatch that does not induce extinction (i.e. reconsolidation condition). Bars represent freezing, expressed as the activity ratio between shock neurons and the sum of shock and non-shock neurons in response to the test cue, before and after a reexposure with either vehicle (dark bars, Hebbian plasticity on) or a protein synthesis inhibitor (light bars, Hebbian plasticity off). Unlike in deconditioning-update, which requires several sessions, freezing decreases after a single reexposure session under protein synthesis blockade. (**B**) Effect of protein synthesis inhibition on regular extinction (red bars) or deconditioning-update (blue bars), as defined by the patterns in ***Figure 5A***. Blockade of Hebbian plasticity inhibits extinction with a pure extinction pattern, which is fully based on learning a new attractor; however, it causes deconditioning-update to initially progress faster due to greater weakening of the original memory. On the long run, however, extinction is impaired in this group as well due to blockade of new learning.

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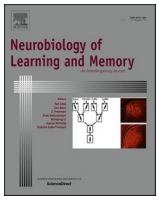

# Characterization of deconditioning-update on fear memory attenuation

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ABSTRACT

Fear memory expression can be attenuated by updating the footshock perception during the plastic state induced by retrieval, from a strong unconditioned stimulus to a very weak one through deconditioning. In this process, the original fear association of the conditioned stimulus with the footshock is substituted by an innocuous stimulus and the animals no longer express a fear response. In the present study, we explore the boundaries of this deconditioning-update strategy by the characterization of this phenomenon. We found that there is an optimal mismatch between the footshock intensity delivered in the training and in the reactivation. Likewise, we characterized the temporal window that the protocol is efficient in hindering fear response. Our findings contribute to a better understanding of the limits in which deconditioning acts in attenuating fear memory, so that an optimized protocol using this strategy can be planned in order to deal with emotional disorders.

## 1. Introduction

Fear memory retrieval might induce a labile state (memory destabilization), allowing changes in memory strength and/or its content. (Nader, 2015; Nader et al., 2000). This process known as reconsolidation allows that remote memories be updated in accordance with the demands of a continuously changing environment (Suzuki et al., 2004). Thus when there is a mismatch between the original memory content and what is presented during retrieval, memory is destabilized and updated (Alfei et al., 2015).

In the last decade, several studies have explored this possibility to modify memory by interfering pharmacologically with reconsolidation in order to hinder fear memory (Monfils et al., 2009). The advantage of interfering with reconsolidation process when compared with extinction-based therapies, relies on the observation that fear reduction is robust and long-lasting (e.g., without the reemergence of the fear response). However, reconsolidation-based strategy presents some limitations if we consider that most of the treatments that interfere with memory reconsolidation are toxic (except few pharmacological interventions such as propranolol (Kindt et al., 2014) and ketamine (Das et al.,2019)) and cannot be readily administered to humans. Moreover, there are some boundary conditions (e.g. memory age and fear memory strength) that constrain memory reconsolidation (Suzuki et al., 2004; Wang et al., 2009).

It has been recently shown a novel approach to erase traumatic memories called deconditioning-update in fear conditioned animals (Popik et al., 2020). In this study, the strong shock information associated with a tone acquired during conditioning was replaced by an extremely low footshock during the plastic state induced by memory retrieval, resulting in a permanent reduction of fear response (Popik et al., 2020). Remarkably, this new strategy broke some boundary conditions, as it was effective in eliminating either a remote or strong traumatic memory. Also, the same beneficial effect was also found in other type of fear-related memories, such as step-through inhibitory avoidance and contextual fear conditioning. Moreover, it was insensitive to renewal, spontaneous recovery, and savings suggesting a permanent update in fear memory.

Although interventions based on reconsolidation mechanisms are attractive from a therapeutic point of view, the use of pharmacological tools commonly precludes their application in humans, while behavioral interventions may be more translational. Thus, the deconditioning-update protocol stands out for its results, and translational potential, without needing pharmacological interventions. In our previous study, we have shown that the deconditioning-update strategy is effective in reducing fear response. Here, we provided a full characterization on the limits in which deconditioning would be effective in attenuating fear

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expression. Thus, we identified the lower and higher footshock intensity range in the training, the interval lengths among the reactivations, and the interval between conditioning and reactivations.

## 2. Material and methods

### 2.1. Subjects in behavioral studies

A total of 163 male Wistar rats (2–3 months old, weighing approximately 350 g) from Center for Reproduction and Experimentation of Laboratory Animals (CREAL) at the Federal University of Rio Grande do Sul (UFRGS) were used for the experiments. They were housed in

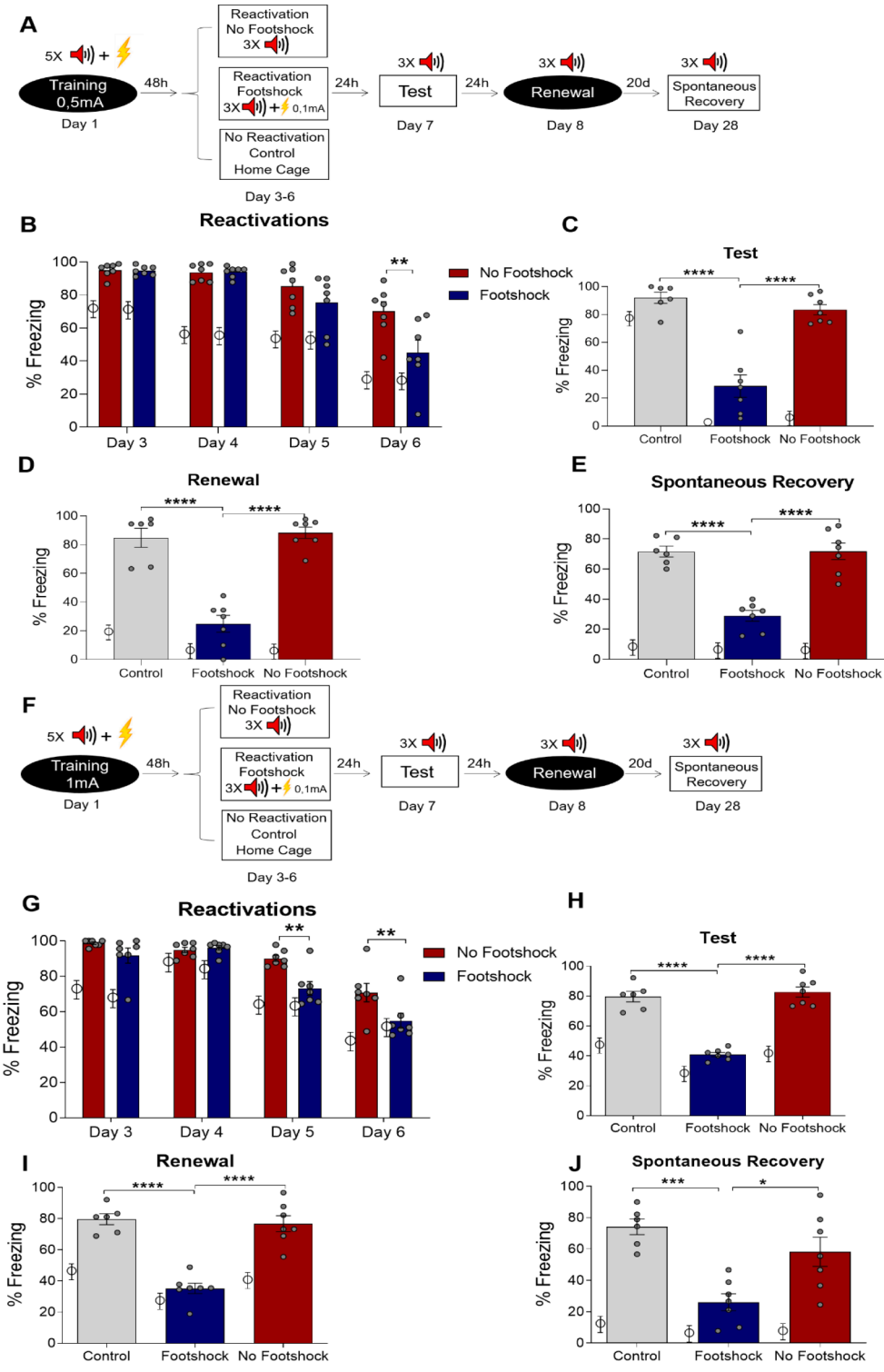


**Fig. 1. Deconditioning attenuates fear memory. (A) Experimental design:** male Wistar rats were conditioned in context A with 5 CS + US of 0.5 mA, 48 h later, 4 daily reactivation sessions (context B), subsequently subjected to test sessions (context B), renewal (context A), and spontaneous recovery (context B). Black circles represent context A, while white rectangles represent context B. **(B)** Levels of freezing during reactivation sessions. The footshock group (deconditioning group) shows a significant reduction in fear behavior during reactivation sessions, maintained during the test **(C)**, renewal **(D)**, and spontaneous recovery **(E)**. **(F)** Experimental design with stronger training (5 CS + US, 1 mA). **(G)** Levels of freezing during reactivation sessions. The footshock group shows a significant reduction in fear behavior during reactivation sessions, maintained during the test **(H)**, renewal **(I)**, and spontaneous recovery **(J)**. Bars represent mean ± SEM. Statistical comparisons were performed using two-way repeated-measures ANOVA followed by a Bonferroni post-hoc (reactivation sessions) or one-way ANOVA followed by Tukey post-hoc (test, renewal, and spontaneous recovery). **p < 0.005; *** p < 0.0005; **** p < 0.0001; ⊕ pre-CS. For full statistics (Table 1) and pre-CS freezing values (Table 1.1), see the supplementary material.

plexiglass boxes, with four animals per cage, with block randomization using the cage as subgroup to ensure that each cage contained at least one animal per experimental group. Animals were kept on 12/12 h light/dark cycle under controlled temperature (21℃ ± 2), with regular food and water available ad libitum and humidity of approximately 65%. All experiments were performed during the light cycle. All procedures followed the Brazilian ethical guidelines for animal research set by the National Council for the Control of Experimental Animal Research (CONCEA).

### 2.2. Auditory fear conditioning

#### 2.2.1. Apparatus

The conditioning chamber (context A) consisted of an illuminated plexiglass box (33 × 22 × 22 cm), with a floor grid of parallel 0.1-cm caliber stainless steel bars spaced 1 cm apart. All context chambers had the same dimensions, but context A had black walls and a different texture (sandpaper glued to the wall), whereas context B was vertically striped in black and white and had a smooth texture.

#### 2.2.2. Training session

Rats were habituated for 2 days in context B (10 min each day), and 24 h later were placed in context A, where they received five conditioning trials consisting of a 30 s presentation of a 5-kHz, 75-dB tone (CS) that co-terminated with a 0.5 mA (0.3 in Figs. 1, 1mA in Fig. 3 or 1.5 mA in Fig. 2), 1 s footshock (US) (five tone-footshock pairings). The first CS was presented 2 min into the session, with an interpairing interval of 1 min between each tone/footshock. One minute after the final pairing, rats were returned to their home cages.

#### 2.2.3. Reactivation sessions

In daily sessions taking place in context B (four reactivation sessions) and starting 48 h after training, animals in the no-footshock group received 3 CS-only, while the footshock group (deconditioning-update) received 3 CSs that co-terminated with a 0.1 mA, 1 s shock. The reaction to the 0.1 mA shock looks like a very small scare/startle. The percentage

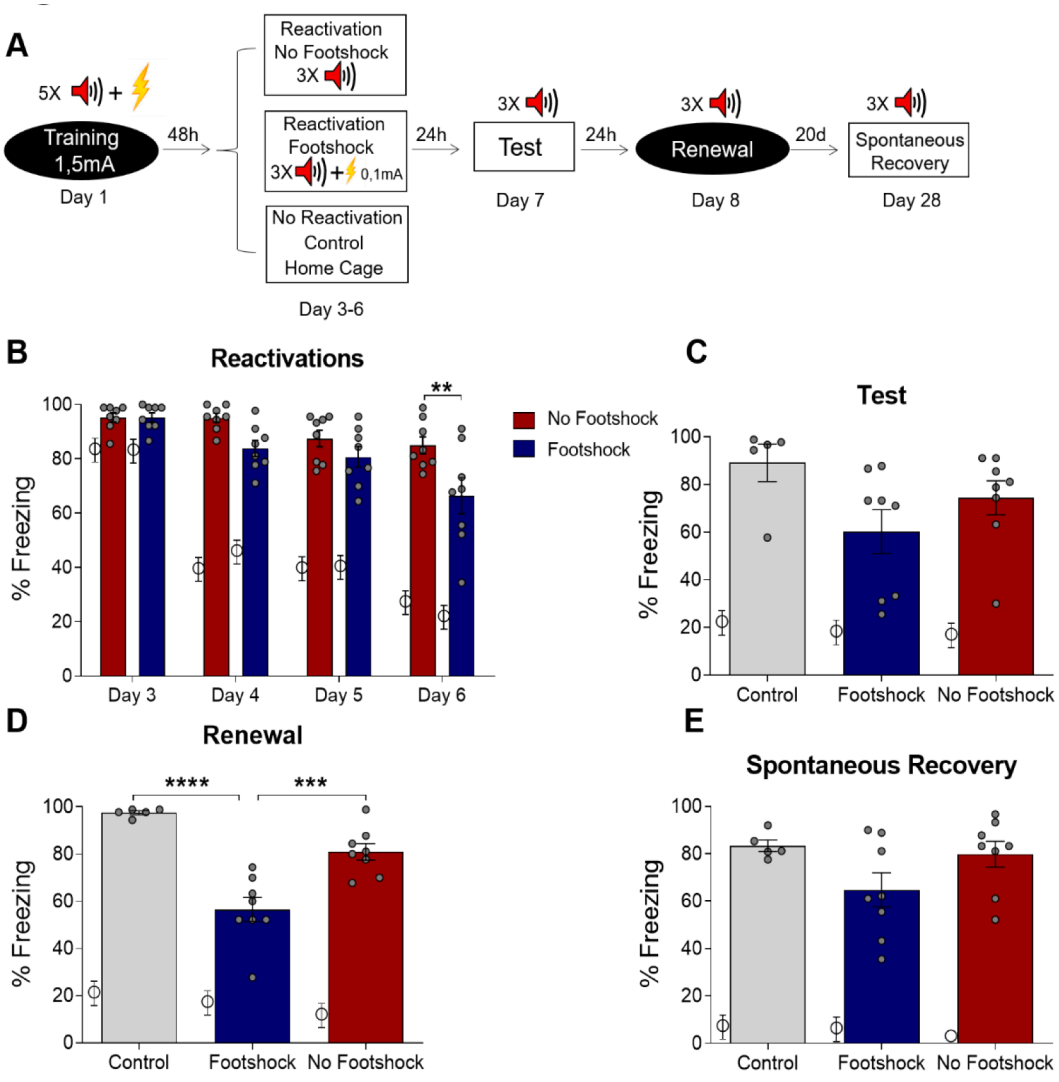


**Fig. 2. Very strong training protocol constrains the deconditioning effect.** (**A**) Experimental design: male Wistar rats were conditioned in context A with 5 CS + US of 1.5 mA, 48 h later, 4 daily reactivation sessions (context B), subsequently subjected to test sessions (context B), renewal (context A), and spontaneous recovery (context B). Black circles represent context A, while white rectangles represent context B. (**B**) Levels of freezing during reactivation sessions. The footshock group shows a significant reduction in fear behavior during reactivation sessions but did not maintain it during the test (**C**), showing a significant difference in renewal (**D**) and no longer existing in spontaneous recovery (**E**). Bars represent mean ± SEM. Statistical comparisons were performed using two-way repeated-measures ANOVA followed by a Bonferroni post-hoc (reactivation sessions) or one-way ANOVA followed by Tukey post-hoc (test, renewal, and spontaneous recovery). **p < 0.005; *** p < 0.0005; **** p < 0.0001; ⊕ pre-CS. For full statistics (Table 2) and pre-CS freezing values (Table 2.1), see the supplementary material.

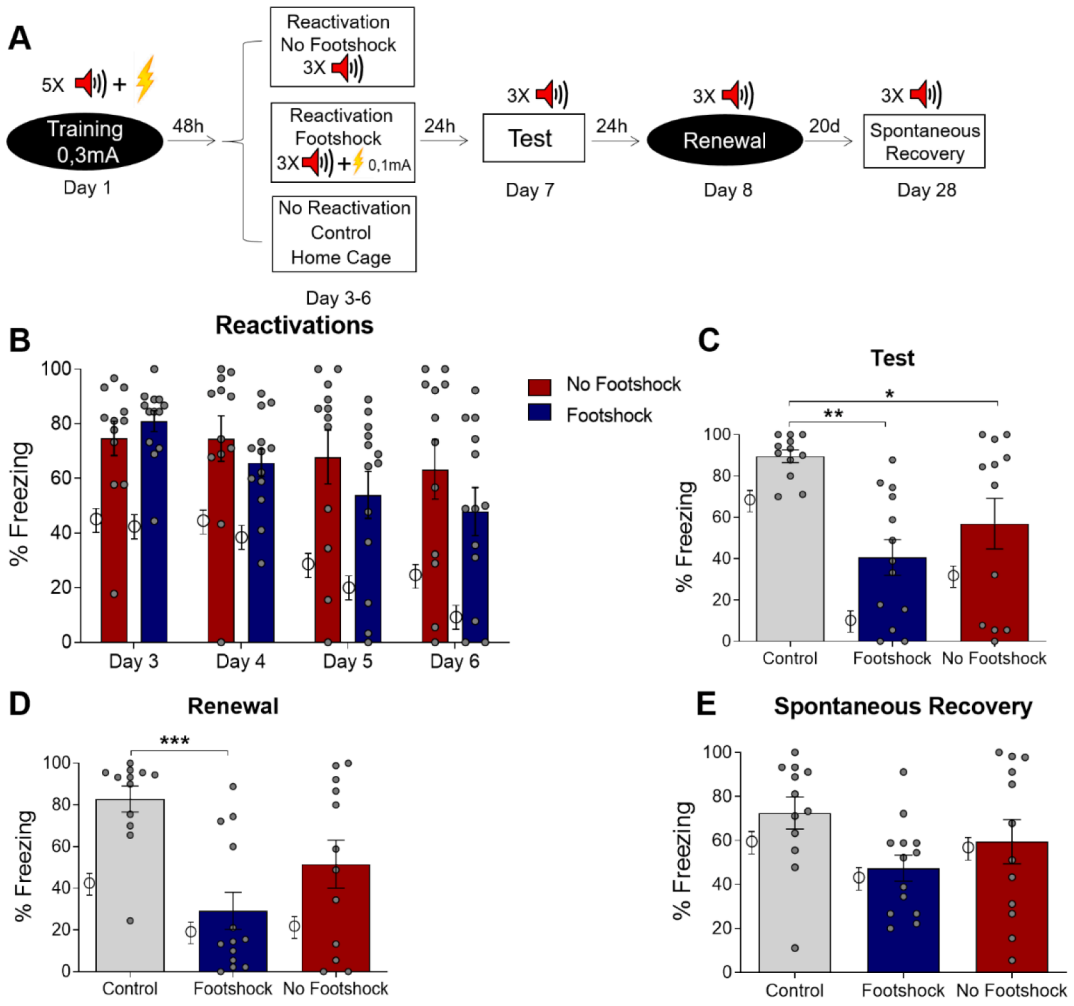


**Fig. 3. Low mismatch generates a lower fear memory reduction.** (**A**) Experimental design: male Wistar rats were conditioned in context A with 5 CS + US of 0.3 mA, 48 h later, 4 daily reactivation sessions (context B), subsequently subjected to test sessions (context B), renewal (context A), and spontaneous recovery (context B). Black circles represent context A, while white rectangles represent context B. (**B**) Levels of freezing during reactivation sessions. The footshock group shows a subtle reduction in fear behavior during the reactivation sessions, being significant during the test (**C**) and renewal (**D**), but not in the spontaneous recovery (**E**). Bars represent mean ± SEM. Statistical comparisons were performed using two-way repeated-measures ANOVA followed by a Bonferroni post-hoc (reactivation sessions) or one-way ANOVA followed by Tukey post-hoc (test, renewal, and spontaneous recovery). *$p < 0.05$; **$p < 0.005$; *** $p < 0.0005$; ⌀ pre-CS. For full statistics (Table 3) and pre-CS freezing values (Table 3.1), see the supplementary material.

of time freezing during each tone presentation was quantified and the mean freezing percentage for the three tones was used as a measure of fear. One minute after the final pairing, rats were returned to their home cages.

### 2.2.4. Test session

One day after the last reactivation session, both groups were presented with 3 CSs in context B. The percentage of time freezing during each tone presentation was quantified, and the average for the three tones was used as a fear measure. One minute after the final pairing, rats were returned to their home cages.

### 2.2.5. Renewal

One day after the test session, animals were placed in context A, where they received 3CSs. The percentage of time freezing during each tone presentation was quantified, and the average percentage was used as a measure of fear recovery. One minute after the final pairing, rats were returned to their home cages.

### 2.2.6. Spontaneous recovery

20 days after the renewal session, animals were placed in context B and received 3 CSs. The percentage of time freezing during each tone presentation was quantified and the average was used as a measure of fear recovery. One minute after the final pairing, rats were returned to their home cages.

### 2.2.7. Single-session extinction

Animals were placed in context B 10 min after training, where they received 12 CSs. In the no-footshock group these tones were not accompanied by the US, while the footshock group received tones that co-terminated with a 0.1-mA footshock. One minute after the final pairing, rats were returned to their home cages.

## 2.3. Behavioral assessment

Freezing behavior was used as a memory index in the fear conditioning tasks, being registered with a stopwatch in real time by an experienced observer that was blinded to the experimental group. Freezing was defined as total cessation of all movements except those required for respiration.

### 2.4. Statistical analysis

Data are expressed as mean ± SEM, always using the animal as the experimental unit. The statistical tests used and their results are detailed for every experiment in Supplementary file; they include two-tailed Student's *t* test; one-way or two-way analysis of variance (ANOVA), followed by Tukey's or Bonferroni's post hoc test. Values of $p < 0.05$ were considered statistically significant. Sample sizes were established in accordance with our previous study (Popik et al., 2020) and based on power analysis, which estimated between 70% and 90% to detect an absolute difference of 40% in freezing, with a standard deviation of 15%.

## 3. Results

### 3.1. Very strong training intensity might constrain deconditioning effect on fear reduction

We have first subjected a batch of rats to our standard deconditioning protocol. Thus, we trained the animals on the auditory fear conditioning task, with 5 tones (CS) paired with 0.5 mA shocks of 1 s each (context A) in the day 1. Animals were then divided into three groups: (1) the control group, which were not subjected to reactivations; (2) the non-shock group, which receives three tones in each reactivation; and (3) the deconditioning group, which the tones were co-terminated with a 0.1 mA shock. The reactivations occurred on days 3 to 6 in the context B. On day 7, all animals were tested in the context B, and 24 h later they were re-tested in context A for renewal, and on day 28 they were re-exposed to context B to assess spontaneous recovery (Fig. 1A).

During the reactivation sessions, both groups showed a decrease in freezing (Two-way RM ANOVA time factor, $F_{(3,36)} = 46.53 P < 0.0001$), but in the footshock group it was more prominent (Two-way RM ANOVA group factor, $F_{(1,12)} = 3.685 P = 0.07$; interaction, $F_{(3,36)} = 5.430 P = 0.003$; Bonferroni post hoc between the groups on day 6 $P = 0.001$; Fig. 1B), likewise demonstrating a marked reduction in freezing compared to the non-shock groups and the homecage group in the test session (One-way ANOVA, $F_{(2,17)} = 36.11 P < 0.0001$; Tukey post hoc: control *vs* footshock $P < 0.0001$, footshock *vs* no footshock; $P < 0.0001$; Fig. 1C). In the same way, animals in the footshock group also showed lower levels of freezing in the renewal (One-way ANOVA, $F_{(2,17)} = 43.31 P < 0.0001$; Tukey post hoc: control *vs* footshock $P < 0.0001$, footshock *vs* no footshock; $P < 0.0001$; Fig. 1D) and spontaneous recovery (One-way ANOVA, $F_{(2,17)} = 32.57 P < 0.0001$; Tukey post hoc: control *vs* footshock $P < 0.0001$, footshock *vs* no footshock; $P < 0.0001$; Fig. 1E) tests compared to the footshock and homecage groups. In this way, we demonstrate that the deconditioning protocol attenuates fear memory in a robust and lasting way, as it rewrites its mnemonic trace (for complete statistics, Table 1 in the supplements).

We next submitted animals to the same protocol, except that the footshock intensity was increased to 1 mA in order to verify whether the deconditioning approach would be efficient to attenuate a stronger fear memory. Even doubling the shock intensity in the training session, the footshock group still showed reduced freezing levels in the reactivation sessions when compared to the footshock group (Two-way RM ANOVA time factor, $F_{(3,36)} = 50.16 P < 0.0001$; group factor, $F_{(1,12)} = 12.79 P = 0.003$; interaction, $F_{(3,36)} = 3.803 P = 0.01$; Bonferroni post hocbetween the groups on day 5 $P = 0.002$, and on day 6 $P = 0.005$; Fig. 1G). These reduced freezing levels were maintained in the test, renewal, and spontaneous recovery sessions (One-way ANOVA; $F_{(2,17)} = 67.86 P < 0.0001$, Tukey post hoc: control *vs* footshock $P < 0.0001$, footshock *vs* no footshock; $P < 0.0001$ Fig. 1H; $F_{(2,17)} = 37.38 P < 0.0001$, Tukey post hoc: control *vs* footshock $P < 0.0001$, footshock *vs* no footshock; $P < 0.0001$ Figure I, and $F_{(2,17)} = 12.00 P < 0.0001$, Tukey post hoc: control *vs* footshock $P = 0.0005$, footshock *vs* no footshock; $P = 0.01$ Figure J, respectively). Thus, we demonstrate that in addition to the protocol being efficient in attenuating a fear memory, it was also efficient in breaking one of the limiting conditions of reconsolidation.

Then, we subjected the animals to a very strong training protocol, in which they received 5 tones accompanied by 1.5 mA footshocks. We observed that the animals form a robust and inflexible fear memory, making it inaccessible to the deconditioning protocol. During reactivations, we noticed a slight decline in freezing expression in the footshock group compared to the footshock group (Two-way RM ANOVA time factor, $F_{(3,42)} = 19.68 P < 0.0001$; group factor, $F_{(1,14)} = 6.429 P = 0.02$; interaction, $F_{(3,42)} = 4.328 P = 0.009$; Bonferroni post hoc between the groups on day 6 $P = 0.001$, Fig. 2B). However, this attenuation of fear behavior in reactivations was not enough to update fear memory, since there are no differences among the groups in the test (One-way ANOVA, $F_{(2,18)} = 2.672 P = 0.09$; Fig. 2C). In the renewal test, the footshock group showed a reduction in freezing (One-way ANOVA, $F_{(2,18)} = 23.34 P < 0.0001$; post hoc Tukey control *vs* footshock $P < 0.0001$, footshock *vs* no footshock; $P = 0.0008$, Fig. 2D), but it was not maintained in spontaneous recovery test (One-way ANOVA, $F_{(2,18)} = 2.661 P = 0.09$; Fig. 2E). Thus, we suggest that this training intensity generates a permanent fear memory that cannot be destabilized/reconsolidated, even with the deconditioning protocol (for complete statistics, Table 2 in the supplements).

#### 3.1.1. Low mismatch generates an inferior fear memory reduction

After reaching the upper training intensity where the deconditioning could attenuate fear memory, we asked whether it would also have a lower intensity level. We predicted that a lower mismatch between the training and the reactivation footshock intensity would limit the deconditioning effect. Then, we used the same behavioral task and the same protocol, but now the animals were trained with (5 CS + US of 0.3 mA) as we can see in Fig. 3A.

Throughout the reactivations, there is a decrease in the freezing levels (Two-way RM ANOVA time factor, $F_{(3,69)} = 10.87 P < 0.0001$; group factor, $F_{(1,23)} = 0.6418 P = 0.4$; interaction, $F_{(3,69)} = 2.743 P = 0.04$; Fig. 3B) However, no statistical differences among the groups. The deconditioning footshock group exhibited a fear reduction in the test (One-way ANOVA, $F_{(2,34)} = 7.967 P = 0.001$, Tukey post hoc: control *vs* footshock $P = 0.001$, control *vs* no footshock $P = 0.03$; Fig. 3C) and renewal (One-way ANOVA, $F_{(2,34)} = 8.757 P = 0.0009$, post hoc Tukey control *vs* footshock $P = 0.0006$; Fig. 3D), but not in the spontaneous recovery (One-way ANOVA, $F_{(2,34)} = 2.585 P = 0.09$; Fig. 3E). It is possible that this smaller effect compared to the fear reduction found in the animals trained with the 0.5 and 1 mA training intensity be due to the lower prediction error between the 0.3 mA in the training and the 0.1 mA delivered during reactivation (for complete statistics, Table 3 in the supplements).

### 3.2. Distinct interval between the reactivations affects the deconditioning effect

After discovering the boundary conditions of deconditioning concerning the mismatch of training intensity and reactivations, we asked whether the interval between each reactivation would affect the deconditioning effect. Thus, animals were trained as in Fig. 1A and, 48 h after, they were submitted to four consecutive reactivations with an interval of 10 min between them (Fig. 4A). Our results show that this short interval is insufficient to reduce fear memory in the test in the deconditioning group ((Two-way RM ANOVA time factor, $F_{(3,36)} = 4.170 P = 0.01$; group factor, $F_{(1,12)} = 1.389 P = 0.2$; interaction, $F_{(3,36)} = 0.3366 P = 0.3$ Fig. 3B; One-way ANOVA, $F_{(2,17)} = 4.671 P = 0.02$, post hoc Tukey control *vs* no footshock $P = 0.01$ Fig. 4C). We then increased the interval between reactivations. Animals were subjected to 2 reactivations with 6 h interval during 2 days (Two-way RM ANOVA time factor, $F_{(3,48)} = 13.89$ $P < 0.0001$; group factor, $F_{(1,16)} = 2.388 P = 0.008$; interaction, $F_{(3,48)} = 3.636 P = 0.01$, post hoc Bonferroni no footshock *vs* footshock 4th $P = 0.003$ Fig. 2E). We have chosen those intervals to guarantee that each reactivation would be consolidated

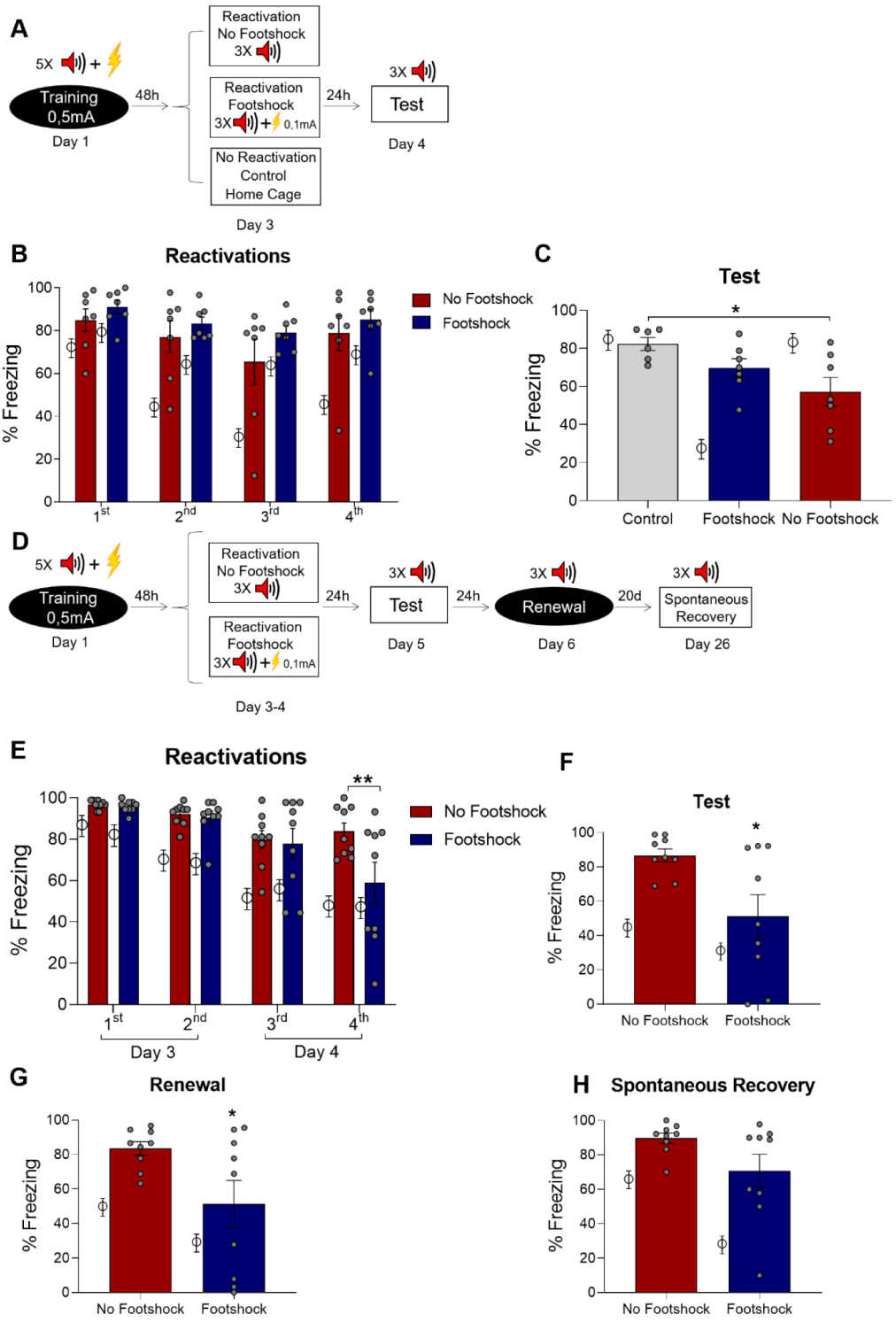


**Fig. 4. Distinct interval between the reactivations affect the deconditioning effect.** (**A**) Experimental design: male Wistar rats were conditioned in context A with 5 CS + US of 0.5 mA, 48 h later, 4 reactivation sessions with an interval of 10 min between them (context B), subsequently subjected to test sessions (context B), renewal (context A), and spontaneous recovery (context B). Black circles represent context A, while white rectangles represent context B. **(B)** Levels of freezing during reactivation sessions. There are no differences between the groups due to the short time between reactivations, maintaining the same behavior in test **(C)**. **(D)** Experimental design: the no footshock and footshock (deconditioning) groups were exposed to 2 reactivation sessions 6 h apart and the following day to 2 more reactivation sessions also 6 h apart (context B). **(E)** Levels of freezing during reactivation sessions. During the reactivation sessions, the footshock group showed a significant reduction in fear behavior, which was maintained during the test **(F)** and renewal **(G)**, but not during spontaneous recovery **(H)**. Bars represent mean ± SEM. Statistical comparisons were performed using two-way repeated-measures ANOVA followed by a Bonferroni post-hoc (reactivation sessions) or one-way ANOVA followed by Tukey post-hoc (test, renewal, and spontaneous recovery). *$p < 0.05$; ** $p < 0.005$; ⊕ pre-CS. For full statistics (Table 4) and pre-CS freezing values (Table 4.1), see the supplementary material.

before being reexposed to the next reactivation. This seems to be the minimum time required for the deconditioning protocol to update the aversive memory, as the footshock group shows a significant reduction in freezing compared to the no footshock group (Fig. 4E). Similar results were found in the test (Student's *t* test, $T_{16} = 2.710 P = 0.01$, Fig. 4F) and renewal (Student's *t* test, $T_{16} = 2.269 P = 0.03$, Fig. 4G). However, this protocol did not prevent spontaneous recovery (Student's *t* test, $T_{16} = 1.889 P = 0.07$, Fig. 4H). For complete statistics, Table 4 in the supplements).

### 3.3. The deconditioning protocol does not affect memory when applied during the time window of consolidation

It has been shown that memory can be erased when a extinction training is performed few minutes after fear memory conditioning (Giustino et al., 2017) or memory reactivation (Monfils et al., 2009) (although many studies have failed to replicate these findings (Chan et al., 2010; Stafford et al., 2013). We then asked whether the deconditioning protocol performed during the consolidation period of recently fear conditioned animals would interfere with the memory formation. Thus, animals were trained as in Fig. 1A, and 10 min later, they were subjected to reactivations for the deconditioning protocol. We used two reactivation protocols: first with 4 reactivations (3 CS each reactivation) with a 10 min interval between each one (Fig. 5A), and the second with a single block of 12 tones (to maintain the same number of tones) (Fig. 5D). In the deconditioning group of each protocol, the tone co-terminated with a 0.1 mA footshock.

We observed that the deconditioning protocol does not modify an unconsolidated fear memory, as there is no attenuation of freezing expression during the reactivations (Two-way RM ANOVA time factor, $F_{(3,36)} = 1.408 P = 0.25$; group factor, $F_{(1,12)} = 0.0294 P = 0.8$; interaction, $F_{(3,36)} = 2.136 P = 0.11$, Fig. 5B) nor in the test (Student's *t* test, $T_{12} = 0.09366 P = 0.9$, Fig. 5C) or renewal (Student's *t* test, $T_{12} = 1.026 P = 0.3$, Fig. 5D). Likewise, the deconditioning protocol did not modify the fear memory during consolidation when applied in a single session (Two-way RM ANOVA time factor, $F_{(5,60)} = 2.394 P = 0.04$; group factor, $F_{(1,12)} = 0.04497 P = 0.8$; interaction, $F_{(5,60)} = 0.6853 P = 0.6$ Fig. 5F; Student's *t* test, $T_{12} = 0.7043 P = 0.49$ Fig. 5G; Student's *t* test, $T_{12} = 1.368 P = 0.19$ Fig. 5H) (for complete statistics, Table 5 in the supplements). That is, the deconditioning protocol does not modify a memory that has not yet been consolidated.

## 4. Discussion

It has been previously demonstrated that the deconditioning approach is an efficient strategy to eliminate fear memory, updating it to a non-aversive level in a permanent form (i.e., without presenting spontaneous recovery, renewal, and savings). Moreover, we showed that it was also effective in distinct fear memory tasks such as inhibitory avoidance and contextual fear conditioning, and breaks some boundary conditions (Popik et al., 2020). In the present study, we explored the limits in which deconditioning would be effective in reducing fear response by analyzing the inferior and superior footshock intensity in the training, the interval ranges among the reactivations, and the interval between conditioning and reactivations.

Our findings stipulate what would be the limit that the weak shocks (0.1 mA) presented during the reactivation sessions that would act as a trigger to destabilize and reconsolidate the mnemonic trace. Considering that the mismatch between what is expected and what actually occurs during retrieval play an important role on memory destabilization, we predicted that a low footshock training could limit the deconditioning effect because of the fact that it would be too similar to the footshock delivered in the reactivation sessions. In fact, we showed a more robust effect in the reduction of the freezing response when animals were trained using a 0.5 mA or 1 mA training (Fig. 1) compared to animals trained with 0.3 mA (Fig. 3). It is possible that the deconditioning group presents a slight/partial fear reduction in this condition that is not sensitive in the test, since the no-footshock group also expressed a reduced freezing behavior (probably by starting the extinction process) due to the lower training intensity.

We also addressed the question of the limits in which the fear conditioning intensity would be effective in order to attenuate the freezing response by the deconditioning-update approach. Then, we submitted the animals to an extremely high training protocol, in which animals received 5 tone-shock pairing using 1.5 mA intensity. We found that the deconditioning-update did not reduce fear response under this extreme circumstances (Fig. 2). This result has been shown in other studies showing that strong aversive memory is immune to undergo reconsolidation (van Schie et al., 2017; Wichert et al., 2013). The main mechanism investigated underpinning this boundary condition is the GluN2B reduction caused by the strong training (Wang et al., 2009). It is noteworthy that although no difference between the groups was found in the test, the deconditioning group presented lower freezing levels in the last reactivation day and in the renewal test, suggesting a possible subtle effect that possibly could be improved by increasing the number of reactivation sessions.

After establishing the footshock training range that the deconditioning is effective, we asked which interval between each reactivation would present a better outcome. We have first tried to combine the 4-days reactivation in a single day, with interval of 10 min between each session. We found that this compact protocol failed to affect fear response in the test. Then, we created an intermediate 2-days reactivation protocol, in which two reactivation sessions were presented in each day (interval of 6 h between them). This intermediate protocol attenuated fear memory response in the tests. It is possible that each deconditioning reactivation requires to be "consolidated" before being reexposed to the next reactivation session in order to be effective in reducing fear expression. In fact, the labile state induced by learning or retrieval lasts few hours, and it is thought that this period is over around 4–6 h (Auchter et al., 2017; Casagrande et al., 2018; Wichert et al., 2013).

Memory consolidation is known to be a period during which interferences can modify the information is still in an unstable form (McGaugh, 2015; Okuda et al., 2004). Numerous studies demonstrate that it is possible to modify (weaken or strengthen) a fear memory when a stimulus is applied in the temporal window of consolidation (Fernandes et al., 2022; Popik et al., 2018; Redondo et al., 2020). We tested the hypothesis that the application of the deconditioning protocol in this consolidation time window would disrupt the fear memory genesis. However, the deconditioning protocol does not seem to interfere with the consolidation process (Fig. 5). Thus, we suggest that the mechanisms by which deconditioning acts are specific to reconsolidation. Interestingly, a recent study has shown that low footshock exposure 24 h after contextual fear conditioning during the reactivation session might increase (2 USs) or reduce (10US) fear expression in the test (Ferrara et al., 2019), suggesting that both the number of US presentation and interval between training and reactivation play an important role on memory updating.

Many studies have shown distinct approaches in order to reduce fear expression. Standard extinction is the most reported form to attenuate fear response. Devaluation of the US, spacing extinction, reactivation-extinction procedure and others have shown positive results in reducing fear relapse (Monfils et al., 2009). We have observed here that deconditioning also provide a promising approach that reduce freezing response in a long-lasting way. One could argue that the deconditioning effect is based on US habituation, revaluation or devaluation. We addressed this possibility by doing two experiments in which the same amount of 0.1 mA US was presented unpaired with the CS or in other context without the tone. In both cases, no fear reduction was observed in the test (Popik et al., 2020), suggesting that the deconditioning effect is due to the updating of the CS-US association. The fact that fear reduction is long-lasting, without spontaneous recovery, renewal and

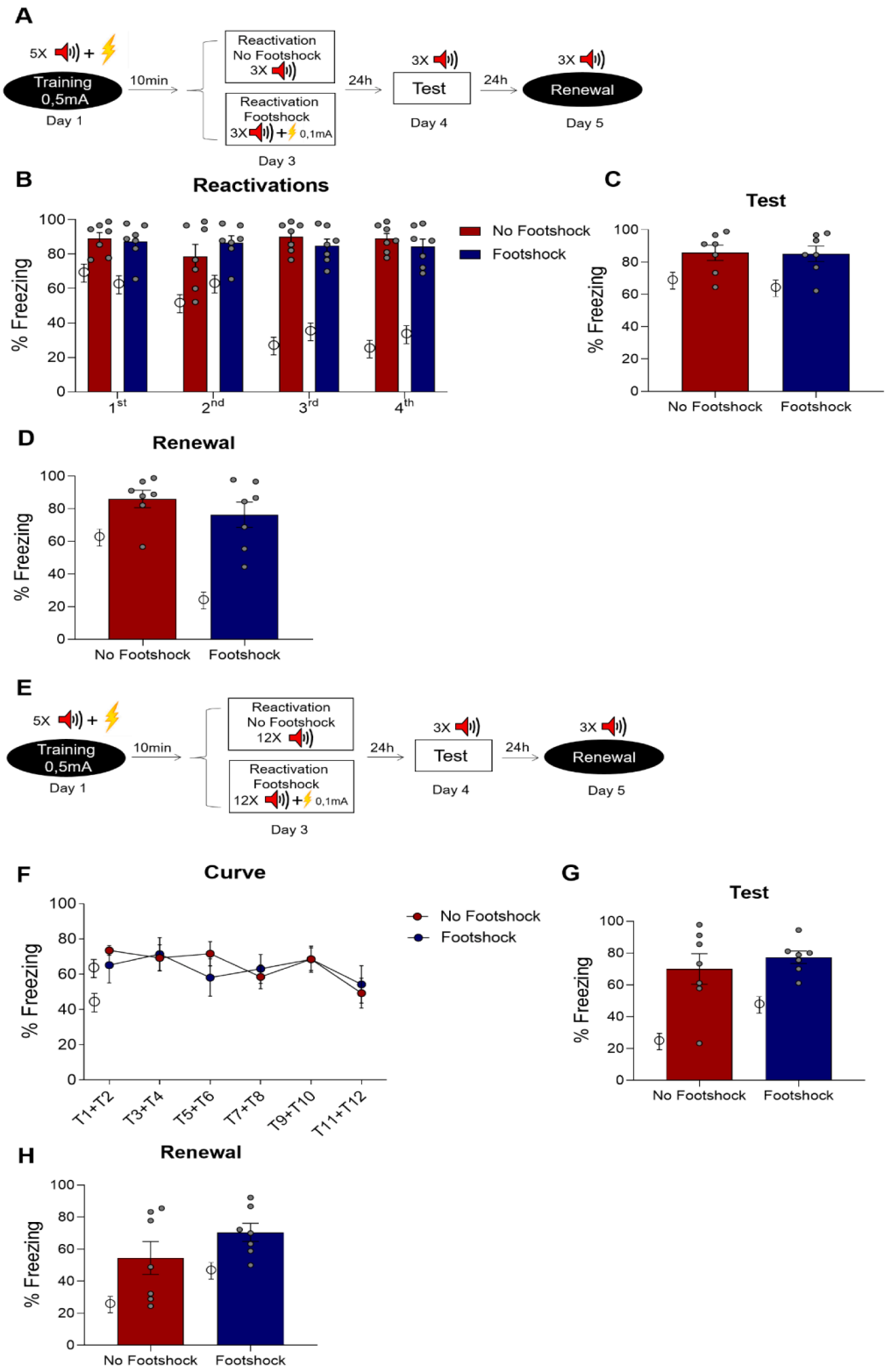


**Fig. 5. The deconditioning protocol does not affect memory when applied during the time window of consolidation.** (**A**) Experimental design: male Wistar rats were conditioned in context A with 5 CS + US of 0.5 mA, 10 min, 4 reactivation sessions with an interval of 10 min between them (context B), subsequently subjected to test sessions (context B) and renewal (context A). Black circles represent context A, while white rectangles represent context B. (**B**) Levels of freezing during reactivation sessions. There are no differences between the groups, similarly, we don't see differences in test **(C)**, and renewal **(D)**. **(E)** Experimental design: 10 min after training, a single reactivation session was applied. **(F)** Levels of freezing during the reactivation session. There are no differences between the groups, likewise, we don't see differences in test **(G)** and renewal **(H)**. Bars represent mean ± SEM. Statistical comparisons were performed using two-way repeated-measures ANOVA (reactivation sessions) or Student *t* test (test, renewal, and spontaneous recovery). ⊕ pre-CS. For full statistics (Table 5) and pre-CS freezing values (Table 5.1), see the supplementary material.

savings also is in line with the hypothesis that the deconditioning-updating effect is mediated by the destabilization/reconsolidation. These properties differ from the extinction assumption that commonly shows fear relapse. Moreover, this fear reduction effect is prevented by LVGCC antagonist (a mechanism closely associated with memory destabilization). However, we cannot rule out the possibility that the fear reduction described here be in consequence of a deepened extinction that prevents fear relapse or the value change in the footshock by modifying the original US representation. In fact a recent study has shown that the weak shock presentation in an alternative context decreases the fear response to the conditioned context (Bortolon et al., 2023). It is also possible that the weak footshock presentation might undergo either memory destabilization/reconsolidation or US devaluation (or none of them), depending on the original training strength and the reactivation peculiarities in which the low US is presented. For instance, weak footshock could lead to deconditioning while a single session containing several weak footshock could undergo US devaluation. Future studies using molecular signatures for these processes might elucidate this question.

It is well established that a discrepancy between what is expected and what actually happens during retrieval (i.e. prediction error) play an essential role on memory destabilization (Agustina López et al., 2016; Sinclair et al., 2021). Here, there is a prediction error when animals are fear conditioned using a 0.5 mA during training and receive a much lower US (0.1 mA) during reactivation. This mismatch might be important to induces the labile state during reactivation (Fernández et al., 2016), and then the previous CS-high US association is updated and replaced by the CS-low US association. The lower prediction error presented in Fig. 3 (0.3 mA in the training and 0.1 mA US in the reactivation) might explain the smaller deconditioning effect.

Taken together, our results demonstrated that the deconditioning protocol is effective in reducing fear memory in distinct circumstances, and that the training intensity range of effectiveness play an important role in the fear reduction outcome. We also set the optimal interval time between reactivation sessions to update memory to a less aversive level. We believe that our new strategy to attenuate traumatic memories is a promising therapeutic avenue that needs to be deeply explored. Some advantages including its high efficacy, its long-lasting beneficial effects, and its drug-free feature make this approach especially appealing to be translated to humans.

## CRediT authorship contribution statement

**Bruno Popik:** Conceptualization, Methodology, Project administration, Formal analysis. **Kétlyn Talise Knak Guerra:** Methodology. **Jordana Griebler Luft:** Methodology. **Henrique Schaan Fernandes:** Methodology. **Lucas de Oliveira Alvares:** Conceptualization, Supervision, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

doi:10.17632/rr8gchm94z.1

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nlm.2023.107763.

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RESEARCH ARTICLE

WILEY

# Molecular mechanisms underpinning deconditioning-update in fear memory

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## Abstract

Traumatic experiences are closely associated with some psychiatric conditions such as post-traumatic stress disorder. Deconditioning-update promotes robust and long-lasting attenuation of aversive memories. The deconditioning protocol consists of applying weak/neutral footshocks during reactivations, so that the original tone-shock association is replaced by an innocuous stimulus that does not produce significant fear response. Here, we present the molecular bases that can support this mechanism. To this end, we used pharmacological tools to inhibit the activity of ionotropic glutamate receptors (NMDA-GluN2B and CP-AMPA), the activity of proteases (calpains), and the receptors that control intracellular calcium storage (IP3 receptors), as well as the endocannabinoid system (CB1). Our results indicate that blocking these molecular targets prevents fear memory update by deconditioning. Therefore, this study uncovered the molecular substrate of deconditioning-update strategy, and, broadly, shed new light on the traumatic memory destabilization mechanisms that might be used to break the boundaries regarding reconsolidation-based approaches to deal with maladaptive memories.

**KEYWORDS**
endocannabinoid system, glutamatergic system, intracellular calcium, protease, update

## 1 | INTRODUCTION

The dynamic nature of memory allows that a well-established memory be modified through the destabilization–reconsolidation cycle induced by retrieval (Nader et al., 2000). This plastic process opens the possibility to change either the memory content or the strength of maladaptive memories such as traumas. Indeed, studies showing that memories associated with traumatic or drug-related events can be manipulated through reconsolidation mechanisms have generated huge interest in the field.

Numerous reports have attempted to interfere with the reconsolidation process in order to attenuate maladaptive memories (Fukushima et al., 2014; Tronel et al., 2005). The great excitement regarding reconsolidation, when compared with extinction, is due to the fact that it induces a long-lasting improvement (e.g., without presenting fear relapse) (Monfils et al., 2009). However, usually reconsolidation is manipulated with pharmacological interference, and most of them are toxic and cannot be used as a treatment in humans.

We have recently demonstrated that it is possible to robustly reduce fear expression by using the deconditioning-update protocol. This strategy relies on the replacement of the strong footshock by a weak footshock previously associated with a CS (such as a tone or context) during reactivation. Importantly, this memory updating does not rely on pharmacological interference. Moreover, it is long-lasting and no fear relapse is observed in renewal, reinstatement, savings, or spontaneous recovery tests (Popik et al., 2020).

In the present study, we provided a molecular characterization on the mechanisms underpinning the deconditioning-update process. Specifically, we selected some main mechanisms that seem to be particularly important to modifying preexistent memories such as $Ca^{++}$

  

influx through CP-AMPA, IP3 receptors and GluN2B-NMDAR, modulation of the endocannabinoid system, and the $Ca^{++}$ dependent protease calpain.

## 2 | MATERIALS AND METHODS

### 2.1 | Subjects in behavioral studies

A total of 94 male Wistar rats (2–3 months old, weighing approximately 350 g) from Center for Reproduction and Experimentation of Laboratory Animals (CREAL) at the Federal University of Rio Grande do Sul (UFRGS) were used for the experiments. They were housed in plexiglass boxes, with four animals per cage, with block randomization using the cage as subgroup to ensure that each cage contained at least one animal per experimental group. Animals were kept on 12/12 h light/dark cycle under controlled temperature (21 ± 2°C), with regular chow and water available ad libitum and humidity of approximately 65%. All experiments were performed during the light cycle. All procedures followed the Brazilian ethical guidelines for animal research set by the National Council for the Control of Experimental Animal Research (CONCEA).

### 2.2 | Auditory fear conditioning

#### 2.2.1 | Apparatus

The conditioning chamber (context A) consisted of an illuminated plexiglass box (33 × 22 × 22 cm), with a floor grid of parallel 0.1-cm caliber stainless steel bars spaced 1 cm apart. All context chambers had the same dimensions, but context A had black walls, whereas context B was vertically striped in black and white.

#### 2.2.2 | Training session

Rats were habituated for 2 days in context B (10 min each), in order to reduce the context-footshock association, and 24 h later were placed in context A, where they received five conditioning trials consisting of a 30 s presentation of a 5-kHz, 75-dB tone (CS) that co-terminated with a 0.5 mA, 1 s footshock (US) (five tone-footshock pairings). The first CS was presented 2 min into the session, with an interpairing interval of 1 min. One minute after the final pairing, rats were returned to their home cages.

#### 2.2.3 | Reactivation sessions

In daily sessions taking place in context B (four reactivation sessions) and starting 48 h after training, animals in the no-footshock group received three CS-only, while the footshock group (deconditioning-update) received 3 CSs that co-terminated with a 0.1 mA, 1 s shock. The percentage of time freezing during each tone presentation was quantified and the mean freezing percentage for the three tones was used as a measure of fear. The first CS was presented 2 min into the session, with an interpairing interval of 1 min. One minute after the final pairing, rats were returned to their home cages. Pre-CS freezing levels were measured for the 30 s immediately preceding the first tone.

#### 2.2.4 | Test session

One day after the last reactivation session, both groups were presented with three CSs in context B. The percentage of time freezing during each tone presentation was quantified, and the average for the three tones was used as a fear measure. The first CS was presented 2 min into the session, with an interpairing interval of 1 min. One minute after the final pairing, rats were returned to their home cages. Pre-CS freezing levels were measured for the 30 s immediately preceding the first tone.

#### 2.2.5 | Renewal

One day after the test session, animals were placed in context A, where they received three CSs. The percentage of time freezing during each tone presentation was quantified, and the average percentage was used as a measure of fear recovery. The first CS was presented 2 min into the session, with an interpairing interval of 1 min. One minute after the final pairing, rats were returned to their home cages. Pre-CS freezing levels were measured for the 30 s immediately preceding the first tone.

#### 2.2.6 | Spontaneous recovery

Twenty days after the renewal session, animals were placed in context B and received three CSs. The percentage of time freezing during each tone presentation was quantified and the average was used as a measure of fear recovery. The first CS was presented 2 min into the session, with an interpairing interval of 1 min. One minute after the final pairing, rats were returned to their home cages. Pre-CS freezing levels were measured for the 30 s immediately preceding the first tone. The protocol was based on Popik et al. (2020) and Popik et al. (2023).

### 2.3 | Behavioral assessment

Freezing behavior was used as a memory index in the fear conditioning tasks, being registered with a stopwatch in real time by an experienced observer that was blinded to the experimental group. Freezing was defined as total cessation of all movements except those required for respiration.

## 2.4 | Statistical analysis

Data are expressed as mean ± SEM, always using the animal as the experimental unit. The statistical tests used and their results are detailed for every experiment in Data S1; they include two-tailed Student's *t*-test; one-way, two-way, or three-way analysis of variance (ANOVA), followed by Tukey's or Bonferroni's post-hoc test. Values of $p < .05$ were considered statistically significant.

## 2.5 | Stereotaxic surgery and cannulae implantation

Rats were anesthetized by intraperitoneal injection with ketamine (75 mg/kg) and xylazine (10 mg/kg) and secured in a Kopf stereotaxic apparatus. Bilateral guide cannulae were targeted for placement directly above the intracerebroventricular (ICV), AP −0.2 mm, LL ±0.16 mm, DV −0.32 mm, from bregma and basolateral nuclei of the amygdala (BLA) AP −2.5 mm, LL ±5.1 mm, DV −7.0 mm, from bregma.

We used meloxicam (analgesic and nonsteroidal anti-inflammatory; 1 mg/kg; via subcutaneous) 20 min before surgery, as well as once a day in the following 2 days. Animals were allowed 5–7 days to recover before experimentation. Following the appropriate behavioral task, animals were euthanized, and their brains were collected to ensure accurate cannula placement. Animals with inaccurate cannula position were excluded from statistical analysis.

## 2.6 | Drugs, virus vector, and microinfusion

2-APB (2-aminoethyl diphenylborinate, Sigma-Aldrich), IP3 receptor antagonist (500 μM, dissolved in 10% dimethyl sulfoxide [DMSO]), and its vehicle (10% DMSO) (Fernandes et al., 2022). NASPM (1-naphthylacetyl spermine trihydrochloride), selective antagonist of calcium permeable AMPA receptors (CP-AMPA) (4 μM, dissolved in 1% DMSO), and its vehicle (1% DMSO) (Torquatto et al., 2019). PD150606 (Sigma-Aldrich), a specific inhibitor of calpain (1 μM; dissolved in 1% DMSO), and its vehicle (1% DMSO) (Popik et al., 2018). These drugs were infused unilaterally into the left or right lateral ventricle (via ICV), in a total volume of 5 μL. At the time of infusion, a 27-gauge needle was fitted to the 22-gauge guide cannula. All infusions were administered at a rate of 30 μL/h over 5 min and with the animals being gently held or restrained by a researcher. Rimonabant was dissolved in DMSO before further dilution in dissolved water (maximum final DMSO concentration, 1%, v/v). All injections were given through the i.p. route of administration in a volume of 1 mL/kg.

We used a viral vector that specifically inhibits the expression of the GluN2B-NMDA subunit. The virus was administered directly into the BLA at a volume of 1 μL per side and a rate of 15 μL/h (for more details, see Acutain et al., 2021). The AAV encoding a specific shRNA against GluN2B (AAVe-shGluN2B, shGluN2B: 5′-GAACGTGG ATGTCGGATCCTT-3′) (Gambrill & Barria, 2011) or a scrambled sequence without a known target in mammalian cells (AAVe-shsc, shsc: 5′-ACGTGACACGTTCGGAGAATT-3′) was as previously described (Ploquin et al., 2013). Each shRNA sequence (Gambrill & Barria, 2011) was cloned into the *Bbs*I site between the ITR sequences in the pBS-GFP-AAV-U6 plasmid. Viral stocks were produced by cotransfection with three plasmids: the pBS-GFP-U6-shGluN2B or pBS-GFP-U6-shsc, the helper, and the rep-cap plasmids in HEK 293SZ cells. The cells and supernatant were collected 48 h posttransfection, and viral stocks were purified by CsCl gradient and quantified by flow cytometry (Ploquin et al., 2013).

## 2.7 | Histology

The animals were anesthetized with ketamine and xylazine and decapitated. The brains were removed and fixed with 4% paraformaldehyde. A scalpel was used to obtain cross-sections of the brain and cannula placement was verified under a light microscope. Animals were only included in the analysis when a blinded experimenter verified that the placement of the cannulas had reached the ICV (see Figure S1). Nine animals were excluded of the experiment.

# 3 | RESULTS

## 3.1 | Inhibition of CB1 receptors blocks fear memory update by deconditioning

It has been recently shown that the CB1 receptors are closely involved in memory destabilization, behavioral flexibility, and updating (Lunardi et al., 2020; Suzuki et al., 2008). Thus, we investigated whether the deconditioning process depends on the endocannabinoid system through the activation of the CB1 receptors. Animals were fear conditioned with five tones (CS) paired with 0.5 mA shocks of 1 s each (context A—represented by a black circle). On subsequent days (day 3 to day 6) the animals were reactivated with three CSs during each reactivation in a different context (context B—represented by a white rectangle). During the reactivation sessions, half of the animals were subjected to the deconditioning protocol, in which a very weak footshock (0.1 mA) was delivered at the end of each tone. On day 7, all animals were tested and, 24 h later, retested in the training context for renewal, and on day 28, they were tested to assess spontaneous recovery (Figure 1a).

The CB1 antagonist rimonabant or vehicle (DMSO 1%) were injected intraperitoneally 30 min before each reactivation. In the course of reactivations, there is a marked decrease in freezing (three-way RM ANOVA: Time Factor $F_{(3,56)} = 23.94$, $p < .0001$; Drug Factor $F_{(1,56)} = 21.04$, $p < .0001$; for statistics see Table S1) in the footshock + vehicle (deconditioning) group when compared to the other groups (Post-hoc Bonferroni, $p = .02$; Figure 1b). The animals from the footshock + vehicle group showed a reduction of more than 50% in freezing compared to the other groups in the test (two-way ANOVA Interaction $F_{(1,28)} = 8.747$, $p = .006$; Drug Factor $F_{(1,28)} = 8.223$,

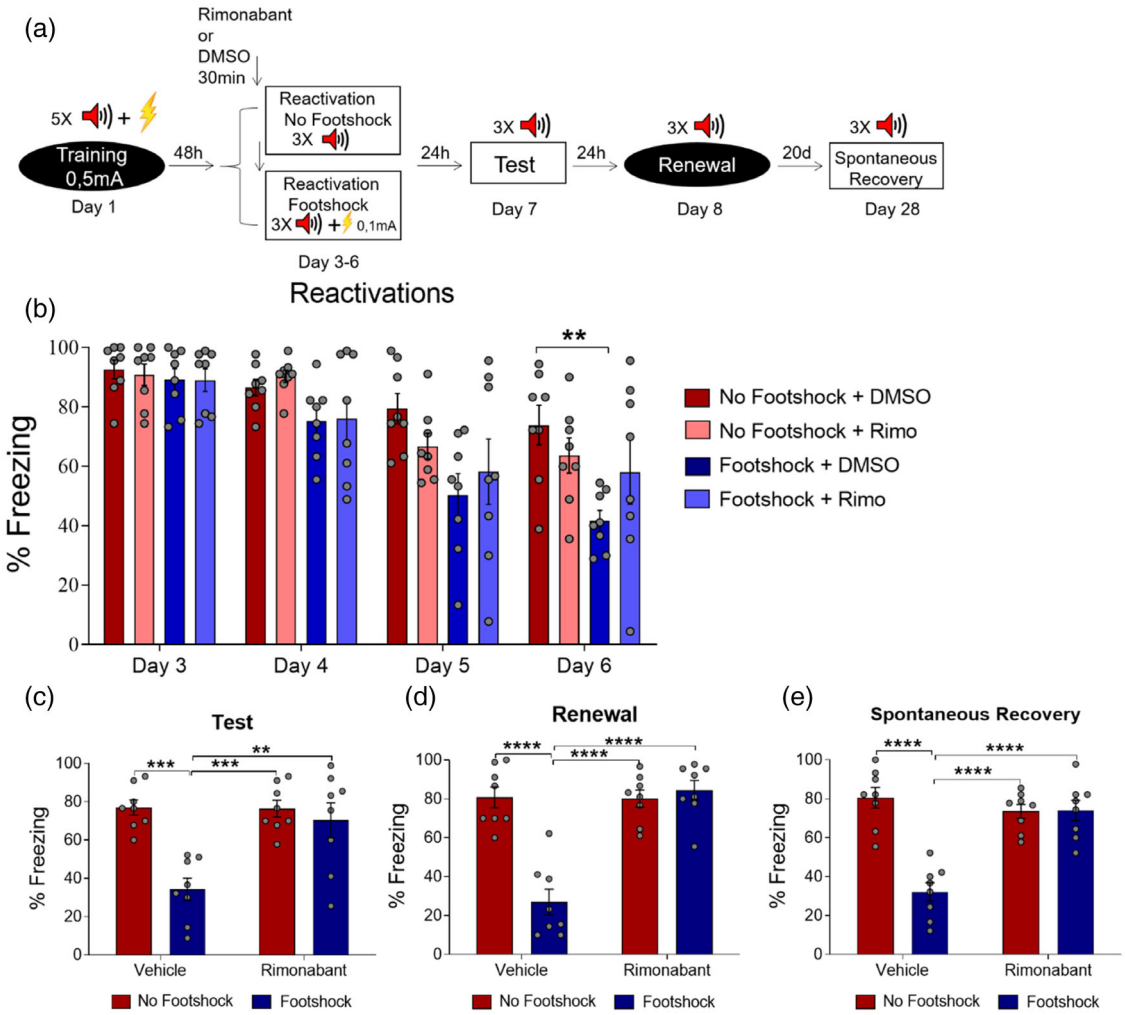


**FIGURE 1** Inhibition of CB1 receptors prevents fear memory update by deconditioning. (a) Experimental design: male Wistar rats were conditioned in context A with five CS + US of 0.5 mA, 48 h later, four daily reactivation sessions (context B), receiving rimonabant (1 mL/kg, i.p.) or vehicle 30 min before each one, subsequently subjected to test sessions (context B), renewal (context A), and spontaneous recovery (context B). Black circles represent context A, while white rectangles represent context B. (b) Levels of freezing during reactivation sessions. The footshock group shows a significant reduction in fear behavior during reactivation sessions. (c) Rimonabant group prevented freezing decrease in the test, renewal (d), and spontaneous recovery (e). Bars represent mean ± SEM. Statistical comparisons were performed using two-way repeated-measures ANOVA followed by a Bonferroni post-hoc (reactivation sessions) or one-way ANOVA followed by Tukey post-hoc (test, renewal, and spontaneous recovery). *N* per group: No Footshock + Vehicle = 8; No Footshock + Rimonabant = 8; Footshock + Vehicle = 8; Footshock + Rimonabant = 8. $^{**}p < .005$; $^{***}p < .0005$; $^{****}p < .0001$. For complete statistics, Table S1. The pre-CS freezing is shown in the Table S1.1. CB1, endocannabinoid system.

$p = .007$; Group Factor $F_{(1,28)} = 15.58$, $p = .0005$; Post-hoc Tukey: Vehicle No Footshock vs. Vehicle Footshock, $p = .0002$; Vehicle No Footshock vs. Rimonabant No Footshock, $p = .99$; Vehicle Footshock vs. Rimonabant Footshock, $p = .001$; Rimonabant No Footshock vs. Rimonabant Footshock, $p = .89$). Moreover, no renewal (two-way ANOVA Interaction $F_{(1,28)} = 28.98$, $p < .0001$; Drug Factor $F_{(1,28)} = 27.62$, $p < .0001$; Group Factor $F_{(1,28)} = 21.04$, $p < .0001$; Post-hoc Tukey: Vehicle No Footshock vs. Vehicle Footshock, $p < .0001$; Vehicle No Footshock vs. Rimonabant No Footshock, $p = .99$; Vehicle Footshock vs. Rimonabant Footshock, $p < .0001$; Rimonabant No Footshock vs. Rimonabant Footshock, $p = .94$) or spontaneous recovery was observed (two-way ANOVA Interaction $F_{(1,28)} = 26.58$, $p < .0001$; Drug Factor $F_{(1,28)} = 13.62$, $p = .001$; Group Factor $F_{(1,28)} = 25.69$, $p < .0001$; Post-hoc Tukey: Vehicle No Footshock vs. Vehicle: Footshock, $p < .0001$; Vehicle No Footshock vs. Rimonabant No Footshock, $p = .73$; Vehicle Footshock vs. Rimonabant Footshock, $p < .0001$; Rimonabant No Footshock vs. Rimonabant Footshock, $p = .99$). These results replicated our previous studies showing the effectiveness of deconditioning to attenuate fear response (Popik et al., 2020, 2023). However, the footshock + rimonabant group exhibited high levels of freezing during the test, renewal, and spontaneous recovery (Figure 1c–e). These results suggest that blocking the CB1 receptors inhibits the memory update by deconditioning.

### 3.2 | The update of fear memory by the deconditioning protocol is dependent on intracellular calcium controlled by the activity of IP3 receptors

Subsequently, we evaluated the participation of intracellular calcium in deconditioning. Thus, we used an antagonist of IP3 receptors (2-APB), administered ICV immediately after each reactivation (Figure 2a). We used the same protocol described above, however, with only two groups, footshock group + vehicle and footshock group + 2-APB. We hypothesized that antagonizing IP3 receptors would prevent the effects of deconditioning, as intracellular calcium favors the underlying processes of synaptic plasticity (Fernandes et al., 2022). Our results show that the IP3 antagonist blocked the effect of deconditioning on reactivations when compared to the footshock + vehicle group (Figure 2b; two-way ANOVA Interaction $F_{(3,48)} = 7.274$, $p = .0004$; Drug Factor $F_{(3,48)} = 46.53$, $p < .0001$; Group Factor $F_{(1,16)} = 12.35$, $p = .002$; Post-hoc Bonferroni Day 6, $p < .0001$), and this robust effect is maintained in subsequent tests (Figure 2c, Student's $t$-test $T_{16} = 2.612$, $p = .01$; Figure 2d, Student's $t$-test $T_{16} = 4.872$, $p = .0002$), showing a difference of approximately 70% in freezing expression between the groups (Figure 2e, Student's $t$-test $T_{16} = 7.490$, $p < .0001$). It suggests that intracellular calcium is intimately involved with the processes of destabilization and restabilization of the memory (for statistics see Table S2).

### 3.3 | The update of fear memories by the deconditioning protocol is dependent on the activity of calpains and CP-AMPA receptors

Depending on the subunits that the AMPAR are composed, it can be $Ca^{++}$ impermeable (CI-AMPARs) or permeable (CP-AMPARs). It has been shown that, after memory reactivation, CP-AMPARs are

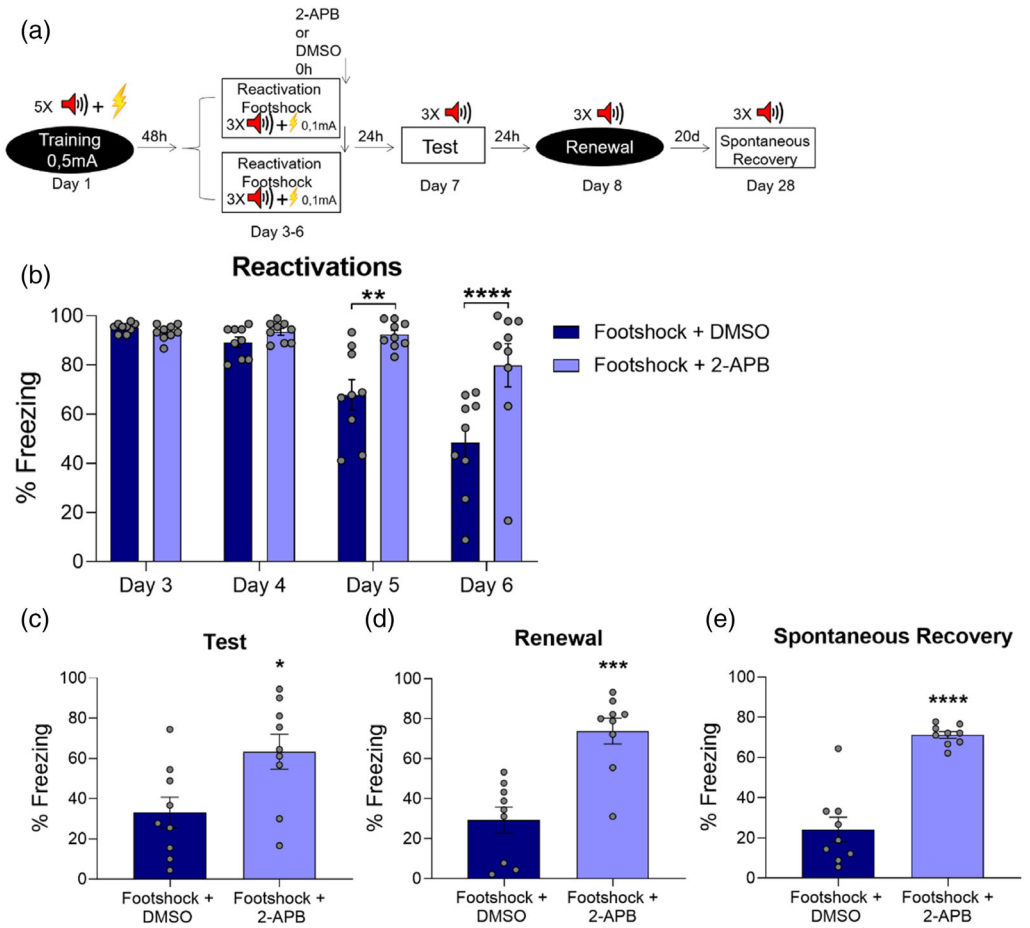


**FIGURE 2** The update of fear memory by the deconditioning protocol is dependent on intracellular calcium controlled by the activity of IP3 receptors. (a) Experimental design: male Wistar rats were conditioned in context A with five CS + US of 0.5 mA, 48 h later, four daily reactivation sessions (context B), receiving 2-APB (500 μM; ICV) or vehicle (DMSO 10%; ICV) immediately after each reactivation, subsequently subjected to test sessions (context B), renewal (context A), and spontaneous recovery (context B). Black circles represent context A, while white rectangles represent context B. (b) Levels of freezing during reactivation sessions. The footshock group shows a significant reduction in fear behavior during reactivation sessions. (c) 2-APB group prevented a freezing decrease in the test, renewal (d), and spontaneous recovery (e). Bars represent mean ± SEM. Statistical comparisons were performed using two-way repeated-measures ANOVA followed by a Bonferroni post-hoc (reactivation sessions) or Student's t-test (test, renewal, and spontaneous recovery). *N* per group: Footshock + DMSO = 9; Footshock + 2-APB = 9. *$p < .05$; **$p < .005$; ***$p < .0005$; **** $p < .0001$. For complete statistics, Table S2. The pre-CS freezing is shown in Table S2.1. DMSO, dimethyl sulfoxide; ICV, intracerebroventricular.

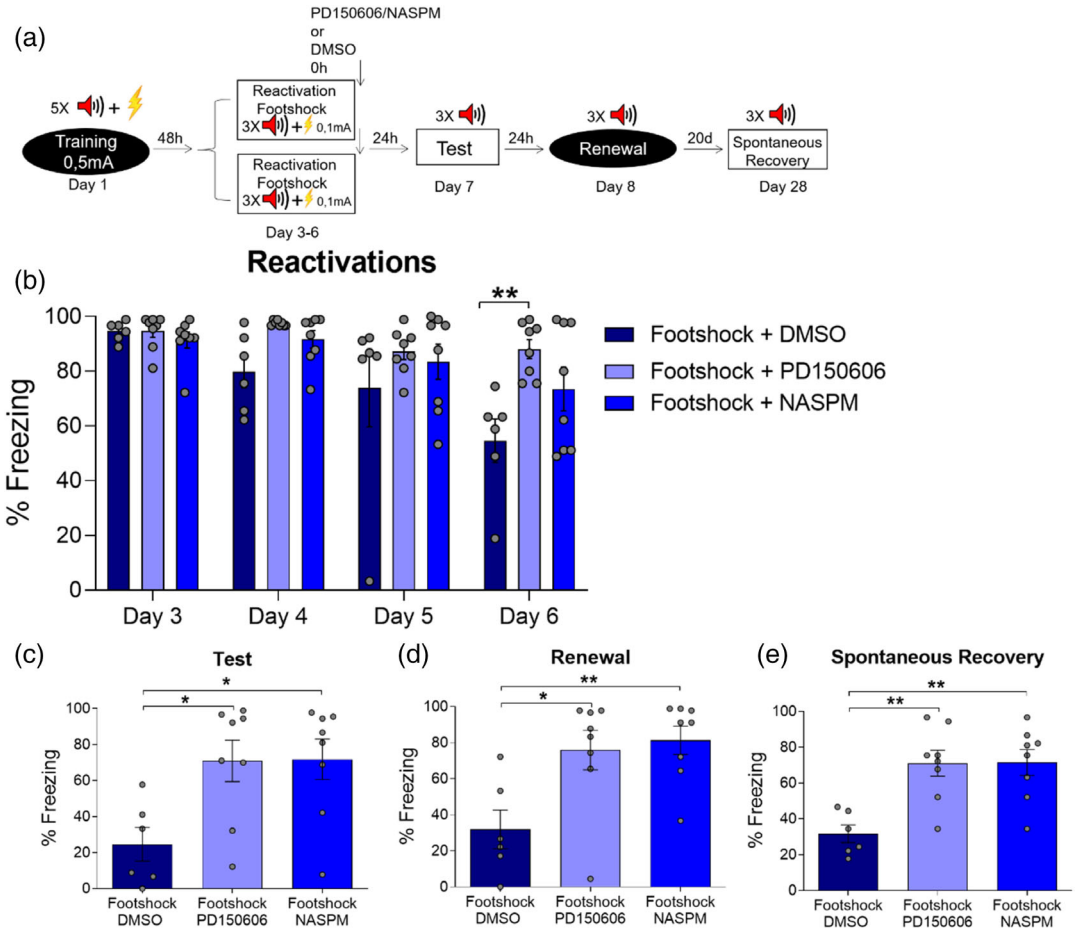


**FIGURE 3** The update of fear memories by the deconditioning protocol is dependent on the activity of calpains and CP-AMPA receptors. (a) Experimental design: male Wistar rats were conditioned in context A with five CS + US of 0.5 mA, 48 h later, four daily reactivation sessions (context B), receiving NASPM (4 μM; ICV), PD150606 (1 μM; ICV), or vehicle immediately after each reactivation, subsequently subjected to test sessions (context B), renewal (context A), and spontaneous recovery (context B). Black circles represent context A, while white rectangles represent context B. (b) Levels of freezing during reactivation sessions. The footshock group + vehicle shows a significant reduction in fear behavior during reactivation sessions. (c) NASPM and PD150606 groups prevented freezing in the test, renewal (d), and spontaneous recovery (e). Bars represent mean ± SEM. Statistical comparisons were performed using two-way repeated-measures ANOVA followed by a Bonferroni post-hoc (reactivation sessions) or one-way ANOVA followed by Tukey post-hoc (test, renewal, and spontaneous recovery). *N* per group: Footshock + DMSO = 6; Footshock + PD150606 = 8; Footshock + NASPM = 8. $^{*}p < .05$; $^{**}p < .005$. For complete statistics, Table S3. The pre-CS freezing is shown in Table S3.1. DMSO, dimethyl sulfoxide; ICV, intracerebroventricular.

transiently elevated in the postsynaptic density (PSD), and such elevation seems to be critical for fear memory updating (Clem & Huganir, 2010; Hong et al., 2013; Torquatto et al., 2019). Calpain is a calcium-dependent protease widely distributed in the brain (Briz & Baudry, 2017). It has a crucial role in synaptic restructuring (Zadran et al., 2010) and in controlling fear memory flexibilization, as it promotes reorganization on dendritic spines and the dynamics of glutamatergic receptors (Lynch et al., 2007; Wu & Lynch, 2006; Zhu et al., 2017). Thus, we predicted that blocking pharmacologically CP-AMPA and calpain (with NASPM and PD150606, respectively) would prevent fear memory attenuation through the deconditioning-update.

Then, animals were trained as described above and, immediately after each reactivation session, the vehicle (DMSO 1%) or the drugs (NASPM or PD150606) were infused. All groups were subjected to the deconditioning protocol (Figure 3a). The inhibition of CP-AMPA receptors and calpain prevented the fear reduction caused by deconditioning (Figure 3b; two-way ANOVA Interaction $F_{(6,57)} = 2.590$, $p = .02$; Drug Factor $F_{(3,57)} = 14.85$, $p < .0001$; Group Factor $F_{(2,19)} = 3.961$, $p = .05$; Post-hoc Bonferroni Day 6, Vehicle Footshock vs. PD150606 Footshock, $p = .001$). That is, the footshock + PD150606 and footshock + NASPM groups showed a higher percentage of freezing compared to the footshock group in all tests (Figure 3c; one-way ANOVA $F_{(2,19)} = 5.314$, $p = .01$; Post-hoc Tukey: Vehicle Footshock vs. PD150606 Footshock, $p = .02$; Vehicle Footshock vs. NASPM Footshock, $p = .02$; Figure 3d; one-way ANOVA $F_{(2,19)} = 6.702$, $p = .006$; Post-hoc Tukey: Vehicle Footshock vs. PD150606 Footshock, $p = .01$; Vehicle Footshock vs NASPM Footshock, $p = .007$; Figure 3e; one-way ANOVA $F_{(2,21)} = 13.88$, $p = .0001$; Post-hoc Tukey: Vehicle Footshock vs. PD150606 Footshock, $p = .0005$; Vehicle Footshock vs NASPM Footshock, $p = .0005$).

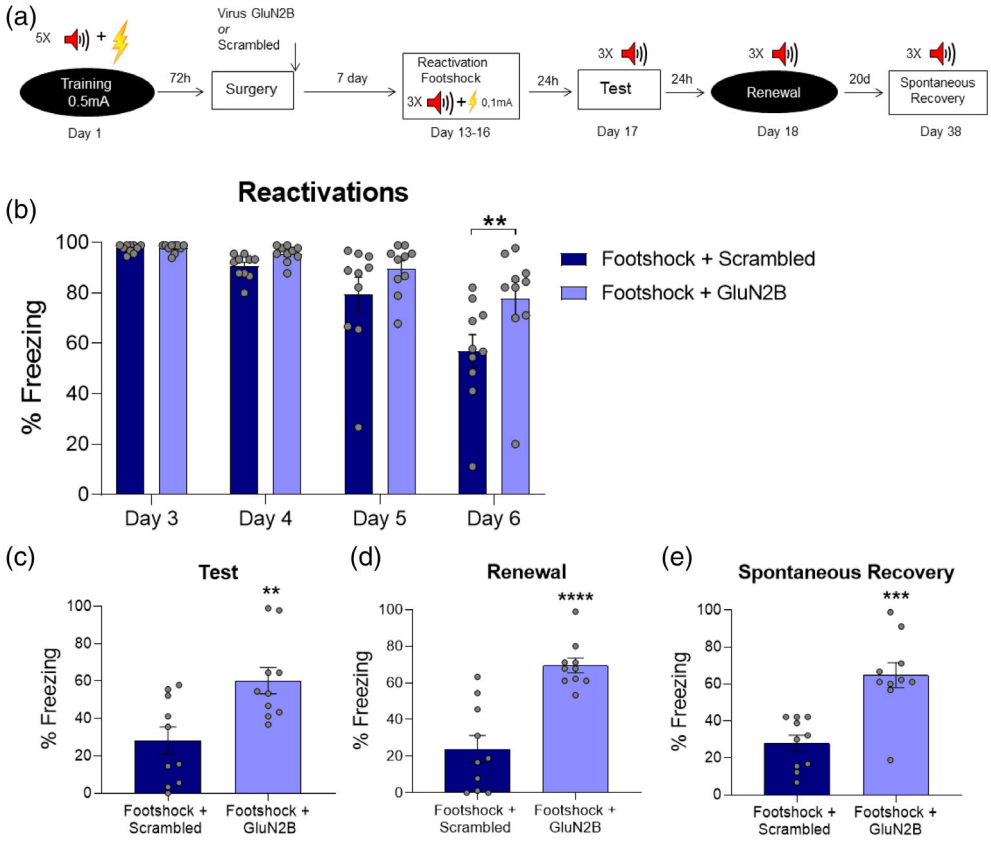


**FIGURE 4** The downregulation of the expression of the NMDA-GluN2B receptors blocks the update of the fear memory by the deconditioning protocol. (a) Experimental design: the viral vector or scrambled was infused and 7 days later male Wistar rats were conditioned in context A with five CS + US of 0.5 mA, 48 h later, four daily reactivation sessions (context B), subsequently subjected to test sessions (context B), renewal (context A), and spontaneous recovery (context B). Black circles represent context A, while white rectangles represent context B. (b) Levels of freezing during reactivation sessions. The footshock + scrambled group shows a significant reduction in fear behavior during reactivation sessions. (c) Footshock + GluN2B group prevented freezing in the test, renewal (d), and spontaneous recovery (e). Bars represent mean ± SEM. Statistical comparisons were performed using two-way repeated-measures ANOVA followed by a Bonferroni post-hoc (reactivation sessions) or Student's *t*-test (test, renewal, and spontaneous recovery). *N* per group: Footshock + Scrambled = 10; Footshock + GluN2B = 10. $^{**}p < .005$; $^{***}p < .0005$; $^{****}p < .0001$. For complete statistics, Table S4. The pre-CS freezing is shown in Table S4.1.

### 3.4 | The downregulation of the expression of the GluN2B receptors blocks fear memory update by the deconditioning protocol

Previous studies have reported that activation of GluN2B-NMDAR is required for destabilization of reactivated memory (Ben Mamou et al., 2006; Haubrich et al., 2015; Wang et al., 2009). That is, blocking these receptors before reactivation prevents memory updating induced by amnestic agents such as anisomycin (Ben Mamou et al., 2006). Thus, we tested the role of GluN2B in the deconditioning protocol. To this end, we infused a viral vector into the BLA (bilateral) that specifically inhibits the expression of the GluN2B subunit. Seven days later, time required for viral expression (Acutain et al., 2021), we applied the protocol described above (Figure 4a).

We demonstrate that knocking-down GluN2B expression in the BLA prevents fear reduction induced by deconditioning in the last reactivation session (Figure 4b; two-way ANOVA Interaction $F_{(3,54)} = 2.630$, $p = .05$; Time Factor $F_{(3,54)} = 24.38$, $p < .0001$; Group Factor $F_{(1,18)} = 5.065$, $p = .03$; Post-hoc Bonferroni Day 6, $p = .005$), test (Figure 4c; Student's *t*-test $T_{18} = 3.181$, $p = .005$), renewal (Figure 4d; Student's *t*-test $T_{18} = 5.382$, $p < .0001$), and spontaneous recovery (Figure 4e; Student's *t*-test $T_{18} = 4.571$, $p = .0002$).

## 4 | DISCUSSION

We have recently proposed a novel and promising approach called deconditioning-update to erase traumatic memories (Popik et al., 2020, 2023). This strategy consists in robustly and permanently eliminating fear memory by updating the fearful information with an extremely low aversive/neutral stimulus during the plastic state induced by memory reactivation. We have shown that the deconditioning-update is effective in eliminating up to 80% of fear responses. Moreover, such effects are long-lasting, and insensitive to renewal and spontaneous recovery, suggesting a permanent update in fear memory. Remarkably, our previous studies have also

demonstrated that deconditioning overcame common boundary conditions that constrain memory updating, such as remote or very strong conditioning protocols. Furthermore, the same beneficial effect was also found in other types of fear-related memories, such as contextual fear memory and inhibitory avoidance (Popik et al., 2020). Following up on these studies, here we propose to investigate the molecular mechanisms involved in the deconditioning-update approach.

In order to be updated, memory must undergo a labile state, which will allow it to be modified. This process is called memory destabilization, and some molecular mechanisms requirements, such as the endocannabinoid system activation, have been described (de Oliveira Alvares & Do-Monte, 2021; Lunardi et al., 2020). CB1 receptors are abundantly expressed in brain areas closely related with stress, emotional arousal, and memory, including the prefrontal cortex, the hippocampus, and the amygdala (Aisenberg et al., 2017; Morena et al., 2014). The CB1 antagonist SR141716A injected after memory reactivation blocks the disruptive effects of protein synthesis inhibitor on memory reconsolidation (Suzuki et al., 2008). Consistently, memory destabilization has been shown to be enhanced after infusion of CB1 agonist (Lee & Flavell, 2014). Together, these results suggest a crucial role of the ECS on memory updating that follows destabilization. We hypothesize that blocking the endocannabinoid effect by infusing a CB1 antagonist would prevent the fear reduction achieved by the deconditioning protocol. Our results corroborate our predictions. The CB1 antagonist rimonabant administered during the reactivation sessions prevented the deconditioning-update effect in fear attenuation in the test, renewal, and spontaneous recovery (Figure 1a–e).

The most important player involved in the memory destabilization process is probably the $Ca^{++}$. It has been described that the $Ca^{++}$ entrance through NMDAR or L-type voltage-gated calcium channels (L-VGCC) impair memory labilization (Ben Mamou et al., 2006; Haubrich et al., 2015; Suzuki et al., 2008). Another important extracellular $Ca^{++}$ source reported to be essential to memory updating is the CP-AMPAR (Clem & Huganir, 2010; Torquatto et al., 2019). Retrieval transiently increases CP-AMPAR at the PSD in order to undergo memory destabilization (Clem & Huganir, 2010; Hong et al., 2013; Rao-Ruiz et al., 2011). We have previously shown that the CP-AMPA antagonist NASPM prevents behavioral flexibility and memory updating (Torquatto et al., 2019). Here, we have also described that interfering with the extracellular $Ca^{++}$ entrance by down-regulating the GluN2B-NMDA receptors (Figure 4) or blocking the CP-AMPAR (Figure 3) prevented the deconditioning-updating effect. Another important $Ca^{++}$ source in the neurons is released by intracellular compartments, such as the endoplasmatic reticulum through the IP3 receptors. It has been recently reported that it participates in memory processes such as consolidation and retrieval (Fernandes et al., 2022). Our results suggest that the IP3 receptors play an important role in memory destabilization, since its inhibition prevented the deconditioning effect (Figure 2a–e).

The increased influx of $Ca^{++}$ through extracellular and intracellular sources activates several targets such as calpain. This $Ca^{++}$-dependent protease is closely involved in the cytoskeleton remodeling that maintains the morphology of the dendritic spines (Lu et al., 2000; Simpkins et al., 2003; Wu & Lynch, 2006). Calpain modulates mnemonic processes such as consolidation, retrieval, and reconsolidation in rodents (Nagayoshi et al., 2017; Popik et al., 2018). Here, we reported that the calpain inhibitor PD150606 blocked the memory updating by deconditioning (Figure 3).

Based on our findings and other studies, we suggest that the mechanisms underlying the deconditioning-update process initiates with the increase in the intracellular calcium levels through GluN2B-containing NMDA receptors, L-VGCC, IP3 receptors, and CP-AMPAR. Then, calcium activates some targets, inducing functional and structural synaptic remodeling through GluA2 internalization and protein degradation by the proteasome, autophagy, and proteases such as calpains. At the presynaptic level, the CB1 receptor is activated in order to modulate the neurotransmission release. These events are thought to promote memory destabilization. Then, the synapses are rebuilt in an updated form, requiring protein synthesis, AMPAR trafficking, and cytoskeleton reorganization (for a detailed review of this model, please see de Oliveira Alvares & Do-Monte, 2021).

While the primary interpretation of the deconditioning-update effect has been largely grounded in reconsolidation, it is important to acknowledge that alternative explanations cannot be excluded. These include possibilities such as footshock habituation, revaluation, new learning (CS-weak US), or devaluation (Pickens & Holland, 2004; Rescorla, 1973; Rescorla & Heth, 1975). Recent studies have also used weak shock in fear-conditioned animals. Remarkably, the impact on memory varies based on the number of weak footshocks delivered during the reactivation session, with the potential for either enhancement or reduction of the fear response (Ferrara et al., 2019). Interestingly, this process seems to be based on updating through reconsolidation, and rely on synaptic modifications within the BLA (Bonanno et al., 2023), such as GluA2 AMPA endocytosis (Ferrara et al., 2019). It is important to note that our experiments exclusively utilized male subjects, thus, we cannot exclude the possibility that a distinct outcome could be found in females.

## 5 | CONCLUSION

Taken together, our results demonstrated several molecular processes underpinning the deconditioning-update effect on the reduction of fear expression. Specifically, we have shown that the CB1 receptors, $Ca^{++}$ increase through GluN2B, CP-AMPA, and IP3R, and the $Ca^{++}$ dependent protease calpain play an important role in memory destabilization that, in the end, allows memory modification. We believe that unraveling the molecular signatures behind strategies that might change the strength or content of maladaptive memories open therapeutic avenues that require to be explored in order to overcome boundary conditions that prevent traumatic memories from being accessed.

### AUTHOR CONTRIBUTIONS

**Bruno Popik**: Methodology; Project administration; Writing; **Jordana Griebler Luft**: Methodology; Writing; **Kétlyn Talise Knak Guerra**: Methodology; **Lucas de Oliveira Alvares**: Supervision; Conceptualization; Funding acquisition; Writing.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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## DISCUSSÃO GERAL

Nas últimas décadas, a nossa compreensão de como as memórias de medo são flexibilizadas avançaram significativamente. Por exemplo, atualmente é amplamente aceito que os mecanismos da reconsolidação agem para flexibilizar uma memória aversiva, tanto para reescrever o seu conteúdo quanto a sua força, podendo inclusive atenuar ou mesmo reforçar a memória aversiva. Esses mecanismos permitem efeitos mais duradouros em relação aos efeitos decorrentes da memória de extinção. Levando isso em consideração, criamos um protocolo chamado descondicionamento que visa atenuar as memórias aversivas pelos mecanismos da reconsolidação. Assim, demonstramos de forma consistente que o descondicionamento atenua uma memória de medo pelos mecanismos da reconsolidação. Desta forma, os nossos resultados são divididos em três tópicos, primeiro caracterizamos o nosso protocolo em diferentes condições experimentais, posteriormente demonstramos os limites pelo qual o descondicionamento permite atualizar uma memória de medo e, para finalizar, sugerimos algumas bases moleculares que sustentaria o descondicionamento.

Para caracterizarmos o descondicionamento utilizamos o aparato de condicionamento ao tom (CAT) e ratos *Wistar* machos, visualizamos uma redução robusta e permanente do comportamento de medo (*freezing*) no teste, da mesma forma, não observamos a renovação (*renewal*) e nem a recuperação espontânea. Além disso, o protocolo preveniu a reaquisição da memória aversiva pelo retreino. Para uma melhor caracterização, testamos o protocolo em fêmeas utilizando o mesmo aparato comportamental, verificamos que não houve diferença nos resultados encontrados entre machos e fêmeas. Os nossos achados demonstram que os mecanismos subjacentes ao descondicionamento não são dependentes de sexo, mesmo que inúmeros estudos relataram que o estradiol modula alguns processos mnemônicos, como a evocação e a flexibilização da memória de medo (Milad et al., 2009; Rivas-arancibia & Vazquez-pereyra, 1994; Yuan & Chambers, 1999; Zeidan et al., 2011) (dados presentes no artigo 1, Fig. 1 – *Shifting from fear to safety through deconditioning-update*).

Em seguida nos perguntamos se o nosso protocolo também funcionaria em outros aparatos comportamentais. Para responder essa pergunta utilizamos o condicionamento aversivo ao contexto (CAC) e a esquiva inibitória (*step-through*). Cabe destacar que cada

aparato comportamental é dependente de uma estrutura encefálica específica, sendo o CAT mais dependente da amígdala (Gogolla et al., 2009; Wang et al., 2009), o CAC do hipocampo (Casagrande et al., 2018; Pedraza et al., 2016) e a esquiva inibitória do córtex pré-frontal (Izquierdo et al., 2016). Deste modo, demonstramos também que o descondicionamento atenua uma memória de medo sob diferentes paradigmas comportamentais (dados presentes no artigo 1, Fig. 3 – *Shifting from fear to safety through deconditioning-update*).

Os nossos dados anteriores corroboram com a nossa hipótese original que o descondicionamento age pelos mecanismos da reconsolidação, pois verificamos essa premissa através dos testes comportamentais de renovação, recuperação espontânea, reinstalação e retreino. Para sustentá-la de forma mais concisa, aplicamos um protocolo clássico de extinção, ou seja, uma única sessão massiva de CS (Fernandes et al., 2022; Merlo et al., 2014; Monfils et al., 2009; Redondo et al., 2020; Suzuki et al., 2004). Desta forma, os animais foram submetidos a 24 tons (CS) e demonstramos que o grupo controle (*no footshock*) apresentou uma memória de extinção no teste, ocorrendo a renovação e a recuperação espontânea, ou seja, ocorreu a extinção da memória. Entretanto, no grupo *footshock* (descondicionamento) não ocorrera a formação da memória de extinção, logo, o nosso protocolo prejudicou os mecanismos da extinção da memória de medo.

Para reforçar a nossa hipótese da reconsolidação, utilizamos uma ferramenta farmacológica que permite distinguir os processos da reconsolidação e extinção, a inibição dos canais de cálcio dependentes de voltagem (Cain et al., 2002; Crestani et al., 2015; Davis & Bauer, 2012; Flavell et al., 2011; Haubrich et al., 2015; Suzuki et al., 2004, 2008). Assim, infundimos o antagonista dos LVGCCs e demonstramos que impede a atualização da memória de medo pelo descondicionamento (artigo 1, Fig. 4). O conjunto dos nossos achados corroboram com a nossa hipótese, pois demonstramos tanto por um protocolo clássico de extinção quanto por uma ferramenta farmacológica que o descondicionamento não age pelos mecanismos subjacentes da extinção, bem como, apontamos que o nosso protocolo não é dependente de contexto, que não ocorre o retorno da memória de medo com passar do tempo ou mesmo por um retreino ou ainda pelo protocolo da reinstalação (premissas da memória de extinção, (Baldi & Bucherelli, 2015; Gogolla et al., 2009; Monfils et al., 2009). Destarte, o conjunto dos nossos dados indicam fortemente que estamos interferindo nos mecanismos da reconsolidação e o nosso

protocolo também se destaca por não utilizar intervenções farmacológicas (dados presentes no artigo 1, Fig. 4 – *Shifting from fear to safety through deconditioning-update*).

Após demonstrarmos que nosso protocolo utiliza os mecanismos da reconsolidação para atenuar uma memória de medo, nos perguntamos se ele é capaz de romper com as condições limitantes da reconsolidação. Vale salientar que esses elementos intrínsecos podem limitar sua reprodutibilidade e aplicabilidade em humanos (Monfils et al., 2009; Schiller et al., 2010; Suzuki et al., 2004; Wang et al., 2009). Para tal, avaliamos duas condições limitantes, a intensidade do treino (treino forte) e a idade da memória (memória remota). Deste modo, utilizamos um treino forte (dobramos a intensidade para 1mA) no CAT e visualizamos que o descondicionamento rompe com esta condição limitante. Posteriormente treinamos os animais e aplicamos o protocolo de descondicionamento 40 dias após para avaliar a memória remota, novamente verificamos que foi possível atualizar a memória de medo, ou seja, rompemos com ambas as condições limitantes da reconsolidação (dados presentes no artigo 1, Fig. 2 – *Shifting from fear to safety through deconditioning-update*).

Cerca de uma década atrás, foi proposto um estudo em que as memórias aversivas eram persistentemente atenuadas sem necessidade de drogas, esses efeitos eram mediados pelos mecanismos da extinção, mas aplicado na janela temporal da reconsolidação. Consequentemente tornou-se promissor, pois impediu algumas condições peculiares que limitam a extinção, como por exemplo, a dependência de contexto e a recuperação espontânea. Entretanto, esses achados não foram facilmente replicados por outros estudos, pois não exploraram as condições limitantes da reconsolidação, que possivelmente limitou a sua reprodutibilidade (Beckers, 2017; Chan et al., 2010; Costanzi et al., 2011; Golkar et al., 2012; Goode et al., 2017; Ishii et al., 2012; Kindt & Soeter, 2013; Luyten & Beckers, 2017). Nesse estudo, Monfils e colaboradores (2009), desenvolveram um protocolo chamado reativação-extinção, onde uma sessão massiva de extinção foi aplicada dentro da janela temporal da reconsolidação. Desta forma, os animais expressavam um baixo nível de *freezing* nas sessões de teste, *renewal* e recuperação espontânea. Demonstrando que a memória de extinção é fortalecida quando utiliza os mecanismos subjacentes da reconsolidação, infelizmente outros estudos que utilizaram o mesmo protocolo experimental não conseguiram demonstrar resultados similares.

Em suma, o protocolo de descondicionamento demonstra resultados promissores e robustos, uma vez que reduziu a expressão da memória do medo de forma eficiente sem depender de agentes farmacológicos clássicos da reconsolidação, como antagonistas dos LVGCCs. Inclusive, os nossos resultados estão de acordo com as previsões de um modelo de rede neural previamente construído para explicar a flexibilidade da memória, basicamente abortando as transições entre reconsolidação e extinção. Embora o modelo seja bastante reducionista, ainda assim foi capaz de explicar algumas variáveis, como por exemplo, a transição da reconsolidação e extinção com o aumento do tempo de reexposição (Suzuki et al., 2004) ou o número de CS (Lee et al., 2006; Sevenster et al., 2014). O modelo também ajuda a explicar por que, diferentemente do bloqueio de reconsolidação farmacológica, a atualização do descondicionamento leva várias sessões para ocorrer, pois o impacto dos mecanismos de labilização em uma memória armazenada é muito mais pronunciado quando os mecanismos Hebbianos são bloqueados concomitantemente (Osan et al., 2011).

Portanto, uma das condições necessárias para induzir a reconsolidação da memória de medo é o erro de predição (*mismatch*). Sendo este um evento caracterizado pela discrepância entre o que é esperado e o que realmente ocorre (Cahill et al., 2019; Sevenster et al., 2013). De acordo com Sevenster et al. (2013), a evocação da memória de medo pode levar a uma expectativa do estímulo incondicionado (US) durante a apresentação do estímulo condicionado (CS), funcionando assim como o erro de predição.

Levando em consideração os experimentos *in silico,* um dos mecanismos que ajuda a explicar os efeitos do descondicionamento é o erro de predição, em que há uma quebra de expectativa entre o estímulo esperado (choque de treinamento) e o que é apresentado nas reativações (choque fraco/neutro). Uma vez que, caracterizamos o protocolo de descondicionamento, agora nos perguntamos quais eram os seus limites tanto de intensidade de treino quanto da sua janela temporal de reativação aplicado na reconsolidação e consolidação. Para tal, utilizamos apenas a tarefa comportamental – CAT – e ratos *Wistar* machos.

Em primeiro momento demonstramos a faixa de intensidade de treino que o protocolo de descondicionamento promove a atualização da memória de medo. Assim, verificamos que só ocorreu o erro de predição que induz a reconsolidação da memória na faixa de discrepância entre o choque apresentado durante as reativações (0.1mA) com as

intensidades do treino de 0.3mA, 0.5mA e 1mA. Todavia, o protocolo não se mostra eficiente em reescrever a memória de medo decorrente de um treino de 1.5mA, ou seja, o traço mnemônico torna-se blindado ao processo da atualização. Em síntese, caracterizamos o protocolo de descondicionamento em relação aos seus limites mínimo e máximo de intensidade de treino para atenuar a memória aversiva (dados presentes no artigo 2, Fig. 1-3 – *Characterization of deconditioning-update on fear memory attenuation*).

A força de uma memória pode ser baseada pela intensidade de treino, constituindo assim um dos elementos intrínsecos que modulam a capacidade de gerar os processos subjacentes da reconsolidação. Por conseguinte, é um percalço da reprodutibilidade e aplicabilidade tanto em modelos animais quanto em seres humanos. Desta forma, sabe-se que tanto a intensidade do treinamento quanto o tempo das sessões de reativação alteram a probabilidade de desestabilizar o traço de memória, condição fundamental para gerar os processos de desestabilização e reestabilização do traço mnemônico (Alberini et al., 2006; Frankland et al., 2006; Kida, 2019; Milekic & Alberini, 2002; Suzuki et al., 2004, 2008; Wang et al., 2009). Em suma, além de demostrarmos que o descondicionamento rompe com as condições limitantes da reconsolidação, também caracterizamos a faixa de discrepância em relação a intensidade de treino que gera a reconsolidação.

Em relação a premissa do tempo dos intervalos das reativações, demonstramos que só ocorre uma atualização robusta da memória quando as sessões de reativações foram espaçadas por um intervalo de 24 horas, corroborando com os experimentos *in silico*. Evidencia-se que só é possível reescrever o traço da memória quando há tempo suficiente para que ocorram os processos de desestabilização e restabilização em cada reativação, pois ao aplicar as quatro sessões de descondicionamento subsequentes, intervalo de apenas dez minutos, não induz a desestabilização do traço mnemônico. Não obstante, ao aplicar um intervalo mínimo de seis horas entre cada reativação, a memória é passível de ser reescrita, porém de forma mais branda, indo de encontro com a literatura que preconiza que a reconsolidação é dependente tanto do número de reativações quanto do tempo das reativações (Auchter et al., 2017; Suzuki et al., 2004; Wichert et al., 2013). Assim, demonstramos o intervalo mínimo da janela de temporal da reconsolidação que o descondicionamento é eficaz.

Levando em consideração esses resultados, agora nos perguntamos se seria possível modificar uma memória ainda não consolidada. Logo, aplicamos o protocolo de descondicionamento na janela temporal da consolidação. Pois esta é caracterizada por ter uma janela temporal plástica, ou seja, permite que as interferências ocorridas neste período modulem as informações que serão consolidadas (McGaugh, 2015; Okuda et al., 2004). Todavia, quando o descondicionamento foi aplicado na janela temporal da consolidação não foi capaz de modificar a memória de medo. Por outro lado, o descondicionamento se mostra extremamente eficiente para reescrever uma memória de medo já consolidada, através da desestabilização do traço mnemônico gerado pelos choques fracos/neutros aplicados nas reativações (dados presentes no artigo 2, Fig. 4 e 5; *The dynamic of deconditioning-update on the reconsolidation of aversive memories*).

O compêndio dos nossos achados auxilia tanto no entendimento de como reescrever uma memória de medo quanto no desenvolvimento de terapias para patologias psiquiátricas como TEPT, ansiedade e depressão, pois estas se baseiam nos períodos plásticos da memória. Esses resultados corroboram para compreender as condições gerais de como os US aplicados durante as reativações induzem a reconsolidação de uma memória de medo.

Finalmente, investigamos alguns eventos moleculares que sustentam o protocolo de descondicionamento. Para tal, foi utilizado ferramentas farmacológicas para avaliar se a inibição desses alvos moleculares impediria a desestabilização/restabilização do traço da memória de medo ocasionada pelo descondicionamento. Para isso, infundimos o rimonabanto (bloqueia os receptores CB1) pela via i.p. 30 minutos antes de cada reativação; 2-APB (bloqueia os receptores IP3), NASMP (bloqueia os receptores CP-AMPA) e PD150606 (bloqueia a atividade das calpaínas) todos pela via ICV imediatamente após cada reativação, e um vetor viral (inibe a expressão dos receptores NMDA-GluN2B) na BLA aplicado sete dias antes de iniciar as reativações da memória de medo no CAT. A inibição farmacológica bloqueou os efeitos do descondicionamento, evitando a atualização e consequentemente a atenuação da memória de medo. Exploramos os possíveis papéis funcionais desses marcadores moleculares na reconsolidação da memória através do descondicionamento (dados presentes no artigo 3 – *Molecular mechanisms that support the deconditioning protocol to induce fear memory reconsolidation*).

Os estudos que exploram o papel dos receptores endocanabidinóides (CBRs; receptores endocanabinóide do tipo 1 e 2, CB1 e CB2 respectivamente) na flexibilidade da memória de medo indicam que a sua atuação pode estar relacionada com os mecanismos moleculares subjacentes à desestabilização, mecanismo intrínseco da reconsolidação do traço mnemônico (Bucherelli et al., 2006; de Oliveira Alvares et al., 2008; Lin et al., 2006; Marsicano et al., 2002; Suzuki et al., 2004, 2008). Entretanto, a sinalização endocanabinóide no processo de reconsolidação se mostra extremamente complexa, inclusive os resultados relatados na literatura são conflitantes, tendo em vista que, agonistas e antagonistas dos receptores CB1 modulam de forma bidirecional a reconsolidação da memória de medo, ou seja, favorecendo ou prejudicando a memória (de Oliveira Alvares et al., 2008; Suzuki et al., 2008).

Os endocanabinóides são neurotransmissores retrógrados que modulam a plasticidade sináptica excitatória e inibitória (Castillo et al., 2012) e desempenham um papel crucial na reconsolidação da memória através da estimulação de receptores endocanabinóides pré-sinápticos tipo 1 (CB1Rs) (Stern et al., 2018). No entanto, os mecanismos fisiológicos pelos quais os CB1Rs modulam a reconsolidação da memória não estão bem elucidados. Propõe-se que atuem através de correntes inibitórias mediadas pelo GABA (ácido gama-aminobutírico), modulando assim os efeitos inibitórios. Vários estudos demonstram que o bloqueio de CB1Rs prejudica a reconsolidação da memória (De Carvalho et al., 2014; Fang et al., 2011; Yu et al., 2009). Aqui, usamos o fármaco rimonabanto para bloquear os receptores CB1 e demonstramos que impediu a atualização da memória de medo pelo descondicionamento. No nosso entendimento, esse efeito foi desencadeado pela inibição do processo de desestabilização do traço mnemônico, pois este é dependente de receptores endocanabinóides (Suzuki et al., 2008). Os dados apresentados aqui são consistentes com trabalhos anteriores que indicam um papel dos CB1Rs nos mecanismos subjacentes a flexibilização da memória do medo (dados presentes no artigo 3, Fig. 1 – *Molecular mechanisms that support the deconditioning protocol to induce fear memory reconsolidation*).

Da mesma forma, nas últimas décadas a nossa compreensão de como as informações são flexibilizadas tiveram avanços notáveis. Por exemplo, agora se sabe que o cálcio armazenado no interior dos neurônios desempenha um papel fundamental na plasticidade sináptica e nos períodos plásticos da memória. O cálcio intracelular está estocado no retículo endoplasmático liso e, os receptores inositol 1,4,5-trifosfato (IP3R)

presentes nessa organela desempenham um dos principais mecanismos de controle citoplasmático desse íon nos neurônios (Brini et al., 2014). Sabemos que a sua ativação se dá tanto em resposta ao $Ca^{2+}$ quanto pela ligação do IP3, um fosfolipídio de membrana que passa a atuar como segundo mensageiro após a hidrólise do fosfatidilinositol bifosfato (PIP2) pela proteína fosfolipase C (PLC) (Foskett et al., 2007).

Um estudo recente demonstra que a inativação deste receptor prejudica a consolidação e a evocação da memória de medo (Fernandes et al., 2022), pois ambos os processos são caracterizados por serem dinâmicos e susceptíveis a modulações (Casagrande et al., 2018; Lopez et al., 2015; Popik et al., 2018). No entanto, ainda há uma carência na literatura que correlaciona os receptores IP3 com os mecanismos da reconsolidação da memória de medo. Assim, nos perguntamos se a atividade desses receptores contribuiriam para os mecanismos de desestabilização da memória gerada pelo descondicionamento.

Os nossos resultados demonstram que a inibição dos receptores IP3 impede os efeitos do descondicionamento, sugerimos que esta inibição da dinâmica do cálcio intracelular impediu o funcionamento adequado de inúmeras cascatas de proteínas responsáveis pela plasticidade da memória, desde a reorganização do citoesqueleto até o tráfego de receptores de membrana. Anteriormente sugerimos que o nosso protocolo é dependente da atividade dos LVGCCs, logo demonstramos que tanto o cálcio extracelular quanto o intracelular controlam os mecanismos subjacentes da desestabilização da memória de medo.

Sabe-se que os receptores ionotrópicos glutamatérgicos também podem controlar o fluxo de cálcio e são cruciais para a desestabilização do traço mnemônico (Clem & Huganir, 2010; Henley & Wilkinson, 2016; Hong et al., 2013; Paoletti et al., 2013; Plant et al., 2006; Wang et al., 2009; Yashiro & Philpot, 2008). Os receptores AMPA desempenham um papel essencial na desestabilização, pois determinam se as sinapses serão maleáveis ou estáveis ou mesmo, resistentes à atualização (Mamou et al., 2006; Rose & Rankin, 2006). Atualmente, foi elucidado que a subunidade CP-AMPAR permite a maleabilidade sináptica, enquanto os CI-AMPAR contribuem para a neurotransmissão basal e a estabilidade sináptica (Hong et al., 2013; Torquatto et al., 2019). Desta forma, a reativação da memória acarreta a substituição momentânea dos CI-AMPAR por CP-AMPAR, o que desencadeia o processo de desestabilização, sendo demonstrado que o bloqueio especifico do CP-AMPA resulta na inibição da desestabilização, enquanto um

inibidor geral de AMPA não demonstra tal efeito (Clem & Huganir, 2010; Ferrara et al., 2019; Hong et al., 2013; Rao-Ruiz et al., 2011). Notavelmente, o bloqueio da endocitose dos receptores CP-AMPA impede a reconsolidação da memória de medo (Ferrara et al., 2019). Esses achados evidenciam o papel essencial dos receptores CP-AMPA na desestabilização.

Estudos anteriores demonstraram também o papel central dos receptores NMDA nas fases de desestabilização e restabilização da memória (Sadler et al., 2007; Warburton et al., 2013; Winters et al., 2009; Wyllie et al., 2013). Tanto no hipocampo quanto na amígdala, o bloqueio dos receptores NMDA ou da subunidade GluN2B impede os mecanismos de desestabilização da memória, processo fundamental para a reconsolidação (Kim et al., 2011; Mamou et al., 2006; Milton et al., 2013). Em contraste, a inibição da subunidade GluN2A parece influenciar seletivamente a fase de restabilização da reconsolidação (Milton et al., 2013). Sabe-se que durante aquisição e consolidação da memória ocorre um aumento na proporção GluN2A/GluN2B (Quinlan et al., 2004). Todavia, as memórias traumáticas (memórias fortes ou treinos fortes) dificilmente sofrem a desestabilização do seu traço quando reativadas e foram correlacionadas com a redução das subunidades GluN2B (Wang et al., 2009). Desta maneira, sugere-se que as subunidades GluN2B estão intimamente correlacionadas com os períodos plásticos da memória, logo a razão das duas subunidades também pode servir como um mecanismo para manter a integridade da memória ao longo do tempo e prevenir que qualquer memória seja desestabilizada e modificada.

Corroborando com a literatura, os nossos resultados com as infusões do fármaco NASPM pela via ICV após cada reativação preveniram a atualização da memória através do protocolo de descondicionamento. Da mesma forma, a inibição dos receptores NMDA através de um vetor viral que inibe especificamente a expressão da subunidade GluN2B demonstrou que impede a atualização da memória de medo. Juntos, esses achados sugerem que ambos os receptores ionotrópicos glutamatérgicos (NMDA-GluN2B e CP-AMPA) participam ativamente para sustentar a reconsolidação da memória de medo induzida pelo descondicionamento.

Examinamos também a atividade de uma protease, as calpaínas, cujo substrato dessa protease são os receptores NMDA e AMPA, bem como o remodelamento do citoesqueleto que mantêm a morfologia os espinhos dendríticos (Lu et al., 2000; Simpkins et al., 2003; Wu et al., 2004). Inúmeros estudos que exploram a atividade das calpaínas

evidenciam o seu papel essencial nos mecanismos que suportam desde a consolidação, evocação até a flexibilização a memória de medo em roedores (Nagayoshi et al., 2017; Popik et al., 2018). Aqui, infundimos um fármaco (PD150606) que inibe a atividade das calpaínas, testamos a nossa hipótese que a sua inibição iria bloquear a atualização da memória pelo descondicionamento. Desta forma, demonstramos que a inibição da atividade dessa protease impede a reconsolidação da memória pelo nosso protocolo.

Assim, demonstramos alguns mecanismos moleculares responsáveis pela atualização da memória de medo através do descondicionamento. Sugerimos que agem pela desestabilização do seu traço mnemônico. Sendo principalmente os receptores NMDA-GluN2B, CP-AMPA, a atividade das calpaínas e dos receptores endocanabinoides – CB1, bem como a dinâmica de cálcio intracelular e extracelular.

## CONCLUSÕES

Desenvolvemos uma inovadora abordagem não tóxica e com potencial translacional para atenuar as memórias aversivas. O protocolo de descondicionamento, como denominamos, consiste em aplicar choques fracos (0,1mA) durante as reativações. Destarte, demonstramos de forma enfática que o protocolo de descondicionamento reduz a expressão do medo sob diferentes circunstâncias, impedindo a *renewal*, a recuperação espontânea e a reaquisição da memória de medo, além de romper com as condições limitantes da reconsolidação. O descondicionamento também foi eficaz em diferentes paradigmas comportamentais (CAC, CAT e esquiva inibitória), bem como para ambos os sexos. Além disso, evidenciamos alguns dos mecanismos moleculares que o sustentaria, como os receptores ionotrópicos de glutamato (NMDA-GluN2B e CP-AMPA), atividade das calpaínas, os receptores IP3, receptores endocanabinóides (CB1) e os canais de cálcio dependente de voltagem (LVGCCs). Ademais, demonstramos tanto a janela temporal da reconsolidação quanto os limites de intensidade de treino necessário para atualizar a memória de medo. Desta forma, os nossos resultados corroboram com a nossa hipótese original, que o descondicionamento age pelos mecanismos da reconsolidação.

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