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**PARTICIPAÇÃO DO SISTEMA CANABINÓIDE ENDÓGENO
HIPOCAMPAL NA CONSOLIDAÇÃO, RECONSOLIDAÇÃO E
EXTINÇÃO DA MEMÓRIA DE RATOS**

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RESUMO

Os receptores canabinóides CB1 estão presentes em grandes quantidades no sistema nervoso central, especialmente no hipocampo, estrutura encefálica essencial para a formação de memórias. Estudos recentes mostram o importante papel do sistema endocanabinóide hipocampal sobre a consolidação e evocação de memórias emocionalmente relevantes ou que envolvam algum grau de aversividade. Na primeira parte desta tese, estudamos os efeitos da administração intra-hipocampal de AM251, um antagonista seletivo para os receptores canabinóides CB1, ou anandamida, um agonista canabinóide endógeno, sobre as fases da reconsolidação e extinção da memória na tarefa do condicionamento aversivo contextual. Nossos resultados mostram que o antagonista CB1 facilitou a reconsolidação, porém inibiu a extinção. A anandamida inibiu a reconsolidação da memória e facilitou a extinção, e esses efeitos foram revertidos pela co-administração de uma dose subefetiva de AM251, mostrando que os efeitos são mediados pelos receptores CB1.

Na segunda parte do trabalho, investigamos os mecanismos por trás do envolvimento do sistema endocanabinóide hipocampal sobre memórias emocionais/aversivas. Foram desenvolvidos dois protocolos com diferentes níveis de aversividade. O antagonista CB1 AM251 inibiu a consolidação da memória no protocolo de alta aversividade (choque forte), mas não no de baixa aversividade (choque fraco). Em seguida, os animais foram submetidos a uma sessão de estresse para mimetizar um aprendizado aversivo e então, treinamos no protocolo fraco. A infusão de AM251 inibiu a consolidação da memória nessas condições. O experimento seguinte avaliou se os

glicocorticóides liberados durante o estresse ou num aprendizado com forte conteúdo emocional é o fator determinante para a ativação do sistema endocanabinóide no hipocampo. O AM251 inibiu a consolidação da memória quando os animais eram tratados com o glicocorticóide sintético dexametasona imediatamente antes do treino com choque fraco. Por fim, avaliamos se o local de ação dos glicocorticóides era o hipocampo. A administração intra-hipocampal de dexametasona não produziu efeito sobre a consolidação, sugerindo que a modulação dos glicocorticóides ocorre de maneira indireta. Esses resultados mostram que existe uma interação sinérgica entre o sistema canabinóide e glicocorticóide sobre a modulação da memória.

ABSTRACT

CB1 cannabinoid receptors are abundantly present in the brain, with large concentration in the hippocampus, an essential structure for the memory processes. Experimental evidence suggests an important role of the endocannabinoid system (ECS) in aversively-motivated memories. Similarly, Glucocorticoids (GC) released in response to stress exposure also modulates memory formation, and both stress and dexamethasone activate the ECS. The scope of the first part is to investigate the potential role of the hippocampal endocannabinoid system on reconsolidation and extinction. Bilateral infusion of CB1 antagonist AM251 into the dorsal hippocampus after memory reactivation facilitated the reconsolidation of the contextual fear conditioning memory. In contrast, the local infusion in CA1 with anandamide blocked memory reconsolidation, an effect that was antagonized after the combined administration of anandamide with a CB1 antagonist, supporting a role of the hippocampal endocannabinoid system in the modulation of the reconsolidation. Local infusion with AM251 into CA1 blocked memory extinction whereas the administration of anandamide facilitated memory extinction. However, when combined with a subthreshold dose of the antagonist the extinction remain unaffected. Our results demonstrated a modulatory role of the hippocampal endocannabinoid system in both processes after retrieval: reconsolidation and extinction

In the second part, we investigated the interaction between the ECS and GCs in the hippocampus in the modulation of fear memory consolidation. Two protocols with different shock intensities were used in order to control the level of aversiveness. Local infusion of AM251 into the CA1 immediately after training was amnesic in the strong, but not in the

weak protocol. Moreover, AM251 was amnesic in animals stressed prior to the weak protocol, reverting the stress-induced facilitating effect. In order to investigate if the stress effect was mediated by glucocorticoid, we performed dexamethasone injection before training. Intrahippocampal AM251 infusion reduced memory in these animals. Finally, we investigated if glucocorticoid action site was in hippocampus. Dexamethasone infused directly in CA1 was not able to mimic the systemic injection effect, suggesting an indirect GC modulation. In conclusion, ECS and GC seem to interact in a synergic way in order to modulate memory consolidation.

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1. INTRODUÇÃO

Dentre todas as funções exercidas pelo encéfalo, a mais fascinante é a sua capacidade de reter informações de fatos, eventos e habilidades. Pois em consequência disso (somado às informações inatas presentes em nosso genótipo) cada um de nós é um indivíduo único. Portanto, é a memória que garante nossa individualidade e personalidade. Além disso, a memória possui um papel fundamental para a nossa sobrevivência, pois permite a retenção de informações oriundas de experiências vividas que irão nortear nossas ações futuras. A existência da memória em organismos filogeneticamente mais antigos, como nos invertebrados, e a sua conservação ao longo da evolução, reflete o grande valor adaptativo da memória, que permite aos organismos adaptarem-se às constantes mudanças do ambiente.

1.1. MEMÓRIA

Pode-se definir memória como o registro da representação de informações adquiridas através de experiências. Apesar do nosso organismo estar constantemente recebendo informações através de nossos sentidos, apenas uma pequena fração será retida de forma duradoura. A intensidade e a duração da memória é determinada pela importância da informação e o grau de atenção e emoção envolvidos no momento da aquisição da memória. Uma vez retida, a informação pode ser evocada. A memória pode ser classificada

de acordo com o tempo de retenção e quanto ao conteúdo da informação, ilustrado resumidamente no quadro I.

Quadro I

CLASSIFICAÇÃO DA MEMÓRIA QUANTO À DURAÇÃO

Tipo	Duração	Características
Memória de Trabalho (Operacional)	Segundos a poucos minutos	Não produz “arquivos” duradouros. Mantém a informação <i>on line</i> , através da atividade de neurônios do córtex pré-frontal enquanto estamos executando determinada tarefa.
Curta Duração	Até cerca de 6 horas após o aprendizado	Mantém a informação enquanto a memória de longa duração está sendo formada. Não requer síntese de novas proteínas.
Longa Duração	Dias, anos, toda a vida	Garante o registro do passado autobiográfico, conhecimento e habilidades do indivíduo.

CLASSIFICAÇÃO DA MEMÓRIA QUANTO À NATUREZA DE SEU CONTEÚDO

Tipo	Características	Principais estruturas encefálicas envolvidas
Declarativa ou Explícita	Podem ser subdivididos em episódicas , que refere-se a eventos autobiográficos, e semânticas , que refere-se a fatos e conceitos.	Formação hipocampal, amígdala, diencéfalo e neocórtex
Não-declarativa ou Implícita (de precedimentos)	Hábitos e habilidades motoras.	Núcleos da base e cerebelo

Nota: Apesar da conceituação e classificação da memória ser um método didaticamente válido, ele não é totalmente preciso. Muitas memórias possuem componentes implícitos e explícitos, dificilmente dissociados, que podem agir de maneira sinérgica ou competitiva. Além disso, não existe consenso entre as classificações, tanto quanto à duração como ao conteúdo, havendo uma grande divergência entre os autores.

Tão importante quanto armazenar informações é a nossa capacidade de esquecer, pois além de não sobrecarregar nosso sistema com memórias que deixaram de ser relevantes, nos permite fazer generalizações e abstrações.

A formação de uma memória não ocorre instantaneamente, logo após o aprendizado, ocorrem modificações moleculares e celulares requeridas para que essa

memória seja formada. Durante esse período, chamado de consolidação, a informação pode ser alterada por eletrochoque convulsivo (ECC), traumas, hipotermia ou tratamentos farmacológicos, antes de se estabilizar. Passada essa “janela temporal”, esses tratamentos não são mais efetivos (Duncan, 1949; McGaugh, 1966, 2000; Sara, 2000). O termo “consolidação” foi cunhado por Muller e Pilzecker em 1900 através de experimentos com humanos, mostrando que um aprendizado pode interferir sobre outro se o intervalo entre eles for curto. Estudos com modelos animais de amnésia retrógrada foram demonstrados a partir de 1949 por Duncan, mostrando que o tratamento com ECC logo após o treinamento prejudicava a consolidação da memória em animais, porém esse fenômeno não ocorria se o ECC fosse apresentado depois de transcorrido um intervalo maior de tempo em relação ao treino. Ou seja, a memória parece possuir uma fase lábil (e, portanto, sujeita a modulações) após a aquisição. Entretanto, depois de consolidada, seria armazenada em um estado estável.

No final da década de 60, foi demonstrado que a memória poderia retornar para um estado lábil após a evocação (Misanin et al., 1968). Nesse trabalho, o grupo do Dr. Lewis demonstrou que o tratamento com ECC após a evocação (usualmente chamada de reativação no jargão de pesquisadores de reconsolidação), prejudicava a memória.

Interessantemente, se o ECC fosse apresentado na ausência da reativação, tal prejuízo não ocorria. Portanto, o ECC só afetava a memória se ocorresse logo após a evocação, mostrando que a memória se relabiliza quando evocada. Esse fenômeno foi chamado posteriormente de *reconsolidação* (apesar de não se tratar exatamente de um simples segundo *round* da consolidação). Apesar da relevância do assunto, esse trabalho não teve a repercussão merecida graças ao forte “dogma” da consolidação vigente na época,

de que a memória após uma vez consolidada, não mais seria passível de mudanças. O tema voltou à tona vigorosamente em 2000, quando um trabalho do laboratório do Dr. Joseph LeDoux, foi publicado na revista *Nature* (Nader et al., 2000). Nesse estudo, a infusão do inibidor de síntese protéica, anisomicina, na amígdala lateral, imediatamente após a reativação, inibiu a reconsolidação da memória. Assim como nos trabalhos de Lewis, a memória tinha que ser reativada para tornar-se sensível ao tratamento.

Durante a evocação, portanto, a memória pode tornar-se lábil novamente, necessitando de processos moleculares e celulares, como transcrição e tradução gênica, ativação de receptores NMDA, entre muitos outros mecanismos necessários para manter-se e/ou atualizar-se (Nader et al., 2000; Lee et al., 2006; Da Silva et al., 2008). Além disso, a reativação da memória por um período prolongado, pode desencadear um outro processo, de conteúdo oposto ao da memória original: a *extinção*.

O fisiologista russo Ivan Pavlov descreveu que quando um estímulo condicionado (EC), o qual inicialmente não produz uma resposta comportamental significativa, é pareado com um estímulo incondicionado (EI) (biologicamente significante, que produz invariavelmente uma resposta), o mesmo passa a produzir uma resposta condicionada. Ou seja, ocorre uma associação entre os dois estímulos. Essa associação foi chamada de condicionamento pavloviano ou clássico. Se o EC for repetidamente apresentado sem o EI, o animal tenderá a produzir uma nova associação: EC sem EI, diminuindo a resposta condicionada, processo chamado de extinção.

Apesar de Pavlov ter demonstrado esse condicionamento em cães, hoje em dia, o mesmo princípio é amplamente utilizado no estudo da neurobiologia da memória em

roedores. Nesse caso, o EC é comumente um determinado contexto, e o EI, um choque nas patas. Após a associação entre o EC e o EI, a exposição ao contexto provoca uma resposta condicionada, usualmente quantificada pela resposta estereotipada de medo, chamada de congelamento (*freezing*, em inglês). A reexposição prolongada ao contexto sem o EI produz a extinção, diminuindo, portanto, a resposta condicionada.

Dois processos antagônicos, portanto, podem derivar da evocação da memória (dependendo do “valor” atribuído à informação): se a informação é importante, essa memória vai ser reconsolidada e mantida, caso contrário ela tenderá a ser extinta, conforme mostra a figura 1. Ou seja, a evocação da memória não é um processo passivo, ela recruta diversas cascatas bioquímicas para “definir” o rumo dessa memória recém-evocada. O sentido biológico da reconsolidação da memória é manter e acrescentar novas informações à memória antiga, enquanto o da extinção é de formar uma nova memória com significado distinto da memória original.

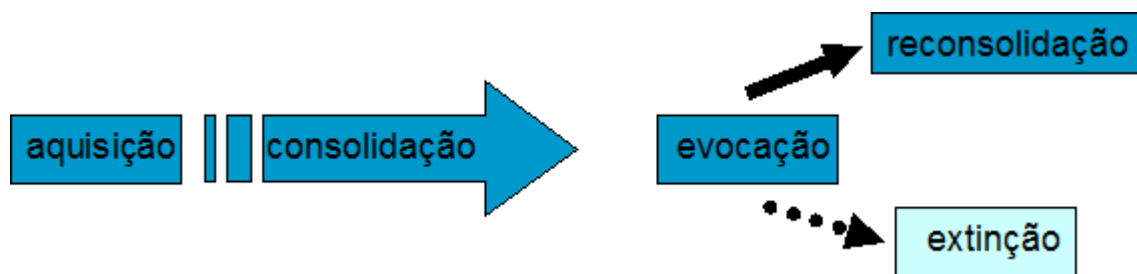


FIGURA 1.

1.2. CANNABIS SATIVA E O SISTEMA ENDOCANABINÓIDE

A *Cannabis sativa* (classificada por Carolus Linnaeus em 1753) (fig. 2), popularmente conhecida como maconha ou haxixe, entre muitos outros nomes, vem sendo utilizada há milhares de anos por seus efeitos psicoativos (Elphick, 2001), tanto para usos medicinais, como recreativos (Wilson e Nicoll, 2002). A folha e a flor da *Cannabis sativa* secretam cerca de 60 compostos terpenofenólicos chamados de canabinóides. Destes, o principal princípio ativo é o Δ^9 -tetrahydrocannabinol (THC), descrito em 1964 (Gaoni & Mechoulam, 1964). Devido à natureza lipofílica do THC, acreditava-se que seu mecanismo de ação seria pela interação com a membrana plasmática, modificando sua fluidez (Hillard et al., 1985). O primeiro indício de que o mecanismo de ação da *cannabis* era mediado por receptores de membrana, surgiu quando Howlett demonstrou que canabinóides diminuíam a quantidade de AMPc em cultura de neuroblastoma, sugerindo que esses receptores eram acoplados a proteína G_i (Howlett, 1984). A busca por uma maior compreensão sobre os receptores canabinóides continuaram, com a confirmação através de *binding* (Devane et al., 1988), localização (Herkenham et al., 1990), e finalmente a clonagem do receptor CB1 (Matsuda et al., 1990).

Depois da identificação de receptores canabinóides específicos, a questão natural a ser pensada era: não possuímos esses receptores para a possibilidade de utilizar a *Cannabis*, provavelmente deve haver algum ligante endógeno que ative esses receptores. Em 1992, utilizando extratos de encéfalo de porcos, isolou-se o primeiro canabinóide endógeno, o N-

araquidonil-etanolamina (Devane et al., 1992), (a qual foi chamada de anandamida, que significa “felicidade”, do sânscrito). Três anos após, um segundo canabinóide endógeno foi identificado, o 2-araquidonilglicerol (2-AG) (Mechoulam et al., 1995). *Cannabis Sativa*



FIGURA 2. *CANNABIS SATIVA*

Nos seres humanos, o THC produz euforia, antinocicepção, dificulta a concentração, entre outros efeitos (Ameri, 1999). Exerce seus efeitos ligando-se aos receptores canabinóides CB1 e CB2 (fig. 3 e 4). Os receptores CB1 estão localizados principalmente no SNC, e os CB2, em células do sistema imunitário - principalmente em linfócitos B e T, mastócitos e macrófagos (Iversen et al., 2001). Entretanto, trabalhos recentes demonstraram a presença de CB2 no tronco encefálico (o qual pode estar envolvido no controle da êmese) (Van et al., 2005) e em outras estruturas encefálicas (Brusco et al., 2008), porém o seu papel fisiológico ainda não é compreendido.

Os receptores CB1 são os receptores metabotrópicos mais abundantes no SNC (Howlett et al., 2002; Herkenham et al., 1991) e estão localizados principalmente nos núcleos da base, córtex, cerebelo e hipocampo (Wilson e Nicoll 2002). Esses pertencem à família de receptores acoplados à proteína $G_{i/o}$, a qual inibe a adenilato ciclase e os canais de cálcio dependentes de voltagem do tipo N e P/Q, e estimulam canais de potássio e a enzima MAPK (Ameri 1999) (fig.6).

Além dos receptores CB1 e CB2, os endocanabinóides podem se ligar nos receptores TRPV1 (Toth et al., 2009; Starowicz et al., 2007), PPAR (O'Sullivan 2007) e GPR55 (Ryberg et al., 2005).

Timeline of Cannabis use and scientific advances

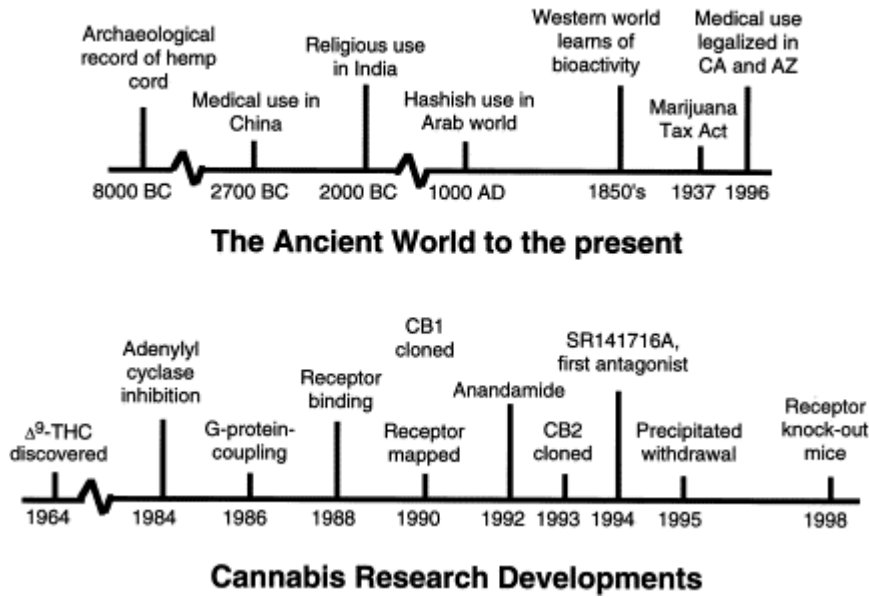


FIGURA 3. HISTÓRICO DO USO E DESCOBERTAS DA CANNABIS. EXTRAÍDO DE CHILDERS 1998.

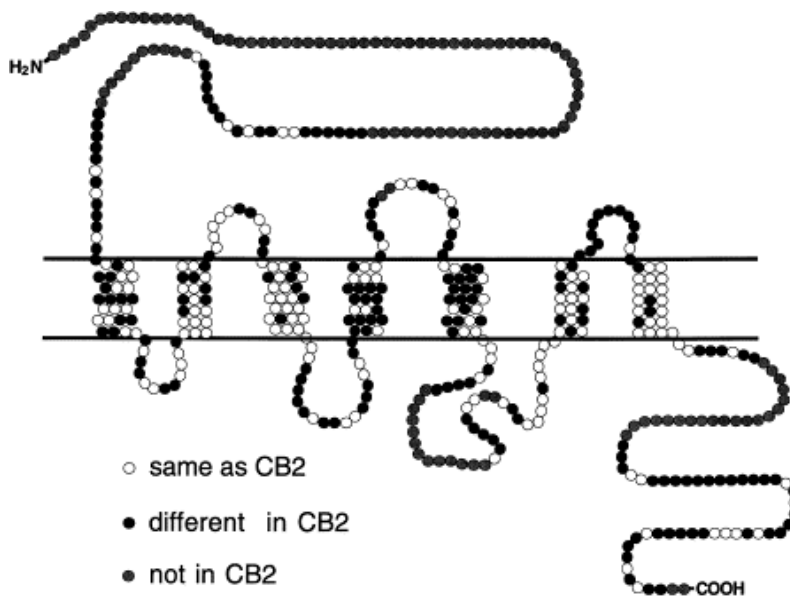


FIGURA 4. RECEPTORES CANNABINÓIDES. ADAPTADO DE CHILDERS 1998.

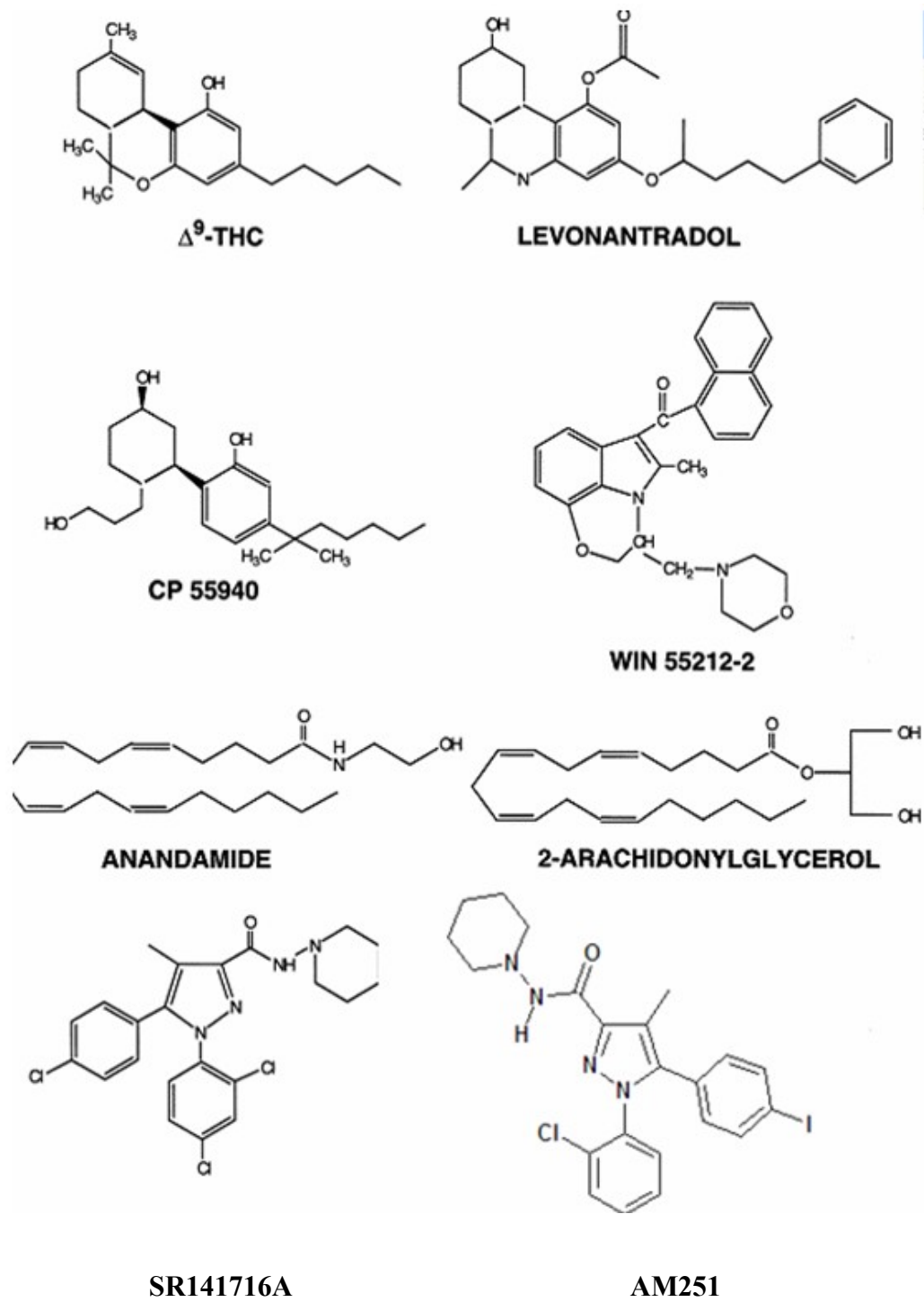


FIGURA 5. AGONISTAS E ANTAGONISTAS (DOIS ÚLTIMOS) CANABINÓIDES. ADAPTADO DE CHILDERS 1998.

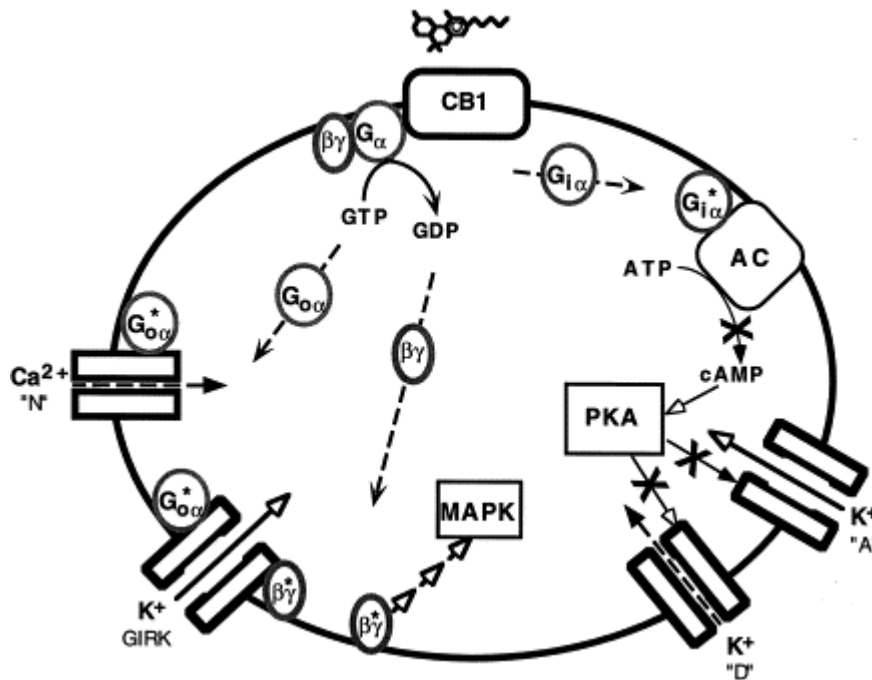


FIGURA 6. MECANISMO DE TRANSDUÇÃO. ADAPTADO DE CHILDERS 1998.

Dois principais canabinóides endógenos, ou endocanabinóides, foram identificados: a n-araquidoniletanolamina (anandamida) (Devane et al., 1992) e 2-araquidnilglicerol (2-AG) (Mechoulam et al., 1995), ambos eicosanóides. Outras moléculas endógenas que se ligam aos receptores já foram identificadas, como a noladina éter (2-araquidnilglicerol éter), a virodamina (O-araquidnil-etanolamina) e a N-araquidnil-dopamina (Bisogno et al., 2005, Hanus et al., 2001, Porter et al., 2002), porém pouco se sabe sobre elas. Os endocanabinóides clássicos (anandamida e 2-AG) são sintetizados a partir de fosfolipídios de membrana e liberados por neurônios pós-sinápticos (mensageiros retrógrados) de forma dependente de cálcio (consequente da atividade neuronal). São inativados principalmente por recaptação (por neurônios e glia) e posteriormente hidrolisados por enzimas específicas: a anandamida é metabolizada pela ácido graxo amida hidroxilase (FAAH) em

etanolamina e ácido araquidônico (Mechoulam *et al.*, 1998) e o 2-AG por uma lipase de monoacilglicerol (embora também pela FAAH) (Goparaju *et al.*, 1999).

Vários achados sugerem que os canabinóides possam modular a liberação e ação de distintos neurotransmissores. Desta forma, foi verificada uma interação entre canabinóides e sistemas gabaérgicos, glutamatérgicos, dopaminérgicos, noradrenérgicos e colinérgicos (Chaperon e Thiebot, 1999).

No hipocampo, os receptores CB1 parecem estar localizados basicamente nos terminais pré-sinápticos de neurônios gabaérgicos, principalmente nas células em cesta que co-liberam colecistocinina (CCK) (Katona *et al.*, 1999; Tsou *et al.*, 1999; Wilson & Nicoll, 2002). De fato, agonistas CB1 reduzem fortemente a liberação de GABA no hipocampo, e que, nesta estrutura, tal efeito é revertido pelo antagonista seletivo CB1 SR141716A (Katona *et al.*, 1999). Três trabalhos recentes mostraram, independentemente, a presença de receptores CB1 em terminais de neurônios piramidais glutamatérgicos no hipocampo (Katona *et al.*, 2006; Takahashi & Castillo, 2006; Kawamura *et al.*, 2006), embora com uma concentração 20 vezes maior em interneurônios inibitórios (Kawamura *et al.*, 2006).

Muitos efeitos atribuídos à maconha em usuários crônicos (muitas vezes por jornalistas e outros leigos, sem conhecimento para interpretar alguns resultados) não foram comprovados em estudos em humanos e modelos animais, além de serem bastante contraditórios: é o caso de muitos relatos acerca do desenvolvimento de tolerância e dependência, toxicidade, lesões em estruturas encefálicas, alterações hormonais ou na produção de gametas, mutagênese e carcinogênese - pelo menos em doses normais, próximas às consumidas por usuários (Iversen, 2001).

1.3 SISTEMA CANABINÓIDE E MEMÓRIA

O déficit de atenção, aprendizado e memória com a administração aguda de canabinomiméticos em mamíferos têm sido mostrados há bastante tempo na literatura. A infusão de agonistas canabinóides sintéticos WIN55212-2, CP55940, HU-210 e naturais como o THC, e mesmo daquelas moléculas que depois vieram a ser detectadas como sendo endocanabinóides, administrados de forma sistêmica antes do treino, tiveram efeitos prejudiciais sobre a memória em diferentes tarefas espaciais: labirinto radial de oito braços (Molina-Holgado et al., 1995, Lichtman et al., 1995 e 1996), alternância espacial no labirinto em T (Jentsch et al., 1997), campo aberto (Ferrari et al., 1999) ou DMTP (Mallet et al., 1996; Hampson et al., 1998, 1999, 2000).

Entretanto esses experimentos não demonstraram conclusivamente até que ponto tal efeito se deve à ação dos canabinóides sobre o hipocampo. Uma exceção foi o trabalho de Lichtman e colaboradores (1995), com administração intra-hipocampal, que encontrou resultados similares com CP55,940 (agonista CB1), porém exclusivamente sobre a memória de trabalho. Estudos com antagonistas canabinóides administrados sozinhos (i.p.) não mostraram efeitos deletérios (Da Silva & Takahashi, 2002; Davies et al., 2002), ou, por outro lado, encontraram até mesmo facilitação da memória de curta duração no labirinto radial de 8 braços (Lichtman et al., 2000). A administração sistêmica pós-treino de anandamida prejudicou a memória na tarefa da esquiwa inibitória em ratos (Murillo-Rodrigues et al., 1998) e camundongos (Castellano et al., 1997, 1999). A administração crônica de THC causou um déficit de memória espacial na tarefa do labirinto radial de oito braços (Stiglick et al., 1982) e, por outro lado, uma facilitação na esquiwa ativa (Stiglick et

al., 1984). Dois trabalhos recentes mostraram uma facilitação da aquisição da memória com a inibição da FAAH na tarefa do labirinto aquático de Morris (Varvel et al., 2006) e um déficit sobre a aquisição e evocação com o antagonista-CB1 AM251 na tarefa do medo condicionado (Arenos et al., 2006).

Em trabalhos anteriores, investigamos o papel do sistema canabinóide endógeno sobre a aquisição, consolidação e evocação da memória, e sobre a indução da potenciação de longa duração (LTP), utilizando o agonista endógeno anandamida e um antagonista seletivo para os receptores CB1, o AM251 (um enfoque um pouco diferente de outros trabalhos, os quais procuravam investigar os efeitos dos canabinóides exógenos sobre a memória). Nossos resultados mostraram uma importante participação do sistema endocanabinóide sobre os processos de memória e indução da LTP (de Oliveira Alvares et al., 2005, 2006 e 2008). Alguns resultados estão representados resumidamente na tabela abaixo.

	Aquisição	Consolidação	Evocação	LTP
Anandamida	ϕ	\Uparrow	ϕ	—
AM251	ϕ	\Downarrow	\Uparrow	\Downarrow

ϕ = sem efeito
 \Uparrow = facilitação
 \Downarrow = prejuízo
 — = não realizado

Estudos recentes mostram um papel-chave do sistema endocanabinóide sobre a extinção da memória em camundongos nocaute para o receptor CB1 (Marsicano et al., 2002). Além disso, a administração sistêmica do agonista canabinóide WIN55212-2 e a inibição da FAAH, facilitam a extinção da memória (Pamplona et al., 2006; Varvel et al., 2006).

Em termos de reconsolidação da memória, existem poucos trabalhos envolvendo os receptores CB1, e seus resultados são aparentemente conflitantes: Suzuki e colaboradores não encontraram efeito com o antagonista CB1 SR141716A administrado i.p. antes da reativação da memória (Suzuki et al., 2004). Outros trabalhos foram feitos com a infusão na amígdala, do agonista WIN55212-2, que inibiu a reconsolidação (Lin et al., 2006), ou do antagonista AM251, que também inibiu a reconsolidação (Bucherelli et al., 2006).

A importância fisiológica do sistema endocanabinóide sobre o aprendizado emocional é reforçada pela grande concentração de receptores CB1 no SNC e a presença de endocanabinóides em estruturas encefálicas envolvidas neste tipo de memória, como o hipocampo (Herkenham et al., 1990). Apesar disso, surpreendentemente pouco se sabe a respeito da função do sistema canabinóide endógeno sobre a *extinção* ou a *reconsolidação* da memória nessa estrutura.

Embora existam muitos trabalhos mostrando a importância do sistema endocanabinóide sobre a formação de memórias emocionais aversivas, esse sistema parece não participar da formação de memórias com menores níveis de alerta e/ou aversividade (Holter et al., 2005; de Oliveira Alvares et al., 2005; Pamplona e Takahashi, 2006; Niyuhire et al., 2007). Entre os hormônios liberados durante um aprendizado com conteúdo

emocional, os glicocorticóides estão entre os que possuem um papel bastante conhecido sobre a memória. Muitos trabalhos mostram que tanto uma situação de estresse, como a administração de corticosterona ou do glicocorticóide sintético dexametasona facilitam a consolidação da memória (Quirarte et al.,1997; Roozendaal et al., 2002; Roozendaal et al., 2006) e prejudicam a evocação (de Quervain et al.,1998). Roozendaal e colaboradores demonstraram que a corticosterona só influencia a consolidação da memória quando o treinamento demanda um nível mínimo de alerta (*arousal*) (Okuda et al.,2004; Roozendaal et al., 2006).

O fato do sistema endocanabinóide e glicocorticóide possuírem o mesmo padrão de modulação sobre memórias que envolvam algum grau de aversividade (facilitando a consolidação e inibindo a evocação, o que é incomum se compararmos com outros sistemas neuromoduladores), sugere uma interação entre esses sistemas. Além disso, estudos recentes mostraram que choques nas patas e a infusão de dexametasona aumentam os níveis dos canabinóides endógenos anandamida e 2-AG (Di et al., 2005; Hohmann et al.,2005). Campolongo e colegas demonstraram que o efeito facilitatório da corticosterona sobre a consolidação da memória era revertido com antagonista CB1 infundido na amígdala basolateral, reforçando a hipótese da interação entre os dois sistemas.

O corpo de resultados desta tese está dividida em dois capítulos, que correspondem aos dois artigos publicados durante o doutorado. O capítulo I refere-se aos experimentos que avaliam o papel do sistema endocanabinóide hipocampal sobre a reconsolidação e extinção da memória. No capítulo II, investigamos a interação do sistema endocanabinóide e glicocorticóide sobre a consolidação da memória.

2. OBJETIVOS

2.1 OBJETIVO GERAL DO CAPÍTULO I:

Verificar a possível participação do sistema endocanabinóide hipocampal sobre os processos de *reconsolidação* e de *extinção* de memórias aversivas empregando o modelo do condicionamento aversivo contextual em ratos Wistar adultos.

2.1.1 Objetivos específicos:

- 1- Verificar os efeitos da administração intra-hipocampal do antagonista seletivo dos receptores CB1, AM251, sobre a reconsolidação da memória no modelo do condicionamento aversivo contextual;
- 2- Verificar os efeitos da administração intra-hipocampal do agonista canabinóide endógeno, anandamida, sobre a reconsolidação da memória no modelo do condicionamento aversivo contextual;
- 3- Verificar os efeitos da administração intra-hipocampal do antagonista seletivo dos receptores CB1, AM251, imediatamente após a sessão de extinção da memória no modelo do condicionamento aversivo contextual;
- 4- Verificar os efeitos da administração intra-hipocampal do agonista canabinóide endógeno, anandamida, imediatamente após a sessão de extinção da memória no modelo do condicionamento aversivo contextual;
- 5- Verificar se os efeitos da administração intra-hipocampal do agonista canabinóide endógeno, anandamida, sobre a reconsolidação é revertido pela infusão de uma dose sem efeito próprio do antagonista seletivo dos receptores CB1, AM251, no modelo do condicionamento aversivo contextual;

- 6- Verificar se os efeitos da administração intra-hipocampal do agonista canabinóide endógeno, anandamida, sobre a extinção é revertido pela infusão de uma dose sem efeito próprio do antagonista seletivo dos receptores CB1, AM251, no modelo do condicionamento aversivo contextual.

2.2 OBJETIVO GERAL DO CAPÍTULO II:

Verificar os mecanismos subjacentes à participação hipocampal do sistema endocanabinóide sobre a formação de memórias emocionais-aversivas, mas não sobre memórias neutras ou com um menor grau de aversividade no modelo do condicionamento aversivo contextual em ratos Wistar adultos.

2.2.1 Objetivos específicos:

- 1- Verificar se o sistema endocanabinóide é recrutado para o processo de consolidação da memória num protocolo de aversividade moderada (com choques de 0,3mA) ou de alta aversividade (choques de 0,7mA) no modelo do condicionamento aversivo contextual, através da administração intra-hipocampal do antagonista CB1, AM251, após o treino;
- 2- Verificar se o sistema endocanabinóide é recrutado para o processo de consolidação da memória num protocolo de aversividade moderada (com choques de 0,3mA) quando os animais são submetidos previamente a uma sessão de estresse, através da administração intra-hipocampal do antagonista CB1, AM251, após o treino;
- 3- Verificar se o sistema endocanabinóide é recrutado para o processo de consolidação da memória num protocolo de aversividade moderada (com choques de 0,3mA) quando os animais são infundidos sistemicamente com glicocorticóide sintético dexametasona i.p., através da administração intra-hipocampal do antagonista CB1, AM251, após o treino;
- 4- Verificar se o local de ação dos glicocorticóides sobre a consolidação da memória é, de fato, o hipocampo, através da infusão intra-hipocampal de

dexametasona, um glicocorticóide sintético, num protocolo de aversividade moderada (com choques de 0,3mA).

3. CAPÍTULO I

Artigo “Opposite action of hippocampal CB1 receptors in memory reconsolidation and extinction” publicado na revista *Neuroscience* 154(4):1648-55, 2008.

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OPPOSITE ACTION OF HIPPOCAMPAL CB1 RECEPTORS IN MEMORY RECONSOLIDATION AND EXTINCTION

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Abstract—Retrieval of a consolidated memory triggers a number of processes which depend, among other factors, on the duration of the reactivation session: reconsolidation requires a brief reactivation session, and extinction, a prolonged one. The scope of this study is to explore the potential role of the hippocampal endocannabinoid system on reconsolidation and extinction processes. Bilateral infusion of the CB1 cannabinoid receptor antagonist, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2, 4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251) into the CA1 region of the dorsal hippocampus of Wistar rats after memory reactivation facilitated the reconsolidation of the contextual fear conditioning memory. The inhibition of protein synthesis with DRB in the same brain region blocked memory reconsolidation. Both effects were persistent, lasting up to 7 days after the first retrieval experience. In contrast, the local infusion of anandamide blocked memory reconsolidation, an effect that was antagonized by the combined administration of anandamide with a subthreshold dose of a CB1 antagonist, supporting a CB1-mediated role of the hippocampal endocannabinoid system in the modulation of the memory reconsolidation. Local infusion of AM251 into CA1 blocked memory extinction whereas the administration of anandamide facilitated it; however, when combined with a subthreshold concentration of the CB1 antagonist, anandamide did not affect the extinction process. The clear-cut, opposite effects observed in each situation suggest a possible role of the hippocampal endocannabinoid system as a switching mechanism deciding which processes will take place, either maintaining the original memory (reconsolidation) or promoting a new learning (extinction). © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: AM251, anandamide, CB1 receptors, memory reconsolidation, memory extinction, dorsal hippocampus.

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Abbreviations: AEA, anandamide; AM251, N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; CS, conditioned stimulus; DCS, D-cycloserine; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; FAAH, fatty-acid amide hydrolase; US, unconditioned stimulus.

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In recent years, numerous authors have shown that memories already established can become transiently labile by a recall session—usually using the conditioned stimulus (CS) as a reminder cue of the original learning presented for a limited period of time (Przybylski and Sara, 1997; Nader, 2003a,b; Debiec and LeDoux, 2004; Duvarci and Nader, 2004). This phase is followed by a stabilization period, usually defined as reconsolidation, which requires de novo protein synthesis, at least in the involved brain structures (Misanin et al., 1968; Przybylski and Sara, 1997; Przybylski et al., 1999; Nader et al., 2000; Eisenberg et al., 2003; Pedreira and Maldonado, 2003; Debiec and LeDoux, 2004; Duvarci and Nader, 2004). For instance, Nader et al. (2000) have shown that anisomycin infused into the basolateral nuclei of the amygdala shortly after memory reactivation (but not in the absence of this session) produces amnesia on later tests, proving that consolidated fear memories, when reactivated, return to a labile state that requires de novo protein, a phenomenon called reconsolidation.

If re-exposure to the CS extends beyond some critical period, the conditioned response gradually decreases in a process called extinction, where the original memory trace is not erased, but transiently replaced by a new active learning: during this acquisition, animals learn that the presentation of the CS no longer predicts the occurrence of the unconditioned stimulus (US) (Bouton, 2004; Bouton et al., 2006; Myers and Davis, 2007). Therefore, reconsolidation demands a brief reactivation session, whereas extinction takes place after longer CS presentation, or after repeated presentations of the CS without the US. Consistent with this view, several authors have proposed that the duration of the re-exposure session to the CS is a decisive factor that critically influence which process will predominate: reconsolidation or extinction (Bustos et al., 2006, 2008; Debiec et al., 2002; Pedreira and Maldonado, 2003; Boccia et al., 2004, 2007; Suzuki et al., 2004; Tronson and Taylor, 2007).

Cannabinoid CB1 receptors are expressed throughout the brain, mainly in the basal ganglia, hippocampus, neocortex, and cerebellum (Davies et al., 2002; Wilson and Nicoll, 2002; Mackie, 2005). Being one of the most abundant class of metabotropic receptors in the brain, it is especially prominent in the hippocampus (Herkenham et al., 1991; Ameri, 1999; Hampson and Deadwyler, 1999), a structure essential for memory formation (Squire, 1992; Izquierdo and Medina, 1995). It is a consensus that glutamatergic synapses are the main responsible for the building of memory traces (see, e.g. Bliss and Collingridge, 1993; Izquierdo and Medina, 1995; Lamprecht and LeDoux, 2004; Izquierdo et al., 2006; Kull-

mann and Lamsa, 2007), so understanding the role of any modulatory system affecting it is of paramount importance. In the hippocampus, CB1 receptors are mostly localized on inhibitory interneurons (Katona et al., 1999; Egertova and Elphick, 2000; Tsou et al., 1999), but also on glutamatergic axon terminals (Katona et al., 2006; Takahashi and Castillo, 2006, and Kawamura et al., 2006), with a density 20 or more times higher in the inhibitory pre-synaptic sites than in the excitatory ones (Monory et al., 2006, 2007; Kawamura et al., 2006; Domenici et al., 2006).

Previous findings from our laboratory have shown that the post-training intra-hippocampal infusion of the CB1 antagonist N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251) *disrupted* memory consolidation of the step-down inhibitory avoidance task (De Oliveira Alvares et al., 2005, 2006, 2008). Surprisingly, a pre-test infusion of the same drug into the same structure and task has *facilitated* memory retrieval at the same dose that was amnesic post-training (for a discussion of that, please refer to De Oliveira Alvares et al., 2008).

In the last years, several reports have been showing that endocannabinoids play an important role in extinction of memories that are mostly aversive (Varvel et al., 2002, 2007; Suzuki et al., 2004; Chhatwal et al., 2005; Marsicano et al., 2002). Some of these works, specially those employing antagonists as pharmacological tools, point to a definite role of the endocannabinoids in these cognitive functions. The difficulty remains in that most of these studies were done using systemic infusions (e.g. Suzuki et al., 2004), and since CB1 receptors are present in different brain structures (involved in different physiological roles), these reports are hardly conclusive in terms of anatomical substrate. However, Kobiljo et al. (2007), infusing SR141716A directly into the insular cortex, have found a disruption of extinction similar to that reported by Suzuki et al. (2004) with i.p. administration: considering they were using different animals (respectively, rats and mice) and behavioral tasks, this is a notable convergence. Also, Varvel et al. (2007), studying knockout mice for fatty-acid amide hydrolase (FAAH), have found a facilitatory effect upon Morris water maze memory extinction: FAAH ($-/-$) mice submitted to an extinction protocol showed an increase in the latencies to reach the position where the hidden platform was located in the training session and a decrease in the percentage of time spent in the target quadrant, i.e. animals in this model of increased endocannabinoid tone extinguished the learned response at a quicker rate than normal FAAH ($+/+$) mice. This is an interesting result since Morris water maze is a less aversive behavioral paradigm.

Concerning memory reconsolidation, however, there are fewer studies focusing on the role of the endocannabinoid system, and conflicting results are not rare. Both Suzuki et al. (2004) and Kobiljo et al. (2007) reported a lack of effect of the CB1 antagonist SR141716A upon the reconsolidation aversive memories, with the important difference that the first one was infused i.p., and the other, in the insular cortex. Lin et al. (2006) found that two cannabinoid agonists infused into the amygdala disrupted reconsolidation of the startle memory, an effect proved to be mediated by CB1 receptors, since it was reverted by a concomitant subthreshold concentration of

AM251. All in all, the participation of this system in the memory reconsolidation remains unclear.

Based on the fact that endocannabinoids seem to modulate several phases of memory formation, and since CB1 receptors are highly concentrated in the hippocampus, a brain structure with a well known role in the control of memory processes, such as extinction or reconsolidation (Bonini et al., 2007; Rossato et al., 2006; Debiec et al., 2002; Vianna et al., 2001; Lee et al., 2006), it seems reasonable to hypothesize that this endogenous system could have an important role as a "route defining" system deciding between these two "routes" of a contextual fear memory continuation. The modulatory role of endocannabinoids in this region, acting both upon glutamatergic synapses and inhibitory GABAergic interneurons, calls for a closer look in terms of its participation in these memory processes.

EXPERIMENTAL PROCEDURES

Animals

One hundred twenty-six male Wistar rats (age 2–3 months, weight 210–300 g) from our breeding colony were used in this experiment. Animals were housed in plastic cages, four to five to a cage, under a 12-h light/dark cycle and at a constant temperature of 24 ± 1 °C, with water and food *ad libitum*.

Surgery

All animals were anesthetized by a mixture of ketamine and xylazine (i.p., 75 and 10 mg/kg, respectively) and bilaterally implanted with a 27-gauge guide cannulae aimed at AP -4.2 mm (from bregma), LL $+3.0$ mm, DV 1.8 mm, aimed 1.0 mm above the CA1 area of the dorsal hippocampus (according to Paxinos and Watson, 1998). After recovery from surgery (1 week), animals were submitted to the behavioral procedure.

Contextual fear conditioning (CFC)

The conditioning chamber consisted in an illuminated Plexiglas box (25.0×25.0 cm grid of parallel 0.1 cm caliber stainless steel bars spaced 1.0 cm apart). In the conditioning trial (day 1), rats were placed in the chamber for 3 min and received two 2-s 0.7 mA foot shocks separated by a 30 s interval. Before returning to the home cages, animals were kept in the conditioning environment for an additional minute. Reconsolidation or extinction protocols were performed 48 h later as follows: animals were re-exposed to the same context for 3 or 25 min, respectively, without receiving a foot-shock. Twenty-four hours later, all animals were tested for 5 min in the same context, and some groups were also retested 5 days later. A control group was submitted to the same procedure, but without the memory reactivation session. Freezing time was registered by an experienced observer that was unaware of the treatments, and used as a memory index.

Drugs

At the time of infusion, immediately after the re-exposure session, a 30-gauge infusion needle was fitted into the guide cannulae, with its tip protruding 1.0 mm beyond the guide cannulae and aimed at the pyramidal cell layer of CA1 in the dorsal hippocampus (Fig. 1). A bilateral infusion of 0.5 μ l was performed at a 20 μ l/h rate. Animals were divided into three groups, each receiving one of these drugs: AM251, a selective CB1 antagonist (Tocris Cookson Inc., Ellisville, MO, USA; concentrations of 5.5 or 0.27 ng per side/hemisphere injected), anandamide (AEA), a CB1 agonist (Tocris, concentrations of

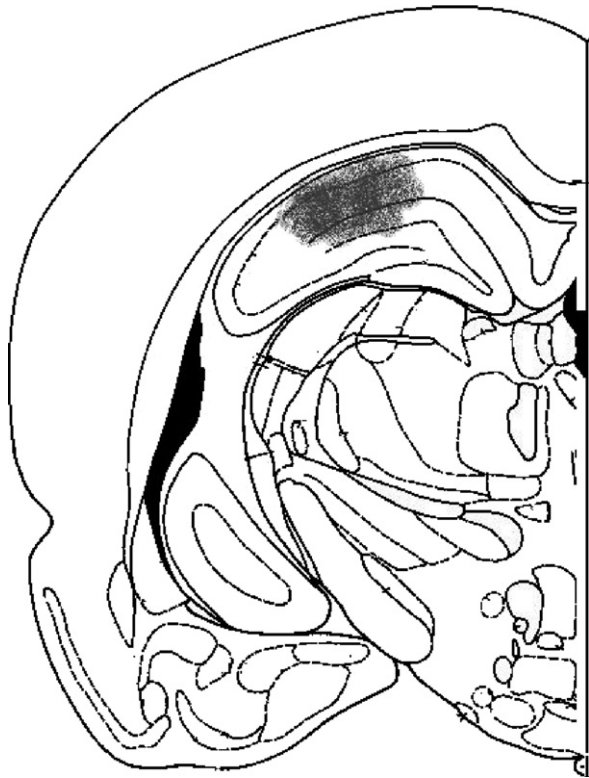


Fig. 1. Drawing representing AP plane -4.2 mm adapted from the atlas of Paxinos and Watson (1998) showing the extent of the area reached by the tip of the infusion cannulae, where our drugs were infused in the rat dorsal hippocampus (stippled area represent average region of acceptance, as dyed by $0.5 \mu\text{l}$ of 2% Methylene Blue in saline infused through the same cannulae right after kill by decapitation).

0.17 ng per side/hemisphere injected), 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), a protein synthesis inhibitor (Calbiochem, 10 ng per side/hemisphere injected) previously shown to block memory consolidation (Igaz et al., 2002), or their vehicle (phosphate-buffered saline with 8% dimethylsulfoxide). Vehicle composition and the selected doses of the drugs were chosen on the basis of previous experiments from our laboratory (De Oliveira Alvares et al., 2005, 2006, 2008). Statistical analysis of the behavioral data was limited to the 107 of 126 animals with correct cannulae placements (see Fig. 1). All experimental procedures were performed in strict accordance to the recommendations of Brazilian Society for Neurosciences (SBNeC), the Brazilian College of Animal Experimentation (COBEA), and the International Brain Research Organization (IBRO), being previously approved by the Ethics on Research Review Committee of our institution, UFRGS, meaning that they are in compliance with the U.S. National Institutes of Health Guide for Care and Use of Laboratory Animals (publication no. 85–23, revised in 1985), the European Communities Council Directive of 24 November 1986 (86/609/EEC), and the Brazilian law (Law no. 6.638/1979). Every effort was made to minimize the number of animals used and their suffering.

RESULTS

Effect of the CB1 antagonist and of the protein synthesis inhibitor on memory reconsolidation

The effects of 5.5 ng/side of both AM251 and DRB administered immediately after a 3 min re-exposure are shown in Fig.

2. The one-way ANOVA test revealed a significant difference among treatments between test 1 and test 2 sessions, the last one taking place 5 days after test 1 ($F(2,23)=41.207$, $P=0.000$ and $F(2,23)=17.471$, $P=0.000$, respectively). All animals performed similar freezing during reactivation ($F(2,23)=0.855$, $P=0.438$). Student-Newman-Keuls post hoc test comparisons indicated that AM251 and DRB rats are different from vehicle-treated animals ($P<0.05$).

Effect of the CB1 antagonist on memory extinction

Fig. 3 depicts the effect of AM251 on the extinction of contextual fear memory. Paired t -test revealed a significant difference between the first 4 min period from the last 4 min of the 25 min re-exposure session in all animals ($P=0.000$ in every group). However, independent t -test revealed no difference between AM251 and vehicle rats during these periods ($F(1,3)=0.14$, $P=0.673$ and $F(1,3)=6.72$, $P=0.919$). In the test, AM251 rats displayed significantly more freezing than control rats ($F(1,3)=0.000$, $P=0.011$). Fear was significantly lower in vehicle animals during the test as compared with the fear observed during the first 4 min period of re-exposure, indicating extinction retention (Paired t -test, $P=0.000$). In contrast, AM251 rats performed similar freezing to that performed by the same animals during the first 4 min of re-exposure, suggesting that AM251 blocked the consolidation of extinction ($P=0.755$).

Effect of the CB1 antagonist and the protein synthesis inhibitor DRB, without re-exposure to the context

Rats injected either with AM251 or DRB, and without re-exposure to the conditioning environment are shown in

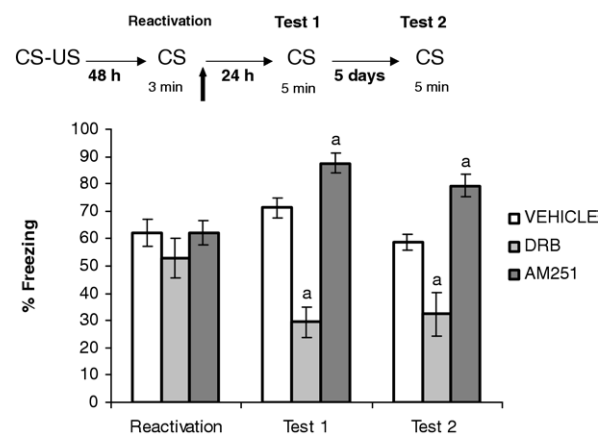


Fig. 2. Effect of AM251 and DRB in the contextual fear conditioning task upon the memory reconsolidation (after a 3 min re-exposure treatment). Diagram above describes the behavioral procedure used in this experiment. Data are the mean \pm S.E.M. percentage of time rats spent freezing in the 3 min reactivation and the 5 min test sessions, each one a re-exposure to the training context. *Ns* per group are 7–11. ANOVA shows no significant difference among reactivation session. (a) Significant difference ($P<0.05$) between the treated groups and those control in the test sessions.

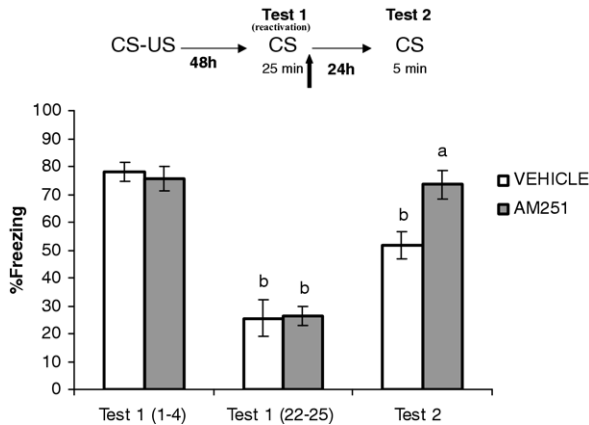


Fig. 3. Effect of AM251 in the contextual fear conditioning task upon the memory *extinction* (after a 25 min re-exposure treatment). Diagram above describes the behavioral procedure used in this experiment. Data are the mean \pm S.E.M. percentage of time rats spent freezing in the first and the last 4 min of the 25 min reactivation session (here named “test 1”) and in the 5 min test session, each of these a re-exposure to the training context. *Ns* per group are 7–8. ANOVA shows no significant difference between the groups in the first and last 4 min of the re-exposure session. (b) Significant difference between the first and the last 4 min, in both groups, showing that they had extinguished (ANOVA, $P < 0.05$). Only the control group kept this difference (between the first 4 min and the test), showing that the AM251 had inhibited the extinction consolidation. (a) Significant difference between the treated groups in the test 2 session (ANOVA $P < 0.05$).

Fig. 4. One-way ANOVA revealed no difference among the groups ($F(2,14) = 0.559$, $P = 0.584$).

Effect of the CB1 agonist and on memory reconsolidation

The effects of AEA, a subthreshold dose of AM251, or both concomitantly administered immediately after a 3 min re-exposure are shown in Fig. 5. One-way ANOVA revealed a significant difference among treatment in the test session

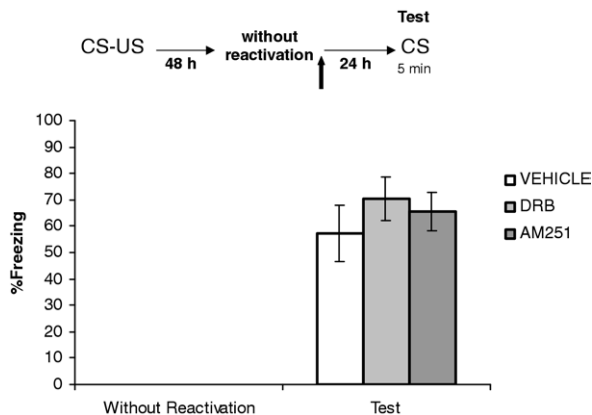


Fig. 4. Effect of AM251 and DRB in the contextual fear conditioning task without exposing the animal to the reactivation session. Diagram above describes the behavioral procedure used in this experiment. Data are the mean \pm S.E.M. percentage of time rats spent freezing in the 5 min test session, actually the first re-exposure to the training context. *Ns* per group are 5–6. ANOVA shows no significant differences among groups in the test session.

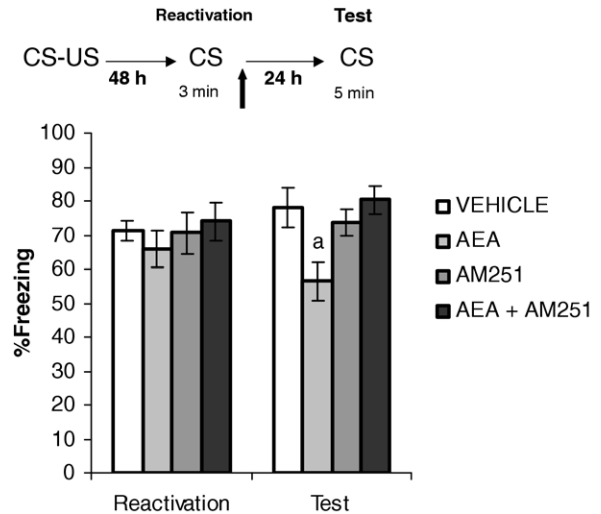


Fig. 5. Effect of AEA in the contextual fear conditioning task upon the memory *reconsolidation* (after a 3 min re-exposure treatment). Data are the mean \pm S.E.M. percentage of time rats spent freezing in the 3 min reactivation and the 5 min test sessions, each one a re-exposure to the training context. *Ns* per group are 6–8. ANOVA shows no significant difference among reactivation session. (a) Significant difference ($P < 0.05$) among the AEA and the other groups in the test session.

($F(3,26) = 5.258$, $P = 0.006$). All animals performed similar freezing during reactivation ($F(3,26) = 0.439$, $P = 0.727$). Student-Newman-Keuls post hoc test comparisons indicated that AEA rats are different from vehicle-treated animals ($P < 0.05$).

Effect of the CB1 agonist on memory extinction

The effects of AEA and AEA with a subthreshold dose of AM251 on the extinction of contextual fear memory are shown in Fig. 6. Paired *t*-test revealed a significant difference between the first 4 min period from the last 4 min of the 25 min re-exposure session in all animals ($P = 0.000$ in every group). However, one-way ANOVA revealed no difference among the groups during these periods ($F(2,27) = 1.352$, $P = 0.285$ and $F(2,27) = 0.188$, $P = 0.831$). In the test, there was a significant difference among the groups ($F(2,27) = 7.606$, $P = 0.004$). Student-Newman-Keuls post hoc test comparisons indicated that AEA rats are different from the other groups ($P < 0.05$). Fear was significantly lower in every group during the test as compared with the fear observed during the first 4 min period of re-exposure, indicating a good extinction retention (paired *t*-test, $P = 0.000$ in every group).

Effect of the CB1 agonist without re-exposure to the context

Rats injected either with AEA, a subthreshold dose of AM251, or both concomitantly administered without re-exposure to the conditioning environment are shown in Fig. 7. One-way ANOVA revealed no difference among the groups ($F(3,19) = 0.048$, $P = 0.986$).

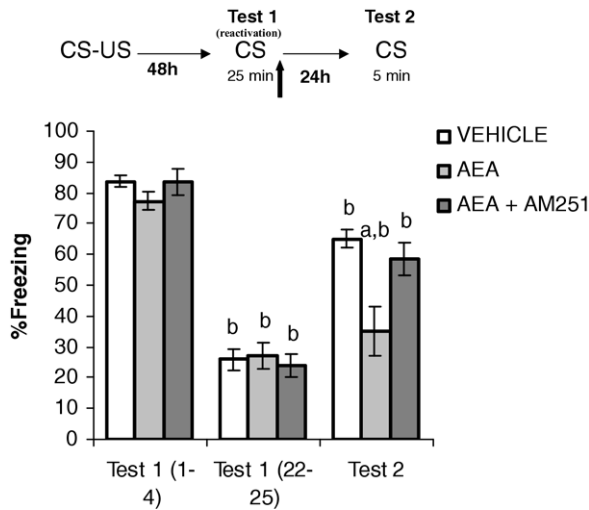


Fig. 6. Effect of AEA in the contextual fear conditioning task upon the memory extinction (after a 25 min re-exposure treatment). Diagram above describes the behavioral procedure used in this experiment. Data are the mean \pm S.E.M. percentage of time rats spent freezing in the first and the last 4 min of the 25 min reactivation session (here named "test 1") and in the 5 min test session, each of these a re-exposure to the training context. Ns per group are 6–7. ANOVA shows no significant difference among the groups in the first and last 4 min of the re-exposure session. (b) Significant difference ($P < 0.05$) between the first and the last 4 min, in every group, showing that they had extinguished. Every group kept this difference (comparing the first 4 min with the test performances). (a) Significant difference between the AEA and control group in the test session.

DISCUSSION

CB1 antagonist AM251 infused intra-hippocampally immediately after a 3 min re-exposure session, caused the enhancement of the freezing response during the test, suggesting a memory reconsolidation *facilitation* (Fig. 2), an effect that was persistent, since a higher fear expression was still present 5 days after test 1. Consistent with previous evidence (Nader et al., 2000; Suzuki et al., 2004), our results also showed that the suppression of protein synthesis employing the transcriptional blocker DRB in CA1 has *blocked* memory reconsolidation (also in Fig. 2): DRB was the drug of choice because of its reversible action and selectivity for RNA polymerase II (Chodosh et al., 1989), and has been employed by other authors as an alternative to other transcriptional inhibitors (e.g. Crow et al., 1997; Igaz et al., 2002). Neither AM251 nor DRB affected fear memory when the reactivation session was omitted (Fig. 4) indicating (a) that these drugs (in these doses) do not influence fear per se, and (b) confirming that the *reactivation* session is necessary to observe the effect of both drugs upon the reconsolidation.

Complementary to that, the local infusion of AEA has *disrupted* memory reconsolidation (Fig. 5), and a combined administration of AEA plus a subthreshold dose of AM251 abolished this effect, supporting the notion that the influence of endocannabinoids on memory reconsolidation is mediated by CB1 receptors in the dorsal hippocampus. Neither AEA nor AEA plus a subeffective

dose of AM251 was effective if the reactivation session was omitted (Fig. 7).

The endocannabinoid hypothesis receives support by the fact that a selective CB1 antagonist has an effect probably due to the displacement of an endogenous pool of ligands. But, as a conjecture it will lose strength if the drug of choice happens to be a partial agonist. Actually, since AM251 is a simple-substitution SR141716A derivative, a proved partial agonist (Landsman et al., 1997; Nakamura-Palacios et al., 1999), the possibility remains. Originally considered a competitive antagonist (Gatley et al., 1996), there is recent, sparse evidence suggesting the reality of an "inverse cannabimimetic" effect of AM251 among other biarylpyrazoles (Pertwee, 2005). In any event, agonist data may also contribute to support an endocannabinoid hypothesis, despite being less conclusive: its infusion, if causing any effect, should be interpreted carefully since this exogenous administration may be adding tonus to a preexisting pool of endogenous ligands and the nature of the effect may be strongly sensitive to the initial state of the endogenous system.

Our evidence also showed that the prolonged exposure to the conditioned environment without the US led to a gradual reduction of the fear response along the reactivation session, presumably indicating that animals began to extinguish their fear response. Consistent with this, animals that were subjected to such re-exposure and later on tested in the associated context, displayed a lower fear compared with the fear observed in animals without the reactivation trial. These observations support the notion that a single but prolonged re-exposure to the context without reinforcement, resulted in extinction in our experimental paradigm.

When AM251 was locally infused in CA1 after a 25 min re-exposure, rats performed a higher fear response if compared with that exhibited by control animals, suggesting that this drug has disrupted the consolidation of extinction

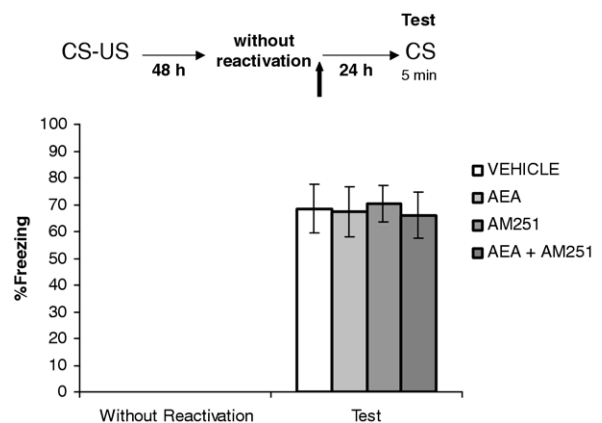


Fig. 7. Effect of AEA in the contextual fear conditioning task without exposing the animal to the memory reactivation on extinction session. Diagram above describes the behavioral procedure used in this experiment. Data are the mean \pm S.E.M. percentage of time rats spent freezing in the 5 min test session, actually the first re-exposure to the training context. Ns per group are 5–6. ANOVA shows no significant difference among the groups in the test session.

(Fig. 3). Confirming the potential role of the hippocampal CB1-mediated endocannabinoid system on the consolidation of extinction, intra-hippocampal administration of exogenous AEA has facilitated extinction whereas the combined infusion with AEA and AM251 has blocked such facilitation (Fig. 6). A similar result was found by Chhatwal et al. (2005) in a study of conditioned fear: using AM404 to increase the tonus of endogenous cannabinoids they detected a facilitation of extinction; SR141716A, by the other side, blocked extinction and, infused along with AM404, reverted the facilitation, supporting the notion of a CB1-mediated effect.

Consistently with previous studies, the current findings showed that the duration of the *reactivation* session is a crucial variable that determines subsequent memory (Debiec et al., 2002; Boccia et al., 2004, 2007; Suzuki et al., 2004; Pedreira and Maldonado, 2003; Bustos et al., 2008). Brief re-exposure leads to reconsolidation whereas a prolonged reactivation session induced extinction. There are relevant functional differences between both processes. It was suggested that reconsolidation can be an updating mechanism conducted to incorporate new information to an already consolidated memory (Tronson and Taylor, 2007; Suzuki et al., 2004). In contrast, the persistent presence of the CS without reinforcement leads to another learning process and results in a new memory that competes with and temporarily suppresses the memory formed during the original association: an extinction (Bouton, 2004; Myers and Davis, 2002). According to our results, the hippocampal endocannabinoid system seems to have opposite modulatory roles on each of these processes: hence, this system may disrupt the emergence of reconsolidation when a brief re-exposure to the CS is used, and with longer re-exposure to the CS extinction, learning takes place and the hippocampal endocannabinoids act to facilitate the consolidation of the extinction memory.

The disruption of reconsolidation observed with the local infusion of AEA could be alternatively interpreted as a facilitatory effect of AEA upon memory extinction after that brief, 3 min re-exposure, an effect that could mimic a reconsolidation disruption. However, we do not consider this likely since such brief re-exposures may not be “intense” enough to be able to initiate a new memory trace necessary to extinguish the previous one: as Fig. 2 shows, no change is detectable in the test session when the reactivation session lasts 3 min for the vehicle-injected groups.

These findings support the importance of the hippocampal endocannabinoid system in the modulation of memory reconsolidation and our results do not conflict directly with any previous study. Suzuki et al. (2004) reported that blockade of CB1 receptors after systemic administration did not affect the reconsolidation of a contextual fear memory, but since they used a systemic infusion, this absence of effect might mean that two or more different brain areas simultaneously affected may have compensated for each other, e.g. neutralizing the particular hippocampal effect. In fact, considering the intracerebral results of Lin et al. (2006) and Kobil et al. (2007), where

reconsolidation of an aversive task was blocked by the infusion of CB1 agonists into the amygdala or the insular cortex, respectively, we could propose that none of these areas is the responsible for the neutralization/compensating effect observed in that systemic study; in any event, each region seems to have its peculiarities, since, e.g. the antagonist caused no effect when infused into the insular cortex (Kobil et al., 2007). Thus, if one of the functional roles of the reconsolidation process is to update information, the incorporation of new information during the reactivation session could, in fact, be under the control of any of these endogenous systems.

Data from Fig. 3 suggest that endocannabinoids are necessary for the “consolidation of extinction,” a concept already used by other authors (Duvarci et al., 2006; Lin et al., 2003; Santini et al., 2001), since AM251 leads the test two response to near-untrained levels, as in test 1 (1–4) (% freezing higher than control group), i.e. the blockage of endocannabinoid normal function disrupts extinction. Recent studies reported not only that an extinction memory attenuation was detected in CB1 knockout mice, but also that blocking CB1 receptor with a specific antagonist disrupts memory extinction in a variety of behavioral tasks (Suzuki et al., 2004; Marsicano et al., 2002; Pamplona et al., 2006; Kobil et al., 2007). Moreover, in accordance with the view that the hippocampal endocannabinoid system is required for the emergence of memory consolidation (de Oliveira Alvares et al., 2005, 2006, 2008), we can tentatively propose that the activation of the endocannabinoid system has a permissive role on the formation of any new memory, including an extinction memory, as reported here and by Marsicano et al. (2002). Thus, during and/or after a new learning, the synthesis and release of endocannabinoids in the CA1 region of the hippocampus may act in order to improve this new memory formation, facilitating the glutamatergic communication, the main thing responsible for the memory trace building (Bliss and Collingridge, 1993; Izquierdo and Medina, 1995; Lamprecht and LeDoux, 2004; Kullmann and Lamsa, 2007).

In fact, Lee et al. (2006) have shown that systemically infused NMDA antagonist MK-801 blocked fear conditioning (FC) extinction, and the NMDA partial agonist D-cycloserine (DCS) potentiated it (systemically and into the amygdala), when administered before a long “extinction training” (reactivation) session; exactly the opposite took place after a brief memory reactivation session: MK-801 impaired, whereas DCS increased, freezing, i.e. they, respectively, impaired and enhanced reconsolidation. Even taking into consideration the fact that hippocampal CB1 receptors are 20 or more times more concentrated in the GABAergic interneurons than in glutamatergic terminals (Monory et al., 2006, 2007; Katona et al., 1999, 2006; Egertova and Elphick, 2000; Tsou et al., 1999; Takahashi and Castillo, 2006; Kawamura et al., 2006; Domenici et al., 2006), endocannabinoids can, by “directly” modulating the effector synapses (i.e. the glutamatergic targets), or “indirectly” (i.e. the GABAergic targets), be acting as the selector of the glutamatergic response: indeed, since (a) most of the behavioral tasks in which this system seems to matter

are of aversive nature, and (b) endocannabinoids were shown to be released by stressful agents in different brain areas (see, e.g. Hohmann et al., 2005), we may suppose that endocannabinoids in the hippocampus are a nice putative “switching mechanism” between memory processes that takes place after reactivation: After a brief re-exposure, endocannabinoids seem to act in order to maintain the original memory, or, in other words, reconsolidating the memory (Sara, 2000); when the animals are re-exposed for a long period in the CS without the US, a new learning occurs, CS/no-US, i.e. an extinction takes place: in this sense, our results may be more than convergent with those of Lee et al. (2006). To this point, however, this is all very conjectural and demands more investigation.

Putative treatments for some severely incapacitating conditions such as posttraumatic stress disorder have been proposed, ranging from the (usually spontaneously reversible) facilitation of extinction (Myers and Davis, 2007) to the most promising reconsolidation impairment (Nader, 2003a,b). Understanding the mechanism that determines the route-choosing between such opposite processes like extinction or reconsolidation may, in time, provide a viable approach for some psychiatric disorders involving aversive memories.

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REFERENCES

- Ameri A (1999) The effects of cannabinoids on the brain. *Prog Neurobiol* 58:315–348.
- Bliss TVP, Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 361:31–39.
- Boccia MM, Acosta GB, Blake MG, Baratti CM (2004) Memory consolidation and reconsolidation of an inhibitory avoidance response in mice: effects of i.c.v. injections of hemicholinium-3. *Neuroscience* 124(4):735–741.
- Boccia MM, Blake MG, Acosta GB, Baratti CM (2007) Post-retrieval effects of icv infusions of hemicholinium in mice are dependent on the age of the original memory. *Learn Mem* 13(3):376–381.
- Bonini JS, Da Silva WC, Bevilacqua LR, Medina JH, Izquierdo I, Cammarota M (2007) On the participation of hippocampal PKC in acquisition, consolidation and reconsolidation of spatial memory. *Neuroscience* 147(1):37–45.
- Bouton ME (2004) A general role for early onset cues and intra-event learning: comment on McDonald and Siegel. *Exp Clin Psychopharmacol* 12(1):18–19.
- Bouton ME, Westbrook FR, Corcoran KA, Maren S (2006) Contextual and temporal modulation of extinction: behavioral and biological mechanisms. *Biol Psychiatry* 60(4):352–360.
- Bustos SG, Maldonado H, Molina VA (2006) Midazolam disrupts fear memory reconsolidation. *Neuroscience* 139(3):831–842.
- Bustos SG, Maldonado H, Molina VA (2008) Disruptive effect of midazolam on fear memory reconsolidation: decisive influence of reactivation time span and memory age. *Neuropsychopharmacology* [Epub ahead of print].
- Chhatwal JP, Davis M, Maguschak KA, Ressler KJ (2005) Enhancing cannabinoid neurotransmission augments the extinction of conditioned fear. *Neuropsychopharmacology* 30(3):516–524.
- Chodosh LA, Fire A, Samuels M, Sharp PA (1989) 5,6-Dichloro-1- β -D-ribofuranosylbenzimidazole inhibits transcription elongation by RNA polymerase II in vitro. *J Biol Chem* 264:2250–2257.
- Crow T, Siddiqi V, Dash PK (1997) Long-term enhancement but not short-term in *Hermissenda* is dependent upon mRNA synthesis. *Neurobiol Learn Mem* 68(3):343–350.
- Davies SN, Pertwee RG, Riedel G (2002) Functions of cannabinoid receptors in the hippocampus. *Neuropharmacology* 42:993–1007.
- Debiec J, LeDoux JE (2004) Disruption of reconsolidation but not consolidation of auditory fear conditioning by noradrenergic blockade in the amygdala. *Neuroscience* 129(2):267–272.
- Debiec J, LeDoux JE, Nader K (2002) Cellular and systems reconsolidation in the hippocampus. *Neuron* 36(3):527–538.
- De Oliveira Alvares L, de Oliveira LF, Camboim C, Diehl F, Genro BP, Lanzotti VB, Quilfeldt JA (2005) Amnesic effect of intrahippocampal AM251, a CB1-selective blocker, in the inhibitory avoidance, but not in the open field habituation task, in rats. *Neurobiol Learn Mem* 83:119–124.
- De Oliveira Alvares L, Genro BP, Breda RV, Pedroso MF, Da Costa JC, Quilfeldt JA (2006) AM251, a selective antagonist of the CB1 receptor, inhibits the induction of long-term potentiation and induces retrograde amnesia in rats. *Brain Res* 1075:60–67.
- De Oliveira Alvares L, Genro BP, Diehl F, Quilfeldt JA (2008) Differential role of the hippocampal endocannabinoid system in the memory consolidation and retrieval mechanisms. *Neurobiol Learn Mem* 2008 Mar 12 [Epub ahead of print; doi:10.1016/j.nlm.2008.01.009].
- Domenici MR, Azad SC, Marsicano G, Schierloh A, Wotjak CT, Dotz HU, Zieglgänsberger W, Lutz B, Rammes G (2006) Cannabinoid receptor type 1 located on presynaptic terminals of principal neurons in the forebrain controls glutamatergic synaptic transmission. *J Neurosci* 26(21):5794–5799.
- Duvarci S, Nader K (2004) Characterization of fear memory reconsolidation. *J Neurosci* 24(42):9269–9275.
- Duvarci S, Mamou CB, Nader K (2006) Extinction is not a sufficient condition to prevent fear memories from undergoing reconsolidation in the basolateral amygdala. *Eur J Neurosci* 24(1):249–260.
- Egertová M, Elphick MR (2000) Localisation of cannabinoid receptors in the rat brain using antibodies to the intracellular C-terminal tail of CB1. *J Comp Neurol* 422:159–171.
- Eisenberg M, Kobilov T, Berman DE, Dudai Y (2003) Stability of retrieved memory: inverse correlation with trace dominance. *Science* 301(5636):1102–1104.
- Gateley SJ, Gifford AN, Volkow ND, Lan R, Makriyannis A (1996) 123 I-labeled AM251: a radioiodinated ligand which binds *in vivo* to mouse brain cannabinoid CB1 receptors. *Eur J Pharmacol* 307:331–338.
- Hampson RE, Deadwyler SA (1999) Cannabinoids, hippocampal function and memory. *Life Sci* 65:715–723.
- Herkenham M, Lynn AB, Johnson MR, Melvin LS, de Costa BR, Rice KC (1991) Characterization and localization of cannabinoid receptors in the rat brain: a quantitative *in vitro* autoradiographic study. *J Neurosci* 11:563–583.
- Hohmann AG, Suplita RL, Bolton NM, Neely MH, Fegley D, Mangieri R, Krey JF, Walker JM, Holmes PV, Crystal JD, Duranti A, Tontini A, Mor M, Tarzia G, Piomelli D (2005) An endocannabinoid mechanism for stress-induced analgesia. *Nature* 435(7045):1108–1112.
- Igaz LM, Vianna MR, Medina JH, Izquierdo I (2002) Two time periods of hippocampal mRNA synthesis are required for memory consolidation of fear-motivated learning. *J Neurosci* 22(15):6781–6789.
- Izquierdo I, Medina JH (1995) Correlation between the pharmacology of long-term potentiation and the pharmacology of memory. *Neurobiol Learn Mem* 63(1):19–32.
- Izquierdo I, Bevilacqua LR, Rossato JI, Bonini JS, Medina JH, Cammarota M (2006) Different molecular cascades in different sites of the brain control memory consolidation. *Trends Neurosci* 29(9):496–505.
- Katona I, Sperlagh B, Sik A, Kafalvi A, Vizi ES, Mackie K, Freund TF (1999) Presynaptically located CB1 cannabinoid receptors regu-

- late GABA release from axonal terminals of specific hippocampal interneurons. *J Neurosci* 19:4544–4558.
- Katona I, Urbán GM, Wallace M, Ledent C, Jung KM, Piomelli D, Mackie K, Freund TF (2006) Molecular composition of the endocannabinoid system at glutamatergic synapses. *J Neurosci* 26(21):5628–5637.
- Kawamura Y, Fukaya M, Maejima T, Yoshida T, Miura E, Watanabe M, Ohno-Shosaku T, Kano M (2006) The CB1 cannabinoid receptor is the major cannabinoid receptor at excitatory presynaptic sites in the hippocampus and cerebellum. *J Neurosci* 26:2991–3001.
- Kobilov T, Hazvi S, Dudai Y (2007) Role of cortical cannabinoid CB1 receptor in conditioned taste aversion memory. *Eur J Neurosci* 25(11):3417–3421.
- Kullmann DM, Lamsa KP (2007) Long-term synaptic plasticity in hippocampal interneurons. *Nat Rev Neurosci* 8(9):687–699.
- Lamprecht R, LeDoux J (2004) Structural plasticity and memory. *Nat Rev Neurosci* 5(1):45–54.
- Landsman RS, Burkey TH, Consroe P, Roeske WR, Yamamura HI (1997) SR141716A is a naive inverse agonist at the human cannabinoid CB1 receptor. *Eur J Pharmacol* 334:R1–R2.
- Lee JL, Milton AL, Everitt BJ (2006) Reconsolidation and extinction of conditioned fear: inhibition and potentiation. *J Neurosci* 26(39):10051–10056.
- Lin CH, Yeh SH, Lu HY, Gean PW (2003) The similarities and diversities of signal pathways leading to consolidation of conditioning and consolidation of extinction of fear memory. *J Neurosci* 23:8310–8317.
- Lin HC, Mao SC, Gean PW (2006) Effects of intra-amygdala infusion of CB1 receptor agonists on the reconsolidation of fear-potentiated startle. *Learn Mem* 13(3):316–321.
- Mackie K (2005) Distribution of cannabinoid receptors in the central and peripheral nervous system. In: *Handbook of experimental pharmacology*, Vol. 168 (Pertwee RG), pp 299–325. Berlin, Germany: Springer.
- Marsicano G, Wotjak CT, Azad SC, Bisogno T, Rammes G, Cascio MG, Hermann H, Tang J, Hofmann C, Zieglgänsberger W, Di Marzo V, Lutz B (2002) The endogenous cannabinoid system controls extinction of aversive memory. *Nature* 418:530–534.
- Misanin JR, Miller RR, Lewis DJ (1968) Retrograde amnesia produced by electroconvulsive shock after reactivation of a consolidated memory trace. *Science* 160(827):554–555.
- Monory K, Massa F, Egertová M, Eder M, Blaudzun H, Westenbroek R, Kelsch W, Jacob W, Marsch R, Ekker M, Long J, Rubenstein JL, Goebbels S, Nave KA, Düring M, Klugmann M, Wölfel B, Dodt HU, Zieglgänsberger W, Wotjak CT, Mackie K, Elphick MR, Marsicano G, Lutz B (2006) The endocannabinoid system controls key epileptogenic circuits in the hippocampus. *Neuron* 51(4):455–466.
- Monory K, Blaudzun H, Massa F, Kaiser N, Lemberger T, Schütz G, Wotjak CT, Lutz B, Marsicano G (2007) Genetic dissection of behavioural and autonomic effects of Delta(9)-tetrahydrocannabinol in mice. *PLoS Biol* 5(10):e269.
- Myers KM, Davis M (2007) Mechanisms of fear extinction. *Mol Psychiatry* 12(2):120–150.
- Myers KM, Davis M (2002) Behavioral and neural analysis of extinction. *Neuron* 36(4):567–584.
- Nader K, Schafe GE, LeDoux JE (2000) Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. *Nature* 406(6797):722–726.
- Nader K (2003a) Memory traces unbound. *Trends Neurosci* 26(2):65–72.
- Nader K (2003b) Neuroscience: re-recording human memories. *Nature* 425(6958):571–572.
- Nakamura-Palacios EM, Moerschbacher JM, Barker LA (1999) The pharmacology of SR141716A: A review. *CNS Drug Rev* 5:43–58.
- Pamplona FA, Prediger RD, Pandolfo P, Takahashi RN (2006) The cannabinoid receptor agonist WIN 55,212–2 facilitates the extinction of contextual fear memory and spatial memory in rats. *Psychopharmacology (Berl)* 188(4):641–649.
- Pamplona FA, Prediger RD, Pandolfo P, Takahashi RN (2006) The cannabinoid receptor agonist WIN 55,212–2 facilitates the extinction of contextual fear memory and spatial memory in rats. *Psychopharmacology (Berl)* 188(4):641–649.
- Paxinos G, Watson C (1998) *The rat brain in stereotaxic coordinates*, 4th ed. San Diego: Academic Press.
- Pedreira ME, Maldonado H (2003) Protein synthesis subserves reconsolidation or extinction depending on reminder duration. *Neuron* 38(6):863–869.
- Pertwee R (2005) Inverse agonism and neutral antagonism at cannabinoid CB1 receptors. *Life Sci* 76(12):1307–1324.
- Przybylski J, Roulet P, Sara SJ (1999) Attenuation of emotional and nonemotional memories after their reactivation: role of beta adrenergic receptors. *J Neurosci* 19(15):6623–6628.
- Przybylski J, Sara SJ (1997) Reconsolidation of memory after its reactivation. *Behav Brain Res* 84(1–2):241–246.
- Rossato JI, Bevilaqua LR, Medina JH, Izquierdo I, Cammarota M (2006) Retrieval induces hippocampal-dependent reconsolidation of spatial memory. *Learn Mem* 13(4):431–440.
- Santini E, Muller RU, Quirk GJ (2001) Consolidation of extinction learning involves transfer from NMDA-independent to NMDA-dependent memory. *J Neurosci* 21(22):9009–9017.
- Sara SJ (2000) Retrieval and reconsolidation: toward a neurobiology of remembering. *Learn Mem* 7(2):73–84.
- Squire LR (1992) Memory and the hippocampus: A synthesis from findings with rats, monkeys and humans. *Psychol Rev* 99:195–231.
- Suzuki A, Josselyn SA, Frankland PW, Masushige S, Silva AJ, Kida S (2004) Memory reconsolidation and extinction have distinct temporal and biochemical signatures. *J Neurosci* 24(20):4787–4795.
- Takahashi KA, Castillo PE (2006) The CB1 cannabinoid receptor mediates glutamatergic synaptic suppression in the hippocampus. *Neuroscience* 139:795–802.
- Tronson NC, Taylor JR (2007) Molecular mechanisms of memory reconsolidation. *Nat Rev Neurosci* 8(4):262–275.
- Tsou K, Mackie K, Sanudo-Pena MC, Walker JM (1999) Cannabinoid CB1 receptors are localized primarily on cholecystokinin-containing GABAergic interneurons in the rat hippocampal formation. *Neuroscience* 93:969–975.
- Varvel SA, Lichtman AH (2002) Evaluation of CB1 receptor knockout mice in the Morris water maze. *J Pharmacol Exp Ther* 301(3):915–924.
- Varvel SA, Wise LE, Niyuhire F, Cravatt BF, Lichtman AH (2007) Inhibition of fatty-acid amide hydrolase accelerates acquisition and extinction rates in a spatial memory task. *Neuropsychopharmacology* 32(5):1032–1041.
- Vianna MR, Szapiro G, McGaugh JL, Medina JH, Izquierdo I (2001) Retrieval of memory for fear-motivated training initiates extinction requiring protein synthesis in the rat hippocampus. *Proc Natl Acad Sci U S A* 98(21):12251–12254.
- Wilson RI, Nicoll RA (2002) Endocannabinoid signaling in the brain. *Science* 296:678–682.

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4. CAPÍTULO II

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Stress response recruits the hippocampal endocannabinoid system for the modulation of fear memory

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Research

Stress response recruits the hippocampal endocannabinoid system for the modulation of fear memory

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The modulation of memory processes is one of the several functions of the endocannabinoid system (ECS) in the brain, with CB1 receptors highly expressed in areas such as the dorsal hippocampus. Experimental evidence suggested an important role of the ECS in aversively motivated memories. Similarly, glucocorticoids released in response to stress exposure also modulates memory formation, and both stress and dexamethasone activate the ECS. Here, we investigate the interaction between the ECS and glucocorticoids in the hippocampus in the modulation of fear memory consolidation. Two protocols with different shock intensities were used in order to control the level of aversiveness. Local infusion of AM251 into the hippocampus immediately after training was amnesic in the strong, but not in the weak protocol. Moreover, AM251 was amnesic in animals stressed 0, but not 30-min prior to the weak protocol, reverting the stress-induced facilitatory effect. Finally, intrahippocampal AM251 infusion reduced memory in animals that received dexamethasone immediately, but not 30 min before training. These results are (1) consistent with the view that the dorsal hippocampus ECS is activated on demand, in a rapid and short-lived fashion in order to modulate the consolidation of an aversive memory, and (2) show that this recruitment seems to be mediated by glucocorticoids, either in the hippocampus or in other brain regions functionally associated with the hippocampus.

In the last decade, growing evidence supports the role of the endocannabinoid system (ECS) as a memory modulator, particularly in mammal brain structures (Davies et al. 2002; Lutz 2007; Viveros et al. 2007; Heifets and Castillo 2009). The involvement of this modulatory system in areas such as the dorsal hippocampus or the basolateral amygdala (BLA) is not only corroborated by receptor density studies (Mackie 2005; Marsicano and Kuner 2008), but is also consistent with several pharmacological results, usually obtained in behavioral tasks that are both aversive and hippocampus dependent. For instance, CB1 antagonists were shown to impair consolidation (de Oliveira Alvares et al. 2005) and extinction (Suzuki et al. 2004; Pamplona and Takahashi 2006; Niyuhire et al. 2007), while having the opposite effect on memory retrieval (de Oliveira Alvares et al. 2008a) and reconsolidation (de Oliveira Alvares et al. 2008b). The same results may not be observable when the administration is performed systemically or under a different experimental protocol (Suzuki et al. 2004; Pamplona and Takahashi 2006; Yim et al. 2008). The ECS may be acting both at the cellular and the systems level, being involved in memory processes that depend on protein synthesis (de Oliveira Alvares et al. 2008b; Heifets and Castillo 2009; Puighermanal et al. 2009) and, on the other side of the spectrum,

is considered a good spike timing/brain oscillations coordinator candidate (Robbe et al. 2006). Although the role of the ECS in long-term memory modulation is better documented for aversive tasks, this system does not appear to be involved in less-aversive memories (Hölter et al. 2005; de Oliveira Alvares et al. 2006; Pamplona and Takahashi 2006; Niyuhire et al. 2007).

Among the several different stress hormones released after an aversive learning paradigm, glucocorticoids (GC) are noticeable for their broad functional and temporal range of effects (Joëls 2008; Joëls et al. 2009). The hippocampus is one of the memory-related targets of these actions, being specifically susceptible to uncontrollable stress through a pathway that involves the amygdala (Kim and Diamond 2002; Akirav and Richter-Levin 2006; Malin and McGaugh 2006). Several convergent studies show that either corticosterone, or the synthetic glucocorticoid dexamethasone, or stress promote the enhancement of memory consolidation (Roosendaal and McGaugh 1996; Roosendaal et al. 1999) while impairing retrieval (de Quervain et al. 1998; Roosendaal et al. 2003). Moreover, recent studies have shown that stress exposure or glucocorticoid administration impair reconsolidation (Maroun and Akirav 2008; Wang et al. 2008) and influence extinction (Cai et al. 2006; Yang et al. 2006) despite some contradictory results (Maroun and Akirav 2008). It is quite uncommon for a drug to exhibit a functional profile structured like that exhibited by agents acting at the ECS, i.e., enhancing consolidation while impairing retrieval, as well as causing exactly opposite effects upon reconsolidation and extinction.

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The parallels between endocannabinoids and glucocorticoids acting upon different phases of memory processing suggest an interaction between both systems, as already shown in other brain areas (Di et al. 2003, 2005; Steiner and Wotjak 2008). A recent report suggests that CB1-mediated signaling in the basolateral amygdala is critically modulated by GCs in emotional memory consolidation (Campolongo et al. 2009). In addition, there are studies showing that both stress and dexamethasone administration increased the level of both endocannabinoids anandamide and 2-arachidonylglycerol (Di et al. 2003, 2005; Hohmann et al. 2005). The aim of the current study was to evaluate the interaction between the ECS and glucocorticoids in the modulation of memory consolidation using the contextual fear conditioning (CFC) paradigm, a well known hippocampus-dependent learning task (Kim and Fanselow 1992). Moreover, the training protocol used allows one to control the strength of the aversive stimulus applied.

Results

Experiment 1: The endocannabinoid system is recruited only with strong fear training

In this first series of experiments, we study whether the ECS is required for memory consolidation when the training used a mild footshock (0.3 mA) or when the training used a strong footshock (0.7 mA). With the strong footshock training, there was a significant difference between the AM251 and the control groups (Fig. 1A, $P < 0.001$, t -test). In contrast, such an effect was not evident with the weak footshock protocol (Fig. 1B, $P = 0.750$, t -test). The fact that the AM251 disruptive effect was absent using the weak protocol suggests that only high levels of aversive stimulus may be able to recruit the hippocampal endocannabinoid system in order to modulate contextual fear memory.

Experiment 2: Prior stress recruits the hippocampal endocannabinoid system for the modulation of fear memory consolidation

Based on the findings obtained in the experiment 1, we next asked whether increasing the aversiveness from another source prior to the weak training protocol would be able to render memory consolidation sensitive to the interference induced by the CB1 antagonist. Figure 2 shows the freezing behavior performed during the test by animals that received a bilateral intrahippocampal infusion of AM251 or its vehicle after the CFC weak training, and with a previous stress session in a different context at two different times. The two-way ANOVA revealed a significant effect for AM251 ($F_{(1,35)} = 11.145$, $P = 0.002$) and an interaction between stress-0-min \times AM251 ($F_{(1,35)} = 4.556$, $P = 0.040$). There was a significant difference between the AM251 and its DMSO control for 0 min (Tukey post-hoc test, $P < 0.001$), but not for 30-min pre-training stress (*idem*, $P = 0.419$); also, the AM251 group for 0 min pre-training stress differed significantly from the 30 min case (*idem*, $P = 0.021$). These results showed that a prior stressful experience strengthens the memory consolidation of a weak conditioning protocol. Moreover, this rapid memory-enhancing effect elicited by stress is modulated by the activation of the hippocampal ECS.

Experiment 3: Glucocorticoids interact with the hippocampal endocannabinoid signaling to modulate fear memory consolidation

In the following experiment, we investigated whether glucocorticoids recruit the hippocampal ECS to influence memory

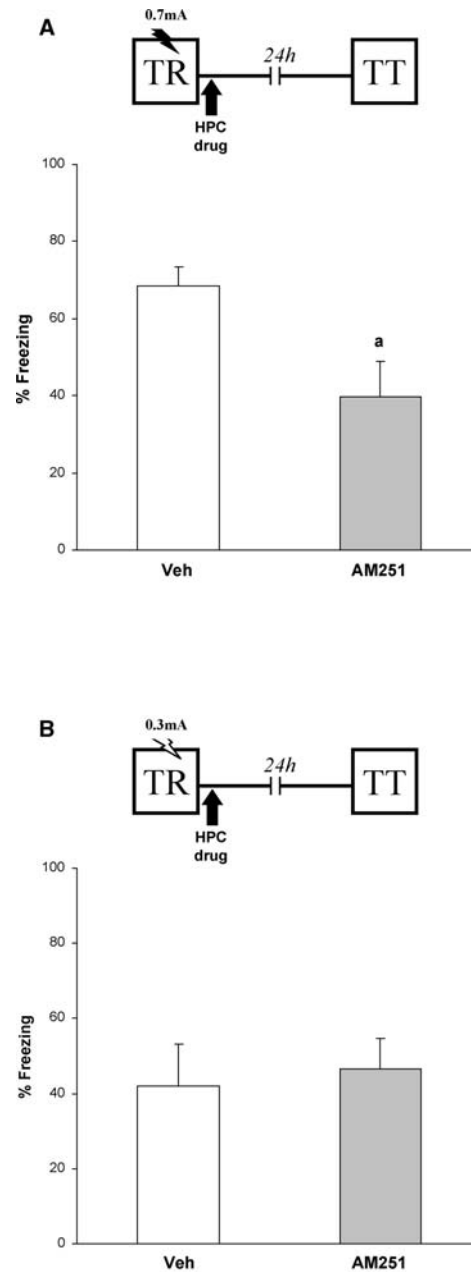


Figure 1. Effect of bilateral intrahippocampal infusion of AM251 or its vehicle (veh: PBS with 8% DMSO) upon percentual freezing time in a test session performed 24 h after a contextual fear conditioning (CFC) training session under (A) a strong (0.7 mA) or (B) a weak (0.3 mA) footshock. The diagram above each histogram depicts the corresponding experimental design. Data expressed as mean \pm SEM of percent of freezing time in a 5-min test session. (a) Significantly different from the control group ($P < 0.001$, t -test), with $n = 11$ and 9 , respectively. In Figure 2 (bottom panel) there were no significant differences between groups ($P = 0.750$, t -test), with $n = 8$ and 8 , respectively.

consolidation following the weak footshock protocol. To this end, we studied the effect of a systemic injection of an exogenous synthetic glucocorticoid injected at 0 or 30 min prior to the CFC weak training. The goal of this experiment was to mimic the effect of the stressful experience observed in experiment 2. For the groups injected with dexamethasone immediately before

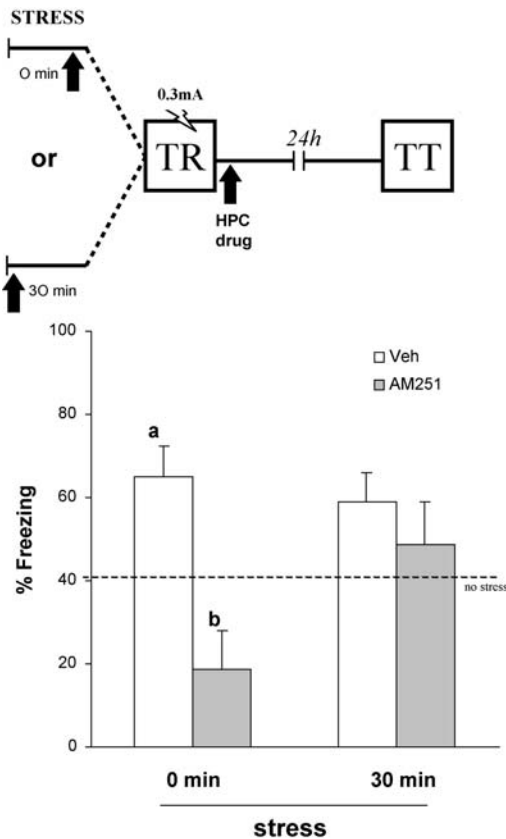


Figure 2. Time-dependent differential responses (percentual freezing) to bilateral intrahippocampal infusion of AM251 or its vehicle (veh: PBS with 8% DMSO), in a test session performed 24 h after a CFC training session under both a weak (0.3 mA) footshock and after a previous stress session in a different context, at different times. The diagram above the histogram depicts the corresponding experimental design. Data expressed as mean \pm SEM of percentual freezing time in a 5-min test session. Dashed line: average value of percent freezing for a group not submitted to stress, shown for illustrative reasons only. Horizontal axis: time of stress before CFC training. Two-way ANOVA indicates a significant effect of treatment (but not of time), and an interaction between treatment and time: (a) significant difference between treatment groups for this time ($P < 0.001$, Tukey post-hoc test); (b) significantly different from the correspondent AM251-infused group stressed at 30 min ($P = 0.021$, Tukey post-hoc test). $n = 11, 10, 10$, and 8 , respectively.

training (Fig. 3A), two-way ANOVA indicated a significant effect of AM251 ($F_{(1,31)} = 5.464$, $P = 0.026$), and an interaction between DEXA and AM251 groups ($F_{(1,31)} = 13.554$, $P < 0.001$): There was a significant difference between both DMSO groups (Tukey post-hoc test, $P = 0.003$); and the AM251 group injected with DEXA was significantly different from its DMSO control (*idem*, $P < 0.001$). For the groups injected with dexamethasone 30 min before training (Fig. 3B), the two-way ANOVA indicates only a significant effect of DEXA ($F_{(1,35)} = 17.051$, $P < 0.001$): There was a significant difference between groups receiving DEXA or its vehicle for each intrahippocampal treatment, be it DMSO (Tukey post-hoc test, $P = 0.003$) or AM251 (*idem*, $P = 0.011$). These data showed that dexamethasone induced a long-lasting facilitatory effect upon memory consolidation, a potentiation that was blocked by AM251 infused into the hippocampus. These results suggest that the rapid memory-enhancing effect of a glucocorticoid upsurge is modulated by the activation of the ECS in the hippocampus. Thus, dexamethasone mimicked the

facilitating influence of a pre-training stress session upon memory consolidation following the weak training protocol.

Experiments 4 and 5: Effect of intrahippocampal dexamethasone on the hippocampal endocannabinoid signaling on the modulation of fear memory consolidation

In order to analyze whether the stress/glucocorticoid-inducing effect upon the hippocampal endocannabinoid system takes place in the hippocampus, we performed two additional experiments. In experiment 4, we investigated whether dexamethasone directly infused into the CA1 area immediately after the CFC training with a weak footshock could lead to the same enhancing effect observed in experiment 3: There was no statistically significant increase in the percent freezing following the weak footshock protocol (Fig. 4). Drug concentrations were similar to those used elsewhere (Abrahám et al. 1996; Ferreira et al. 2000; Di et al. 2003) and pretraining infusions were also ineffective (data not shown). Thus, intrahippocampal dexamethasone infusion failed to mimic the facilitating influence of a pretraining stress session or systemic dexamethasone on fear memory consolidation following the weak training protocol.

In additional experiments we evaluated the effect of mifepristone (RU486), a glucocorticoid cytoplasmatic receptor antagonist, in concentrations used earlier (Calfa et al. 2007), directly infused into the CA1 area immediately before the CFC training with a strong footshock. Since there is no membrane/rapid/nongenomic GC receptor (mGCR) antagonist available yet (Di et al. 2005), we checked whether at least the slow, genomic-mediated GC hippocampal receptors might contribute to the observed enhancing effect induced by the strong shock (see Fig. 1A). The findings of this experiment showed comparable levels of freezing among the different groups ($P = 0.947$, one-way ANOVA), indicating that the intrahippocampal infusion of this drug at two different doses does not affect fear memory consolidation. As in experiment 1, all groups were run in parallel, at the same time, and the means were: Vehicle: 39.2 ± 5.5 sec; Mifepristone $6 \text{ ng}/\mu\text{L}$: 42.4 ± 8.1 sec; Mifepristone $60 \text{ ng}/\mu\text{L}$: 39.9 ± 10.0 sec ($n = 9, 8$, and 5 , respectively). The smaller freezing time of the control group compared with that in Figure 1A (with same shock intensity) might be due to natural variations among samples, since the experiments were performed in different months of the year.

Discussion

The current study examined the role of the hippocampal ECS in the modulation of memory consolidation induced by different footshock intensities during fear conditioning. The amnesic effect of the CB1 antagonist AM251 on consolidation following the strong footshock protocol (Fig. 1A) is consistent with previous data from our group using both the CFC and the step-down inhibitory avoidance tasks (de Oliveira Alvares et al. 2005, 2006, 2008a,b). In contrast, such effect was absent in animals subjected to weak footshock training (Fig. 1B). These findings suggest that a strong emotionally arousing experience is a necessary condition for the involvement of the hippocampal ECS on fear memory consolidation.

The view that hippocampal ECS requires some level of aversiveness in order to be recruited is also supported by studies showing that memory motivated by mildly aversive tasks, such as the open field habituation, systematically fails to respond to CB1 agents (de Oliveira Alvares et al. 2005, 2008a). Although the generality of this supposition might be disputed (see, e.g.,

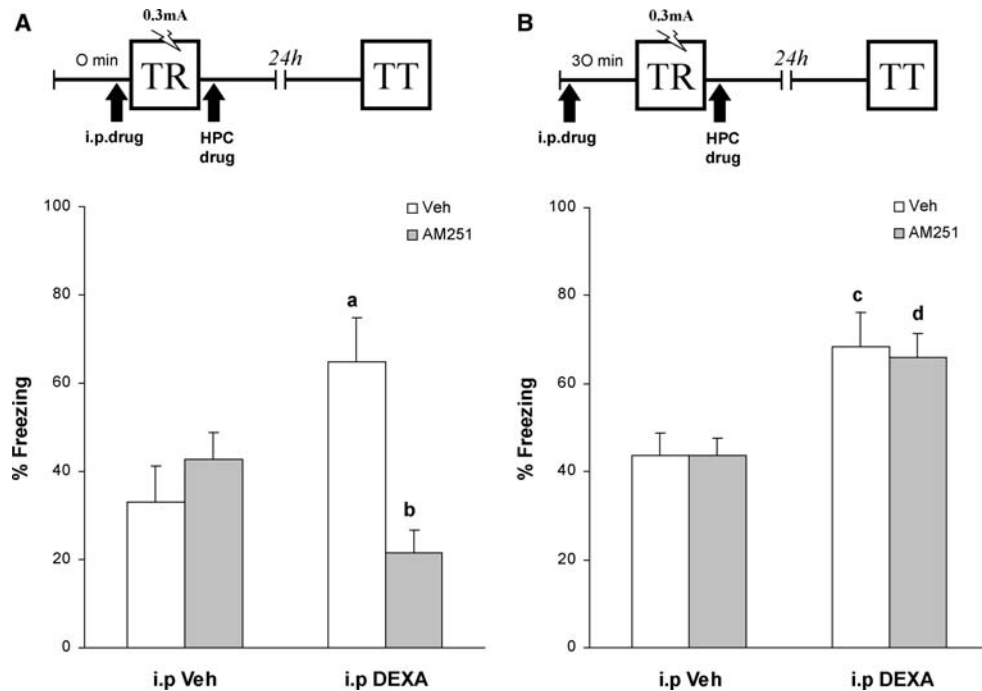


Figure 3. Interdependency between intrahippocampal treatment (AM251 or its vehicle) and dexamethasone systemically infused (A) immediately (0 min) or (B) 30 min before the CFC training session under a weak (0.3 mA) footshock. The diagram above each histogram depicts the corresponding experimental design. Data expressed as mean \pm SEM of percentual freezing time in a 5-min test session performed 24 h after the CFC training session. Horizontal axis: The two intrahippocampal infusion groups (Veh: PBS with 8% DMSO; AM251: 5.5 ng/side) are grouped according to the systemic treatment—first the control group (i.p. Veh: saline \pm 2.5% ethanol) and, next, the dexamethasone group (i.p. DEXA: 0.01 mg/Kg). In A the two-way ANOVA indicates a significant effect of intrahippocampal, but not of systemic treatment, and an interaction between these treatments: (a) significantly different from the intrahippocampal vehicle-injected of the i.p. Veh systemic group ($P = 0.003$, Tukey post-hoc test); (b) significantly different from the correspondent vehicle-injected group of the same systemic (i.p. DEXA) group ($P < 0.001$, Tukey post-hoc test). $n = 9, 7, 10$, and 9 , respectively. In B, the two-way ANOVA indicates a significant effect of systemic (but neither of intrahippocampal treatment nor an interaction between the two treatments): There is a significant difference between systemic groups for each intrahippocampal treatment, (c) Veh ($P = 0.003$, Tukey post-hoc test) or (d) AM251 ($P = 0.011$, Tukey post-hoc test), with $n = 11, 9, 10$, and 9 , respectively.

Kamprath et al. 2006; Jacob et al. 2009), it seems consistent with the literature indicating that ECS involvement is task-specific (de Oliveira Alvares et al. 2005; Höltner et al. 2005; Niyuhire et al. 2007). Recently, Kamprath et al. (2009) have demonstrated a dependency of endocannabinoid action on the intensity of the footshock used in a fear-conditioning task that associates tone response with previous shock treatment.

It is widely known that stressful events, such as an aversive learning paradigm, or the release of hormones functionally associated with threatening events, play a critical role in memory processes (Roosendaal and McGaugh 1996; de Quervain et al. 1998; Roosendaal et al. 1999, 2003; Okuda et al. 2004). It is noticeable how the effects of these stimuli resemble the endocannabinoid system on its influence on different memory phases (Suzuki et al. 2004; de Oliveira Alvares et al. 2005, 2008a,b; Cai et al. 2006; Pamplona and Takahashi 2006; Yang et al. 2006; Niyuhire et al. 2007; Maroun and Akirav 2008; Wang et al. 2008). Actually, the release of endocannabinoids such as anandamide and 2-AG have been demonstrated in the hippocampus (Kamprath et al. 2006), the amygdala (Marsicano et al. 2002), and other brain structures, such as the periaqueductal gray matter (Hohmann et al. 2005) and the midbrain (Di et al. 2005), always in response to an aversive situation. Another suggestive clue comes from the fact that cannabinoids can influence synaptic events taking place in areas such as the hippocampus, particularly after an aversive stimulation (Wilson et al. 2001; Carlson et al. 2002).

Consonant with this view, the present study shows that intrahippocampus infusion of AM251 blocked the memory-

enhancing effect of both pre-training treatments, a single stress session (Fig. 2) or a single dexamethasone injection (Fig. 3A), suggesting an activation of the hippocampal ECS by the glucocorticoid system. Endocannabinoids would, in turn, suppress the local interneuronal GABAergic control (Katona et al. 1999; Wilson and Nicoll 2002) and, through this mechanism, disinhibit the memory trace building excitatory pathway as we have suggested elsewhere (de Oliveira Alvares et al. 2005, 2006).

In the last few years, it became clear that the Hypothalamus–Pituitary–Adrenal (HPA) axis activity is controlled by an endocannabinoid *tonus* (Cota 2008; Steiner and Wotjak 2008); more specifically, subcortical brain areas expressed CB1 receptors that seem to be involved in stress-induced GC release (Steiner et al. 2008). The existence of a two-way interdependence between endocannabinoid and GC systems began to be uncovered after the demonstration of the existence of rapid/nongenomic/membrane-bound glucocorticoid receptors (Tasker et al. 2006); and corticosterone, an endogenous GC released by the adrenal cortex in response to a stressful stimulus, can act upon several targets in the brain after freely crossing the blood-brain barrier (Joëls 2008). It was suggested that the fact that this effect takes place in a few minutes was in conflict with the well-known slow, genomic effects mediated by the two cytoplasmic types of GC receptors. This evidence led to the proposal of a fast, functional G-protein membrane-bound receptor (mGCR), first shown in the hypothalamic PVN area (Di et al. 2003, 2005): Postsynaptic mGCRs in parvocellular neurons can promote a fast feedback inhibition of further hormone release, a mechanism mediated

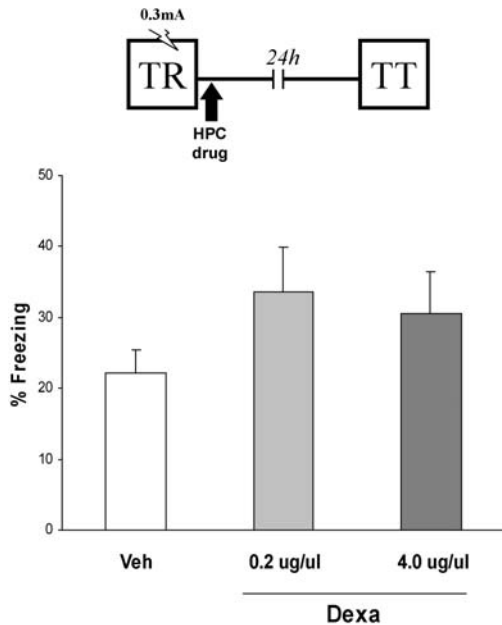


Figure 4. Effect of dexamethasone (Dexa: 0.2 and 4 $\mu\text{g}/\mu\text{L}$) or its vehicle (Veh: PBS) directly infused into the dorsal hippocampus (area CA1) immediately after the CFC training under a weak (0.3 mA) shock. Data expressed as mean \pm SEM of percentual freezing time in the 5-min test session performed 24 h after the CFC training session. There were no significant differences between the groups ($P = 0.332$, one-way ANOVA). $n = 12, 13,$ and 14 , respectively.

by the production and release of endocannabinoids acting presynaptically to reduce glutamate release (Di et al. 2003). Similarly, GCs can reduce glutamate and increase GABA release in magnocellular neurons through endocannabinoids that will retrogradely act upon two different cell types (Di et al. 2005), using two divergent G-protein pathways (Di et al. 2009). This putative fast-signaling receptor might explain the different results we have observed between the two time points selected (0 or 30 prior to training) for stress exposure (Fig. 2), or dexamethasone administration (Fig. 3A,B), suggesting that the intrahippocampal infusion of the CB1 antagonist disrupted memory consolidation only at the beginning of the GC system activation.

In order to conclude whether the GC-dependent hippocampal endocannabinoid recruitment takes place in the hippocampus itself, we performed experiments 4 and 5. These results, however, are inconclusive, and the “link” may still be in the hippocampus—possibly mediated by the putative, fast mGCRs—or in another brain structure. In this regard, two recent reports showed that local AM251 infusion into the BLA may interfere with the memory-enhancing effect of stress or GCs (Campolongo et al. 2009; Ganon-Elazar and Akirav 2009). In the first study, the memory-enhancing effect of GCs was attributed to an ECS-mediated disinhibitory influence on noradrenaline release, a mechanism that facilitated the formation of the aversive memory trace (Campolongo et al. 2009; Hill and McEwen 2009). In the second work, intra-BLA AM251 disrupted an avoidance memory extinction and the agonist WIN55,212-2, administered either into the BLA or systemically, modulated the behavioral enhancement effect of stress, prompting a small increase in plasma corticosterone levels (Ganon-Elazar and Akirav 2009). Thus, not only the dorsal hippocampus and the basolateral amygdala may share an analogous mechanism, but they might even be functionally connected in order to modulate a cognitive process

such as the formation of a new contextual fear memory trace (Akirav et al. 2001; Kim and Diamond 2002; Akirav and Richter-Levin 2006; Malin and McGaugh 2006). Further experiments are necessary to investigate such a possibility.

The dexamethasone i.p. dose selected in the present study enhances memory consolidation in a long-lasting way (Fig. 3A,B). Despite the fact that this synthetic glucocorticoid has a different time profile as compared with corticosterone (Bohus and De Kloet 1981), experiment 3 was able to effectively mimic the stress situation, again supporting the idea that the stress-induced enhancing effect involves the recruitment of hippocampal endocannabinoids (Fig. 3A), either directly or through an external relay as mentioned above. Furthermore, this ECS recruitment appears to vanish 30 min after dexamethasone administration (Fig. 3B), similar to the effect we observed when stress exposure was performed 30 min prior to the CFC training session (Fig. 2). This was also consistent with evidence showing that endocannabinoids are rapidly released on demand and have a brief half-life of circa 5 min (Di Marzo et al. 1994, 2005).

Since the stress-enhancing effect took place only when presented immediately before (or during) training, a time course of action for the stress-released endogenous agent that mediates the phenomenon is suggested. The fact that the effect is no longer evident when animals are subjected to the environmental demand 30 min prior to training is consistent with the time course of several stress hormones (Joëls 2008). In this line of reasoning, corticosterone may be a good candidate, even considering the fact that its hippocampal peak takes place 30 min after stress (Pfaff et al. 1971; Joëls 2008) and similar times may be expected for nearby anatomical targets. Thus, its receptor-mediated effects, both rapid and/or slow, might be induced early in the time curve, and the receptor activation/inactivation cycle might be completed well before the GCs have reached their maximum levels.

In summary, our results suggest that the activation of the ECS in the hippocampus requires a certain level of aversiveness or emotional status to exert its modulatory role on fear memory consolidation. This negative emotional state may be provided (1) by the task stimulus itself (e.g., a strong shock; Fig. 1A), or (2) by a previous stress session (Fig. 2), or, alternatively, (3) by a hormone functionally associated with a stressful stimulus, such as the glucocorticoids (Fig. 3A). Based on these findings we propose that glucocorticoids may be the putative endogenous mediators of this aversive-dependent hippocampal ECS recruitment, despite the fact that this functional link may be taking place in the same or in adjacent brain structures. To our knowledge, this is the first demonstration that the hippocampal ECS can functionally interact with glucocorticoids in order to modulate the formation of a contextual fear memory.

Materials and Methods

Animals

Two-hundred and thirty-one male Wistar rats (age 2–3 mo, weight 250–320 g) from our breeding colony were used in these experiments. Animals were housed in plastic cages, four to five in a cage, under a 12-h light/dark cycle and at a constant temperature of $24 \pm 1^\circ\text{C}$, with water and food ad libitum.

Stereotaxic surgery and cannulae placement

All animals were anesthetized by a mixture of ketamine and xylazine (i.p., 75 and 10 mg/kg, respectively) and bilaterally implanted with a 27-gauge guide cannulae aimed at AP -4.2 mm (from bregma), LL ± 3.0 mm, DV 1.8 mm, just 1.0 mm above the CA1 area of the dorsal hippocampus (according to Paxinos and Watson 2007). After a 1-wk recovery from surgery, animals were submitted to the behavioral procedures. Following the

behavioral experiments, all subjects were sacrificed and their brains dissected and preserved in 10% formaldehyde to verify for cannulae position under low magnification (Fig. 5): 210 out of 231 animals had correct cannulae placements, so their data were included in the statistical analysis.

Contextual fear conditioning (CFC)

The conditioning chamber consisted of an illuminated Plexiglas box (25.0 × 25.0-cm grid of parallel 0.1-cm caliber stainless steel bars spaced 1.0 cm apart). In the conditioning session (training), rats were placed in the chamber for 3 min for habituation, and only after this, they received two 2-sec footshocks, either of 0.3 or 0.7 mA, separated by a 30-sec interval. Before returning to their home cages, animals were kept in the conditioning environment for an additional minute. Twenty-four hours later, all animals were tested for 5 min in the same context.

Stress vs. glucocorticoid injection

The stress session consisted of receiving two 1-mA inescapable footshocks in a different context (10 × 10-cm plastic box, grid floor), delivered 10 sec after being put there. Both stress exposure or dexamethasone i.p. administration (0.01 mg/kg) were either applied 30 min or immediately before training in the CFC task. In order to avoid any association with the grid floor itself, the very few animals (less than five) that presented freezing behavior during the 3-min habituation phase of the conditioning session (before receiving the conditioning shocks) were excluded from the analyses.

Intrahippocampally infused drugs

At the time of infusion, a 30-gauge infusion needle was fitted into the guide cannulae, with its tip protruding 1.0 mm beyond the guide cannula end and aimed at the pyramidal cell layer of CA1 of the dorsal hippocampus (see Fig. 5). A volume of 0.5 μ L was bilaterally infused at a slow rate (20 μ L/h) and the needle was removed only after another additional 30 sec. In experiments 1–3 animals were divided into two groups, each receiving one of the following drugs: AM251, a selective CB1 antagonist (5.5 ng/side; Tocris Cookson, Inc.), or its vehicle (phosphate-buffered saline with 8% dimethylsulfoxide). Vehicle composition and the doses/concentrations used were selected based on previous experiments from our laboratory (de Oliveira Alvares et al. 2006, 2008b). Dexamethasone (0.2 and 4 μ g/ μ L) and the GCR antagonist mifepristone/RU486 (Sigma; 6 and 60 ng/ μ L) for the intrahippocampal infusions were dissolved in the same vehicle as described above for AM251.



Figure 5. Typical acceptable needle placement, aimed at the CA1 region (according to Paxinos and Watson 2007) of the rat dorsal hippocampus (formol thionine technique).

Experimental design

Experiment 1

Animals were trained in the CFC with either a strong (0.7 mA) or a weak (0.3 mA) footshock; immediately after the training session, rats received the intrahippocampal infusion of AM251 (5.5 ng/side) or its vehicle (PBS + 8% DMSO), and the test was performed 24 h later (see the diagram above Fig. 1A,B).

Experiment 2

Animals were submitted to a stressful event in a different context at different time points, either 0 or 30 min prior to the training session, and then trained in the weak shock protocol (0.3 mA); immediately after training, animals received locally in the dorsal hippocampus either AM251 or its vehicle (as in experiment 1); test was performed 24 h later (see the diagram above Fig. 2).

Experiment 3

Animals were injected i.p. with dexamethasone (DEXA, 0.01 mg/Kg) or its vehicle (ETOH, PBS + 2.5% ethanol) at different time points, either 0 or 30 min prior to the training session, and then trained using the weak shock protocol (0.3 mA); immediately after training, animals received locally in the dorsal hippocampus either AM251 or its vehicle (as in experiment 1); test was performed 24 h later (see diagram above Fig. 3A,B).

Experiment 4

Experiment 4 was similar to experiment 1, with dexamethasone (0.2 and 4 μ g/ μ L) directly infused into the dorsal hippocampus (area CA1) after the training session with the weak (0.3 mA) shock (see diagram above Fig. 4).

Experiment 5

Experiment 5 was also similar to experiment 1, with mifepristone (6 and 60 ng/ μ L) directly infused into the dorsal hippocampus (area CA1) before the training session with the strong (0.7 mA) shock.

Statistical analysis

Since CFC data (percent freezing) in all experimental groups have both reached $P > 0.200$ in the Kolmogorov–Smirnov test with Lilliefors' correction and passed the Equal Variance test, normality and homoscedasticity were ensured and only parametric tests were used (data expressed as mean \pm SEM). Differences between groups receiving intrahippocampal post-training infusions of AM251 or its vehicle after a weak or a strong footshock were identified by *t*-test for independent samples (experiment 1); when more groups were involved, one-way (experiments 4 and 5) or two-way (experiments 2 and 3) ANOVAs was used and differences sorted by Tukey all-pairwise multiple comparison post-hoc test. Statistical analysis of the behavioral data was limited to the 210 out of 231 animals with correct cannulae placements (Fig. 5), and only P of <0.05 were considered significant.

Ethical aspects

All experimental procedures in living animals (rats) were performed in strict accordance to the recommendations of the Brazilian Society for Neurosciences (SBNeC), the Brazilian College of Animal Experimentation (SBCAL), and the International Brain Research Organization (IBRO), being previously approved by our Institutional (UFRGS) Committee for Ethics in Research, meaning that they are in compliance with the U.S. National Institutes of Health Guide for Care and Use of Laboratory Animals (publication no. 85-23, revised in 1985), the European Communities Council Directive of 24 November 1986 (86/609/EEC), and the Brazilian law (Federal Law no 11.794/2008). Every effort was made to minimize the number of animals used and their suffering.

Acknowledgments

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References

- Abrahám I, Juhász G, Kékesi KA, Kovács KJ. 1996. Effect of intrahippocampal dexamethasone on the levels of amino acid transmitters and neuronal excitability. *Brain Res* **733**: 56–63.
- Akirav I, Richter-Levin G. 2006. Factors that determine the non-linear amygdala influence on hippocampus-dependent memory. *Dose Response* **4**: 22–37.
- Akirav I, Sandi C, Richter-Levin G. 2001. Differential activation of hippocampus and amygdala following spatial learning under stress. *Eur J Neurosci* **14**: 719–725.
- Bohus B, de Kloet ER. 1981. Adrenal steroids and extinction behavior: Antagonism by progesterone, deoxycorticosterone and dexamethasone of a specific effect of corticosterone. *Life Sci* **28**: 433–440.
- Cai WH, Blundell J, Han J, Greene RW, Powell CM. 2006. Postreactivation glucocorticoids impair recall of established fear memory. *J Neurosci* **26**: 9560–9566.
- Calfa G, Bussolino D, Molina VA. 2007. Involvement of the lateral septum and the ventral Hippocampus in the emotional sequelae induced by social defeat: Role of glucocorticoid receptors. *Behav Brain Res* **181**: 23–34.
- Campolongo P, Roozendaal B, Trezza V, Hauer D, Schelling G, McGaugh JL, Cuomo V. 2009. Endocannabinoids in the rat basolateral amygdala enhance memory consolidation and enable glucocorticoid modulation of memory. *Proc Natl Acad Sci* **106**: 4888–4893.
- Carlson G, Wang Y, Alger BE. 2002. Endocannabinoids facilitate the induction of LTP in the hippocampus. *Nat Neurosci* **5**: 723–724.
- Cota D. 2008. The role of the endocannabinoid system in the regulation of hypothalamic-pituitary-adrenal axis activity. *J Neuroendocrinol* **20**: 35–38.
- Davies SN, Pertwee RG, Riedel G. 2002. Functions of cannabinoid receptors in the hippocampus. *Neuropharmacology* **42**: 993–1007.
- de Oliveira Alvares L, de Oliveira LF, Camboim C, Diehl F, Genro BP, Lanzotti VB, Quilfeldt JA. 2005. Amnesic effect of intrahippocampal AM251, a CB1-selective blocker, in the inhibitory avoidance, but not in the open field habituation task, in rats. *Neurobiol Learn Mem* **83**: 119–124.
- de Oliveira Alvares L, Genro BP, Vaz Breda R, Pedrosa MF, Da Costa JC, Quilfeldt JA. 2006. AM251, a selective antagonist of the CB1 receptor, inhibits the induction of long-term potentiation and induces retrograde amnesia in rats. *Brain Res* **1075**: 60–67.
- de Oliveira Alvares L, Genro BP, Diehl F, Quilfeldt JA. 2008a. Differential role of the hippocampal endocannabinoid system in the memory consolidation and retrieval mechanisms. *Neurobiol Learn Mem* **90**: 1–9.
- de Oliveira Alvares L, Pasqualini Genro B, Diehl F, Molina VA, Quilfeldt JA. 2008b. Opposite action of hippocampal CB1 receptors in memory reconsolidation and extinction. *Neuroscience* **154**: 1648–1655.
- de Quervain DJ, Roozendaal B, McGaugh JL. 1998. Stress and glucocorticoids impair retrieval of long-term spatial memory. *Nature* **394**: 787–790.
- Di S, Malcher-Lopes R, Halmos KC, Tasker JG. 2003. Nongenomic glucocorticoid inhibition via endocannabinoid release in the hypothalamus: A fast feedback mechanism. *J Neurosci* **23**: 4850–4857.
- Di S, Malcher-Lopes R, Marcheselli VL, Bazan NG, Tasker JG. 2005. Rapid glucocorticoid-mediated endocannabinoid release and opposing regulation of glutamate and γ -aminobutyric acid inputs to hypothalamic magnocellular neurons. *Endocrinology* **146**: 4292–4301.
- Di S, Maxson MM, Franco A, Tasker JG. 2009. Glucocorticoids regulate glutamate and GABA synapse-specific retrograde transmission via divergent nongenomic signaling pathways. *J Neurosci* **29**: 393–401.
- Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz JC, Piomelli D. 1994. Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature* **372**: 686–691.
- Di Marzo V, De Petrocellis L, Bisogno T. 2005. The biosynthesis, fate and pharmacological properties of endocannabinoids. *Handb Exp Pharmacol* **168**: 147–185.
- Ferreira VM, Takahashi RN, Morato GS. 2000. Dexamethasone reverses the ethanol-induced anxiolytic effect in rats. *Pharmacol Biochem Behav* **66**: 585–590.
- Ganon-Elazar E, Akirav I. 2009. Cannabinoid receptor activation in the basolateral amygdala blocks the effects of stress on the conditioning and extinction of inhibitory avoidance. *J Neurosci* **29**: 11078–11088.
- Heifets BD, Castillo PE. 2009. Endocannabinoid signaling and long-term synaptic plasticity. *Annu Rev Physiol* **71**: 283–306.
- Hill MN, McEwen BS. 2009. Endocannabinoids: The silent partner of glucocorticoids in the synapse. *Proc Natl Acad Sci* **106**: 4579–4580.
- Hohmann AG, Suplita RL, Bolton NM, Neely MH, Fegley D, Mangieri R, Krey JF, Walker JM, Holmes PV, Crystal JD, et al. 2005. An endocannabinoid mechanism for stress-induced analgesia. *Nature* **435**: 1108–1112.
- Hölter SM, Kallnik M, Wurst W, Marsicano G, Lutz B, Wotjak CT. 2005. Cannabinoid CB1 receptor is dispensable for memory extinction in an appetitively motivated learning task. *Eur J Pharmacol* **510**: 69–74.
- Jacob W, Yassouridis A, Marsicano G, Monory K, Lutz B, Wotjak CT. 2009. Endocannabinoids render exploratory behaviour largely independent of the test aversiveness: Role of glutamatergic transmission. *Genes Brain Behav* doi: 10.1111/j.1601-183X.2009.00512.x.
- Joëls M. 2008. Functional actions of corticosteroids in the hippocampus. *Eur J Pharmacol* **583**: 312–321.
- Joëls M, Krugers HJ, Lucassen PJ, Karst H. 2009. Corticosteroid effects on cellular physiology of limbic cells. *Brain Res* doi: 10.1016/j.brainres.2009.03.036.
- Kamprath K, Marsicano G, Tang J, Monory K, Bisogno T, Di Marzo V, Lutz B, Wotjak CT. 2006. Cannabinoid CB1 receptor mediates fear extinction via habituation-like processes. *J Neurosci* **26**: 6677–6686.
- Kamprath K, Plendl W, Marsicano G, Deussing JM, Wurst W, Lutz B, Wotjak CT. 2009. Endocannabinoids mediate acute fear adaptation via glutamatergic neurons independently of corticotropin-releasing hormone signaling. *Genes Brain Behav* **8**: 203–211.
- Katona I, Sperlagh B, Sik A, Kafalvi A, Vizi ES, Mackie K, Freund TF. 1999. Presynaptically located CB1 cannabinoid receptors regulate GABA release from axonal terminals of specific hippocampal interneurons. *J Neurosci* **19**: 4544–4558.
- Kim JJ, Fanselow MS. 1992. Modality-specific retrograde amnesia of fear. *Science* **256**: 675–677.
- Kim JJ, Diamond DM. 2002. The stressed hippocampus, synaptic plasticity and lost memories. *Nat Rev Neurosci* **3**: 453–462.
- Lutz B. 2007. The endocannabinoid system and extinction learning. *Mol Neurobiol* **36**: 92–101.
- Mackie K. 2005. Distribution of cannabinoid receptors in the central and peripheral nervous system. *Handb Exp Pharmacol* **168**: 299–325.
- Malin EL, McGaugh JL. 2006. Differential involvement of the hippocampus, anterior cingulate cortex, and basolateral amygdala in memory for context and footshock. *Proc Natl Acad Sci* **103**: 1959–1963.
- Maroun M, Akirav I. 2008. Arousal and stress effects on consolidation and reconsolidation of recognition memory. *Neuropsychopharmacology* **33**: 394–405.
- Marsicano G, Kuner R. 2008. Anatomical distribution of receptors, ligands and enzymes in the brain and in the spinal cord: Circuitries and neurochemistry. In *Cannabinoids and the Brain* (ed. A Kofáľvi), pp. 161–201. Springer, Heidelberg, Germany.
- Marsicano G, Wotjak CT, Azad SC, Bisogno T, Rammes G, Cascio MG, Hermann H, Tang J, Hofmann C, Zieglgänsberger W, et al. 2002. The endogenous cannabinoid system controls extinction of aversive memories. *Nature* **418**: 530–534.
- Niyuhire F, Varvel SA, Thorpe AJ, Stokes RJ, Wiley JL, Lichtman AH. 2007. The disruptive effects of the CB1 receptor antagonist rimonabant on extinction learning in mice are task-specific. *Psychopharmacology* **191**: 223–231.
- Okuda S, Roozendaal B, McGaugh JL. 2004. Glucocorticoid effects on object recognition memory require training-associated emotional arousal. *Proc Natl Acad Sci* **101**: 853–858.
- Pamplona FA, Takahashi RN. 2006. WIN 55212–2 impairs contextual fear conditioning through the activation of CB1 cannabinoid receptors. *Neurosci Lett* **397**: 88–92.
- Paxinos G, Watson C. 2007. *The rat brain in stereotaxic coordinates*. Academic Press, San Diego, CA.
- Pfaff DW, Silva MT, Weiss JM. 1971. Telemetered recording of hormone effects on hippocampal neurons. *Science* **172**: 394–395.
- Puigherman E, Marsicano G, Busquets-García A, Lutz B, Maldonado R, Ozaita A. 2009. Cannabinoid modulation of hippocampal long-term memory is mediated by mTOR signaling. *Nat Neurosci* **12**: 1152–1158.
- Robbe D, Montgomery SM, Thome A, Rueda-Orozco PE, McNaughton BL, Buzsáki G. 2006. Cannabinoids reveal importance of spike

- timing coordination in hippocampal function. *Nat Neurosci* **9**: 1526–1533.
- Roozendaal B, McGaugh JL. 1996. Amygdaloid nuclei lesions differentially affect glucocorticoid-induced memory enhancement in an inhibitory avoidance task. *Neurobiol Learn Mem* **65**: 1–8.
- Roozendaal B, Nguyen BT, Power AE, McGaugh JL. 1999. Basolateral amygdala noradrenergic influence enables enhancement of memory consolidation induced by hippocampal glucocorticoid receptor activation. *Proc Natl Acad Sci* **96**: 11642–11647.
- Roozendaal B, Griffith QK, Buranday J, De Quervain DJ, McGaugh JL. 2003. The hippocampus mediates glucocorticoid-induced impairment of spatial memory retrieval: Dependence on the basolateral amygdala. *Proc Natl Acad Sci* **100**: 1328–1333.
- Steiner MA, Wotjak CT. 2008. Role of the endocannabinoid system in regulation of the hypothalamic-pituitary-adrenocortical axis. *Progress Brain Res* **170**: 397–432.
- Steiner MA, Marsicano G, Wotjak CT, Lutz B. 2008. Conditional cannabinoid receptor type 1 mutants reveal neuron subpopulation-specific effects on behavioral and neuroendocrine stress responses. *Psychoneuroendocrinology* **33**: 1165–1170.
- Suzuki A, Josselyn SA, Frankland PW, Masushige S, Silva AJ, Kida S. 2004. Memory reconsolidation and extinction have distinct temporal and biochemical signatures. *J Neurosci* **24**: 4787–4795.
- Tasker JG, Di S, Malcher-Lopes R. 2006. Minireview: Rapid glucocorticoid signaling via membrane-associated receptors. *Endocrinology* **147**: 5549–5556.
- Viveros MP, Marco EM, Llorente R, López-Gallardo M. 2007. Endocannabinoid system and synaptic plasticity: Implications for emotional responses. *Neural Plast* doi: 10.1155/2007/52908.
- Wang XY, Zhao M, Ghitza UE, Li YQ, Lu L. 2008. Stress impairs reconsolidation of drug memory via glucocorticoid receptors in the basolateral amygdala. *J Neurosci* **28**: 5602–5610.
- Wilson RI, Nicoll RA. 2002. Endocannabinoid signaling in the brain. *Science* **296**: 678–682.
- Wilson RI, Kunos G, Nicoll RA. 2001. Presynaptic specificity of endocannabinoid signaling in the hippocampus. *Neuron* **31**: 453–462.
- Yang YL, Chao PK, Lu KT. 2006. Systemic and intra-amygdala administration of glucocorticoid agonist and antagonist modulate extinction of conditioned fear. *Neuropsychopharmacology* **31**: 912–924.
- Yim TT, Hong NS, Ejaredar M, McKenna JE, McDonald RJ. 2008. Post-training CB1 cannabinoid receptor agonist activation disrupts long-term consolidation of spatial memories in the hippocampus. *Neuroscience* **151**: 929–936.

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5. DISCUSSÃO

5.1 Participação dos receptores CB1 na reconsolidação e extinção da memória

Os resultados apresentados no capítulo I mostram a participação do sistema endocanabinóide hipocampal nos processos de reconsolidação e extinção da memória na tarefa do condicionamento aversivo contextual em ratos.

A infusão bilateral do antagonista seletivo dos receptores CB1, AM251, no hipocampo dorsal imediatamente após a sessão de reativação da memória de 3 min facilitou a reconsolidação da memória. Esse efeito parece ser duradouro e/ou permanente, já que a memória dos animais tratados com AM251 permaneceu facilitada depois de 5 dias (Figura 2). O agonista canabinóide endógeno anandamida apresentou um efeito oposto ao AM251, inibindo a reconsolidação da memória (Figura 5). Esse efeito foi revertido quando administrado concomitantemente com uma dose sub-efetiva de AM251, mostrando que seu efeito é mediado pelos receptores CB1 (Figura 5).

Esses estudos sobre o processo de reconsolidação foram pioneiros no laboratório, e, portanto, foi necessário validar o protocolo para poder interferir no processo de reconsolidação (especialmente para ajustar a intensidade do choque e o tempo de reexposição durante a reativação, que varia muito de um laboratório para outro). Para esse fim, utilizamos o inibidor de síntese protéica, que é classicamente utilizado para inibir a reconsolidação da memória. Usualmente são utilizadas drogas que inibem a tradução gênica, como anisomicina ou ciclohexamida, entretanto, nesse trabalho foi utilizado o

inibidor de transcrição gênica DRB, que atua reversivelmente sobre a RNA polimerase II. O que inicialmente teria um mero papel de validação acabou tendo um resultado original, já que foi o primeiro trabalho a demonstrar o efeito do DRB sobre a reconsolidação em memórias aversivas (num intervalo de poucas semanas foi publicado um outro trabalho com efeitos similares sobre a memória espacial, Da Silva et al., 2008).

A infusão de AM251, anandamida ou DRB só produziu efeito sobre a memória quando estas foram administradas imediatamente após a sessão de reativação da memória (Figura 4), mostrando que (a) essas drogas não possuem um efeito *per se*, e que (b) a exposição a dicas que induzem a evocação da memória é fundamental para a labilização da mesma, tornando-a assim vulnerável a interferências.

Esses resultados corroboram outros trabalhos que demonstraram que agonistas dos receptores CB1 inibem a reconsolidação da memória no córtex insular (Kobilo et al., 2007) e na BLA (Lin et al., 2006). Um resultado aparentemente contraditório, no qual a infusão intra-hipocampal do antagonista CB1, SR 141615A (uma molécula extremamente parecida com o AM251, a ponto de serem utilizadas freqüentemente nas mesmas concentrações), não possuiu efeito sobre a reconsolidação da memória quando infundido sozinho, porém, previniu a labilização da memória, tornado-a insensível a anisomicina (Suzuki et al., 2008). Uma possível explicação para essa discrepância foi a diferença nas concentrações utilizadas. Nesse trabalho, foram utilizadas concentrações de 400 a 800 vezes mais altas do que as utilizadas nos experimentos aqui apresentados, podendo, portanto, estar atuando em sítios inespecíficos.

Além de ter abordado a reconsolidação, o primeiro capítulo desta tese também verificou a participação do sistema endocanabinóide hipocampal sobre a extinção da memória. O AM251 inibiu a consolidação da extinção quando administrado imediatamente após a exposição de 25 minutos ao contexto (Figura 3). A anandamida, por outro lado, facilitou a extinção da memória (Figura 6). Esse efeito parece ser mediado pelos receptores CB1, pois o mesmo foi bloqueado por uma dose sub-efetiva de AM251 infundida de forma concomitante (Figura 6).

Esse efeito inédito encontrado no hipocampo, parece ser um mecanismo geral do sistema endocanabinóide na extinção, pois foram mostrados resultados similares com camundongos nocaute para receptores CB1, onde apresentavam um enorme déficit sobre a extinção (Marsicano et al., 2002), uma facilitação da extinção com o agonista CB1 WIN55212-2 (Pamplona et al., 2006), ou uma inibição da extinção com o antagonistas CB1 (Suzuki et al., 2004 e 2008; Pamplona et al., 2006; Kobilko et al., 2007).

Em trabalhos anteriores demonstramos que os processos de consolidação e de evocação são modulados pela atividade do sistema endocanabinóide. A infusão intra-hipocampal do antagonista CB1, AM251, imediatamente após o treino, prejudicou a consolidação da memória (de Oliveira Alvares et al., 2005) e também sobre seu análogo eletrofisiológico, a LTP (de Oliveira Alvares et al., 2006), enquanto que concentrações baixas do canabinóide endógeno anandamida facilitaram a formação da memória (de Oliveira Alvares et al., 2008). Para nossa surpresa, obtivemos resultados opostos com o tratamento pré-teste, ou seja, a facilitação da evocação da memória com o bloqueio dos receptores CB1. Esta assimetria sugere a ocorrência de uma alteração plástica que modificaria a sensibilização e/ou a expressão de receptores CB1 hipocampais em função do

aprendizado (i.e., durante o processo de consolidação), e que fica evidente no momento da evocação, 24h mais tarde (de Oliveira Alvares et al., 2008).

Interessantemente, encontramos agora essa mesma assimetria entre os processos de reconsolidação e extinção (i.e. efeitos opostos entre os dois processos). Essas diferenças são coerentes com a idéia de que no processo de reconsolidação da memória, interfere-se no próprio “traço” de memória, enquanto que na extinção seria um novo traço, o qual compete com a memória original. Ou seja, no processo de reconsolidação (onde já ocorreu o aprendizado, e, portanto, a eventual alteração plástica modulada pelo sistema canabinoide) o antagonista CB1 possui um papel facilitador do sistema (o mesmo que ocorre durante a evocação), enquanto que na extinção da memória, onde está se formando um novo aprendizado, o antagonista CB1 possui um efeito prejudicial, assim como ocorre durante a consolidação.

5.2 Interação entre o sistema endocanabinóide e glicocorticóide no hipocampo

Conforme já havia sido demonstrado não só em nosso laboratório (de Oliveira Alvares et al., 2005) mas também por outros grupos (Holter et al., 2005; Pamplona e Takahashi, 2006; Niyuhire et al., 2007), o sistema endocanabinóide parece estar envolvido na formação de memórias emocionais, mas não sobre as memórias neutras. Neste capítulo, estudou-se os possíveis mecanismos envolvidos em memórias emocionais que recrutam o sistema endocanabinóide para sua participação na consolidação da memória.

No primeiro experimento, desenvolveu-se um protocolo onde fosse possível dissociar a participação do sistema endocanabinóide na consolidação da memória. A variável alterada entre os protocolos foi a intensidade do choque nas patas, 0,3mA no protocolo de intensidade moderada (choque fraco) ou 0,7mA no protocolo de intensidade alta (choque forte). Se o sistema endocanabinóide fosse recrutado no treino de alta intensidade, então a infusão de um antagonista CB1 deveria interferir sobre a consolidação da memória. No protocolo de intensidade moderada, por outro lado, hipotetizamos que o antagonista CB1 não deveria influenciar na formação da memória, já que o sistema endocanabinóide não estaria sendo demandado nessas condições.

Esses resultados sugerem que o sistema endocanabinóide participa na consolidação da memória no protocolo com choque forte, mas não no protocolo de intensidade moderada (choque fraco). O antagonista AM251 administrado no hipocampo imediatamente após o treino com choque forte apresentou um efeito amnésico. Porém, não houve efeito quando administrado após o treino com choque fraco.

Esses resultados mostraram que o sistema endocanabinóide necessita de um estímulo com um nível de aversividade mínimo para ser recrutado. No experimento seguinte testamos se o estresse inerente de um aprendizado emocional aversivo, como a associação de um contexto com choques de intensidade alta, estaria envolvido na síntese e/ou liberação dos canabinóides endógenos sobre a consolidação da memória. Hohmann e colegas (2005) já haviam demonstrado que ocorria um aumento da liberação dos endocanabinóides anandamida e 2-AG em resposta a choques nas patas.

Para testar essa hipótese, submetemos os animais a uma sessão de estresse em dois tempos, 30 minutos ou imediatamente antes do treino, com o protocolo de choque de intensidade moderada (choque fraco). Se a sessão de estresse fosse suficiente para o recrutamento de endocanabinóides, então (a) os animais deveriam apresentar uma facilitação sobre a consolidação da memória devido à ação dos endocanabinóides (e outros neuromoduladores liberados pelo estresse que não podem ser negligenciados) e (b) a administração de um antagonista CB1 após o treino com choque fraco deveria inibir a consolidação da memória.

Os resultados mostram que os ratos submetidos à sessão de estresse imediatamente, mas não 30 minutos antes do treino com choque fraco, apresentaram uma facilitação da formação da memória. O antagonista CB1, AM251, teve um efeito amnésico sobre a consolidação da memória nos ratos que passaram pela sessão de estresse imediatamente antes do treino, mas não 30 minutos antes.

Esses resultados sugerem que o estresse induz a formação de endocanabinóides, os quais participariam da consolidação da memória no sentido de facilitá-la. De fato, já

hávamos demonstrado esse efeito em trabalhos anteriores, onde a infusão intra-hipocampal de anandamida facilitou a consolidação da memória (de Oliveira Alvares et al., 2008). A ausência de efeito sobre a memória e a sensibilidade ao AM251, quando a sessão de estresse ocorria 30 minutos antes do treino, mostra a ação rápida e a subsequente inativação do sistema endocanabinóide. Essa característica de uma meia-vida curta dos endocanabinóides, já havia sido descrita em outros trabalhos (Di Marzo et al., 1994; 2005; Hohmann et al., 2005).

O estresse produz uma enorme gama de efeitos fisiológicos sobre o organismo. No experimento seguinte, investigamos qual molécula liberada pelo estresse estaria atuando sobre o sistema endocanabinóide. Di e colaboradores (2005) mostraram que o glicocorticóide sintético dexametasona aumenta os níveis dos canabinóides endógenos anandamida e 2-AG *in vitro*. Motivados por esse trabalho, injetamos dexametasona i.p. imediatamente ou 30 minutos antes da sessão de treino. Se os efeitos do estresse sobre o sistema endocanabinóide fossem mediados pela liberação de glicocorticóides, então a infusão de um glicocorticóide exógeno deveria facilitar a consolidação da memória, e tornar esses animais sensíveis ao antagonista CB1 no treino com choque fraco.

Nossos resultados mostraram que a infusão sistêmica de dexametasona imediatamente antes do treino facilitou a formação da memória. Coerentemente com os achados anteriores, a infusão intra-hipocampal de AM251 apresentou efeito amnésico no grupo que recebeu dexametasona imediatamente antes do treino com choque fraco. Um recente trabalho abordou a interação entre o sistema glicocorticóide e endocanabinóide na amígdala: Campolongo e colaboradores demonstraram que o efeito facilitatório da infusão sistêmica de glicocorticóides dependia da transmissão canabinérgica na BLA. A infusão do

antagonista CB1, AM251, na BLA reverteu o efeito facilitatório do glicocorticóide sobre a consolidação da memória (Campiono et al., 2009). Esses resultados encontrados na BLA corroboram nossos achados no hipocampo, sugerindo que essa interação sinérgica possa ser um mecanismo geral no encéfalo entre os dois sistemas.

No experimento em que a infusão de dexametasona ocorreu 30 minutos antes do treino, a infusão de AM251 não apresentou efeito, porém, a dexametasona continuou facilitando a consolidação da memória. Esses resultados sugerem que o glicocorticóide sintético dexametasona está envolvido com a síntese e/ou liberação de endocanabinóides no hipocampo durante a aquisição e começo da consolidação, já que se mostrou sensível ao bloqueio dos receptores CB1 no grupo onde recebeu o tratamento com dexametasona imediatamente antes do treino. A ausência de efeito do AM251 sobre o grupo que recebeu a infusão de dexametasona 30 minutos antes do treino indica que (a) os endocanabinóides são sintetizados quando o sistema detecta um aumento na concentração de glicocorticóides, (b) esse feito é efêmero e não persistente, mesmo que ainda existam glicocorticóides circulantes, já que se acredita que durante o treino ainda haja uma concentração razoável de dexametasona devido à injeção sistêmica. Ademais, esses resultados mostram que os glicocorticóides atuam sobre outro alvo de maneira a influenciar a consolidação da memória, já que, apesar de não ser sensível ao AM251, facilitou a formação da memória.

Especula-se que os efeitos tardios (30 minutos) e precoces (imediatamente pré-treino) dos glicocorticóides são mediados por receptores distintos. Embora os hormônios esteróides sejam classicamente descritos como ligantes de receptores citoplasmáticos, e portanto, de ação lenta, novos estudos vêm mudando essa visão. Estudos com anfíbios já haviam demonstrado esses efeitos rápidos de glicocorticóides, os quais eram mediados por

receptores de membrana acoplados à proteína G (Moore e Orchinich, 1994). Porém, em mamíferos, esse tipo de receptor glicocorticóide está apenas começando a ser compreendido. Em um elegante trabalho do grupo do Dr. Tasker, Di e colaboradores (2005) mostraram que a administração de corticosterona ou dexametasona inibe a transmissão glutamatérgica *in vitro* de maneira rápida (cerca de 3 minutos), sugerindo, portanto, que o efeito seria mediado por receptores de membrana ao invés dos clássicos receptores citoplasmáticos. Para testar essa hipótese, administraram dexametasona conjugada com albumina, de modo que essas moléculas não pudessem entrar na célula e, portanto, o possível efeito, se existisse, seria sobre os receptores de membrana, e não citoplasmáticos. O resultado desse experimento foi a manutenção dos efeitos encontrados com a dexametasona e corticosterona, demonstrando que realmente eram mediados por receptores de membrana. Ademais, mostraram que esses efeitos eram produzidos por receptores metabotrópicos, pois eram revertidos pela administração de GDP- β -S, um bloqueador da proteína G.

Esses resultados corroboram nossos achados de que a interação dos glicocorticóides com os endocannabinóides ocorre de maneira rápida, estando ausente depois de 30 minutos. Os resultados, contudo, não excluem a participação dos glicocorticóides sobre a consolidação da memória através de receptores esteróides clássicos, afinal, a injeção de dexametasona 30 minutos antes do treino produziu efeito facilitatório, apesar não ter sido revertido pelo AM251, sugerindo mecanismos independentes que atuam em paralelo.

Em todos os casos estudados até agora, o aumento de glicocorticóides era sistêmico. Para testar se os efeitos dos glicocorticóides eram sobre o hipocampo, fizemos um experimento administrando dexametasona diretamente nesta estrutura, utilizando o

protocolo com choque fraco. Se o efeito dos glicocorticóides fosse no hipocampo, a infusão de dexametasona facilitaria a consolidação da memória.

Os resultados mostraram, porém, que a dexametasona infundida no hipocampo não produziu qualquer efeito sobre a memória, sugerindo que a atuação dos glicocorticóides sobre os endocanabinóides no hipocampo seja indireta. Especulamos que o hipocampo receba aferências de outras estruturas encefálicas que são sensíveis aos glicocorticóides, possivelmente a amígdala. De fato, essa interação entre a amígdala basolateral e o hipocampo dorsal já foi demonstrada por Roozendaal e McGaugh, onde a lesão na BLA reverteu os efeitos modulatórios de glicocorticóides no hipocampo, tanto na consolidação como na evocação da memória (Roozendaal e McGaugh 1997).

Os experimentos realizados nesse trabalho mostraram que o sistema endocanabinóide hipocampal requer um grau mínimo de conteúdo emocional/aversividade para participar efetivamente do processo de formação da memória. Alternativamente a um “treino forte”, uma sessão de estresse ou a mera administração de glicocorticóide exógeno parece ser capaz de recrutar o sistema endocanabinóide para a modulação da memória. Apesar da liberação de endocanabinóides ocorrer no hipocampo, a ação do glicocorticóide parece ser indireta, estimulando a síntese/liberação de anandamida e 2-AG através aferências hipocampais.

6. CONCLUSÕES

6.1 Participação dos receptores CB1 na reconsolidação e extinção da memória:

No primeiro capítulo, verificou-se a participação do sistema endocanabinóide hipocampal sobre os processos de *reconsolidação* e de *extinção* de memórias aversivas empregando o modelo do condicionamento aversivo contextual em ratos Wistar adultos. Abaixo estão as conclusões:

- a) A administração bilateral de **AM251**, antagonista seletivo dos receptores canabinóides CB1, imediatamente após a reativação da memória, **facilita a reconsolidação** da memória na tarefa do condicionamento aversivo contextual em ratos;
- b) A administração bilateral de **anandamida**, agonista endógeno dos receptores canabinóides CB1, imediatamente após a reativação da memória, **inibe a reconsolidação** da memória na tarefa do condicionamento aversivo contextual em ratos;
- c) A administração bilateral de **AM251**, antagonista seletivo dos receptores canabinóides CB1, imediatamente após a sessão de extinção, **inibe a consolidação da extinção** da memória na tarefa do condicionamento aversivo contextual em ratos;
- d) A administração bilateral de **anandamida**, agonista endógeno dos receptores canabinóides CB1, imediatamente após a sessão de extinção, **facilita a consolidação da extinção** da memória na tarefa do condicionamento aversivo contextual em ratos;

e) A administração bilateral de uma **dose sub-efetiva de AM251**, antagonista seletivo dos receptores canabinóides CB1, imediatamente após a reativação da memória, **reverte os efeitos da anandamida sobre a reconsolidação** da memória, demonstrando que esses efeitos são mediados pelos receptores CB1.

f) A administração bilateral de uma **dose sub-efetiva de AM251**, antagonista seletivo dos receptores canabinóides CB1, imediatamente após a sessão de extinção, **reverte os efeitos da anandamida sobre a extinção** da memória, demonstrando que esses efeitos são mediados pelos receptores CB1.

6.2 Interação entre o sistema endocanabinóide e glicocorticóide no hipocampo

No segundo capítulo, verificou-se a interação entre o sistema endocanabinóide e glicocorticóide no hipocampo sobre a modulação da consolidação da memória no modelo do condicionamento aversivo contextual em ratos Wistar adultos. Abaixo estão as conclusões:

a) A infusão intra-hipocampal de AM251, antagonista seletivo dos receptores CB1, prejudicou a consolidação da memória no protocolo de alta aversividade (choques de 0,7mA), mas não no protocolo de aversividade moderada (com choques de 0,3mA) no modelo do condicionamento aversivo contextual, demonstrando que o **sistema endocanabinóide é recrutado durante um aprendizado que envolva um grau mínimo de conteúdo emocional e/ou aversividade;**

b) A infusão intra-hipocampal de AM251, antagonista seletivo dos receptores CB1, prejudicou a consolidação da memória no protocolo de aversividade moderada (com choques de 0,3mA) no modelo do condicionamento aversivo contextual, quando um evento estressor era apresentado imediatamente antes do treino, demonstrando que **o estresse é capaz de recrutar o sistema endocanabinóide para a modulação da memória;**

c) A infusão intra-hipocampal de AM251, antagonista seletivo dos receptores CB1, prejudicou a consolidação da memória no protocolo de aversividade moderada (com choques de 0,3mA) no modelo do condicionamento aversivo contextual, quando o glicocorticóide sintético dexametasona é injetado sistemicamente logo antes do treino,

demonstrando que **os glicocorticóides são capazes de recrutar o sistema endocanabinóide para a modulação da memória;**

d) A infusão de dexametasona, um glicocorticóide sintético, no hipocampo não mimetizou os efeitos do estresse ou da injeção sistêmica da mesma sobre a consolidação da memória no protocolo de aversividade moderada (com choques de 0,3mA) no modelo do condicionamento aversivo contextual, sugerindo que **a modulação dos glicocorticóides no hipocampo ocorrem de forma indireta.**

7. REFERÊNCIAS BIBLIOGRÁFICAS

Ameri A. (1999) The Effects Of Cannabinoids On The Brain. *Prog Neurobiol* 58: 315-48

Arenos JD, Musty RE, Bucci DJ (2006) Blockade of cannabinoid CB1 receptors alters contextual learning and memory. *Eur J Pharmacol* 539:177-183.

Bisogno T, Ligresti A, Di Marzo V. (2005) The endocannabinoid signalling system: biochemical aspects *Pharmacol Biochem Behav.* 81(2):224-38

Brusco A, Tagliaferro PA, Saez T, Onaivi ES. (2008) Ultrastructural localization of neuronal brain CB2 cannabinoid receptors. *Ann N Y Acad Sci.*1139:450-7.

Bucherelli C, Baldi E, Mariottini C, Passani MB, Blandina P. (2006) Aversive memory reactivation engages in the amygdala only some neurotransmitters involved in consolidation. *Learn Mem.*13(4):426-30.

Campolongo P, Roozendaal B, Trezza V, Hauer D, Schelling G, McGaugh JL, Cuomo V.(2009) Endocannabinoids in the rat basolateral amygdala enhance memory consolidation and enable glucocorticoid modulation of memory. *Proc Natl Acad Sci U S A.*106(12):4888-93.

Castellano C, Cabib S, Palmisano A, Di Marzo V, Puglisi-Allegra S. (1997). The Effects Of Anandamide On Memory Consolidation In Mice Involve Both D1 And D2 Dopamine Receptors. *Behavioral Pharmacology.* 8: 707–712.

Castellano C, Ventura R, Caibb S, Puglisi-Allegra S. (1999).Strain-Dependent Effects Of Anandamide On Memory Consolidation In Mice Are Antagonized By Naltrexone. *Behavioral Pharmacology,* 10:453–457.

Chaperon F & Thiebot MH. (1999) Behavioral Effects Of Cannabinoid Agents In Animals. *Crit Rev Neurobiol.* 13(3):243-81.

- Childers SR, Breivogel CS. (1998) Cannabis and endogenous cannabinoid systems. Drug and alcohol dependence. 51, 173-87.
- Da Silva GE, Takahashi, RN. (2002) SR 141716A Prevents Delta 9-Tetrahydrocannabinol-Induced Spatial Learning Deficit In A Morris-Type Water Maze In Mice. Prog Neuropsychopharmacol Biol Psychiatry 26(2):321-5
- Da Silva WC, Bonini JS, Bevilaqua LR, Medina JH, Izquierdo I, Cammarota M. (2008) Inhibition of mRNA synthesis in the hippocampus impairs consolidation and reconsolidation of spatial memory. Hippocampus 18 (1):29-39.
- Davies SN, Pertwee RG, Riedel G. (2002) Functions Of Cannabinoid Receptors In The Hippocampus. Neuropharmacology, 42: 993-1007.
- De Oliveira Alvares L, de Oliveira LF, Camboim C, Diehl F, Genro BP, LANZIOTTI VB, Quillfeldt JA. (2005) Amnesic effect of intrahippocampal AM251, a CB1-selective blocker, in the inhibitory avoidance, but not in the open field habituation task, in rats. Neurobiol Learn Mem 83:119-124.
- De Oliveira Alvares L, de Oliveira LF, Camboim C, Diehl F, Genro BP, LANZIOTTI VB, Quillfeldt JA. (2008) Differential role of the hippocampal endocannabinoid system upon memory consolidation and retrieval in the inhibitory avoidance task . Neurobiol Learn Mem.90(1):1-9
- De Oliveira Alvares L, Genro BP, Breda RV, Pedroso MF, Da Costa JC, Quillfeldt JA. (2006) AM251, a selective antagonist of the CB1 receptor, inhibits the induction of long-term potentiation and induces retrograde amnesia in rats. Brain Res 1075:60-67.
- De Quervain DJ, Roozendaal B, McGaugh JL. (1998) Stress and glucocorticoids impair retrieval of long-term spatial memory. Nature 394(6695):787-90.
- Devane WA, Dysarz FA 3rd, Johnson MR, Melvin LS, Howlett AC. (1988) Determination and characterization of a cannabinoid receptor in rat brain. Mol Pharmacol. 34(5):605-13.

- Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A & Mechoulam R. (1992) Isolation And Structure Of A Brain Constituent That Binds To The Cannabinoid Receptor. *Science*. 258(5090):1946-9
- Di Marzo V, De Petrocellis L, Bisogno T. (2005) The biosynthesis, fate and pharmacological properties of endocannabinoids. *Handb Exp Pharmacol*. (168):147-85.
- Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz JC, Piomelli D. (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature*. 372(6507):686-91.
- Di S, Malcher-Lopes R, Marcheselli VL, Bazan NG, Tasker JG. (2005) Rapid glucocorticoid-mediated endocannabinoid release and opposing regulation of glutamate and gamma-aminobutyric acid inputs to hypothalamic magnocellular neurons. *Endocrinology* 146:4292–4301.
- Diehl F, Furstenuau LO, Sanchez G, Camboim C, de Oliveira Alvares L, Lanziotti VB, Cervenansky C, Kornisiuk E, Jerusalinky D, Quillfeldt JA. (2007) Facilitatory effect of the intra-hippocampal pre-test administration of MT3 in the inhibitory avoidance task. *Behav Brain Res*. 177(2):227-31.
- Duncan CP. (1949) The retroactive effect of electroshock on learning. *J Comp Physiol Psychol*. 42(1):32-44.
- Elphick, MR, Egertová, M. (2001) The neurobiology and evolution of cannabinoid signalling. *Phil. Trans. R. Soc. Lond. B*. 356, 381-408.
- Ferrari F, A Ottani, R Vivoli D. (1999) Learning Impairment Produced In Rats By The Cannabinoid Agonist HU210 In A Water-Maze Task. *Pharmacology Biochemistry And Behavior* 64, 555–561.
- Gaoni Y, Mechoulam R.(1964) Isolation, Structure And Partial Synthesis Of Na Constituent Of Haxish. *J. Am. Chem. Soc*.86, 1647-47

- Goparaju SK, Ueda N, Taniguchi K, Yamamoto S. (1999) Enzymes of porcine brain hydrolyzing 2-arachidonoylglycerol, an endogenous ligand of cannabinoid receptors. *Biochem Pharmacol.* 57(4):417-23.
- Hampson AJ, Bornheim LM, Scanziani M, Yost CS, Gray AT, Hansen BM, Leonoudakis DJ, Bickler PE. (1998) Dual Effect Of Anandamide On NMDA Receptors-Mediated Responses And Neurotransmission. *J Neurochem* 70: 671-676.
- Hampson, RE. Deadwyler, SA. (1999) Cannabinoids, Hippocampal Function And Memory. *Life Sciences* 65, 715–723.
- Hampson, RE. Deadwyler, SA. (2000) Cannabinoids Reveal The Necessity Of Hippocampal Neural Encoding For Short-Term Memory In Rats. *Journal Of Neuroscience* 20, 8932–8942
- Hanus L, Abu-Lafi S, Fride E, Breuer A, Vogel Z, Shalev DE, Kustanovich I, Mechoulam R. (2001) 2-arachidonyl glyceryl ether, an endogenous agonist of the cannabinoid CB1 receptor. *Proc Natl Acad Sci U S A.* 98(7):3662-5.
- Herkenham M, Lynn AB, Johnson MR, Melvin LS, de Costa BR, Rice KC. (1991). Characterization and localization of cannabinoid receptors in the rat brain: a quantitative in vitro autoradiographic study. *J Neurosci* 11:563-583.
- Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, de Costa BR, Rice KC. (1990) Cannabinoid receptor localization in brain. *Proc Natl Acad Sci U S A.* 87(5):1932-6.
- Hillard CJ, Harris RA, Bloom AS. (1985) Effects of the cannabinoids on physical properties of brain membranes and phospholipid vesicles: fluorescence studies. *J Pharmacol Exp Ther.* 232(3):579-88.
- Hohmann AG, Suplita RL, Bolton NM, Neely MH, Fegley D, Mangieri R, Krey JF, Walker JM, Holmes PV, Crystal JD, Duranti A, Tontini A, Mor M, Tarzia G, Piomelli D.

- (2005). An endocannabinoid mechanism for stress-induced analgesia. *Nature* 435(7045):1108-12
- Hölter SM, Kallnik M, Wurst W, Marsicano G, Lutz B, Wotjak CT. (2005) Cannabinoid CB1 receptor is dispensable for memory extinction in an appetitively-motivated learning task. *Eur J Pharmacol.* 510(1-2):69-74.
- Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R, Pertwee RG. (2002) International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Ver.*54(2):161-202.
- Howlett AC. (1984) Inhibition of neuroblastoma adenylate cyclase by cannabinoid and nantadol compounds. *Life Sci.* 35(17):1803-10.
- Iversen L. (2001) *Marihuana, Conocimiento Científico Actual.* Ariel.
- Izquierdo I. (2002) *Memória.* Editora Artmed. Porto Alegre. 95p.
- Jentsch, JD, Andrusiak E, Tran A, Bowers MB, Roth RH.(1997) Δ 9-Tetrahydrocannabinol Increases Prefrontal Cortical Catecholaminergic Utilization And Impairs Spatial Working Memory In The Rat: Blockade Of Dopaminergic Effects By HA966. *Neuropsychopharmacology* 16, 426–432.
- Katona I, Sperlagh B, Magloczky Z, Santha E, Kofalvi A, Czirjak S, Mackie K, Vizi ES & Freund TF. (2000) Gabaergic Interneurons Are The Targets Of Cannabinoid Actions In The Human Hippocampus. *Neuroscience.* 100(4):797-804.
- Katona I, Sperlagh B, Sik A, Kafalvi A, Vizi ES, Mackie K, Freund TF. (1999) Presynaptically Located CB1 Cannabinoid Receptors Regulate GABA Release From Axonal Terminals Of Specific Hippocampal Interneurons. *J Neurosci* 19: 4544–58.

- Katona I, Urban GM, Wallace M, Ledent C, Jung KM, Piomelli D, Mackie K, Freund TF. (2006) Molecular composition of the endocannabinoid system at glutamatergic synapses. *J Neurosci.* 26(21):5628-37.
- Kawamura Y, Fukaya M, Maejima T, Yoshida T, Miura E, Watanabe M, Ohno-Shosaku T, Kano M. (2006) The CB1 cannabinoid receptor is the major cannabinoid receptor at excitatory presynaptic sites in the hippocampus and cerebellum. *J Neurosci* 26:2991-3001.
- Kobilo T, Hazvi S, Dudai Y. (2007) Role of cortical cannabinoid CB1 receptor in conditioned taste aversion memory. *Eur J Neurosci.* (11):3417-21.
- Lee JL, Milton AL, Everitt BJ. (2006). Reconsolidation and extinction of conditioned fear: inhibition and potentiation. *J Neurosci.* 26(39):10051-6.
- Lichtman AH, Dimen KR, Martin BR. (1995) Systemic Or Intrahippocampal Cannabinoid Administration Impairs Spatial Memory In Rats. *Psychopharm* 119: 282–90.
- Lichtman AH, Martin BR. (1996) Δ 9-Tetrahydrocannabinol Impairs Spatial Memory Through Cannabinoid Receptor Mechanism. *Psychopharmacology* 126,125–131.
- Lichtman AH. (2000) SR141716A Enhances Spatial Memory Assessed In A Radial-Arm Maze Task In Rats. *European Journal Of Pharmacology* 404, 175–179.
- Lin HC, Mao SC, Gean PW. (2006) Effects of intra-amygdala infusion of CB1 receptor agonists on the reconsolidation of fear-potentiated startle. *Learn Mem.*(3):316-21.
- Mac Donalds CJ, Vaughan CW. (2001) Cannabinoids act backwards. *Nature.* 410, 527-30.
- Mallet RJ, Beninger S. (1996) The Endogenous Cannabinoid Receptor Agonist Impairs Memory In Rats. *Behavioral Pharmacology* 7, 276–284.
- Marsicano G, Wotjak CT, Azad SC, Bisogno T, Rammes G, Cascio MG, Hermann H, Tang J, Hofmann C, Zieglgänsberger W, Di Marzo V, Lutz B. (2002) The Endogenous Cannabinoid System Controls Extinction Of Aversive Memory. *Nature* 418,:530-34.

- Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI. (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature*. 346(6284):561-4.
- McGaugh JL. (2000) Memory – a century of consolidation. *Science*. 287, 248-51.
- McGaugh, JL. (1966) Time-dependent processes in memory storage. *Science*. 153, 1351-1358.
- Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S, Martin BR, Compton DR. (1995) Identification Of An Endogenous 2-Monoglyceride Present In Canine Gut, That Binds To Cannabinoid Receptors. *Biochem Pharmacol*. 50(1):83-90.
- Mechoulam R., Fride E., Di Marzo V.(1998) Endocannabinoids. *Eur. J. Pharmacol*.359:1-18.
- Misanin JR, Miller RR, Lewis DJ. (1968) Retrograde amnesia produced by electroconvulsive shock after reactivation of a consolidated memory trace. *Science*.160(827):554-5
- Molina-Holgado F, Gonzalez MI, Leret ML. (1995) Effect Of Δ^9 -Tetrahydrocannabinol On Short-Term Memory In The Rat. *Physiology And Behavior* 57: 177–179.
- Moore FL, Orchinik M. (1994) Membrane receptors for corticosterone: a mechanism for rapid behavioral responses in an amphibian. *Horm Behav*. 28(4):512-9.
- Muller GE, Pilzecker A. (1900). Experimentelle Bietrage zur Lehre vom Gedachtnis. *Z. Ergänzungsband*. 1, 1-300
- Murillo-Rodriguez E, Sanchez-Alvarez M, Navarro L, Martinez-Gonzaluz D, Drucker-Colin R, Prospero-Garcia O. (1998) Anandamide Modulates Sleep And Memory In Rats. *Brain Research* 812: 270–274.

- Nader K, Schafe GE, Le Doux JE. (2000) Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. *Nature* 406(6797):722-726.
- Niyuhire F, Varvel SA, Thorpe AJ, Stokes RJ, Wiley JL, Lichtman AH. (2007) The disruptive effects of the CB1 receptor antagonist rimonabant on extinction learning in mice are task-specific. *Psychopharmacology (Berl)*191(2):223-31.
- Okuda S, Roozendaal B, McGaugh JL. (2004) Glucocorticoid effects on object recognition memory require training-associated emotional arousal. *Proc Natl Acad Sci U S A*.101(3):853-8.
- Pamplona FA, Prediger RD, Pandolfo P, Takahashi RN. (2006) The cannabinoid receptor agonist WIN 55212-2 facilitates the extinction of contextual fear memory and spatial memory in rats. *Psychopharmacology (Berl)*.188(4):641-9.
- Pamplona FA, Takahashi RN. (2006) WIN 55212-2 impairs contextual fear conditioning through the activation of CB1 cannabinoid receptors. *Neurosci Lett* 397:88-92.
- Porter AC, Sauer JM, Knierman MD, Becker GW, Berna MJ, Bao J, Nomikos GG, Carter P, Bymaster FP, Leese AB, Felder CC. (2002) Characterization of a novel endocannabinoid, virodhamine, with antagonist activity at the CB1 receptor. *J Pharmacol Exp Ther*. 301(3):1020-4.
- Quirarte GL, Roozendaal B, McGaugh JL. (1997) Glucocorticoid enhancement of memory storage involves noradrenergic activation in the basolateral amygdala. *Proc Natl Acad Sci U S A*. 94(25):14048-53.
- Roozendaal B, McGaugh JL. (1997) Basolateral amygdala lesions block the memory-enhancing effect of glucocorticoid administration in the dorsal hippocampus of rats. *Eur J Neurosci*. 9(1):76-83.
- Roozendaal B, Okuda S, Van der Zee EA, McGaugh JL. (2006) Glucocorticoid enhancement of memory requires arousal-induced noradrenergic activation in the basolateral amygdala. *Proc Natl Acad Sci U S A*. 103(17):6741-6.

- Rooszendaal B. (2002) Stress and memory: opposing effects of glucocorticoids on memory consolidation and memory retrieval. *Neurobiol Learn Mem.* 78(3):578-95.
- Sara SJ. (2000). Retrieval and reconsolidation: toward a neurobiology of remembering. *Learn Mem.*(2):73-84.
- Stiglick A, Kalant H. (1982) Learning Impairment In The Radial-Arm Maze Following Prolonged Cannabis Treatment In Rats. *Psychopharmacology* 77:117–123.
- Stiglick A, Llewelly ME, Kalant H. (1984) Residual Effects Of Prolonged Cannabis Treatment On Shuttle-Box Avoidance In The Rat. *Psychopharmacology* 84: 476–479.
- Suzuki A, Josselyn SA, Frankland PW, Masushige S, Silva AJ, Kida S. (2004) Memory reconsolidation and extinction have distinct temporal and biochemical signatures. *J Neurosci.* 24(20):4787-4795.
- Suzuki A, Mukawa T, Tsukagoshi A, Frankland PW, Kida S. (2008) Activation of LVGCCs and CB1 receptors required for destabilization of reactivated contextual fear memories. *Learn Mem.* 15(6):426-33.
- Takahashi KA, Castillo PE. (2006) The CB1 cannabinoid receptor mediates glutamatergic synaptic suppression in the hippocampus. *Neuroscience* 139:795-802.
- Tsou K, Mackie K, Sanudo-Pena MC, Walker JM. (1999) Cannabinoid CB1 receptors are localized primarily on cholecystokinin-containing GABAergic interneurons in the rat hippocampal formation. *Neuroscience* 93:969-975.
- Van Sickle MD, Duncan M, Kingsley PJ, Mouihate A, Urbani P, Mackie K, Stella N, Makriyannis A, Piomelli D, Davison JS, Marnett LJ, Di Marzo V, Pittman QJ, Patel KD, Sharkey KA. (2005) Identification and functional characterization of brainstem cannabinoid CB2 receptors. *Science.* 310(5746):329-32.

Varvel SA, Wise LE, Niyuhire F, Cravatt BF, Lichtman AH. (2006) Inhibition of Fatty-Acid Amide Hydrolase Accelerates Acquisition and Extinction Rates in a Spatial Memory Task. *Neuropsychopharmacology*. 32(5):1032-41

Wilson RI, Nicoll RA (2002). Endocannabinoid Signaling In The Brain. *Science* 296: 678-82.

ANEXO I

Realizei o estagio de doutorado no exterior (sanduíche) no laboratório do Dr. Karim Nader, na universidade McGill, Montreal, Qubec, Canadá, de maio de 2008 até fevereiro de 2009. Essa experiência foi extremamente proveitosa para meu desenvolvimento científico (e pessoal). O Dr. Karim Nader é o principal pesquisador sobre o tema da reconsolidação da memória em atividade, foi o que me motivou a procurá-lo para estagiar em seu laboratório.

Nas próximas páginas dessa tese, está o artigo “Cellular and systems mechanisms of memory strength as a constraint on auditory fear reconsolidation”, fruto do trabalho realizado durante o doutorado sanduiche publicado na revista Nature Neuroscience (2009)12(7):905-12.

Cellular and systems mechanisms of memory strength as a constraint on auditory fear reconsolidation

Szu-Han Wang, Lucas de Oliveira Alvares & Karim Nader

Memory reconsolidation has been demonstrated in various tasks and species, suggesting it is a fundamental process. However, there are experimental parameters that can inhibit reconsolidation from occurring (boundary conditions). These conditions and their mechanisms remain poorly defined. Here, we characterize the ability of strong training to inhibit reconsolidation at the behavioral, systems and molecular levels. We demonstrate that strong memories in rats initially are resistant to reconsolidation, but after sufficient time will undergo reconsolidation, suggesting that boundary conditions can be transient. At the systems level, we show that the hippocampus is necessary for inhibiting reconsolidation in the amygdala. At the molecular level, we demonstrate that NR2B NMDA-receptor subunits which are critical for the induction of reconsolidation of auditory memories in the amygdala, are downregulated only under conditions when strong memories do not undergo reconsolidation. This suggests that one molecular mechanism for mediating boundary conditions is through downregulation of reconsolidation induction mechanisms.

Memories not only undergo a time-dependent process of stabilization after the initial learning, which is called consolidation¹; they can also undergo another restabilization process after reactivation that typically entails presentation of a training-related stimulus to call up the memory^{2,3}, which is now called reconsolidation^{4–7}. The memory process induced by reactivation of consolidated memories meets the standards of being a consolidation process⁸. There have been many demonstrations of reconsolidation across species, tasks and amnesic agents, suggesting that it is a fundamental process. However, reconsolidation is not ubiquitous. There are experimental conditions under which reconsolidation does not seem to occur. These conditions, which we define as boundary conditions on reconsolidation, have recently drawn research attention.

A number of boundary conditions have been suggested, such as trace dominance between cues that no longer predict the occurrence of a reinforcement (extinction) and reconsolidation^{9–11}, memory age^{12,13}, directly versus indirectly activated memories¹⁴ and training strength¹². However, comprehensive descriptions of the behavioral conditions and the mechanisms for inhibiting reconsolidation under specific parameters are limited. Further, for each condition—extinction^{15,16}, strength of training^{17,18} and age¹⁹—there are contradictory findings (for review see ref. 8).

One source probably contributing to the observed inconsistencies is that the typical logic used to conclude that a boundary condition exists is through challenging a memory's sensitivity to post-activation amnesic agents under one set of experimental parameters. If memory disruption is not observed, then it is concluded that the memory does not undergo reconsolidation under those conditions. Several reports, however, have demonstrated that a memory may undergo reconsolidation only under specific reactivation conditions^{12,20,21}. The implication

of these findings is that it is extremely difficult to conclude on the basis of behavioral studies that a memory never undergoes reconsolidation. Do the negative effects upon which the boundary conditions are based imply that a given memory never undergoes reconsolidation, or is the memory still capable of undergoing reconsolidation with another reactivation protocol? Given that the parameter space of possible reactivation procedures is essentially infinite, a real boundary condition is very difficult to prove at the behavioral level. This is likely to be part of why there is so much inconsistency in the field of boundary conditions⁸.

Here we have taken a complementary approach to identify some of the molecular mechanisms by which boundary conditions inhibit reconsolidation from occurring. If a molecular or conceptual definition of how they are manifested in the brain could be identified, then we could make strong predictions concerning when we should see these molecular mechanisms expressed. For example, if strong memories represent real boundary conditions, then the putative mechanisms used to inhibit reconsolidation from occurring should only be expressed after strong but not weak training. This strategy would significantly complement the behavioral studies described above in their search for true boundary conditions and help resolve some of the conflicting findings in the field.

An understanding of how boundary conditions are mediated across levels of analysis is critical because targeting reconsolidation of traumatic memories has been proposed to be a potential treatment for many psychopathologies, including post-traumatic stress disorder (PTSD)^{19,22}. For PTSD, blocking the reconsolidation of traumatic memories might weaken the long-term maintenance of these traumatic memories, in turn reducing PTSD pathology. However, if strong aversive experiences act as a boundary condition on reconsolidation¹²,

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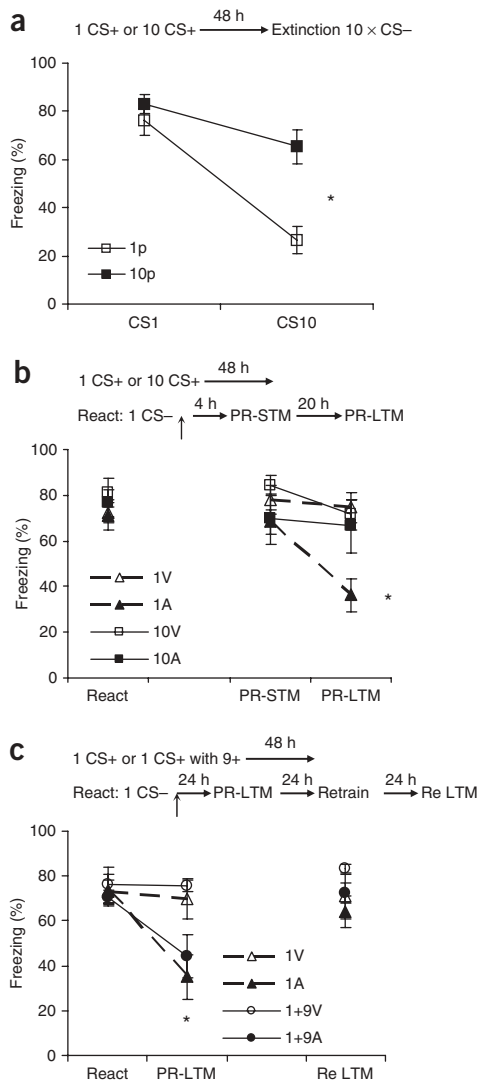


Figure 1 Strong auditory fear memories are insensitive to anisomycin 2 d after training. **(a)** Top: behavioral protocol. Separate groups of rats received one or ten tone–shock pairings (1P/1CS+ or 10P/10CS+). Two days after training they received extinction sessions (CS–). At the end of the extinction session, rats trained with 10P had significantly more freezing. Freezing percentage was defined as percentage of time during tests that the animal stayed immobile except for breathing. **(b)** Top: behavioral protocol. LBA-cannulated rats received either 1P or 10P. Two days after training, the memory was reactivated (React) with one unreinforced tone (1CS–) immediately followed by intra-LBA infusions (vertical arrow) of vehicle (V) or anisomycin (A). PR-STM and PR-LTM tests were done at 4 h and 24 h after reactivation, respectively. All groups (1V, 1A, 10V, $N = 7$ per group and 10A, $N = 8$) froze similarly during reactivation and PR-STM. Although anisomycin blocked PR-LTM in the 1P group (1V > 1A), it, however, did not impair PR-LTM in the 10P group (10V \approx 10A). **(c)** Top: behavioral protocol. Rats received either 1P or 1P followed by nine unsignaled footshocks (1 CS+ with 9+). Post-reactivation anisomycin infusion blocked both groups' PR-LTM compared to vehicle controls (1V and 1+9V > 1A and 1+9A; $n = 6, 7, 6, 8$, respectively). When rats were retrained, they were capable of maintaining the retrained long-term memory (Re LTM). * $P < 0.05$. Means \pm s.e.m.

RESULTS

Recent strong memories do not undergo reconsolidation

We first determined whether the strength of auditory fear memories acquired with ten tone–shock pairings (10P) was stronger than with one pairing (1P). Separate groups of rats were conditioned with either 1P or 10P and then received multiple extinction trials in a single session (see Online Methods). Extinction reduced freezing significantly more in the 1P group than in the 10P group (group by trial interaction $F_{1,6} = 9.28$, $P < 0.05$; **Fig. 1a**). This indicates that the 10P memory was stronger than the 1P memory.

To test whether these stronger memories underwent reconsolidation, 2 d after conditioning the auditory fear memory was reactivated in a context different from the context used for training and followed by intra-LBA infusion of anisomycin or its vehicle. Post-reactivation short-term memory (PR-STM) and post-reactivation long-term memory (PR-LTM) tests, assayed by conditioned freezing²⁵, were given 4 and 24 h later, respectively. The results showed that the strong memory was not sensitive to anisomycin challenge (**Fig. 1b**). A two-way analysis of variance (ANOVA) on reactivation performance showed no significant training effect (1P versus 10P), drug effect (vehicle versus anisomycin), or training by drug interaction (all $F_{1,25} < 1.5$, $P > 0.25$). A three-way, one-repeated ANOVA comparing training, drug and test (PR-STM versus PR-LTM, repeated measure) showed a significant three-way interaction ($F_{1,25} = 7.68$, $P = 0.01$). Further analyses revealed that all groups had comparable PR-STM scores (all $F_{1,25} < 2.5$, $P > 0.1$). At PR-LTM, however, only the 1P-anisomycin group showed significantly impaired performance compared to the other groups (*post hoc* tests, all $P < 0.02$) which did not differ from each other (all $P > 0.4$). These data are consistent with the possibility that strong training either inhibited the memory from undergoing reconsolidation or made it more difficult for auditory fear memories to undergo reconsolidation 2 d after training¹². The negative finding in the 10P group demonstrates that anisomycin infusion did not induce damage that was sufficient to compromise behavioral functions²⁶.

The boundary condition is due to increased associative strength

We asked whether the inability of 10P memories to undergo reconsolidation was due to the learning or to some non-associative factor caused by multiple footshocks. We trained two groups of rats with a single tone–footshock pairing followed by nine un-signaled, unpaired footshocks (that is, 1+9UP). Two more groups were trained with 1P

then this would suggest that the traumatic memories in PTSD patients may be resistant to undergoing reconsolidation, negating reconsolidation as a therapeutic target. Therefore, it is critical to determine what the optimal conditions are to allow an extremely strong fear memory to undergo reconsolidation.

To this end, we show that strong auditory fear memories initially did not undergo reconsolidation but did over time, suggesting that the boundary condition induced by strong training is transient. The time course resembled the time course over which contextual fear memories are thought to be transformed from a hippocampus-dependent to hippocampus-independent memory²³. We hypothesized and found that the hippocampus inhibited the auditory fear memory from undergoing reconsolidation in the lateral and basal amygdala (LBA). On the basis of our previous findings²⁴, we hypothesized that one principle that could mediate boundary conditions is downregulation of the mechanisms that allow memories to undergo reconsolidation. Using two complementary methods, we demonstrated that NR2B expression in the LBA, which is critical for the induction of fear reconsolidation but not the expression of fear²⁴, was reduced under conditions when memories did not undergo reconsolidation and was normal when memories underwent reconsolidation.

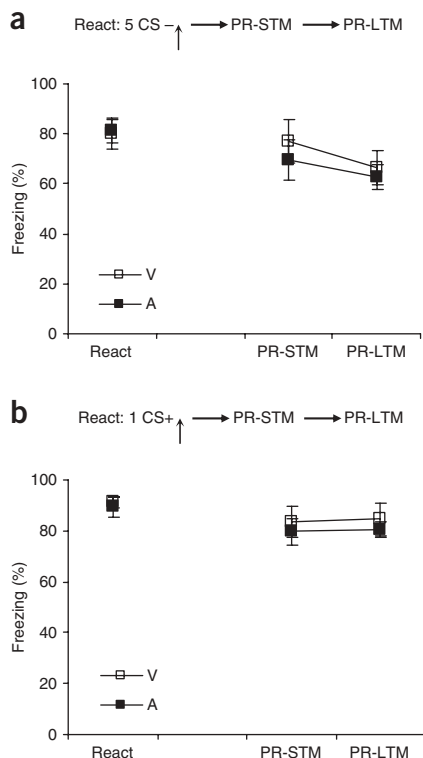


Figure 2 Alternative reactivation protocols are not sufficient to make the strong auditory fear memories sensitive to anisomycin. Top subpanel of each panel represents the behavioral protocol, which is similar to **Figure 1b** except for the reactivation procedure. All LBA-cannulated rats received ten tone-footshock pairings. The memory was reactivated 2 d later with five unreinforced tone presentations (5CS-, $n = 7$ per group) (**a**) or 1 tone-footshock pairing (1CS+, $n = 6$ per group) (**b**). In both cases, post-reactivation anisomycin (A), compared to vehicle (V), infusion did not block post-reactivation short-term and long-term memory in the strongly trained rats. Means \pm s.e.m.

similar performance at reactivation (that is, no training effect, drug effect or training by drug interaction, all $F < 1$). An analysis of PR-LTM showed that both anisomycin groups were impaired compared to both vehicle groups ($F_{1,24} < 15.93$, $P < 0.001$). There was neither main effect of training nor an interaction between training and drug (both $F < 1$).

All rats were retrained with 1P. The long-term memory for this relearning was tested on the next day. All four groups showed similar long-term memory of relearning (that is, no effect of training history, drug history, or training by drug interaction, all $F_{1,24} < 2.9$, $P > 0.1$). This again demonstrates that memory impairment caused by anisomycin is unlikely due to LBA damage²⁶.

These results demonstrate that auditory fear memories formed after 1P followed by nine unsignaled footshocks is as labile as memories formed after 1P alone. We conclude that the resistance of 10P memories to undergoing reconsolidation is due to the stronger association (**Fig. 1c** versus **Fig. 1b**).

Other reactivation protocols do not induce reconsolidation

The above findings suggest that either strong memories do not undergo reconsolidation or it is harder to induce reconsolidation of strong memories. To partially address this, we asked whether reconsolidation of strong memories could be induced with other reactivation protocols. One reactivation protocol was to extend the tone presentation by giving five tone presentations without footshock¹². The second protocol used

and served as positive controls. If greater associative strength inhibits memories from undergoing reconsolidation, then the 1+9UP memory, sharing a similar associative strength with the 1P memory, should undergo reconsolidation. Results showed that the 1+9UP memory underwent reconsolidation (**Fig. 1c**). A three-way, one-repeated ANOVA comparing training, drug and test (reactivation versus PR-LTM, repeated measure) showed a significant drug by test interaction ($F_{1,24} = 11.98$, $P = 0.0002$). Further analyses showed all groups had

Figure 3 Strong memories undergo reconsolidation at 30 and 60 d, but not 7 d, after training. Top subpanel of each panel represents the behavioral protocol. (**a–c**) Separate groups of rats were LBA-cannulated and trained with ten tone-footshock pairings. The memory was reactivated at 7 (**a**), 30 (**b**) or 60 (**c**) days after training. Intra-LBA anisomycin infusion (vertical arrow) after memory reactivation with 1 tone (1CS-) impaired the PR-LTM only when the memory was reactivated at 30 and 60 d but not 7 d after training. In all cases, the reactivation was similar and PR-STM was intact in the anisomycin (A) rats compared to the vehicle (V) rats. (**d**) Strong memories undergo reconsolidation over time. For the purposes of comparison, the data in **Figures 3a–c** and **1b** were converted to a freezing ratio, $(\text{PR-LTM} - \text{PR-STM})/\text{PR-STM} \times 100\%$. Intra-LBA anisomycin infusion impaired the PR-LTM only when the strong memory was reactivated at 30 and 60 d after training. Each data point represents separate groups of rats (data for 2 d were adapted from **Figure 1b**; $n = 7$ per group for 7 d; $n = 5$ (10V), 7 (10A) for 30 d; $n = 8$ per group for 60 d). * $P < 0.05$. Means \pm s.e.m.

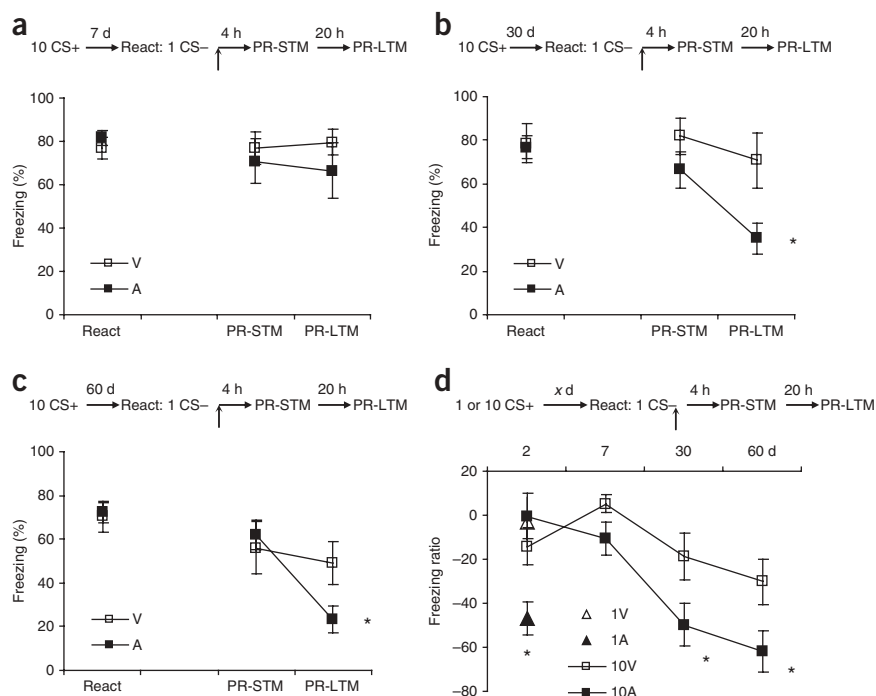


Figure 4 Pretraining dorsal hippocampus lesions cause strong fear memory to undergo reconsolidation in the LBA. Top: behavioral protocol. All rats received electrolytic dorsal hippocampus lesion when cannulae were implanted. After recovery from surgery, they received 10 tone-footshock pairings. (a) Post-reactivation anisomycin (A, $n = 7$) infusion in LBA did not block PR-STM but did impair PR-LTM compared to the vehicle infusion (V, $n = 7$). (b) When the memory reactivation was omitted before the drug infusion, the PNR-LTM was comparable in vehicle (V, $n = 6$) and anisomycin (A, $n = 4$) groups. The same rats further received memory reactivation and were divided in two subgroups in a counterbalanced manner ($n = 5$ per group for vehicle or anisomycin infusion). The group assignments yielded a comparable baseline (see the text). The PR-LTM then was impaired by intra-LBA anisomycin when the drug was contingent on the memory reactivation (React). (c) Rats received hippocampus or sham lesion ($n = 7$ per group) followed by 10 pairings and an extinction session (10CS-). The two groups showed comparable extinction rates. $*P < 0.05$. Means \pm s.e.m.

was a reinforced trial that has been shown to induce reconsolidation in the LBA²⁷. This should be a very strong reactivation because the LBA neurons mediating the memory will be reactivated by sensory input from both tone and footshock afferents to the LBA.

Results showed that neither reactivation protocol was sufficient to detect an anisomycin impairment (Fig. 2). When the reactivation trial contained five tone presentations, the PR-LTM was still normal in the anisomycin group ($F < 1$, Fig. 2a) as it was when another pairing was used to reactivate the memory ($F < 1$, Fig. 2b).

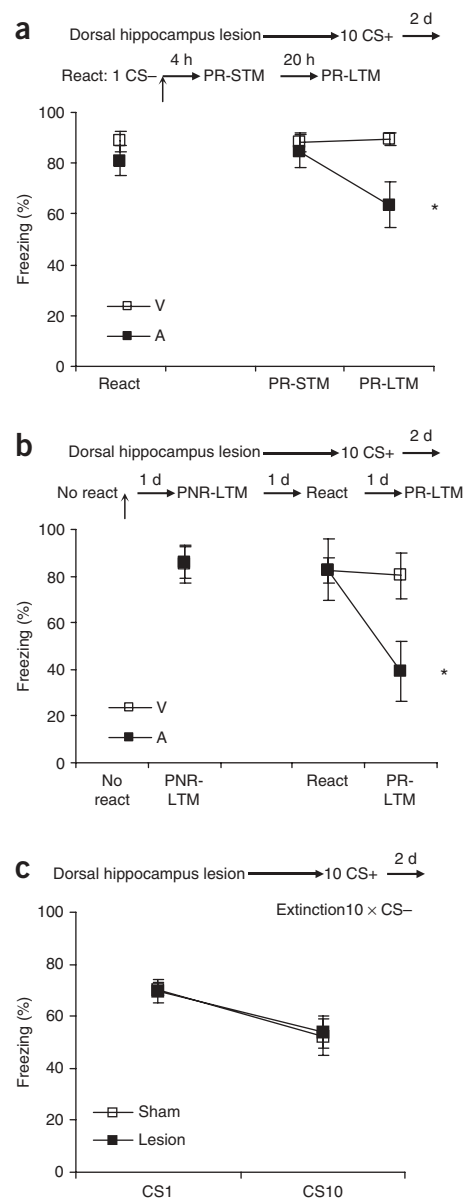
Strong training boundary condition is transient

In clinical settings, PTSD patients have experienced extremely aversive past events. Often years will have passed between the trauma and the opportunity for intervention (R. Pitman, personal communication). Therefore, we asked whether the time between training and memory reactivation would interact with a strong memory's inability to undergo reconsolidation. To this end, we increased the time between strong training and reactivation. When the strong memory was reactivated 7 d after training, post-reactivation anisomycin infusions did not impair PR-LTM ($F < 1$, Fig. 3a). However, when the memory was reactivated 30 or 60 d after training, anisomycin infusions induced a behavioral impairment at PR-LTM (30 d: $F_{1,10} = 7$, $P < 0.02$, Fig. 3b; 60 d: $F_{1,14} = 5.19$, $P < 0.04$, Fig. 3c) but not PR-STM. To summarize the relationship of the interval between training and reactivation and whether the strong memory undergoes reconsolidation, we standardized the behavioral results as a freezing ratio (Fig. 3d). Anisomycin did not induce any detectable impairment 2 or 7 d after training but did 30 and 60 d after training (a significant group by day interaction, $F_{3,49} = 2.83$, $P < 0.05$). *Post hoc* tests showed that, compared to day 2, the significant group differences only emerged at days 30 and 60, not day 7 ($P < 0.03$, $P < 0.01$ and $P > 0.5$, respectively).

The freezing percentage during reactivation at 2, 7, 30 or 60 d after strong training did not change significantly and did not differ between vehicle and anisomycin groups (both $F < 1$). Moreover, the freezing elicited by the test context before the onset of the tone is also consistent across days and between groups (both $F < 1$, Supplementary Fig. 1 online). This suggests that the age of a memory at the time of reactivation interacts with its strength and that this interaction determines the memory's susceptibility to reconsolidation.

Dorsal hippocampus is necessary for the boundary condition

If strong training indeed transiently inhibits a fear memory from undergoing reconsolidation, why would this time course resemble the time course of systems consolidation, in which the hippocampus has been proposed to play a time-limited role^{23,28–30}? Specifically, a lesion of the dorsal hippocampus 1 d after training impairs contextual,



but not auditory, fear conditioning. However, the same lesion has no effect on memory retention if made 28 d after training²³. Auditory fear conditioning usually also leads to contextual fear³¹. Therefore, it is possible that the strong contextual fear, acquired with strong training, would inhibit the ability of memories in the LBA to undergo reconsolidation for as long as the contextual memory is hippocampus dependent. Once the contextual memory is putatively hippocampus independent, this could allow the strong fear memory in the LBA to undergo reconsolidation. This inhibition could be mediated through the LBA's connections with the hippocampus^{32,33}. The ability of the hippocampus to modulate the amygdala's plasticity has been proposed³⁴.

To test whether the systems consolidation of contextual fear memory imposes the strong training boundary condition, we applied electrolytic dorsal hippocampus lesions to the rats before strong training. We chose this lesion method on the basis of its effect on causing temporally-graded amnesia of contextual, but not auditory, freezing^{17,23}. We predicted that if the dorsal hippocampus is critical for inhibiting new strong fear memories from undergoing

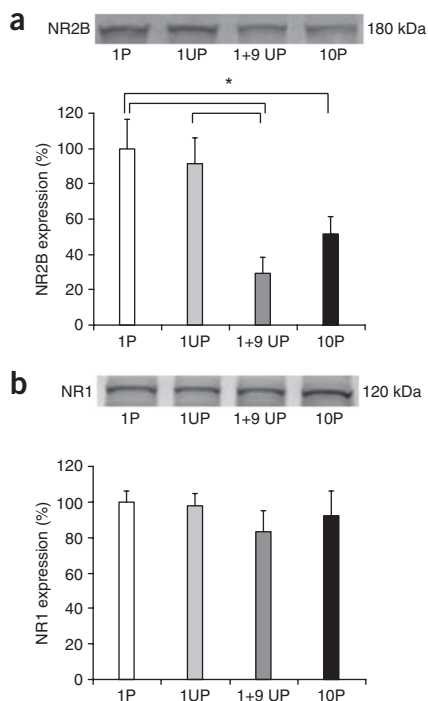


Figure 5 NR2B-subunit abundance is inversely related to the ability of strong memories to undergo reconsolidation over time. **(a)** Western blot and quantification of NR2B subunits in the LBA 2 d after training. Rats received one pairing (1P, $n = 7$), one unpaired tone and shock presentation (1UP, $n = 4$), ten pairings (10P, $n = 7$) or one pairing followed by nine (1+9UP, $n = 4$) unpaired foot shocks. Rats receiving 10P or 1+9UP showed less NR2B than rats receiving 1P or 1UP. **(b)** Western blot and quantification of NR1 subunits in the LBA 2 d after training. Rats received one pairing (1P, $n = 4$), one unpaired tone and shock presentation (1UP, $n = 4$), ten pairings (10P, $n = 4$) or one pairing followed by nine unpaired foot shocks (1+9UP, $n = 4$). No differences were found. $*P < 0.05$. Means \pm s.e.m.

reconsolidation, then strong memories should undergo reconsolidation 2 d after training in dorsal hippocampus-lesioned rats, a time when the memory does not undergo reconsolidation in intact rats. Dorsal hippocampus lesions did not impair auditory fear memory given comparable freezing at reactivation in unlesioned rats that received strong training (Fig. 4a versus Fig. 1b, $F_{1,25} < 1.1$, $P > 0.3$; Supplementary Figs. 2 and 3 online).

We found that strong memories in lesioned rats were sensitive to post-reactivation anisomycin infusions 2 d after training (Fig. 4a). Specifically, both anisomycin and vehicle groups had similar freezing percentages at reactivation and PR-STM test (both $F < 1$), whereas the anisomycin group froze significantly less during PR-LTM test than the vehicle group ($F_{1,12} = 6.95$, $P < 0.03$). This is in contrast to results in intact rats, in which the strong memory remained insensitive to anisomycin when it was reactivated 2 d after training.

We then performed the identical experiment in different rats but omitting memory reactivation. Anisomycin had no effect on the post-non-reactivation long-term memory (PNR-LTM) test (Fig. 4b, $F < 1$). These data demonstrate that reconsolidation of a strong memory 2 d after training occurs in rats with dorsal hippocampus lesions and is dependent on memory reactivation. We then used these rats, which did not receive a reactivation session, to replicate the reactivation-dependent reconsolidation. One day after the PNR-LTM test, these rats received a reactivation session and were infused with either vehicle or anisomycin in a counterbalanced manner (that is, the vehicle group consisted of equal numbers of animals that had received vehicle and anisomycin in the previous experiment, and vice versa). The group assignment showed comparable performance between groups (rats that were to receive vehicle versus those that were to receive anisomycin) in the previous PNR-LTM test ($F < 1$). The reactivation result showed that both groups had similar freezing to the conditioned tone ($F < 1$). However, a significant impairment of PR-LTM was observed in the anisomycin group ($F_{1,8} = 6.48$, $P < 0.04$). This replicates the previous experiment.

These findings suggest that the dorsal hippocampus actively inhibits strong memories from undergoing reconsolidation 2 d after training. However, if the absence of an anisomycin effect in normal rats is due to

a ceiling effect 2 d after training, perhaps the anisomycin sensitivity after lesioning might be due to the hippocampus lesions decreasing freezing to a range in which a putative anisomycin impairment could be behaviorally detected. To directly test this possibility, two groups of rats received either sham or electrolytic lesions of the dorsal hippocampus, followed by 10P and extinction. The lesion and the sham groups showed comparable extinction rates 2 d after strong training (Fig. 4c, group by trial interaction $F < 1$). This rules out a ceiling-effect interpretation of our findings.

The molecular mechanism for the boundary condition

What could be the molecular mechanism in the LBA that inhibits reconsolidation of strong memories for up to 30 d after training? Our group recently demonstrated that NMDA receptors containing NR2B subunits are necessary in transforming stable, consolidated memories into labile ones during reactivation²⁴. New strong memories show similar properties: normal expression of freezing during reactivation but insensitivity to post-reactivation anisomycin. We reasoned that strong training may downregulate NR2B expression in the LBA, thereby making the memory insensitive to post-reactivation anisomycin infusions but capable of being expressed normally.

The ability of robust fear conditioning to strongly affect NMDA receptor subtypes has already been described³⁵. An earlier finding used electrophysiological recordings to show that there is a postsynaptic decrease of NMDA receptors during the maintenance phase of strong fear conditioning. Furthermore, this study used western blots to show that protein expression in the amygdala of NR2B, but not total NR1 NMDA-receptor subunit, is reduced after strong fear conditioning³⁵. We measured NR2B in the LBA using quantitative western blots and immunohistochemistry (IHC) under conditions that prevent (2 d) or allow (60 d) reconsolidation to occur after training.

Four groups (1P, 1UP, 1+9UP and 10P) were killed 2 d after training, a time when strong memories do not undergo reconsolidation. Western blot results demonstrated that strong training reduced NR2B expression (Fig. 5a, Supplementary Fig. 4a online). A one-way ANOVA showed a significant group effect ($F_{3,17} = 5.92$, $P < 0.01$). *Post hoc* tests showed that NR2B expression decreased after both 10P and 1+9UP compared with the 1P control group ($P < 0.05$). We further found NR1 expression comparable for all groups (Fig. 5b, Supplementary Fig. 4b, $F_{3,12} = 0.501$, $P > 0.6$). This result shows that the decrease of NR2B was selective in the LBA, replicating the previous report³⁵.

Using IHC, we counted NR2B-containing cells within the lateral and basal amygdala separately (Supplementary Fig. 5a online). A two-way ANOVA showed significant group effect ($F_{2,9} = 19.86$, $P < 0.001$), area effect (lateral > basal amygdala, $F_{1,9} = 82.02$, $P < 0.001$) and group by area interaction ($F_{2,9} = 7.76$, $P = 0.01$). *Post hoc* tests revealed that the 1P group had similar NR2B-positive cells compared to the 1UP group ($P > 0.5$). However, strong training significantly reduced NR2B-positive cells compared to either the 1P or 1UP group (both $P < 0.01$).

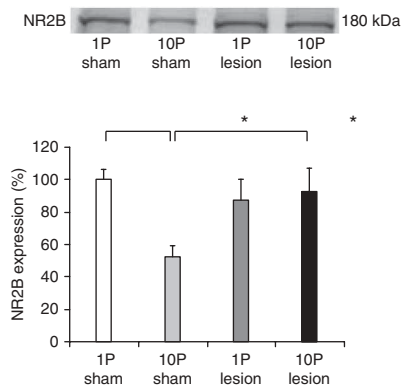


Figure 6 Pretraining dorsal hippocampus lesions prevent the downregulation of NR2B in strongly trained rats. Rats received a pretraining electrolytic dorsal hippocampus lesion or sham lesion. After recovered from the surgery, they were trained with 1 or 10 tone–footshock pairings (1P and 10P, respectively). Two days after training, tissue was extracted from the amygdala for NR2B quantification. The 1P sham ($n = 6$), 1P lesion ($n = 7$) and 10P lesion ($n = 7$) all had comparable NR2B subunit expression. The 10P sham ($n = 7$) group showed less expression of NR2B in the LBA, an effect that was not present in 10P lesion group. * $P < 0.05$. Means \pm s.e.m.

We next asked whether this decrease was reversed 60 d after strong training, a time when strong memories undergo reconsolidation. Western blot results showed that NR2B downregulation disappeared 60 d after training ($t_7 = 1.98$, $P > 0.05$, **Supplementary Fig. 6** online), at a time when the strong memory can undergo reconsolidation (**Fig. 5** and **Supplementary Figs. 5** and **6**). IHC results confirmed this (**Supplementary Fig. 5b**). A two-way ANOVA (1P versus 10P in lateral versus basal amygdala) detected insignificant group difference or group by area interaction (both $F < 1$). Overall results demonstrated an inverse relationship between NR2B abundance and the ability of a memory to undergo reconsolidation.

NR2B levels functionally relate to reconsolidation

If the NR2B abundance in the LBA had a functional relationship with whether fear memories undergo reconsolidation, then we predicted that manipulations that allow reconsolidation of strong memories 2 d after training should also prevent NR2B downregulation. We applied pretraining dorsal hippocampus lesions, which allows new strong memories to undergo reconsolidation (**Fig. 4**). Four groups of rats received sham or electrolytic lesions and 1P or 10P.

Western blot results confirmed the prediction (**Fig. 6** and **Supplementary Fig. 7** online): a one-way ANOVA showed significant difference among groups ($F_{3,23} = 4.001$, $P = 0.019$). *Post hoc* tests showed that only 10P-sham group had less NR2B expression than the 1P-sham and 10P-lesion groups ($P < 0.05$). Using IHC (**Supplementary Fig. 8** online), a two-way ANOVA showed significant group ($F_{2,9} = 6.57$, $P < 0.05$) and area effects (lateral > basal amygdala, $F_{1,9} = 75.21$, $P < 0.001$). *Post hoc* tests showed that the 10P-sham group had fewer NR2B-positive cells ($P < 0.05$) than the 1P-sham group. This again replicated the downregulation (**Fig. 5a** and **Supplementary Fig. 5a**). However, the downregulation was absent in the 10P-lesion group.

DISCUSSION

Previous work has demonstrated that reconsolidation is a fundamental phenomenon, but it is not ubiquitous. There are reports of boundary conditions^{9,12,13,36}. Here we show that strong training-induced boundary conditions can (i) be transient, (ii) require a separate brain system and (iii) be manifested by downregulation of a mechanism mediating the induction of reconsolidation, which in the case of fear conditioning in the LBA is the NR2B NMDA receptor subunit (**Supplementary Figs. 9** and **10** online). The reduction in mechanisms necessary for reconsolidation is likely to be a graded phenomenon and would be maximally reduced under conditions when the memory is resistant to undergoing reconsolidation using a variety of reactivation protocols. Under moderate conditions—for example, after 5 tone–shock pairings—the NR2B reduction could be more modest, which might

leave enough NR2B receptors for the memory to undergo reconsolidation with alternative reactivation protocols.

One possible alternative interpretation of our conclusion that strong training boundary conditions are transient would posit that strong auditory fear memories are initially amygdala independent and then become amygdala dependent over time. However, the amygdala is thought to be always critical for acquisition and consolidation of pavlovian fear memories^{37,38}. Even strong memories acquired with 75 shocks are dependent on the LBA³⁹. In addition, this alternative interpretation cannot explain why lesions of the dorsal hippocampus make the auditory fear memories sensitive to anisomycin challenge in the LBA.

A second alternative interpretation of the transient boundary condition would suggest that strong memories undergo reconsolidation; however, the freezing level might reach a ceiling and this prevents us from detecting a positive effect of anisomycin at 2 and 7 d after strong training. However, to explain the positive findings at 30 and 60 d after training, this interpretation would have to posit that the levels of freezing decline over 30 d to allow an anisomycin-induced deficit to be detectable. This interpretation has more difficulty explaining the findings that strong memories in rats with dorsal hippocampus lesion are sensitive to post-reactivation anisomycin. This is because lesions of the dorsal hippocampus are not thought to affect the level of auditory freezing²³. To explain the anisomycin impairment in the dorsal hippocampus lesioned rats, the ceiling effect interpretation would have to posit that the lesions substantially decreased auditory fear to levels at which an anisomycin impairment could be detected. We directly tested this and found no change in the rate of extinction (**Fig. 4c**), suggesting that the lesions did not affect the strength of the memory. This last finding rules out the ceiling effect interpretation of our data.

Lastly, the pattern of findings cannot be explained by nonspecific effects, such as state-dependent learning, or by toxicity, such as apoptosis, due to anisomycin infusions²⁶. This is because PR-STM was always intact, and the identical infusions have both negative and positive effects on the long-term memory, depending on the training protocol, the reactivation time after training and the presence or absence of hippocampus lesions. Thus, the most parsimonious interpretation of the data is that the strong fear memory remains consolidated in the LBA and over time can again begin to undergo reconsolidation.

We found that the strong training-induced boundary condition was due to associative effects of the shocks. Memories for a single pairing followed by nine unsignaled footshocks (1+9UP) underwent reconsolidation. This suggests that at the behavioral level, it is the change in associative strength acquired with the ten pairings that induces the boundary condition. At the molecular level, both of these groups showed decreases in NR2B subunit abundance. It could be argued that the ability of the auditory fear memory to undergo reconsolidation in the 1+9UP group, while it also reduces NR2B subunit abundance in the LBA, dissociates amounts of the NR2B subunit from a memory's ability to undergo reconsolidation. In turn, it could be argued that on

the basis of this finding the decrease in NR2B subunit is nonspecific. However, if this interpretation is correct, then there is no reason why a nonspecific effect would reverse over time (60 d after strong straining), be subunit specific (decreased NR2B but not NR1) and be reversed by lesions of the dorsal hippocampus.

An alternative specific interpretation of the decreased NR2B expression in the 1+9UP group is that the protocol induces strong contextual fear conditioning. The decrease in NR2B subunit would serve to inhibit reconsolidation of the contextual memory. Projections of auditory and contextual information are thought to be acquired by different populations of neurons within the LBA⁴⁰. The tissue analyzed in our experiments included both regions. We assume that the reduction in NR2B subunit after 10P occurs predominantly at LBA afferents relaying the 5-kHz frequency of the conditioned stimulus and adjoining frequencies. Similarly, the decreased NR2B expression in the 1+9UP group would predominantly be reduced at the afferents mediating the contextual memory within the LBA. In this case, the 1+9UP should decrease the total NR2B subunits (induced at the afferents mediating the contextual memory), but the abundance of NR2B on the afferents mediating auditory fear conditioning would be sufficient to permit the auditory memory to undergo reconsolidation.

The dorsal hippocampus has been previously shown to be mostly dispensable for the acquisition and expression of auditory fear conditioning^{23,41,42}. For this reason, it is unexpected that lesions of the dorsal hippocampus allowed strong memories to undergo reconsolidation in the amygdala 2 d after training. This is not due to nonspecific effects of the lesions, as the rats showed freezing levels comparable to those in unlesioned rats during reactivation, with intact PR-STM scores, and the impairment was only seen when the memory was reactivated. Any nonspecific effects of the lesion, such as increased locomotion, that could compete with freezing would have led to a decrease in freezing during reactivation in both the reactivated and non-reactivated groups. Further evidence for the specificity of dorsal hippocampus lesions on the mechanisms associated with the boundary condition was that the abundance of NR2B subunits in these lesioned rats was comparable to that in sham-lesioned rats with weak 1P training. This cross-region regulation of reconsolidation needs future studies to identify which stage of the training experience the hippocampus is critical for. It is possible that the hippocampus is only involved in the initial training in order to inhibit reconsolidation. Alternatively, the dorsal hippocampus may only be involved in the maintenance of the boundary condition. Attempts to answer these questions are under way.

Because of the novelty of the ability of the hippocampus to affect reconsolidation in the amygdala, we can only speculate as to the nature of the information mediated by the hippocampus that is inhibiting strong new memories from undergoing reconsolidation in the amygdala. Current models of hippocampus functions suggest the involvement of the dorsal hippocampus in the time-dependent reorganization of contextual memories²⁸; but see ref. 43). It is possible that during the reorganization of the contextual memory into a remote memory, the strong auditory fear memories consolidated in the LBA are inhibited from undergoing reconsolidation. Over time, however, the memory is thought to become independent of the hippocampus and dependent on the anterior cingulate cortex²⁹. Once the memory has become hippocampus independent, it would cease to inhibit reconsolidation within the LBA.

Reconsolidation experiments entail two processes. First, reactivation induces the consolidated memory to return to a labile state. Second, the memory must be reconsolidated from this labile state^{8,24}. Recent findings suggest that NR2B subunits must be activated in the LBA during reactivation for the consolidated auditory fear memory to return to a labile state²⁴. We found a clear relationship between NR2B

expression and the ability of a strong auditory fear memory to undergo reconsolidation in the LBA. NR2B downregulation coincides with time points at which strong memories do not undergo reconsolidation and returns to normal (either passively by the passage of time or by dorsal hippocampus lesion) at times when the strong memories can undergo reconsolidation. It is unlikely that the initial decrease in NR2B subunits was due to increased cellular stress from strong training because (i) NR1 expression was normal in rats that received strong training and (ii) NR2B expression was normal in hippocampus-lesioned rats. Last, the finding that dorsal hippocampus lesion did prevent both the strong training boundary condition and the decrease in NR2B expression demonstrates a functional relationship between these two factors.

The reduced NR2B but normal NR1 expression is congruent with an earlier report using physiological recording and western blot³⁵. The authors suggested that one protective effect on the strong memory that results from the downregulation of NMDA receptors would be that it would prevent the acquisition of new fear memories that could interfere with the original strong memory. This downregulation may be a homeostatic response to overstimulation⁴⁴. Another effect on a recently acquired strong fear memory of decreasing NR2B subunits in the LBA is that it would prevent the strong fear memory from returning to a labile state during which it could be changed or weakened. Thus, the very strong memory is protected for some time from interference. Substantial downregulation of NMDA receptors is also seen during development, often at the end of a critical period^{45,46}. Therefore, decreasing NMDA receptor abundance could be a general mechanism by the brain to preserve the learned experience and reduce the potential interference from future events. This reduction would, theoretically, compromise any computations, memory-related or not, performed by afferents with a very low abundance of NR2B subunits.

The insensitivity of the strong memory to anisomycin 2 d after training could be interpreted as a memory that does not undergo reconsolidation or as one that is harder to induce to undergo reconsolidation. The finding that three different reactivation procedures (Figs. 1b and 2) did not induce any amnesia suggests that strong memories initially do not undergo reconsolidation. It is always possible that some other protocol would be effective. However, 2 d after training, the receptor mechanisms critical for inducing reconsolidation are downregulated. Because the mechanisms that are necessary for inducing a consolidated memory to enter a labile state are extremely reduced, we could consider strong memories as a real boundary condition in the LBA.

Our suggested role of the NR2B subunits in regulating when fear memory in the LBA will undergo reconsolidation may not generalize to all memory systems or types of memory. Thus far, there are four studies that have examined the mechanisms involved in transforming a consolidated memory into one in a labile state. While we have demonstrated that NR2B subunit is critical for memories to return to a labile state within the LBA for fear conditioning²⁴, NMDA receptors in the hippocampus for fear memories and within the amygdala for appetitive memories are thought to play a role in restabilization process^{47,48}. In the hippocampus, voltage-gated calcium channels (VGCC)⁴⁷ and protein degradation⁴⁹ are critical for return of a memory to a labile state. Thus, boundary conditions within the hippocampus may work by decreasing VGCC abundance or by preventing protein degradation or any molecular mechanism initiated by VGCC activation that will putatively lead to protein degradation. For each system, the specific molecules mediating boundary conditions are likely to change, but the conceptual mechanisms should remain the same: boundary conditions inhibit reconsolidation by downregulating a mechanism that is critical for transformation of a memory from a stable to a labile state.

In summary, these results begin to describe the training-strength boundary conditions on reconsolidation from the perspectives of behavioral variables, brain system dynamics and molecular mechanisms. These data provide new insights into the nature of the mechanisms that constrain reconsolidation: (i) they can be transient, (ii) different brain areas can be necessary for the boundary conditions on other brain areas and (iii) one conceptual mechanism mediating boundary conditions is the downregulation of the mechanisms mediating the induction of reconsolidation. These findings contain important clinical implications: treating PTSD too soon after the memory has consolidated may be fruitless as the memory is less likely to undergo reconsolidation.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureneuroscience/>.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

K.N. and S.-H.W. designed and developed this study. S.-H.W. conducted the behavioral, pharmacological, lesion and immunohistochemical experiments, performed the statistical and histological analyses and wrote the paper with K.N. L.d.O.A. conducted the western blot and lesion with extinction experiments and performed related data analyses. K.N. supervised this project.

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- Müller, G.E. & Pilzecker, A. Experimentelle Beiträge zur Lehre vom Gedächtnis [Experimental contributions to the theory of memory]. *Z. Psychol. Ergänzungsband (Z. Psychol. suppl.)* **1**:300 (1900).
- Misanin, J.R., Miller, R.R. & Lewis, D.J. Retrograde amnesia produced by electroconvulsive shock after reactivation of a consolidated memory trace. *Science* **160**, 554–555 (1968).
- Schneider, A.M. & Sherman, W. Amnesia: a function of the temporal relation of footshock to electroconvulsive shock. *Science* **159**, 219–221 (1968).
- Alberini, C.M. Mechanisms of memory stabilization: are consolidation and reconsolidation similar or distinct processes? *Trends Neurosci.* **28**, 51–56 (2005).
- Nader, K., Schafe, G.E. & LeDoux, J.E. The labile nature of consolidation theory. *Nat. Rev. Neurosci.* **1**, 216–219 (2000).
- Dudai, Y. Reconsolidation: the advantage of being refocused. *Curr. Opin. Neurobiol.* **16**, 174–178 (2006).
- Sara, S.J. Retrieval and reconsolidation: toward a neurobiology of remembering. *Learn. Mem.* **7**, 73–84 (2000).
- Nader, K. & Hardt, O. A single standard for memory: the case for reconsolidation. *Nat. Rev. Neurosci.* **10**, 224–234 (2009).
- Eisenberg, M., Kobilo, T., Berman, D.E. & Dudai, Y. Stability of retrieved memory: inverse correlation with trace dominance. *Science* **301**, 1102–1104 (2003).
- Nader, K. Memory traces unbound. *Trends Neurosci.* **26**, 65–72 (2003).
- Pedreira, M.E. & Maldonado, H. Protein synthesis subserves reconsolidation or extinction depending on reminder duration. *Neuron* **38**, 863–869 (2003).
- Suzuki, A. *et al.* Memory reconsolidation and extinction have distinct temporal and biochemical signatures. *J. Neurosci.* **24**, 4787–4795 (2004).
- Milekic, M.H. & Alberini, C.M. Temporally graded requirement for protein synthesis following memory reactivation. *Neuron* **36**, 521–525 (2002).
- Debiec, J., Doyere, V., Nader, K. & LeDoux, J.E. Directly reactivated, but not indirectly reactivated, memories undergo reconsolidation in the amygdala. *Proc. Natl. Acad. Sci. USA* **103**, 3428–3433 (2006).
- Stollhoff, N., Menzel, R. & Eisenhardt, D. Spontaneous recovery from extinction depends on the reconsolidation of the acquisition memory in an appetitive learning paradigm in the honeybee (*Apis mellifera*). *J. Neurosci.* **25**, 4485–4492 (2005).
- Duvarci, S., Mamou, C.B. & Nader, K. Extinction is not a sufficient condition to prevent fear memories from undergoing reconsolidation in the basolateral amygdala. *Eur. J. Neurosci.* **24**, 249–260 (2006).
- Debiec, J., LeDoux, J.E. & Nader, K. Cellular and systems reconsolidation in the hippocampus. *Neuron* **36**, 527–538 (2002).
- Lee, J.L., Di Ciano, P., Thomas, K.L. & Everitt, B.J. Disrupting reconsolidation of drug memories reduces cocaine-seeking behavior. *Neuron* **47**, 795–801 (2005).
- Debiec, J. & LeDoux, J.E. Disruption of reconsolidation but not consolidation of auditory fear conditioning by noradrenergic blockade in the amygdala. *Neuroscience* **129**, 267–272 (2004).
- DeViets, T.L. & Holliday, J.H. Retrograde amnesia produced by electroconvulsive shock after reactivation of a consolidated memory trace: a replication. *Psychon. Sci.* **29**, 137–138 (1972).
- Bozon, B., Davis, S. & Laroche, S. A requirement for the immediate early gene *zif268* in reconsolidation of recognition memory after retrieval. *Neuron* **40**, 695–701 (2003).
- Przybylski, J., Roulet, P. & Sara, S.J. Attenuation of emotional and nonemotional memories after their reactivation: role of beta adrenergic receptors. *J. Neurosci.* **19**, 6623–6628 (1999).
- Kim, J.J. & Fanselow, M.S. Modality-specific retrograde amnesia of fear. *Science* **256**, 675–677 (1992).
- Ben Mamou, C., Gamache, K. & Nader, K. NMDA receptors are critical for unleashing consolidated auditory fear memories. *Nat. Neurosci.* **9**, 1237–1239 (2006).
- Blanchard, R.J. & Blanchard, D.C. Crouching as an index of fear. *J. Comp. Physiol. Psychol.* **67**, 370–375 (1969).
- Rudy, J.W., Biedenkapp, J.C., Moineau, J. & Bolding, K. Anisomycin and the reconsolidation hypothesis. *Learn. Mem.* **13**, 1–3 (2006).
- Duvarci, S. & Nader, K. Characterization of fear memory reconsolidation. *J. Neurosci.* **24**, 9269–9275 (2004).
- Frankland, P.W. & Bontempi, B. The organization of recent and remote memories. *Nat. Rev. Neurosci.* **6**, 119–130 (2005).
- Frankland, P.W., Bontempi, B., Talton, L.E., Kaczmarek, L. & Silva, A.J. The involvement of the anterior cingulate cortex in remote contextual fear memory. *Science* **304**, 881–883 (2004).
- Zola-Morgan, S.M. & Squire, L.R. The primate hippocampal formation: evidence for a time-limited role in memory storage. *Science* **250**, 288–290 (1990).
- Blanchard, D.C., Blanchard, R.J. & Fukunaga, K.K. Environmental control of defensive reactions to footshock. *Bull. Psychon. Soc.* **8**, 129–130 (1976).
- McDonald, A.J. Cortical pathways to the mammalian amygdala. *Prog. Neurobiol.* **55**, 257–332 (1998).
- Pitkanen, A., Pikkarainen, M., Nurminen, N. & Ylinen, A. Reciprocal connections between the amygdala and the hippocampal formation, perirhinal cortex, and postrhinal cortex in rat: a review. *Ann. NY Acad. Sci.* **911**, 369–391 (2000).
- Richter-Levin, G. & Akirav, I. Amygdala-hippocampus dynamic interaction in relation to memory. *Mol. Neurobiol.* **22**, 11–20 (2000).
- Zinebi, F. *et al.* NMDA currents and receptor protein are downregulated in the amygdala during maintenance of fear memory. *J. Neurosci.* **23**, 10283–10291 (2003).
- Morris, R.G. *et al.* Memory reconsolidation: sensitivity of spatial memory to inhibition of protein synthesis in dorsal hippocampus during encoding and retrieval. *Neuron* **50**, 479–489 (2006).
- Fanselow, M.S. & Gale, G.D. The amygdala, fear, and memory. *Ann. NY Acad. Sci.* **985**, 125–134 (2003).
- Schafe, G.E., Nader, K., Blair, H.T. & LeDoux, J.E. Memory consolidation of Pavlovian fear conditioning: a cellular and molecular perspective. *Trends Neurosci.* **24**, 540–546 (2001).
- Maren, S. Neurotoxic basolateral amygdala lesions impair learning and memory but not the performance of conditional fear in rats. *J. Neurosci.* **19**, 8696–8703 (1999).
- Reijmers, L.G., Perkins, B.L., Matsuo, N. & Mayford, M. Localization of a stable neural correlate of associative memory. *Science* **317**, 1230–1233 (2007).
- Phillips, R.G. & LeDoux, J.E. Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behav. Neurosci.* **106**, 274–285 (1992).
- Ambrogio Lorenzini, C.G., Baldi, E., Bucherelli, C., Sacchetti, B. & Tassoni, G. Neural topography and chronology of memory consolidation: a review of functional inactivation findings. *Neurobiol. Learn. Mem.* **71**, 1–18 (1999).
- Moscovitch, M., Nadel, L., Winocur, G., Gilboa, A. & Rosenbaum, R.S. The cognitive neuroscience of remote episodic, semantic and spatial memory. *Curr. Opin. Neurobiol.* **16**, 179–190 (2006).
- Turrigiano, G.G. & Nelson, S.B. Hebb and homeostasis in neuronal plasticity. *Curr. Opin. Neurobiol.* **10**, 358–364 (2000).
- Franks, K.M. & Isaacson, J.S. Synapse-specific downregulation of NMDA receptors by early experience: a critical period for plasticity of sensory input to olfactory cortex. *Neuron* **47**, 101–114 (2005).
- Joshi, I. & Wang, L.Y. Developmental profiles of glutamate receptors and synaptic transmission at a single synapse in the mouse auditory brainstem. *J. Physiol. (Lond.)* **540**, 861–873 (2002).
- Suzuki, A., Mukawa, T., Tsukagoshi, A., Frankland, P.W. & Kida, S. Activation of LVGCCs and CB1 receptors required for destabilization of reactivated contextual fear memories. *Learn. Mem.* **15**, 426–433 (2008).
- Milton, A.L., Lee, J.L., Butler, V.J., Gardner, R. & Everitt, B.J. Intra-amygdala and systemic antagonism of NMDA receptors prevents the reconsolidation of drug-associated memory and impairs subsequently both novel and previously acquired drug-seeking behaviors. *J. Neurosci.* **28**, 8230–8237 (2008).
- Lee, S.H. *et al.* Synaptic protein degradation underlies destabilization of retrieved fear memory. *Science* **319**, 1253–1256 (2008).

ONLINE METHODS

Subjects. Adult male Sprague-Dawley rats bred at Charles River were used in this study. Both food and water were provided *ad libitum*. The 12-h light-dark cycle began at 7 am daily. All experiments were conducted at the light cycle and followed the protocols approved by McGill University Animal Care Center.

Surgery. Rats were anesthetized with sodium pentobarbital (60 mg ml⁻¹), injected with atropine to prevent the obstruction of the respiration and placed in stereotaxic frames. Guide cannula (22 gauge) were bilaterally implanted and aimed at lateral and basal nuclei of the amygdala (LBA). The coordinates were 3 mm posterior, 5.3 mm lateral, and 8 mm ventral from the bregma based on rat brain atlas. Three jewelry screws were implanted into the skull and acrylic cement was applied to stabilize the cannula. The rats were then allowed 7–10 d to recover from the surgery. During the recovery period, rats were handled daily.

For dorsal hippocampus lesions, rats were given electrolytic or sham lesions at the time of LBA cannula implantation. The screws were first anchored. The electrodes were then placed at two sites of dorsal hippocampus in each hemisphere. The coordinates were 2.8 mm posterior, 2 mm lateral and 4 mm ventral; 4.2 mm posterior, 3 mm lateral and 4 mm ventral from the bregma. Stainless steel microelectrodes (FHC, model KK1) with 500 μm of the tip insulation removed were lowered through an incision in the dura into the target area. Lesions were made by passing a positive current (1.0 mA, 20 s) through a lesion-making device (Ugo Basile). Sham lesioned rats underwent a similar surgery procedure except for the electrolytic current being omitted.

Drugs and micro-infusions. Anisomycin (125 μg μl⁻¹, Sigma-Aldrich) was dissolved in 1 M HCl and normal physiological saline. The pH was adjusted to 7.4 with 1 M NaOH. Normal physiological saline was used as vehicle solution. For intra-LBA microinfusion, injectors (28 gauge, extending 1.5 mm below the guide cannula) were connected to microsyringe (5 μl, Hamilton) with polyethylene tubes. The solution (0.5 μl per side) was infused with a pump (Harvard) over the course of 2 min. The injector was left for an additional minute to allow the complete diffusion.

Histology. After completing all behavioral procedures, rats were transcardially perfused with physiological saline followed by 10% formalin-saline. The brains were then cryosectioned at 50-μm thickness and stained with formal-thionin to identify cannula placement.

Behavioral procedures. *Habituation.* After recovery from the surgery, rats were given 2 d of habituation in the training and testing chambers, which had different olfactory, tactile and visual properties from each other. On day 1, half of the rats were habituated to the training contexts for 30 min and 5 h later, they were habituated to the testing contexts for 30 min. On the next day, the same rats received a reversed order of habituation (that is, testing context first and then training context). The remaining half rats received the reverse sequence of habituation.

Training. The day after habituation rats were conditioned. After 3 min of acclimation, one tone (5 kHz, 75 dB) was presented for 30 s and it coterminated with a scrambled footshock (1.5 mA, 1 s). In the strong training paradigm, 10 tone-footshock pairings were given. The interpairing interval was variable with an average of 4 min. One minute after the final pairing, rats were returned to their home cages.

Reactivation. Reactivation entailed one 30-s tone presentation in the testing box. One minute after the offset of the tone, rats were removed from the testing chamber. Half of the rats were immediately infused with anisomycin and the remaining were infused with vehicle. They were then returned to the home cage. Four hours later, they were given a post-reactivation short-term memory (PR-STM) test. The test session was 8 min long and composed of three presentations of the 30-s tone. Twenty-four hours after reactivation, rats were

given post-reactivation long-term (PR-LTM) memory test which was 8 min long and composed of three presentations of the 30-s tone.

Extinction. Rats were habituated and trained with 1 pairing or 10 pairings as described above. Two days later, rats received 10 presentations of the tones (30 s each) without any footshocks in the testing context. The intertone interval was varied between 2 and 5 min (average 3 min).

Western blots and antibodies. The rats were deeply anesthetized with urethane (50 mg ml⁻¹) and put to death and their brains were rapidly removed and frozen. Amygdala punches were obtained with a neuro punch (1 mm; Fine Science Tools) from frozen brains. The punches included the lateral amygdala and the basal nucleus and possibly portions of the lateral central nucleus. The samples were homogenized in cold lysis buffer with protease inhibitors. Equal amounts of protein (15 μg) were resolved using 7.5% SDS-PAGE and transferred to nitrocellulose membranes as previously described⁵⁰. The protein blots were incubated with primary antibodies (NR2B, 1:300 (Zymed) or NR1, 1:1,000 (Chemicon)), followed by incubation with horseradish peroxidase-conjugated antibody to goat IgG. For quantification of immunoblots, they were scanned and analyzed using ImageQuant software (Amersham).

Immunohistochemistry. Two days after training, the rats were deeply anesthetized with urethane (50 mg ml⁻¹). They were then transcardially perfused with cold PBS followed by 4% paraformaldehyde in phosphate buffer. Brains were removed and postfixed in the same fixative overnight. Brains were then sliced with vibratome (Leica) at 50-μm thickness. Sections were collected from the region around 2.8 to 3.5 mm posterior to the bregma, where it contains amygdalar structures. IHC was done using a free-flotation method. Selected sections were then incubated in 0.3% H₂O₂ to quench endogenous peroxidase activity, blocked in PBS containing 1% bovine serum with 0.2% Triton X-100 and incubated in antibodies to NR2B (rabbit polyclonal antibody, 1:500; Upstate) in the same blocking buffer at room temperature (23–26 °C) overnight. After washing with PBS, slices were then incubated in biotinylated goat anti-rabbit antibodies (1:1,000, Vector) for 1 h at room temperature, washed with PBS and incubated in ABC (Elite kit, Vector). The color development was done with 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) for 2 min. After a series of dehydration procedures, sections were mounted on coated slides and coverslipped.

Quantification of NR2B-labeled cells. In each rat, cell counts were taken from two to three sections, separated by 200 μm, from 2.8 to 3.2 mm posterior to the bregma. The boundary of lateral amygdala and basal amygdala was defined under the microscope (Olympus, IX81) using a ×20 objective. Cell counting was done under a ×40 objective. We used ImagePro software (Media Cybernetics) to identify circular, stained objects that were substantially darker than the background. We later verified that these objects were cell bodies. A region of interest (ROI; dimensions, a 210 μm × 150 μm rectangle) was randomly selected ~0.2 mm below the tip of the lateral amygdala or below the boundary of the lateral and basal amygdala. In total, eight similar ROIs were randomly collected within lateral and basal amygdala. The cell numbers within these ROIs were later averaged for statistic analysis.

Statistics. We used one-way independent, two-way independent, and two-way or three-way with one repeated measure ANOVA for behavioral data analysis. *Post hoc* tests were further used to identify the critical differences that contributed to significant interaction. Type-one error rate was set at 0.05. Behavioral data entered statistical analysis only when the cannula correctly targeted LBA bilaterally.

50. Lengyel, I. *et al.* Autonomous activity of CaMKII is only transiently increased following the induction of long-term potentiation in the rat hippocampus. *Eur J. Neurosci.* **20**, 3063–3072 (2004).

Supplementary Figures

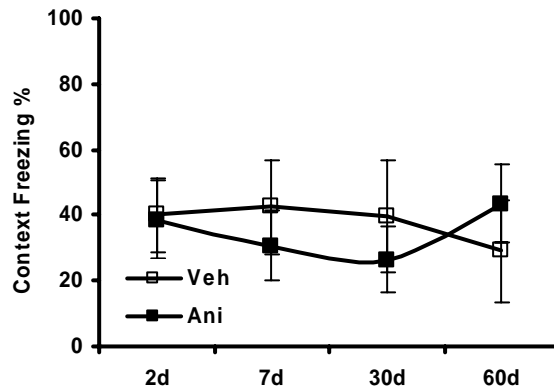


Figure S1 Freezing to the context during reactivation does not change over time. The percentage of freezing to the test context was assessed by the duration of freezing during the 30 seconds before the onset of the CS. Veh (open squares) or Ani (black squares) refers to the animals that would receive intra-LBA infusion of vehicle or anisomycin after reactivation. Each data point is represented in mean \pm s.e.m.

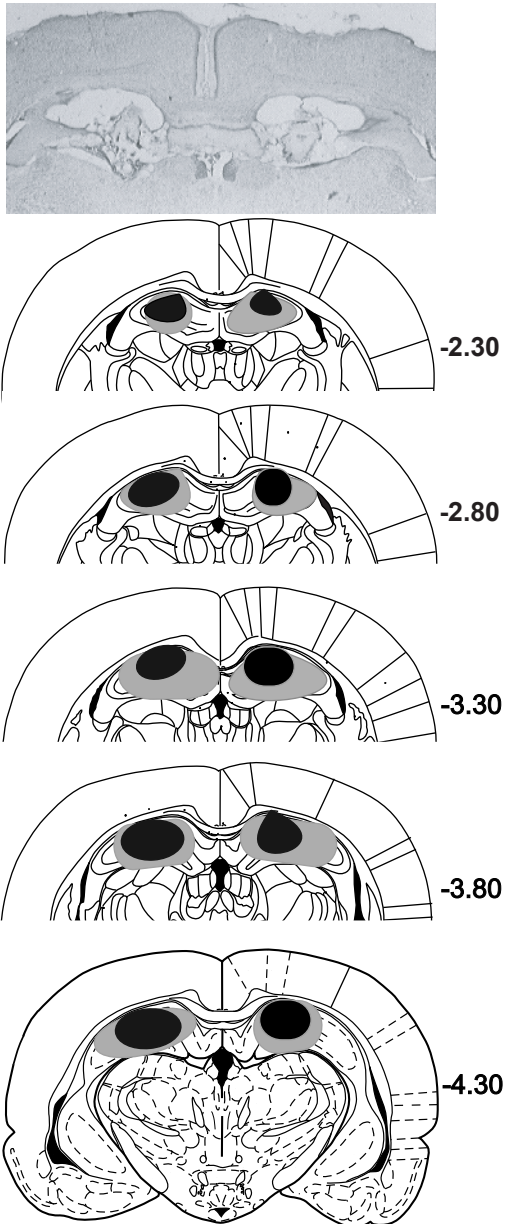


Figure S2 Electrolytic lesion of dorsal hippocampus. Top panel: The picture represents a typical brain section with dorsal hippocampus lesion. Bottom panel: The schematic representation shows the extent of the lesion. The number attached to each brain section indicates its distance (in mm) posterior to the bregma (based on Paxinos and Watson atlas). Shaded area shows the largest lesion while the black area shows the smallest lesion. This figure represents the animals in Fig. 4a study.

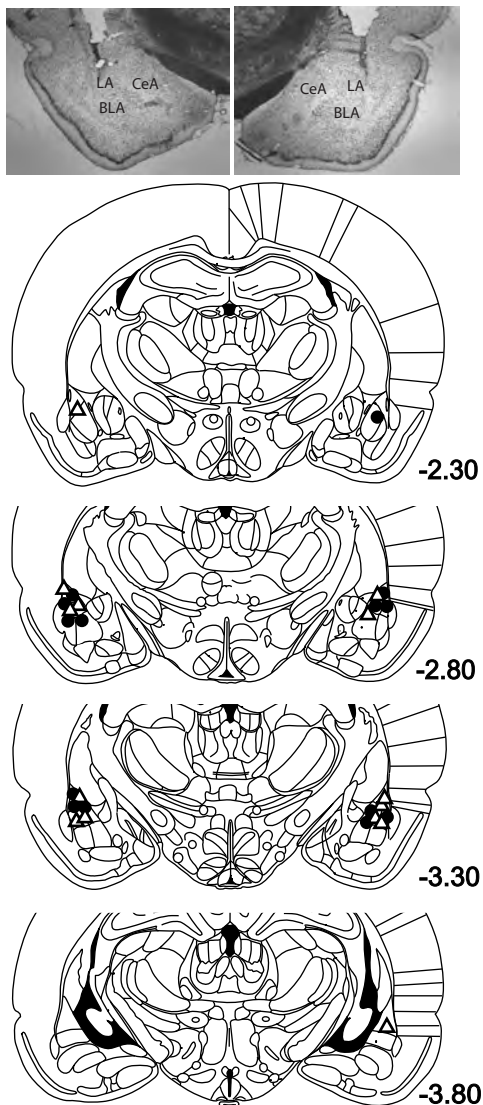
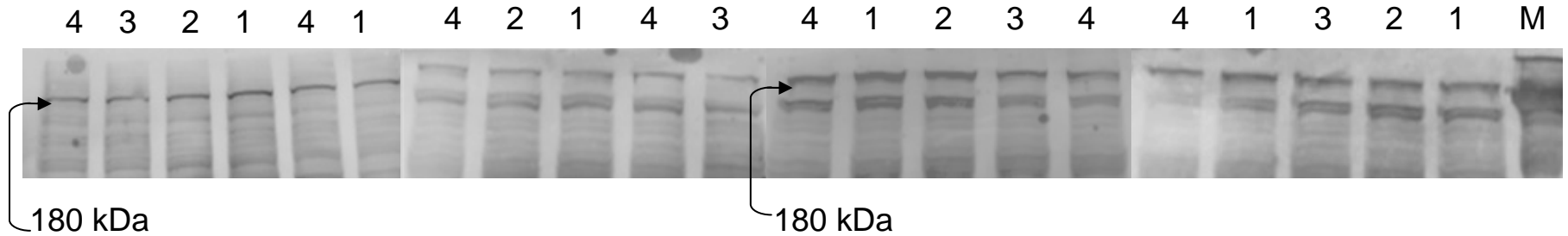


Figure S3 Lateral and basal amygdala cannula placement. Top panel: The picture represents a typical brain section showing cannula aimed at lateral amygdala. Bottom panel: the schematic representation shows the placement of the tip of injectors which can be clearly identified under microscopy by tracing the scar trace. The number attached to each brain section indicates its distance (in mm) posterior to the bregma (based on Paxinos and Watson atlas). This figure represents the animals in Fig. 4a study.

a 2 d: NR2B level



b 2 d: NR1 level

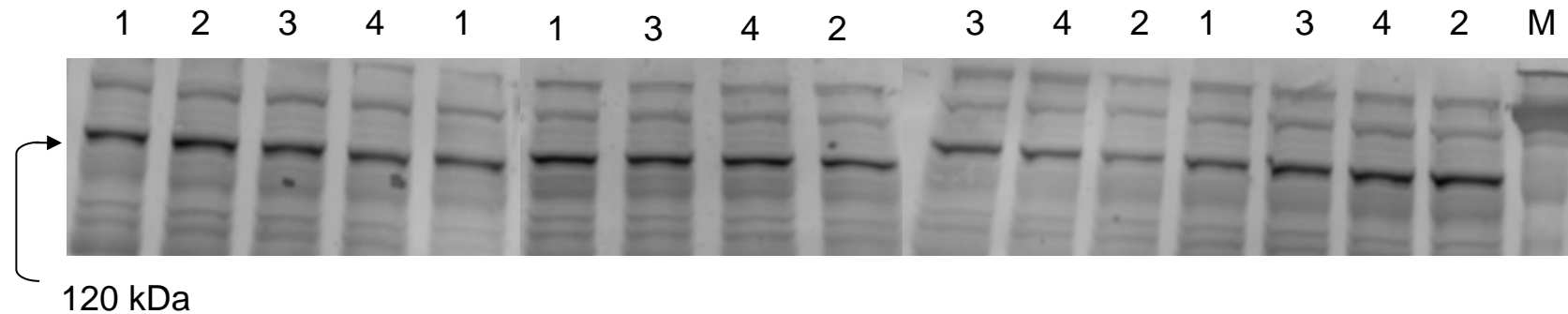


Figure S4 Western blots of Fig. 5. The number above each lane represents the group identify: 1: 1 pairing, 2: 1 unpairing, 3: 1 pairing + 9 unpaired shocks, 4: 10 pairings. 'M' indicates markers of protein molecular weight.

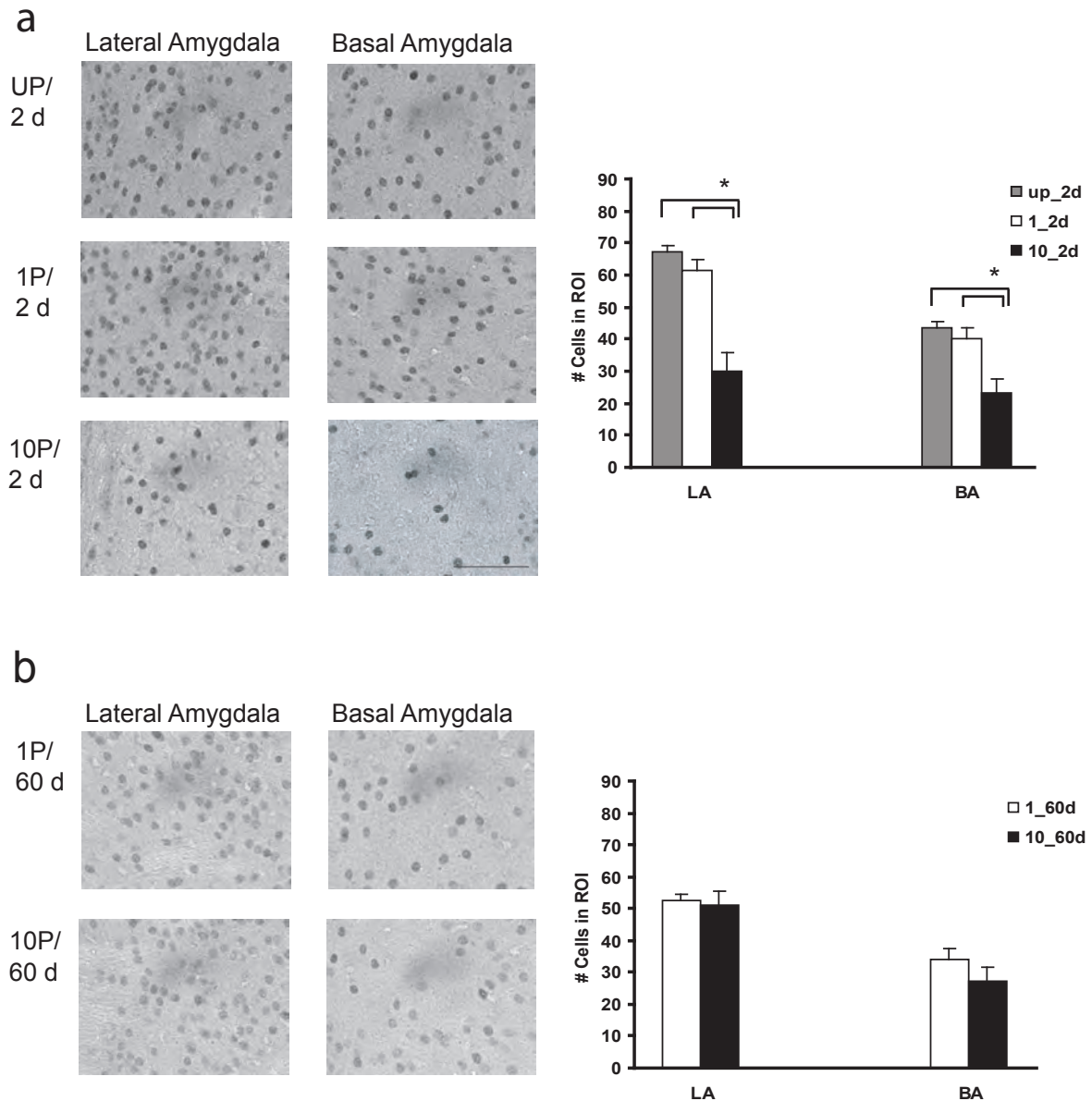
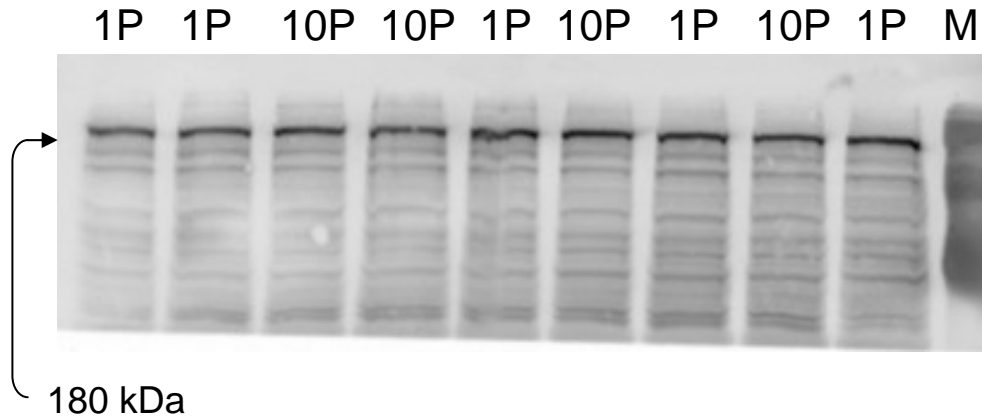


Figure S5 NR2B-subunit levels, assessed by immunohistochemistry (IHC), are inversely related to the ability of the strong memories to undergo reconsolidation over time. **(a)** Animals received 10 tone-footshock pairings (10P), 1 pairing (1P), or 1 footshock followed by an unpaired tone (UP). They were sacrificed 2 days after training, a time when the memory does not undergo reconsolidation, and their brains were later processed for IHC. The left panel represents the actual staining in regions of interest (ROI) in lateral and basal amygdala (LA, BA) in individual groups (n=4/group). The graph shows the quantification of NR2B-positive cell numbers in each ROI. While 1P and UP animals showed similar level of NR2B-immunostained cells, 10P animals showed significantly less stained cells in either LA or BA. The asterisk (*) indicates significant group differences. **(b)** Animals received either 10P or 1P. They were sacrificed 60 days after training, a time when the memory does undergo reconsolidation, and their brains were later processed for IHC. Both groups show similar level of NR2B-positive cells in LA and BA. The scalar bar represents 80 μ m. All pictures in the left panel are in the same scale. Each data point is represented in mean \pm s.e.m.

a

60 d: NR2B level



b

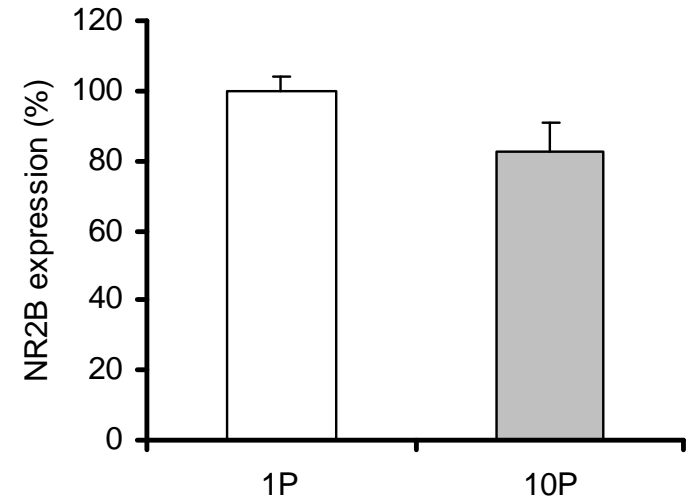


Figure S6 Strong memory induced down-regulation of NR2B subunits dissipates over time. **(a)** Western blot of NR2B subunits in amygdala tissue. Animals were trained with 1 ($n = 5$) or 10 ($n = 4$) tone-footshock pairings (1P or 10P) and sacrificed 60 days later, a time when the memory does undergo reconsolidation. 'M' indicates markers of protein molecular weight. **(b)** Quantification of the levels of NR2B subunits which was comparable between strong and weak training when tested 60 days after training. Each data point is represented in mean \pm s.e.m.

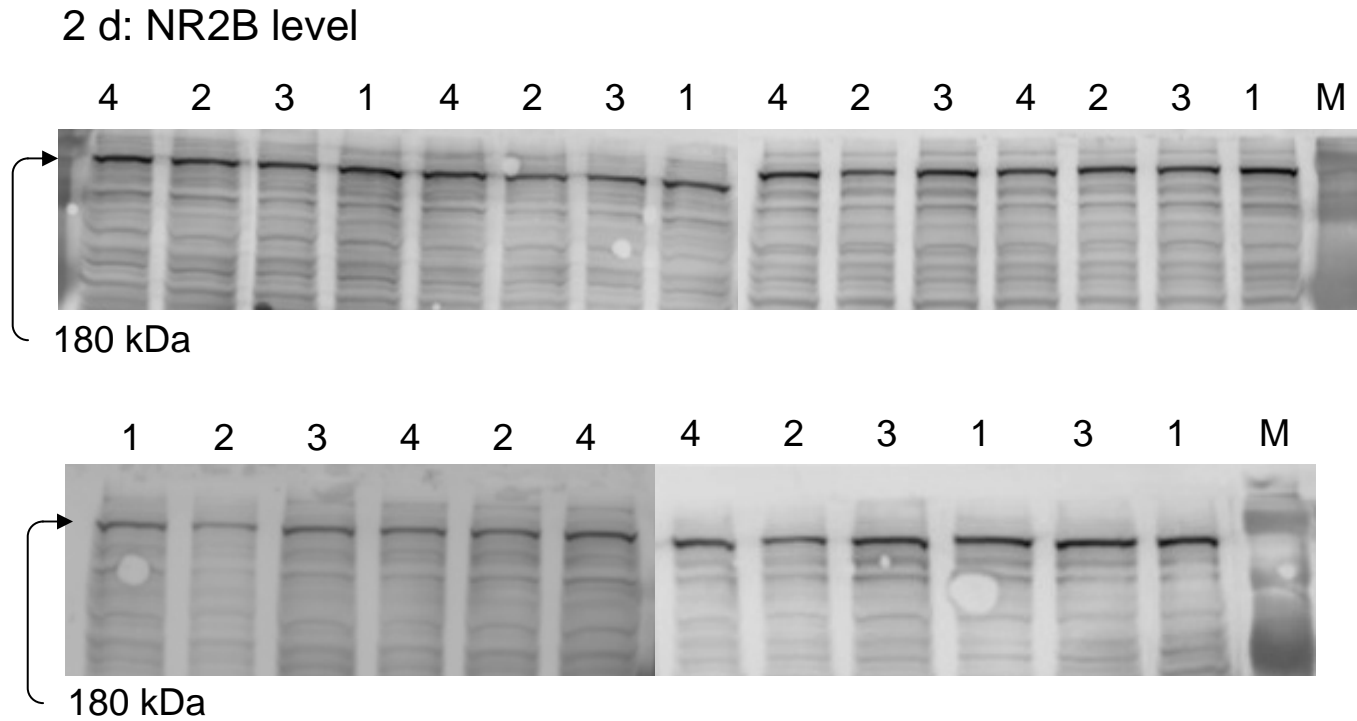


Figure S7 Western blots of Fig. 6. The number above each lane represents the group identify: 1: 1 pairing with sham dorsal hippocampus (dHPC) lesion, 2: 10 pairing with sham dHPC lesion, 3: 1 pairing with electrolytic dHPC lesion, 4: 10 pairing with electrolytic dHPC lesion. 'M' indicates markers of protein molecular weight.

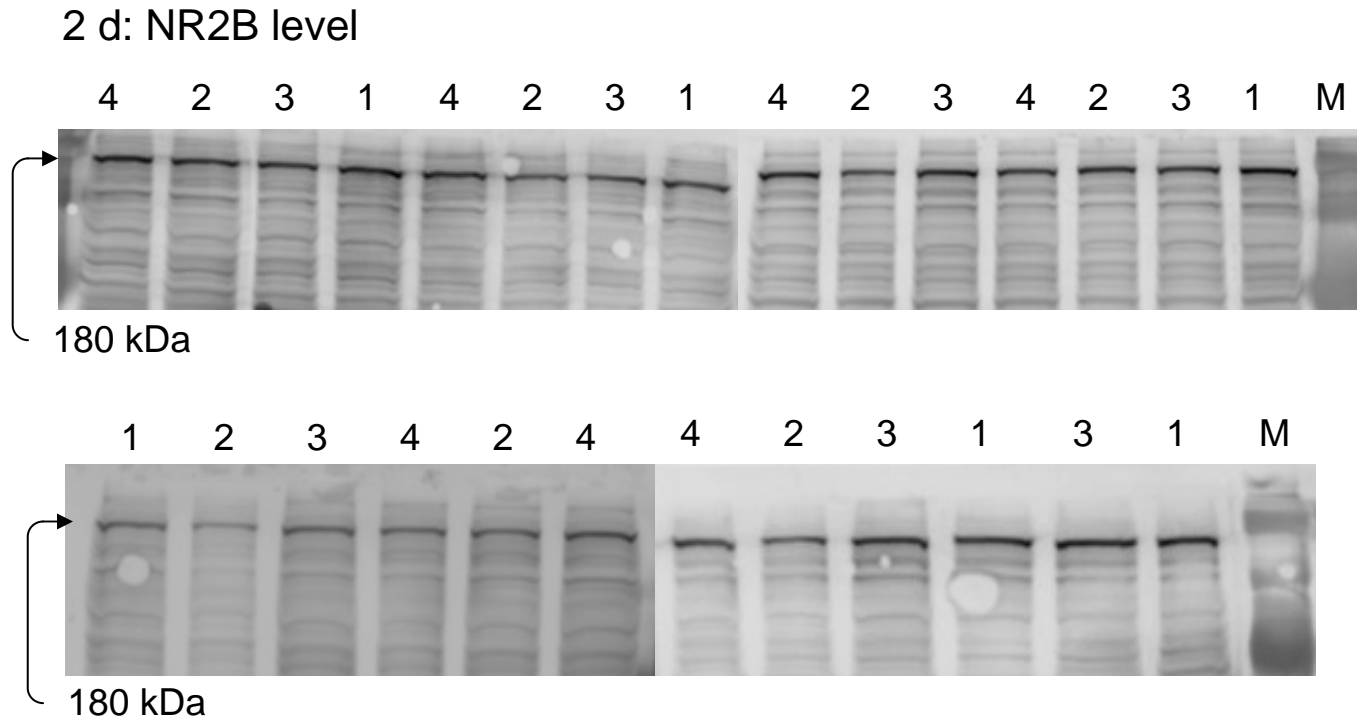


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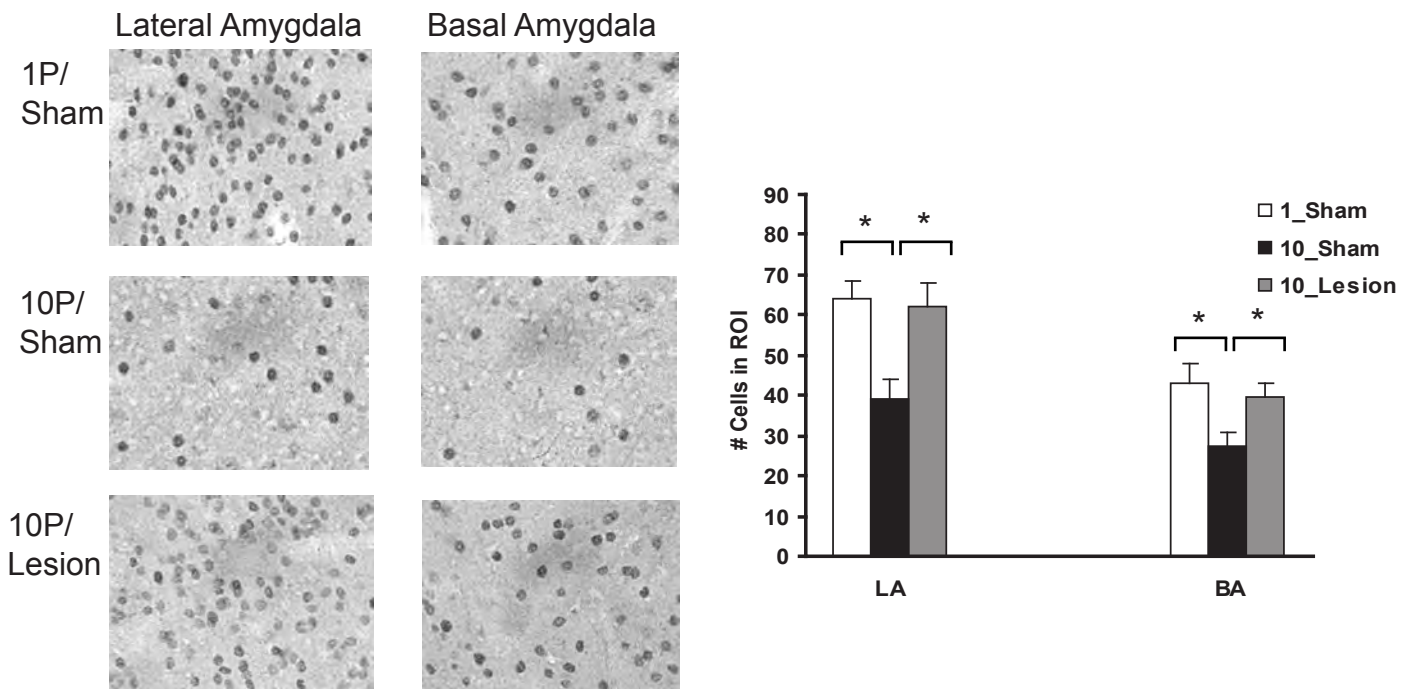


Figure S8 Immunohistochemical analysis of the effects of pre-training dorsal hippocampus lesion on the down regulation of NR2B-positive cells in strongly trained animals. Animals received pre-training, electrolytic dorsal hippocampus lesion or sham lesion at the time of implanting LBA cannula. They were trained with either 10 tone-footshock pairings (10P) or 1 pairing (1P). They were sacrificed 2 days after training and their brains were processed for immunohistochemistry. The sham-lesioned, strong training group (10p_sham) showed less NR2B-positive cells than those in sham-lesioned, 1 pairing group (1p_sham). Dorsal hippocampus lesions prevented the reduction in NR2B positive cells induced by normally induced by strong training (10p_lesion) to a level that is comparable to the regular training group (1p_sham). The asterisk (*) indicates significant group differences. Each data point is represented in mean +/- s.e.m.

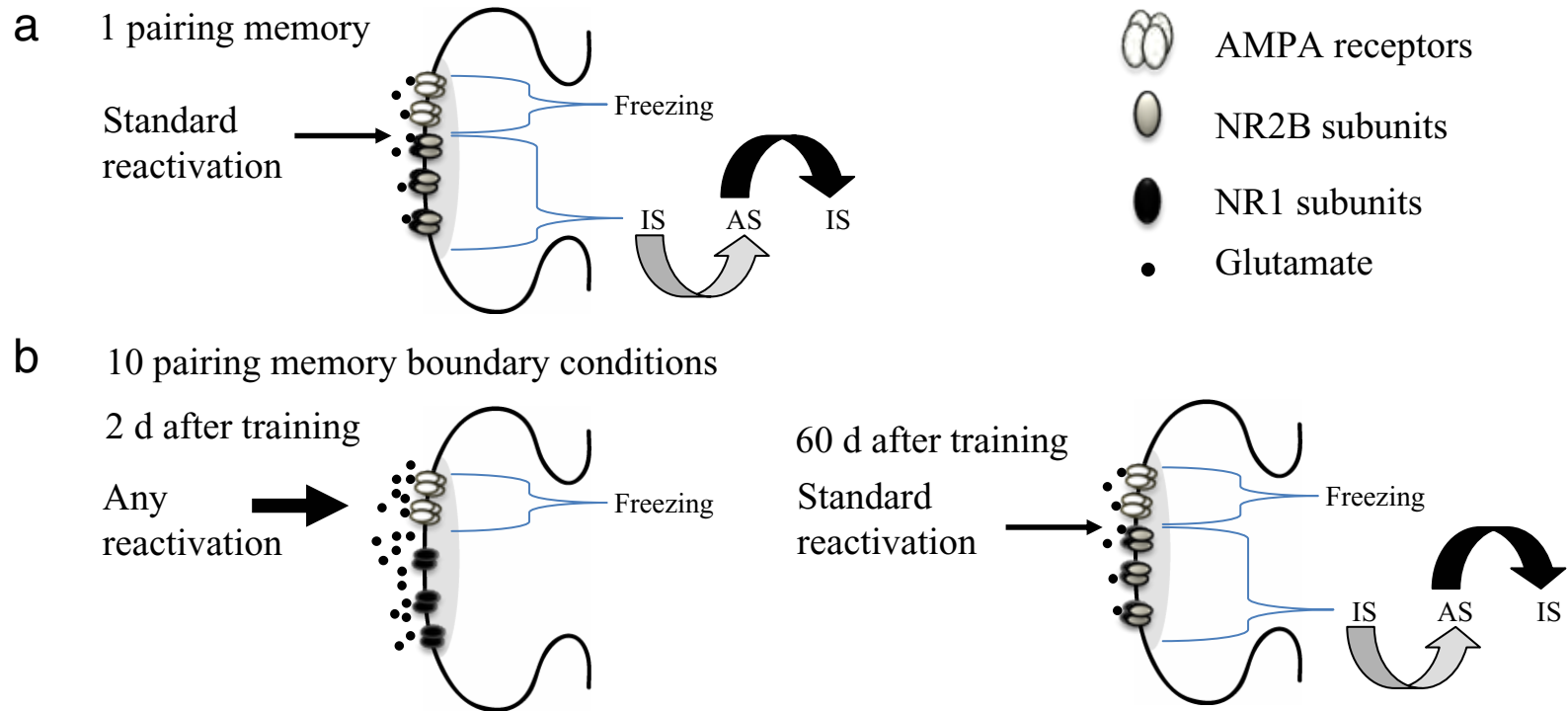
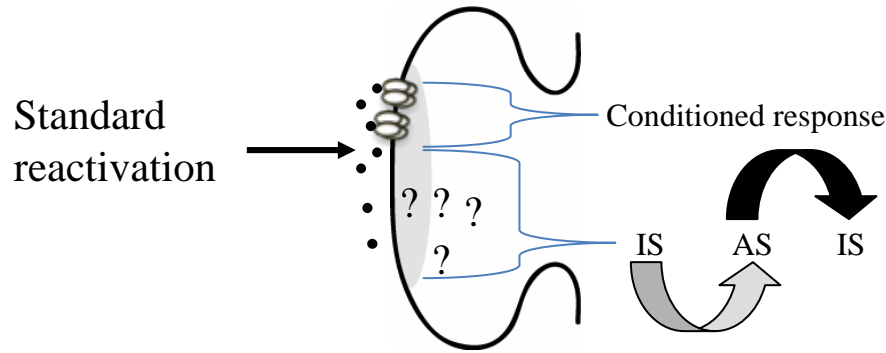


Figure S9 A diagram summarizing how strength of auditory fear memory can influence a mechanism that allows reconsolidation to occur at the LBA synapses. **(a)** After 1 pairing, NR2B containing NMDA receptors are activated for the memory to go from a consolidated inactive state (IS) to a labile active state (AS) (gray curved arrow). The labile memory then undergoes reconsolidation and return to IS (black curved arrow). Independently of this mechanism, AMPA receptors are critical for the expression of freezing behavior²⁴. After 1 pairing, there are sufficient NR2B subunits at the synapse for the memory to undergo reconsolidation when standard reactivation procedures are used. **(b)** After 10 pairings, via hippocampus mediated mechanisms (not shown), levels of the NR2B, but not NR1, are reduced 2 days after training. NR2B reduction eliminates a necessary mechanism for the memory to undergo reconsolidation. Hence, reactivation by standard or alternative protocols should be unable to induce reconsolidation. Meanwhile, the existing AMPA receptors will mediate normal expression of freezing. NR2B level returns to normal 60 days after training. Thus, standard reactivation procedures are now sufficient to induce reconsolidation.

a Conditions permitting a memory to undergo reconsolidation



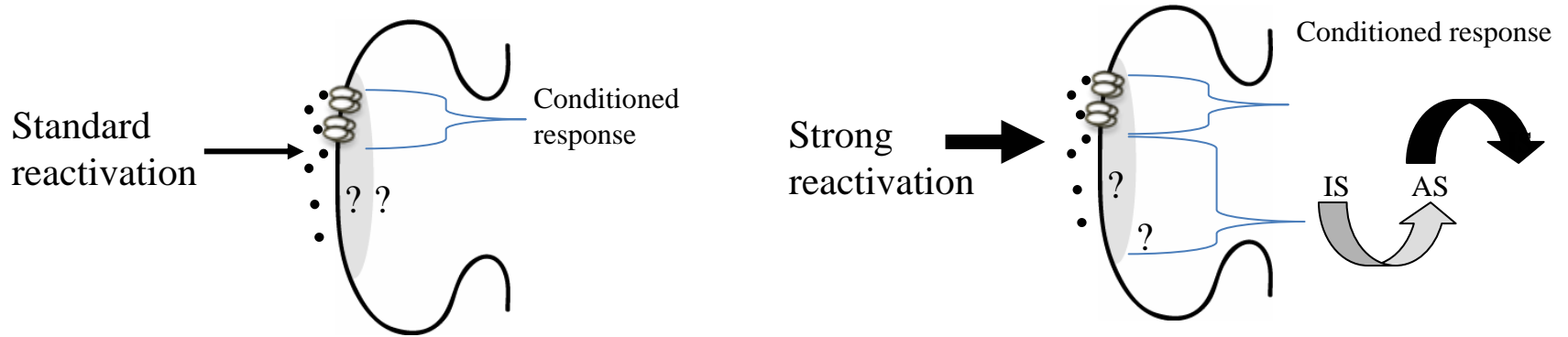
AMPA receptors

?

Mechanisms that transform a consolidated memory to a labile state

- Glutamate

b Conditions that begin to inhibit reconsolidation from occurring



c Conditions that prevent reconsolidation from occurring

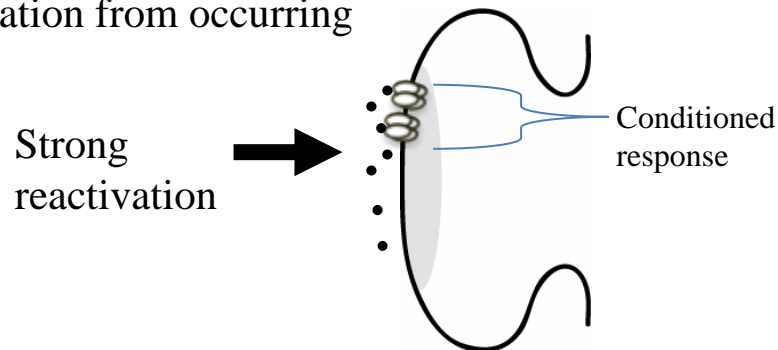


Figure S10 (see next page)

Figure S10 Conceptual diagram demonstrating how boundary conditions could inhibit memories from undergoing reconsolidation across memories types and memory systems. **(a)** Under experimental conditions when a memory undergoes reconsolidation, the mechanisms allowing a memory to be transformed from a consolidated inactive state (IS) to a labile active state (AS), must be present and functional at the synapse (? in figure). These mechanisms, of course, will involve more than surface receptors and will likely include a number of molecular processes that have yet to be identified. One other molecular mechanism is protein degradation, which is required for a consolidated memory to return to a labile state⁴⁹. These mechanisms will likely not be identical from all memory systems. **(b)** Experimental conditions that begin to inhibit memories from undergoing reconsolidation may lead to a partial reduction in a mechanism that is critical for the induction of reconsolidation. The partial reduction might be sufficient to prevent the induction of reconsolidation when a standard protocol is used. However, there may still be sufficient amounts of this mechanism to permit the memory to undergo reconsolidation when a stronger reactivation is used. **(c)** Under conditions when the memory does not undergo reconsolidation, a boundary condition, a necessary mechanism for the induction of reconsolidation is reduced to the point that alternative reactivation protocols cannot induce the memory to undergo reconsolidation.