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INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS: FISIOLOGIA ANIMAL COMPARADA

IMPORTÂNCIA DA PAK 1 E 3 NA REGIÃO CA1 DO HIPOCAMPO DE RATOS WISTAR, RELACIONADA À RECONSOLIDAÇÃO, EXTINÇÃO E REAQUISIÇÃO DA MEMÓRIA AVERSIVA

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Fisicamente, habitamos um espaço, mas, sentimentalmente,

somos habitados por uma memória.

José Saramago

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A conclusão desta tese representa o fim de uma etapa muito importante dentre tantas outras que deverão compor o processo de construção de um pesquisador. Processo que sempre estará em pleno andamento, munido de muitas questões, buscas, respostas e, finalmente, novas questões. E, sobretudo, provido de um "espaço" chamado dúvida, onde o conhecimento possa sempre se desenvolver livremente.

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Sumário

Lista de Abreviaturas

AMPA - a-amino-3-hidroxi-5-metil-4-isoxazolproprionato

AMPc – Adenosina Monofosfato Cíclico

CA1 – Corno de Amon 1

CPF – Córtex Pré-frontal

CPFm – Córtex Pré-frontal medial

CaMK - cinase dependente cálcio calmodulina

Cdc42 - cell division cycle 42

CIT – Células Intercaladas

CREB - proteína ligada ao elemento de resposta ao AMPc

CRIB - Cdc/Rac interative binding

Erk - cinase regulada por sinal extracelular

GTP – Guanosina Tri-fosfato

GDP – Guanosina Di-fosfato

LIMK – LIM cinase

NMDA - N-metil-D-aspartato

PAK – Cinase ativada por p21

PBD - p21 binding domain

PDK – Cinase dependente de fosfoinositideo

PKA - cinase ativada por AMPc

PKC - proteína cinase dependente de cálcio

PRP – Proteínas Relacionadas a Plasticidade

p38MAPK - p38 da subfamília de proteínas quinases activadas mitogen

Rac - Ras-related C3 botulinum toxin substrate

Raf - proto-oncogene c-Raf

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Resumo

A reconsolidação e a extinção da memória aversiva são dependentes dos mecanismos de síntese de novas proteínas e estabilização do citoesqueleto de actina. Já, a reaquisição da memória aversiva não necessita da síntese de novas proteínas, mas é dependente do processo de rearranjo de citoesqueleto de actina. Os mecanismos de síntese de novas proteínas e alterações no citoesqueleto são decorrentes da atividade das Rho GTPases Cdc42 e Rac1 e suas efetoras cinase ativada por p21 isoformas 1 e 3 (PAK1/3). Nesta tese, primeiramente foi realizado uma revisão de literatura sobre a PAK 1 e 3 onde discorremos sobre os seguintes aspectos: estrutura, mecanismos de ação, localização no sistema nervoso central de mamíferos, substratos alvos, regulação do citoesqueleto e participação nos processos de neuroplasticidade. Num segundo momento nós avaliamos a importância da PAK 1 e 3 na região CA1 do hipocampo de ratos, relacionada à reconsolidação, extinção e reaquisição da memória aversiva no medo condicionado contextual (CFC – do inglês contextual fear conditioning). Para tanto, ratos machos adultos foram submetidos à cirurgia estereotáxica para implante de cânulas na região CA1 do hipocampo para infusão de DMSO 3% (veículo) ou IPA-3 (inibidor alostérico da PAK 1 e 3). Para verificar a reconsolidação; no dia 1 os animais foram treinados no CFC; dia 2 sofreram reativação e imediatamente ou 3h após receberam as infusões; dia 3 foram testados no CFC. Para avaliar extinção: dia 1 os animais foram treinados no CFC; dia 2 e 3 foram treinados para extinção e imediatamente ou 3h após receberam as infusões; dia 4 foram testados no CFC. Para verificar reaquisição: dia 1 os animais foram treinados no CFC; dia 2, 3 e 4 treinados para extinção; dia 5 foram novamente treinados no CFC e imediatamente ou 3 h após receberam as infusões. Os resultados mostram que o bloqueio da PAK 1 e 3 em CA1 não prejudica a reconsolidação da memória aversiva. O bloqueio da PAK 1 e 3 em CA1 imediatamente após o primeiro treino de extinção retarda esse processo, mas não impede que a memória aversiva seja extinta. No entanto, a infusão do IPA-3 3h após o treino de extinção não afeta esse processo mnemônico. Quanto a reaquisição, o bloqueio da PAK 1 e 3 imediatamente, mas não 3 h, após o seu treino (dia 5) impede que essa memória seja fortalecida novamente. Assim, podemos sugerir que o bloqueio da PAK pode afetar os mecanismos moleculares subjacentes a extinção e reaquisição da memória de CFC, mas não tem nenhum efeito sobre os mecanismos relacionados com à reconsolidação.

Abstract

The stabilization of memory depends on molecular and morphological changes at synapses activated during mnemonic training in key regions such as CA1 of the hippocampus. The reconsolidation and extinction of aversive memory mechanisms are dependent on new protein synthesis and stabilization of the actin cytoskeleton. Since the reacquisition of aversive memory does not require synthesis of new proteins, but is dependent on the actin cytoskeleton rearrangement process. The mechanisms of synthesis of new proteins and changes in the cytoskeleton are due to the activity of Rho GTPases Cdc42 and Rac1 and its effector kinase activated by p21 isoforms 1 and 3 (PAK1 / 3). This thesis was first conducted a literature review of the PAK 1 and 3 which carry on about the following aspects: structure, mechanisms of action, located in the central nervous system of mammals, substrates targets, cytoskeletal regulation and participation in neuroplasticity processes. Secondly we value the importance of PAK 1 and 3 in the CA1 region of the hippocampus, related to reconsolidation, extinction and reacquisition of aversive memory in contextual fear conditioning (CFC). For this purpose, male adult rats underwent stereotaxic surgery to implant cannulae in the hippocampus CA1 region for infusion of 3% DMSO (vehicle) or IPA-3 (allosteric inhibitor of PAK 1 and 3). To check the reconsolidation; on day 1, the animals were trained in CFC; day 2 suffered reactivation and immediately or 3 hours after receiving infusions; day 3 were tested in CFC. To assess extinction: day 1 the animals were trained in the CFC; day 2 and 3 were trained to extinction and immediately or 3 hours after receiving infusions; day 4 were tested in CFC. To check reacquisition: day 1 the animals were trained in the CFC; day 2, 3:04 trained to extinction; day 5 were again trained in the CFC and immediately or 3 hours after receiving infusions. The results show that blocking the PAK 1 and 3 in CA1 not affect the reconsolidation of aversive memory. The lock 1 and 3 PAK in CA1 immediately after the first extinction training slows this process, but does not prevent the aversive memory is extinguished. However, infusion IPA-3 3h after extinction training mnemonic does not affect this process. The reacquisition, the lock 1 and 3 PAK immediately, but not 3 h after training (day 5) prevents this memory is strengthened again. Thus, we suggest that blocking PAK can affect the molecular mechanisms underlying the extinction and reacquisition of CFC memory, but has no effect on the mechanisms related to the reconsolidation.

Introdução

Aprendizado e Memória

Memória é a propriedade que nos permite aprender ao longo da nossa existência, seja pelas experiências que vivenciamos diretamente ou por fatos e eventos que observamos ou sobre os quais lemos e/ou escutamos. Nosso comportamento, nossas reações diante de determinadas situações, os valores e critérios que consideramos para guiar nossas atitudes, são fortemente influenciados pelo que aprendemos a partir da nossa educação e pelas experiências que vivemos. Assim, memória pode ser definida como a propriedade que nos permite adquirir, consolidar e gerenciar informações, de maneira que podemos evocá-las no futuro (Izquierdo, 2002).

A todo momento, em qualquer ambiente estamos expostos a informações de diversas naturezas, tais como cores, sons, formas, cheiros, dentre outros que, juntos ou isolados, tem uma importância maior ou menor para nós. Determinadas informações em certas situações podem não ter muita importância, como a cor da camiseta de um balconista que nos atende num mercado. Porém, é importante sabermos o significado das cores de um semáforo, caso contrário nossa vida estará em risco. Poder lembrar e associar fatos nos permite reviver momentos de valor inestimável, como o de escutar a voz daquela vó que já não está mais aqui, seus passos lentos e cuidadosos pelo chão da cozinha e, até mesmo, sentir o cheiro daquele café recém-feito por ela, simplesmente por saboreamos uma fatia de pão caseiro quente semelhante ao que ela fazia. Por causa dessa lembrança somos arremessados ao passado ou o buscamos até nós, sentindo a estranha mistura de falta e prazer chamada saudade.

A memória é a propriedade que define nossos valores, personalidade, fraquezas e poderes, fazendo de nós quem somos, nos permitindo existir e compor a sociedade de maneira muito particular.

A formação da memória se dá por eventos neurofisiológicos que levam ao fortalecimento da conexão sináptico de determinados grupos neuronais em certas regiões do encéfalo. Algumas memórias podem ser guardadas por um período muito curto, como algumas poucas horas apenas, sendo chamadas de memória de curta duração (MCD). Como exemplo podemos citar o horário exato em que tomamos um café na tarde do dia anterior (Figura 1). Essas memórias são formadas independentemente de síntese de novas

proteínas, de maneira que não fica estabelecida a formação de um traço forte, estável e duradouro onde a informação pode ser buscada por ocasião da evocação.

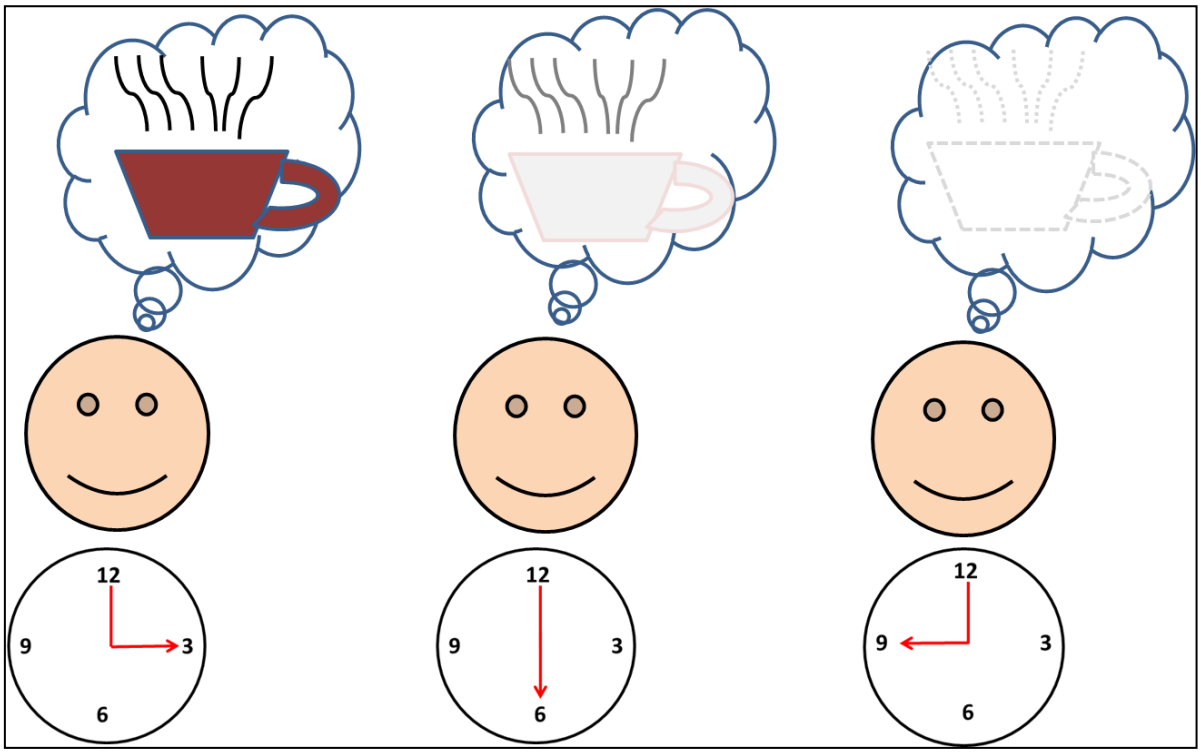

Figura 1. Memória de Curta duração. A MCD não forma um traço estável e tente a desaparecer em poucas horas. Conforme podemos observar na imagem da xícara de café, a MCD não é armazenada por um período maior que 4-6hs.

Outras memórias são armazenadas por um período mais longo, como dias, meses, anos e, até mesmo por toda a vida, que conhecemos como memória de longa duração (MLD). Esse é o caso de informações como o nome da cidade onde passamos parte da nossa infância e cursamos o ensino fundamental. Diferentemente das MCD, a MLD é formada de maneira dependente de síntese de novas proteínas, dando origem a um traço estável e duradouro de onde a informação pode ser acessada por ocasião da evocação (Izquierdo & Medina, 1995, 1997) (Figura 2).

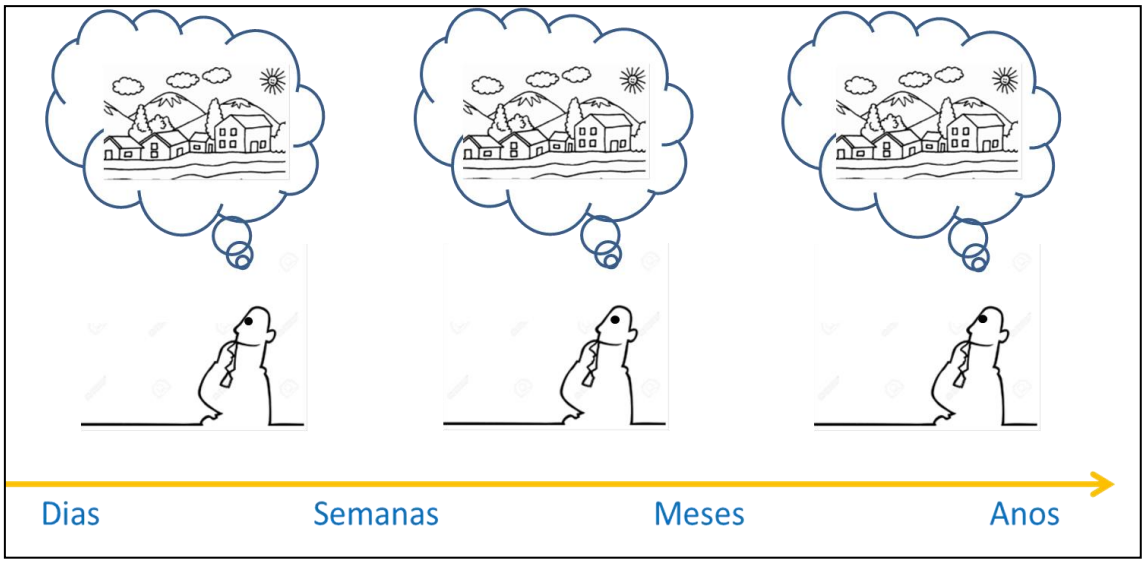

Figura 2. Memória de Longa Duração. Adaptado de http://fr.123rf.com/photo. A MLD decorre da formação de um traço estável que pode ser evocada mesmo depois de muitos anos.

Mecanismos neurobiológicos da formação da memória

O processo de formação da memória decorre de mecanismos celulares tanto em nível molecular quanto morfológico em determinados grupos neuronais de diversas regiões encefálicas. Após a aquisição da memória, mudanças na atividade neuronal nas sinapses envolvidas levarão a formação da MCD. Mas, ao longo das seis próximas horas após a aquisição a rede neural ativada pela informação recebida sofrerá alterações morfológicas e funcionais que estabilizarão a memória adquirida e formará a MLD, processo denominado consolidação sináptica da memória (Lamprecht & LeDouxes, 2004) (Figura 3).

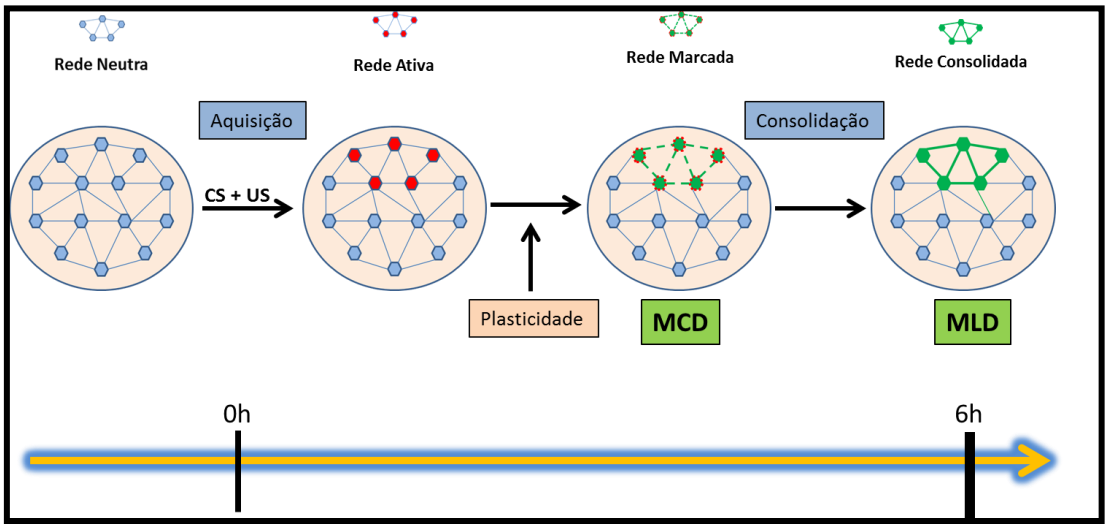

Figura 3. Formação da memória ao longo do tempo. Ativação, marcação e consolidação da rede neural, ao longo das seis primeiras horas após a aquisição da memória.

Durante a fase de consolidação a informação adquirida está instável e susceptível a interferências. Os eventos moleculares subjacentes à consolidação da memória envolvem a atividade de diversas moléculas, tais como cinases, segundos mensageiros e fatores de transcrição, relacionados com a neuroplasticidade. Essa cascata de eventos leva a síntese de novas proteínas, incremento do citoesqueleto de actina, aumento na densidade de receptores na superfície celular, especialmente do tipo AMPA (a-amino-3-hidroxi-5-metil-4-isoxazolproprionato), de maneira a fortalecer a atividade nas sinapses estimuladas pelo aprendizado (He et al., 2014; Holcman & Triller, 2006).

O mecanismo de gatilho do processo de plasticidade se dá com ativação de receptores glutamatérgicos dos tipos AMPA e NMDA (N-metil-D-aspartato) possibilitando o influxo celular de Na^{+} abertura de canais de Ca^{++} voltagem dependente (Paoletti et al., 2013; Yashiro & Philpot, 2008). No seguimento desencadeia-se uma cascata de sinalização onde há a ativação de diversas proteínas cinases, dentre elas a proteína cinase dependente cálcio calmodulina (CaMK), cinase ativada por cAMP (PKA) e proteína cinase dependente de cálcio (PKC), que levam às alterações estruturais e consequente aumento da eficiência da sináptica (Frankland et al., 2001; Izquierdo et al., 2006; Tanaka & Nishizuka, 1994) (Figura 4). As mudanças estruturais resultam no aumento da densidade pós-sináptica (PDS) pela inserção de novas proteínas, como receptores e proteínas estruturais do citoesqueleto, bem como formação de novas sinapses nos neurônios piramidais da região CA1 do hipocampo. Além disso, durante o processo de plasticidade também é verificada a translocação de poliribossomos às sinapses, o que facilitaria a síntese e inserção de novas proteínas a sua maquinaria (Barria & Malinow, 2005; Kennedy, 1997).

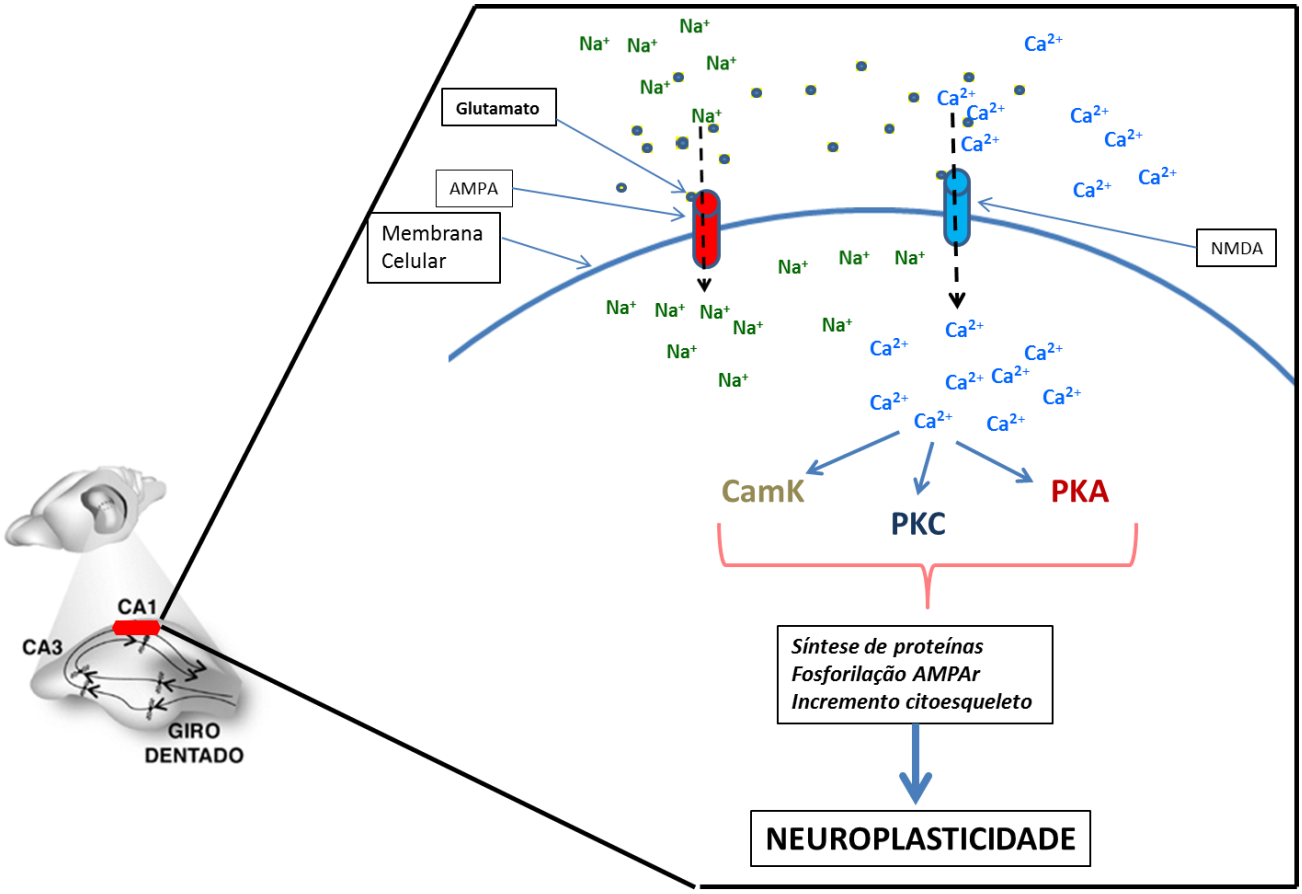

Figura 4. Sinápses glutamatérgicas na região CA1 do hipocampo. Atividade dos receptores AMPA com influxo de sódio e despolarização da células seguido da ativação dos receptores NMDA e influxo de Ca++ e ativação das cinases CamK, PKC e PKA.

A repetida estimulação das sinapses alvo nos neurônios piramidais da região CA1 do hipocampo levam a potenciação de longa duração (LTP – do inglês Long term potentiation), fenômeno eletrofisiológico que resulta na amplificação da força sináptica, aumentando a amplitude da corrente excitatória pós-sináptica (EPSC) (Lisman et al., 2012; Lømo, 2003; Malenka & Bear, 2004). A LTP tem algumas particularidades em comum com o processo de consolidação da memória e é importante para que esse processo mnemônico seja bem sucedido. Já está bem demonstrado que a LTP tem uma fase precoce (E-LTP), que dura cerca de 3h e não depende de síntese de novas proteínas, e uma fase tardia (L-LTP), que pode durar até dias e depende da síntese de novas proteínas. A MCD ocorre em paralelo com a E-LTP, durante as primeiras 2-3hs a pós o estímulo disparado pela aquisição. Se esse estímulo for forte o suficiente para induzir a síntese de novas proteínas, a L-LTP ocorrerá e o traço mnemônico será consolidado em MLD. Mas, se o estímulo não for suficiente para induzir a síntese proteica, o potencial decai em níveis basais. Dessa forma, a LTP não se manterá e o aprendizado não se estabilizará (Makino & Malinow, 2009; Malenka & Bear, 2004; Schafe & LeDoux, 2001).

Durante a LTP há o aumento da fosforilação do sitio Serina 845 (Ser845) das subunidades GluR1 de AMPA por PKA, ainda no citoplasma, seguido pela translocação dessa subunidade para a membrana plasmática. Já na membrana, GluR1 é fosforilado também em Serina 818 (Ser818), por PKC, o que eleva a condutância através do AMPA e sustenta a LTP. O contrário acontece durante a redução prolongada da atividade neuronal, conhecido como Depressão de Longa Duração (LTD – do inglês long term depression), diminuindo a fosforilação e translocação de GluR1. Esse processo de fosforilação e translocação de GluR1, do citosol para a membrana plasmática, representa um evento chave para os mecanismos de plasticidade que medeiam os processos mnemônicos. (Derkach et al., 2007; Newpher & Ehlers, 2008; Huganir & Nicoll, 2013).

As alterações morfológicas no citoesqueleto de actina são moduladas por eventos que controlem a polimerização dos monômeros de actina. Esta proteína encontra-se no citoplasma na forma de G-actina (monômero) e F-actina (filamento polimerizado) e sua polimerização é modulada negativamente pela Cofilin. Por outro lado, a Lim Cinase (LIMK) inibe a atividade da Cofilin através da fosforilação do seu resíduo Serina 3 (Ser 3), favorecendo a polimerização do citoesqueleto de actina. (Edwards et al., 1999; Honkura et al., 2008; Lamprecht & Ledoux, 2004). Assim, a razão de F-actina/G-actina aumenta, resultando no incremento da cinética, forma e tamanho da estrutura celular, favorecendo o tráfego e aumento na densidade de receptores de membrana, aumentando os espinhos dendríticos em número e tamanho, eventos primordiais aos mecanismos de plasticidade sinápticas subjacentes aos processos de consolidação mnemônica (Lamprecht & Ledoux, 2004; Hotulainen et al., 2009).

A estimulação extracelular que resulta no rearranjo do citoesqueleto, levando a manutenção da LTP e consolidação da memória, requer a participação de um grupo de moléculas conhecidas como Rho GTPases, especialmente as pequenas GTPases Cdc42 (do inglês cell division cycle 42) e Rac1 (do inglês Ras-related C3 botulinum toxin substrate 1). Essas moléculas agem como um interruptor de sinalização subsequente ao estímulo extracelular, e se alternam entre a forma ativa, ligada a uma molécula de GTP, e inativa, ligada a uma molécula de GDP (Hall, 1998; Luo, 2000). As Rho GTPases aumentam a razão de fosforilação da proteína ligada ao elemento de resposta ao AMPc (CREB), fator de transcrição essencial a síntese de novas proteínas relacionadas com a plasticidade (PRP) envolvidas na manutenção da LTP e consolidação da memória (Martin et al., 1997; Milner, 1998). O mecanismo pelo qual Rac1 e Cdc42 estimulam a fosforilação de CREB ocorre, principalmente, através das cinases da via da proteína

cinase regulada por sinal extracelular (Erk) e da cinase ativada por p21 (PAK), as principais efetoras das Rho GTPases nos processos de neuroplasticidade (Kida & Serita, 2014; Koth et al., 2014; Ortega-Martínez, 2015) (Figura 5). Assim, interferências sobre a atividade dessas moléculas podem prejudicar as alterações morfológicas e a manutenção da LTP, comprometendo a estabilização do aprendizado.

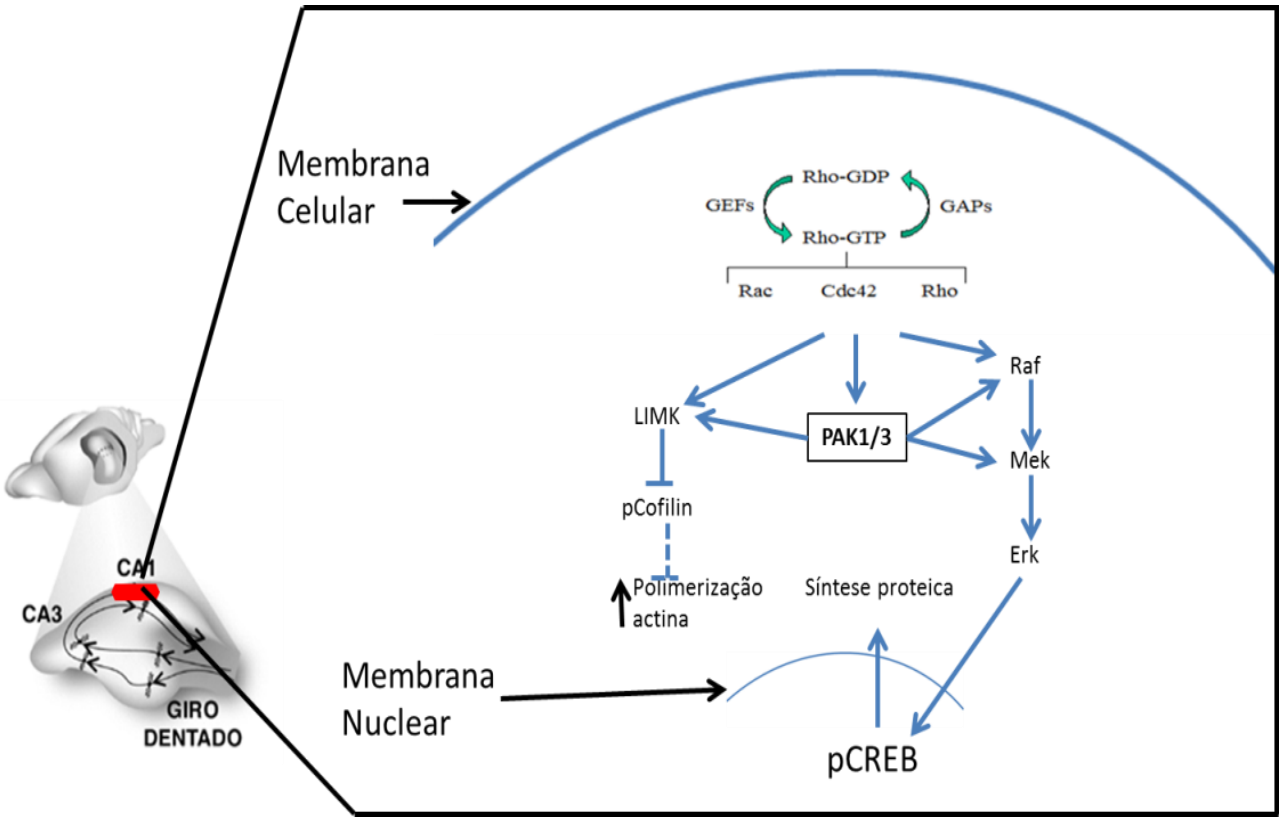

Figura 5. Rho GTPases e suas efetoras LIMK, PAK e cinases da via da Erk, nos neurônios da região CA1 do hipocampo. LIMK inativa Cofilin, estabilizando F-actina. Cinases Raf, Mek e Erk fosforilam CREB e estimulam síntese de proteínas. PAK atua tanto fosforilando e estimulando LIMK quando fosforilando e estimulando Raf e Mek.

Condicionamento pavloviano e o circuito do medo

O condicionamento pavloviano (CP) é um importante modelo de aprendizado e é extensamente utilizado nos estudos experimentais sobre memória. De acordo com o CP o indivíduo aprende a associar uma pista, tal como um som ou um lugar, com um evento de recompensa ou de perigo. Essa pista seria neutra até ser apresentada juntamente com o estímulo que causou a sensação de recompensa ou aversão (LeDoux, 2014; Maren, 2001). Seguindo esse princípio do CP temos o método de CFC um paradigma bastante utilizado nos estudos de memória aversiva. No medo condicionado contextual (CFC – *do inglês contextual fear conditioning*) o animal aprende a parear um estímulo anteriormente neutro (Estímulo condicionado – EC), tal como um contexto (caixa), a um estímulo aversivo (Estimulo incondicionado – EI), como um choque (em torno de 0,7mA) nas patas conduzido pelas grades que compõe o piso da caixa - EC. Quando o animal é colocado novamente dentro da caixa onde recebeu o choque, ele reconhece o ambiente, lembra que ali recebeu choques nas patas e apresenta um comportamento de medo conhecido como congelamento, do inglês *freezing* (imobilidade quase total, exceto pelos movimentos respiratórios), o que caracteriza uma resposta condicionada (RC) (Maren, 2001, 2008; Schafe *et al*., 2001) (Figura 6).

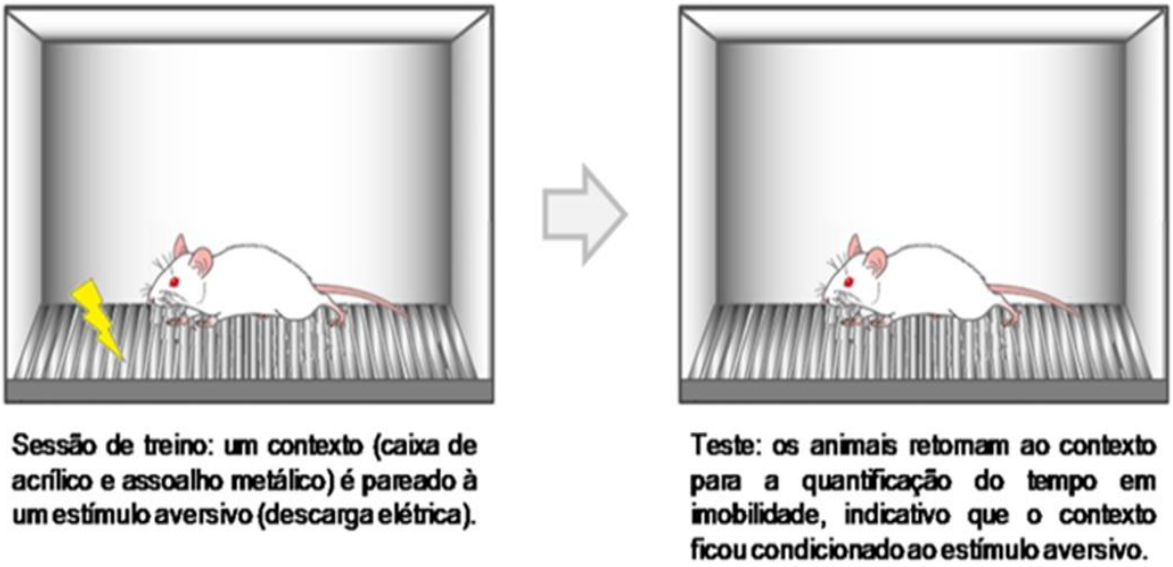

Figura 6. Experimento comportamental de Medo Condicionado Contextual. Adaptado de http://btc.bol.ucla.edu/fear.htm.

A memória de CFC envolve a ativação de determinados grupos neuronais dentro de locais já bem determinados no encéfalo, em especial o hipocampo, amígdala e o córtex pré-frontal medial (CPFm). Essas regiões são conectadas por aferências e eferências,

tanto excitatórias quanto inibitórias, formando um circuito neurológico para a memória de medo (Marek et al., 2013) (Figura 7).

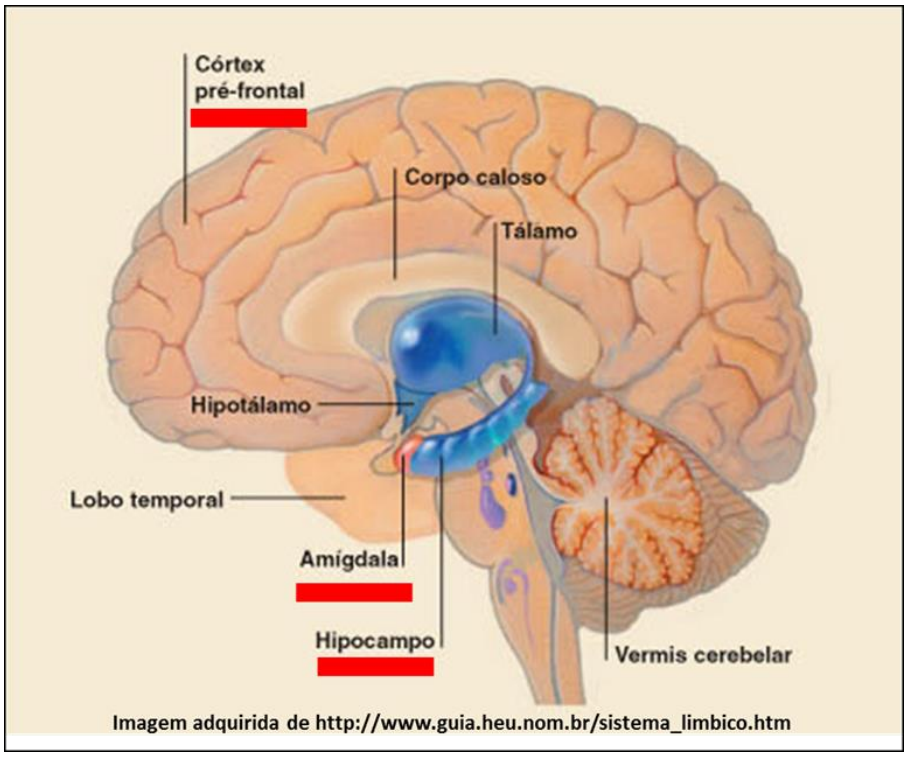

Figura 7. O Hipocampo, CPF e Amidala, as três principais áreas componentes do circuito do medo, que modulam a formação da memória aversiva e expreção do comportamento de medo, aparecem grifadas em vermelho.

O CPFm é subdividido em duas pequenas regiões que atuam de maneira antagônica sobre a consolidação da memória de CFC, denominadas CPF pré-límbico (CPFPL) e CPF infra-límbico (CPFIL). A amígdala, por sua vez, também é subdividida em regiões chaves ao condicionamento aversivo e modulação do medo, sendo as principais a amígdala basolateral (ABL – a principal via de entrada da amígdala) e o núcleo central da amígdala (AC – a principal via de saída da amígdala). No caso da memória de condicionamento contextual, a importância do hipocampo está diretamente relacionada com o armazenamento de informações que formam a representação contextual, quando e onde o evento aversivo aconteceu (Corcoran et al., 2005; Matus-Amat et al., 2004). Assim, quando o EC é pareado com o EI, e o animal aprende que em determinado contexto levará choques nas patas, projeções glutamatérgicas partindo do CPFPL e do hipocampo estimulam neurônios glutamatérgicos da ABL que se projetam para a AC. Essa via estimulatória ativa as projeções excitatórias da AC para regiões como a substância cinzenta periaquedutal (PAG) e hipotálamo, deflagrando o comportamento de medo e as

respostas fisiológicas ao estresse (Marek et al., 2013). Por outro lado, por ocasião da extinção da memória de CFC aumenta a atividade do CPFIL, que envia projeções glutamatérgicas para um grupo de interneurônios gabaérgicos conhecido como células intercaladas (CIT). Neste caso, as CIT emitem projeções gabaérgicas inibindo a atividade da AC, reduzindo a saída do estímulo da amígdala para a substancia cinzenta periaquedutal e ao hipotálamo, resultando numa diminuição ou desaparecimento no comportamento de medo (Janak & Tye, 2015; Johansen et al., 2011) (Figura 8).

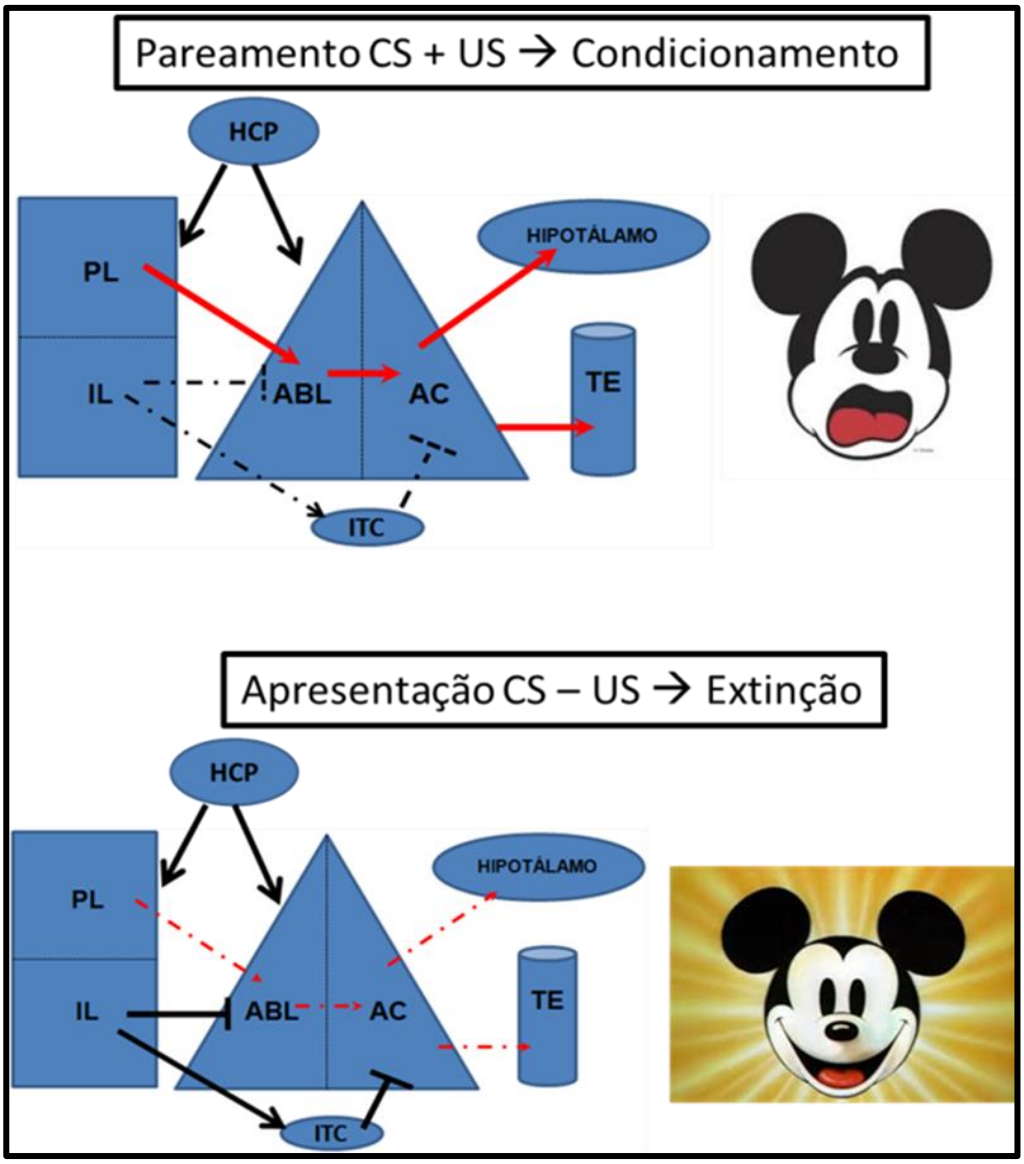

Figura 8. Condicionamento ou extinção da memória aversiva. CPFPL, ABL e AC estão ativos no condicionamento do medo e modulam positivamente o comportamento de medo. CPFIL, e ITC inibem a ABL e AC na extinção do medo, inibindo o comportamento de medo. O HCP é o principal local de armazenamento da informação sobre o contexto onde o condicionamento ou extinção ocorrem.

Reconsolidação da memória aversiva

Após a aquisição do aprendizado, uma série de eventos moleculares ocorre para que a memória adquirida se estabilize e permaneça por um longo período. A ativação de fatores de transcrição e o aumento na expressão gênica levam à síntese de proteínas relacionadas com a plasticidade (PRP), resultando no incremento das sinapses estimuladas e fortalecimento do traço mnemônico (Rogerson et al., 2014). No entanto, quando a memória é reativada através da sua evocação o traço formado e consolidado sofre alterações que desorganizam o citoesqueleto nas sinapses estimuladas e a memória aprendida volta a um estado instável (Nader et al., 2000; Sara, 2000) (Figura 9). Uma situação experimental bem demonstrada para a reativação da memória aversiva consiste em reexpor um animal treinado no CFC ao EC. Essa reexposição permite ao animal evocar a situação desconfortável causada pelo choque recebido nas patas durante o treino, o que consiste no mecanismo de reativação. Esse período lábil induzido pela reativação ocorre de maneira dependente da degradação de proteínas através da via proteolítica ubiquitina-proteossoma, cuja principal importância é a fragilização do traço, principalmente pela desorganização do citoesqueleto (Bustos, 2009; Kaang et al., 2009; Lee et al., 2008). Este estado "frouxo" da estrutura sináptica possibilita a renovação e atualização da informação aprendida, tornando possível a adição de novos fatores ao aprendizado original. No entanto, para que a memória original volte a se estabilizar, o que denominamos reconsolidação, é necessário a ativação de fatores de transcrição com nova expressão gênica e nova síntese de PRP. Assim, durante o período de labilização, fatores que afetam a polimerização da actina ou a síntese de PRP podem prejudicar a reorganização do traço e impedir a reconsolidação do aprendizado (Figura 9). Por outro lado, o processo de labilização também depende de alguns fatores, tais como idade da memória, força do estímulo e a duração da reativação. Por exemplo, memórias mais antigas são menos susceptíveis à fragilização do traço, sendo mais resistentes à reativação (Cassini et al., 2013; Lee, 2010). Também já é bem demonstrado que é necessário um tempo mínimo de duração da reativação. Conforme Bustos (2009) uma reativação de um minuto não é suficiente para causar a fragilização do traço do aprendizado de CFC, mas se a reativação perdurar por 3 minutos a desorganização do traço é bem sucedida e o processo de reconsolidação deve ocorrer (Bustos, Maldonado & Molina, 2009).

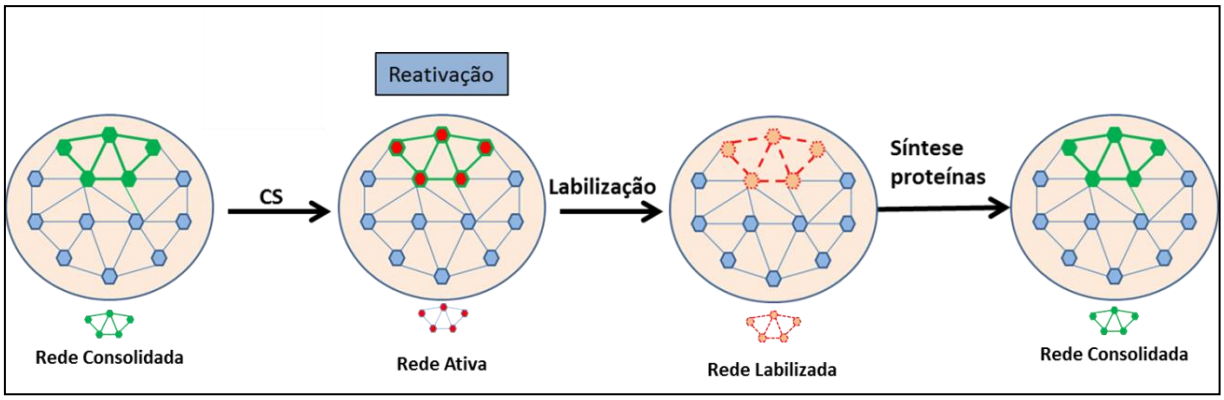

Figura 9. A reativação da rede consolida da a torna lábil novamente. Se houver nova síntese de proteínas, o citoesqueleto reorganiza-se e a memória reconsolida-se.

Extinção da memória aversiva

Os eventos moleculares e estruturais disparados pela reativação de uma memória previamente adquirida podem ser vistos como uma fronteira a partir da qual a experiência aprendida será mantida, atualizada ou enfraquecida. O desarranjo estrutural no citoesqueleto de actina nas sinapses relacionadas com a memória que está sendo reativada poderá ou não ser reorganizado. Ou seja, nem toda a reativação leva a manutenção do aprendizado original (Hardt et al., 2013; Roesler et al., 2014). Conforme já comentado, o que determina se a reativação conduzirá ao reforço do traço original ou a sua extinção ainda não são claros, mas sabe-se que a força do estímulo e o tempo de duração da reexposição são fatores importantes nos eventos pós-reativação (Cassini et al., 2013; Furini et al., 2014). O processo de extinção da memória de CFC pode ser disparado por repetidas reexposições ao EC na ausência do EI, de maneira que um novo aprendizado se formará indicando que determinadas condições não necessariamente precedem ou estão associadas a um evento aversivo, tal como um choque (Almeida-Corrêa, Amaral, 2014) (Figura 10). A extinção não consiste em apagar a memória original, mas na formação de uma nova memória onde a informação armazenada indica que o EC não necessariamente precede ou está associado ao EI (Furini et al., 2014) (Figura 10). Se considerarmos o CFC, administrar o choque – EI - nas patas do rato quando ele está no interior da caixa – CS – faz com que o animal associe o EC ao EI. Quando o animal é colocado novamente na caixa do MCC ele reconhece o contexto, lembra do choque e sente medo – comportamento de *freezing*. No entanto, se continuarmos a colocar o animal na caixa e não apresentar o choque, ele tenderá a não mais associar o contexto em que está ao

choque, extinguindo a memória original e diminuindo o comportamento de *freezing* (Almeida-Corrêa & Amaral, 2014; Furini et al., 2014; Pavlov, 1927).

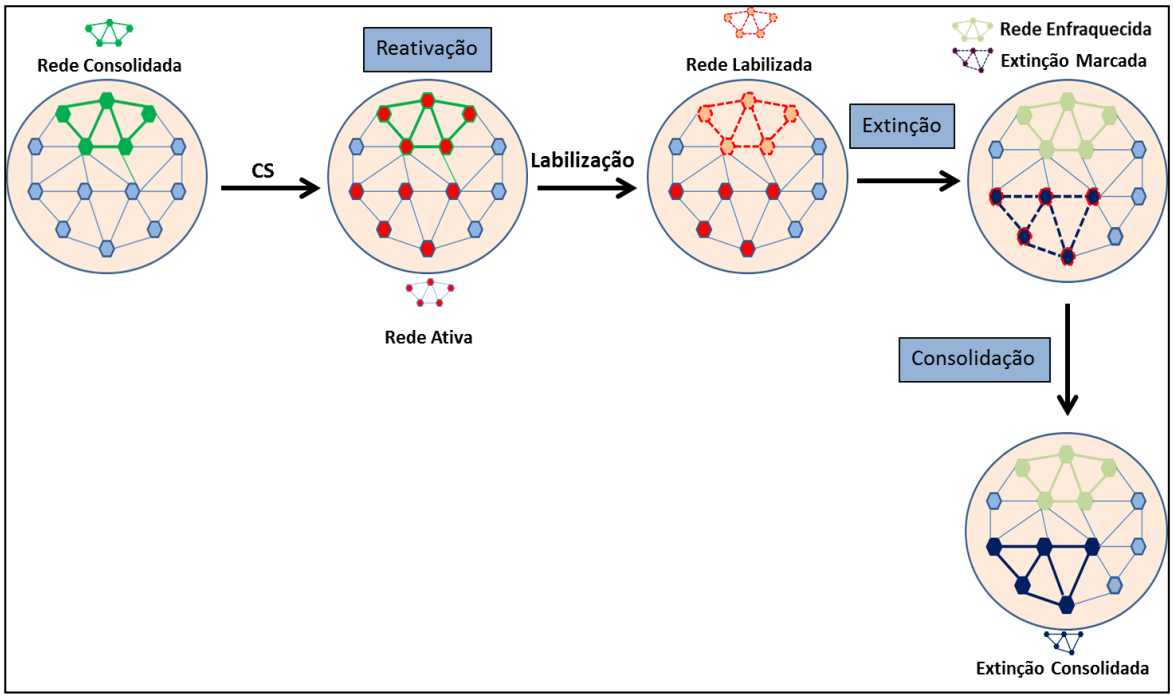

Figura 10. Formação e consolidação da memória de extinção. Após a reativação prolongada o traço original sofre enfraquecimento, enquanto a memória de extinção se fortalece. Apesar da extinção resultar na formação de uma nova memória, a memória original não é apagada, apenas é enfraquecida.

Conforme já comentamos, a extinção da memória de CFC envolve atividade de um circuito neurológico composto principalmente, pela amígdala CPFm e hipocampo. Ao contrário do que acontece no condicionamento do medo contextual, na extinção da memória de medo contextual a AC é inibida por projeções gabaérgicas que parte da ABL e da CIT, diminuindo o comportamento de medo. Essa atividade é modulada por projeções glutamatérgicas que partem do CPFIL diretamente para os neurônios gabaérgicos da CIT, e pela conexão recíproca entre a ABL e o hipocampo, cuja principal contribuição é a informação sobre o contexto envolvido no aprendizado (Bouton et al., 2006; Marek et al., 2013; Sotres-Bayon et al., 2006). O processo de extinção tem algumas semelhanças com outras formas de memória, tal como a consolidação. Para que a nova memória de extinção se consolide e se torne estável é necessária transcrição gênica e síntese de novas proteínas após o treino, em regiões como a amígdala, hipocampo e CPFm.

Assim, durante o período em que a memória de extinção está sendo consolidada fatores que interferem nos processos de síntese proteica podem prejudicar a estabilização dessa memória. Por exemplo, a infusão de anisomicina (um inibidor de síntese proteica) na região CA1 do hipocampo de ratos, 10 minutos antes ou imediatamente após o treino de extinção na esquiva inibitória, assim como a infusão de α-amanitin ou DRB (dois importantes inibidores da RNA polimerase) antes do treino de extinção, prejudicam a extinção da memória aversiva (Vianna et al., 2001; Vianna et al., 2003). A extinção da memória aversiva também é dependente da atividade dos receptores glutamatérgicos tipo NMDA, de maneira que o bloqueio desses receptores, tanto na amígdala quanto no hipocampo, pode prejudicar a extinção da memória de medo, indicando que esse receptor tem um papel chave no processo de extinção (Falls et al., 1992; Furini et al., 2014; Vianna et al., 2004). Ademais, já é bem demonstrado que moléculas chaves aos mecanismos de plasticidade subjacentes a consolidação da memória aversiva, tais como a Erk, PKA e CaMKII, também são importantes durante a consolidação da extinção do medo. A atividade dessas cinases no hipocampo e amígdala estão associadas com os mecanismos de síntese de proteínas durante a consolidação da extinção, sendo que sua inibição prejudica a estabilização da extinção e a redução do comportamento de medo (Vianna et al., 2004; Szapiro et al., 2003).

Reaquisição da memória aversiva

A reaquisição da memória aversiva consiste em adquirir novamente uma memória que foi previamente aprendida e, posteriormente, extinta. As repetidas reexposições ao EC na ausência do EI leva a extinção da memória aversiva original, de maneira que a apresentação do EC passa a não mais indicar que um evento aversivo está para vir (Pavlov, 1927). A extinção da memória aversiva consiste na formação de uma nova memória sem o pareamento dos EC e EI e não em apagar o traço da memória original. Assim, por ocasião da extinção a memória original acaba tendo seu traço enfraquecido, mas ele continua armazenado (Bouton, 1993, 2004) (Figura 10). Uma memória extinta pode ser revertida e o traço original ser fortalecido espontaneamente ou por ocasião de um novo pareamento entre os EC e EI, resultando no retorno da resposta de medo (Vervliet et al., 2013; Willcocks & McNally, 2014) (Figura 11). O processo de reaquisição da memória aversiva é relativamente rápido quando comparado com a

consolidação ou extinção (Anokhin et al., 2002; Rescorla, 2001). Igualmente a consolidação e a extinção da memória aversiva, a sua reaquisição se estabiliza de maneira dependente do rearranjo do citoesqueleto de actina, tanto no hipocampo quanto na amígdala, mas diferentemente da consolidação e extinção, a reaquisição não depende de síntese de novas proteínas. Assim, a infusão de citocalasina na região CA1 hipocampal e na ABL de ratos prejudica a reaquisição da memória aversiva no CFC, mas a indusão de anisomicina nas mesmas regiões não afetam esse processo mnemônico (Motanis &Moroun, 2012). Os mecanismos moleculares subjacentes à reaquisição da memória aversiva incluem a participação da p38 da subfamília de proteínas quinases activadas mitogen (p38MAPK). Isso foi bem demonstrado em estudo realizado por Rossato et a.l, (2006), em que ratos submetidos à extinção da memória aversiva na esquiva inibitória foram, posteriormente, treinados para reaquisição com a reapresentação do choque e imediatamente ou 3h após esse treino, foram infundidos na região CA1 hipocampal com um inibidor de p38MAPK. Foi verificado que os animais infundidos imediatamente, mas não 3h após o treino impediu a reaquisição da memória aversiva, demonstrando que p38MAPK é uma molécula chave na fase precoce da reaquisição da memória aversiva, mas não mais tardiamente (Rossato et al., 2006).

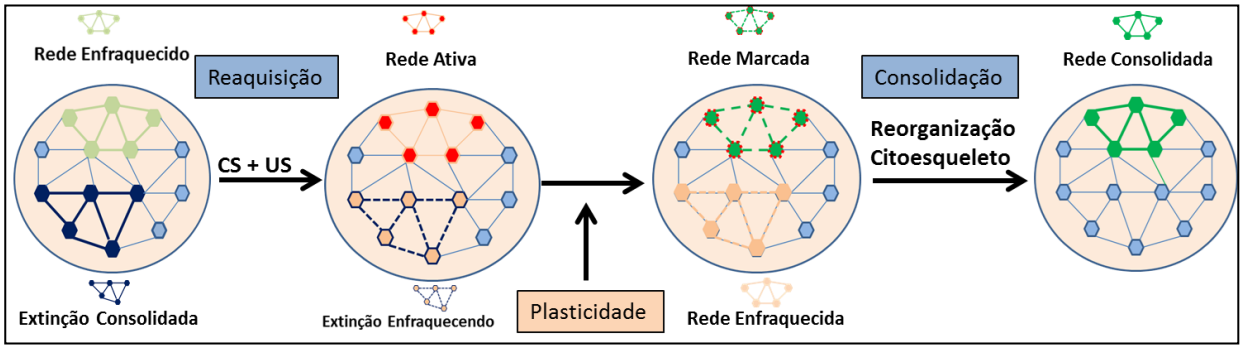

Figura 11. Reaquisição da memória. A reativação do traço original enfraquecido durante a extinção, leva à reorganização e fortalecimento da rede original.

Proteína cinase ativada por p21 (PAK)

As PAKs são cinases serina/treonina constituintes de dois grupos de seis isoformas, tais como: Grupo I (isoformas 1, 2 e 3) e grupo II (isoformas 4, 5 e 6). As PAKs 1, 2 e 3 são as mais importantes aos estudos relacionados a neuroplasticidade, possuem 93% de similaridade na sequência de seus aminoácidos e pesam 68, 62 e 65 kDa, respectivamente (Bokoch, 2003; Hofmann et ai, 2004; Manser et al, 1995; Shin et al., 2013). Essas cinases são estruturalmente similares entre si, apresentando um domínio regulatório N-terminal

e um domínio catalítico C-terminal, ambas regiões chaves para a regulação da atividade das PAKs (Manser et al., 1994). Na região N-terminal está o domínio de ligação de p21 (PBD – *do inglês p21 binding domain*), onde se ligam as pequenas GTPases da família Rho, moléculas chaves na regulação alostérica das PAKs 1, 2 e 3 (Figura 12). Dentro do PBD está um sítio denominado ligação interativa de Cdc42 / Rac (CRIB – *do inglês Cdc/Rac interative binding*), onde ligam essas duas moléculas, as principais GTPases responsáveis pela modulação alostérica da PAK 1, 2 e 3 (Figura 12). É também em N-terminal que está o domínio auto-inibitório (AID), presentes apenas nas PAKs do grupo I, onde a própria molécula modula sua atividade (Coniglio et al., 2008; Deacon et al., 2008; Coleman & Kissil, 2012) (Figura 12). Na região C-terminal está o domínio cinase, onde a PAK é fosforilada por outras cinases ou por outra isoformas da sua família. Dentre esses domínios está o treonina 423 (Thr423), sítio de fosforilação chave para ativação da atividade cinase das PAKs (Arias-Romero & Chernoff, 2008; Wang et al., 2011) (Figura 12).

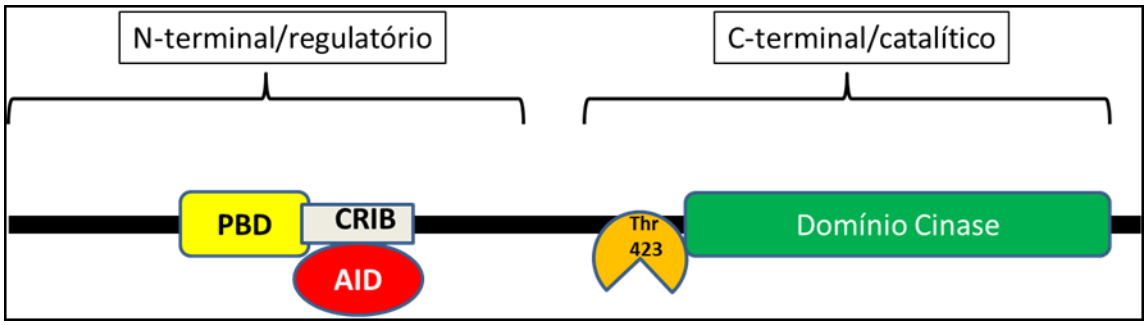

Figura 12. Estrutura molecular da PAK 1/2/3. Na região N-terminal (Regulatória) esta o PBD, o CRIB e o AID. Na região C-terminal (catalítico) esta o sítio Thr 423 e o domínio catalítico.

As PAKs se encontram na forma inativa, associadas em homodímeros ou heterodímeros, onde a extremidade regulatória de uma molécula sobrepõe-se à extremidade catalítica da outra molécula, de maneira que uma inibe a exposição do sítio de fosforilação Trh423 da outra, se inativando mutuamente (Figura 13). Para que essa cinase seja ativada são necessários dois processos consecutivos. Primeiramente as Rho GTPases ligam-se ao sítio CRIB e causam uma regulação alostérica no dímero, de maneira que as duas moléculas se separam e exibem seu sítio de fosforilação Trh423 (Figura 13). Em seguida, o sítio Trh423 pode ser fosforilado por outras cinases, tais como PDK1 e por outras isoformas da família da PAK (Bokoch, 2003; Koth et al., 2014) (Figura

13). As PAKs do grupo I são altamente expressas no sistema nervoso central, com uma distribuição relativamente distinta entre as três isoformas. Enquanto a PAK1 encontra-se principalmente no hipocampo, cerebelo, medula, córtex piriforme e núcleo talâmico, PAK3 também está nessas mesmas regiões e no bulbo olfatório, núcleo pré-óptico medial, amígdala, hipotálamo, tálamo e núcleo da rafe. PAK2, no entanto, é igualmente distribuída nos tecidos relacionados (Manser et al., 1995; Allen et al., 1998; McPhie et al., 2003; Boda et al., 2006). Já é bem demonstrado que PAKs do grupo I tem papel importante nos eventos moleculares e mecanismos intracelulares relacionados como síntese de proteínas, alterações do citoesqueleto e movimentos celulares. Diversos estudos demonstram evidencias da ligação das PAKs com patologias como o câncer e desordens neurológicas tais como Alzheimer, Retardo Mental e Doença de Huntington (Bokoch, 2003; Kumar et al., 2006; Luo et al., 2008; Ma et al., 2008; Minden, 2012; Ye & Field, 2012).

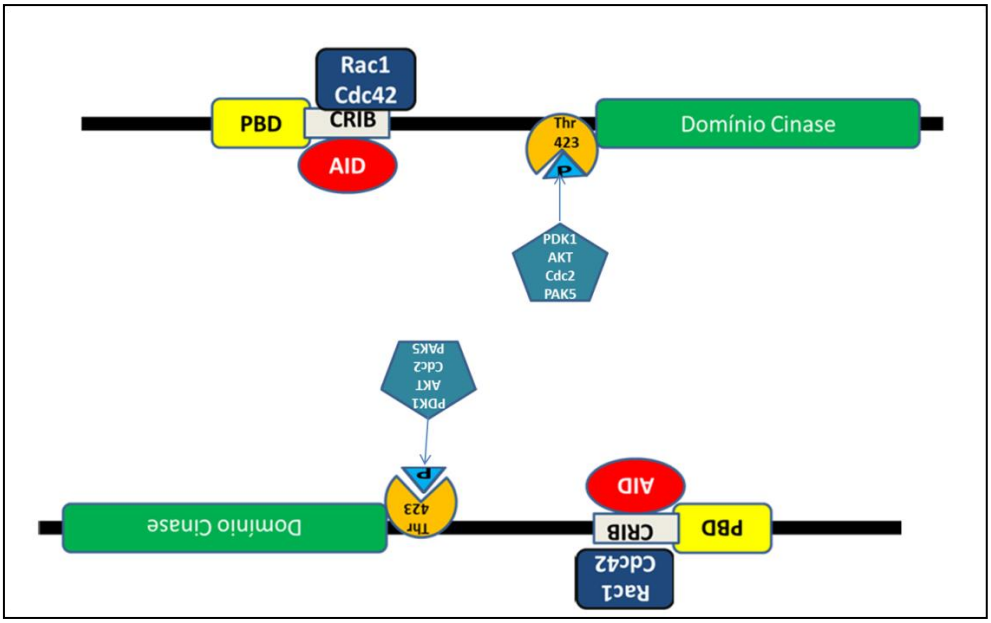

Figura 13. O dímero formado por duas moléculas de PAK que se inibem reciprocamente; A ligação da Cdc42 e Rac no domínio CRIB é seguida pela separação do dímero e exposição do sítio Thr423, que poderá ser fosforilado por outras cinases.

PAK e neuroplasticidade

As PAKs do grupo I, especialmente as isoformas 1 e 3, estão implicadas nos mecanismos celulares relacionados com a plasticidade neuronal. Os eventos moleculares que modulam a síntese de proteínas, polimerização do citoesqueleto de actina de espinhos dendríticos em neurônios glutamatérgicos da região CA1 do hipocampo decorrem de cascatas de sinalização nas quais as PAKs 1 e 3 tem atividade chave (Hayashi et al., 2004; Manser, 2012). A PAK pode interagir com cinases envolvidas com o processo de remodelamento da morfologia sináptica como LIMK, sendo esta a principal moduladora da enzima coafilin, que regula negativamente a polimerização da actina (Asrar et al., 2008; Kreis et al., 2009; Saneyoshi et al., 2010) (Figura 14). Na forma desforforilada cofilin é ativa a atua despolimerizando a F-actina, tirando os monômeros de actina dos filamentos. Cofilin é fosforilada em Ser3 e inativada por LIMK, mecanismo que inibe a despolimerização do citoesqueleto de actina PAK, por sua fez, fosforila LIMK em Thr508, estimulando sua atividade cinase sobre Cofilin (Edwards, 1999) (Figura 14). Assim, PAK se mostra um importante estabilizador do citoesqueleto de actina, uma vez que sua atividade sobre a LIMK previne indiretamente a labilização do citoesqueleto de actina pela Cofilin (Luo., 2000) (Figura 14).

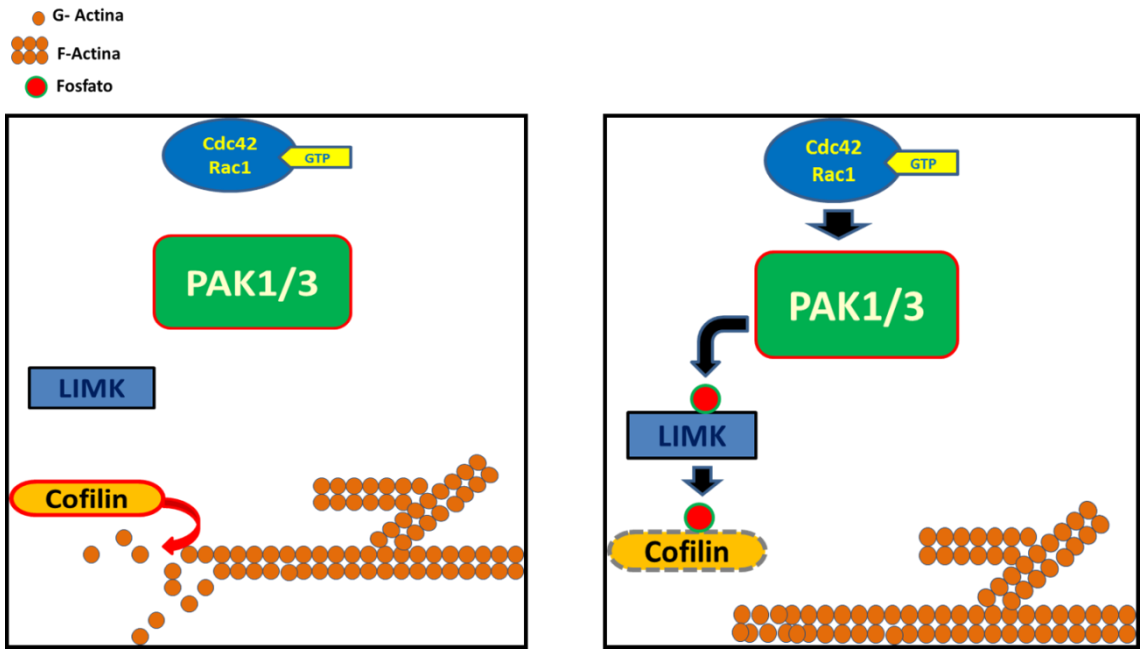

Figura 14. PAK estabiliza o citoesqueleto de actina. PAK fosforila LIMK, que fosforila e inibe Cofilin, prevenindo a despolimeração do citoesqueleto de actina.

Estudos mostram que a PAK leva a fosforilação de Raf-1 (MAPKKK) no resíduo Serina-338 (Ser338) melhorando seu acoplamento Ras-Raf, acarretando em sua máxima atividade. Também foi observada a capacidade da PAK de fosforilar a Mek1(MAPKK) no resíduo Serina-298 (Ser298) induzindo a autofosforilação em seu loop de ativação tornando-a ativa independentemente da via Ras/Raf (Figura 15). As cinas Raf, Mek e Erk (MAPKKK, MAPKK e MAPK) compõe uma cascata de sinalização intracelular conhecida como via da Erk, importentes moduladoras positivas da fosforilação do fator de transcrição CREB (Frost et al., 1997; Hofmann., 2004) (Figura 15). O aumento nos níveis de fosforilação de CREB estimulam a transcrição gênica e síntese de novas proteínas (Figura 15), possibilitando o encremento estrutural da célula nervosa e aumento da eficiencia na transmissão sináptica (Kida., 2014; Ortega-Martínez., 2015. Estas observações tornam a PAK um importante regulador da Erk (Frost et al., 1997; King et al., 1998; Slack-Davis et al., 2003 Parket et al., 2007) e dos mecanismos de transcrição gênica. Posteriormente em camundongos *knockouts* (KO) para PAK 3 foi demonstrada uma diminuição da fosforilação do fator de transcrição CREB, o que sugere o envolvimento dessa cinase na regulação do incremento do citoesqueleto e desenvolvimento da plasticidade sináptica e memória (Meng et al., 2005).

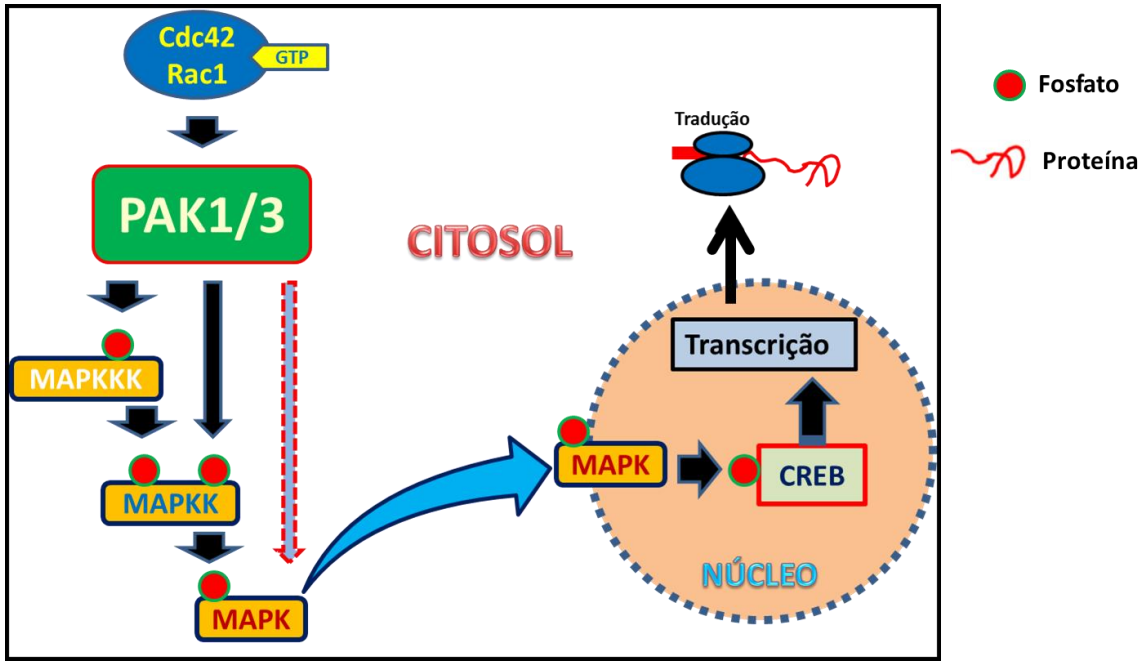

Figura 15. PAK afeta a transcrição gênica, via fosforilação das MAPKs e indução da fosforilação do CREB.

Outros dois importantes mecanismos pelos quais a PAK influencia as alterações estruturais do citoesqueleto é pela sua atividade cinase sobre a Miosina de Cadeia Leve II (MLC) e a Miosina de cadeia Leve Cinase (MLCK) (Dummler et al., 2009; Zhang et al., 2005) (Figura 16). A MLCII atua estimulando a atividade contrátil da miosina sobre a F-actina, favorecendo o deslocamento e projeção desses filamentos ao longo do citosol (Figura 16). A MLCK, por sua vez, fosforila MLC em Thr 18 e Ser 19, estimulando sua atividade sobre a miosina (Kneussel & Wagner, 2013) (Figura 16). É bem demonstrado que PAK 1/2/3 são importantes catalizadoras da fosforilação de MLC em Ser 19, e a forma inativa da PAK 1 e 3 reduzem os níveis de MLC fosforilada em Ser 19, bem como diminuem o tamanho das espeinhas dendriticas (Dummler et al., 2009; Zhang et al., 2005) (Figura 16). Os mecanismos celulares que levam às modificações estruturais do citoesqueleto e incremento morfológico das sinapses relacionadas com a formação da memória dependem da capacidade de deslocamento das moléculas ao longo do citosol. O deslocamento da F-actina até a superfície dos espinhos, bem como a capacidade dos filamentos de se contraírem ou relaxarem são ecenssiais para as alterações da dinâmica e da estrutura celular (Lamprecht, 2014). Também, a capacidade motora do citoesqueleto de actina suporta a atividade de deslocamentode receptores ao longo do citosol até a membrana, bem como sua inserção na membrana celular. Essas alterações resultam no aumento no tamanho dos espinhos, bem como no enriquecimento da densidade pos-sinptica, levando a uma maior eficiencia na comunicação entre os neurônior que compõe um engrama mnemônico (Kneussel & Wagner, 2013; Lamprecht, 2014). Por outro lado, PAK 1, 2 e 3 inibe a atividade cinase de MLCK sobre MLC, pela sua fosforilação em Ser 439 e Ser 991 (Dummler et al., 2009; Sanders et al., 1999) (Figura 16). Atravéz desse mecanismo, a atividade da PAK pode conduzir à inibição do processo contrátil entre a miosina e F-áctina (Goeckeler et al., 2000; Li et al., 2005) (Figura 16).

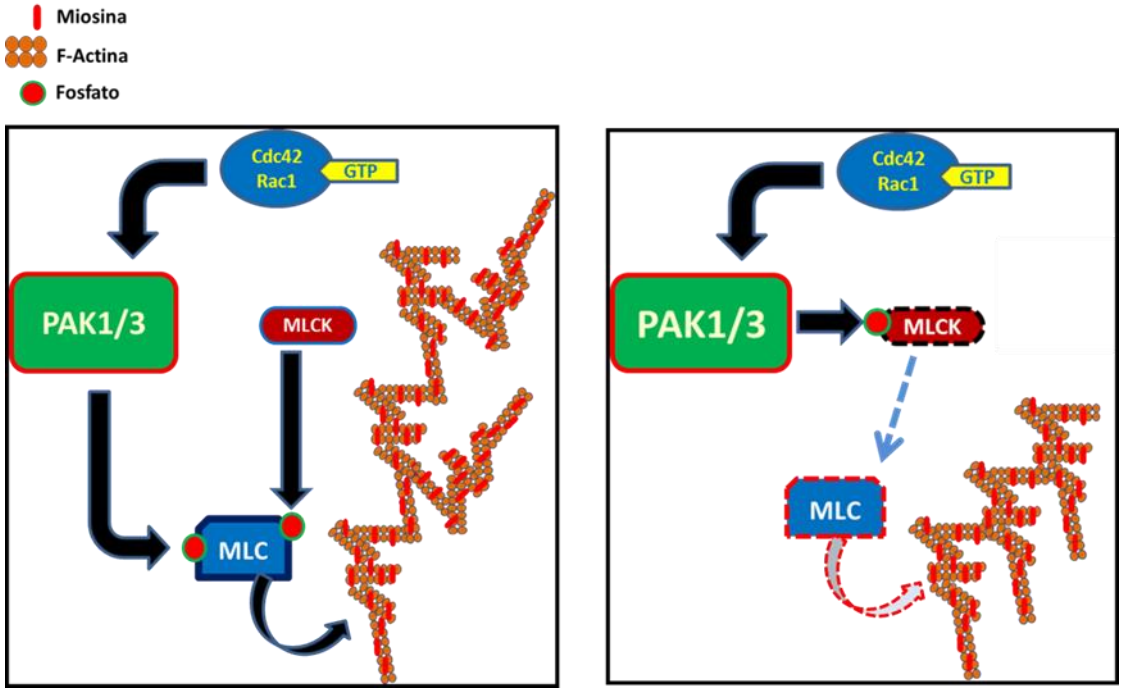

Figura 16. PAK fosforila MLC e MLCK, estimulando e inibindo a atividade contrátil de miosina sobre F-actina, respectivamente.

A PAK1 afeta as alterações morfológicas do citoesqueleto também por sua interação com a Filamina A (FLNa) (Bokoch, 2003) (Figura 17). A FLNa é uma proteína que se localiza próximo a membrana celular e medeia a interação de outras moléculas citoplasmáticas, com a membrana (MacPherson and Fagerholm, 2010; Muriel et al., 2011). As F-actina pode ligar-se a sítios específicos na FLNa e, assim, usá-la como âncora para estabilizar-se próximo a membrana celular. A FLNa também tem um papel importante na estabilização de receptores de membrana, coordenando a interação desses receptores ao citoesqueleto de actina (MacPherson and Fagerholm, 2010; Parton et al., 2013). Assim, a FLNa consiste numa proteína andaime para outras moléculas, permitindo a interação entre proteínas intracelulares e componentes da membrana, bem como a interação de receptores e o citoesqueleto e a colocalização de proteínas relacionadas com a transdução de sinal, participando diretamente dos mecanismos de rearranjo do citoesqueleto de actina como resposta ao estimulo extracelular (MacPherson and Fagerholm, 2010; Muriel et al., 2011; Parton et al., 2013). A PAK1, por sua vez, tem um papel importante na atividade de FLNa, uma vez que fosforila essa proteína no sítio Ser 2152, tanto em vitro quanto em vivo (Mendoza et al., 2011; Vadlamudi et al., 2002) (Figura 17). Esta demonstrado que essa fosforilação de FLNa em Ser 2152, por PAK1 é necessária para que FLNa atue adequadamente no processo de modificação estrutural do

citoesqueleto de actina, sendo essencial para a formação das chamadas membranas de ruffling, importantes progeções do citoesqueleto de actina que permitem a motilidade da célula (Mendoza et al., 2011; Vadlamudi et al., 2002). Por outro lado, a atividade da PAK1 na ausência da atividade de FLNa, falha no processo de formação dessas membranas de rufling, indicando que ambas as proteínas tem dependência mutua nos mecanismos relacionados com as modificações morfológicas do citoesqueleto (Vadlamudi et al., 2002).

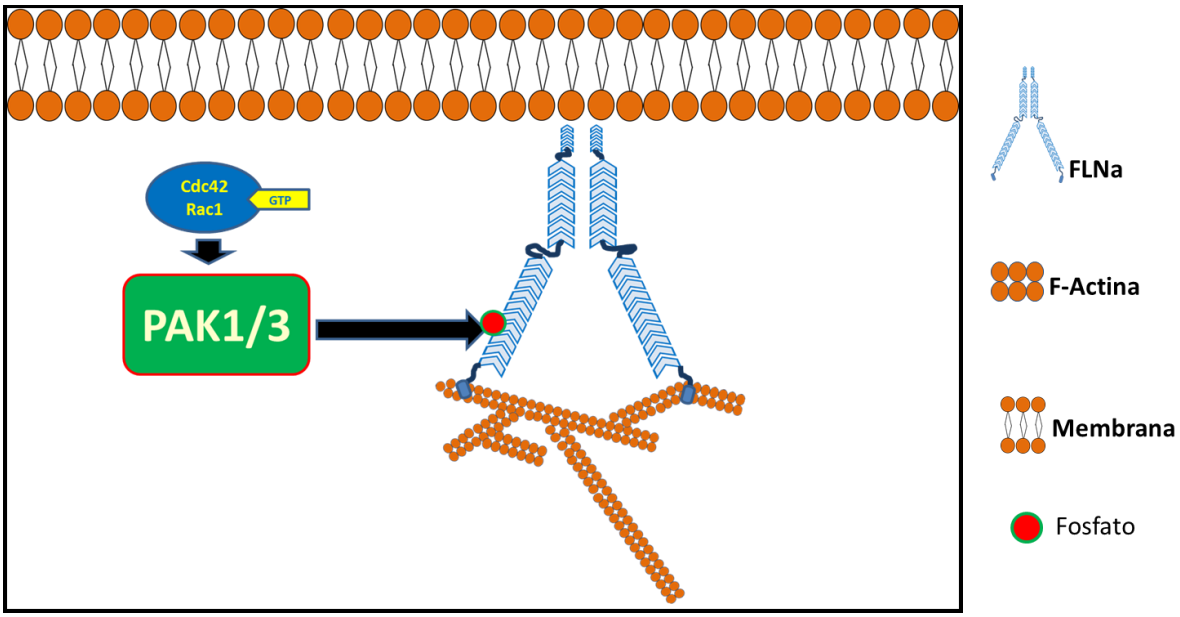

Figura 17. PAK interage com FLNa. PAK fosforila FLNa em Ser 2152, estimulando a atividade de FLNa no seu papel de "molécula âncora" para outras moléculas próximo à membrana.

Dessa forma, esta bem demonstrada a importância da PAK nos mecanismos de modificações na atividade molecular e na estrutura da célula, por ocasião de um estimulo extracelular. A relação da PAK com outras moléculas tais como LIMK, MAPKs, MLC, MLCK e FLNa evidenciam a sua participação em mecanismos essenciais as modificações morfológicas da célula, a motilidade celular e ao deslocamento de proteínas ao longo do citosol, sendo todos esses processos muito importantes para os mecanismos plásticos que estão subjacentes a formação e/ou enfraquecimento de um traço mnemônico.

Assim, podemos sugerir que a PAK é potencialmente importante para a formação da memória e aprendizado. Pensamos que interferência farmacológica que iniba a atividade da PAK na região CA1 do hipocampo durante o fortalecimento de uma memória

contextual poderia prejudicar a estabilização dessa memória. Nesta tese de doutorado visamos entender sobre a importância da PAK 1 e 3 nos mecanismos mnemônicos, com foco na reconsolidação, extinção e reaquisição da memória aversiva de Medo Condicionado Contextual.

Nesta tese de doutorado visamos entender sobre a importância da PAK 1 e 3 nos mecanismos mnemônicos, com foco na reconsolidação, extinção e reaquisição da memória aversiva de Medo Condicionado Contextual.

Objetivos

Objetivo Geral

Escrever uma revisão bibliográfica sobre as PAKs do grupo I e avaliar a importância dessas cinases aos mecanismos de reconsolidação, extinção e reaquisição da memória aversiva na região CA1 do hipocampo de ratos Wistar.

Objetivos Específicos

- Escrever uma revisão bibliográfica sobre a PAK isoformas 1, 2 e 3, com ênfase em sua estrutura molecular, mecanismos de ativação, localização no sistema nervoso central de mamíferos, substratos alvos, seu papel na regulação do citoesqueleto e sua participação na neuroplasticidade.

- Verificar o papel da PAK 1 e 3 na região CA1 do hipocampo de ratos Wistar, às 0h ou 3h após o treino de reconsolidação da memória aversiva no CFC;

- Avaliar o papel da PAK 1 e 3 na região CA1 do hipocampo de ratos Wistar, às 0h ou 3h após o treino de extinção da memória aversiva no CFC;

- Avaliar a importância da PAK 1 e 3 na região CA1 do hipocampo de ratos Wistar, às 0h ou 3h após o treino de reaquisição da memória aversiva no CFC;

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Participation of group I p21-activated kinases in neuroplasticity.

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Author information

Abstract

PAKs are a family of serine/threonine protein kinases activated by small GTPases of the Rho family, including Rac and Cdc42, and are categorized into group I (isoforms 1, 2 and 3) and group II (isoforms 4, 5 and 6). PAK1 and PAK3 are critically involved in biological mechanisms associated with neurodevelopment, neuroplasticity and maturation of the nervous system, and changes in their activity have been detected in pathological disorders, such as Alzheimer's disease, Huntington's disease and mental retardation. The group I PAKs have been associated with neurological processes due to their involvement in intracellular mechanisms that result in molecular and cellular morphological alterations that promote cytoskeletal outgrowth, increasing the efficiency of synaptic transmission. Their substrates in these processes include other intracellular signaling molecules, such as Raf, Mek and LIMK, as well as other components of the cytoskeleton, such as MLC and FLNa. In this review, we describe the characteristics of group I PAKs, such as their molecular structure, mechanisms of activation and importance in the neurobiological processes involved in synaptic plasticity.

1. Introduction:

Kinase activated by p21 (PAK) PAKs are proteins involved in several intracellular processes, such as signal transduction, cellular motility and cytoskeletal protein synthesis (Bokoch, 2003; Kumar et al., 2006). These proteins were first identified by Manser 1994 as effectors of the Rho family of small GTPases, specifically cell division cycle 42 (Cdc42) and Ras-related C3 botulinum toxin substrate (Rac). The Rho family of small GTPases becomes inactivated when bound to guanosine diphosphate (GDP) and activated when bound to guanosine triphosphate (GTP). In the latter form, Rho GTPases drive several intracellular molecular activities and directly bind to PAKs,allosterically modifying their structure and facilitating their phosphorylation via a third protein (Ong et al., 2002; Shin et al., 2013; Wang et al., 2013).

PAKs are a serine/threonine kinase protein family consisting of two relatively distinct subfamilies: group I, consisting of isoforms 1, 2 and 3, and group 2, consisting of isoforms 4, 5 and 6. The expression of PAK isoforms from both groups have been detected in the mammalian brain and other tissues, but aside from their structural and genetic similarities, these groups of PAKs are distinct and have different functions (Manser et al., 1995; Hofmann et al., 2004; Shin et al., 2013). Group I PAKs display high amino acid sequence similarity, and many pieces of evidence gathered in recent years suggest that this group plays unique roles in brain ontogenesis, neuronal differentiation and synaptic plasticity (Kreis et al., 2007).

Small GTPases promote allosteric regulation of PAKs in a manner that facilitates their phosphorylation and, therefore, their activation (Edwards et al., 1999; King et al., 2001). Once this occurs, PAKs perform their kinase activity on other serine/threonine kinases, such as the proto-oncogene c-Raf (Raf), extracellular signal-regulated kinase (ERK) kinases (MEKs), LIM kinase (LIMK) and myosin light chain kinase (MLCK), positively regulating actin protein synthesis and polymerization, essential events in the process of neuroplasticity (Goeckeler et al., 2000; King et al., 2001; Somlyo and Somlyo, 2003; Zang et al., 2008).

Several studies have revealed evidence linking the PAKs to pathological disorders, such as cancer, Alzheimer's disease, mental retardation and Huntington's disease (Luo et al., 2008; Ma et al., 2008; Minden, 2012; Ye and Field, 2012). PAK is also implicated in type 2 diabetes, since islets from type 2 diabetic individuals contain 80% less PAK1 protein than non-diabetic individuals (Kalwat et al., 2013). Moreover, a recent study demonstrated that this kinase plays an important role in gene expression modulations that were associated with DNA damage induced by ionizing radiation (Motwani et al., 2013).

In this review, we aim to provide information about the group I PAKs, isoforms 1, 2 and 3, concerning the following topics: their molecular structure, activation, localization in the neural system, substrates and importance in neural plasticity and learning.

2. Molecular structure

The group I PAKs include tree isoforms: PAK1, 2 and 3. These isoforms display 93% similarity in the amino acid sequence (Bokoch, 2003). Isoforms 1, 2 and 3 have a molecular weight of 68, 62 and 65 kDa, respectively. PAKs from both groups (I and II) contain an N-terminal regulatory domain and a C-terminal catalytic domain, which are the key sites for the regulation of their kinase activity by Rho GTPases and other kinases that phosphorylate (Manser et al., 1994; Bokoch, 2003).

The N-terminal region of PAKs contain a p21-binding domain (PBD), which possesses the binding site of the Rho family of small GTPases, including Cdc42 and Rac, termed the Cdc42/Rac interactive binding (CRIB) motif, as well as several polyproline (PXXP) motifs, which serve as binding sites of proteins that possess a Src homology 3 (SH3) domain, such as the Nck adaptor proteins, interacting exchange factor (bPIX) and growth factor receptor-bound protein 2 (Grb2) (Coniglio et al., 2008). The SH3 domain is commonly found in proteins that interact with other proteins to mediate the assembly of specific signaling complexes typically by binding to proline-rich regions of a binding partner (Coniglio et al., 2008; Deacon et al., 2008; Coleman and Kissil, 2012).

In the PAK C-terminal (catalytic) region, there is a highly conserved kinase domain that allows PAK to phosphorylate its substrates (Manser et al., 1998; Hofmann et al., 2004; Wang et al., 2011). Furthermore, in the N-terminal (regulatory) region of only group I PAKs, a self-inhibitory domain (AID), which is the source of the self-inhibitory capacity of PAK. In the inactive form, the AID of PAK binds to and downregulates the activity of the catalytic domain of other PAK molecules, acting as a trigger that regulates the basal activity of the kinase (Chong et al., 2001; Arias-Romero and Chernoff, 2008; Wang et al., 2011).

When inactive, the group I PAKs are organized as homodimers; in other words, two identical PAK isoforms are fused. This homodimer remains stable, with the N-terminal region of one PAK molecule overlying the C-terminal region of the other one (Bokoch, 2003). Under this condition, the PBD of each molecule comprising the dimer is partially twisted behind its AID, preventing the phosphorylation of Thr423 in the activation loop by other kinases (Chong et al., 2001; Arias-Romero and Chernoff, 2008; Wang et al., 2011). PAK must undergo an allosteric conformational modification to expose the Thr423 motif, which allows for its phosphorylation by other kinases to activate the kinase. Therefore, the important role of these signaling pathways is clear.

3. Mechanism of action

PAKs are kinases that serve as important effectors of GTPases, especially Cdc42 and Rac (Boda et al., 2006; Arias-Romero and Chernoff, 2008; Chi et al., 2013). The Rho family of GTPases participates in intracellular signaling pathways as a molecular trigger with many downstream targets. Among them, PAKs (Ong et al., 2002) are importantly associated with cellular morphological alterations, such as protein synthesis processes, actin polymerization and lamellipodia and filopodia projections (Etienne-Manneville and Hall, 2002; Sinha and Yang, 2008).

GTPases become activated when bound to GTP and inactivated when bound to GDP. GTPases are crucial for several cellular processes, such as cellular differentiation and proliferation, cytoskeletal organization, vesicle trafficking and nuclear transport (Etienne-Manneville, 2004). The mechanism of action of GTPases begins when a guanine exchange factor (GEF) disrupts the molecular interaction between the GTPase and GDP, facilitating the interaction between GTP and the GTPase. The opposite occurs when a GTPase-activating protein (GAP) promotes the hydrolysis of GTP to form GDP, inactivating the Rho GTPase (Etienne- Manneville and Hall, 2002; Bokoch and Zhao, 2006; Sinha and Yang, 2008; Chi et al., 2013).

When bound to GTP, Cdc42 and Rac associate with homodimer PAK molecules in the N-terminal region of the CRIB domain, causing a conformational alteration and destabilizing the dimerization, leading to the decoupling of the monomers (Galisteo et al., 1996; Bokoch, 2003; Li et al., 2003; Coleman and Kissil, 2012). This destabilization of the homodimer relies on the release of the AID via the binding of a Rho GTPase to the PBD of a molecule comprising the homodimer, which then dissociates from the catalytic region of the other molecule. Both molecules undergo this allosteric modification induced by Cdc42 or Rac to mutually release themselves (Coleman and Kissil, 2012; Shin et al., 2013).

Several PAK phosphorylation sites, such as Ser149, Ser198, Ser203 and Thr423 are associated with AID release (Zenke et al., 1999). This modification occurs due to the conformational change in the dimer caused by its interaction with Cdc42 and Rac. Thr423 has been considered as the most critical site that inhibits AID binding and stabilizes the molecule in its active phosphorylated form. The exposure of the Thr423 site in the activation loop provides a target for other kinase molecules via the key effector function

of phosphoinositide-dependent kinase-1 (PDK1) (Zenke et al., 1999; Lei et al., 2000; Coleman and Kissil, 2012; Eswaran et al., 2012).

PAK1 is also stimulated by phosphorylation at Ser21 and Thr212. Akt phosphorylates PAK1 at Ser21 independently of the binding of Rho GTPases; this phosphorylation induces the release of the Nck adapter protein from the N-terminal region of PAK1 and its translocation to the cytosol (Thiel et al., 2002; Zhou et al., 2003). Residue Thr212 of PAK1 is known to be a common target of cyclin-dependent protein kinase (Cdc2) and cyclin-dependent kinase 5 (Cdk5). Phosphorylation by Cdc2 at Thr212 is detected in mitotic cells and is critically associated with the morphological changes involved in cell division (Thiel et al., 2002), whereas phosphorylation of the same residue by Cdk5 in neurons appears to regulate the activity of PAK, a key event in the cytoskeletal remodeling of growing axons (Rashid et al., 2001).

4. Localization in the nervous system of mammals

Previous studies have demonstrated that group I PAKs are differentially distributed among tissues. Whereas PAK1 is present in the brain and the spleen, PAK2 is equally distributed throughout several tissues and PAK3 is predominantly expressed in the brain (Manser et al., 1995; Teo et al., 1995; Rousseau et al., 2003).

In the brain, PAK1 is found to be most strongly expressed in the hippocampal region, primarily in the CA1 region, the cortex (primarily in layers IV and V), the cerebellum, the medulla, the piriform cortex and the ventral/lateral thalamic nuclei. PAK3 is also expressed in these regions, but it is most strongly expressed in the hippocampal dentate gyrus and layers II, III and V of the cortex, as well as the olfactory bulb, the piriform cortex, the medial preoptic nucleus, the hypothalamus, the thalamus, the amygdala and the raphe nuclei (Manser et al., 1995; Allen et al., 1998; McPhie et al., 2003; Boda et al., 2006). PAK3 was detected in cells during their division cycle in the cerebral ventricles and in the subventricular zone (Teo et al., 1995). Previous studies have demonstrated that PAK isoforms 1 and 2 are more strongly expressed in oligodendrocytes, whereas PAK3 is more strongly expressed in neurons, and all three isoforms are strongly detected in the brain (Arias-Romero and Chernoff, 2008; Cahoy et al., 2008; Kreis et al., 2008).

The group I PAK isoforms are also differentially localized within neurons. The presence of this kinase has been detected in dendritic spines, and its active form is highly

elevated in the postsynaptic density (Hayashi et al., 2002, 2004). In hippocampal and cortical neurons, PAK1 is highly concentrated in axons and dendrites, whereas PAK3 is more highly concentrated in cell bodies and dendrites of larger diameter (Hayashi et al., 2002; Ong et al., 2002). In a study performed using cultured N1E – 115 neuroblastoma cells, PAK1 expression predominated along the neurite axes and the cell center, PAK2 expression predominated in filopodia and filopodial activity areas, and PAK3 expression was found in areas containing lamellipodia and membrane ruffling, such as the newly polymerized actin mesh (Marler et al., 2005). The expression levels of these kinases are influenced by various factors under both normal and pathological conditions (Kumar et al., 2006).

The expression of PAK1 is elevated in primary cortical neurons derived from mouse embryos, and its expression level is maintained until adulthood; alternatively, the expression of the PAK 3 is upregulated during neuronal differentiation and migration of GABAergic interneurons in the rat cortex (Souopgui et al., 2002; Cobos et al., 2007; Causeret et al., 2009).

Analysis of brain tissue from post-stroke survivors 2 or 6 days after ischemia revealed upregulation of PAK1 (Mitsios et al., 2007). The same result was detected in tissues obtained within 4 weeks after the ischemic event. PAK1 was also upregulated in rat brain tissues obtained 1, 12 or 24 h after ischemia, but the expression level of this kinase returned to baseline 7 days after the ischemic event (Mitsios et al., 2007). In another study, it was found that in genetically modified rats more prone to depression, PAK1 was downregulated in the frontal cortex compared animals resistant to depression (Nakatani et al., 2007).

5. Target substrates

PAK is associated with several intracellular mechanisms that are involved in many biological processes. The participation of PAK in those processes occurs via many molecules that act upstream and downstream of it. PAK becomes activated by the allosteric regulatory mechanism described above or by phosphorylation in the activation loop, which leads to the activation of other signaling molecules via the kinase activity of PAK (Bokoch, 2003; Boda et al., 2006; Eswaran et al., 2012).

The Erk pathway is an important pathway that is associated with PAK. Rho GTPases upregulate the Erk pathway, which consists of three kinases which are arranged from the cell membrane toward the nucleus in the following order: Raf, Mek and Erk, such that Raf phosphorylates Mek, and Mek phosphorylates Erk. The Erk pathway is a modulator of the cytoskeletal morphological changes in several tissues, including nervous tissue (King et al., 1998; Zang et al., 2002; Yi et al., 2010). This pathway is activated by Rho GTPases bound to GTP, leading to an increase in Erk activity via cyclic adenosine monophosphate response element binding protein (CREB). When phosphorylated, CREB induces the transcription of genes important for neuronal plasticity processes (Frost et al., 1997; Park et al., 2007; Cao et al., 2012).

Many studies have demonstrated that Mek1 and Raf1 are two direct substrates of PAK. Raf1 becomes activated upon phosphorylation at Ser 338, and phosphorylation of Mek1 at Ser298 facilitates signal transduction from Raf to Mek. Thus, Mek1 becomes activated (King et al., 1998; Zang et al., 2002; Yi et al., 2010; Field and Manser, 2012). Co-expression of constitutively active PAK and Raf can replace Rho GTPases as an activator of Erk, thus increasing the phosphorylation levels of this molecule (Frost et al., 1997). PAK also directly phosphorylates Mek at Ser298 in vitro and in vivo and elevates the level of human Mek phosphorylation at Thr292; both of these sites are essential for the Raf-Mek interaction (Frost et al., 1997; Park et al., 2007; Yi et al., 2010; Wang et al., 2013).

It was also demonstrated that inhibition of group 1 PAKs reduces Raf phosphorylation at Ser338 and Mek phosphorylation at Ser298 via platelet-derived growth factor (PDGF) and epithelial growth factor (EGF) (Manser and Zhao, 2012). PAK is also associated with the activity of LIMK, a serine/threonine kinase that promotes actin polymerization via cofilin inhibition (Edwards et al., 1999). While dephosphorylated, cofilin is active and downregulates actin polymerization. This condition is reversed by LIMK, which phosphorylates cofilin, inactivating it and preventing actin depolymerization (Moon and Drubin, 1995; Lamprecht and LeDoux, 2004; Rubio et al., 2012; Deo et al., 2013).

Prior studies have found that PAK can activate LIMK via Thr508 phosphorylation, increasing the capacity of LIMK to phosphorylate cofilin at Ser3 (Edwards et al., 1999). Therefore, the Rac and Cdc42 GTPases are associated with the actin cytoskeletal structure

by regulating its polymerization level via the kinase activity of PAK and LIMK on cofilin (Bokoch, 2003; Rubio et al., 2012; Aslan and McCarty, 2013; Deo et al., 2013).

Another group of mechanisms in which PAKs appears to be involved is the dynamic regulation of actin (Boda et al., 2006). Several studies have demonstrated that PAK plays a key role in myosin light chain (MLC) activity modulation in hippocampal dendritic spines as well as in mammal fibroblasts (Wirth et al., 2003; Zhang et al., 2005). MLC is found in dendritic spines and converts the energy from ATP hydrolysis to actin–myosin kinetic force. PAK and MLC kinase (MLCK) phosphorylate MLC at Ser19, activating myosin ATPase (Goeckeler et al., 2000; Wirth et al., 2003; Deo et al., 2013). MLC phosphorylation is a critical factor in the longterm structural stability of the actin cytoskeleton and underlies molecular processes that lead to long-term memory consolidation (Zhang et al., 2005; Rubio et al., 2012). Such findings suggest the importance of PAKs in synaptic morphogenesis and cellular motility (Sells et al., 1999).

On the other hand, PAK also indirectly inhibits MLC activity via direct MLCK phosphorylation. When MLCK is phosphorylated by PAK at Ser 439 and 991, its upregulatory effect on MLC is decreased, thereby reducing stress fiber formation and limiting isometric tension development in smooth muscle and non-muscle cells (Goeckeler et al., 2000; Coniglio et al., 2008; Szczepanowska, 2009). However, the mechanism by which PAK regulates these pathways in a temporal and spatial manner during cell motility is not well understood (Coniglio et al., 2008; Szczepanowska, 2009).

PAK1 has well known importance in stabilizing actin in the cellular membrane via the phosphorylation of filamin A (FLNa) at Ser2152. FLNa binds to actin and organizes it into orthogonal filaments attached to the cell membrane in motile cells. Because it appears that FLNa may be anchored to the CRIB domain of PAK1, stimulating its kinase activity, it can be concluded that this interaction contributes to the local activation of PAK1 (Vadlamudi et al., 2002; Szczepanowska, 2009; MacPherson and Fagerholm, 2010).

6. Cytoskeletal regulation

Several studies have provided evidence for the important roles of PAK in cytoskeletal dynamics due to its activity in pathways related to actin polymerization, novel protein synthesis and intracellular movement (Arias-Romero and Chernoff, 2008). The earliest

evidence that PAK could be involved in the regulation of the cytoskeletal dynamics arose in 1997, when Sell induced PAK overexpression in Swiss 3T3 quiescent cells and detected an increase in lamellipodia and filopodia formation. Furthermore, the expression of PAK in its constitutively active form leads to a reduction in actin stress fibers and an increase in cellular motility (Bloom et al., 2003; Smith et al., 2008; Szczepanowska, 2009).

When PAK is activated by Rac/Cdc42, it induces the formation or remodeling of lamellipodia, filopodia, membrane ruffles, stress fibers and focal adhesion complexes (Bokoch, 2003). The interaction between PAK and Rac/Cdc42 triggers mechanisms that lead to alterations in the properties of proteins involved in cytoskeletal restructuring, including actin/myosin intermediary filaments, microtubules, integrins among other proteins (Brzeska et al., 1997; Kelly and Chernoff, 2011; Chi et al., 2013).

Actin is an essential cellular component to the maintenance of cellular dynamics. Thus, it is crucial for many processes ranging from motility, cell division and morphogenesis, as well as intracellular protein trafficking. The actin cytoskeleton plays a key role in neuronal development and synaptogenesis. Additionally, actin is the most predominant component of the presynaptic and postsynaptic cytoskeleton of mature neurons (Landis et al., 1998; Hirokawa et al., 1989; Bloom et al., 2003). In addition, actin is expressed at high levels in dendritic spines, the postsynaptic structure specialized for excitatory synaptic transmission (Matus, 2000; Capani et al., 2001; Yuste and Bonhoeffer, 2004).

The participation of PAK in the mechanism of cytoskeletal rearrangement has been typically considered as a result of indirect kinase activity on cell structural components. PAK phosphorylates LIMK at Thr508, increasing its phosphorylation and cofilin inactivation (Bokoch, 2003; Cingolani and Goda, 2008; Kelly and Chernoff, 2011). LIMK phosphorylates cofilin at Ser3, inactivating and preventing its coupling to the F-actin bearded end, which facilitates actin polymerization (Manser et al., 1995; Cingolani and Goda, 2008; Szczepanowska, 2009; Sit and Manser, 2011), strongly highlighting the importance of PAK in the pathway of actin regulation in neurons.

There are many PAK substrates that participate in its modulatory effect on cytoskeleton rearrangement. Some of these substrates are proteins that comprise the cytoskeleton. Among these cytoskeletal components which are targets of PAK are the

myosins, which belong to a protein superfamily that shares the same actin– interacting domain, hydrolyze ATP and generate movement (Sellers, 2000; Redowicz, 2007).

The MLC is an essential component of this family that is responsible for cytoskeletal dynamics for communication, migration and cell division (Sellers, 2000; Redowicz, 2007; Hartman et al., 2011) Myosin has an important role in the processes related to structural stabilization and cellular dynamics, rendering the cell membrane resistant to potential deformations. Furthermore, myosin is involved in actin-enabled motor mechanisms and the organization of actin in the intracellular space (Hartman et al., 2011; Kneussel and Wagner, 2013).

FLNa, a substrate of PAK, is also an important cellular structural component. FLNa associates with actin and organizes its filaments as orthogonal networks bound to the cellular membrane, providing the necessary actin stabilization during cellular motor activities. FLNa is phosphorylated by PAK1 at the Ser2152 residue, a process that is necessary to attain its full activation and its efficacy to interact with actin. The benefits of the interaction between PAK1 and FLNa are mutual, as FLNa also anchors to the CRIB domain of PAK and stimulates its kinase activity, thus highlighting the importance of this interaction to the local activation of PAK (Goeckeler et al., 2000; Szczepanowska, 2009; MacPherson and Fagerholm, 2010).

In mammalian neurons, PAK1 and 3 phosphorylate MLC at Ser19, promoting dendritic spine morphogenesis via local actin network stabilization (Ramos et al., 1997; Chew et al., 1998; Van Eyk et al., 1998; Kneussel and Wagner, 2013). Accordingly, previous studies using cultured hippocampal cells have demonstrated that constitutively activated PAK1 and 3 lead to an increase post synaptic density (PSD) formation. The number of dendritic spines in neurons constitutively active expressing PAK1 was increased by 39% compared to control neurons, whereas that of dendritic protrusions were increased by 176%. Furthermore, there was a concomitant increase in the number of PSD protein clusters, suggesting an increase in the formation of excitatory synapses (Zhang et al., 2005).

Nevertheless, the expression of PAK isoforms 1 and 3 containing a mutation in their kinase domain caused a significant decrease in the number of dendritic spines and PSDs (Zhang et al., 2005). These mutants most likely do not efficiently bind to GTPases, such

as Rac or Cdc42, and in addition, their activation and interaction with other effectors involved in synaptic plasticity is impaired (Sells et al., 1997; Tang et al., 1997).

Cytoskeletal rearrangement processes, as well as the outgrowth of cellular machinery, such as increased expression of signaling proteins and intracellular trafficking to their sites of activity, depend on transcription factor activation, gene expression and novel protein synthesis. Therefore, PAK plays a key role by phosphorylating Raf and Mek, two important kinases whose activities are dependent on Rho GTPases that phosphorylate CREB phosphorylation via Erk, the molecule downstream of Raf and Mek (Frost et al., 1997; King et al., 1998; Shin et al., 2002; Smith et al., 2008). Mek phosphorylation at Ser298 by PAK1 is essential for the synergistic effect of Rho GTPases on the Erk pathway and the increase in the upregulatory effect of Raf on Mek (Frost et al., 1997; King et al., 1998). Moreover, Raf1 phosphorylation by PAK1, 2 or 3 at Ser338 is critical for Erk stimulation via Raf-1(Chaudhary et al., 2000; Zang et al., 2002).

A study using organotypic cultures of hippocampal pyramidal neurons has demonstrated that in PAK3 knockout cultures or in cultures treated with an antisense oligonucleotide to PAK3, abnormal dendritic spine and filopodia formation and damage to mushroom-type spine maturation were detected. These effects reproduce described cases of the human mental retardation phenotype (Purpura, 1974). Such abnormalities are also associated with reduced spontaneous activity and altered a-amino-3- hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamatergic receptors, as well as LTP deficits. Altogether, this study demonstrated that transient suppression of PAK3 expression in pyramidal neurons resulted in an increase in the proportion of immature and non-functional dendritic spines, a defect in the establishment of synaptic contact, and stabilization of PSDs (Boda et al., 2006).

7. Participation of PAK in neuroplasticity

Synaptic plasticity is the cellular basis for learning and memory and involves mechanisms such as synaptic circuit remodeling, via either novel synapse formation or old synapse elimination to selectively strengthen or weaken the existing synaptic contacts (Yuste and Bonhoeffer, 2001; Sudhof and Malenka, 2008). These processes involve adaptive changes in presynaptic active zones that control vesicle fusion and enable

neurotransmitter release. The postsynaptic region also undergoes alterations, such as increases or decreases in the number of dendritic spines and modulation of the number of cell surface receptors, among others (Stevens, 2004; Newpher and Ehlers, 2009; Kasai et al., 2010; Kneussel and Wagner, 2013).

The processes of synaptic plasticity depend, among other factors, on the actin cytoskeletal dynamics, including the abundance of actin near the synaptic region. Actin filaments in dendritic spines are highly dynamic; they undergo continuous polymerization and depolymerization, constantly reorganizing themselves due to the consolidation of more stable networks and plasticity processes, such as long-term potentiation (LTP) (Hotulainen and Hoogenraad, 2010; Racz and Weinberg, 2012; Kneussel and Wagner, 2013).

The actin cytoskeleton also supports the trafficking of cell surface receptors in the postsynaptic cell and is associated with the recycling of synaptic vesicles in the presynaptic region (Hotulainen and Hoogenraad, 2010; Racz and Weinberg, 2012; Kneussel and Wagner, 2013). These mechanisms, which rely on the interactions between various cellular structural components, such as FLNa, myosin and actin, to provide structural stability, motor function and an efficient intracellular transport system for the cells which compose the synapse (Hotulainen and Hoogenraad, 2010; Korobova and Svitkina, 2010; Racz and Weinberg, 2012; Kneussel and Wagner, 2013).

PAK participates in the processes of stabilization and remodeling of cytoskeletal structures, such as lamellipodia, filopodia and stress fibers. These processes are indirectly upregulated by PAK via its activity on other molecules that participate in these processes, such as Mek, Raf and LIMK, leading to protein synthesis and actin polymerization, as well as directly regulated by PAK via its activity on structural components of the cytoskeleton, such as MLC and FLNa, as described above (Vadlamudi et al., 2002; Boda et al., 2008; Smith et al., 2008).

A mutation in PAK3 causes nonsyndromic intellectual disability (ID), or mental retardation (RM), because the inactivity of this kinase leads to changes in the morphology and function of dendritic spines. Furthermore, PAK3 gene silencing in hippocampal organotypic slice cultures resulted in a reduction in the number of mature dendritic spines, which provides strong evidence that the deficit in PAK3 activity caused a reduction in LTP (Fiala et al., 2002; Boda et al., 2004). Moreover, in another study by Boda et al.

(2008), it was found that constitutively active PAK1 reversed the damage to spines caused by the inhibition of PAKs activity (Boda et al., 2008). This abnormal phenotype of dendritic spines is observed in the neocortex of mice model RM. An increase in the proportion of immature and thin spines has been detected, as well as a reduction in the proportion of mature spines in the hippocampal dentate gyrus of these animals (Grossman et al., 2006, 2010; von Bohlen and Halbach, 2009).

A more recent study demonstrated a reduction in PAK activity in dendritic spines of mental retardation model mice. These animals were stimulated with hippocampal theta burst, and the activity of PAK and Rac1 was analyzed. Both proteins displayed decreased activity compared to control animals. A possible explanation is that these proteins are involved in the stabilization of newly formed actin filaments (Chen et al., 2010). Meng et al. (2005) did not detect phenotypic alterations in dendritic spines from brain tissue of PAK3 KO animals but in the same tissues, they detected a significant reduction in the late phase of LTP, an electrophysiological process importantly associated with learning and memory (Meng et al., 2005).

According to a study performed by Kreis (2007) the R67C, R419X and A365E mutations determine changes in different functional PAK3 regions and underlie mental retardation. The R67 mutation impairs PAK3 activation by Cdc42 and affects its subsequent activation by this GTPase. The R419X and A365E mutations completely abrogate PAK3 kinase activity. Both the R419X and A365E mutants slightly decrease the number of spines, but profoundly alters spine morphology, whereas expression of the R67C mutant dramatically decreases the spine density in rat hippocampal slices. These results highlight the importance of the combined activity of Cdc42 and PAK3 in the mechanisms of dendritic spine formation and synaptic plasticity (Kreis et al., 2007; Kelly and Chernoff, 2011).

In a study performed by Rex et al. (2009) evidence of the importance of the interaction between group I PAKs and Rac in LTP consolidation was provided. This study demonstrated that inhibition of the Rac-PAK interaction increases the period in which LTP is sensitive to disruption by latrunculin A after theta pulse stimulation (TBS) (Rex et al., 2009). PAK is a known actin filament regulator (Bokoch, 2003), and mice deficient in PAK3 exhibit impaired late phase LTP (Meng et al., 2005). It was also found that F-

actin polymerization is stabilized via Rac-PAK signaling over a period of 2– 10 min post-TBS (Rex et al., 2009; Panja and Bramham, 2013).

The group I PAKs participate in the processes of synaptic plasticity and are important for the mechanisms of spinogenesis and postnatal brain development (Kreis and Barnier, 2009;. Huang et al., 2011). Dysfunction of the group I PAKs has been detected in the brain of patients exhibiting mental retardation, and genetically altered mice not expressing PAK3 exhibited deficits in neuronal signaling. PAK3 knockout animals exhibited significant deficits in neuronal plasticity processes, such as LTP, and reduced learning and memory. However, the mechanism by which PAK3 regulates synaptic transmission and plasticity remains largely unknown (Thévenot et al., 2011; Ma et al., 2012).

A significant change in the levels of PAK1 and PAK3 have also been detected in brain tissue samples from patients suffering from Alzheimer's disease as well as animal models of this disease (Engler et al., 2006). Both PAK and PAK1-3 are typically diffusely distributed throughout the cell bodies and dendrites, but their expression levels are reduced in the cytoplasm of tissue from AD model animals. This result suggests that signaling disruption of both PAKs may play an important role in dendritic spine deficits, synapse dysfunction and cognitive abnormalities in AD (Engler et al., 2006; Zhang et al., 2013).

Similar to that found for MR, in AD, spine morphology defects are early events that exert a negative effect on relevant circuit memory function, causing learning and cognitive deficits. In the brain of individuals suffering from AD, significant loss of PAK1 (35 ± 6%) and PAK3 (55–69%) were detected in the hippocampus, and the expression PAK3 was also significantly decreased in AD temporal cortex (63–77%) (Engler et al., 2006). In some cases, theremwas an increase in the total levels of PAK1-3 during the early stages of Alzheimer's disease, but a reduction in both the total cytoplasmic and phosphorylated PAK1 expression levels was detected during the final, AD stage, very serious (Duyckaerts et al., 2008; Zhang et al., 2013).

In cultured hippocampal neurons treated with b-amyloid, abnormal activation and decreased levels of PAK protein were observed in the cytoplasm, which was accompanied by a rapid loss of F-actin and dendritic spines. Along with this morphological change is the finding that hippocampal AD tissue also displays cells containing increased active

cofilin, whereas the level of phosphorylated PAK is progressively reduced. The explanation for this rapid reduction in F-actin and dendritic spines is described above (Jonsson et al., 2013; Zhang et al., 2013).

8. Conclusion

As described in this review, it is clear that group I PAKs, especially isoforms 1 and 3, are importantly involved in the morphological regulation and the molecular behavior of neurons, with a key role in the processes of neuroplasticity. PAKs participate in the mechanisms of cytoskeletal regulation by interacting with cellular structural components, such as MLC and FLNa, and its activity modulates the dynamics and stability of the cellular structure. PAKs also participate in molecular cascades, such as the pathways of LIMK and ERK, resulting in increased levels of actin polymerization and synthesis of new proteins. This effect of PAKs on the cytoskeleton of neurons highlights its importance for the normal function of neurons and the effectiveness of neural networks involved in learning and many other cognitive functions. Therefore, changes in the activity of these kinases are potentially associated with deficits in learning and memory and diseases such as mental retardation, Huntington's disease and Alzheimer's disease. Therefore, we conclude that a better understanding of the function of PAKs relative to both their upstream and downstream signaling partners, as well as the physiological conditions in which its activity is relevant, can significantly contribute to a better understanding of various diseases or neurological deficits and also provides new information that can contribute to the diagnosis and treatment of these disorders.

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Manuscrito 2: Role of PAK 1 and 3 to reconsolidation, extinction and reacquisition of contextual conditioned fear memory in the CA1 region of the hippocampus of rats.

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Research Paper

Running title: PAK – Reconsolidation, extinction and reacquisition of memory

Title: Role of PAK 1 and 3 to reconsolidation, extinction and reacquisition of contextual conditioned fear memory in the CA1 region of the hippocampus of rats.

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Abstract

The stabilization of memory depends on molecular and morphological changes at synapses activated during mnemonic training in key regions such as CA1 of the hippocampus. The reconsolidation and extinction of aversive memory mechanisms are dependent on new protein synthesis and stabilization of the actin cytoskeleton. Since the reacquisition of aversive memory does not require synthesis of new proteins, but is dependent on the actin cytoskeleton rearrangement process. The mechanisms of synthesis of new proteins and changes in the cytoskeleton are due to the activity of Rho GTPases Cdc42 and Rac1 and its effector kinase activated by p21 isoforms 1 and 3 (PAK1 / 3). Here we avaluated the importance of PAK 1 and 3 in the CA1 region of the hippocampus, related to reconsolidation, extinction and reacquisition of aversive memory in contextual fear conditioning (CFC). For this purpose, male adult rats underwent stereotaxic surgery to implant cannulae in the hippocampus CA1 region for infusion of 3% DMSO (vehicle) or IPA-3 (allosteric inhibitor of PAK 1 and 3). To check the reconsolidation; on day 1, the animals were trained in CFC; day 2 suffered reactivation and immediately or 3 hours after receiving infusions; day 3 were tested in CFC. To assess extinction: day 1 the animals were trained in the CFC; day 2 and 3 were trained to extinction and immediately or 3 hours after receiving infusions; day 4 were tested in CFC. To check reacquisition: day 1 the animals were trained in the CFC; day 2, 3:04 trained to extinction; day 5 were again trained in the CFC and immediately or 3 hours after receiving infusions. The results show that blocking the PAK 1 and 3 in CA1 not affect the reconsolidation of aversive memory. The lock 1 and 3 PAK in CA1 immediately after the first extinction training slows this process, but does not prevent the aversive memory is extinguished. However, infusion IPA-3 3h after extinction training mnemonic does not affect this process. The reacquisition, the lock 1 and 3 PAK immediately, but not 3 h after training (day 5) prevents this memory is strengthened again. Thus, we suggest that blocking PAK can affect the molecular mechanisms underlying the extinction and reacquisition of CFC memory, but has no effect on the mechanisms related to the reconsolidation.

Introduction

The reactivation of a previously consolidated memory induces a desorganization in the actin cytoskeleton, and the consolidated trace returns to a labile state (Milekic & Alberini, 2002; Przybyslawski & Sara, 1997). Following labilization, new proteic

synthesis is required to rearrange the actin cytoskeleton to strenghten the original memory again, a process called reconsolidation (Kida et al., 2002; Rossato, Bevilaqua, Izquierdo, Medina & Cammarota, 2010). This process leads to the strengthening of the original memory, or even its updade, by the addition of new information during the reactivation period (De Oliveira Alvares, Crestani, Cassini, Haubrich, Santana & Quillfeldt, 2013; Lee, 2010), but the disrruption of the mechanisms that promote protein synthesis impairs the reconsolidation, ultimately weakening the original memory (Johansen, Cain, Ostroff & LeDoux 2011; Sara, 2000; Thonson & Taylor, 2007). If the reactivation period is prolonged or repeated a few times, the original trace is weakened and suffers extinction. (Suzuki, Josselyn, Frankland, Masushige, Silva & Kida, 2004; Rossato, Bevilaqua, Lima, Medina, Izquierdo & Cammarota, 2006).

In the contextual fear conditioning (CFC) behavioral experiment, the repeated re-exposure to the conditioned stymulus (CS) without the unconditioned stymulus (US) leads to the extinction of the previously acquired aversive memory (Cammarota, Bevilaqua, Kerr, Medina & Izquierdo, 2003; Cammarota, Bevilaqua, Medina & Izquierdo, 2004). Extinction is the formation of a new memory where the CS and the US are not associated, and it is also dependent on molecular events that lead to new proteic synthesisand citoesqueleton increment. However, the original aversive memory is not erased and a new re-exposure to the US in the same context of the original memory can lead to a rapid recovery of the fear memory, strengthening the conditioned response (CR) (Bouton, Westbrook, Corcoran & Maren, 2006; Denniston & Miller, 2003; Revillo, Castello, Paglini & Arias, 2014 Robleto, 2004).

The molecular events required for the CFC memory extinction and reconsolidation depend on the activity of N-methyl-D-aspartate receptor (NMDAr). Blocking this receptor affect the process of extinction (Lee & Hynds, 2013; Sotres-Bayon & Quirk, 2010). The protein kinase regulated by extracellular signals 1/2 (ERK 1/2), has been identified as necessary for extinction of memories, both in the hippocampus and in other regions (Cannich, Wotjak, Kamprath, Hermann, Lutz & Marsicano, 2004; Rossato et al., 2006). Inhibition of ERK 1/2 in the prefrontal cortex and hippocampus impairs extinction of aversive memory in the inhibitory avoidance test (Hugues, Chesse, Lena, Marsault & Garcia, 2006; Szapiro, Vianna, McGaugh, Medina & Izquierdo, 2003). The kinase-dependent calcium and calmodulin II (CaMKII) and mitogen activated protein kinase agents (MAPK) are also key molecules in the molecular mechanisms underlying

extinction. Infusion of CaMK II or MAPK inhibitors in the hippocampal CA1 region after the extinction training prevents the decrease of the aversive memory (Szapiro, Viana, McGaugh, Medina & Izquierdo, 2003).

The plasticity mechanisms associated with learning are dependent on the activation of a complex molecular cascade, among which are the Rac-1 GTPase and Cdc42 and its effector p21 cinasis activity (PAK), especially the isoforms 1 and 3 (Kreis et al, 2009). PAK is inactive when it is in the form of a dimer (two PAK molecules in equal or different isoforms), as its catalytic site is inhibited. PAK 1 and 3 affect the protein synthesis and polymerization of the actin cytoskeleton specially by the stimulation of LIM kinase (LIMK) and ERK (Bokoch, 2003, 2008). PAK 1 phosphorylates LIMK in the Thr508 site, reducing the cofilin-mediated depolymerization of F-actin (Edwards, Sanders, Bokoch & Gill, 1999). It is also well demonstrated that PAK1 phosphorylates MEK in Ser298 and Raf in Ser338, improving the interaction between Raf and Mek in the ERK pathway. In fact, PAK1 downregulation reduces the phosphorylation level of MEK in Ser298 and Raf in Ser338 (Frost, Steen, Shapiro, Lewis, Ahn, Shaw & Cobb, 1997; Manser & Zhao, 2012).

In a study of brain tissue dendritic spines using PAK3 knock-out mice, phosphorylated CREB and late LTP both were significantly reduced, both necessary to plastic events that stabilize memory (Meng, Meng, Hanna, Janus & Jia, 2005). In a work with organotypic culture of hippocampal slices, the silencing of PAK3 expression lead to the formation of immature-like thin and elongated dendritic spines (Boda, Alberi, Nikonenko, Node-Langlois, Jourdain, Moosmayer, Parisi-Jourdain & Muller, 2004). On the other hand, in 2008 Boda et al. showed that the constitutively active expression of PAK 1 can reverse the spine formation deficit caused by downregulation of PAK 1 (Boda, Jourdain & Muller, 2008).

Considering the importance of the PAK in plasticity related mechanisms, we think that the pharmacological inhibition of this kinase in the hippocampus could impair the processing of the aversive memory generated in the CFC. Thus, in this work we verified the importance of PAK 1 and 3 during the reconsolidation, extinction and reacquisition processes of CFC aversive memory. To this end, we infused IPA-3 (allosteric regulator of PAK 1, 2 and 3) (Deacon, Beeser, Fukui, Rennefahrt, Myers, Chernoff & Perterson, 2008) in the hippocampal CA1 region of Wistar rats in different time points after

reconsolidation, extinction and reacquisition training sections. Our results show that PAK inhibition PAK delays extinction and impairs the reacquisition of aversive memory in CFC, but does not affect its reconsolidation.

Materials and Methods

Animals and stereotactic surgery

The animal model used in this study was *Rattus norvegicus*, of the Wistar strain. Male rats (n = 170; age 2-4 months) were obtained from the breeding colony of Universidade Federal do Rio Grande – FURG - Rio Grande, RS, Brazil. The animals were kept in groups of five per cage, with a 12 h light/dark cycle, at a temperature of 22 ºC ± 1 ºC, with food and water *ad libitum*. After a week of acclimation, the animals were submitted to a stereotactic surgery for a bilateral cannulae implantation in the CA1 region of the hippocampus (see details, Barros, Ramirez, Dos Reis & Izquierdo, 2004), under ketamin (62.5 mg/kg) and xylazin (13 mg/kg) anesthesia. The guide cannulae were fixed with acrylic resin 1 mm above CA1 region of dorsal hippocampus, following the coordinates relative to bregma: AP, 4.3 mm; ML, 3.0 mm; DV, 1.8 mm (Paxinos & Watson, 1997). At the end of the surgery, in order to prevent infections, the animals were treated with an antibiotic association (Pentabiótico, Brazil). This work has been approved by the ethics committee for animal experimentation of FURG, under the register 039/2013.

Treatments and microinjections

Firstly we performed a pilot study to identify the appropriate concentration of IPA-3 (PAK 1, 2, and 3 inhibitor, Sigma) for use in this study, as described in Experiment 1. As described below, we have adopted IPA-3 at the concentration of 1.0 mM. Animals were infused either with DMSO 3% (CTR-vehicle, Sigma) or with IPA-3 at concentrations of 1.0 mM bilaterally into the CA1 region of the hippocampus. For the infusion, we used a gingival needle linked to a Hamilton syringe to enter to 1.0 mm beyond the guide cannulae. After the slow injection of 1 µL of solution, the infusion cannula was left in its place for an additional 15 s in order to avoid backflow. Infusions were carried out first on one side and then on the other. The treatments were administered either 0 h or 3 h after the reconsolidation training session, 0 h or 3 h after extinction training session, and 0 h or 3 h after reacquisition training session, in order to verify the

participation of PAK 1 and 3 in reconsolidation, extinction, and reacquisition of fear memory in rats submitted to CFC (Bevilaqua, da Silva, Medina, Izquierdo & Cammarota, 2005; Bustos, Maldonado & Molina, 2009; Rossato, Bevilaqua, Izquierdo, Medina & Cammarota, 2010).

Behavioral tests

Contextual fear conditioning

The CFC apparatus consisted in an acrylic box (25 X 25 X 25 cm), whose floor consisted of parallel stainless steel bars spaced 1.0 cm apart from each other. During the training session, the animals were placed in the apparatus for a period of 5 min. After the first 3 minutes of habituation, the animals underwent to a sequence of 3 footshocks at 0.7 mA, each lasting 2 s. The CFC memory is a result of the association of the CS (environmental pairing) and the US (aversive stimulus of the footshock). After the shocks, the animals were kept in the device until the completion of 5 min since the test beginning. Test session procedure was similar to the training, but the footshock was absent (CS+ and US-). In this session, the total time of 'freezing' behavior was registered. Freezing was defined as the time during which the animals showed no motion except for breathing movements (Bekinschtein, Cammarota, Igaz, Bevilaqua, Izquierdo & Medina, 2007).

Experiment 1 – Pilot study for the identification of IPA-3 dose to be used in this study:

On day 1, the animals were trained to acquire the CFC memory (CS + and US+), as previously described, and immediately after the animals were infused with 1 µL of one of the following solutions: Saline (control), DMSO 3% (vehicle control), IPA-3 0.01 Mm (Deacon, 2008), IPA-3 0.1 mM, IPA-3 1.0 mM, or IPA-3 10.0 mM; on day 2 the animals returned CFC box for the test session (CS + and US-), as described above. As seen in Figure 1, IPA-3 at 1.0 mM and 10.0 mM caused deficit in aversive memory when compared to saline controls, whereas DMSO 3% and IPA-3 in lower concentrations did not affect memory in this behavioral test. Then, we adopted IPA-3 1.0 mm for our experimental procedures.

Experiment 2 - Reconsolidation of aversive memory in the CFC:

2A – This experiment was performed to evaluate the role of PAK 1 and 3 to the initial phase of CFC memory. On day 1, the animals were trained to acquire the CFC memory (CS+ and US+), as previously described; on day 2 the animals returned to the CFC box, where they remained for only 3 min without negative stimuli (CS+ and US-), in order to reactivate the memory acquired on day 1 and induce its reconsolidation; intracerebral infusions were performed immediately after the reactivation session; on day 3, the animals returned to the CFC apparatus for the test session (CS+ and US-) as described above.

2B - For the assessment of the role of PAK 1 and 3 in the intermediate phase of the CFC memory, we adopted a protocol similar to 2A, except for the infusion times, which were carried out 3 h after reactivation sessions.

Experiment 3 - Extinction of aversive memory in the CFC:

3A – In this experiment we investigated the importance of PAK 1 and 3 in the initial phase of CFC memory extinction. For this end, the infusions were carried out immediately after each extinction session; on day 4, the animals were returned to the CFC box for the test session (CS+ and US-).

3B - With this experiment we assessed the importance of PAK 1 and 3 to the intermediate phase of CFC memory extinction. The experimental procedure was similar to Experiment 2A, but infusions were performed 3 h after extinction session.

Experiment 4 - Reacquisition of CFC aversive memory:

Experiment 4A - with this experiment we evaluated the importance of PAK 1 and 3 in the initial stage of the CFC memory reacquisition: On day 1, the animals were trained to acquire the CFC memory (CS+ and US+); on days 2, 3, and 4 the animals returned to the CFC box, where they remained for 5 min without the aversive stimuli (CS+ and US-), in order to train the extinction of the memory acquired on 1; on day 5, the animals were trained to reacquire the CFC memory (same procedure as Day 1). Immediately after this training session, the animals received IPA-3; On day 6, the animals returned to the CFC box for the test session (CS+ and US-), as described above.

Experiment 4B - This experiment was performed to evaluate the importance of PAK 1 and 3 in the intermediate phase of the CFC memory reacquisition. The experimental

procedure was similar to Experiment 3A, but the infusions were carried out 3 h after reacquisition sessions.

Statistical Analysis

Results obtained from contextual fear conditioning are shown in freezing time (seconds), expressed in mean ± SEM. After verifying the normality and homogeneity of variance, statistical analysis from experiment 1 was made through analysis of variance (ANOVA) followed by Dunnett's test ($P < 0.05$ was considered to indicate statistical significance). Data from Experiment 2, 3, and 4 were analyzed using Student's t-test, followed by Mann-Whitney post-hoc. In all cases, significance was set to $p < 0.05$.

Results

The hippocampal inhibition of PAK 1 and 3 did not affect the CFC aversive memory reconsolidation:

In this experiment, we tested if blocking PAK 1 and 3 by IPA-3 could affect the CFC aversive memory reconsolidation. In the first phase of this experiment, the animals were treated immediately or 3 h after the reactivation session (Fig. 2A and 2B, respectively), and were tested on day 3. In both conditions, no statistically significant differences were found between the treated groups and the controls. (DMSO 3%: 205.03 ± 16.70; 1.0 mM: 171.4±25.92; $p > 0.05$; n = 8-14)(DMSO 3%: 192.9 ± 22.67; 1.0 mM: 172.1 ± 25.72; $p > 0.05$; n=9). These results indicate that PAK 1 and 3 do not have an essential role in the initial and in the late aversive memory reconsolidation stage, suggesting that the inhibition of this kinase does not affect the molecular dynamics in the CA1 region of the hippocampus, which underlies the reconsolidation of CFC aversive memory.

Allosteric inhibition of PAK 1 and 3 retards the extinction of CFC aversive memory:

Here we investigated the importance of PAK 1 and 3 in the CFC aversive memory extinction. On day 3, the animals treated with IPA-3 0 h after extinction training, showed a significantly higher freezing time if compared to controls ((DMSO 3%: 36.88 ± 15.53;

1.0 mM: 176.8 ± 20.07; $p < 0.05$; n = 9-11)Figure 3A), indicating a deficit in memory extinction caused by IPA-3. However, no significant differences were observed in freezing time between treated groups and controls on day 4(DMSO 3%: 46.88 ± 14.91; 1.0 mM: 46.06 ± 18.01; $p > 0.05$; n = 9-11), demonstrating that the extinction was successful for all groups. Regarding infused groups 3 h after extinction training, no significant differences were found between the treated animals and controls on day 3 (Figure 3B) (DMSO 3% 86.39: ± 22.02; 1.0 mM: 89.99 ± 37.40; $p > 0.05$) and day 4 (DMSO 3%: 25.59 ± 12.01; 1.0 mM: 34.16 ± 18.53; $p > 0.05$)(n = 6-10)Our data show a progressive reduction in freezing time over the days 3 and 4 for all groups. Our results in this test show an intriguing effect of PAK 1 and 3 in memory extinction. Inhibition of PAK 1 and 3 immediately after the extinction training caused a delay in this process, but we found that the termination is successful when the freezing time was measured on day 4. On the other hand, when the inhibition by IPA-3 were carried out 3 h after extinction training, no impairments were detectable.

The reacquisition of aversive memory CFC depends on the activity of PAK 1 and 3:

For these experiments, we performed an experiment to see if PAK 1 and 3 inhibition can affect CFC memory reacquisition. Therefore, on day 1, the animals were trained to acquire a CFC memory . On days 2, 3, and 4, we proceed with the extinction training by the re-exposure to the original CFC context. On Day 5, the memory was reactivated by US in the same CFC context. DMSO 3% (control) or IPA-3 were infused in the CA1 region, immediately or 3 h after reacquisition training and on day 6 the animals were tested in the CFC. During the CFC test on day 6, the treated animals infused 0 h after training (DMSO 3%: 165.5 ± 26.69; 1.0 mM: 79.47 ± 17.69; $p < 0.05$; n = 9-11) showed significantly lower freezing times if compared to controls (Fig. 4A). Conversely, animals treated 3 h after reacquisition training there was no significant difference between the treated and control group (DMSO 3%: 212.1±17.01; 1.0 mM: 184.7 ± 23.93; $p > 0,05$; n = 9-10)(Fig. 4B). The results found in this trial indicate an important role for PAK 1 and 3 in the early phase of the CFC aversive memory reacquisition process, since the PAK inhibition immediately after reacquisition training caused a deficit in the aversive memory 24 h later. These data suggest that the PAK have a key role in the molecular events leading to the recovery of a memory that had been extinguished.

Discussion

Our results show that blocking PAK 1 and 3 in the CA1 region of Wistar rats does not affect the reconsolidation of CFC aversive memory, but can slow down the process of memory extinction. Furthermore, PAK 1 and 3 seem to participate in the reacquisition of previously extinguished aversive memories, since the inhibition of PAK 1 and 3 in the hippocampal CA1 region prevented reacquisition.

The reconsolidation of CFC aversive memory, is not affected by blocking PAK 1 and 3 in the CA1 region:

First, we consider the potential contribution of infusion IPA-3 at 0h in labilization process due to the reactivation of memory, since the PAK indirectly inhibits Cofilin stabilizes the cytoskeleton (Edwards & Gill, 1999; Wang et al., 2013). It is known that a reactivated memory is primarily labilized by a derangement in the cytoskeletal actin, which is determinant in whether this memory will or will not be reconsolidated. The foregoing reconsolidation labilization and stabilization of actin filaments by infusion phalloidin, which prevents the depolymerization in the basolateral amígdala 0.5h after reactivation affect reconsolidation of the CFC memory (Rehberg, Bergado-Acosta, Koch & Stork 2010). PAK 1 activates LIMK by phosphorylation at Thr508 site. In fact. Edwards et al. (1999) reported that the addition of a constitutively active PAK 1 enhanced cofilin phosphorylation in Ser3, while PAK 1 inhibition PAK 1 blocked the cytoskeletal changes induced by LIMK (Edwards, Sanders, Bokoch & Gill , 1999).

After reactivation, proteic synthesis is required for the reconsolidation of the original trace (Hall, Thomas & Everitt, 2001; Kida et al., 2002). In a study conducted by Kida et al. (2002), mice were trained to acquire CFC memory and infused with a CREB inhibitor 6 h before LTM reactivation, the LTM had significantly reduced freezing time compared to controls during the retention test to 24 hours after reactivation, but not at 2 hours after the reactivation.The role of PAK in the positive modulation of the ERK pathway and CREB phosphorylation are well known (Meng, Meng, Hanna Janus & Jia, 2005). However, IPA-3 infusion in CA1 region 0 h or 3 h after reactivation did not affect the consolidation of the original memory. Furthermore, it was demonstrated that ERK is not determinant for memory reconsolidation in the dorsal hippocampus. In a study by Lee (2013), the ratio of phosphorylated ERK did not increase in the dorsal hippocampus of mice following the reactivation of the CFC memory. Also, ERK inhibition in dorsal hippocampus 30 min prior to CFC memory reactivation did not impair reconsolidation

24 h after reactivation (Lee & Hynds, 2013). Our results indicate that, despite the importance of PAK in cytoskeleton increment and proteic synthesis underlying memory consolidation, PAK in the CA1 region immediately or 3 h after reactivation did not affect the CFC aversive memory reconsolidation. Thus, we consider the importance of further studies to better understand the behavior of PAK 1 and 3 in the reconsolidation of aversive memory.

PAK 1 and 3 inhibition retards the CFC aversive memory extinction:

Blocking PAK 1 and 3 0 h after training for extinction resulted in the maintenance CFC memory to the test day 3 but not on day 4, since the freezing time was kept high on day 3, but significantly reduced on the day 4, when compared to controls. However, administration of IPA-3 3 h after the extinction training did not affect its consolidation, since treated animals and controls did not differ significantly in their freezing time during the retention test. As the reconsolidation, the extinction also results from the re-exposition to CS without the US (Izquierdo, Furini and Myskiw, 2016; Quirk & Mueller, 2008). During reactivation, various molecular events are triggered and change its course during re-exposure, which may result in reconsolidation or extinction of the memory, as the two processes compete with each other, a process that begins from the reactivation of an already stable memory (Furini, Myskiw & Izquierdo., 2014, Quirk & Mueller, 2008).

According to a report by Sananbenesi et al. (2007), Rac1 activity in the dorsal hippocampus is important for the consolidation of the extinction of the CFC aversive memory. Results from the same study suggest that PAK 1 is the effector of Rac1 in the extinction process, demonstrating that PAK 1 phosphorylation levels are high after extinction training, and the expression of PAK 1 in its inactive form impairs extinction. Moreover, it was found that the activation of Cdk5, a inhibitor for PAK1, difficult the CFC memory extinction, and its inhibition facilitates the process of extinction (Sananbenesi, Fischer, Wang, Schrick, Neve, Radulovic and Tsai, 2007). The ERK pathway, positively modulated by PAK, has a well stablished role in the memory consolidation and extinction (Fischer, Radulovic, Schrick, Sananbenesi, Godovac-Zimmermann & Radulovic, 2007; Frost et al., 1997; Hofmann., 2004). In a study with mice knockouts to PAK 3, there was observed a significant reduction in phosphorylated CREB, a transcription factor modulated by Erk that is essential for gene transcription events for memory consolidation (Meng, Meng, Hanna, Janus & Jia, 2005). Thus, IPA-3

infusion immediately after the extinction training possibly disturbed the synthesis of proteins required for extinction. Extiction impairment might have allowed that the original memory was reconsolidated on the first day of re-exposure to CS, once reconsolidation and extinction are concurrent processes and, in addition, this study did not observe any impairments to the reconsolidation caused by PAK inhibition. However, as our method of inhibition is effective for a limited time period, unlike a knockout model for PAK, it is possible that the first extinction training (d 2) started a response that was reinforced after the second training (d 3), so that the extinction of the new memory prevailed over the original memory. Evocation of new knowledge prevailed in the retention test on day 4, which strengthened extincion, leading to a reduction in freezing time. These results strongly indicate the involvement of PAK 1 and 3 in the extinction process, which is agreement with previous findings (Sananbenesi et al. 2007). However, data is insufficient to elucidate how and to what extent PAK plays a role in this process.

The reacquisition of CFC memory depends on the activity of PAK 1 and 3:

We tested if the IPA-3 infusion in CA1 can affect the reacquisition of aversive CFC memory. We found that among the animals infused immediately after the reacquisition session the IPA-3 treated animals freezing behavior was much less frequent 24 h after training if compared to the control animals. This was verified in 24 retention test after the reacquisition test, where the animals treated with IPA-3 1.0 mM showed a significantly reduced freezing time if compared to controls. It is well demonstrated that the extinction of a memory is neither a replacement nor an exlusion of apre-existing (Bouton, 1993, 2004). Rather, memory extinction corresponds to a new learning. Thus, the re-exposure to a CS with the reactive US induces the strengthening of the the original trace (Pavlov, 1927; Rescorla, 1975; Vervliet, Craske & Hermans , 2013; Willcocks & McNally, 2014). Memory reacquisition, like memory consolidation, also depends on the actin cytoskeleton rearrangement. However, unlike consolidation, restoration of a pre-existing memory does not depend on the synthesis of new proteins to stabilize the cytoskeleton (Motanis & Moroun, 2012). Furthermore, it is well known that cytoskeletal events induced by F-actin polymerization during the stabilizationcan affect the mnemonic consolidation (Rudy, 2015). Interestingly, the inhibition of PAK 1 and 3 immediately after the reacquisition training impaired the retention of knowledge, but not when this kinase was blocked 3 h after training. It is known that Rac-PAK and Cdc42-PAK cascade plays a key role in cytoskeletal changes related to LTP stabilization actin cytoskeleton

reorganization (Rex et al., 2009). Consequently, any event that affects PAK activity can not prevent the polymerization of actin filaments, but does not allow the polymer to stabilize (Chen et al., 2007, Honkura et al., 2008). The CFC memory reacquisition deficit observed in our study when IPA-3 infusion was performed 3 h after training suggests that the PAK 1 and 3 do not have an determinant role in the reacquisition period. This can be explained by the rapid strengthening of the existing trace. As it does not require the synthesis of new proteins, it may be able to stabilize 3 h after the reacquisition training.

Conclusion

Our data suggests that the PAK 1 and 3 have a key role in hippocampal CA1 during the early stages of extinction and reacquisition of CFC aversive memory, since its inhibition immediately after extinction and reacquisition training impaired memory retention. However, PAK does not appear to have a relevant participation in the reconsolidation process of CFC memory, once inhibition after reactivation did not affect the performance of the animals during the retention test. We believe that the PAK 1 and 3 are important molecular target for future efforts on fear memory, such as those related Post Traumatic Stress Disorder.

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Figures

Figure 1

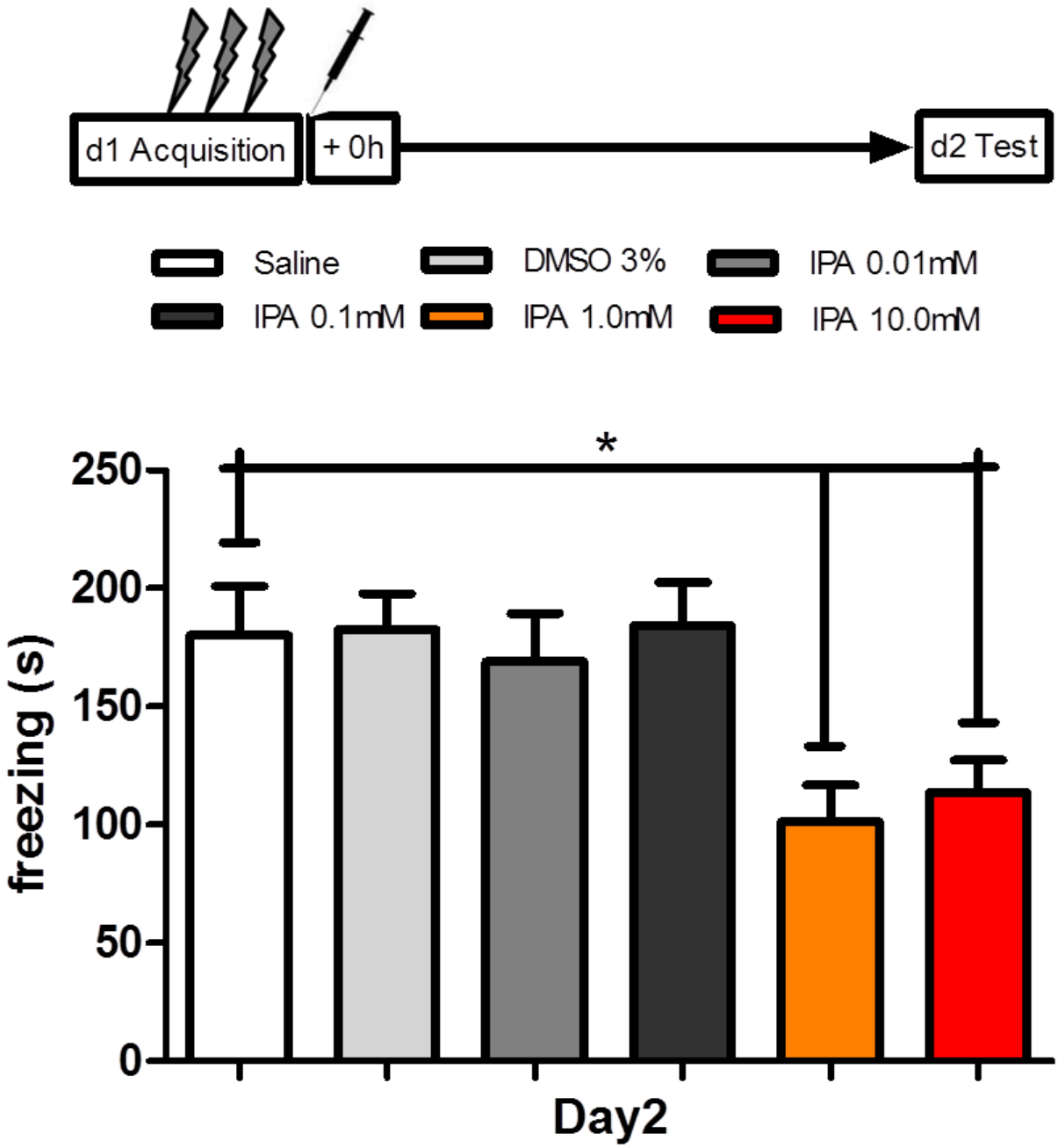
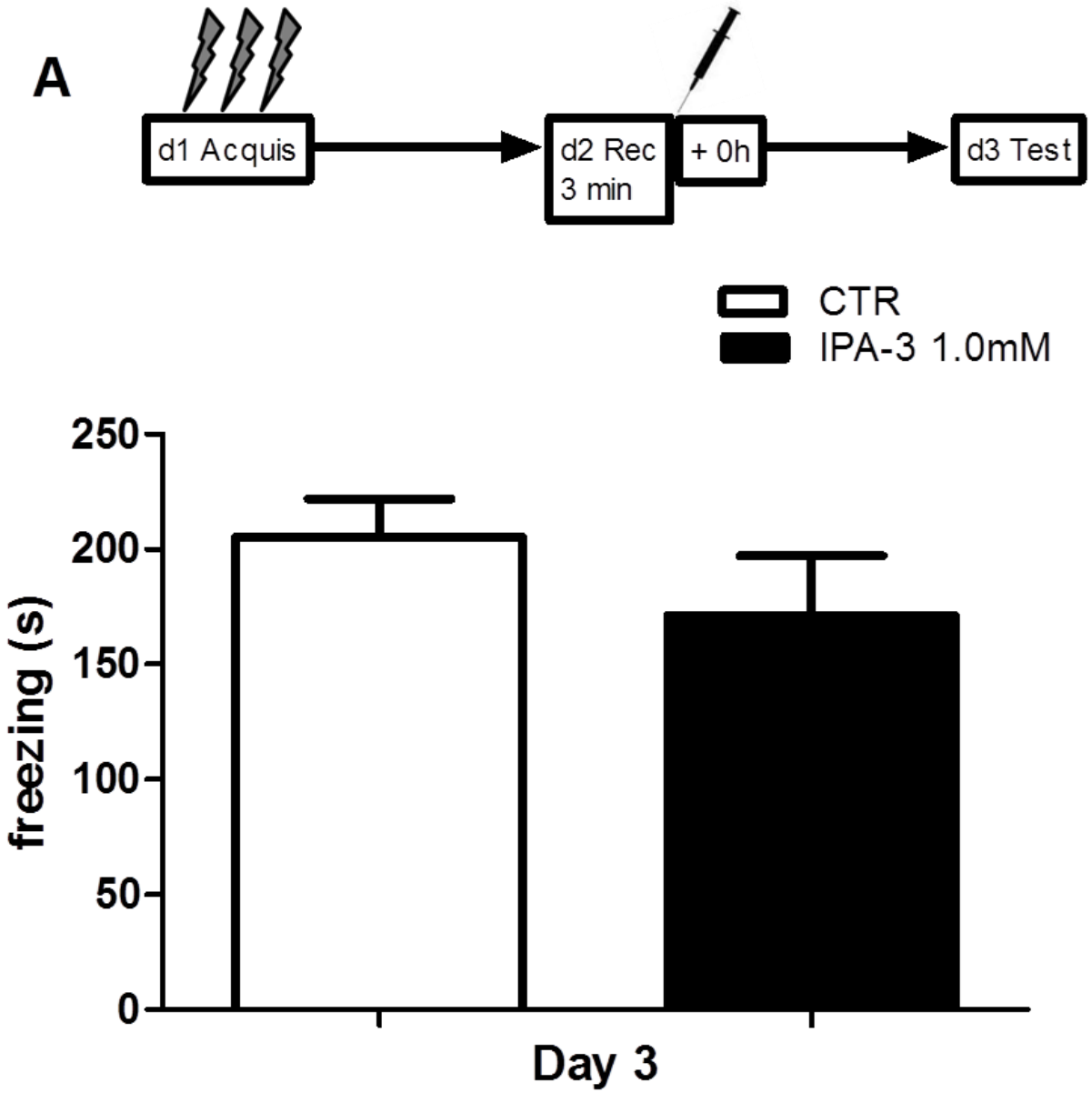


Figure 2 A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Day 3

Figure 2 B

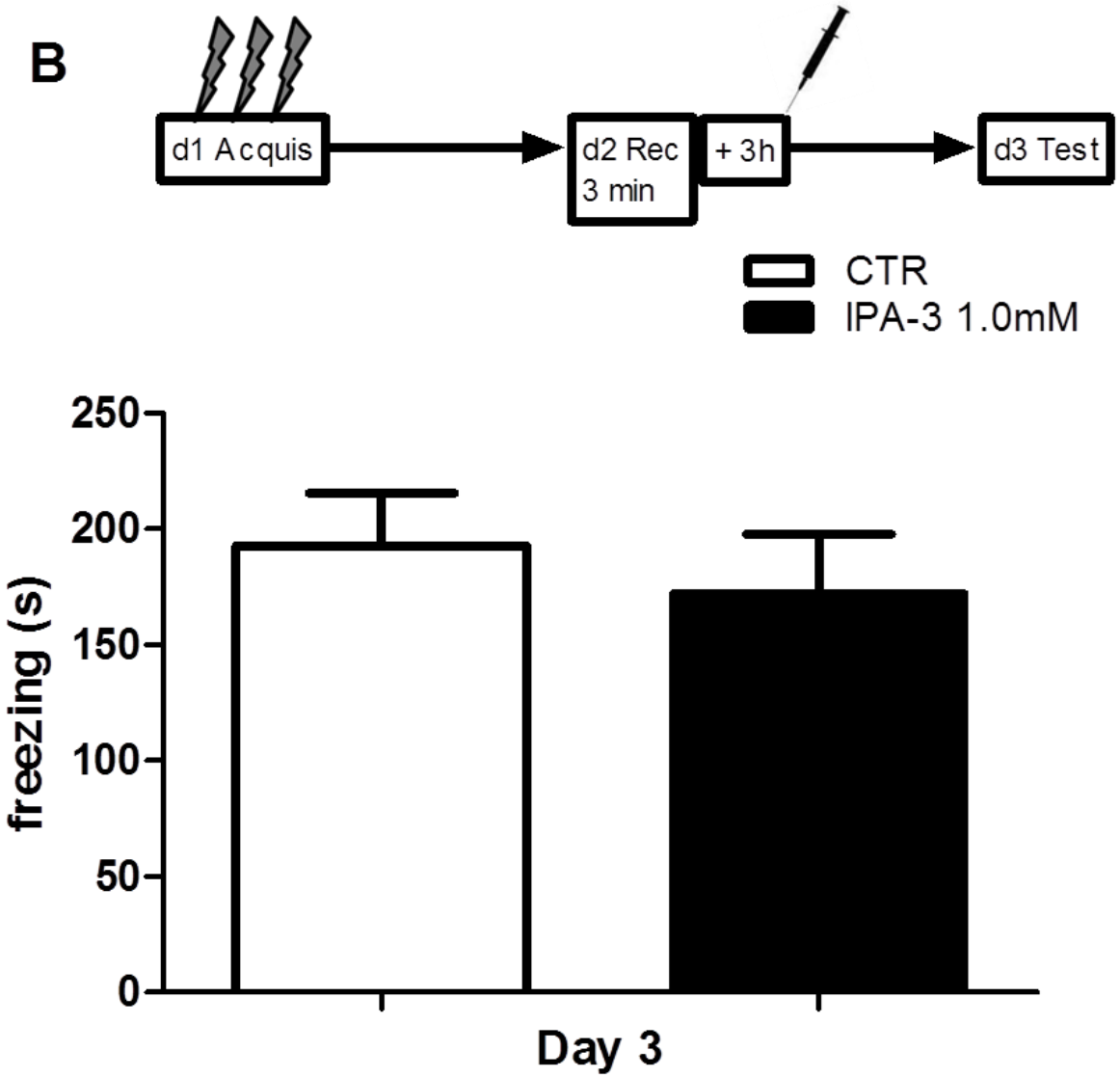
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Figure 3 A

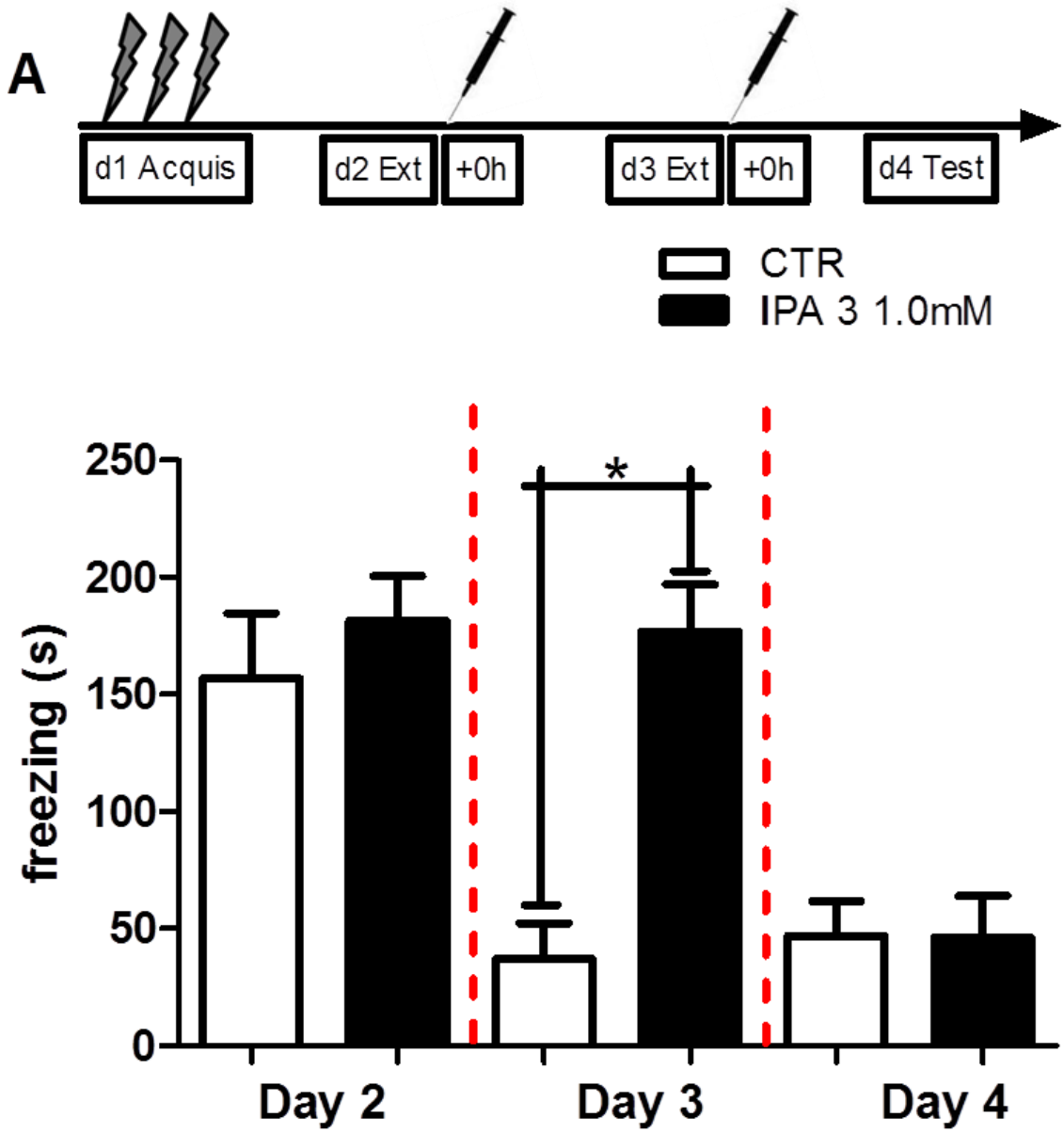
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Figure 3 B

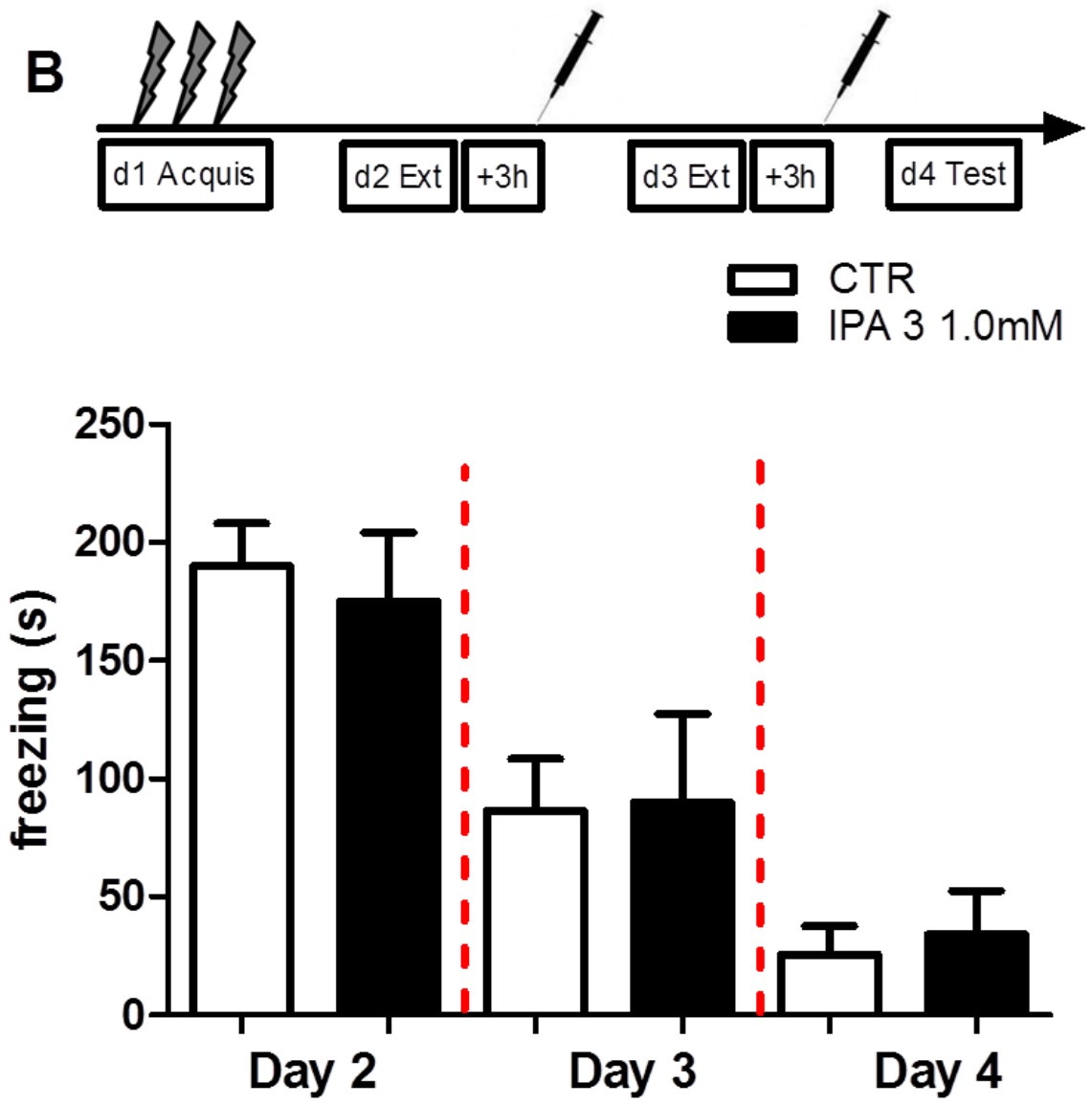
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Figure 4 A

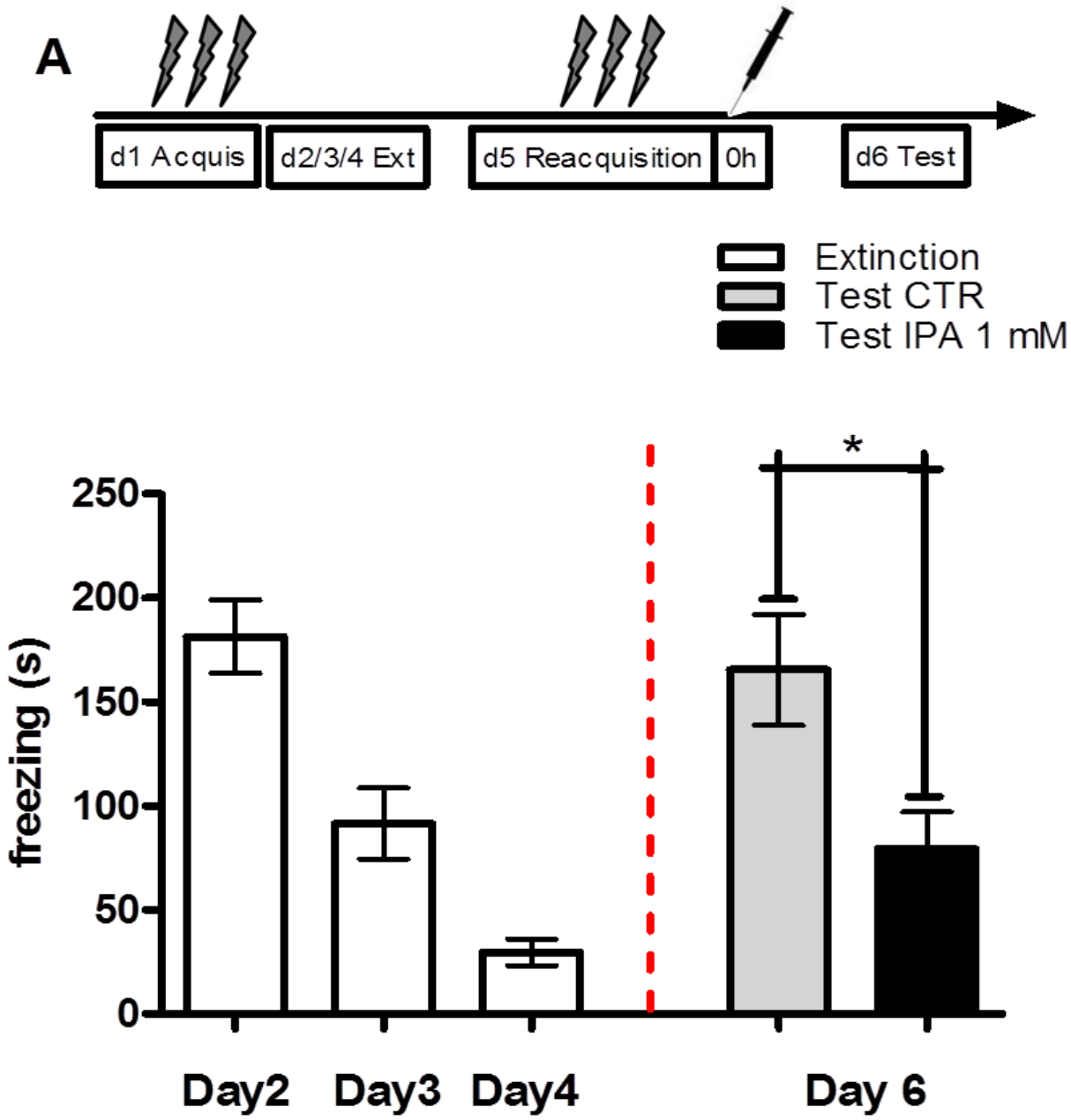
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Figure 4 B

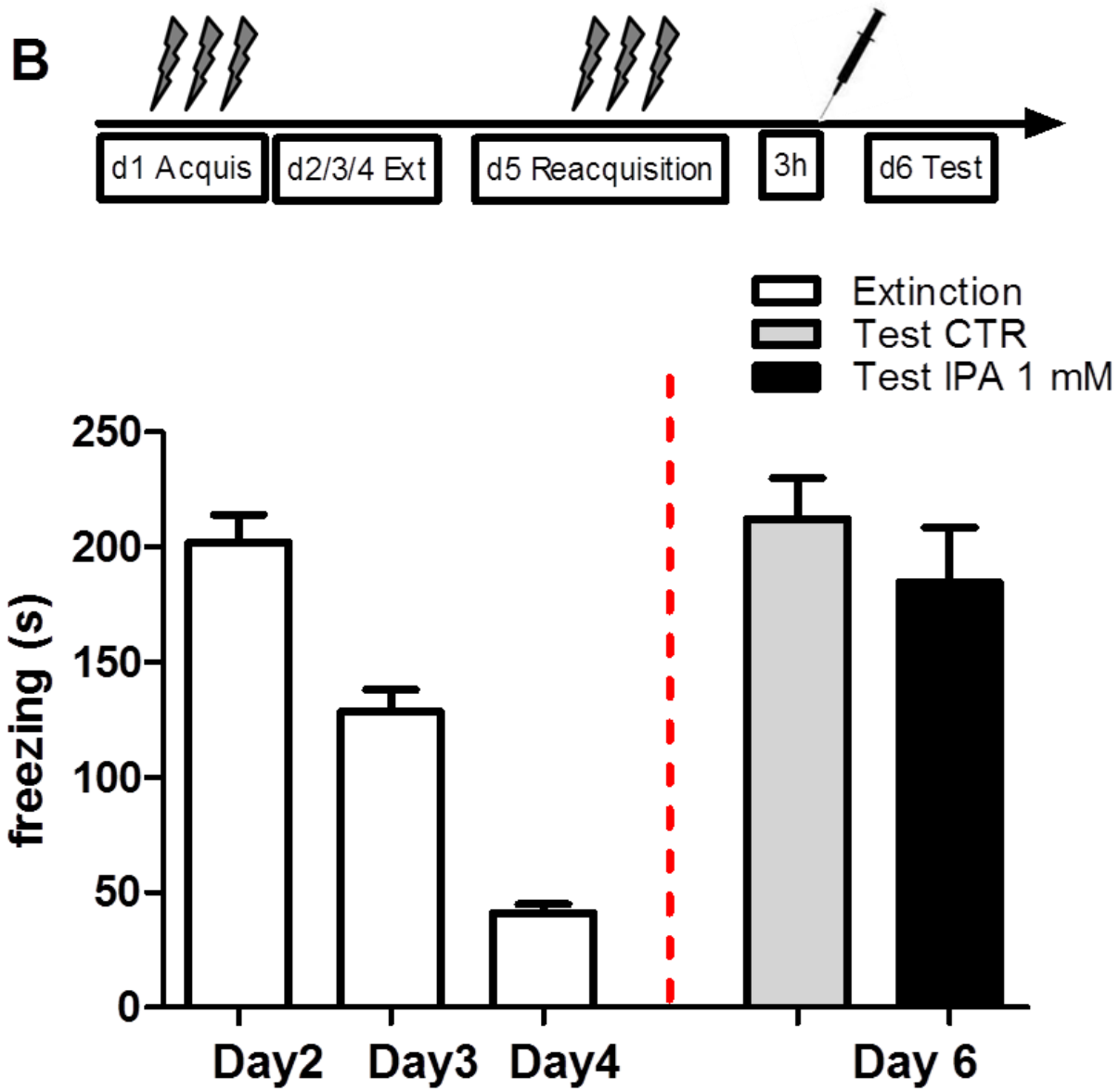
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Fig. 1. IPA-3 concentration test. On day 1, the animals were trained in the CFC and immediately after training were infused with IPA-3 into the CA1 region, with 1 µL of saline, DMSO 3%, or IPA-3 at 0.01 mM, 0.1 mM, 1.0 mM, or 10.0 mM. On day 2, the animals were tested in the CFC by quantifying the time the animals remained on freezing behavior. Only animals treated with IPA-3 1.0 mM and 10.0 mM showed a freezing time significantly lower if compared to control ($p<0.05$). As DMSO 3% did not alter the animal normal behavior, we adopted the DMSO 3% and IPA-3 1.0 mM to infuse in the control and treated animals, respectively.

Fig. 2. Inhibition of PAK 1 and 3 does not affect the aversive memory reconsolidation as tested on the CFC method: (A) On day 1 the animals were trained in the CFC for the acquisition of the aversive memory; on day 2, the reactivation of the acquired memory was induced. After that, the animals were immediately infused into the CA1 region of the hippocampus with 3% DMSO or 1.0 mM IPA-3; on day 3, aversive memory of these animals was tested by measuring the freezing time in the CFC, where there was no significant difference between the control animals and those treated with IPA-3 ($P> 0.05$ vs. controls; n=8-14). (B) Same as (A), but the IPA-3 infusions were performed 3 h after the reactivation of the memory. Again, no significant differences were observed between the control animals and those treated with IPA-3 ($p > 0.05$ vs. controls; $n = 9$).

Fig. 3. Inhibition of PAK can slow the extinction of the aversive memory acquired in CFC: (A) On day 1 the animals were trained to acquire the CFC aversive memory; on day 2 and 3 they were trained for extinction in the CFC and immediately after training sessions were infused into the CA1 region of the hippocampus, with 3% DMSO (control) or IPA-3 1.0 mM; on day 4, animals had their aversive memory tested by measuring the freezing time in the CFC. The animals treated with IPA-3 1.0 mM exhibited a freezing time significantly higher than controls on day 3 ($p < 0.05$ vs. controls; n=9-11), but had a time decrease in freezing behavior on the test day 4, when there was no significant difference if compared to controls ($p > 0.05$ vs. controls). (B) The experimental design was the same as above, (A), except for the infusions, which were made 3 hours after the extinction training. There was no difference in freezing behavior time between treated and control animals ($p > 0.05$ vs. controls; n=6-10). Additionally, our results show that similarly reduced the freezing time in tests 3 and 4.

Fig. 4. Blocking PAK affect the reacquisition of CFC aversive memory in CFC: On day 1, the animals were trained to acquire the CFC aversive memory; on days 2, 3, and 4, were trained for (white bars); On day 5, the animals were trained for the reacquisition of CFC memory and immediately (A) or 3 h (B) later were infused with 3% DMSO (controls) or 1.0 mM IPA-3 in the CA1 region of the hippocampus; On day 6, the animals had CFC memory assayed by quantification of time showing freezing behavior. (A) The animals treated with IPA-3 0 h after reacquisition training had significantly reduced *f*reezing time compared to controls ($p < 0.05$ vs. controls; n=9-11), suggesting CFC memory reacquisition deficit caused by treatment with IPA-3. (B) When the animals were treated 3 h after the reacquisition training, there was no significant difference in *freezing* time between the treated and controlanimals ($p>0,05$ vs. controls; n=9-10).

Considerações Finais

As PAK 1 e 3 são cinases que estão implicadas em mecanismos intracelulares relacionados com os processos de neuroplasticidade subjacentes a formação e estabilização da memória. Diversos estudos têm demonstrado a importância dessas

cinases nos eventos associados à síntese de novas proteínas e estabilização do citoesqueleto de actina em estruturas encefálicas chaves aos processos mnemônicos, entre elas o hipocampo. A atividade da PAK como modulador positivo na cascata da Erk estimula indiretamente a fosforilação e ativação do fator de transcrição CREB, o que resulta em incremento na atividade de síntese proteica. A PAK também esta implicada na estabilização do citoesqueleto de actina por inibir a atividade da cofilin, principal moduladora negativa do processo de polimerização do citoesqueleto de actina. Para tanto, PAK fosforila e ativa LIMK, importante moduladora negativa da atividade de cofilin, impedindo a despolimerização dos filamentos de actina e estabilizando o citoesqueleto.

A formação e estabilização do traço mnemônico são processos que dependem dos mecanismos que levam a síntese de novas proteínas e estabilização do citoesqueleto de actina nas sinapses ativadas por ocasião do aprendizado. Após a reativação de uma memória previamente aprendida, nova síntese de proteínas relacionadas com plasticidade (PRP) e reorganização do citoesqueleto é necessário para que a memória original se reconsolide. Se houver alguma interferência que impeça a síntese de novas proteínas ou a reorganização do citoesqueleto, o traço da memória original não se reconsolidará e será extinto. A extinção da memória, que se trata de um novo aprendizado, também necessita de novas proteínas sintetizadas e estabilização do citoesqueleto de actina para que seja consolidada. Mas, com a formação da memória de extinção o traço original não é apagado, sendo apenas enfraquecido. Com o estimulo adequado, aquela memória original que havia sido extinta pode ser novamente adquirida através de um processo denominado reaquisição. Desta vez ainda é necessário à reorganização e estabilização do citoesqueleto para que a memória readquirida volte a se estabilizar, mas esse processo não é dependente de nova síntese de proteínas.

Neste estudo nós avaliamos a importância da PAK 1 e 3 à reconsolidação, extinção e reaquisição da memória aversiva, na região CA1 do hipocampo de ratos Wistar. Para tanto, realizamos experimentos onde infundimos o IPA-3 na região CA1 do hipocampo dos ratos em diferentes momentos, em relação ao treino no experimento comportamental CFC. Os experimentos realizados estão resumidos no diagrama a baixo (Fig 18):

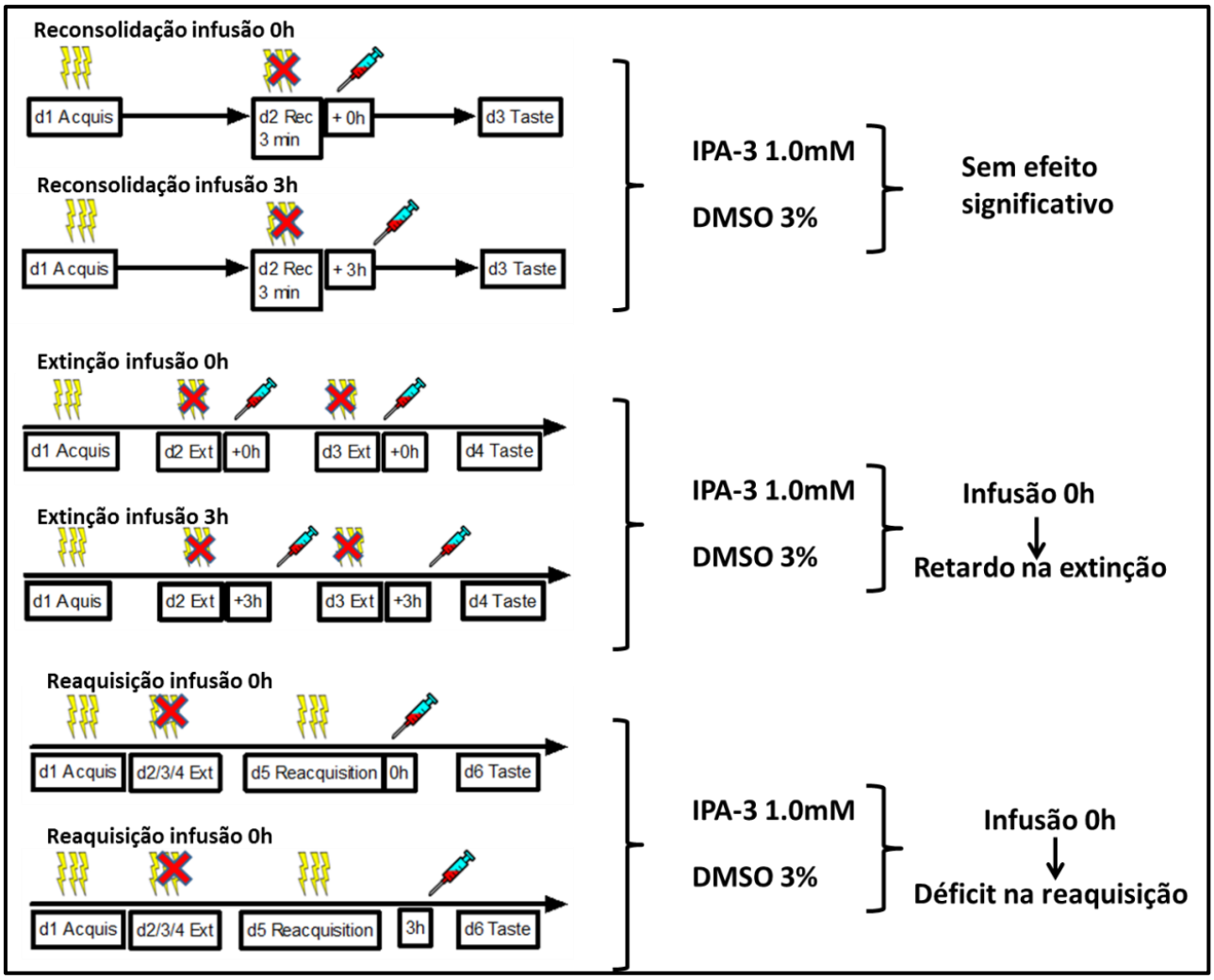

Figura 18 – Resumo do desenho experimental e dos resultados.

Como podemos observar no diagrama, a infusão do IPA-3 0h ou 3h após a reativação não impediu que a memória aversiva de CFC se reconsolidasse, indicando que a PAK 1 e 3 não tem um papel importante durantes os eventos moleculares subjacentes ao processo de rearranjo do traço fragilizado pela reativação, na região CA1 do hipocampo. Por outro lado, vemos que o processo de extinção da memória de CFC é afetada pelo bloqueio da PAK 1 e 3 em CA1, às 0h após o treino de extinção no dia 2, visto que no dia 3 o *freezing* dos animais tratados é elevado em comparação como os animais controles. No entanto, no dia 4 os animais tratados não se comportaram diferentes dos controles, reduzindo o tempo de *freezing* em níveis significativamente iguais aos dos animais controles; neste experimento deduzimos que o bloqueio da PAK 1 e 3 no hipocampo dorsal pode retardar a extinção da memória aversiva, mas não impede que ela aconteça.

Ademais, quando as infusões foram realizadas 3h após o treino de extinção, não houve diferença significativa entre os animais tratados e controles, visto que o tempo de *freezing* de ambos os grupos foi baixo no dia 4, quando comparado com os dias anteriores. A reaquisição da memória, por sua vez, se mostrou dependente da atividade da PAK 1 e 3 em CA1 a partir do início de sua consolidação, mas não a partir da 3ª hora da sua consolidação. A inibição da PAK 1 e 3 ás 0h após o treino de reaquisição prejudicou a sua consolidação, visto que no teste de CFC 24h depois os animais tratados apresentaram um tempo de *freezing* significativamente menor que os controles. Os resultados alcançados sugerem fortemente a importância da PAK 1 e 3 nos eventos moleculares necessários aos processos mnemônicos, na região CA1 do hipocampo. A importância da PAK1 e 3 na formação da memória se deve, provavelmente, ao seu papel nos mecanismos intracelulares que levam a síntese de novas proteínas e estabilização do citoesqueleto de actina, eventos necessários aos mecanismos de plasticidade que aumentam a eficácia nas sinapses envolvidas na formação do traço mnemônico. No entanto, ainda são necessários estudos complementarem que conduzam a novas informações sobre a atividade das PAK 1 e 3 nos processos de plasticidade e memória.

Perspectivas

Estudos que possam trazer maior acurácia acerca do tempo necessário para a razão de PAK1e3 fosforilada/PAK1e3 total aumentar significativamente após o treino no medo condicionado, bem como quanto tempo é necessário para que essa razão volte aos valores basais, são de extrema importância para melhor conhecer o papel dessa cinase nos mecanismos de plasticidade que participam da formação da memória. Também, conhecer melhor o papel de outras moléculas chaves aos mecanismos de plasticidade, em função da atividade ou bloqueio da PAK1 e 3 seria extremamente esclarecedor. Por exemplo, avaliar a densidade de receptores AMPA, especialmente a sua subunidade GluR1, na superfície da membrana de neurônios piramidais da região CA1 do hipocampo, ou a translocação da Arc para próximo da membrana celular, em função da atividade ou inibição da PAK1 e 3 traria informações mais claras sobre o quanto, onde e em que momento a PAK1 e 3 se mostra importante a diversas fases da formação da memória.

Ademais, avaliar a atividade da PAK1 e 3 em função da atividade do glutamato e do óxido nítrico, dois mensageiros de importância absoluta aos mecanismos de neuroplasticidade e implicados em diversas desordens neurológicas, tais como Alzheimer

e Esquizofrenia, poderiam indicar o potencial dessas cinases no desenvolvimento da fisiopatologia dessas doenças. Para tanto, trabalhos com tecidos neurológicos e/ou de células neuronais de cultura primária, associados a técnicas experimentais tais como Western Blotting, imunofluorescência e PCR, são essenciais e de grande valia para demonstrar a quantificação e localização moleculares e associação entre a PAK1 e 3 e outros fatores morfológicos indicativos de plasticidade.

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