UNIVERSIDADE ESTADUAL DO NORTE FLUMINENSE DARCY RIBEIRO

FREDERICO VELASCO COSTA SANGUEDO

ESTUDO FARMACOLÓGICO SOBRE A ATIVAÇÃO DA PROTEÍNA ERK NO CONDICIONAMENTO EM ESTRUTURAS CEREBRAIS RELACIONADAS À DEPENDÊNCIA QUÍMICA

Campos dos Goytacazes
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Tese apresentada ao Centro de Ciências e Tecnologias Agropecuárias da Universidade Estadual do Norte Fluminense Darcy Ribeiro, como requisito parcial para a obtenção do grau de Doutor em Ciência Animal, na Área de Concentração de Produção, Reprodução e Saúde Animal.

ORIENTADORA: Prof.ª Drª. Marinete Pinheiro Carrera

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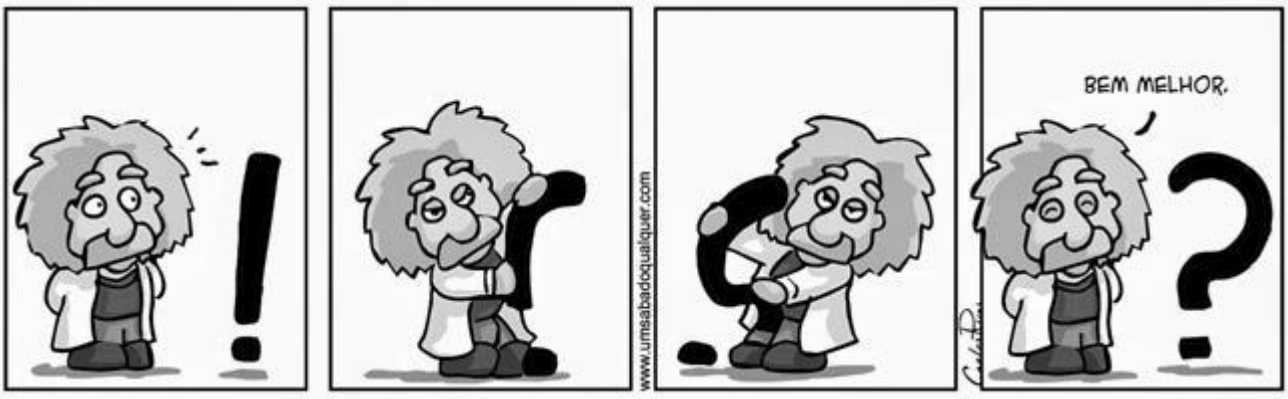
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RESUMO

O tratamento repetido com agonistas dopaminérgicos, como a apomorfina, produz o desenvolvimento dos processos de condicionamento e sensibilização comportamental. Esses processos produzem a ativação da proteína quinase regulada por sinais extracelulares (ERK) em estruturas cerebrais relacionadas à dependência química, tais como a parte rostral do lobo frontal, também chamado de “córtex pré-frontal” e o “núcleo *accumbens*”. O trabalho teve como objetivo avaliar a ativação da ERK no “córtex pré-frontal” e no “núcleo *accumbens*” na resposta locomotora condicionada decorrentes de diferentes tratamentos com uma dose elevada de apomorfina (2,0 mg/ kg). O primeiro objetivo foi construir uma curva temporal de ativação da ERK-P, após administração aguda de apomorfina (2,0 mg/ kg) ou veículo, após os tempos de 5, 15, 30 e 60 min. Em seguida, avaliar a ativação da ERK-P, após um teste de condicionamento associado ao ambiente com duração de 30 min, outro teste não associado ao ambiente com duração de 30 min, outro teste associado ao ambiente com duração de 5 min e um último teste, utilizando-se pós-tratamento investigando seu efeito na reconsolidação da memória. Para isso, diferentes experimentos foram realizados. Antes do início dos tratamentos, os animais passaram por um período de habituação de três dias no qual receberam apenas solução do veículo. No primeiro experimento, foi examinado o efeito do condicionamento na ativação da ERK em um teste em campo aberto. Os ratos passaram por um período de indução de cinco dias em que receberam apomorfina (2,0 mg/ kg) ou veículo imediatamente antes da colocação na arena experimental para registro da locomoção por 30 min. Após um período de retirada de dois dias, foi realizado o teste de condicionamento de 30 min. Imediatamente após o teste, foi feita a imunoistoquímica para medir a ativação de ERK. Os resultados mostraram que, no experimento 1, o tratamento com apomorfina produziu o desenvolvimento da sensibilização comportamental e uma resposta condicionada. A imunoistoquímica mostrou o aumento da ativação ERK no “córtex pré-frontal”, mas não no “núcleo *accumbens*”. No segundo experimento, um protocolo de teste semelhante foi realizado. Porém, os animais receberam o tratamento farmacológico não associado ao ambiente de teste. Os resultados mostraram que não

houve resposta condicionada, nem diferença de ativação de ERK em comparação com os grupos tratados com veículo nas estruturas cerebrais em estudo. Juntos, esses resultados sugerem a participação do “córtex pré-frontal” nas alterações associativas neuroadaptativas induzidas pela estimulação dopaminérgica. No terceiro experimento, dois grupos de ratos tratados ou com apomorfina (2,0 mg/ kg) ou com o veículo, foram submetidos à fase de indução em que receberam apomorfina ou veículo durante cinco dias em sessões de 30 minutos para registro da locomoção. Posteriormente ao período de retirada de 2 dias, os animais passaram por um teste de condicionamento de cinco minutos, no qual o grupo tratado com apomorfina mostrou uma resposta condicionada. A imunoistoquímica mostrou ativação de ERK tanto no córtex quanto no “núcleo *accubens*”. No quarto experimento houve também sensibilização, os ratos passaram por uma fase de indução de cinco dias, um dia de teste de condicionamento, dois dias de retirada e um dia de reindução, para serem, então, subdivididos em dois grupos veículo e dois grupos apomorfina. Os grupos tratados com o veículo receberam o veículo imediatamente (VI) ou 15 minutos (VD) após dois dos três testes de condicionamento, e os grupos tratados com a apomorfina (AI e AD) seguiram o mesmo protocolo. No primeiro teste de condicionamento, ambos os grupos tratados com apomorfina exibiram respostas condicionadas equivalentes. No terceiro teste, somente houve resposta condicionada do grupo AI, enquanto que no grupo AD não houve resposta condicionada. Imediatamente após o terceiro teste de condicionamento, iniciou-se o protocolo para medir a ativação da ERK no “córtex pré-frontal” e no “núcleo *accumbens*”. A ativação de ERK ocorreu tanto no “córtex pré-frontal” quanto no “núcleo *accumbens*” no grupo apomorfina imediato (AI). Esses resultados sugerem que a atividade de ERK está envolvida com a resposta condicionada da apomorfina associada à ausência de atividade nos grupos que receberam tratamento após 15 min. O trabalho sugere que a ativação da ERK é um indicador de atividade dopaminérgica em sistemas cerebrais importantes na aprendizagem e memória e que tratamentos pós-teste imediatos podem modificar a reconsolidação do comportamento condicionado.

Palavras-chave: condicionamento; ERK; apomorfina; sensibilização comportamental; memória; dependência química.

ABSTRACT

Repeated treatment with dopaminergic agonists, such as apomorphine, leads to the development of behavioral conditioning and sensitization processes. These processes produce the activation of extracellular signal-regulated protein kinase (ERK) in chemical structures related to addiction, such as the rostral part of the frontal lobe, also called the prefrontal cortex and the nucleus accumbens. The aim of the study was to evaluate the activation of ERK in the "prefrontal cortex" and the "nucleus accumbens" in the conditioned locomotor response resulting from different treatments with a high dose of apomorphine (2.0 mg / kg). The first aim was to construct a time curve of ERK-P activation after acute administration of apomorphine (2.0 mg / kg) or vehicle after the 5, 15, 30 and 60 min times. Then, to evaluate the activation of the ERK-P, after a conditioning test associated to the environment with duration of 30 min, another test not associated to the environment with duration of 30 min, another test associated to the environment with duration of 5 min and one the last test using post-treatment investigating its effect on memory reconsolidation. For this, different experiments were performed. Before the start of the treatments, the animals went through a three-day habituation period in which they received only vehicle solution. In the first experiment, the effect of conditioning on ERK activation was examined in an open field test. Rats underwent a five-day induction period receiving apomorphine (2.0 mg / kg) or vehicle just prior to placement in the experimental arena for locomotion recording for thirty minutes. After a two-day withdrawal period, the 30-min conditioning test was performed. Immediately after the test, immunohistochemistry was performed to measure ERK activation. The results showed that, in experiment 1, treatment with apomorphine produced the development of behavioral sensitization and a conditioned response. Immunohistochemistry showed increased ERK activation in the "prefrontal cortex", but not in the "nucleus accumbens". In the second experiment, a similar test protocol was performed. However, the animals received pharmacological treatment not associated with the test environment. The results showed that there was no conditioned response or difference in ERK activation compared to vehicle-treated groups in the brain structures under study. Together, these results suggest the participation of the

"prefrontal cortex" in the neuroadaptive associative changes induced by dopaminergic stimulation. In the third experiment, two groups of rats treated with apomorphine (2.0 mg / kg) or vehicle were subjected to the induction phase in which they received apomorphine or vehicle for five days in thirty-minute sessions to record the locomotion. After the 2-day withdrawal period, the animals underwent a five-minute conditioning test, in which the apomorphine treated group showed a conditioned response. Immunohistochemistry showed activation of ERK in both the cortex and the "nucleus accubens". In the fourth experiment there was also sensitization, the rats underwent a five-day induction phase, one conditioning test day, two days withdrawal, one day of re-induction, to be subdivided into two vehicle groups and two apomorphine groups. Vehicle-treated groups received the vehicle immediately (VI) or 15 minutes (RV) after two of the three conditioning tests, and the apomorphine-treated groups (AI and AD) followed the same protocol. In the first conditioning test, both groups treated with apomorphine exhibited equivalent conditioned responses. In the third test, there was only conditioned response of the AI group, whereas in the AD group there was no conditioned response. Immediately after the third conditioning test, the protocol was started to measure the activation of ERK in the "prefrontal cortex" and in the "nucleus accumbens". ERK activation occurred in both the prefrontal cortex and the nucleus accumbens in the immediate apomorphine (AI) group. These results suggest that ERK activity is involved in the conditioned response of apomorphine associated with absence of activity in the groups receiving treatment after 15 min. The work suggests that ERK activation is an indicator of dopaminergic activity in brain systems important in learning and memory, and that immediate post-test treatments can modify the reconsolidation of conditioned behavior.

Keywords: conditioning; ERK; apomorphine; behavioral sensitization; memory; addiction.

SUMÁRIO

1. INTRODUÇÃO

A dependência química é um grave problema de saúde pública que apresenta consequências físicas, mentais e sociais incalculáveis. Um dos problemas clínicos relacionados à dependência química é a recaída dos dependentes. O sistema dopaminérgico é considerado a chave para a compreensão das alterações produzidas pelos psicoestimulantes e a proteína quinase regulada por sinais extracelulares (ERK), tem sido explorada na tentativa de atenuar ou reverter os mecanismos da dependência.

Uma das explicações para a recaída é o desenvolvimento do processo de sensibilização comportamental, que é caracterizado pelo aumento progressivo de uma determinada resposta comportamental, quando a mesma dose da droga é administrada repetidamente (ROBINSON e BERRIDGE, 1993). É inegável a contribuição da sensibilização comportamental e do condicionamento na gênese e manutenção da dependência.

O condicionamento é um tipo de aprendizagem associativa, na qual uma mudança comportamental apresentada pelo indivíduo é resultado da interação desse indivíduo com o ambiente. Na farmacodependência, o condicionamento se estabelece quando os efeitos das substâncias psicoativas são associados ao ambiente, fazendo com que pistas ambientais (estímulos inespecíficos, como sons, cheiros, luzes) que frequentemente estão associadas ao uso da droga tornem-se preditoras dos seus efeitos. Operacionalmente, no condicionamento, um ambiente específico (estímulo condicionado) é associado com a administração de um fármaco psicoativo (estímulo incondicionado) tal que, quando o animal é reexposto ao ambiente na ausência do fármaco, ele exibirá uma resposta comportamental semelhante à resposta produzida pelos efeitos do fármaco (resposta condicionada).

A dependência química, enquanto um problema multifatorial, tem recebido destaque ao ser tratada como um estado suscetível a modificações reguladas pelo sistema dopaminérgico. O condicionamento induzido por drogas é de grande importância para a identificação dos substratos neurais relacionados aos processos de aprendizagem e memória. Este fato pode ser evidenciado pela expressão de uma resposta condicionada semelhante àquela induzida pela droga, mesmo quando o estímulo incondicionado (droga) não está presente.

Os processos de condicionamento e sensibilização comportamental dependente do contexto são dois fenômenos comportamentais relacionados entre si e têm sido demonstrados em animais geralmente após a administração de psicoestimulantes, com o estímulo da atividade dopaminérgica. Em razão de suas propriedades associativas, ambos têm sido relacionados ao desenvolvimento e à expressão da sensibilização comportamental. Assim, a atenuação e/ou bloqueio desses processos, sensibilização e condicionamento, são desejáveis para o tratamento da dependência química.

De acordo com o exposto, é possível inferir que processos que produzam um enfraquecimento, ou mesmo um bloqueio da associação entre uma substância psicoativa e o ambiente no qual o seu efeito é experimentado possam atenuar ou reverter às consequências da dependência química. Várias vias de sinalização dopaminérgica e marcadores moleculares têm sido explorados para decifrar seus mecanismos. Acredita-se que a proteína quinase regulada por sinais extracelulares (*extracellular signal-regulated kinase* ERK) tenha grande importância na neuroplasticidade de longa duração. A ERK é uma das proteínas quinases da cascata de proteínas quinases ativadas por mitógeno (*mitogen activated protein kinase* MAPK), que pode ser ativada por estimulação dos receptores dopaminérgicos D_1 e D_2.

Estudos da literatura científica mostraram o papel da ERK nos processos de sensibilização e condicionamento usando-se agonistas dopaminérgicos indiretos, tais como a cocaína e as anfetaminas. A escolha da apomorfina é interessante, pois é um agonista direto de receptores dopaminérgicos D_1 e D_2. A apomorfina possui uma ação dual, em termos de condicionamento de drogas, na qual a apormofina apresenta efeitos mais pronunciados, mas opostos sobre a neurotransmissão de dopamina, dependendo da dose. Em doses elevadas (> 1,0 mg/kg) atua preferencialmente nos receptores dopaminérgicos pós-sinápticos produzindo aumento da atividade locomotora em ratos. Em doses baixas (< 0,1 mg /kg), atua preferencialmente nos receptores pré-sinápticos, D_2 produzindo diminuição da atividade locomotora. Em trabalhos anteriores do nosso grupo de pesquisa (BLOISE et al., 2007; BRAGA et al., 2009a, b; DIAS et al., 2010; DE MATOS et al., 2010), mostrou-se que tratamentos repetidos de altas doses de apomorfina induzem ao aumento da locomoção e que essa resposta comportamental sofre sensibilização dependente do contexto e, além disso, gera efeitos estimulantes

locomotores condicionados (BRAGA et al., 2009a, b; DIAS et al., 2010; DE MATOS et al., 2010). Em nossos primeiros relatos sobre a relação da ativação da ERK com a atividade dopaminérgica induzida pela apomorfina, mostramos que a apomorfina produziu ativação da forma fosforilada da ERK (ERK-P), no córtex pré-frontal, após o desenvolvimento da sensibilização comportamental dependente do ambiente (SANGUEDO et al., 2014) e que a amígdala teve ativação da ERK relacionada ao efeito do ambiente (SANGUEDO et al., 2016).

Estruturas cerebrais relacionadas à formação das emoções são importantes constituintes do sistema límbico. Em consonância com esses resultados, relatamos que a sensibilização à apomorfina potencializa seletivamente a resposta da ERK induzida pela apomorfina no córtex pré-frontal (SANGUEDO et al., 2014). Na medida em que também se mostrou que a sensibilização à apomorfina é específica do contexto (DE MATOS et al., 2010), nossos trabalhos sugeririam que o “córtex pré-frontal” está envolvido em respostas associativas de drogas. O “córtex pré-frontal” controla a memória, atenção, é importante no reconhecimento de objetos e associação de memórias com o ambiente e está ligado a outra importante estrutura cerebral relacionada à dependência química, o “núcleo *accumbens*” (estriado ventral), uma estrutura fortemente envolvida com a emoção, recompensa e prazer.

Diante do exposto, o presente trabalho se propôs a investigar o efeito dos processos de condicionamento induzidos pela apomorfina sobre a ativação da proteína ERK em estruturas cerebrais dopaminérgicas por meio da avaliação imunoistoquímica da ERK-P. Os resultados deste trabalho podem contribuir para a melhor compreensão dos mecanismos moleculares e a busca por novas estratégias para reverter ou atenuar a dependência química.

2. REVISÃO DE LITERATURA

2.1 - O PROCESSO DE CONDICIONAMENTO INDUZIDO POR DROGAS

Entre os problemas decorrentes da dependência química está a recaída, que pode ocorrer quando os indivíduos são reexpostos a um ambiente associado ao consumo de psicoestimulantes. Esse fenômeno pode ser explicado pelo processo de condicionamento. O condicionamento é uma forma de aquisição de novos conhecimentos, por meio da qual ocorrem modificações comportamentais, que resultam das influências ambientais (SIEGEL, 1988; O'BRIEN et al., 2006, BEAVER, 2001).

Pode-se dividir didaticamente o condicionamento em operante e clássico. No condicionamento operante, um determinado evento produz um resultado que pode influenciar na ocorrência da resposta futura. Por exemplo, o resultado punitivo diminui a chance de que o evento ocorra novamente, acarretando ainda efeitos secundários. Já o resultado reforçador aumenta a chance de que o evento ocorra posteriormente (SKINNER, 1982).

O fisiologista Ivan Petrovitch Pavlov foi responsável pelo desenvolvimento do condicionamento clássico, por meio do qual criou-se uma associação entre um estímulo dito neutro (condicionado), associado a um outro estímulo (incondicionado), que produziria um determinado efeito passível de mensuração. Por exemplo, a luz associada à presença do alimento aumentaria a salivação de um cão (ATKINSON et al., 1995).

Pavlov testou os efeitos produzidos pela administração de apomorfina em cães por via subcutânea no condicionamento clássico. Neste teste, era emitido um som após alguns minutos da administração da droga (Pavlov, citado por McKIM, 2000). As associações entre os estímulos condicionados (som) e o estímulo incondicionado (droga) foram feitas de modo que, depois de um tempo, observou-se que a simples apresentação do estímulo condicionado era capaz de produzir os mesmos efeitos relacionados à apomorfina. Os efeitos da apomorfina nos cães são: êmese, agitação e salivação excessiva. Os mesmos efeitos foram notados quando o animal foi exposto ao som sem que houvesse a administração da droga. É mostrado que houve uma

associação dos fatores ambientais com os efeitos condicionais produzidos pela droga. Essa aprendizagem associativa seria responsável pelos mesmos efeitos encontrados na administração da apomorfina apenas, o que reforça a importância dos estímulos ambientais na expressão dos efeitos comportamentais (DREW e GLICK, 1988).

No condicionamento clássico, os estímulos produzidos por uma droga, denominados incondicionados, produzem as mesmas respostas em indivíduos da mesma espécie. A resposta condicionada é aquela que resultou do estímulo aprendido, sendo esse processo denominado condicionamento clássico ou Pavloviano. Por outro lado, o estímulo neutro, denominado condicionado, não é capaz de produzir sozinho, uma mesma resposta nos indivíduos da mesma espécie. No entanto, quando o estímulo incondicionado é associado a um ambiente, a resposta gerada pela droga é evocada quando apenas o estímulo neutro é apresentado ao indivíduo (Pavlov, citado por McKIM, 2000).

Por isso, o condicionamento clássico induzido por drogas é de grande importância para a identificação dos substratos neurais relacionados aos processos de aprendizagem e memória. Este fato é notado pela expressão de uma resposta condicionada semelhante àquela induzida pela droga, mesmo quando o estímulo incondicionado (droga) não está presente. Dessa forma, é possível que os mesmos substratos neurais envolvidos na resposta à droga sejam ativados durante à exposição ao estímulo condicionado (DAMIANOPOULOS e CAREY, 1992).

Estudos anteriores demonstraram que psicoestimulantes que atuam de forma indireta na liberação da dopamina, tais como cocaína e anfetaminas, e de forma direta, como a apomorfina, desencadearam o processo de condicionamento (BROWN et al., 1992; FONTANA et al., 1993). Após as associações entre o ambiente e a droga, o estímulo ambiental foi capaz de gerar uma resposta semelhante àquela observada pela administração da droga. Dessa forma, esse processo reforça a interação entre o condicionamento e a ação das drogas de abuso sobre o sistema dopaminérgico (BROWN et al., 1992; FONTANA et al., 1993).

Na literatura científica está bem estabelecido que a ativação do sistema dopaminérgico estaria envolvida com o processo de condicionamento (SCHIFF, 1982; PERT et al., 1990). Carrera e colaboradores (1998) e Dias e colaboradores (2006)

verificaram que administrações de apomorfina intraestriatais levaram ao desenvolvimento da atividade locomotora condicionada, sugerindo o envolvimento do estriado dorsal no condicionamento. Em conformidade aos dados da literatura, diversos trabalhos do nosso laboratório têm mostrado que a administração crônica de psicoestimulantes induzem tanto a sensibilização dependente do ambiente como os efeitos condicionados que são reforçados pela apomorfina (BLOISE et al., 2007; BRAGA et al., 2009a, 2009b; DIAS et al., 2010; DE MATOS et al., 2010).

2.2 - A SENSIBILIZAÇÃO COMPORTAMENTAL E A ERK

A sensibilização comportamental é o processo em que a administração crônica de psicoestimulantes produz o aumento significativo da atividade locomotora em resposta às administrações diárias e em doses fixas (constantes) do psicoestimulante (ROBINSON e BECKER, 1986; CADOR et al., 1995).

Esse fenômeno pode ser compreendido como fenômeno oposto ao processo de tolerância[1], pois se por um lado algumas drogas têm seu efeito diminuído com o uso crônico, como os analgésicos opioides, outras apresentam o aumento dos seus efeitos com o uso crônico (sensibilização comportamental), o que poderia ser denominado de tolerância reversa (CADOR et al., 1995; HYMAN et al., 2006).

O processo de sensibilização comportamental caracteriza-se por desviar a curva dose-resposta para a esquerda de maneira que para certa dose, observa-se uma resposta locomotora maior do que a gerada pela dose inicial. A sensibilização comportamental tem sido atribuída às alterações intracelulares, como fatores de transcrição, função proteica e enzimática (NESTLER, 2001).

A sensibilização comportamental pode ser encontrada com a utilização de diferentes drogas. Dentre elas estão: a cocaína (MISERENDINO e NESTLER, 1995), a apomorfina (BLOISE et al., 2007; BRAGA et al., 2009a) e as anfetaminas (FRAIOLI et al., 1999). Uma característica do processo de sensibilização comportamental é que os animais permanecem sensibilizados mesmo após longos períodos de abstinências (meses ou anos), mesmo cessada a administração da droga, além de ocorrer o reforço

[1]Redução do efeito da droga após a sua administração repetida (O´BRIEN, 2006).

da autoadministração de drogas (HYMAN et al., 2006; HOPE et al., 2006; CARLEZON e NESTLER, 2002; ROBINSON e BECKER, 1986). É bem estabelecido que a sensibilização comportamental seja produzida pelo aumento na liberação de dopamina (ROBINSON e BECKER, 1986).

Este processo pode ser resultante da ação de agonistas indiretos que aumentam os níveis extracelulares de dopamina no "núcleo *accumbens*" (KALIVAS e DUFFY, 1989) ou, também, por agonistas dopaminérgicos diretos, como a apomorfina (MATTINGLY et al., 1991).

Entre as hipóteses que buscam explicar a sensibilização comportamental está a teoria do condicionamento, da tolerância dos autorreceptores e da neuroquímica (ROBINSON e BECKER, 1986). Na teoria do condicionamento, o aumento da locomoção estaria relacionado a uma resposta locomotora condicionada a um estímulo neutro (ambiente). Uma explicação para a hipótese da tolerância dos autorreceptores seria a de que os receptores se tornariam menos sensíveis com a exposição repetida aos agonistas dopaminérgicos levando a uma diminuição do efeito inibitório sobre a síntese e liberação da dopamina, ou seja, ocorreria o aumento da liberação de dopamina e, consequentemente, da resposta locomotora (ROBINSON e BECKER, 1986; BERKE e HYMAN, 2000). Segundo a hipótese neuroquímica, a sensibilização ocorreria pelo aumento da liberação de dopamina induzida por agonistas dopaminérgicos indiretos, como a cocaína e as anfetaminas, que atuam inibindo a recaptação de dopamina na fenda sináptica (KALIVAS e DUFFY, 1989; MATTINGLY et al., 1991).

É possível que o papel do ambiente seja efeito da interação entre o estímulo incondicionado e condicionado, cujo aumento ocorre à medida que o animal experimenta o efeito do psicoestimulante mais vezes (CADOR et al., 1995; HYMAN et al., 2006). Uma característica importante na sensibilização dependente do contexto é que o animal experimente os efeitos no mesmo ambiente do desenvolvimento da sensibilização (VALJENT et al., 2006). Por isso, a resposta da expressão da sensibilização seria diferente, caso o animal fosse colocado em um ambiente novo, diferente daquele no qual ocorreu a indução.

Uma modificação sutil no contexto/ambiente em que as drogas são administradas exerce uma forte influência sobre a resposta neurocomportamental dos animais (SANCHIS-SEGURA e SPANAGEL, 2006). O desenvolvimento da sensibilização comportamental também depende de fatores relacionados ao procedimento experimental. Por exemplo, uma variação do tempo de permanência do animal no ambiente experimental pode determinar o aumento ou a diminuição da resposta locomotora. A presença e as ações típicas do manipulador, tais como os procedimentos de manipulação e de administração podem influenciar o estado de alerta dos animais (TODTENKOPF e CARLEZON, 2006).

Segundo Valjent e colaboradores (2006), ratos permaneceram sensibilizados no teste de expressão da sensibilização mesmo usando-se um antagonista da ERK, ou seja, impedir a ativação da ERK no teste de expressão da sensibilização não afetou a sensibilização previamente desenvolvida. No entanto, também foi demonstrado que o uso de antagonista de ERK no núcleo *accumbens* pode inibir sua ativação e, consequentemente, a expressão da sensibilização comportamental induzida por cocaína. Assim, a ativação da ERK estaria relacionada à expressão da sensibilização no núcleo *accumbens* (KIM et al., 2011). A sensibilização produzida pela cocaína e dependente do contexto foi acompanhada pelo aumento do número de núcleos imunorreativos a ERK em um grupo pequeno de neurônios do núcleo *accumbens*. Esses neurônios seriam os mesmos relacionados com o aprendizado de associação entre os efeitos da droga com o ambiente (MARIN et al., 2009).

2.3 ATIVIDADE FUNCIONAL DO “CÓRTEX PRÉ-FRONTAL” E DO “NÚCLEO *ACCUMBENS*”

O córtex cerebral (sensitivo e motor) é dinâmico. A mudança contínua sugere que cada indivíduo apresente uma representação somatotópica única dependente do uso (ex.: aprendizagem motora, lesões). O córtex cerebral tem capacidade plástica, importante em situação de lesão, se a perda não ultrapassar os limites anatômicos

requeridos para que neurônios vizinhos tenham a capacidade de se mudar para as áreas adjacentes (SERUCA, 2013; ONGUR e PRICE, 2000).

A plasticidade cortical é visível após períodos de treino. Por exemplo: aumento de uma habilidade motora após períodos de prática intensiva. Quando uma tarefa implica o uso seletivo de uma parte específica, a área cortical correspondente a esta zona sofre hipertrofia, levando à invasão das zonas vizinhas comprometidas, devido ao aumento da carga cognitiva sobre estas. Este mecanismo resulta da excitação simultânea dos neurônios pré e pós-sinápticos, levando à amplificação sináptica (PASCUAL-LEONE, 2005).

O córtex cerebral é a fina camada de substância cinzenta que reveste o centro branco medular do cérebro. No córtex, chegam os impulsos provenientes de todas as vias de sensibilidade e lá são interpretados. No córtex, existem neurônios, células neurogliais e fibras. A divisão córtex em lobos não corresponde a uma divisão funcional ou estrutural, pois em um mesmo lobo temos áreas corticais de funções e estruturas diferentes (SERUCA, 2013; ONGUR e PRICE, 2000).

As áreas funcionais do córtex cerebral podem ser divididas entre: áreas de projeção (primárias) sensitivas e motoras; áreas de associação secundárias e terciárias (memórias, processos simbólicos e pensamento abstrato). As áreas sensitivas primárias se subdividem entre: somestésica, que está localizada no giro pós-central correspondente às áreas 3,2,1 do mapa de Brodman; área visual, que está localizada nos lábios do sulco calcarino (área de 17 de Brodman); área auditiva, que está localizada no giro temporal transverso anterior, áreas 41 e 42 de Brodman; área olfatória, correspondente à área 34 de Brodman e área gustativa, que está localizada na área 43 de Brodman, porção inferior do giro pós-central (PASCUAL-LEONE, 2005).

A área “pré-frontal” compreende a parte anterior não motora do lobo frontal. Ela recebe fibras das áreas de associação do córtex ligando-se ao sistema límbico. As principais funções são: escolha das opções e estratégias comportamentais mais adequadas à situação física e social do indivíduo; capacidade de alterá-las quando tais situações se modificam; manutenção da atenção (seguir sequência ordenada de pensamentos); controle do comportamento emocional juntamente com o hipotálamo e o

restante do sistema límbico; comportamento, linguagem e raciocínio; funções cognitivas como a conceptualização; flexibilidade mental (SERUCA, 2013).

O “córtex pré-frontal” preenche cerca de um quarto do córtex cerebral. Compreende três áreas distintas: orbital ou inferior, medial/cingulada e lateral. A área orbital do “córtex pré-frontal”, pela sua proximidade física na região ventral ou inferior com as órbitas oculares subdividida nas áreas 11, 12, 13 e 14, tem ligações com o lobo temporal e recebe estímulos límbicos menores. Apresenta projeções subcorticais para o hipotálamo e complexo amigloide. Recebe informações sensoriais do processamento visual, olfativo e do paladar (SERUCA, 2013; ONGUR e PRICE, 2000).

A região medial do “córtex pré-frontal” abarca as áreas 25 e 32 e tem ação no sistema motor e sistema límbico. Ela tem projeções para estruturas dos núcleos da base (caudado ventromedial, “putâmen” ventral, núcleos “*accumbens*” e tubérculo olfatório) com ação no comportamento motivado. A área dorsolateral engloba as áreas 9 e 46, a área 9 lateral e área 9-46, devido às semelhanças citoarquitetônicas entre ambas. A área 9 tem conexões com áreas pré-motoras (6 e 8), parte do córtex orbital (11), ínsula e partes do sistema límbico. As áreas 9/46 têm conexão com a área 11 orbitofrontal e área 19 medial no lobo occipital. Suas funções são: organização, planejamento, atenção e mediando a organização de informação (SERUCA, 2013; ONGUR e PRICE, 2000).

O núcleo *accumbens* (estriado ventral) está localizado na cabeça do núcleo caudado, anterior ao “putâmen”, lateralmente ao septo pelúcido. Essa estrutura é importante na captação/percepção dos estímulos ambientais (dicas associadas à droga). O núcleo *accumbens* pode ser dividido histologicamente em dois compartimentos: o núcleo (*core*) e a concha (*shell*). Essas duas subdivisões formam juntas uma importante estrutura cerebral relacionada às funções motora, recompensadora e emocional (ZAHM e BROG, 1992; BERENDSE et al., 1992; PAXINOS e WATSON, 2004; BASSAREO e DICHIARA, 1999). A diferença entre as duas porções ocorre principalmente pelo padrão diferente de inervação que apresentam (BASSAREO e DICHIARA, 1999). Enquanto a porção *core* apresenta semelhanças ao estriado dorsal e não se distingue histologicamente do resto do corpo estriado, a porção

shell se projeta para estruturas diencefálicas, como o hipotálamo lateral (BERENDSE et al., 1992).

A ligação da porção *shell* com o hipotálamo contribui para estabelecer associações entre as emoções e as dicas ambientais (ITO et al., 2004). Essa ligação com as emoções produz resposta dopaminérgica sobre novos estímulos de recompensa (ROBBINS e EVERITT, 1992; BERRIDGE e ROBINSON, 1998; ITO et al., 2004). Por outro lado, o compartimento *core* medeia a resposta comportamental frente a dicas do contexto associado à droga (KALIVAS e VOLKOW, 2005).

Os estímulos incondicionados contribuem para o aumento da liberação da dopamina no "núcleo *accumbens*", especialmente na porção *shell*, responsável pela aprendizagem e regulação dos estímulos incondicionados (CARDINAL et al., 2002). Bassareo e Dichiara (1999) relataram que os níveis da dopamina aumentaram na porção *shell* em resposta a estímulos incondicionados (cocaína) (ITO et al., 2000). Ao contrário da porção *core* do "núcleo *accumbens*", que está relacionada aos estímulos condicionados (dicas do ambiente) (ITO et al., 2000; BASSAREO e CHIARA, 1999). Por fim, ambos os compartimentos, *core* e *shell,* induzem a locomoção e a estimulação dopaminérgica (SWANSON et al., 1997).

A transmissão dopaminérgica no "núcleo *accumbens*" induz o processo de sensibilização induzida pela administração crônica de psicoestimulantes (DICHIARA, 2002). O papel da dopamina sobre o "núcleo *accumbens*" produz efeitos de recompensa e motivacionais, importantes na farmacodependência (BERRIDGE e ROBINSON, 1998). É o alvo principal dos neurônios dopaminérgicos da via mesolímbica, que se origina a partir da área tegmental ventral (BASSAREO e DICHIARA, 1999).

2.4 PROTEÍNA QUINASE REGULADA POR SINAIS EXTRACELULARES

A ERK, proteína quinase regulada por sinais extracelulares, pertence à família das proteínas quinases ativadas por mitógenos, as MAPK, pertencentes a um grupo de enzimas da família das proteínas serina-treonina quinases (ALIAGA et al., 1999, HERSKOWITZ, 1995). Elas são responsáveis pela transmissão de sinais mitogênicos,

geração de respostas adaptativas frente às mudanças ambientais, regulação da expressão genética, síntese de proteínas, movimento, metabolismo, crescimento, diferenciação celular e apoptose (JOHNSON e LAPADAT, 2002; SHIFLETT e BALLEINE, 2011).

As ERKs são conhecidas como ERK 1 e ERK 2, sendo também denominadas de p44 e p42 de acordo com o seu peso molecular, respectivamente (BERHOW et al., 1996; ADAMS e SWEATT, 2002). No entanto, a isoforma ERK 2 tem sido mais relacionada a estudos comportamentais de farmacodependência (GIRAULT et al., 2006). A fosforilação de ERK 2 é maior do que a de ERK 1, mas a ERK 2 pode ser inibida por ERK 1 na competição da fosforilação da MEK. A ativação da ERK 2 aumenta a plasticidade neuronial (GIRAULT et al., 2006; LU et al., 2006) e está envolvida na retenção de memórias espaciais, tarefas de medo condicionado, comportamentos duradouros e tem papel no desenvolvimento da sensibilização locomotora (SANGUEDO et al., 2014; SHIFLETT e BALLEINE, 2011).

Entre as principais funções da ERK estão o controle/diferenciação do crescimento celular (SHIFLETT e BALLEINE, 2011), neuroplasticidade sináptica resultante da exposição repetida à droga e estabilidade celular (GIRAULT et al., 2006; LU et al., 2006). A ERK é considerada um parâmetro de aprendizagem e transdução da memória em nível molecular (ADAMS e SWEATT, 2002). Em nível celular, a ERK fosforilada controla substratos, canais iônicos, proteínas do citoesqueleto e liberação de neurotransmissores. Quando translocada para o núcleo, estimula a transcrição genética (HYMAN et al., 2006).

A exposição crônica aos psicoestimulantes (cocaína, anfetaminas) produz uma forte ativação e aumento dos níveis da ERK, quando comparadas aos estímulos naturais (VALJENT et al., 2004a, 2004b). A ativação da ERK pode desencadear o processo de neuroplasticidade, pois tem a propriedade de converter um estado celular de baixo para elevado potencial, rapidamente. Além disso, o ambiente/contexto pode ser um elemento de reforço na ativação da ERK em certas estruturas cerebrais (ADAMS e SWEATT, 2002; KIM et al., 2011). O papel bioquímico da ERK no armazenamento da memória ocorre pela integração dos sinais celulares, e a consolidação da memória sofre influência das fosfatases induzidas pela ERK, que são

elementos importantes no bloqueio das sinapses que não estão suscetíveis à plasticidade (ADAMS e SWEATT, 2002; KIM et al., 2011).

A transdução da ERK resulta da fosforilação dos aminoácidos tirosina e treonina. A sua fosforilação ocorre através das tirosinas quinases, como por ativação dos receptores acoplados à proteína G, GTP (guanosina 5-trifosfato) e da família Ras-Raf. Os receptores Trk ativam a proteína Ras acoplada à GTP, que, por sua vez, ativa a proteína Raf (proteína quinase ativada por mitógeno), responsável por ativar a MEK (uma proteína quinase ativada por mitógeno) e, por último, ativa a ERK (BERHOW et al., 1996; RADWANSKA et al., 2005).

As ERKs fosforiladas atuam diretamente sobre canais iônicos, transportadores, fatores de transcrição (*c-fos*) e quinases, como a RSK (quinase ribossomal S6 de 90 kDa) e a MSK (proteína quinase ativada por mitógenos e *stress*). Ambas proteínas podem fosforilar CREB (elemento proteico regulado por AMPc) (DEAK et al., 1998; FRODIN et al., 1999).

Os psicoestimulantes ativam a ERK na área mesolímbica do encéfalo, tanto em administrações agudas quanto crônicas (GIRAULT et al., 2006; VALJENT et al., 2000). A ativação dos receptores D_1 e D_2 (principalmente D_1) pela dopamina desencadeia a fosforilação da ERK (SALZMANN et al., 2003; VALJENT et al., 2004a). Os receptores D_1 estão em sua maioria acoplados à enzima adenilato-ciclase do tipo 5 (AC 5) através da proteína G. Após a ligação da dopamina ao receptor D_1, os eventos subsequentes levam a conversão de adenosina trifosfato (ATP) em AMPc. Em seguida, o aumento de AMPc ativa a proteína quinase A (PKA), levando à ativação da ERK, embora o próprio aumento do nível de AMPc também possa aumentar a sua ativação (ADAMS e SWEATT, 2002, BERHOW et al., 1996).

Os processos decorrentes da ativação dos receptores dopaminérgicos promovem uma alteração conformacional dos canais de cálcio (Ca^{2+}) voltagem dependentes propiciando o aumento do influxo cálcio para o meio intracelular (BERHOW et al., 1996). Esse aumento é fundamental para a ativação da cascata Ras-Raf-MEK, que ativa a forma MEK. Em seguida, formas ativas da MEK fosforilam

quinases da família MAP, entre elas a ERK e a proteína quinase Jun[2] (JNK) (CHERRIER et al., 2005; RADWANSKA et al., 2005).

Ainda existem outras vias ativadoras da ERK. No corpo estriado, é necessária a participação do DARPP-32[3], uma fosfoproteína também responsável pela fosforilação da ERK e sua ativação. O DARPP-32 é fosforilado pela PKA, tornando-se assim um agente potente na inibição da proteína fosfatase 1 (PP1). O DARPP-32 inibe a PP1 e as fosfatases STEP ficam fosforiladas (STEP-P), permitindo o aumento da concentração de ERK fosforilada (ERK-P) (LU et al., 2006; VALJENT et al., 2004b). Segundo Valjent e colaboradores (2004b), mutações no DARPP-32 provocam alterações na sensibilização locomotora induzida pela administração de psicoestimulantes.

Depois de ativada, a ERK tem muitos alvos celulares e influencia um grande grupo de funções celulares (SHIFLETT e BALLEINE, 2011). A fosforilação da ERK leva à produção de fatores de transcrição, proteínas que atuam direta ou indiretamente sobre o DNA em regiões genéticas promotoras para estimular ou reprimir a transcrição genética (CHERRIER et al., 2005). Alguns dos produtos como o CREB e Elk-1, desencadeiam a ação de proteínas quinases nucleares, como a p90 através da ação de MSK 1 e 2, e por RSK, através da ação da proteína quinase ribossomal S6 (SALZMANN et al., 2003; VALJENT et al., 2006). A ativação dos fatores de transcrição tem papel importante para a indução e transcrição de genes imediatos, como o *c-fos* e o *zif-268* (RADWANSKA et al., 2005). Estes são alvos importantes na caracterização dos efeitos sobre a plasticidade de longa duração induzida pelas drogas, pois a regulação genética é considerada essencial para o estudo das mudanças comportamentais duradouras (SALZMANN et al., 2003).

A proteína ERK ativa indiretamente o CREB e o Elk-1, responsáveis pela transcrição de fatores, como o *c-fos* e *zif-268*. O CREB está envolvido na plasticidade da memória, síntese proteica e resulta da fosforilação no resíduo de serina 133 por diferentes quinases (PKA, RSK2 e CaMK I/IV) (SALZMANN et al., 2003). O fator complexo ternário Elk-1 é um elemento de transcrição e de resposta sobre vários genes

[2] Proteína quinase cuja atividade é regulada em resposta a citocinas ou "*stress*".
[3] Fosfoproteína de 32 kDa modulada por AMPc responsável pela regulação de cascatas de fosforilação.

imediatos (IEG) (*c-fos* e *zif-268*) e, também, um dos primeiros alvos nucleares da ERK (ADAMS e SWEATT, 2002).

Por fim, a ERK possui papel direto e indireto na transcrição genética, fosforilando os fatores de transcrição e os componentes da cromatina (RADWANSKA et al., 2005). A ERK fosforilada, ERK-P, fosforila canais de K^+ aumentando a excitabilidade celular (SHIFLETT e BALLEINE, 2011). Outro efeito da ERK-P é a inserção de ácido alfa-amino-3-hidroxil-5-metil-4-isoxazolepropiônico (AMPA) nas subunidades receptoras transmembrana ionotrópicas para glutamato (diferentes dos receptores NMDA), responsáveis pela mediação da transmissão sináptica rápida no sistema nervoso central (SNC) (LU et al., 2006).

2.5 A APOMORFINA

A apomorfina (APO) é uma substância alcaloide derivada da morfina (não-narcótica) pertencente à classe das dibenzoquilonas com amplo uso em medicina. A sua obtenção ocorreu a partir do aquecimento da morfina em solução de ácido clorídrico e não apresenta características narcóticas da sua precursora. É uma droga lipofílica suscetível a oxidações pela exposição à luz ou ao ar (LEWITT, 2004).

A apomorfina ativa principalmente os receptores dopaminérgicos (D_1, D_2, D_3, D_4 e D_5) de forma não seletiva, mas, também, receptores de serotonina e alfa-adrenérgicos. (LEWITT, 2004; LI et al., 2012; RIBARIC, 2012). Em doses elevadas (> 1.0 mg/kg) ativa tanto receptores dopaminérgicos pré e pós-sinápticos (MATTINGLY e GOTSICK, 1988).

A estrutura química da APO explica suas atividades farmacológicas, pois apresenta grande homologia à molécula da dopamina. A sua estrutura policíclica e terciária de amina possui porção homóloga a da molécula de dopamina. Além de sua ação reguladora através de receptores, a APO possui atividade antioxidante (LEWITT, 2004; LI et al., 2012; RIBARIC, 2012). A APO apresenta um início de ação que varia em torno de 7 a 14 minutos, efeito de duração breve e o tempo de meia vida variando de 30 a 90 minutos, independente da via de administração intravenosa (LEWITT, 2004)

As vias enterais de administração da APO são contraindicadas, pois a APO sofre intenso metabolismo hepático de primeira passagem. Suas ações farmacodinâmicas duram até 30 minutos, a partir do momento em que se inicia o declínio. Em relação aos aspectos de excreção, a APO sofre metabolização hepática, principalmente com reações de glicuronidação e sulfatação e outras vias não enzimáticas (LEWITT, 2004; LI et al., 2012; RIBARIC, 2012).

Em relação à sua aplicação clínica, a APO começou a ser utilizada pelo fim do século XIX para o tratamento de doenças como depressão, insônia e esquizofrenia. Também se utilizou a APO por suas propriedades fortemente eméticas (LEWITT, 2004). Com o passar dos anos, passou a ser empregada para o tratamento da doença de Parkinson, na qual se sugeriu que a APO ativasse os receptores D_2 dos núcleos caudado e putâmen, contribuindo para a redução dos efeitos parkinsonianos. Em seres humanos, teve seu uso substituído pela levodopa devido a seus efeitos adversos. Na medicina veterinária, possui aplicação para a indução de vômito e no tratamento das intoxicações (LEWITT, 2004; MATTINGLY e GOTSICK, 1988).

Os tratamentos repetidos com os agonistas dopaminérgicos, como a apomorfina, resultam em um reforço na atividade comportamental medida pelo aumento da atividade locomotora. A APO produz o aumento da atividade locomotora em doses elevadas (BLOISE et al., 2007; BRAGA et al., 2009a; BRAGA et al., 2009b) possivelmente pela sua ação sobre os receptores D_1 e D_2, modulada pelo “núcleo *accumbens*” e demais estruturas do corpo estriado (MATTINGLY e GOTSICK, 1988). As doses baixas produzem ativação dos autorreceptores D_2 mais do que os receptores D_2 pós-sinápticos promovendo, assim, a diminuição da atividade locomotora (MOLLER et al., 1987). De acordo com Li e colaboradores (2012), a APO estimula os receptores D_1 ativando a via de sinalização da ERK, na qual promove o aumento da fosforilação das proteínas através do aumento de concentrações dos segundos mensageiros AMPc e PKA.

3. HIPÓTESE

O condicionamento induzido pela apomorfina aumentaria a ativação da ERK no “córtex pré-frontal” e no “núcleo *accumbens”*.

4. OBJETIVOS GERAIS

1) Construir uma curva temporal de ativação da ERK-P, após administração aguda de apomorfina (2,0 mg/ kg) ou veículo após os tempos de 5, 15, 30 e 60 min;

2) Avaliar a ativação da ERK-P, após um teste de condicionamento associado ao ambiente com duração de 30 min;

3) Avaliar a ativação da ERK-P, após um teste de condicionamento não associado ao ambiente com duração de 30 min;

4) Avaliar a ativação da ERK-P, após um teste de condicionamento associado ao ambiente com duração de 5 min;

5) Avaliar a ativação da ERK-P, após um teste de condicionamento utilizando-se testes de pós-tratamento, investigando seu efeito na reconsolidação da memória.

5. MATERIAIS E MÉTODOS

As figuras apresentadas mostram o desenho experimental dos experimentos desenvolvidos no presente trabalho que estão detalhados nos artigos publicados.

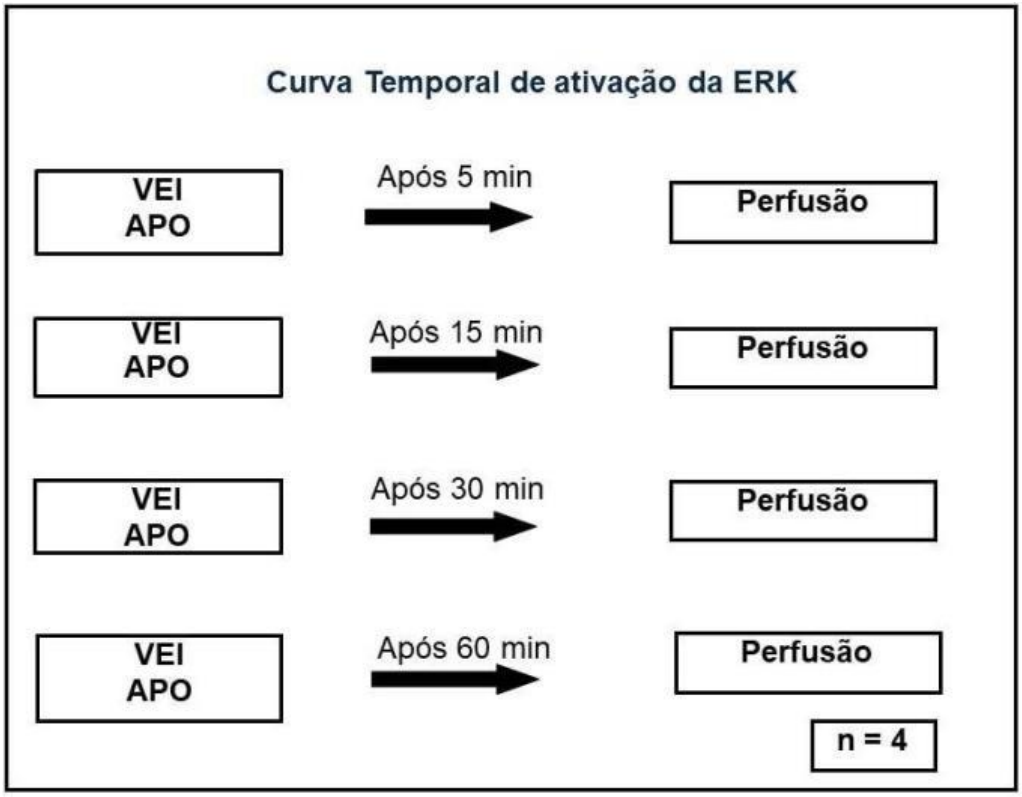

Figura 1: Esquema do desenho experimental da curva temporal de ativação da ERK após a administração aguda de veículo ou apomorfina (2,0 mg/ kg), nos tempos de 5, 15, 30 e 60 min.

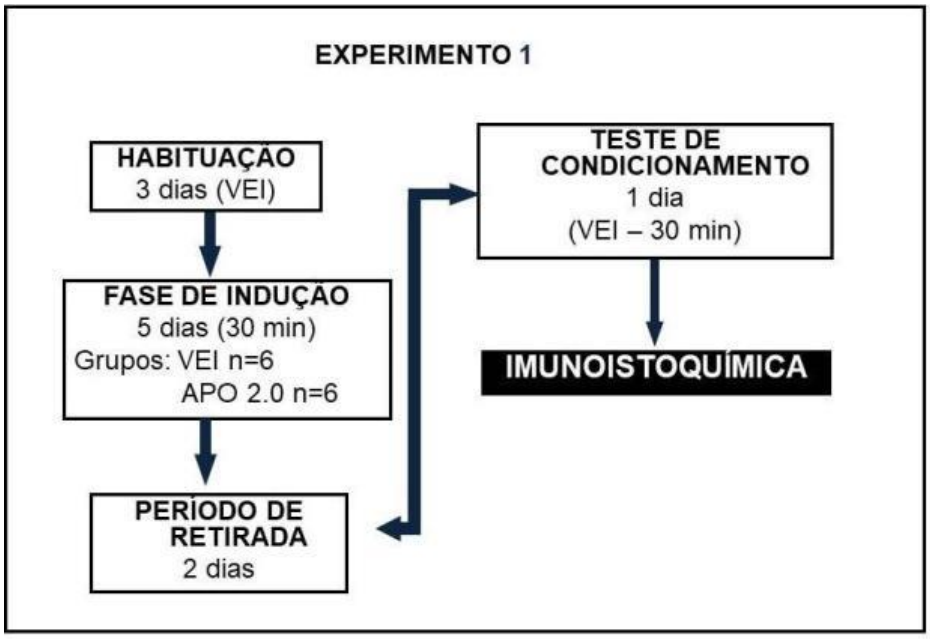

Figura 2: Esquema do desenho experimental do experimento 1 de ativação da ERK após teste de condicionamento de 30 min, associado ao ambiente.

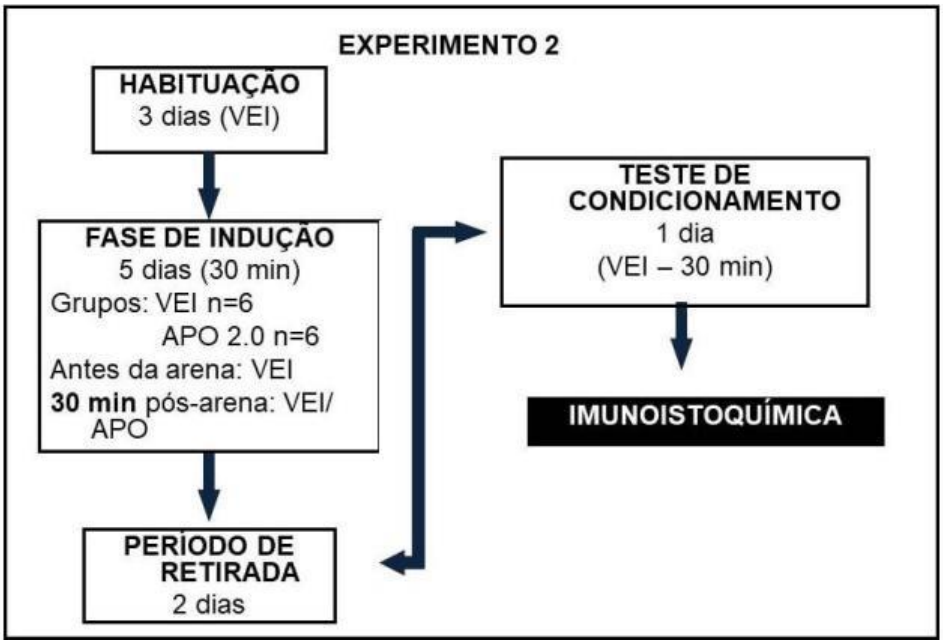

Figura 3: Esquema do desenho experimental do experimento 2 de ativação da ERK após teste de condicionamento de 30 min, não associado ao ambiente.

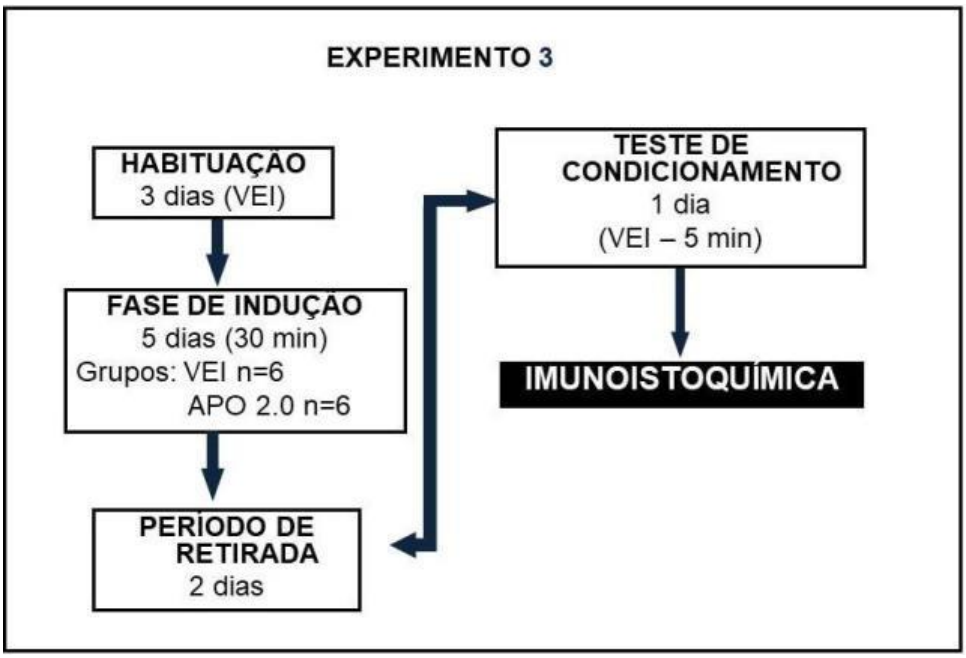

Figura 4: Esquema do desenho experimental do experimento 3 de ativação da ERK após teste de condicionamento de 5 min, associado ao ambiente.

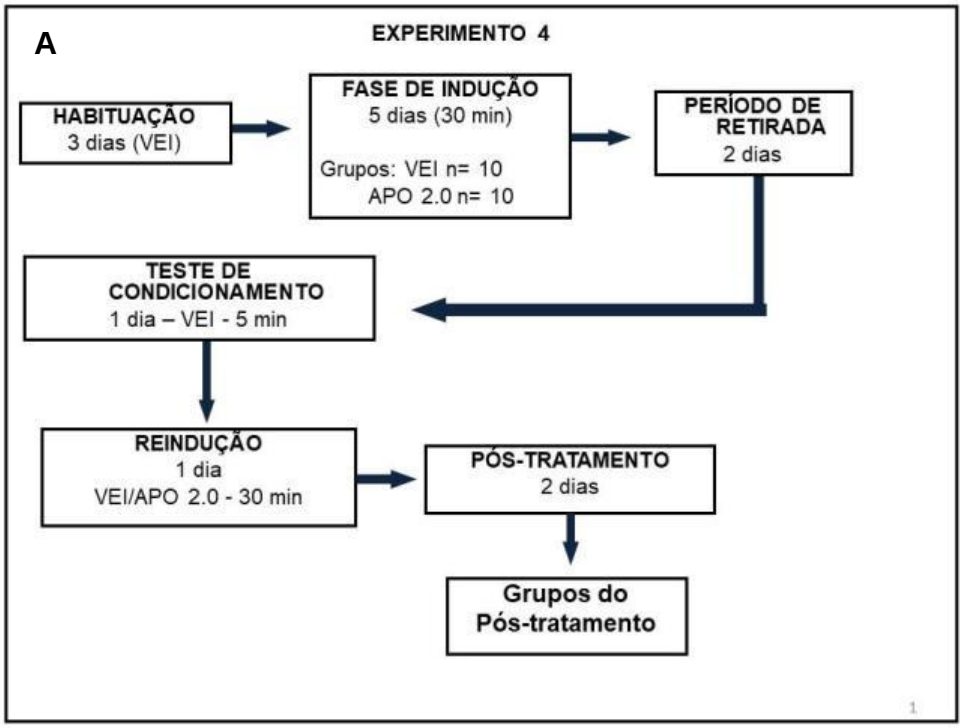

Figura 5: Esquema do desenho experimental do experimento 4 de ativação da ERK após teste de condicionamento com pós-tratamentos. (A) Primeira sequência de etapas do experimento 4.

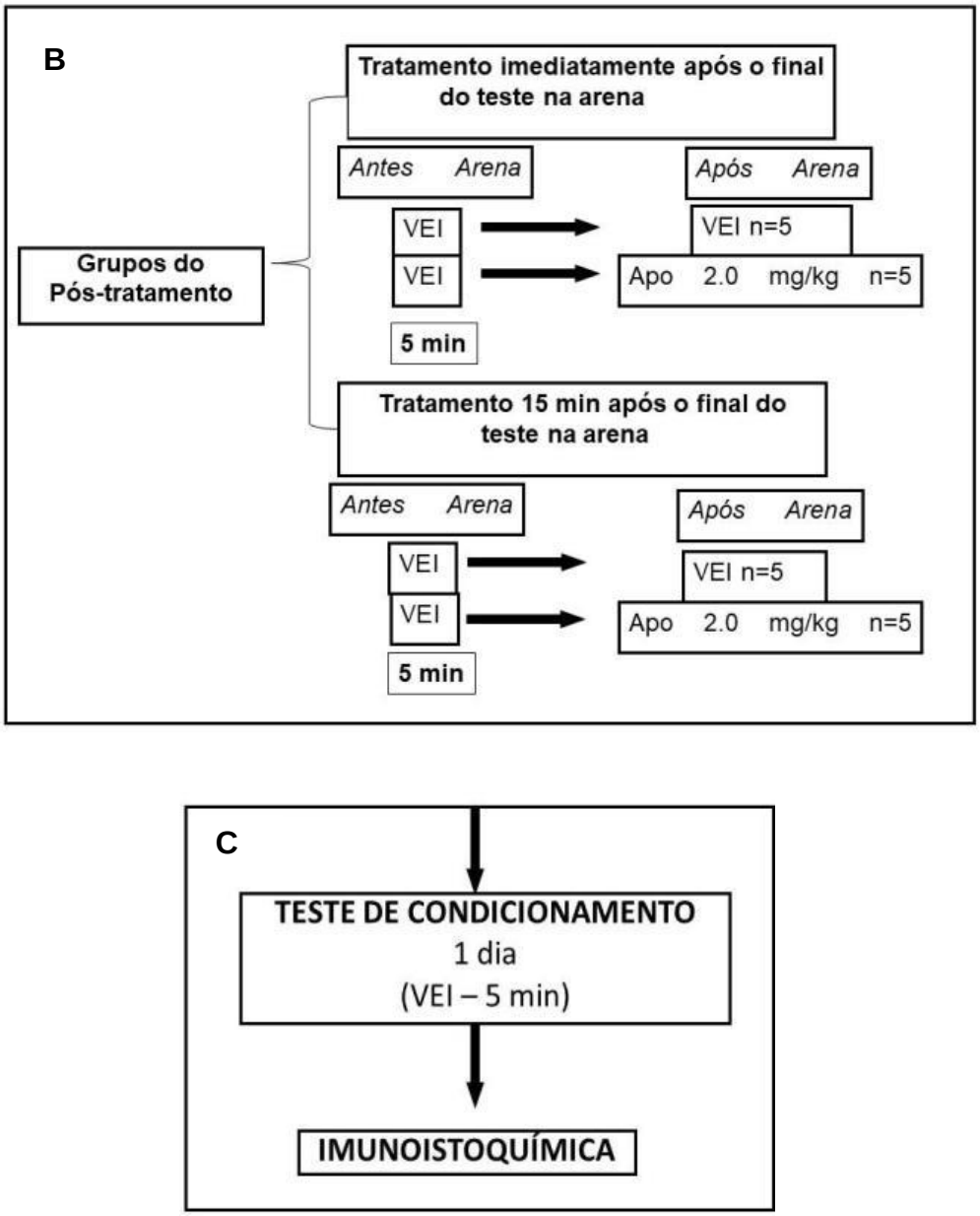

Figura 6: Esquema do desenho experimental do experimento 4 de ativação da ERK após teste de condicionamento com pós-tratamentos. (B) Segunda sequência de etapas do experimento 4. (C) Terceira sequência de etapas do experimento 4.

6. ARTIGOS

Os dois artigos publicados com os dados desta tese serão apresentados em dois capítulos. No primeiro capítulo, será apresentado o artigo "*ERK activation in the prefrontal cortex by acute apomorphine and apomorphine conditioned contextual stimuli"*, publicado na Revista "*Pharmacology Biochemistry and Behavior*".

No Segundo capítulo, será apresentado o artigo "*Medial prefrontal cortex ERK and conditioning: Evidence for the association of increased medial prefrontal cortex ERK with the presence/absence of apomorphine conditioned behavior using a unique post-trial conditioning/extinction protocol"* submetido à Revista "*Behavioural Brain Research*".

6.1 PRIMEIRO CAPÍTULO

ERK activation in the prefrontal cortex by acute apomorphine and apomorphine conditioned contextual stimuli

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Research article

ERK activation in the prefrontal cortex by acute apomorphine and apomorphine conditioned contextual stimuli

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ABSTRACT

The activation of extracellular signal-regulated kinase protein (ERK) has been linked to the adaptive responses to environmental changes and memory. The aim of this study was to measure ERK activation in primary dopamine projection areas namely, the prefrontal cortex and the nucleus accumbens, following a conditioned dopaminergic drug response. Initially, the effect of unconditioned apomorphine (2.0 mg/kg) administration on ERK activation was measured and the results showed an increase in ERK for both brain regions. Subsequently, two experiments were conducted to assess ERK activation in these two areas following apomorphine conditioned contextual stimuli. In experiment 1, rats received 5 daily injections of 2.0 mg/kg apomorphine or vehicle immediately prior to placement in an open-field. After a withdrawal period of two days, a conditioning test was conducted, in which rats received a 30 min non-drug test. Immediately after completion of the test, an immunohistochemical protocol was carried out to measure ERK activation. In experiment 2, a similar test protocol was performed except that the treatments were administered 30 min following open-field tests (post-trial experiment). The results showed that the repeated apomorphine treatments given prior to testing induced conditioned effects. An increase in ERK activation was seen in the prefrontal cortex but not in the nucleus accumbens. There was no conditioning response observed in the post-trial experiment and no differential ERK activation. These observations implicate the prefrontal cortex in the associative neuro-adaptive changes induced by dopaminergic stimulation.

1. Introduction

With repeated treatments, the behavioral effects of psychostimulant drugs such as apomorphine are potentiated (Damianopoulos and Carey, 1993; Mattingly et al., 1997; Rowlett et al., 1997; de Matos et al., 2010; Coelho et al., 2011; Mattingly et al., 2001; Braga et al., 2009c; Sanguedo et al., 2014). In that psychostimulant behavioral sensitization effects persist long after drug withdrawal, they are considered to represent an enduring drug induced alteration of the nervous system. Furthermore, this sensitization phenomenon has been repeatedly demonstrated for a number of psychostimulant drugs with addictive properties and indeed sensitization has been considered an important contributor to the addictive liability of these drugs (Robinson and Berridge, 1993; Stewart and Badiani, 1993; Carey and Damianopoulos, 2006).

It is now well established that dopamine systems are strongly implicated in psychostimulant conditioning and sensitization processes. The involvement of dopamine systems in this type of conditioning has been demonstrated frequently in drug conditioning studies in which dopaminergic drugs are used as unconditioned stimuli to induce conditioned drug effects. Repeated pairings of the drug treatments with placement in a specific test environment commonly results in context specific drug conditioning and sensitization effects (Mazurski and Beninger, 1991; Carey and Gui, 1998; Bloise et al., 2007; Braga et al., 2009a,b). In several previous reports, we have shown that repeated high dose apomorphine treatments induce hyper locomotion and that this behavioral response undergoes sensitization that is context specific (Bloise et al., 2007; Braga et al., 2009a,b; Dias et al., 2010; de Matos et al., 2010) and in addition, generates conditioned locomotor stimulant effects (Braga et al., 2009a,b; Dias et al., 2010; de Matos et al., 2010).

An important pharmacological characteristic of apomorphine is that

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its effect upon the dopamine system can be either inhibitory or facilitatory depending upon the administrated dose. Low doses (< 0.1 mg/kg) of apomorphine preferentially stimulate dopamine auto-receptors and thus inhibit dopamine neurons (Aghajanian and Bunney, 1973; DiChiara et al., 1977; Missale et al., 1998). When dose levels are increased (> 0.2 mg/kg), apomorphine increases post-synaptic dopamine receptor stimulation and functions as a dopamine agonist and psychostimulant drug (Mattingly et al., 1988a,b; Rowlett et al., 1997). This apomorphine functional duality when acting on the dopamine system means that it can be considered as either a pro-dopamine or an anti-dopamine treatment depending upon dosage.

In the search for the identity of cellular and molecular changes in the brain areas pertinent to the sensitization effects of repeated psychostimulant drug treatments, ERK has generated significant interest (Adams and Sweatt, 2002; Valjent et al., 2004, 2005; Radwanska et al., 2005; Girault et al., 2007; Lu et al., 2006; Shiflett and Balleine, 2011). Furthermore, psychostimulant drugs such as cocaine produce an increased ERK response in striatal dopaminergic projection areas (Valjent et al., 2005; DiRocco et al., 2009; Janes et al., 2009; Fricks-Gleason and Marshall, 2011), including the nucleus accumbens (Marin et al., 2009), frontal cortex (Li et al., 2008) and amygdala (Radwanska et al., 2005). In line with thesefindings, we recently reported (Sanguedo et al., 2014) that apomorphine sensitization selectively potentiates the apomorphine induced ERK response in the prefrontal cortex. In that we have also shown that apomorphine sensitization is context specific (de Matos et al., 2010), our ERK sensitization findings suggested that the prefrontal cortex is involved in associative drug responses. In the present investigation, we initially induced apomorphine sensitization and then conducted a non-drug test for conditioned effects. Following the conditioning tests, we measured ERK in the prefrontal cortex as well as another major dopamine projection site namely the nucleus accumbens. The present report details the changes in ERK associated with conditioned apomorphine behavior.

2. Methods

2.1. Subjects

Male Wistar albino rats provided by the State University of North Fluminense, initially weighing 250 300 g were housed in individual plastic cages (25 × 18 × 17 cm) until the end of the experiment. Food and water were freely available at all times. The vivarium was maintained at a constant temperature (22 + 2 °C), humidity controlled and a 12/12 h light/dark cycle (lights on at 07:00 h and offat 19:00 h). All experiment occurred between 9:00 and 14:00 h. For 7 days prior to all experimental procedures each animal was weighed and handled daily for 5 min. All experiments were conducted in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2. Drugs

Apomorphine-HCl (Sigma, St. Louis, MO, USA) was dissolved in 0.1% ascorbate/saline solution at a concentration of 2.0 mg/ml and injected subcutaneously in the nape of the neck at a dose of 2.0 mg/kg. All solutions were freshly prepared and administered in a volume of 1 ml/kg.

2.3. Apparatus and environmental context of drug treatment

The behavioral measurements were conducted in a black openfield chamber (60 × 60 × 45 cm). A closed-circuit video camera (IKEGAMI, model ICD-49), mounted 60 cm above the arena was used to record behavioral data. Locomotion, measured as distance traveled (m), was automatically analyzed by EthoVision software (Noldus, The Netherlands). The complete test procedure was conducted automatically without the presence of the experimenter in the test room. All behavioral testing was conducted under dim red light to avoid the possible aversive quality of white light and to enhance the contrast between the white subject and dark background of the test chamber. Testing under red light conditions is less stressful and also favors locomotor activation as the rats are transferred from the ambient light of the vivarium to the red light of the testing room (Nasello et al., 1988). Masking noise was provided by a fan located in the experimental room that was turned on immediately prior to placing the animal in the experimental arena and turned offupon removal of the animal from the experimental arena (i.e. test chamber).

2.4. Experimental protocol

2.4.1. Apomorphine time course experiment

In order to assess the magnitude and time course of the ERK induced by the apomorphine treatment (2.0 mg/kg) used in the present study, we initially measured the ERK activation following the 2.0 mg/kg apomorphine acute treatment. Eight groups of rats were given either vehicle (4 groups, n = 4 for each group) or 2.0 mg/kg apomorphine (4 groups; n = 4 for each group) and returned to their home-cage and either 5, 15, 30 or 60 min later were euthanized and ERK measurements were made in the prefrontal cortex and the nucleus accumbens.

2.4.2. Conditioning experiment 1

Initially all rats received three 30 min habituation sessions conducted on consecutive days. The habituation protocol was conducted so that a stable baseline of locomotor behavior could be established prior to the start of the drug treatments. The animals were administered vehicle, placed in the experimental arena and locomotor activity was measured. After the third habituation test session, the animals were assigned to groups equated on locomotor activity over the three test sessions ($p > 0.05$). There were two treatment groups: an apomorphine group and a vehicle treatment group. In the apomorphine group, rats received injections of 2.0 mg/kg apomorphine (APO-2.0; n = 6) immediately before placement in the test arena and vehicle administration 30 min after the end of the arena test. The vehicle group (VEH; n = 6) was treated in the same way as the apomorphine group except that the animals received only injections of vehicle. These treatments were administered for 5 consecutive days, one trial per day and served as the conditioning induction phase. The induction phase was designed to establish an apomorphine sensitized response effect selectively in the apomorphine treatment group. After a period of 2 days without injections or behavioral testing (withdrawal period), in order to insure an effective drug washout for the short duration acting apomorphine, the conditioning test was performed in which the animals received vehicle prior to being placed into the test environment and locomotion was recorded for 30 min. Immediately after the end of the conditioning test, the animals were deeply anesthetized with sodium pentobarbital (50 mg/kg I.P.), perfused transcardially and the brains removed and stored (as described in Section 2.5) for subsequent immunohistochemical phosphorylated-ERK-P analysis.

2.4.3. Conditioning experiment 2

This experiment was a replication of conditioning experiment 1 except that during the 5 day induction phase both the apomorphine (2.0 mg/kg) and the vehicle groups received vehicle prior to testing. Thirty min after completion of the arena tests, there was a post-trial treatment (P), in which the apomorphine group received apomorphine (APO-P, n = 6) and the vehicle group received vehicle (VEH-P, n = 4). In this experiment, the apomorphine group received the same apomorphine exposure as the apomorphine group in conditioning experiment 1, but not in association with the test arena. The assumption of conditioning experiment 1 was that an effect of the apomorphine treatment on the conditioning test was attributable to the association of the drug effect with the test arena cues for this experiment controlled

during repeated exposure to apomorphine per-se. The conditioning test was also conducted 2 days after the completion of the induction phase. Immediately after the end of the conditioning test, the animals were deeply anesthetized with sodium pentobarbital (50 mg/kg I.P.), perfused transcardially and the brains removed and stored (as described in Section 2.5) for subsequent immunohistochemical phosphorylated -ERK-P analysis.

2.5. Immunohistochemistry

The immunohistochemistry protocol was conducted as previously described bySanguedo et al. (2014, 2016)and adapted from that described byMarin et al. (2009). In brief, rats were rapidly anesthetized by intraperitoneal injection of thiopental (50 mg/kg) prior to intracardiac perfusion of 4% paraformaldehyde (500 ml) in 0.1 M sodium phosphate buffer (pH 7.4) for 30 min. The brains were removed and post-fixed for 2 h in 4% paraformaldehyde solution before transfer to 20% sucrose in 0.1 M sodium phosphate buffer (pH 7.4) for 48 h at 4 °C. Brains were placed on an aluminum paper base and cryoprotected by a solution of water soluble glycols and resins (Tissue Tek®O. C. T. Sakura Finetek®, USA). The brains were then frozen and maintained in liquid nitrogen until being processed for immunohistochemistry.

Two brain areas sampled for the immunohistochemical analysis were the same ones used in our previous report in which we observed increases in frontal cortical ERK associated with apomorphine induced context specific sensitization (Sanguedo et al., 2014): prefrontal cortex (medial and lateral approximately 4.20 2.70 mm from bregma) and the nucleus accumbens (core and shell approximately +1.70 1.32 mm from bregma). The coordinates adopted as the reference were obtained from Paxinos and Watson (2004). Four slices were collected sequentially from each brain structure of each animal and sectioned in a cryostat (Zeiss, Germany) at a thickness of 30μm. The sections were placed onto previously gelatinized microscopy slides to allowfixation. For immunohistochemistry, sections were rinsed three times for 10 min in phosphate-buffered saline (PBS) and placed in blocking buffer (3% normal goat serum and 0.25% Triton X-100 in PBS) for 1 h at 22 °C. Sections were then incubated for 24 h at 4 °C in 1:500 dilution of anti-phosphorylated-ERK antibody diluted in blocking buffer as previously described (Cat # 9101, Cell Signaling Technology®, Boston, MA, USA). After the end of the incubation time, sections were washed 3 times for 10 min each in PBS and incubated at 22 °C with a 1:100 dilution of biotinylated goat anti-rabbit IgG secondary antibody (BA-1000, Vector Laboratories®, CA, USA) in 1% normal goat serum and 0.25% Triton X-100 in PBS. Sections were then washed three times for 10 min in PBS and processed using an ABC Elite kit (Vector Laboratories®, Burlingame, CA, USA). In the next step, sections were washed again in PBS and processed with a DAB substrate kit for peroxidase (SK-4100, Vector Laboratories®, Burlingame, CA, USA) and incubated in DAB substrate simultaneously and timed precisely at 22 °C for 3 min for color development of signal intensity. After drying, the slides were mounted with DPX (Sigma®, USA).

Photomicrographs of brain sections were obtained using a CCD camera (Nikon Photometrics Cool Snap) attached to a Nikon microscope. The labeled nuclei in two hemispheres from each brain structure of each animal were observed in the ROI (region of interest) and quantified using the Image J® software multi-point tool. Negative control slices were incubated with normal serum instead of primary antibody (data not shown). In order to minimize any potential bias in the scoring, two experimenters unaware of the treatment groups independently performed the labeled nuclei counts. The counts obtained by each experimenter for each brain area and for each group were compared using student *t*-tests and no statistically significant differences ($p > 0.05$) were found.

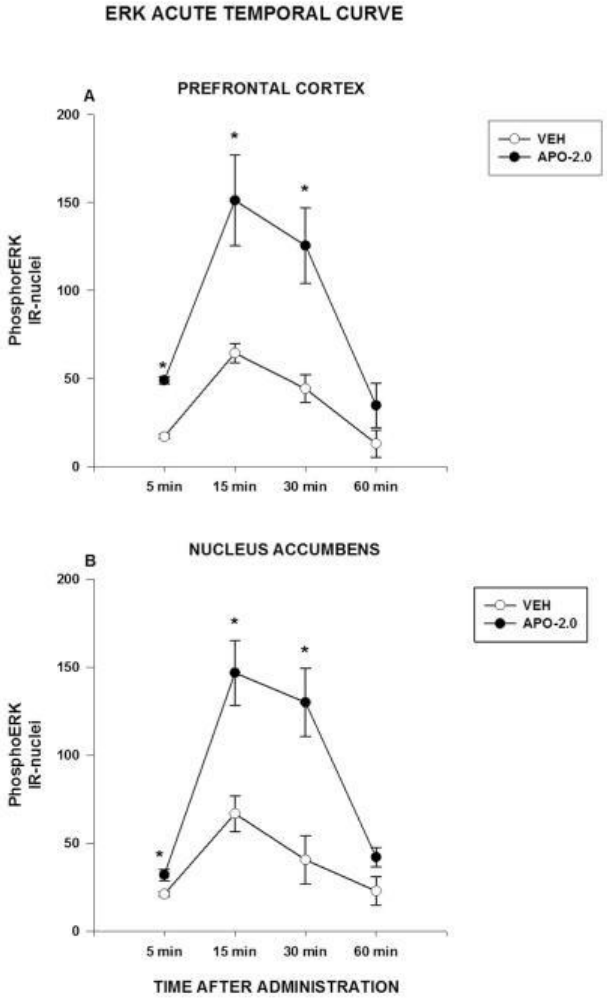

Fig. 1. The effects of injections of 2.0 mg/kg apomorphine and vehicle on ERK activation in the prefrontal cortex (A) and nucleus accumbens (B) at 5, 15, 30 and 60 min post-injection. *p < 0.5 versus vehicle.

2.6. Statistics

For the induction phase of the conditioning experiments, a repeated two-way analysis of variance (ANOVA) was used in order to determine the group effect, day effect, as well as the interactions between variables. For the conditioning test, total time of locomotor activity in the arena (30 min) was divided into 12 intervals of 2.5 min each. A repeated two-way analysis of variance (ANOVA) was used to evaluate locomotion over time to determine the group effect, interval effect, as well as the interaction between variables. For the apomorphine induced ERK activation experiment, the time course effect was assessed using ANOVA and for the ERK results obtained following the conditioning experiments, independent*t*-tests were used to compare ERK differences between the prefrontal cortex and the nucleus accumbens apomorphine groups and their respective vehicle control groups.

3. Results

Fig. 1 shows ERK activation in the prefrontal cortex and nucleus accumbens 5, 15, 30 or 60 min after being injected with either vehicle or 2.0 mg/kg of apomorphine. As can be seen inFig. 1, apomorphine markedly and similarly increased ERK in the prefrontal cortex and the

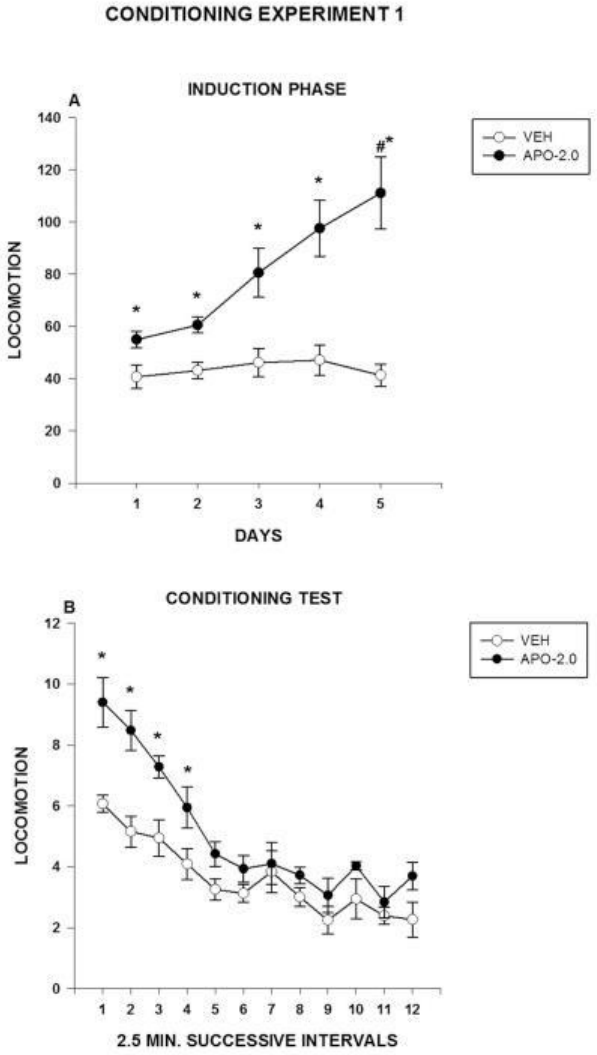

Fig. 2. Means and S. E. M. of locomotor activity for the 2.0 mg/kg apomorphine and vehicle groups during the induction phase (A) and conditioning test (B) of conditioning experiment 1. *Denotes higher locomotor activity than the vehicle group. #Denotes higher locomotor activity for the apomorphine group during the last day of the induction phase when compared to the first day of the induction phase ($p < 0.05$; ANOVA followed by Duncan's multiple range test).

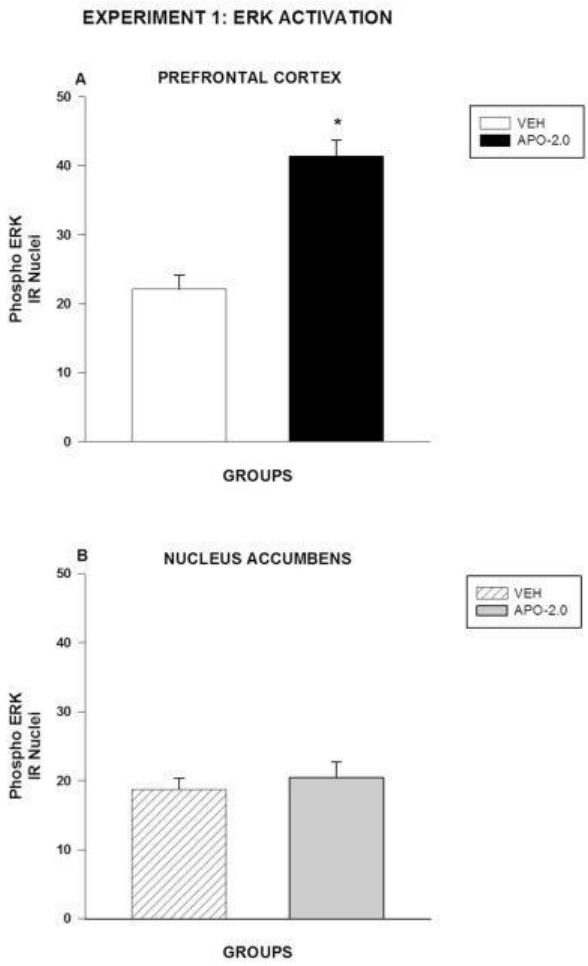

Fig. 3. Quantification of immunohistochemical results for ERK phosphorylation in prefrontal cortex (A) and nucleus accumbens (B) after the conditioning test for apomorphine 2.0 mg/kg and vehicle groups in conditioning experiment 1. Data represent mean ± SEM (n = 6 rats per group). *Denotes higher immunoreactive nuclei in the apomorphine group when compared to the vehicle group in the same brain area ($p < 0.05$).

nucleus accumbens and that ERK activation was inversely related to the post-injection time. In fact, by 60 min there was either no difference or a very modest difference between the apomorphine treated and the vehicle treated groups for both the prefrontal cortex and nucleus accumbens, a result consistent with the duration of action of apomorphine. Fig. 1A shows the ERK results for the prefrontal cortex and a two-way ANOVA showed an interaction groups × time [$F(3, 32) = 3.10$; $p < 0.05$], an effect of groups [$F(1, 32) = 34.35$; $p < 0.01$] and an effect of time post injection [$F(3, 32) = 18.34$; $p < 0.01$]. Fig. 1B shows the ERKfindings for the nucleus accumbens and as was the case for the prefrontal cortex a two-way ANOVA showed an interaction groups × time [$F(3, 32) = 5.94$; $p < 0.01$], an effect of groups [$F(1, 32) = 36.1$; $p < 0.01$] and an effect of time [$F(3, 32) = 22.60$; $p < 0.01$].

Fig. 2 presents the results of conditioning experiment 1. For the induction phase (Fig. 2A), a repeated two-way ANOVA shows that there was an interaction of groups × days [$F(4, 40) = 11.90$; $p < 0.01$], an effect of groups [$F(1, 10) = 20.41$; $p < 0.01$] and an effect of days [$F(4, 40) = 13.50$; $p < 0.01$]. The results showed that from day 1 until the end of the induction phase, the apomorphine group had higher locomotion than the vehicle group ($p < 0.05$) and there was a difference between thefirst and the last day of the induction phase only for the apomorphine group ($p < 0.05$). For the conditioning test analysis (Fig. 2B), a repeated two-way ANOVA shows that there was an interaction of groups × intervals [$F(11, 110) = 2.25$; $p < 0.05$], an effect of groups [$F(1, 10) = 29.30$; $p < 0.01$] and an effect of days [$F(11, 110) = 23.83$; $p < 0.01$]. The results showed that from interval 1 until interval 5, the apomorphine group had higher locomotion than the vehicle group ($p < 0.05$).

Fig. 3 shows the number of phosphorylated-ERK immunoreactive nuclei in the pre-frontal cortex (3A) and nucleus accumbens (3B) following the 30 min conditioning test (conditioning experiment 1). The results showed that for the pre-frontal cortex, the apomorphine group had a higher number of phosphorylated-ERK immune-reactive nuclei than the vehicle group [$t(10) = 6.30$; $p < 0.01$]. For the nucleus accumbens, the differences between the apomorphine and vehicle groups were not statistically significant [$t(10) = 0.61$; $p > 0.05$].

Figs. 4 and 5 show examples of the sections used for counting

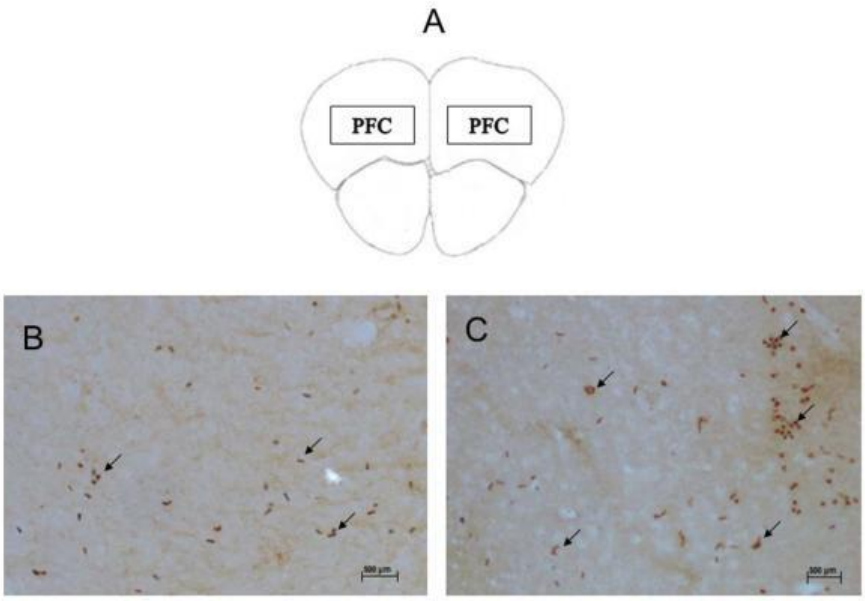

Fig. 4. Schematic representation of the prefrontal cortex (A) and immunohistochemistry images for apomorphine conditioning experiment 1 from this region. Drawings of coronal sections were obtained from the Paxinos and Watson (2004) atlas. ERK-P-immunoreactive cells in the prefrontal cortex for vehicle (B) and apomorphine groups (C) are show with arrows. The number of arrows does not represent the total number of immunoreactive nuclei. Scale bar = 500μm.

phosphorylated-ERK-immunoreactive cells in the prefrontal cortex and nucleus accumbens in the apomorphine and vehicle groups following the 30 min conditioning test (conditioning experiment 1).

Fig. 6shows the locomotor activity during the induction phase and conditioning test for the vehicle and apomorphine 2.0 mg/kg post-test groups (conditioning experiment 2). For the induction phase (Fig. 6A), a repeated two-way ANOVA showed that there was no effect of groups [$F(1, 8) = 0.30$; $p > 0.05$], no effect of days [$F(4, 32) = 0.50$; $p > 0.05$] and no interaction of groups × days [$F(4, 32) = 0.51$; $p > 0.05$]. For the 2.5 min interval of the conditioning test analysis (Fig. 6B), a repeated two-way ANOVA shows that there was only an effect of intervals [$F(11, 88) = 9.20$; $p < 0.01$]; there was no effect of groups [$F(1, 8) = 2.30$; $p > 0.05$] and no interaction of groups × intervals [$F(11, 88) = 0.90$; $p > 0.05$].

Fig. 7 shows the number of phosphorylated-ERK immunoreactive nuclei in the pre-frontal cortex and nucleus accumbens after the 30 min conditioning test for vehicle and apomorphine post-test groups (conditioning experiment 2). There were no differences between the prefrontal apomorphine group and its vehicle control group [$t(8) = 0.33$; $p > 0.05$] (Fig. 7A) or between the nucleus accumbens group and its vehicle control group (Fig. 7B) [$t(8) = 1.25$; $p > 0.05$].

4. Discussion

In the initial time course experiment, we found that 2.0 mg/kg apomorphine produced a marked ERK activation in both the prefrontal cortex and the nucleus accumbens and that the increases and the time course of the apomorphine induced ERK activation were similar in both brain areas. In that both areas are major projection sites for dopamine neurons this finding was to be expected. It is relevant to the conditioning experiments in that it suggests that both projection sites received comparable apomorphine stimulation over the course of the experiments. In our previous report (Sanguedo et al., 2014), we showed that ERK activation following an apomorphine (2.0 mg/kg)

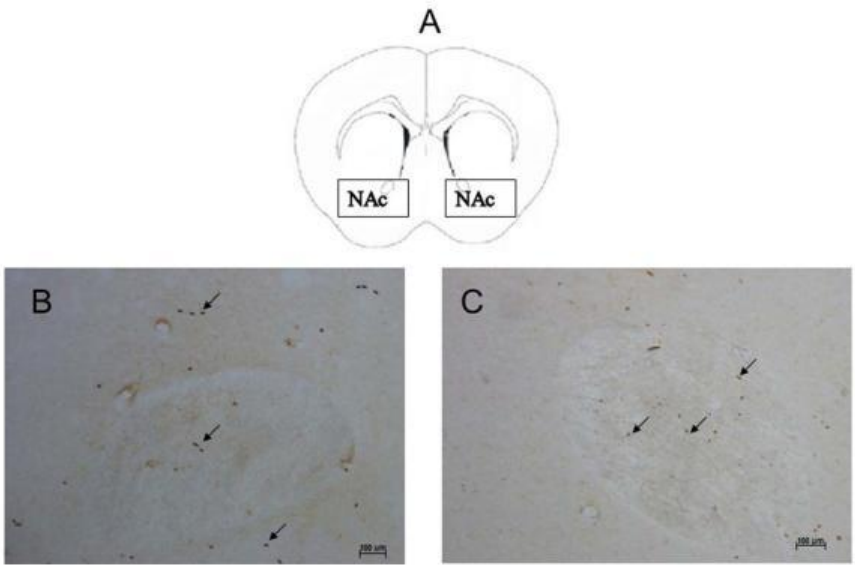

Fig. 5. Schematic representation of the nucleus accumbens (A) and immunohistochemistry sections for apomorphine conditioning experiment 1 obtained from this region. ERK-P-immunoreactive cells in the nucleus accumbens for vehicle (B) and apomorphine groups (C) are indicated using arrows. Scale bar = 100μm.

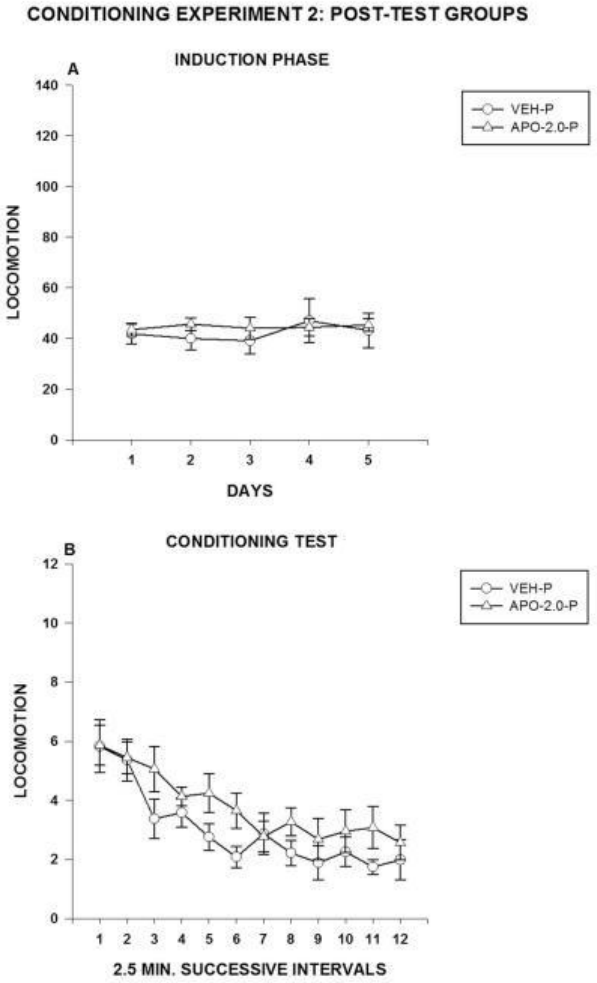

Fig. 6. Means and S. E. M. of locomotor activity for the 2.0 mg/kg apomorphine and vehicle groups during the induction phase (A) and conditioning test (B) in conditioning experiment 2.

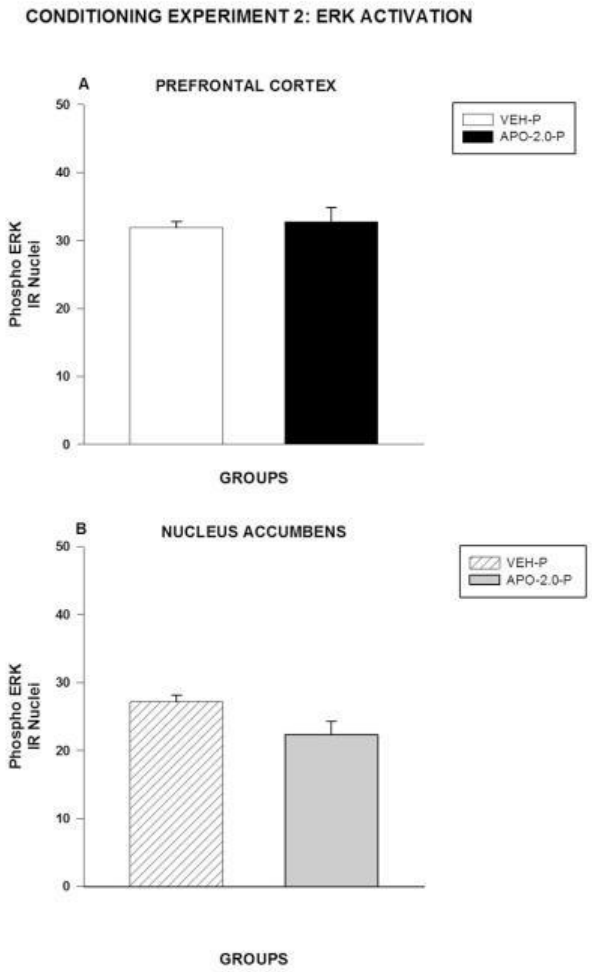

Fig. 7. Quantification of immunohistochemical results for ERK phosphorylation in the prefrontal cortex (A) and nucleus accumbens (B) after the conditioning test for apomorphine 2.0 mg/kg and vehicle post-test treatment groups. Data represent mean ± SEM (n = 6 for apomorphine group; n = 4 for vehicle group).

sensitization protocol was greater in the prefrontal cortex than the nucleus accumbens. As shown in our previous studies (Bloise et al., 2007; Braga et al., 2009a,b; Coelho et al., 2011; Dias et al., 2010; de Matos et al., 2010), apomorphine sensitization is context specific, indicative of associative processes. Linking the increased ERK activation in the prefrontal cortex to associative effects, we suggested that the enhanced ERK activation in the prefrontal cortex reflected a combination of apomorphine induced-activation plus conditioned apomorphine activation. Thefindings obtained in the conditioning experiments are consistent with this proposition in that an increased ERK activation was observed in the prefrontal cortex but not the nucleus accumbens and only in the experiment in which an apomorphine conditioned response was observed.

It is also of interest that in Pavlovian conditioning, the conditioned response (CR) is a replica of the unconditioned response (UCR) but of a more limited duration (Pavlov, 1928, 1929). This apparent CR/UCR identity is typically limited to behavioral similarities and in the present study the behavioral comparability was evident in the hyper-locomotion induced by apomorphine and the conditioned apomorphine hyperlocomotion response were similar albeit that the conditioned response was a fractional response of the apomorphine induced hyperactivity. The added dimension provided in the present experiment was that the ERK increase in the prefrontal cortex observed with the apomorphine conditioned response (CR) was similarly a fractional response of the ERK increase in the prefrontal cortex induced by apomorphine (UCR).

Significantly, the ERK activation increase induced by the conditioned apomorphine response occurred in the neocortical dopamine projection site thought to mediate dopamine effects upon learning and memory processes (Bäckman et al., 2006; Chao et al., 2013; Cools and D'Esposito, 2011; D'Ardenne et al., 2012; Glickstein et al., 2005; Goldman-Rakic, 1998). Furthermore, the importance of the frontal cortex in psychostimulant neuro-plastic effects has recently been shown by Muñoz-Cuevas et al. (2013), in which they found that cocaine increases dendritic spine growth within hours of exposure to the drug. Given this association to an increase in ERK in the prefrontal cortex, it would be of importance to determine whether, with an extinction protocol, the conditioned behavioral response and the ERK activation would decline in parallel and in addition following a non-test interval, whether spontaneous recovery would occur in both the behavioral and ERK activation. Thus, the opportunity to assess the relationship between the conditioned response and the ERK activation provides a way to evaluate the degree of alignment of ERK activation with conditioning principles.

Another potential application of ERK activation would be to use a drug substitution protocol (Damianopoulos and Carey, 1993; Emmett-

Oglesby et al., 1989; Overton et al., 1999). The objective would be to ascertain if other psychostimulant drugs (e.g. cocaine) with significant dopaminergic activity evoke an enhanced ERK activation in the prefrontal cortex in animals that had previously been sensitized to apomorphine. Presumably, a drug such as cocaine given acutely to drug naïve animals would not elicit a selectively enhanced ERK activation in the prefrontal cortex, whereas in animals previously sensitized to apomorphine the ERK foundation established in the prefrontal cortex may redirect the cocaine response. This line of investigation has clear-cut relevance to drug substitution and may also be pertinent to poly drug abuse. Seemingly, if a change in brain organization as manifested in selective ERK activation in a specific brain area has been induced by repeated use of an addictive drug then other drugs as well as conditioned drug stimuli may be able to trigger this ERK activation and become linked into the web of addiction. Given the importance of the MAPK/ERK pathway in mediating cell surface signals to gene expression, its effects are relevant to neurite growth (Schloesser et al., 2007).

The sustained activation of this pathway provides a possible mechanism for the induction of neuroplastic changes in brain. Seemingly, the persistent activation of the MAPK/ERK pathway in dopaminergic cortical target areas could provide a basis for a lasting alteration in brain organization that could contribute to the maintenance of drug directed behavior. While this is an admittedly speculative approach to drug use and poly drug abuse, the ERKfinding in the present experiment suggest a novel line of investigation to this complex issue.

In conclusion, the presentfindings linking an increase in prefrontal cortex ERK with a conditioned dopaminergic drug response indicates the potential utility of ERK activation as a tool to localize changes in the brain related to associative processes. Clearly, this line of investigation needs to be expanded to additional brain structures and conditioned effects induced by different drug treatments and regimens.

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6.2 SEGUNDO CAPÍTULO

Medial prefrontal cortex ERK and conditioning: Evidence for the association of increased medial prefrontal cortex ERK with the presence/absence of apomorphine conditioned behavior using a unique post-trial conditioning/extinction protocol

Medial prefrontal cortex ERK and conditioning: Evidence for the association of increased medial prefrontal cortex ERK with the presence/absence of apomorphine conditioned behavior using a unique post-trial conditioning/extinction protocol.

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Abstract

Increases in Medial Prefrontal cortex ERK have been linked to learning and memory processes. In the present study separate groups of rats initially underwent testing in an open-field paired with either 2.0mg/kg apomorphine or vehicle injections. Subsequently, in a brief conditioning 5 min test the paired apomorphine group manifested a conditioned hyperactivity response. The vehicle/apomorphine groups were then subdivided into two vehicle and two apomorphine subgroups matched for their activity scores in this conditioning test. Following another apomorphine/vehicle pairing in the test environment the groups received 3 additional 5 min non-drug conditioning tests, in which the groups received post-trial vehicle/apomorphine treatments. The vehicle groups received vehicle either immediately or 15 min after the first two of the three conditioning tests and the apomorphine groups received 2.0 mg/kg either immediately or 15 min after the first two of the three conditioning tests. In the first conditioning test both of the apomorphine groups exhibited equivalent conditioned responses. By the third test the conditioned response of the immediate post-trial apomorphine group remained robust whereas conditioned response of the 15 min apomorphine post-trial group extinguished. Immediately following the third conditioning test the groups were sacrificed and ERK was measured in the medial prefrontal cortex and the nucleus accumbens. ERK was enhanced in both brain areas selectively in the immediate apomorphine post-trial group. Increased ERK activity linked to the presence of the apomorphine conditioned response coupled with the absence of increased ERK activity following extinction of the apomorphine conditioned response suggests that ERK activity immediately following a conditioning test is an indicator of dopaminergic activity in brain systems important in learning and memory. It also is consistent with the proposition and that immediate post-trial dopaminergic drug treatments can modify the re-consolidation of conditioned behavior.

Key Words: Conditioning, extinction, post-trial, re-consolidation, ERK, apomorphine.

1. Introduction

The drug apomorphine is of interest in terms of drug conditioning in that this drug can have pronounced but opposite effects upon dopamine neurotransmission depending upon dose level. In the low dose range in rats (<0.1 mg/kg) apomorphine can induce a profound inhibition of movement presumably by a preferential stimulation of dopamine auto-receptors and thereby inhibiting dopamine activity in brain [1, 2, 3]. At higher dose levels (>0.5 mg/kg) apomorphine increasingly stimulates dopamine post-synaptic receptors and is a potent behavioral stimulant manifested as hyper-locomotion [4, 5, 6]. In line with the importance of the role of dopamine in the modulation of stimulus-response (S-R) associations, it has been reliably reported that the repeated pairing of a high dose of apomorphine with a specific environment induces context dependent sensitization and conditioning of the hyper-locomotion elicited by apomorphine [7, 8, 9, 10, 11, 12]. Interestingly, both low and high dose apomorphine treatments have behavioral inhibitory/excitatory effects when given post-trial [13, 14, 15]. Post-trial treatment effects have been long known to influence the consolidation [16] or re-consolidation memory process [17, 18].

The underlying assumption of consolidation/re-consolidation is that the brain activity initiated during the acquisition trial/re-consolidation trial continues to persist briefly after completion of the trial and it is during this post-trial period that immediate post-trial treatments can influence consolidation/re-consolidation. The post-trial apomorphine modulatory behavioral effects were effective following brief exposure to a novel environment [19] or brief exposure to a

dopaminergic drug conditioned environment [14, 15]. To account for these post-trial inhibitory/excitatory effects of apomorphine it has been proposed that the post-trial apomorphine drug treatments attenuate/enhance the dopamine trace that persists post-trial [19]. In the case of exposure to a novel environment there is evidence that a dopamine activation state persists briefly post-trial [20] but with regard to post-trial apomorphine effects following brief exposure to drug conditioned contextual cues there is no direct evidence for a post-trial dopamine trace. In these studies, a conditioned hyper-activity response was induced using a high dose of apomorphine (2.0 mg/kg). Subsequently the animals were given a brief non-drug exposure to the test environment and a conditioned stimulant response was elicited selectively in rats that had previously experienced the high dose apomorphine treatment in the test environment. Immediately following this brief CS exposure the rats were administered either the same high dose of apomorphine (2.0 mg/kg), a low behavioral inhibitory dose of apomorphine (0.05 mg/kg) or vehicle. In the group given the high apomorphine dose immediately post-trial, the conditioned hyperactivity response was potentiated whereas in the group given the low apomorphine dose immediately post-trial, the conditioned response was suppressed and the conditioned response in the vehicle treated groups underwent extinction. The same apomorphine treatments given after a 15 min. post-trial delay to similarly conditioned groups or immediately post-trial to unpaired groups were without effect on subsequent tests. These findings appear to be in keeping with the proposition that the post-trial apomorphine treatments impacted memory reconsolidation by increasing/decreasing the level of dopamine activity during re-consolidation.

The present study was undertaken to more directly evaluate the occurrence of a dopamine activation state immediately post-trial following a brief exposure to an environment previously associated with a high dose (2.0 mg/kg) apomorphine treatment. In previous reports, we have found that an acute high dose (2.0 mg/kg) apomorphine treatment increases extracellular signal-

regulated kinase protein (ERK) widely in brain including the medial prefrontal cortex [21, 22, 23]. With repeated high dose apomorphine treatments context specific sensitization is induced and ERK is selectively potentiated in the medial prefrontal cortex [22]. In line with these findings, an increased ERK response in the medial prefrontal cortex was observed following a 30 min. conditioning test selectively in the paired group but not in the unpaired group [22]. Guided by these observations, the present study was undertaken to assess medial prefrontal cortex and nucleus accumbens ERK activity immediately following a brief conditioning test of a conditioned/extinguished apomorphine response. The objective was to use ERK activity as a marker for dopamine activity in dopamine target areas considered relevant to learning and memory during the immediate post-trial re-consolidation period.

2. Methods

2.1. Subjects

Male Wistar albino rats provided by the State University of North Fluminense, initially weighing 250-300 g were housed in individual plastic cages (25 X 18 X 17 cm) until the end of the experiment. Food and water were freely available at all times. The vivarium was maintained at a constant temperature (22 + 2 ºC), humidity controlled and a 12/12 h light/dark cycle (lights on at 0700 h and off at 1900 h). All experiment occurred between 9:00 and 14:00 h. For 7 days prior to all experimental procedures each animal was weighed and handled daily for 5 min. All experiments were conducted in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2. Drugs

Apomorphine-HCl (Sigma, St. Louis, MO, USA) was dissolved in 0.1% ascorbate/saline

solution at a concentration of 2.0 mg/ml and injected subcutaneously in the nape of the neck at a dose of 2.0 mg/kg. All solutions were freshly prepared and administered in a volume of 1 ml/kg.

2.3. Apparatus and environmental context of drug treatment

The behavioral measurements were conducted in a black open field chamber (60 X 60 X 45 cm). A closed-circuit video camera (IKEGAMI, model ICD-49), mounted 60 cm above the arena was used to record behavioral data. Locomotion, measured as distance traveled (m), was automatically analyzed by EthoVision software (Noldus, The Netherlands). The complete test procedure was conducted automatically without the presence of the experimenter in the test room. All behavioral testing was conducted under dim red light to avoid the possible aversive quality of white light and to enhance the contrast between the white subject and dark background of the test chamber. Testing under red light conditions is less stressful and also favors locomotor activation as the rats are transferred from the ambient light of the vivarium to the red light of the testing room [24]. Masking noise was provided by a fan located in the experimental room that was turned on immediately prior to placing the animal in the experimental arena and turned off upon removal of the animal from the experimental arena (i.e. test chamber).

2.4. Behavioral procedure

Two experiments were conducted. In the experiment 1, initially, all rats received three 30 min habituation sessions (habituation phase), conducted on consecutive days. The animals were administered physiological saline and placed in the experimental arena and locomotor activity was measured. On the next day, the animals were assigned to groups equated on baseline locomotor activity and were submitted to the induction pharmacological treatment phase, in which one group received apomorphine 2.0 mg/kg (APO-2.0, n=6) and the other group received

vehicle (VEH; n=6). The animals received their treatment (apomorphine 2.0 mg/kg or vehicle) immediately before being placed into the experimental arena. These treatments were administered daily for 5 days, one trial per day and locomotion was recorded for 30 minutes. After 2 days of withdrawal period, the animals received vehicle prior to being placed into the test environment in a conditioning test. Locomotion was recorded for 5 min immediately after the end of the conditioning test, the animals were deeply anesthetized with sodium pentobarbital (50 mg/kg I.P.), perfused transcardially and the brains removed and stored for subsequent immunohistochemical phosphorylated-ERK-P analysis. Table 1 presents the experimental timeline. The objective of this first experiment was to determine whether changes in medial prefrontal cortex and nucleus accumbens ERK occur following a 5 min conditioning test. In that experimente it was showed that changes in ERK occur selectively in the apomorphine group that exhibited a conditioned response a more detailed second experiment was undertaken. This experiment was designed to assess whether the ERK response linked to the conditioned behavior is altered by extinction and if the ERK and conditioned response can be maintained by immediate apomorphine post-trial treatments.

In the initial phase of the second experiment, the objective was to induce a conditioned apomorphine response. To accomplish this, a conventional drug conditioning protocol was used similar to experiment 1. After the completing the habituation phase, the animals were divided into two groups in which one group (n=10) received vehicle (VEH) and the other group (n=10) received 2.0 mg/kg apomorphine (APO) immediately prior to a 30 min test in the open-field. Both groups were tested for 5 successive daily test sessions (induction phase). After the withdrawal period, both groups were given a non-drug 5 min test in the open-field to assess for conditioned apomorphine effects. Based on this test, the groups were subdivided into two VEH groups (n=5 each) and two APO groups (n=5 each) equated for locomotor distance scores in the

5 min conditioning test and then given another single induction treatment (re-induction day) in order to insure the conditioned response was maintained. In the next phase of the experiment, all groups received vehicle immediately before the test in the open-field and the treatments (VEH and APO) were administered after the 5 min. test in the open-field. One VEH and one APO subgroup received the injections immediately (I) after being removed from the test environment. The second VEH and APO subgroups were given their respective injections 15 min delay (D) after removal from the test arena. The groups received 5 min. tests on each of three successive days. Immediately after the third final test no injections were administered and all groups were deeply anesthetized, euthanized and their brains sectioned and prepared for ERK analysis. The final experimental groups were named in terms of 2 treatments: (a) their induction treatment + (b) their post-trial treatment. Thus, the groups of the experiment 2 were: VEH+VEH-I (n=5), VEH+VEH-D (n=5), APO-2.0+APO-2.0-I (n=5) and APO-2.0+APO-2.0-D (n=5). Table 2 and Table 3 present, respectively, the experimental timeline and the experimental groups.

2.5. Immunohistochemistry

The immunohistochemistry protocol was conducted as previously described by Sanguedo and co-workers [21, 22, 23] and adapted from that described by Marin and co-workers [25]. In brief, rats were rapidly anaesthetized by intraperitoneal injection of thiopental (50 mg/kg) prior to intracardiac perfusion of 4% paraformaldehyde (500 ml) in 0.1 M sodium phosphate buffer (pH 7.4) for 30 minutes. The brains were removed and post-fixed for 2 h in 4% paraformaldehyde solution before transfer to 20% sucrose in 0.1 M sodium phosphate buffer (pH 7.4) for 48 h at 4 ^{o} C. Brains were placed on an aluminum paper base and cryoprotected by a solution of water soluble glycols and resins (Tissue Tek® O. C. T. Sakura Finetek®, USA). The brains were then

frozen and maintained in liquid nitrogen until being processed for immunohistochemistry.

The two brain areas sampled for the immunohistochemical analysis were the same that in our previous report in which we observed increases in frontal cortical ERK associated with context specific sensitization induced by apomorphine [21]: prefrontal cortex (medial and lateral approximately 4.20–2.70 mm from bregma) and nucleus accumbens (core and shell approximately +1.70 – 1.32 mm from bregma). The coordinates adopted as the reference were obtained from Paxinos and Watson [26]. Four slices were collected sequentially from each brain structure of each animal and sectioned in a cryostat (Zeiss, Germany) at a thickness of 30 µm. The sections were placed onto previously gelatinized microscopy slides to allow fixation. For immunohistochemistry, sections were rinsed three times for 10 minutes in phosphate-buffered saline (PBS) and placed in blocking buffer (3% normal goat serum and 0.25% Triton X-100 in PBS) for 1 h at 22 ^{o}C. Sections were then incubated for 24 h at 4 ^{o}C in 1:500 dilution of anti-phosphorylated-ERK antibody diluted in blocking buffer as previously described (Cat # 9101, Cell Signaling Technology®, Boston, MA, USA). After the end of the incubation time, sections were washed 3 times for 10 minutes each in PBS and incubated at 22 ^{o}C with a 1:100 dilution of biotinylated goat anti-rabbit IgG secondary antibody (BA-1000, Vector Laboratories®, CA, USA) in 1% normal goat serum and 0.25% Triton X-100 in PBS. Sections were then washed three times for 10 minutes in PBS and processed using an ABC Elite kit (Vector Laboratories ®, Burlingame, CA, USA). In the next step, sections were washed again in PBS and processed with a DAB substrate kit for peroxidase (SK-4100, Vector Laboratories®, Burlingame, CA, USA) and incubated in DAB substrate simultaneously and timed precisely at 22 $^{\circ}$ C for 3 minutes for color development of signal intensity. After drying, the slides were mounted with DPX (Sigma ®, USA).

Photomicrographs of brain sections were obtained using a CCD camera (Nikon Photometrics Cool Snap) attached to a Nikon microscope. The labeled nuclei in two hemispheres from each brain structure of each animal were observed in the ROI (region of interest) and quantified using the Image J® software "multi-point" tool. Negative control slices were incubated with normal serum instead of primary antibody (data not shown). In order to minimize any potential bias in the scoring two experimenters unaware of the treatment groups independently performed the labeled nuclei counts. The counts obtained by each experimenter for each brain area and for each group compared using student t-tests and no statistically significant differences ($p>0.05$) were found.

2.6. Statistics

In the experiment 1, for the induction phase, a repeated measure two-way analysis of variance (ANOVA) was used in order to determine the group effect, day effect, as well as the interactions between variables. When a significant effect of interaction was recorded, data were statistically evaluated further by an independent t-test or by one-way ANOVA followed by the Duncan post-hoc (test $p<0.05$ as the criterion for statistical significance). For the conditioning test and ERK activation, an independent t-test was used. In the experiment 2, for the induction phase, a repeated two-way analysis of variance (ANOVA) was used in order to determine the group effect, day effect, as well as the interactions between variables. When a significant effect of group versus day interaction was recorded, data were further evaluated by one-way ANOVA followed by the Duncan post-hoc test using $p<0.05$ as the criterion for statistical significance. For the conditioning tests, re-induction test and ERK activation experiment, a one-way analysis of variance (ANOVA) was used in order to determine the group effect. Wherever indicated by the ANOVA (group effects with p-values <0.05), possible differences among groups were further

analyzed by Duncan's multiple range test.

3. Results

Prior to the start of the experimental treatment phases, the animals underwent 3 days of habituation/familiarization with the testing apparatus. The statistical analysis using a one-way ANOVA indicated a significant decrease in locomotion over days for experiment 1 [$F(2, 35) = 37.74$; $p<0.01$] and for experiment 2 [$F(2, 59) = 21.41$; $p<0.01$]. The Duncan's test showed that day 1 had higher locomotor activity than day 2 and day 3 ($p<0.05$) for both experiments (data not shown). Importantly, prior to the initiation of the conditioning protocol, there were no differences ($p>0.05$) among the treatment groups in any experiment.

3.1 Experiment 1

Figure 1 shows the locomotor activity during the induction phase and conditioning test for the vehicle and apomorphine 2.0 mg/kg groups for the experiment 1. For the induction phase (Fig. 1A), a repeated two-way ANOVA shows that there was an interaction groups X days [$F(4, 40) = 15.0$; $p<0.01$], an effect of groups [$F(1, 10) = 37.55$; $p<0.01$] and an effect of days [$F(4, 40) = 20.10$; $p<0.01$]. The results also showed that from day 1 until the end of the induction phase, the apomorphine group had higher locomotion than the vehicle group ($p<0.05$). For the conditioning test (Fig. 1B), an independent t-test using the 5 min total test showed that the apomorphine group had higher locomotion than the vehicle group [$t(10) = 4.56$; $p<0.01$].

Figure 2 shows the number of phosphor ERK immunoreactive nuclei in the pre-frontal cortex and nucleus accumbens. For the pre-frontal cortex (Fig. 2A), an independent t-test showed that the apomorphine group had higher number of phosphor ERK immunoreactive nuclei than the vehicle group [$t(10) = 2.60$; $p<0.05$]. For the nucleus accumbens (Fig. 2B), an independent t-test

showed that the apomorphine group had higher number of phosphor ERK immunoreactive nuclei than the vehicle group [t (10) = 2.30; $p<0.05$].

Figures 3 and 4 show examples of the sections used for counting phosphorylated-ERK-immunoreactive cells in the prefrontal cortex (Fig. 3) and nucleus accumbens (Fig. 4) in the apomorphine and vehicle groups following the 5 min. conditioning test (conditioning experiment 1).

3.2 Experiment 2

Figure 5 presents the locomotor activity during the induction phase, conditioning test and re-induction day for the groups of the experiment 2. For the induction phase of (Fig. 5A), a repeated two-way ANOVA shows that there was an interaction of groups X days [F (12, 64) = 34.12; $p<0.01$], an effect of groups [F (3, 16) = 50.50; $p<0.01$] and an effect of days [F (4, 64) = 137.35; $p<0.01$]. The results showed that from day 1 through day 5, all the apomorphine groups had higher locomotion than the vehicle groups ($p<0.05$) in the 20 min. test and there was a difference between the first and the last day of the induction phase for the apomorphine groups ($p<0.05$). For the conditioning test 1 (Fig. 5B), a one-way ANOVA shows that there was an effect of groups [F (3, 16) = 27.23; $p<0.01$] and Duncan's test showed that the apomorphine groups had higher locomotion than the vehicle groups ($p<0.05$). For the re-induction day (Fig. 5C), a one-way ANOVA shows that there was an effect of groups [F (3, 16) = 37.84; $p<0.01$] and Duncan's test showed that the apomorphine groups had higher locomotion than the vehicle groups ($p<0.05$).

Figure 6 shows the locomotor activity during the two 5 min. conditioning tests that were followed by immediate (I) or 15 min. delayed (D) post-trial treatments with vehicle or apomorphine 2.0 mg/kg. For conditioning test 2 (Fig. 6A), the one-way ANOVA showed that

there was an effect of groups [$F(3, 16) = 10.10$; $p<0.01$] and Duncan's test showed that the apomorphine groups had higher locomotion than the vehicle groups ($p<0.05$). For conditioning test 3 (Fig. 6B), the one-way ANOVA showed that there was difference among the experimental groups [$F(3, 16) = 14.23$; $p<0.01$] and Duncan's test showed that APO-2.0+APO-2.0-I had higher locomotion than all other groups ($p<0.05$). The results also showed that the APO-2.0+APO-2.0-D group had higher locomotion than the vehicle groups ($p<0.05$).

Figure 7 shows the locomotor activity during the final 5 min. conditioning test. The one-way ANOVA showed that there was an effect of groups [$F(3, 16) = 18.80$; $p<0.01$] and Duncan's test showed that the APO-2.0+APO-2.0-I had higher locomotion than the other groups ($p<0.05$).

Figure 8 shows the number of phosphor ERK immunoreactive nuclei in the pre-frontal cortex and nucleus accumbens immediately following the final 5 min. conditioning test. For the prefrontal cortex (Fig. 8A), a one-way ANOVA showed that there was difference among the groups [$F(3, 16) = 10.92$; $p<0.01$] and Duncan's test showed that the apomorphine group that received immediate 2.0 mg/kg apomorphine as post-trial treatment (APO-2.0+APO-2.0-I) had a higher number of phosphor ERK immune-reactive nuclei than the other groups ($p<0.05$). For the nucleus accumbens (Fig. 8B), a one-way ANOVA showed that there was difference among the groups [$F(3, 16) = 15.70$; $p<0.01$] and Duncan's test showed that the APO-2.0+APO-2.0-I had a higher number of phosphor ERK immune-reactive nuclei than the other groups ($p<0.05$).

Figures 9 and 10 show examples of the sections used for counting phosphorylated-ERK-immunoreactive cells in the prefrontal cortex (Fig. 9) and nucleus accumbens (Fig. 10) in the immediate and delay apomorphine and vehicle groups following the final 5 min. conditioning test (conditioning experiment 2).

4. Discussion

There are two particularly important facets of the present ERK findings relevant to drug conditioning. One is the strong ERK signal observed in the medial prefrontal cortex and the nucleus accumbens during the immediate post-trial interval following the third conditioning test in the immediate apomorphine post-trial group that manifested a conditioned response. The second is that the group that had undergone the same conditioning protocol and received the same apomorphine treatments but delayed by 15 min. in the post-trial treatment phase of the experiment exhibited extinction of the conditioned response and loss of the ERK response on the third final conditioning test. Together these findings support a dopamine activation effect elicited by the conditioned apomorphine behavior in this paradigm of drug conditioning. The results are also consistent with the proposition that immediate post-trial apomorphine administration can interact with the post-trial dopamine activation state initiated by the conditioned response and become incorporated into the re-consolidation process. In several previous reports [10, 13, 27, 28] we have suggested that post-trial dopaminergic drug treatments administered during consolidation/reconsolidation in which dopamine systems have been activated can modulate this post-trial dopamine trace and consequently increase/decrease the incentive/salience value of the association. As a corollary, the loss of an ERK response in the dopamine target areas following extinction of the conditioned response in the apomorphine post-trial delay group in the present experiment suggests that the immediate post-trial apomorphine treatments would be ineffective if they were subsequently administered after extinction in that there would be no post-trial dopamine activation trace to modify. A determination of this possibility awaits direct experimental validation.

The findings of increased ERK in the medial prefrontal cortex in our previous apomorphine context specific sensitization and conditioning studies [21, 22] and the present

results appear in line with evidence that this neocortical dopamine projection site mediates dopamine effects upon learning and memory processes [29, 30, 31, 32, 33, 34]. In the search for the identity of cellular and molecular changes in the brain areas pertinent to the sensitization effects of repeated psychostimulant drug treatments, ERK has generated significant interest [35, 36, 37, 38 39, 40, 41]. Furthermore, psychostimulant drugs such as cocaine produce an increased ERK response in striatal dopaminergic projection areas [41, 42, 43, 44], including the nucleus accumbens [25], frontal cortex [45] and amygdala [38]. It also has been reported [46] that cocaine increases dendritic spine growth within hours of cocaine exposure in the frontal cortex. This observation is in line with an earlier report [47] that the MAPK/ERK pathway in mediating cell surface signals to gene expression effects is relevant to possible growth effects such as neurite growth. Notwithstanding the impact of drugs such as cocaine on neurite growth these findings have not differentiated associative versus non-associative drug effects. In the present study, the ERK changes were observed under non-drug conditions and were selective to conditioned drug cues and no longer evident following extinction. At this stage, it is unclear whether neurite changes are present in non-drug tests or whether they undergo extinction. The recent findings by Kobrin and co-workers [48] indicate that changes in dendritic complexity in the nucleus accumbens core are associated with the presence/absence of conditioned morphine behavior. In the present study, the conditioned apomorphine ERK effects undergo extinction. It will be important to extend the present experimental protocol to conditioned effects to ERK effects induced by drugs such as cocaine and morphine.

In terms of drug conditioning effects that are linked to external stimulus cues as opposed to drug induced changes in physical effects on brain such as neurite growth it seems likely that conditioned responses induced by drugs such as cocaine with prominent pro-dopamine effects would elicit a post-trial dopamine trace. As such, further cocaine use during re-consolidation

following exposure to the cocaine conditioned cues could strengthen and potentiate the cocaine conditioning [49]. Cocaine has effects on multiple neurotransmitter system such as serotonin and norepinephrine so it is presumptuous to limit post-trial cocaine impact to dopamine [50]. Regardless, the use of addictive drugs such as cocaine during re-consolidation is of clinical importance in that cocaine conditioned cues are long known to contribute to the maintenance of cocaine use and thwart the addiction treatment in that exposure to these cues even during abstinence can trigger craving and relapse [51, 52].

Another important consideration with respect to drug conditioning suggested by the present findings is the observation of an increased though attenuated ERK response on the second extinction trial. This finding suggests that if the immediate apomorphine post-trial treatments were delayed until after the second extinction trial that a sufficient dopamine trace was still present that could be strengthened by the apomorphine treatment such that a conditioned response would be restored and manifested on the subsequent third extinction trial. This possibility raises the question as to the potential efficacy of post-trial treatments given on an intermittent schedule. In the present study, the post-trial dopamine response expressed as ERK activation can be seen as a response that was enhanced by the immediate post-trial apomorphine treatments. When the dopamine trace is viewed as a response that can be modified by post-trial apomorphine treatments then it is possible to consider the use of the post-trial drug treatments on an intermittent schedule. This consideration appears relevant to drug conditioning and drug addiction in that psychostimulant dopaminergic drugs are addictive and conditioned drug effects are an important contributor to the addiction and are difficult to eliminate [51, 52]. In typical addictive drug usage, it seems unlikely that exposure to conditioned cues are always followed by drug rather, it is more likely that cue exposure is linked to drug use on an intermittent basis. In conditioning, it has been long established that intermittent reinforcement substantially increases

resistance to extinction [53]. When drug conditioning is considered from this perspective the resistance of the conditioned drug responses of addictive drugs appears explicable. Until this possibility is experimentally assessed, however, it is speculative but remains of heuristic value.

As was mentioned earlier the enhancement of the dopamine trace by post-trial apomorphine is not a direct enhancer of the very complex consolidation process but instead it impacts the level of dopamine activation that is associated with the consolidation or in the case of the present study associated with re-consolidation. Viewed in this way the immediate apomorphine post-trial enhancement of the dopamine trace does not generate the association per-se but rather alters expression of conditioning by modulation of the incentive/salience level of the association [54, 55].

In conclusion, the initial induction of an apomorphine conditioned response by a conventional drug/environment pairing is consistent with Pavlovian drug conditioning. This conditioning can subsequently be maintained by the post-trial pairing of apomorphine with the conditioned response/dopamine trace during re-consolidation indicative of an additional conditioning process that can substantially strengthen the initial drug induced Pavlovian conditioned drug response.

Acknowledgements

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Reference

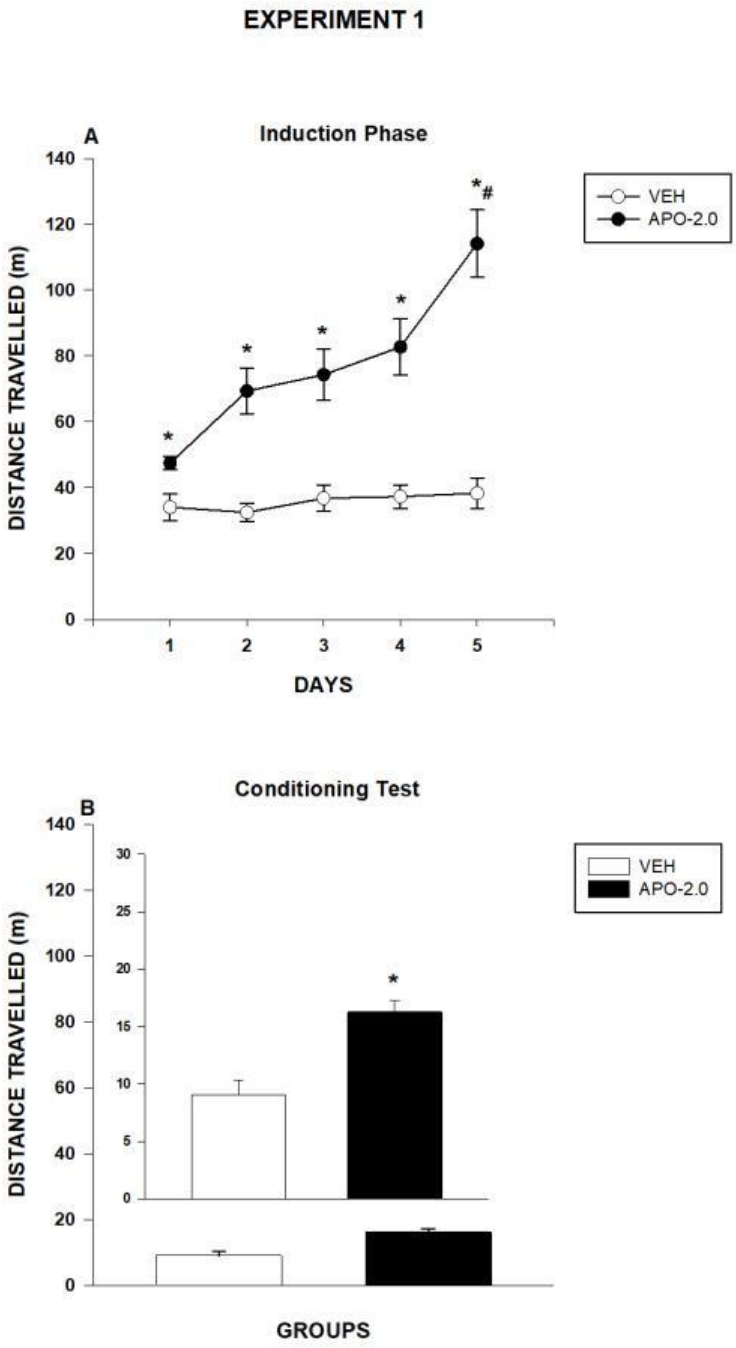

Fig. 1: Means and S. E. M. of locomotor activity for the 2.0 mg/kg apomorphine and vehicle groups during the induction phase (A) and conditioning test (B) of experiment 1. * Denotes significantly higher locomotor activity than the vehicle group; # denotes that for the apomorphine group, the locomotor activity on the 5th day was significantly higher than on the 1st day ($p<0.05$; repeated ANOVA followed by Duncan's multiple range test in A and independent t-test in B). The insert shows the same locomotion data on a different scale.

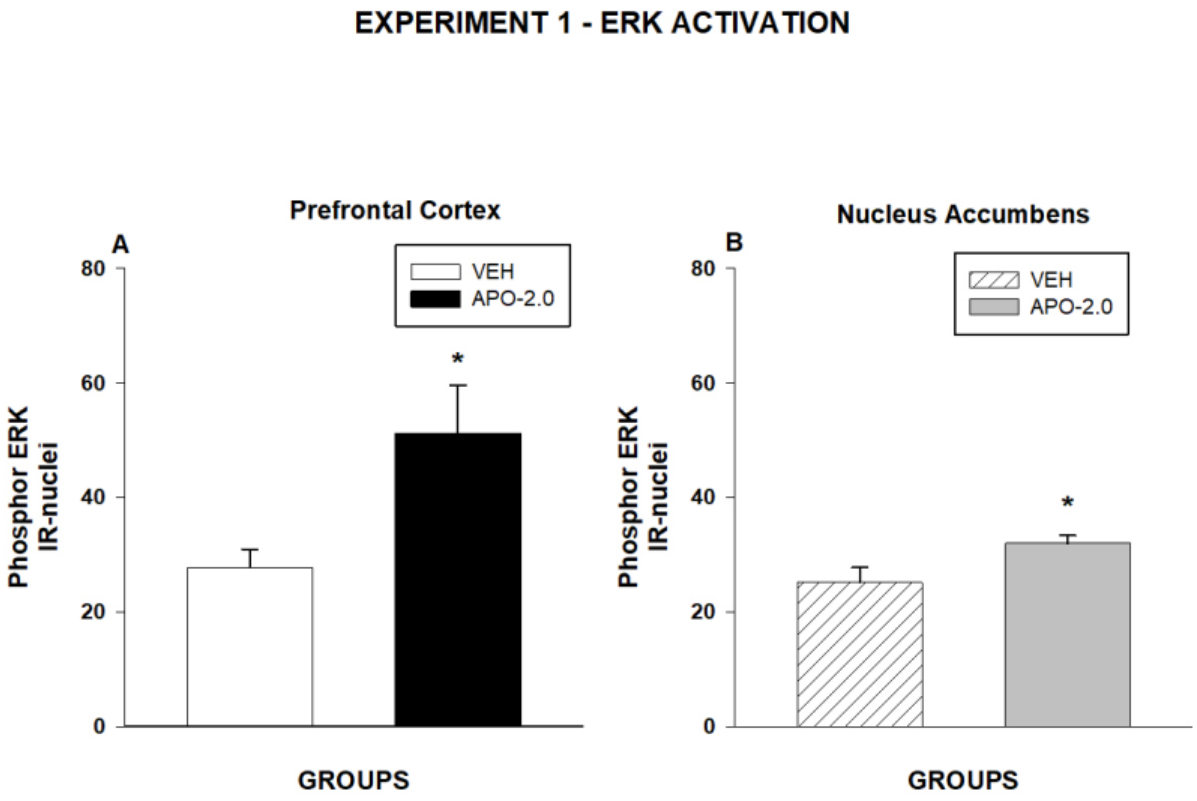

Fig. 2: Quantification of immunohistochemical results for ERK phosphorylation in the prefrontal cortex (A) and nucleus accumbens (B) following the apomorphine 2.0 mg/kg conditioning test and vehicle groups in experiment 1. Data represent mean ± S.E.M. * Denotes significantly higher numbers of immunoreactive nuclei in the apomorphine group when compared to the vehicle group in the same brain area ($p<0.05$; independent t-test).

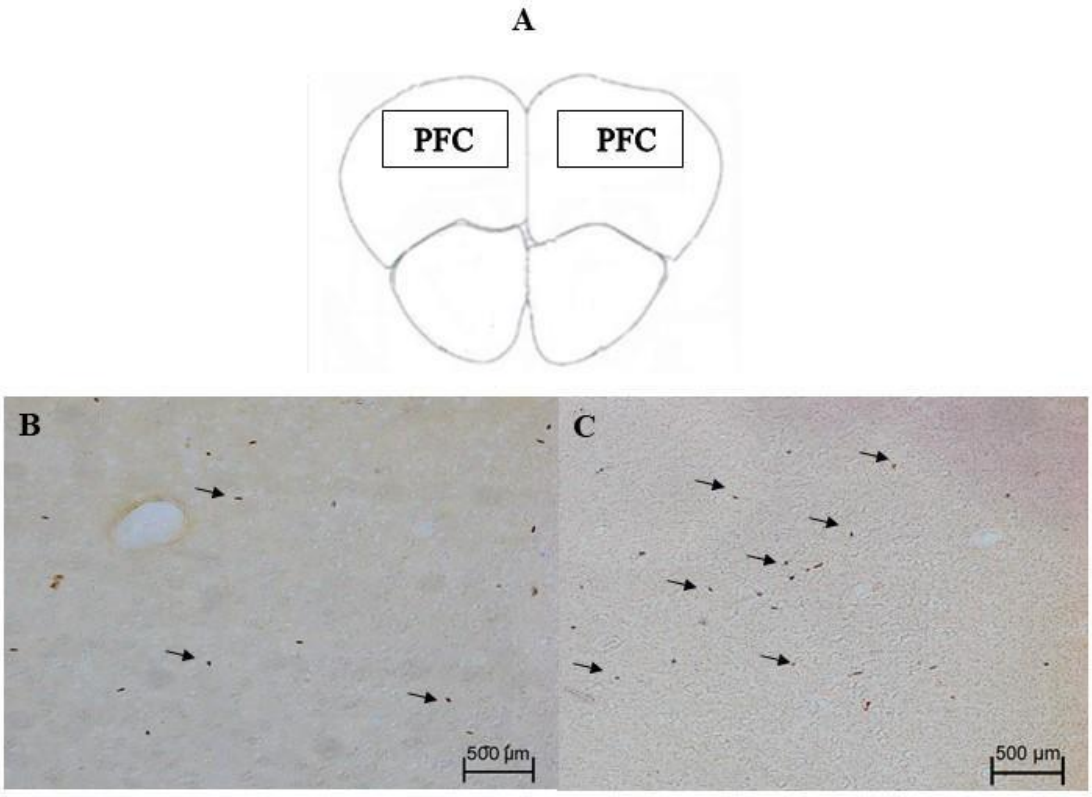

Fig. 3: Schematic representation of the áreas from which immunohistochemistry images related to experiment 1. Prefrontal cortex (A). Photomicrographs in B and C show ERK-P-immunoreactive cells arrows in the vehicle (B) and ERK-P-immunoreactive cells in apomorphine groups (C). Scale bar = 500 µm. Drawings of coronal sections were obtained from the Paxinos and Watson atlas (2004).

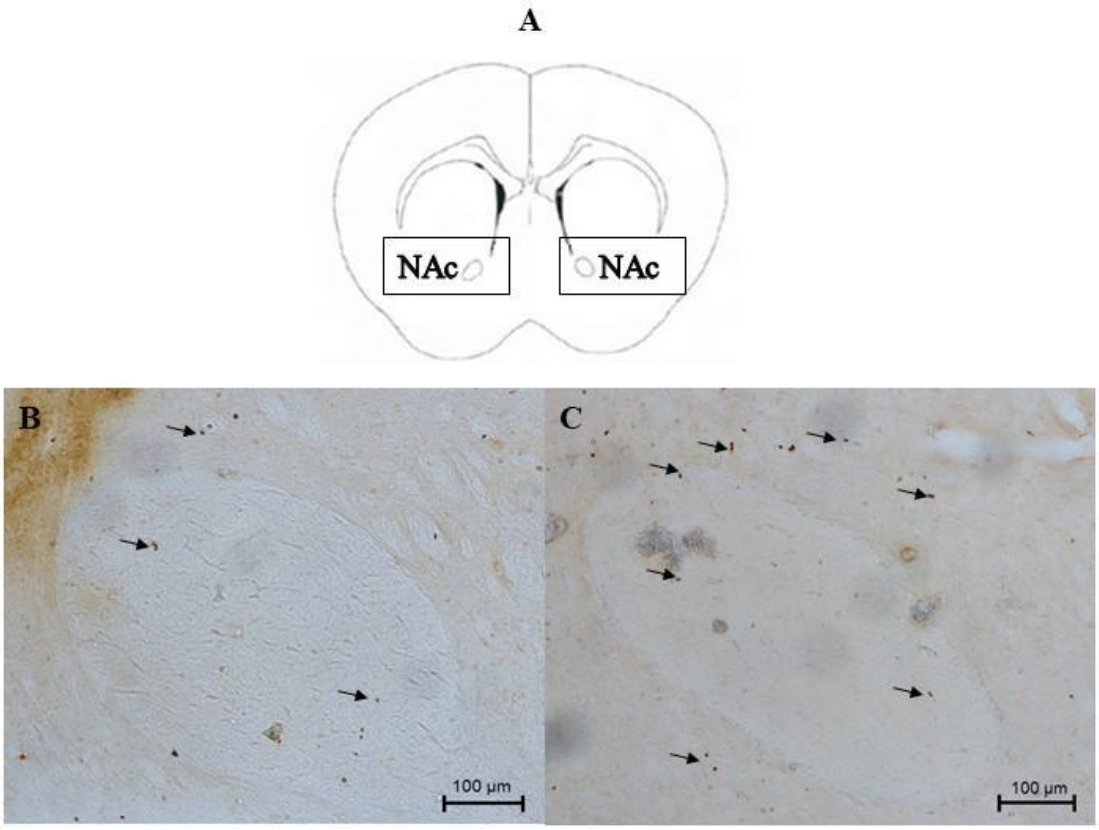

Fig. 4: Schematic representation of the áreas from which immunohistochemistry images related to experiment 1. Nucleus accumbens (A), ERK-P-immunoreactive cells arrows in the vehicle (B) and ERK-P-immunoreactive cells in the apomorphine groups (C). Scale bar = 100 µm. Drawings of coronal sections were obtained from the Paxinos and Watson atlas (2004).

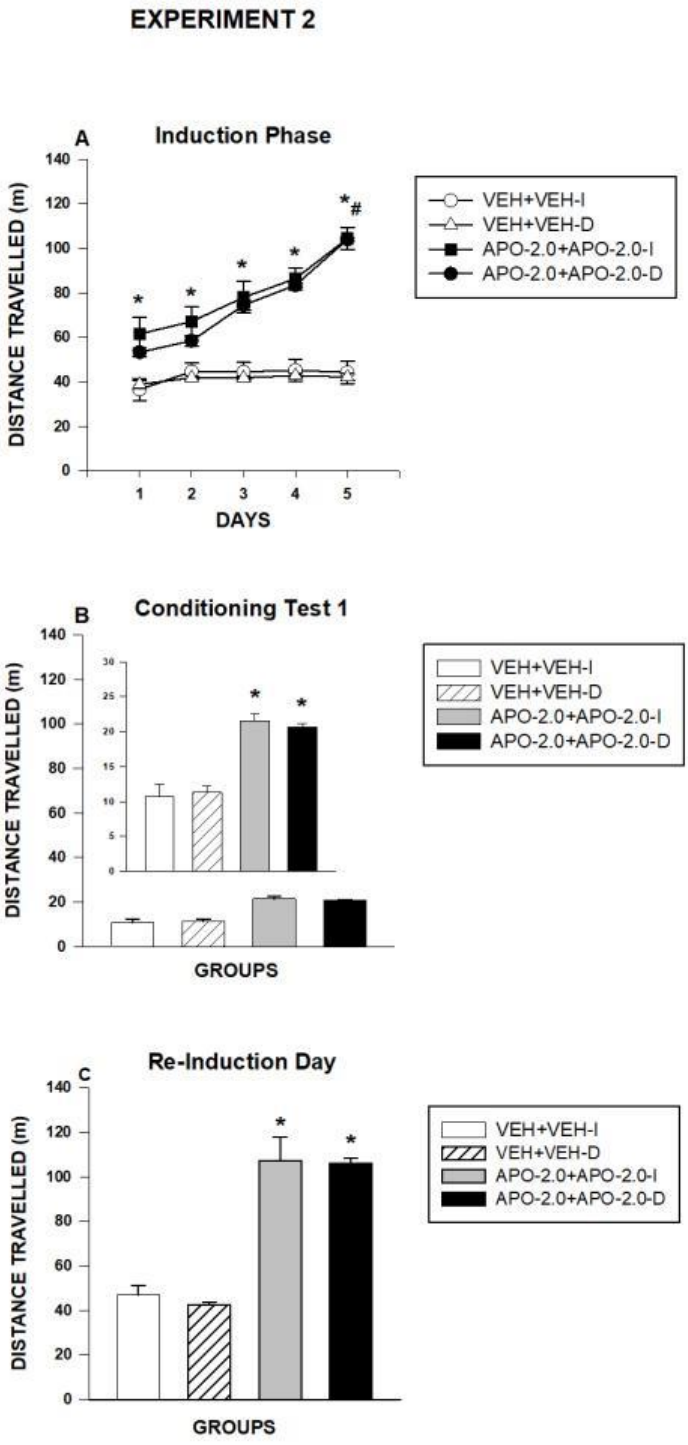

Fig. 5: Means and S. E. M. of locomotor activity for the 2.0 mg/kg apomorphine and vehicle groups during the induction phase (A), conditioning test 1 (B) and re-induction day (C) of experiment 2. * Denotes significantly higher locomotor activity than the vehicle groups. # Denotes that for the apomorphine groups the locomotor activity on the 5th day was significantly higher than on the 1st day. $P<0.05$; according repeated to measure two-way ANOVA followed by Duncan's multiple range test. The insert shows the same locomotion data on a different scale.

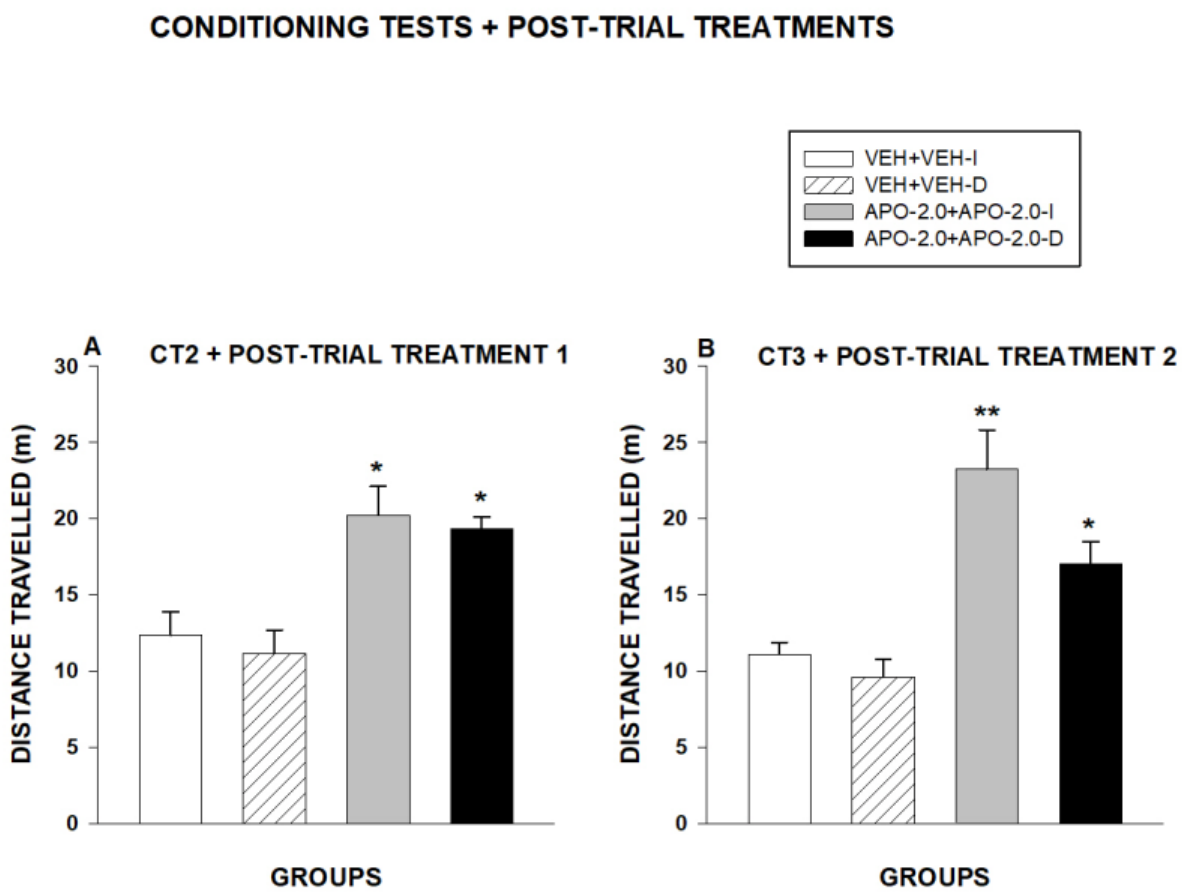

Fig. 6: Means and S. E. M. of locomotor activity for the 2.0 mg/kg apomorphine and vehicle treated groups during conditioning test 2 + post-trial treatment (A) and conditioning test 3 + post-trial treatment (B) of experiment 2. ** Denotes significantly higher locomotor activity than all other groups. * Denotes significantly higher locomotor activity than the vehicle groups ($p<0.05$; one-way ANOVA followed by Duncan's multiple range test).

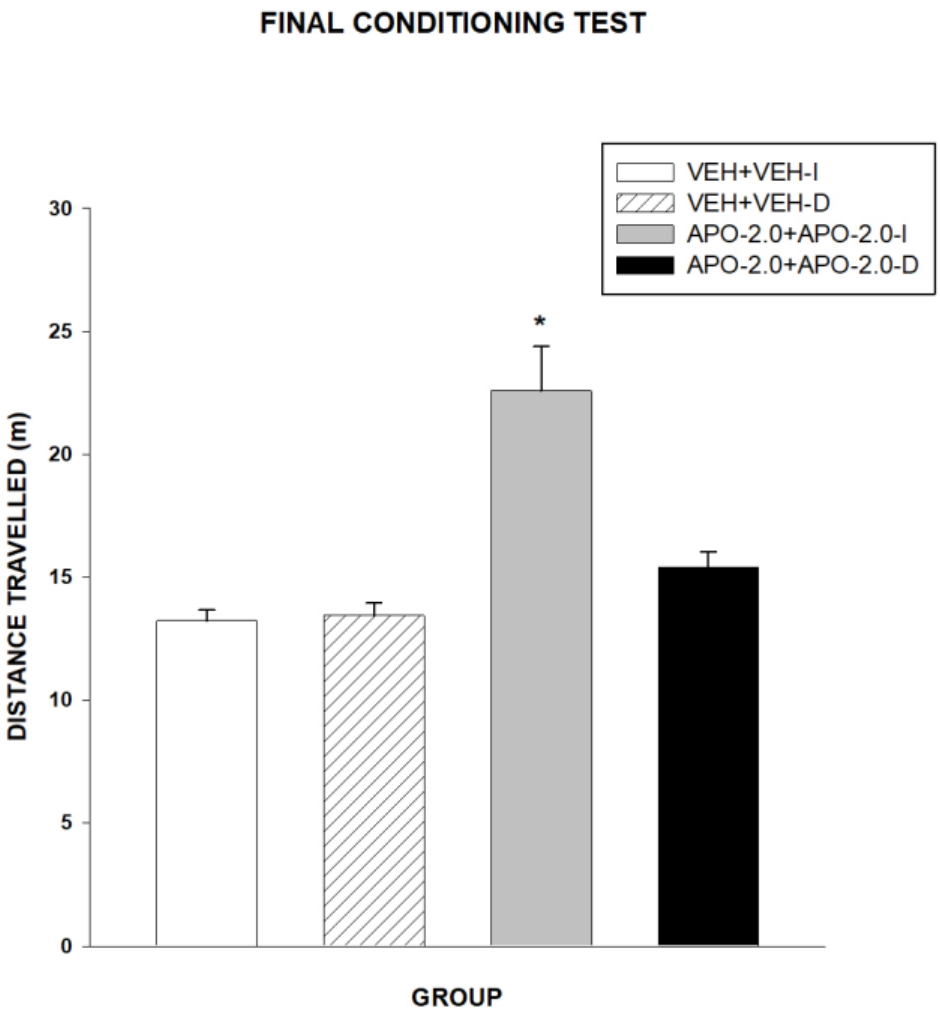

Fig. 7: Means and S. E. M. of locomotor activity for the final conditioning test of experiment 2. * Denotes significantly higher locomotor activity than all other groups ($p<0.05$; one-way ANOVA followed by Duncan's multiple range test).

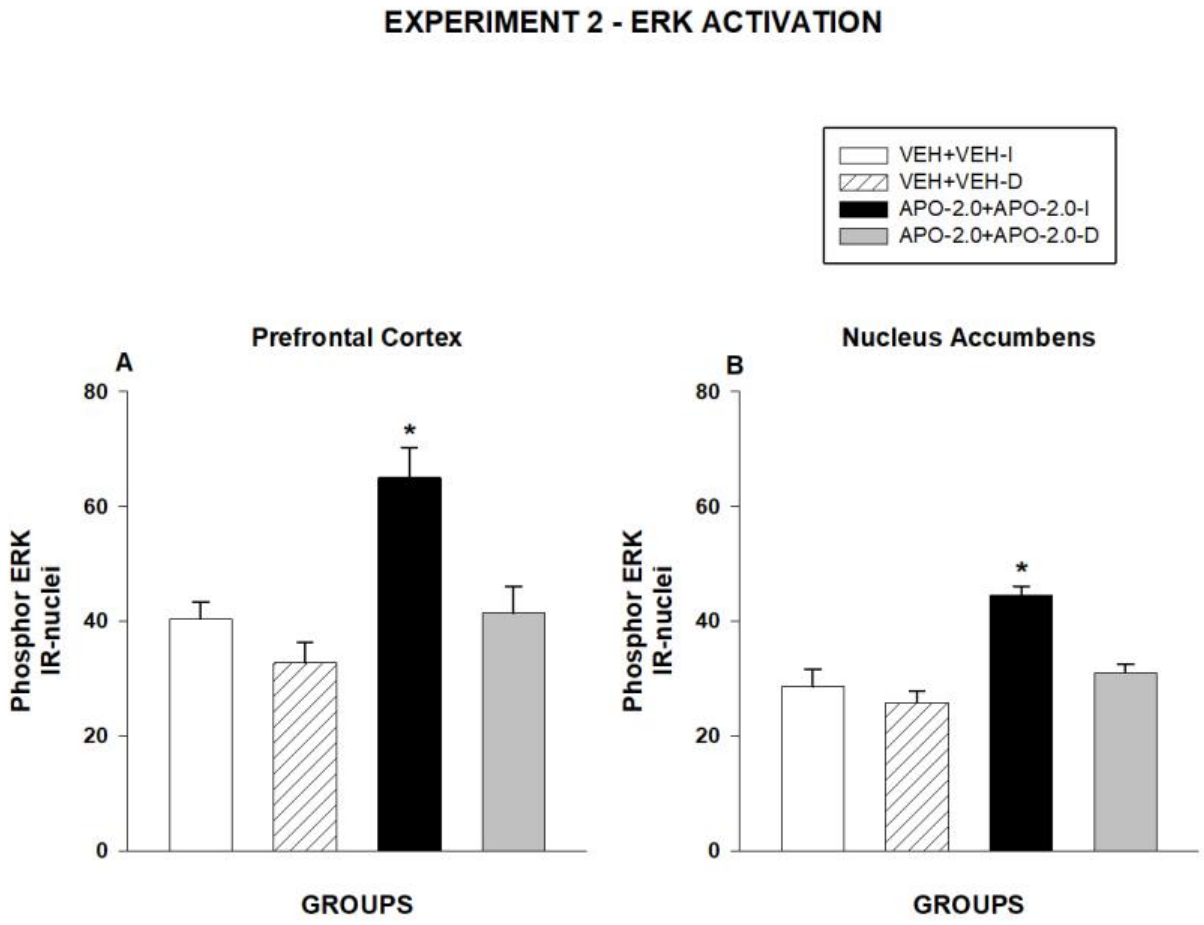

Fig. 8: Quantification of immunohistochemical results for ERK phosphorylation in the prefrontal cortex (A) and nucleus accumbens (B) after the final conditioning test for experiment 2. Data represent mean ± S.E.M. * Denotes significantly higher numbers of immunoreactive nuclei in the apomorphine group when compared to the vehicle treated group for the same brain area ($p<0.05$; one-way ANOVA followed by Duncan's multiple range test).

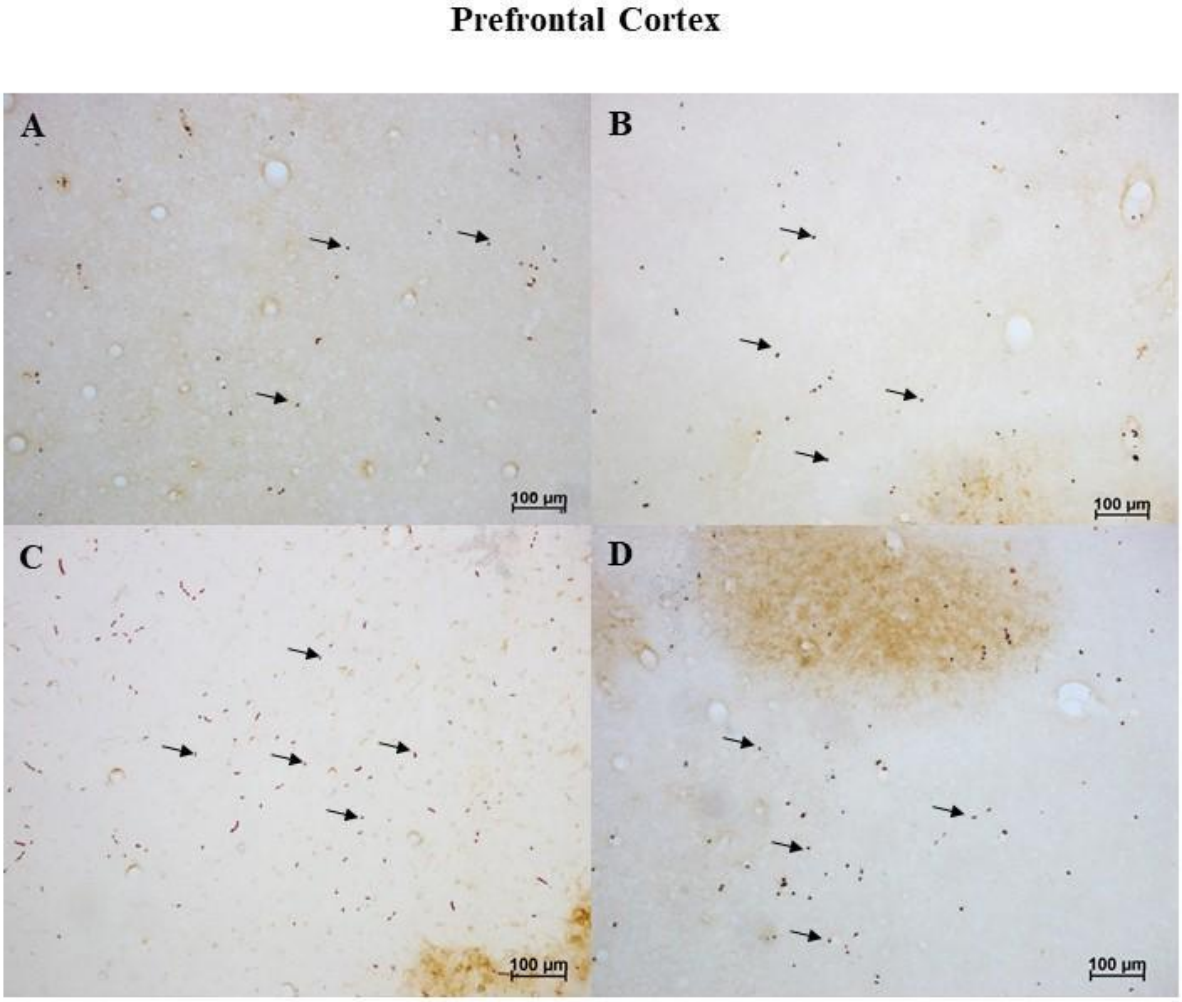

Fig. 9: Photomicrographs of tranverse sections of the prefrontal córtex of rats submitted to the experiment 2 shows ERK-P-immunoreactive cells arrows for vehicle immediate (A), vehicle delay (B), apomorphine immediate (C) and apomorphine delay (D). Scale bar = 100 µm.

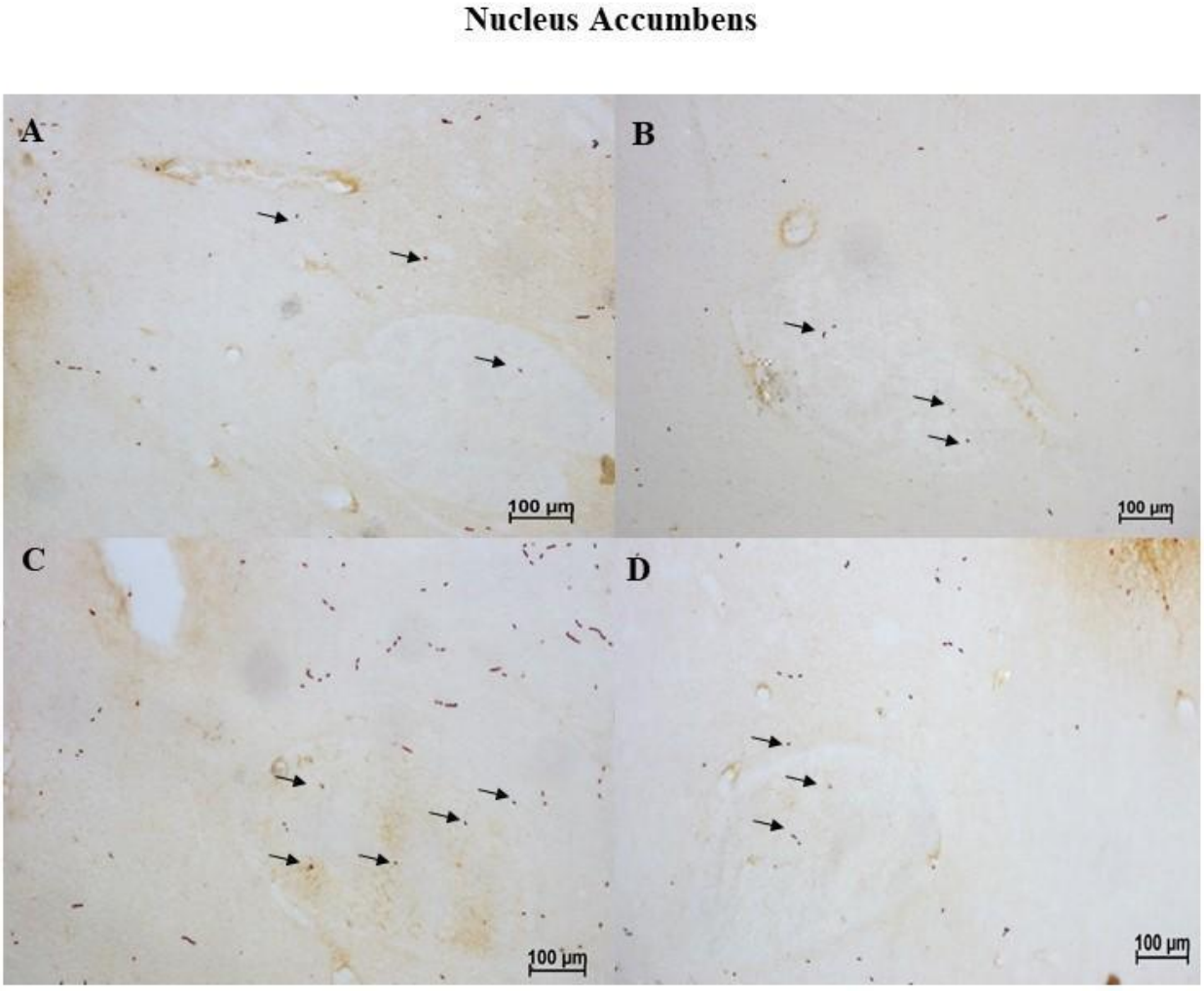

Fig. 10: Photomicrographs of tranverse sections of the nucleus accumbens of rats submitted to the experiment 2 shows ERK-P-immunoreactive cells arrows for vehicle immediate (A), vehicle delay (B), apomorphine immediate (C) and apomorphine delay (D). Scale bar = 100 µm.

Table 1: Timeline of the experiment 1.

Days	1	2	3	4	5	6	7	8	9	10	11	ERK Essay
	Habituation Phase			Induction Phase					WP		CT	

WP= withdrawal period; CT=conditioning test

Table 2: Timeline of the experiment 2.

Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	ERK
	Habituation Phase			Induction Phase					WP		CT1	RI	CT + Post-trial Treatments		CT Final	

WP= withdrawal period; CT=conditioning test; RI=re-induction day.

Table 3: Groups of experiment 2.

Initial Groups	Induction Phase	CT1	Re-induction	CT + Post-trial		CT Final	Final Groups
				Before	After		
VEH (n=10)	VEH	VEH	VEH →	VEH	VEH-I	VEH	VEH+VEH-I (n=5)
			→	VEH	VEH-D	VEH	VEH+VEH-D (n=5)
APO-2.0 (n=10)	APO-2.0	VEH	APO-2.0 →	VEH	APO-2.0-I	VEH	APO-2.0+APO-2.0-I (n=5)
			→	VEH	APO-2.0-D	VEH	APO-2.0+APO-2.0-D (n=5)

VEH=vehicle; APO=apomorphine 2.0 mg/kg; CT= conditioning test; I=post-trial treatment administered immediately after conditioning test; D=post-trial treatment administered 15 min. after conditioning test.

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7. DISCUSSÃO GERAL

Em nosso primeiro trabalho (SANGUEDO et al., 2014), investigamos a relação entre a ativação da ERK-P com o desenvolvimento da sensibilização comportamental, utilizando-se o agonista dopaminérgico apomorfina (2,0 mg/ kg) em uma dose capaz de ativar os receptores dopaminérgicos D1 (MATTINGLY et al., 1988a; ROWLETT et al., 1997) e mostramos que houve ativação da ERK-P no "córtex pré-frontal" naquele regime de tratamento. Tendo em vista que, tanto a sensibilização comportamental como o condicionamento, são processos envolvidos na gênese e manutenção da dependência química, investigamos nesse trabalho o efeito do condicionamento na ativação da ERK-P.

A ERK-P tem sido explorada como marcador molecular nos estudos de dependência química (GIRAULT et al., 2006; LU et al., 2006). No primeiro artigo apresentado neste trabalho mostramos maior ativação de ERK-P no "córtex pré-frontal" do grupo tratado com apomorfina, porém, no "núcleo *accumbens*" não houve diferença entre os grupos veículo e apomorfina. O córtex pré-frontal é uma área de projeção neocortical da dopamina importante na mediação dos processos de aprendizagem e memória. Na tentativa de explicar esse fato, pode-se sugerir que o "córtex pré-frontal" esteja mais diretamente envolvido com a resposta condicionada do que o "núcleo *accumbens*", pois a estimulação do "córtex pré-frontal" desencadeia a ativação no "núcleo *accumbens*" (CHAO et al., 2013; GLICKSTEIN et al., 2005).

O córtex pré-frontal está relacionado ao fenômeno da neuroplasticidade, capacidade do sistema nervoso de mudar, adaptar-se e moldar-se em nível estrutural e funcional ao longo do desenvolvimento neuronial. Esta característica única faz com que os circuitos neuroniais sejam maleáveis e está na base da formação das memórias e da aprendizagem (DUFFAU, 2006; HAASE e LACERDA, 2004; MAY, 2011). Trabalhos anteriores do nosso laboratório mostraram que a sensibilização e o condicionamento são dependentes do ambiente (BLOISE et al., 2007; BRAGA et al., 2009a, 2009b; DIAS et. al, 2010; DE MATOS et al., 2010). O ambiente está normalmente ligado à neuroplasticidade porque apresenta novas experiências ligadas à adaptação. Os ambientes enriquecidos melhoram as interações e capacidades cognitivas, sensitivas e

motoras, o que potencializa a aprendizagem e memória. Ambientes enriquecidos favorecem ao aumento do fator de crescimento nervoso (NFG), do fator neurotrófico derivado da glia (GDNF) e do fator neurotrófico derivado do cérebro (HART e BALLEINE, 2016; PASCUAL-LEONE, 2005).

Considerando-se que a via de sinalização da ERK está envolvida na manutenção das alterações das memórias de longa duração no cérebro, a sua ativação nas áreas de projeção dopaminérgica poderia contribuir para a neuroplasticidade. O comportamento de busca pela droga ("*craving*") poderia ser afetado pelas alterações neuroplásticas induzidas pela ativação da ERK nas áreas alvo da dependência.

De acordo com o protocolo adotado nos experimentos do primeiro artigo, observamos que o "córtex pré-frontal" foi ativado no condicionamento de 30 minutos associado ao ambiente. Esses resultados reforçam os dados da literatura que sugerem que a ativação da ERK-P contribui para a neuroplasticidade (GIRAULT et al., 2006; LU et al., 2006).

Na tentativa de expansão dos resultados obtidos, adotamos outros regimes de tratamentos utilizando-se a apomorfina para a avaliação dos efeitos da ERK-P sobre a resposta condicionada. Os dados apresentados no segundo artigo deste trabalho mostraram que o "córtex pré-frontal" e o "núcleo *accumbens*" foram ativados pela ERK-P, após o terceiro teste de condicionamento nos grupos "apomorfina imediatos" e que a resposta condicionada induzida pela apomorfina foi extinguida nos grupos apomorfina que tiveram tratamento após o tempo de 15 min. Uma possível explicação para isso é que houve uma extinção da resposta condicionada nos animais que tiveram um pós-tratamento, ou seja, é possível que essa resposta se mantenha durante o tempo de 5 minutos, mas que seja extinguida depois.

As memórias, uma vez estabelecidas, permanecem em um estado inativo até serem evocadas. Durante a evocação pode haver a desestabilização da memória original para permitir a incorporação de alterações nos traços da memória original. Esse processo é chamado de reconsolidação da memória (CESTARI et al., 2013; KRAWCZYK et al., 2013). Acredita-se que neste período, a memória esteja em um estado lábil, em que diante da evocação do estímulo condicionado induzido pela droga, seja possível uma reconsolidação. Neste processo, a reativação da memória é

reconsolidada. Embora ainda não existam tratamentos específicos para a reconsolidação da memória, tais como inibidores de síntese proteica ou choque eletroconvulsivo, é notório que a dopamina é um alvo de interesse para o entendimento dos mecanismos cerebrais através dos quais os psicoestimulantes exercem sua influência (CARRERA et al., 2011; 2012).

Na tentativa de expandir os dados disponíveis sobre o efeito dos testes de pós-tratamento sobre a reconsolidação da memória, utilizamos no segundo artigo deste trabalho uma dose elevada de apomorfina para aumentar a atividade dopaminérgica cerebral. Essa ativação foi fortemente potencializada pela resposta condicionada induzida pela apomorfina em comparação com o tratamento pós-teste com veículo. Sugerimos que a ativação da ERK no grupo tratado com dose elevada de apomorfina tenha ocorrido pela ativação de uma resposta locomotora condicionada induzida pela apomorfina. Essa resposta estaria envolvida com a evocação de um processo de reconsolidação da memória quando o animal experimenta rapidamente o efeito no ambiente associado às pistas (efeito condicionado). O efeito de exposição às dicas do ambiente permanece por um período (reconsolidação) após a remoção do animal do ambiente de teste. Nesse momento, a dose elevada de apomorfina ativa o sistema dopaminérgico e mantém a associação entre as pistas/dicas do ambiente com a ativação dopaminérgica. Essa associação sugere que a reconsolidação crie condições para reforçar a neuroplasticidade (CARRERA et al., 2010; 2011; 2012).

Trabalhos da literatura mostraram a relação entre a ativação da MAPK com o processo de reconsolidação, utilizando-se outros psicoestimulantes (MILLER e MARSHALL, 2005; BESNARD et al., 2013, KRAWCZYK et al., 2016). A administração do inibidor da MAPK, o U0126, injetado no “núcleo *accumbens*” no primeiro dia de teste, bloqueou a “memória de recaída à cocaína” em teste de condicionamento por preferência por lugar (CPP), durante os 14 dias seguintes, o que pode ser interpretado como uma interferência na reconsolidação, pois o bloqueio da transcrição/translocação proteica MEK ocorreu quando a memória foi evocada e estava lábil. Esse tratamento resultou na diminuição da ativação da ERK comparado aos grupos controles (MILLER e MARSHALL, 2005). Besnard e colaboradores (2013) propuseram que a ativação da

ERK é necessária no teste condicionamento a partir da evocação da memória e que a expressão do gene zif 268 regula a estabilização da memória.

De forma geral, notamos que a interação “neocórtex-*accumbens*” é um fator importante na ativação dopaminérgica. As respostas celulares nas subáreas do “córtex pré-frontal”, que recebem estímulos específicos do núcleo *accumbens*, sugerem um papel específico das interações córtico-estriatais nas respostas comportamentais a psicoestimulantes (VANDERSCHUREN et al., 2008). A região frontal do córtex estaria mais relacionada à procura por drogas do que a porção dorsal, o que poderia ser atribuído à sua ligação ao núcleo *accumbens* (KOYA et al., 2009).

A extinção da resposta condicionada nos grupos apomorfina de atraso (15 min) não aumentou a ativação da ERK-P no “córtex pré-frontal” e “núcleo *accumbens*”. Esses dados levantam uma interessante linha de investigação sobre o efeito dos testes de atraso (pós-testes), do efeito do tempo na reconsolidação da memória e nas estratégias para atenuar ou reverter a resposta condicionada (CARRERA et al., 2011; 2012; 2013; DE MELLO et al., 2014).

Sendo, então, a resposta condicionada mantida nos grupos imediatos, cabe ressaltar a importância da interação do ambiente com o condicionamento, de acordo com o modelo pavloviano. A resposta condicionada inicial induzida pela apomorfina poderia ser fortalecida pela aplicação de pós-testes com fármacos/estimulação dopaminérgica. É possível que a resposta condicionada sofresse uma redução ou extinção, caso ocorressem modificações no contexto ambiental, no qual os animais foram pré-sensibilizados com a droga (CROMBAG et al., 2001; BADIANI et al., 1995).

De acordo com Robinson e colaboradores (1998), a sensibilização comportamental não seria uma consequência inevitável da administração do psicoestimulante em doses diárias e fixas, mas resultaria de interações entre as manipulações farmacológicas e o seu ambiente de administração (ANAGNOSTARAS e ROBINSON, 1996; CROMBAG et al., 2001).

Os dados deste trabalho indicam que o “córtex pré-frontal” e o “núcleo *accumbens*” estão envolvidos no condicionamento induzido por apomorfina em animais previamente sensibilizados. A ativação da ERK-P é um indicador da sinalização dopaminérgica em tais estruturas e alterações neuroplásticas produzidas por fatores

como ambiente, tempo, tratamentos com fármacos podem interferir no efeito da resposta condicionada.

Os resultados do presente trabalho reforçam ainda a funcionalidade das conexões entre o “córtex pré-frontal” e o “núcleo *accumbens*” e estendem a importância da via mesolímbica na resposta condicionada induzida pela apomorfina. No entanto, estudos futuros são necessários para avaliar a ativação da ERK-P, utilizando-se outros regimes de tratamentos e psicoestimulantes.

8. CONCLUSÃO

- O tratamento com apomorfina (2,0 mg/kg) produziu sensibilização comportamental dependente do contexto e resposta condicionada.

- Apenas animais sensibilizados com apomorfina (2,0 mg/kg) tiveram ativação de ERK-P no “córtex pré-frontal” e no “núcleo *accumbens”*.

- Os animais sensibilizados com apomorfina imediato (2,0 mg/kg) tiveram ativação de ERK-P no terceiro teste de condicionamento no “córtex pré-frontal” e no “núcleo *accumbens”*.

- O “córtex pré-frontal” está associado com mudanças neuro-adaptativas induzidas por estimulação dopaminérgica.

- A ativação de ERK-P no “córtex pré-frontal” está relacionada com a reposta condicionada induzida pela apomorfina (2,0 mg/kg) juntamente com a ausência de ativação, após a extinção da resposta condicionada.

- A ERK-P é um indicador da atividade dopaminérgica e importante na aprendizagem e memória.

- Os animais sensibilizados com apomorfina (2,0 mg/kg) que passaram por testes pós-tratamento após 15 min tiveram extinção da resposta condicionada.

- Os tratamentos pós-teste podem ser utilizados na reconsolidação do comportamento condicionado.

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