UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE – UFCSPA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

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A INFLUÊNCIA DO AMBIENTE ENRIQUECIDO E DA ESCOLHA NO CONSUMO E NA BUSCA PELO EFEITO DA COCAÍNA

Porto Alegre

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Tese submetida ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Doutor (a)

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Porto Alegre
2016

Catalogação na Publicação

Freese, Luana
A INFLUÊNCIA DO AMBIENTE ENRIQUECIDO E DA ESCOLHA NO CONSUMO E NA BUSCA PELO EFEITO DA COCAÍNA / Luana Freese. -- 2016.
139 p. : il., graf. ; 30 cm.

Tese (doutorado) -- Universidade Federal de Ciências da Saúde de Porto Alegre, Programa de Pós-Graduação em Ciências da Saúde, 2016.

Orientador(a): Helena Maria Tannhauser Barros ; coorientador(a): Rosane Gomez.

1. Cocaína. 2. Sacarina. 3. Condicionamento de Preferência de Lugar. 4. Autoadministração. I. Título.

Sistema de Geração de Ficha Catalográfica da UFCSPA com os dados fornecidos pelo(a) autor(a).

Agradecimentos

Contemplar a todos que fizeram parte desta importante e longa etapa da minha vida não é tarefa fácil. Finalizo a tese e ainda não sei bem como fazê-lo. Cada vez mais tenho certeza que a forma como progredimos profissionalmente está diretamente ligada aos desdobramentos da nossa vida pessoal. Muitas pessoas fizeram parte de maneira significativa da minha vida nestes anos de formação. E a vida, que não é exata, não precisa de significância estatística pra ser relevante. Ela precisa ser vivida e a sua relevância não é medidas pelo tempo de permanência, pela média, pela frequência; ela é medida pela intensidade, pelo sentimento gerado e que permanece de tudo aquilo que foi vivido, que nos torna o que somos. E essa medida, subjetiva como é, só pode ser demonstrada pela lembrança, que volta, na hora de pensar sobre aqueles que estiveram comigo durante esta etapa.

Primeiro, como não poderia deixar de ser, agradeço a minha mãe, cujo tempo passado ao meu lado foi curto, mas cuja intensidade se prolonga e me acompanha em tudo que faço até hoje. Obrigada por todo o amor que me deste e demonstraste em todas as oportunidades que tiveste. Carrego comigo o valor de saber sentir e demonstrar amor. E só tenho agradecer por isso. Ao meu pai, por me ouvir semanalmente enquanto estive fora Obrigada por ser meu exemplo de trabalho, amor e dedicação à docência. Por sempre confiar em mim e pelo apoio firme e seguro que me deste, mesmo longe.

Obrigada ao meu grandessíssimo amor, Ramiro Barcos Nunes, por compor ao meu lado, uma linda e verdadeira história de amor. Obrigada pelas tantas afinidades que temos, a qual destaco a vontade imensa de sermos melhores, também um para o outro. Obrigada pelas preciosas visitas à Bordeaux, que me recarregavam de energia. E claro, pelas viagens incríveis que fizemos juntos. Sou muito grata por dividirmos juntos as nossas vidas. Obrigada pela paciência e pela segurança e exemplo que és pra mim. Agradeço também a tua família por todo o carinho, comemorações na casa da Ruth, pelas crianças brincando, pela cultura das conversas à mesa enfim, por serem exatamente como desejaria que fossem.

Agradeço a minha orientadora Helena Barros, primeiro, pela oportunidade de trabalhar a seu lado, em grandes projetos. Tenho muito orgulho de fazer parte deles, tanto no laboratório de neuropsicofarmacologia quanto no Ligue 132. São 12 anos de um aprendizado imenso. Ainda, muito obrigada pelo exemplo de mulher que és. Isso, da mesma forma, que orgulha muito.

Agradeço a professora Rosane Gomez, também, como mulher, pela fibra e pela luta pra conquistar teu espaço. Agradeço também pela amizade, pela simplicidade, pela disponibilidade, pelas jantinhas e, principalmente, por ter os braços abertos, sempre. Obrigada pela amizade, que me enche de satisfação e gratidão.

Obrigada ao meu supervisor do doutorado sanduíche Serge Ahmed, pela oportunidade, pela paciência com o *english-french* do início. Obrigada por ser tão incentivador e motivador e ensinar que "*the science is long and hard"*. Agradeço imensamente a oportunidade de vê-lo trabalhar com ciência. A oportunidade que me deste fará sempre do minha história.

Obrigada a Maristela Ferigolo, pelo reconhecimento, pelos ensinamentos, pela rigorosidade dos métodos, fundamentais para o meu desenvolvimento. Obrigada pela confiança e oportunidade.

Obrigada à veterinária Joana, do biotério da UFCSPA, pela ajuda preciosa com o bem estar dos animais. Obrigada a Inês, pela simpatia, pelo sorriso fácil e por sempre me ajudar nas trocas dos animais quando precisei; e à Dina, por ajudar na sujeira que os animais do ambiente enriquecido faziam no biotério.

Obrigada ao querido Mário, pela disponibilidade em ajudar nos "dias finais" dos experimentos e por fazer tudo com uma alegria contagiante. Tuas histórias farão parte sempre do acervo de "alegrias" da farmacologia da UFCSPA.

Obrigada aos ICs Nicolas, Thábata, pela ajuda com o planejamento do ambiente enriquecido no início do doutorado, ao Tierre e ao Thiago, por ajudar com as cirurgias de canulação, onde era tudo bastante intuitivo, mas muito preciso. Obrigada à Núbia, ao Paulo, ao Alan e ao Luiz pela ajuda com as trocas de animais e com a execução dos protocolos comportamentais e neuroquímicos. Obrigada a professora Dinara pela energia e motivação em aceitar participar desse trabalho, disponibilizando seu tempo raro e suas preciosas alunas. À competentíssima Luiza, pela segurança demonstrada na execução de vários testes. À Aline, pela ajuda e calma ao trabalhar com o cometa.

Obrigada às minhas queridas colegas de laboratório e amigas Laísa, Priscila, Greice pelas conversas deliciosas, pelas opiniões compartilhadas, pelas lágrimas de insegurança e pelas palmas de incentivo que sempre fizeram parte da nossa relação. Junto com o Felipe, formamos um grupo incrivelmente bom de trabalhar, que me enche de orgulho de fazer parte. Felipe, aliás, obrigada pelo companheirismo do processo de finalização e pelas pequenas conversas e cafés em meio às nossas correrias pelo lab. e pelo mundo. Agradeço ainda à Marilise, por ser um exemplo essencial de jovem pesquisadora e hoje, de profissional. À

Natividade, pelo humor fino compartilhado acerca de pessoas e situações. Ao Maurício, por ser uma figura tão importante dentro do laboratório e por tentar sempre não deixar de participar, ainda que a vida tenha tomado tantos outros merecidos caminhos.

Obrigada a grande equipe do ligue 132, cujo agradecimento que dali advém, são profundos. O projeto, do qual tive a oportunidade de participar, em todas as suas fases, desde a concepção às mudanças relativas ao momento político que vive o nosso país, são de uma importância e grandeza na minha vida, que se reflete tanto pelos consultores, supervisores e secretários que por ali passaram, quanto nas pessoas que foram atendidas e que compartilharam conosco as suas lutas. Sinto-me imensamente grata por fazer parte desse projeto. Obrigada a toda a equipe, sem exceção.

Obrigada às minhas queridas amigas e aos seus filhotinhos amados, Mariana e Gabriel, queridas pessoinhas tão doces e tão cheias de personalidade. Muito obrigada por tanta companhia e por saber tanto se fazer presente em todos os momentos que precisei. Vocês são imprescindíveis. Nádia e o nosso amado Bento, que espera por mais atenção da sua dinda, que está chegando, logo término da tese. Obrigada a Deisi e à Milena, cuja saudade é sentida no olhar sempre que nos encontramos. À Claudia Mazoni e sua família, pela visita que recebi e por ser tão orgulhosa do que você plantou em mim. Obrigada às queridíssimas amigas Taís e Hildinha, exemplos de dedicação e responsabilidade. Compartilhar qualquer momento com vocês é sempre um carinho na alma e no coração.

Obrigada às secretárias queridíssimas e muitíssimo prestativas do PPG Cris, Maristela e à incrivelmente especial Luciane Spencer, que tive a sorte de contar com a competência durante processo relativo ao doutorado sanduíche.

Por fim, obrigada à equipe do Institut des maladies neurodégénératives - Prise de décison pathologique de l'addiction de Bordeaux, França. Obrigada ao Paul, por toda ajuda dos primeiros dias e pela presteza de sempre; a Audrey, por toda delicadeza, a Karine, a Caroline, ao Demian, pelo bom humor, pelas piadas, que muitas eu não entendia, mas morria rindo, à Marina, pela pelas conversas e viagenzinhas a Lacanau dos finais de semana que guardarei pra sempre no meu coração. Margaux, querida e pacienciosa, pra quem tive a oportunidade de retribuir os ensinamentos sobre o laboratório de autoadministração. Obrigada a toda a equipe. Vocês foram muito generosos e serão sempre um exemplo.

Obrigada à CAPES, CNPq e SENAD pelo imprescindível apoio financeiro.

RESUMO

A exposição repetida à cocaína causa neurotoxicidade e pode resultar no desenvolvimento de dependência. O desenvolvimento da dependência, por sua vez, implica numa convergência de fatores, que podem ser tanto ambientais como individuais. Do porto de vista ambiental, a criação de ratos em um ambiente enriquecido (AE) tem revelado um papel neuroprotetor, o que poderia diminuir o risco de adição. Possibilitar a animais de laboraório realizar uma escolha entre uma injeção de cocaína ou beber uma solução doce tem sido estudada para entender o papel das influências individuais na escolha pelo efeito da droga. O objetivo geral deste estudo é verificar se 1) o AE e a presença de sacarina alteram o comportamento de condicionamento à cocaína em ratos submetidos a um protocolo adaptado de condicionamento de preferência de lugar (CPP) e, ainda, determinar o papel neuroprotetor do AE no hipocampo e no córtex pré-frontal dos animais após exposição à cocaína. 2) Testar se existe mudanças na predição dos ratos por sacarina ou cocaína, após injeções passivas de cocaína em modelo de autoadministração e escolha. Para responder aos objetivos, foram realizados dois experimentos: 1) Cinqüenta ratos Wistar machos foram divididos em grupos padrão (ST) ou AE do dia 21 pós-natal (PND) aos 50 PND. Os animais foram então testados em um protocolo de condicionamento de preferência de lugar (CPP) padrão e outro adaptado para medir a preferência por cocaína (15 mg/kg; i.p.) ou sacarina [0,2%]. Após, os animais foram decaptados e o hipocampo e córtex pré-frontal foram dissecados para posterior análise de neurotoxicidade. No experimento 2) foram realizadas cirurgias para implantação de cânula na veia jugular de ratos Wistar machos (n=14) para posterior autoadministração endovenosa de cocaína. Foi conduzida então uma série de experimentos: autoadministração prolongada à cocaína (0,25 mg por injeção durante 4,2s); seguido de um esquema de escolha entre obter uma injeção de cocaína ou poder beber uma solução de sacarina [0.2%] e;, por fim; um regime de escolha na autoadminitração, intercalado por injeções passivas de cocaína (0,75 mg) para testar a preferência do animal estando sob efeito da droga. Os resultados obtidos nos experimentos 1) foram que os animais do grupo AE apresentaram menor interesse pelo compartimento pareado com a cocaína no CPP. Ainda, todos os animais, para os quais foi dada a opção de escolha, foram menos propensos a mostrar preferência pelo lado pareado com cocaína. Nas análises de neurotoxicidade, o AE diminuiu o estresse oxidativo e o dano ao DNA induzido por cocaína no hipocampo e córtex pré-frontal dos animais. Importantemente, demonstramos que os efeitos da cocaína, tanto comportamentais quanto de neurotoxicidade, podem ser diferentes, dependendo dos fatores ambientais envolvidos. No estudo 2) encontramos evidências que apoiam nossa hipótese de que estar sob efeito de cocaína leva a optar por continuar intoxicado. No entanto, houve também uma variação individual interessante, onde mesmo sob efeito da cocaína, alguns animais foram capazes de aumentar ainda mais sua preferência por sacarina. Analisados em conjunto, os nossos resultados demonstram que tanto o ambiente enriquecido como a presença de solução adocicada podem favorecer escolhas outras que não o uso de uma droga altamente “viciante”como a cocaína, mas que isso pode depender de fatores individuais. De maneira geral, confiando nos modelos animais de dependência como fundamentais para o avanço do conhecimento acerca dos efeitos comportamentais e neuroquímicos das drogas de abuso sobre os indivíduos, entendemos que pesquisas futuras devem ser dedicadas a melhor explorar modelos que considerem cada vez mais a mulfatoriedade envolvida na dependência química.

ABSTRACT

Repeated cocaine exposure causes neurotoxicity and may result in the addiction development. The dependence, in turn, implies a convergence of factors, which can be both environmental and individuals. From the port of environmental view, the environmental enrichment (EE) has revealed a neuroprotective role, which could attentuate the risk of addition. To offer for laboratory rats a possibity to make a choice between cocaine and another reward has been studied to better understand the role of individual influences in drug choice. The general objective of this study is to verify if 1) the EE and the presence of saccharin can to alter the behavior of cocaine conditioning in rats submitted to an adapted protocol of conditioning preference place (CPP) and also to determine the neuroprotective role of EE on the hippocampus the pre-frontal cortex of animals exposed to cocaine. 2) To test if there are changes in the prediction of rats by saccharin or cocaine, after passive injections of cocaine in a model of self-administration and choice. In order to respond to the objectives, two experiments were performed: 1) Fifty male rats were divided on the 21st day postnatal (PND) in a standard (ST) or AE group. They were reared in EE until 50 PND. Then, the animals were tested in a standard or an adapted conditioning preference place (CPP) protocol to measure a preference for cocaine (15 mg / kg, i.p.) or saccharin [0.2%]. After, the animals were decapitated and hippocampus and prefrontal cortex were dissected for later neurotoxicity analysis. In the experiment 2) surgeries were performed for a cannula implantation in the jugular vein of male Wistar rats (n = 14) for subsequent intravenous cocaine self-administration (0.25 mg per injection for 4.2 seconds). A series of experiments were then conducted, in sequence: prolonged cocaine self-administration - Scheme to choose between a cocaine injection or to drink a saccharin solution (choice self-administration) - Schem of choice self-administration, interspersed by passive injections of cocaine to test the choice of animal under the effect of the drug. The results obtained in the experiments 1) were that the animals of group EE presented less interest in the compartment paired with cocaine in CPP. Still, all animals which the choice procedure on CPP was made were less likely to show preference for the side paired with cocaine. In neurotoxicity analyzes, the EE group decreased oxidative stress and cocaine-induced DNA damage in the hippocampus and prefrontal cortex of the animals. Summarizing, we have demonstrated that the effects of cocaine, both behavioral and neurotoxicity, may be different, depending on the environmental factors involved. In second study we found evidence that supports our hypothesis that being under cocaine effects leads to opting to remain intoxicated. However, there was also an interesting individual variation, where even under the effect of cocaine, some animals were able to further increase their preference for saccharin. Taken together, our results demonstrate that both the enriched environment and the presence of sweetened solution may favor choices other than using a highly "addictive" drug such as cocaine, but that this may depend on individual factors. In general, relying on animal models of dependence as fundamental for advancing knowledge about the behavioral and neurochemical effects of drugs of abuse on individuals, we understand that future research should be devoted to better explore models that increasingly consider the multifariousness involved in the addiction development.

Lista de Abreviações do Referencial Teórico

AE – Ambiente Enriquecido

APA – *American Psychological Association*; Associação Americana de Psicologia

AS – Ambiente Social

CAT – Catalase

COC – Cocaína

CPF – Córtex pré-frontal

CPP – *Conditioned Place Preference*; Condicionamento de Preferência de Lugar

DA – Dopamina

DNA – Ácido desoxirribonucleico

DSM – Manual Diagnóstico de Transtornos Mentais

H_2O_2 – Peróxido de Hidrogênio

IC – *Isolated Condition*; Isolamento Social

O_2 – Superóxido

PR – *Progressive Ratio*; Razão Progressiva

RF – Razão Fixa

ROS - *Reactive Oxygen Species;* Espécies Reativas de Oxigênio

SAC – Sacarina

SNC – Sistema Nervoso Central

VMAT-2 - *Vesicular monoamine transporter 2* - Transportador de monoamina vesicular 2

SOD – Superóxido Dismutase

Lista de Publicações

Neuroprotection by environmental enrichment in cocaine place preference

Freese, L.; Almeida, F.B.; Heidrich, N.; Steffens, L.; Moura, D.J.; Gomez, R.; Barros, H.M.T.

Differential time evolution of cocaine's anorexic and reward satiating effects influences individual drug choice

Freese, Luana; Durand, Audrey; Guillem, Karine; Ahmed, Serge.

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Lista de figuras do referencial teórico

SUMÁRIO

1 INTRODUÇÃO

1.1 Dependência: conceito e prevalência

O uso de substâncias psicotrópicas para modificar a percepção, o humor, os sentidos e o comportamento sempre foi presente na sociedade, independente do tempo. Drogas como álcool, cafeína, nicotina, cocaína, maconha, opiáceos e outras substâncias tem sido usadas em uma variedade de culturas no mundo para socialização, cerimônias, rituais ou como um breve escape para uma existência difícil (NATHAN; CONRAD; SKINSTAD, 2016). Os conceitos com relação ao uso de drogas, da mesma maneira, são relativos ao contexto e ao conhecimento prevalente em cada época. Acerca disso, muitas questões derivam da tentativa de entender a complexidade que permeia o uso de drogas, desde a antiguidade até os dias atuais.

> "A complexa etiologia da dependência química está refletida nas frequentes oscilações entre atitudes opostas pra temas que atualmente seguem sendo debatidos, como: se a dependência é pecado ou doença; se tratamento deve ser moral ou médico; se o vício é causado pela substância, pela vulnerabilidade do indivíduo, pela sua psique ou por fatores sociais; e se as substâncias devem ser regulamentadas ou devem estar livremente disponíveis" (CROCQ, 2007).

É de fato impressionante observar que muitas das mesmas questões continuam a ser debatidas desde os tempos antigos até hoje (NATHAN; CONRAD; SKINSTAD, 2016). No entanto, entender o conceito de dependência como uma desordem psiquiátrica é uma preocupação central para o estabelecimento de um modelo animal de adição. Assim, a primeira parte da introdução será usada para definir o conceito de doença psiquiátrica, colocando-a em um breve contexto histórico.

1.1.1 A evolução de conceitos: do julgamento moral à doença crônica

A história do tratamento de desordens mentais perpassa fases de não assimilação de insanidade ou culpa, doença ou impureza, manifestações da mente ou pecado. Entre os hebreus, na Babilônia, durante muitos anos antes de Cristo (A.C.), a doença, física ou mental,

era entendida como uma manifestação do pecado e o uso de penitências manifestava uma ideia de purificação. No mundo grego e romano, a saúde era uma função do equilíbrio dos humores e as patologias, incluindo a psíquica, ocorriam em função de mudanças no ambiente interno do indivíduo. A teoria humoral é estabelecida por Hipócrates, na Grécia, cerca de quatro séculos A.C.. Ainda, durante o longo período entre o fim do Império Romano do Ocidente (476 dC) e a queda de Constantinopla (1453), a ideia predominante na sociedade é que a loucura era um castigo divino, quando não era um sinal de possessão demoníaca. O tratamento, a partir deste entendimento, combinava exorcismo, ritos sagrados e orações. O início do Renascimento é marcado por tragédias terríveis, onde vítimas de doenças mentais, bem como um grande número de pessoas saudáveis, eram vistas como praticantes de bruxarias. Alguns médicos, protestando contra a prática, acreditavam que eles devessem ser tratados com cuidado, embora não houvesse esperança real de tratamento e recuperação (CROCQ, 2007; NATHAN et al., 2016).

No final do século XVIII (1806), o psiquiatra francês Philippe Pinel propôs uma taxonomia dos transtornos. A nosologia de Pinel foi a primeira tentativa de classificar as doenças mentais. Pinel aventurou, por exemplo, que a melancolia, uma de suas classes de transtorno mental era por vezes provocada pelo abuso crônico do álcool (PINEL, 1962), uma conexão feita ainda até os dias de hoje.

Benjamin Rush (1745-1813) era outro defensor precoce de uma concepção mais médica da dependência. Enquanto a prática comum em seu tempo era a punição dos crimes causados por usuários, Rush viu esses crimes como um sintoma de uma doença subjacente. Entre outras realizações, ele fundou a primeira instituição dedicada exclusivamente ao tratamento de alcoolistas em Boston, nos Estados Unidos (NATHAN et al., 2016).

Após progressões discretas acerca do estabelecimento claro da dependência como doença e ainda refletindo a influência das teorias psicanalíticas da etiologia dos transtornos mentais, após a Segunda Guerra Mundial, em 1952, surge a primeira edição do Manual Diagnóstico e Estatístico de Transtornos Mentais (DSM), publicada pela Associação de Psiquiatria Americana (APA). A segunda versão (DSM-II) foi publicada em 1968 e estendeu a classificação para um número maior de doenças. Nestas duas edições não havia distinção clara entre normalidade e anormalidade e todas as doenças mentais identificadas foram consideradas reações a eventos ambientais. Nestas duas versões preliminares do DSM havia a distinção apenas entre duas formas de transtornos psiquiátricos: psicoses e neuroses (MACK et al., 1994).

1.1.2 Concepções modernas da dependência

Muitos psiquiatras e historiadores na área da saúde mental acreditam que o psiquiatra alemão Emil Kraepelin (1856-1926) fundou a psiquiatria científica moderna por seus esforços para desenvolver a psicofarmacologia, a genética psiquiátrica, a classificação e o diagnóstico das doenças mentais. Para este fim, o DSM-III e seus sucessores são algumas vezes referidos como neo-Kraepelinianos porque exigem observação cuidadosa do comportamento do paciente e completa identificação de sinais e sintomas da desordem para o diagnóstico. A definição moderna de dependência como um transtorno psiquiátrico foi introduzida no DSM-III. O lado clínico é bem caracterizado por um uso compulsivo de drogas, que o indivíduo não pode controlar. Tolerância e sintomas de abstinência podem estar presentes, mas não indicam necessariamente uma dependência. Os traços característicos da dependência são a presença de comportamentos persistentes e recorrentes de uso da droga em detrimento de outras atividades habituais e prazerosas. Esta definição pressupõe, mesmo ainda sem total esclarecimento, que a dependência é uma neuropatologia, comum a muitas drogas, mesmo que farmacologicamente diferentes (WILSON, 1993).

O DSM-IV e o DSM-V ratificam e aprimoram essa visão biológica do processo da dependência; O DSM-V é bastante explícito ao apontar para crescentes descobertas envolvendo mecanismos cerebrais específicos no processo de dependência. Hoje, a dependência a drogas psicoativas, também chamada adição, é considerada uma doença psiquiátrica crônica associada a um elevado risco de recaída pela *American Psychological Association*, que publica o DSM (APA, 2014). Embora conscientes de que ainda há debate sobre o uso adequado de termos como adição ou dependência (AHMED, S. H., 2010; MADDUX; DESMOND, 2000), e mesmo o termo adição não sendo muito usado no Brasil, podendo ser traduzido como vício, usaremos ao longo desta tese os termos igualmente para descrever o mesmo fenômeno.

Nos dias atuais pode-se considerar que existem principalmente duas concepções da dependência: de um lado, caracterizando como uma doença e de outro, como uma desordem de escolha. Na primeira, a toxicodependência é considerada uma doença crônica (AMERSON; SMITH, 2001; HYMAN, 2005;2007), que acaba com a capacidade do indivíduo de agir livremente, de forma voluntária e racional. Ela poderia ser caracterizada por uma diminuição na capacidade de controlar o uso de drogas, mesmo com o reconhecimento de fatores que deveriam motivar a parada do uso (HYMAN, 2007). Como descrito acima, este

conceito de dependência é defendido pela APA no seu manual de classificação das doenças mentais.

Por outro lado, há um segundo ponto de vista, defendido por outros pesquisadores, que definem a dependência como uma escolha voluntária (HEYMAN, GENE M., 2009; HEYMAN, G. M., 2013), mas não no sentido de uma decisão racional de sua vida, como algumas teorias da dependência racional (SCHALER, 2000). Neste caso, o usuário não escolhe antecipadamente e racionalmente tornar-se dependente. A transição do uso recreativo à dependência acontece de forma inesperada e como uma consequência não intencional de uma soma de escolhas diárias, não necessariamente sábias, embora consideradas "normais". Por exemplo, tomar decisões impulsivas (isto é, fazendo a escolha da melhor opção imediata, sem considerar as consequências em longo prazo) é algo considerado comum entre as pessoas (sejam jovens ou adultos). No entanto, este comportamento pode, em algumas circunstâncias, levar à dependência quando, por exemplo, existe um aumento da disponibilidade da droga combinada com uma falta de oportunidades e opções alternativas. De acordo com esta teoria, não haveria perda patológica de controle ou de qualquer disfunção cerebral que privaria o indivíduo de sua capacidade de fazer uma escolha livre e voluntária. Mesmo no auge de sua dependência, o indivíduo dependente continuaria tendo a capacidade de exercer controle sobre seu uso de drogas e seria até mesmo capaz de parar de usar, com ou sem ajuda profissional, se as circunstâncias forem favoráveis (HEYMAN, GENE M., 2009). A dependência, sendo conceituada como uma escolha voluntária, mas impulsiva, sustentada por uma série de escolhas ruins, é suportada pelas altas taxas de cura espontânea observada entre dependentes e tem boa compatibilidade com a eficácia de terapias, tais como as comportamentais utilizadas em dependentes de cocaína, como aqueles que defendem o uso de *vouchers* ou prêmios em troca da manutenção da abstinência (HEYMAN, GENE M., 2009).

No entanto, parece surpreendente que, após mais de um século de debate e pesquisa, a comunidade científica ainda não tenha encontrado um consenso sobre a natureza da dependência e que os dois conceitos melhor detalhados até hoje, possam coexistir. Em verdade, os argumentos científicos são sólidos e consistentes em ambos os lados e, na ausência de evidência clara da natureza da neuropatologia nos seres humanos, é difícil favorecer um ou outro destes modelos. Entendemos que, acima de tudo, existem também fatores anteriores que predispõe um indivíduo ao uso. Estes fatores vão desde questões individuais, como a resiliência, a toxicocinética da droga no organismo e, ainda, tamanho do valor do efeito da droga, que pode ser mensurado a partir de um repertório comparativo muito

particular; até, justamente, de qual repertório se está falando. É um repertório que representa um fator de proteção ou de risco para o uso de drogas.

De maneira geral, não se pode negar que exista sim uma visão consensual, a de que a dependência química seja determinada a partir da interação de múltiplos fatores (Figura 1). Estes fatores estão sempre presentes influenciando não só o aparecimento, mas também os processos de abstinência e recaída, que permeiam o círculo de uso – abuso – dependência – abstinência e recaída da adição. Considera-se, neste conceito multifatorial, muito importantemente, o fato de que uma grande porcentagem da população experimenta os efeitos das drogas de abuso e apenas uma pequena parte desenvolve a síndrome patológica compulsiva típica da dependência química (KOOB; VOLKOW, 2016; KREEK et al., 2005; PIAZZA; LE MOAL, 1996).

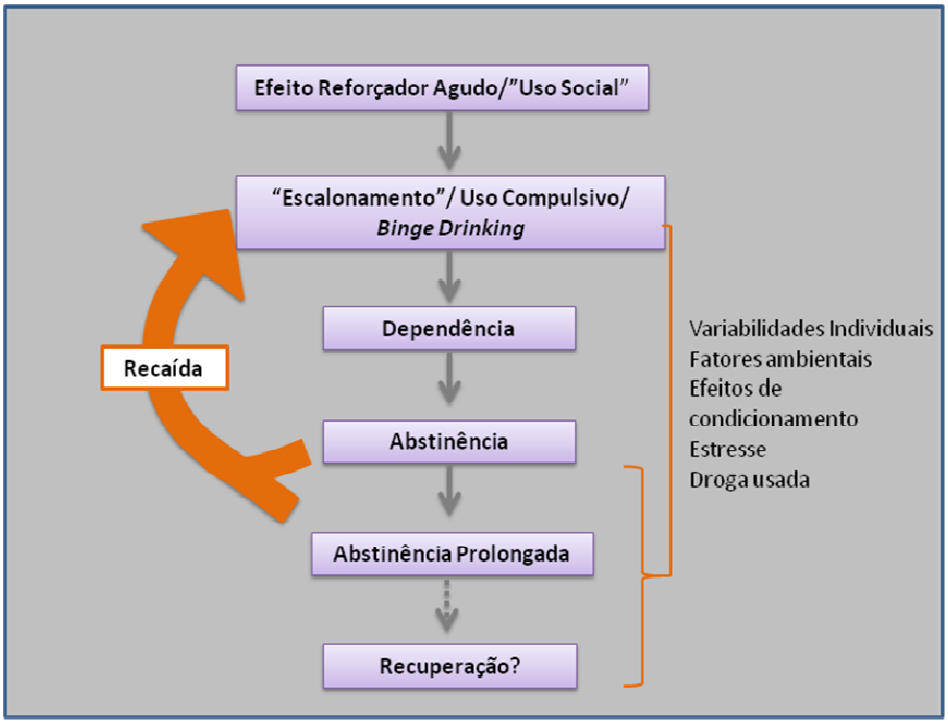

Figura 1: Estágios de dependência a drogas. O uso compulsivo começa invariavelmente a partir de um uso "social" e em seguida, mas não exclusivamente, se movem para um padrão de uso problemático, com aumento das doses e frequência até a dependência. Em seguida, seguem estágios de abstinência até a abstinência prolongada. A recaída promove então uma nova repetição do ciclo. Fatores genéticos, ambientais estresse e condicionamento contribuem para a variabilidade individual para iniciar o ciclo de abuso e dependência, bem como para recair dentro dele (Figura adaptada/traduzida de Koob & Le Moal, 2007).

Por outro lado, estão os fatores neurobiológicos da dependência, que estão baseados nas propriedades reforçadoras das drogas psicoativas, que ocorrem em grande parte, pela ativação do sistema dopaminérgico mesocorticolímbico (KALIVAS; VOLKOW, 2005). Neste contexto, a dependência pode ser explicada também em parte, pela habilidade das

drogas de abuso em reorganizar regiões cerebrais envolvidas no reforço e na motivação de repetir o uso (ROBINSON; BERRIDGE, 2008). Neste sentido, a habilidade de uma droga em relação à chance de se tornar um indivíduo dependente diminui, seguindo esta ordem: tabaco > heroína > cocaína > álcool > maconha (VSEVOLOZHSKAYA; ANTHONY, 2016).

Mas antes de abordar as questões relativas à habilidade da cocaína em reorganizar regiões cerebrais de maneira a tornar o indivíduo dependente, foco deste trabalho, é importante lembrar algumas figuras-chave para mensurar o problema de saúde que o uso dessa substância representa.

1.1.3 Dados Epidemiológicos

O consumo de drogas é uma das principais preocupações na saúde pública em todo o mundo. De acordo com as últimas estimativas fornecidas pelo Escritório das Nações Unidas contra a Droga e o Crime (UNODC), cerca de 247 milhões de pessoas, com 15 anos ou mais, usaram uma droga ilícita em 2014 (UNODC, 2016). Destes, um em cada dez, ou seja, quase 29,5 milhões de pessoas em todo o mundo, são considerados usuários problemáticos de drogas (UNODC, 2016) (Figura 2). Este grupo particular de usuários, em que os efeitos nocivos do consumo de droga tendem a progredir para um estado mais grave, também conduzirão aos mais elevados custos humanos (saúde, violência, etc.) e econômicos para a sociedade.

A cocaína é utilizada por cerca de 17 milhões de pessoas em todo o mundo, o que corresponde a aproximadamente 0,4 por cento da população global com idade entre 15 a 64 anos (UNODC, 2015). O uso é mais prevalente na América do Norte (5,3 milhões de pessoas; 1,7 por cento da população) e América Central e do Sul (3,5 milhões de pessoas; 1,2 por cento) e na Europa Ocidental e Central (3,2 milhões de pessoas; 1,0 por cento). Aproximadamente um quinto dos 4,2 milhões de usuários nos EUA que relataram ter feito uso da droga no último ano atendiam aos critérios diagnósticos do DSM-V para abuso ou dependência de cocaína. O consumo de cocaína é menor na África Ocidental e Central, no Leste e Sudeste Asiático e na Europa Oriental e Sudeste (UNODC, 2015). Esse padrão pode ser devido a fatores de oferta e não de demanda, devido à dificuldade em obter cocaína de sua única fonte na América do Sul e à pronta disponibilidade de estimulantes sintéticos alternativos, como anfetaminas ou seus derivados (DEGENHARDT et al., 2008).

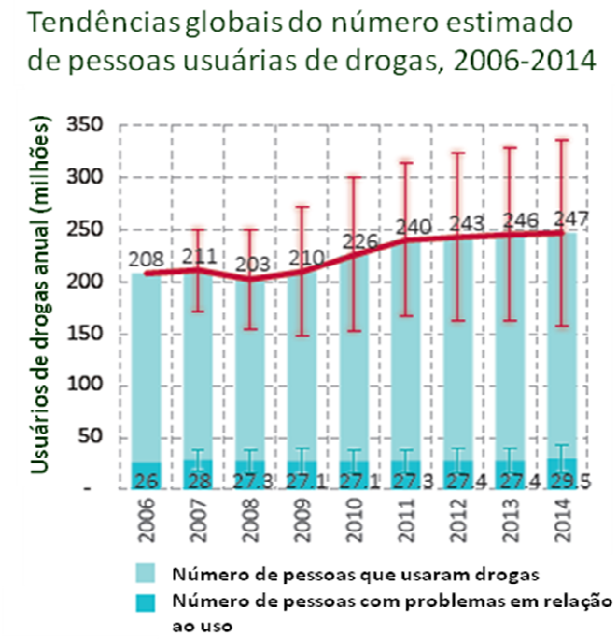

Figura 2: Estimativa do número de usuários/usuários problemático de drogas. As estimativas são para adultos (15-64 anos) com base no uso do ano passado. Fonte: respostas ao questionário do relatório anual (UNODC, 2016).

No Brasil, estima-se que haja 0,9 milhões de usuários de cocaína. O uso no país aumentou significativamente nos últimos anos, tendência esta que não é observada nos EUA e Europa, onde há uma estabilização e até queda no consumo e apreensões da droga. O levantamento domiciliar feito em 2005, que incluiu 7.939 pessoas com idade superior a 15 anos, moradores de cidades com mais de 200 mil habitantes, o uso relatado de cocaína ao longo da vida foi de 2,9% e de crack de 0,7% (GALDUROZ et al., 2005).

1.2 Fatores envolvidos no desenvolvimento da dependência química

A vulnerabilidade individual à dependência advém de interações complexas entre a droga, o indivíduo e um ambiente, o qual optamos por chamar aqui, de vulnerabilizante, ou seja, capaz de predispor o indivíduo ao uso de drogas (KREEK et al., 2005).

1.2.1 Influências ambientais

O termo “ambiente” inclui uma ampla variedade de fatores que vão desde as condições socioeconômicas da família, relacionamentos com seus pares e compreende a totalidade das experiências de vida pré e pós-natal que podem influenciar o cérebro e as respostas comportamentais. Uma revisão feita por Hopfer et al (2003) demonstrou alguns dos fatores não genéticos que influenciariam na suscetibilidade individual as drogas. O contexto social e ambiental afeta a vulnerabilidade ao abuso de drogas de pelo menos duas maneiras. Primeiro, o contexto social associado ao desenvolvimento da infância (por exemplo, o contexto da família, os relacionamentos entre pares) influencia a vulnerabilidade do abuso de drogas. Em segundo lugar, o contexto em que ocorre o uso de drogas também influencia os efeitos funcionais das drogas de abuso, impactando diretamente a aquisição de comportamentos de consumo de drogas e, portanto, a vulnerabilidade ao abuso e dependência.

Os fatores ambientais, como acesso a atividades extraclasses e sociais e um ambiente rico em estímulos serviriam como fatores de proteção ao uso e abuso de drogas bem como aumentariam as chances de sucesso durante a abstinência em adolescentes e adultos. Até certo ponto, experiências de vida positivas podem ser mimetizadas no laboratório por meio do enriquecimento ambiental (EA). Experimentos usando enriquecimento ambiental são capazes de alterar circuitos mesocorticolímbicos, sugerindo que essas experiências prévias inibem a ativação cerebral que motiva o comportamento de busca por cocaína (FRITZ et al., 2011).

1.2.2 Fatores individuais

É bem evidenciado que humanos dispõe de vastas diferenças individuais na suscetibilidade a dependência às drogas. Algumas pessoas ficam dependentes a partir de uma única exposição, enquanto outras podem ser resistentes, mesmo após várias exposições repetidas a altas doses de uma determinada substância (LICHTI et al., 2014).

Estudos que examinam a vulnerabilidade ao abuso de drogas a partir de uma concepção baseada na diferença genética entre indivíduos (isto é, estudos de herdabilidade) estabeleceram um papel importante no entendimento da dependência (KOOB; LE MOAL, 2008). A vulnerabilidade ao desenvolvimento da dependência de drogas e álcool varia com o grau de herança compartilhada (isto é, gêmeos idênticos têm taxas de concordância mais altas que os gêmeos fraternos, mesmo quando as influências ambientais compartilhadas são controladas) (BARDO; NEISEWANDER; KELLY, 2013). Estudos recentes de biologia molecular começaram a identificar quais genes contribuem para a vulnerabilidade ao abuso de drogas, bem como seus mecanismos de ação (KOOB; LE MOAL, 2008), estabelecendo as

influências genéticas sobre os processos neurobiológicos que medeiam a sensibilidade às drogas e, portanto, desempenhando um papel crítico na aquisição do comportamento e abuso de drogas (COMER et al., 2010). No entanto, é igualmente importante reconhecer o papel independente e interativo das influências ambientais sobre a vulnerabilidade ao abuso de drogas (SWENDSEN; LE MOAL, 2011). Desenvolvimentos recentes na epigenética, por exemplo, estabeleceram mecanismos pelos quais a experiência ambiental pode modificar a expressão genética (GODINO; JAYANTHI; CADET, 2015; MAZE; NESTLER, 2011).

Um dos fatores de diferença individual mais crítico, que prediz o uso de drogas entre os seres humanos, é a busca por novidades ou a busca por sensações (BARDO et al., 2013; KOSTEN; BALL; ROUNSAVILLE, 1994). A busca de sensações pode ser definida como um traço determinado pela busca por experiências variadas, novas, complexas e intensas e a disposição em assumir riscos físicos, sociais, legais e financeiros em prol dessas experiências (ZUCKERMAN, 1994). Estudos neurobiológicos indicam que o neurocircuito subjacente associado à busca por sensações envolve, pelo menos em parte, o núcleo accumbens (BARDO et al., 2013; HOLMES et al., 2016). A anfetamina produz uma maior liberação de DA no núcleo accumbens entre os indivíduos que possuem essa característica (LEYTON et al., 2002). Ao visualizar imagens altamente excitantes, os indivíduos com “alta sensibilidade” mostram ativação aumentada em regiões envolvidas na indução e recompensa emocional, bem como na redução da ativação de regiões envolvidas na regulação emocional (JOSEPH et al., 2009). Assim, os sistemas cerebrais alterados pela exposição a estímulos salientes compartilham uma ligação comum com o sistema de recompensa à drogas, o que pode mediar a associação entre a procura por sensações e o uso de drogas.

Outro fator de diferença individual que pode prover o uso de drogas é a impulsividade, definida amplamente como a tendência para engajar-se em comportamento prematuro, inapropriado ou desadaptado sem antecipação (DALLEY; EVERITT; ROBBINS, 2011). Embora a impulsividade seja um traço de base biológica amplamente definida, que aparece na maioria das principais teorias da personalidade, pode ser analisado em diferentes facetas (LYNAM; WIDIGER, 2001). Os indivíduos impulsivos, determinados usando critérios baseados na personalidade ou no desempenho em testes específico, iniciam o uso de drogas em idades mais precoces, progridem para uso pesado e transitam para abuso e dependência mais rapidamente, sendo também menos propensos a permanecerem abstinentes após o tratamento comparados com indivíduos não impulsivos (DALLEY et al., 2011; DICK et al., 2010). Assim, indivíduos com alto grau de impulsividade provavelmente se envolverão

em uma variedade de comportamentos de risco, incluindo o uso de drogas, e são mais sensíveis ao reforço e outros efeitos farmacodinâmicos das drogas após o uso inicial, tornando-os mais propensos a permanecer usando a droga (BARDO et al., 2013).

Paralelamente a alguns dos trabalhos descrito em seres humanos, não há dúvida de que o uso de drogas em animais de laboratório também envolve fatores individuais. As técnicas de reprodução seletiva, linhagens recombinantes, knock-out e silenciamento de genes são poderosas ferramentas para examinar a herdabilidade genética das diferenças individuais no uso de drogas (BARDO et al., 2013). Por exemplo, foram utilizados ratos para demonstrar que as diferenças individuais em resposta à novidade ou busca por novidade estão associadas a diferenças individuais na autoadministração de estimulantes (CUMMINGS et al., 2011; MEYER et al., 2010). No entanto, este trabalho prepara o cenário para o estudo dos mecanismos neurocomportamentais envolvidos nas principais diferenças individuais e influências sociais subjacentes à vulnerabilidade ao abuso de drogas. Coletivamente, esses resultados sugerem sobreposição dos mecanismos neurobiológicos subjacentes.

2 NEUROBIOLOGIA DA DEPENDÊNCIA À COCAÍNA

Farmacologicamente, a cocaína é classificada como um potente psicoestimulante, supressor do apetite e bloqueador de canais de sódio com voltagem não específica, o que, por sua vez, faz com que produza anestesia em doses baixas (DAWSON; MOFFATT, 2012). Esta droga geralmente ocorre em forma de um pó branco, mas pode ser encontrado como cristais sólidos chamados “crack”. O crack é a forma mais potente da cocaína, que é processada para formar um cristal ou “pedra” (também chamado "*freebase*") que pode ser fumado. Os cristais são aquecidos para produzir vapores que são absorvidos na corrente sanguínea através dos pulmões (ESTROFF, 2001; PEREIRA; ANDRADE; VALENTAO, 2015). Uma vez presente na corrente circulatória, ao atingir o cérebro, a cocaína é capaz de bloquear a recaptação de monoaminas no cérebro, principalmente noradrenalina e dopamina. Os primeiros sinais clínicos de toxicidade da cocaína são geralmente palpitações, sudorese, ansiedade, tremores, espasmo muscular e hiperventilação (NNADI et al., 2005). A facilitação da neurotransmissão noradrenérgica periférica produz os efeitos simpatomiméticos, como vasoconstrição, aumento da pressão arterial, excitabilidade cardíaca, hipertermia, arritmias e até convulsões. Pode-se seguir depressão bulbar com parada respiratória e colapso cardiovascular (WEINSHENKER; SCHROEDER, 2007). O quadro de euforia e o desejo por

novas experiências de uso podem ser atribuídos à ação da cocaína no bloqueio da recaptação de dopamina no sistema de recompensa do cérebro (KOOB; VOLKOW, 2016).

1.1 Efeito inicial: acumulo de dopamina no sistema de recompensa

Em geral, os psicoestimulantes são uma classe de drogas abusadas pelas sensações de euforia e bem-estar que produzem. A via dopaminérgica é o substrato fundamental do sistema de incentivo que produz saliência comportamental para estímulos reforçadores relevantes (VOLKOW et al., 2014).

A dopamina (DA) é sintetizada a partir do aminoácido tirosina, através da ação da tirosina hidroxilase. Este primeiro passo conduz à formação de 3,4-di-hidroxifenilalanina (DOPA). DA tem a sua origem na descarboxilação de DOPA. Depois disso, ele é armazenado nas vesículas de terminais pré-sinápticas para ser liberado na fenda sináptica após um estímulo neuronal. A DA é inativada por oxidação (catalisada pela enzima monoamina oxidase-MAO) e metilação (catalisada por catecol-O-metiltransferase-COMT), dando dois metabolitos principais, ácido 3,4-dihidroxifenilacético (DOPAC) e ácido homovanílico, respectivamente (figura 3). O uso crônico de cocaína parece levar à desregulação do sistema dopaminérgico cerebral (HOU et al., 2014). Embora a cocaína seja capaz de inibir a reabsorção de DA, de noradrenalina e serotonina, acredita-se que seu efeito poderoso resulte de suas ações no transportador de dopamina (DAT) nos terminais neuronais, causando um aumento da concentração e da intensidade de ação da DA nos receptores pós-sinápticos (GOWRISHANKAR; HAHN; BLAKELY, 2014; MARTIN et al., 2011). Além da ação em DAT a cocaína também interage com o transportador de monoamina vesicular 2 (VMAT-2) (Fig. 1), favorecendo o armazenamento de catecolaminas dentro de vesículas sinápticas (FLECKENSTEIN; VOLZ; HANSON, 2009). Esta ação desencadeia um aumento da quantidade de DA em cada vesícula antes da sua liberação, levando a uma alteração na proporção de DA citoplasmática para vesicular. Este efeito sobre o VMAT-2 contribui para um aumento adicional da DA sináptica, sobre um estímulo despolarizante (FLECKENSTEIN et al., 2009). Estes efeitos são decorrentes da potencialização da neurotransmissão dopaminérgica no circuito mesocorticolímbico. Seus mecanismos envolvem interações complexas entre estruturas telencefálicas corticais e subcorticais e projeções oriundas do

tronco encefálico. Tais estruturas estão envolvidas com as respostas reforçadoras naturais (BERRIDGE, 2006).

O núcleo accumbens ou especificamente, corpo estriado ventral, tem papel fundamental nos circuitos responsáveis por comportamentos orientados por objetivos (ROBINSON & BERRIDGE, 1993; DAFNY & YANG, 2006; ESPEJO, 2006). Esses comportamentos são gerados por projeções glutamatérgicas que se originam na amígdala basolateal, no hipocampo e no córtex pré-frontal, inervando os neurônios do estriado. A amígdala basolateral e o hipocampo seriam importantes para estabelecer associação entre estímulo, ambiente e o efeito reforçador da droga (NAKANO, 2000). O córtex pré-frontal é relacionado com o controle executivo do comportamento com base na avaliação da relação entre o valor do estímulo e o efeito esperado. Estes circuitos glutamatérgicos são regulados pela dopamina para determinar a intensidade da excitação dos neurônios GABAérgicos de projeção do núcleo accumbens (ROBSINSON & BERRIDGE, 1993; DAFNY & YANG, 2006; ESPEJO, 2006). Um dos principais alvos desta última projeção é o globo pálido ventral que, junto com outros núcleos, cerebelo e córtex, tem participação na coordenação dos movimentos (KALIVAS & HU, 2006). Desta forma, o núcleo accumbens funciona como uma interface entre os sistemas límbico e motor (GRAEFF, 2008). Este efeito sobre o sistema mesocorticolímbico, ainda que o efeito proeminente seja no sistema monoaminérgico, psicoestimulates, bem como a cocaína, são capazes de mobilizar também outros sistemas neurotransmissores, como o sistema GABAérgico e isso acontece independente do sexo (FREESE et al., 2012; SOUZA et al., 2014). A partir disso, poder-se-ia inferir que a neurotoxicidade da cocaína implica em diversos sistemas neurotransmissores.

1.2 A neurotoxicidade da cocaína

O mecanismo pelo qual a cocaína causa danos neurológicos é complexo e envolve interações da droga com vários sistemas neurotransmissores, mas principalmente pelo aumento dos níveis extracelulares de dopamina e radicais livres e modulação de fatores de transcrição. Assim, pode-se dizer que os efeitos neurotóxicos da cocaína estão fortemente ligados às concentrações excessivas de dopamina, mecanismo esse têm sido associado cada vez mais à indução de danos de DNA nas células do SNC (PEREIRA et al., 2015). Concentrações excessivas de DA podem ser neurotóxicas e as catecolaminas demonstraram causar morte neuronal em culturas de tecidos (GANDHI et al., 2012). Em estudo prévio de

nosso laboratório (DE SOUZA et al., 2014), mostramos que a cocaína aumentou o dano do DNA em diferentes áreas cerebrais de ratas. Além disso, Alvarenga et al demonstraram que uma única exposição à cocaína pode induzir danos genéticos em múltiplos órgãos de roedores (ALVARENGA et al., 2010; ALVARENGA et al., 2011).

A toxicidade DA é devida à formação de espécies reativas de oxigênio (ROS) resultantes do seu metabolismo (por auto-oxidação ou ação da MAO), que, por sua vez, leva ao estresse oxidativo (BANERJEE et al., 2014) e posteriormente, a apoptose. Conforme referido acima, a MAO catalisa a conversão de DA em DOPAC e peróxido de hidrogênio (H_2O_2), o qual, através da reação de Haber-Weiss/Fenton, pode reagir com íons de transição, originando o radical hidroxila altamente tóxico ($\cdot OH$) (CAUDLE et al., 2007). Por outro lado, a auto-oxidação da DA pode ocorrer no meio extracelular, levando à geração de superóxido ($O_2^{\bullet -}$) e quinonas tóxicas (BANERJEE et al., 2014). Além disso, $O_2^{\bullet -}$ reage com o óxido nítrico, um produto da oxidação da arginina, formando o peroxinitrito altamente tóxico (CADET; BRANNOCK, 1998; MURIACH et al., 2010) no hipocampo após uma injeção intraperitoneal diária de cocaína (15 mg/kg), durante 20 dias, desencadeando a formação de mais produtos de oxidação. Estes produtos podem danificar lipídios, proteínas e DNA, afetando a sobrevivência celular (SURENDRAN; RAJASANKAR, 2010). Outros estudos também demonstraram que a exposição à cocaína induziu a alterações nas enzimas antioxidantes, como diminuição da atividade da catalase (CAT) (MACEDO et al., 2005) bem como um aumento das atividades de superóxido dismutase (SOD) e glutationa peroxidase (DIETRICH et al., 2005) no córtex e no estriado. Superóxido dismutase (SOD) e catalase (CAT) são enzimas importantes para a proteção do tecido cerebral do estresse oxidativo. O $O_2^{\bullet -}$ é um ROS primário formado nas células e é convertido enzimaticamente em peróxido de hidrogênio (H_2O_2) por SOD, que pode reagir com íons livres para produzir radicais hidroxila altamente reativos e tóxicos. Subsequentemente, o peróxido reativo (H_2O_2) pode ser destruído por reações de CAT ou glutationa peroxidase (JANG et al., 2015).

Além disso, a neurotoxicidade induzida pela cocaína pode também ser mediada pela libertação descontrolada de glutamato (Glu) (PEREIRA, 2015). Isso desencadeia uma sobre-ativação de receptores Glu, levando a uma excitação excessiva de neurônios e a um aumento de Ca^{2+} intracelular, que estimula o estresse do retículo endoplasmático, podendo induzir morte neuronal (LAU; TYMIANSKI, 2010).

Outra via possível para a apoptose induzida pela cocaína foi demonstrada por Ahn et al. (2007), que observaram que a administração aguda ou repetida de cocaína aumentou a

expressão de caspase-12, um mediador crítico de morte celular, no estriado dorsal de rato. Sabe-se também que o estresse no retículo endoplasmático aumenta a expressão de caspase-12 em neurónios e células gliais (SHIMOKE et al., 2004). Em conjunto, estes achados sugerem que a cocaína está intimamente relacionada com o aumento da libertação de Glu, levando à indução de proteínas de stress no retículo endoplasmático, contribuindo para a apoptose das células neuronais (PEREIRA, 2015).

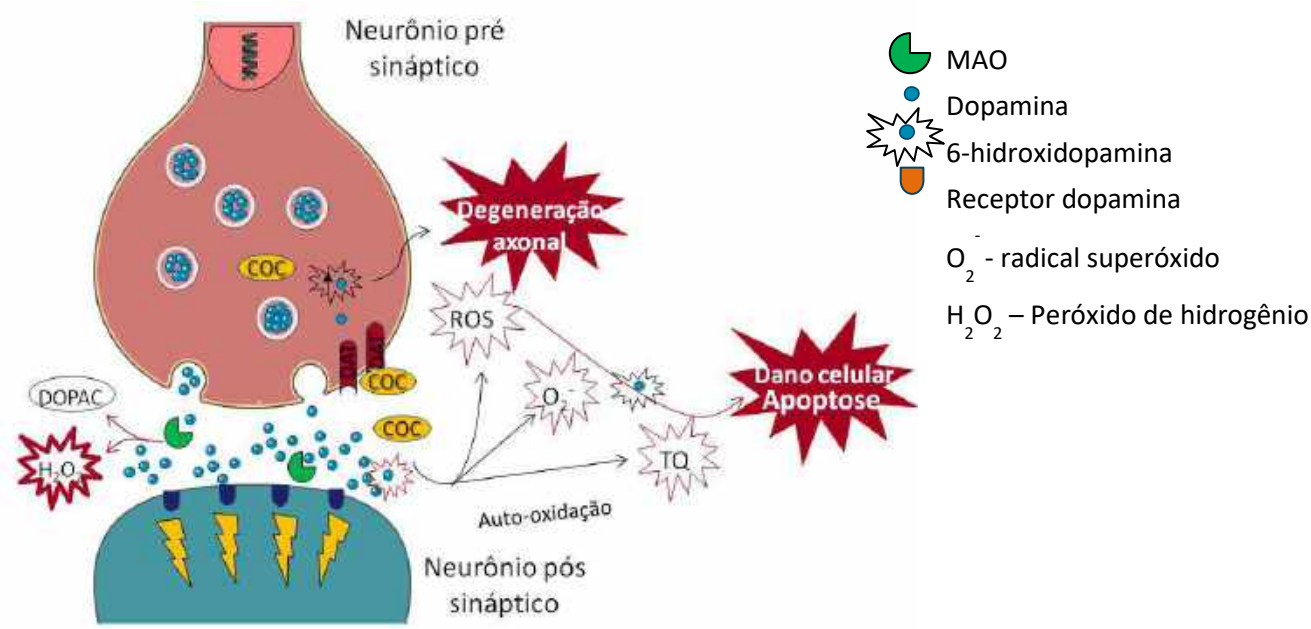

Figura 3: Mecanismo de indução ao estresse oxidativo da cocaína no sistema dopaminérgico.MAO= monoaminaoxidase; COC= cocaína; DAT= Transportador de dopamina; DOPAC= 3,4-dihidroxifenilacético.

3 MODELOS ANIMAIS

Durante as últimas cinco décadas, os estudos sobre drogas em modelos animais por meio de modelos de autoadministração, condicionamento de preferência de lugar e auto-estimulação intracraniana resultaram em uma grande quantidade de dados acerca dos substratos neurais do sistema de recompensa e reforço ligados ao uso de drogas (KOOB, 2009; KOOB; LE MOAL, 2005; LYNCH et al., 2006; O'CONNOR et al., 2011). O reconhecimento desse fato tem inspirado o surgimento de ainda mais pesquisas na última década ou duas, em que os pesquisadores tentaram (e, como desejamos argumentar, conseguiram um grau considerável) capturar aspectos genuínos do comportamento da dependência usando animais de laboratório (AHMED, S. H.; KOOB, 2005; VANDERSCHUREN; EVERITT, 2004).

3.1 Modelos animais em dependência química

Por muito tempo, a comunidade científica pensou que a dependência a drogas era exclusivamente um fenômeno humano que não podia ser reproduzido e modelado em animais (SPRAGG, 1940). Contudo, as limitações inerentes à testagens em seres humanos levaram ao desenvolvimento de modelos animais de dependência.

Como vamos ver em detalhe, os primeiros modelos animais concentraram-se essencialmente em medir os efeitos de recompensa das drogas.

3.1.1 Autoadministração

O modelo de autoadministração de drogas é apresentado como um dos melhores para representar, em laboratório, os mecanismos reforçadores da dependência química. Sem dúvida, este modelo possui boa aplicação e validades preditivas aceitáveis para o entendimento de aspectos comportamentais e neurobiológicos do uso de drogas em seres humanos.

Este modelo consiste em expor o animal à possibilidade de se autoadministrar uma dose da droga, controlando a frequência e quantidade ingerida (oral) ou injetada (intravenosa). Na Autoadministração por Condicionamento Operante utiliza-se uma caixa com duas barras, uma das quais libera a droga e está pareada com algum estímulo luminoso ou sonoro (Figura 4). A outra barra deve estar pareada com um estimulo diferente da barra “correta”. Para a fase de condicionamento, pode-se usar algum alimento palatável ou deixar os animais em restrição de alimento ou água antes do condicionamento, dando-lhes o alimento ou água em troca do bater na barra correta. Existem ainda protocolos chamados de *autoshaping*, onde o animal é condicionado com a própria droga ao invés do uso de alimentos. Está bem estabelecido na literatura que animais se autoadministram psicoestimulantes como cocaína, anfetamínicos e nicotina, etanol, opiáceos, benzodiazepínicos, entre outras substâncias. Quanto maior o potencial de reforço da substância, mais o animal irá “trabalhar”, batendo na barra, para obter a dose da droga.

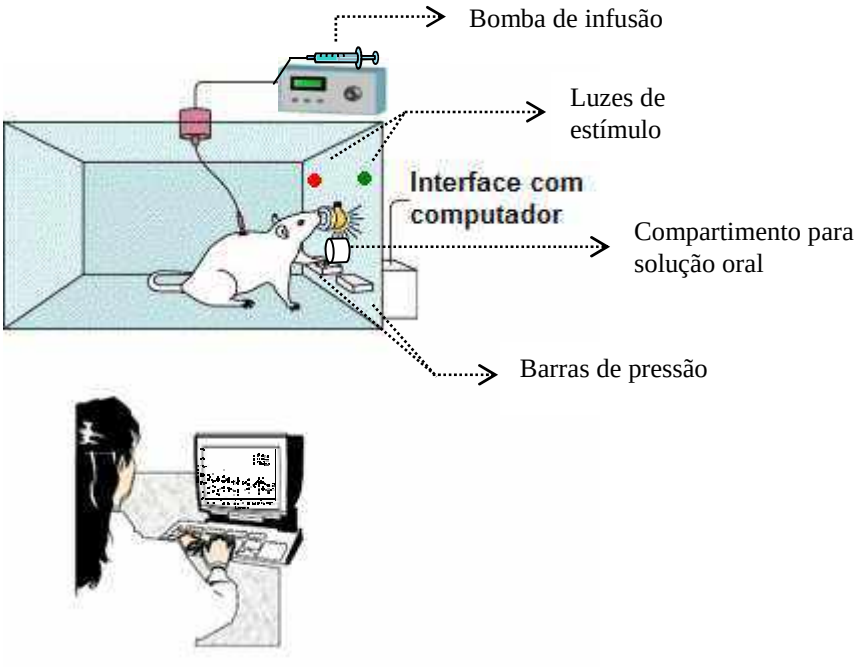

Figura 4. Equipamento de autoadministração. Caixa: Disposição da barra de pressão, das luzes de estímulo, do compartimento para solução oral e da bomba de infusão para controle e velocidade de infusão das doses intravenosas. Monitoramento e controle por meio de software acoplado ao sistema Adaptado de Cryan e cols. (CRYAN et al., 2003).

Quanto às vias de administração, no início, a autoadministração de drogas em animais era feita exclusivamente por meio da via oral. Desenvolvido principalmente para o estudo da dependência do álcool, esta via de administração foi estendido para muitas substâncias de abuso, tais como benzodiazepínicos, opióides, estimulantes, barbitúricos e anestésicos dissociativos. No entanto, quando muito distante dos modos de administrações encontrados em humanos, a via oral não é provavelmente o modelo mais adequado para o estudo de todas as drogas. Foi neste contexto de modelação melhorada da adição humana que a autoadministração i.v. foi considerada e desenvolvida. A técnica, no entanto, implica na realização de uma delicada cirurgia de canulação do animal, onde uma cânula é inserida da veia jugular e atravessando, sob a pele, para sair nas costas ou na cabeça do animal, onde ele não tenha como acessar e roer (figura 4).

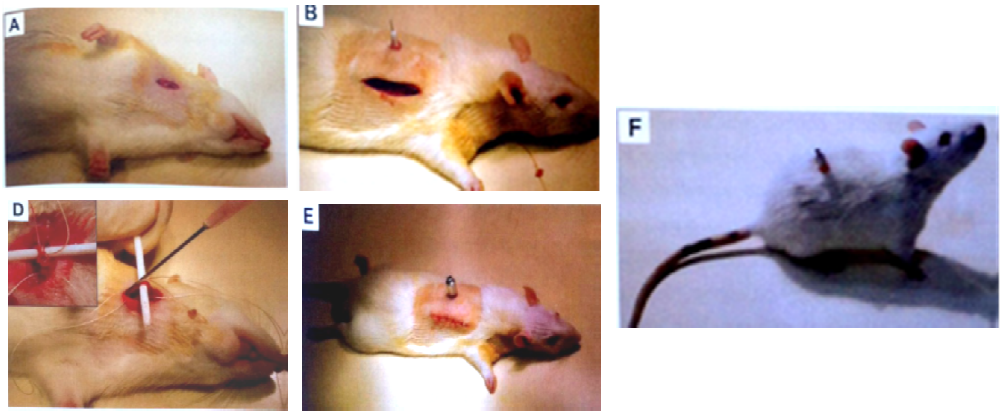

Figura 5: Sequência da cirurgia de canulação da veia jugular para experimento de autoadministração. (Fotos provenientes de arquivos do Instituto de doenças neurodegenerativas - Universidade Victor Segalen, Bordeaux- França).

No início dos anos 1960, os investigadores demonstraram pela primeira vez que o rato de laboratório (WEEKS, 1962) e macacos (THOMPSON; SCHUSTER, 1964) eram capazes de se autoadministrar morfina, uma substância altamente viciante em seres humanos, por via intravenosa. Subsequentemente, numerosos estudos demonstraram que os animais de laboratório podem autoadministrar drogas mais consumidas em seres humanos, e, em geral, as drogas mais autoadministradas são aquelas que têm um elevado potencial de abuso (SCHUSTER; THOMPSON, 1969). Esta relação é tão forte que a autoadministração de drogas foi considerada um modelo preditivo da potência aditiva de uma substância de abuso (COLLINS et al., 1984) e foi sugerido que os ensaios pré-clínicos poderiam ser usados para a detecção do potencial de causar dependência de novas substâncias (JOHANSON; BALSTER, 1978).

O procedimento de autoadministração pode ser realizado com diferentes regimes de reforços. No procedimento de razão fixa (FR), que é um procedimento de reforço contínuo, o fármaco é administrado após o animal ter completado um número fixo de respostas necessárias estipuladas pelo experimentador. Por exemplo, se o animal precisar pressionar duas vezes a barra ou colocar o nariz duas vezes no buraco para uma infusão de drogas, falamos de FR2. Portanto, o número de infusões de droga obtido pelo animal é diretamente proporcional ao número de ações que o animal realiza, em outras palavras, o quanto ele trabalha pra receber a droga. Os resultados obtidos com este plano de construção mostra que os animais desenvolvem uma frequência muito estável de comportamento, obtendo injeções regulares de uma sessão para outra (JOHANSON; SCHUSTER, 1975; LYNCH; CARROLL,

2001; YOKEL; PICKENS, 1973). Além disso, a taxa de injeção (número de infusões obtidas por hora) é fortemente influenciada pela concentração da droga, com injeções menos frequentes conforme a concentração é elevada (GERBER; WISE, 1989). Resultados semelhantes foram também mais recentemente observados em indivíduos dependentes de cocaína (LYNCH et al., 2006; SUGHONDHABIROM et al., 2005), indicando que o animal, tal como o homem, regula a sua ingestão, para manter seus efeitos de recompensa em um nível ideal.

3.1.2 Condicionamento de preferência de lugar

Em 1954, Olds e Milner estiveram entre os primeiros autores que demonstraram a existência do fenômeno de preferência de lugar no animal, quando observaram que os ratos estimulados por um eletrodo intracraniano voltavam ao lugar onde haviam recebido esta estimulação (OLDS; MILNER, 1954). O condicionamento de preferência de lugar (CPP) foi extensivamente utilizado para demonstrar a existência dos efeitos recompensadores (bem como dos efeitos aversivos) das drogas de abuso. O CPP é um procedimento de condicionamento essencialmente do tipo Pavloviano (com um componente Skinneriano) que permite avaliar a intensidade da lembrança do valor hedônico que os efeitos de uma substância deixam ao animal.

Classicamente, os animais são colocados em uma caixa que comporta dois compartimentos principais, que devem distinguir-se entre si pela textura do solo e pelas cores e motivos das paredes; e mais um compartimento neutro, menor, também com cor e textura do solo diferente das demais (Figura 6). A experiência desenrola-se em três fases: 1) Pré-condicionamento; fase durante a qual o animal pode entrar cada um dos ambientes e explorá-los à sua vontade. Esta fase serve de controle a fim de verificar se existe uma preferência espontânea do animal para o um dos dois compartimentos principais. 2) Condicionamento; onde o animal é confinado só um em compartimento, de maneira alternada, no qual recebe uma injeção passiva de droga em um lado e uma solução salina para o outro. Esta fase permite assim associar os efeitos da droga a um ambiente específico. 3) Teste ou pós-condicionamento; fase onde o animal é reintroduzido na caixa acesso livre a todos os compartimentos (e sem receber nenhuma injeção). Se o animal passa então mais tempo no compartimento associado à injeção passiva de droga (e exprime assim, uma preferência de lugar condicionada), a droga é considerada como uma recompensa para o animal (reforço

positivo). Pelo contrário, a substância será aversiva (e o animal exprimirá uma aversão de lugar condicionada) quando há uma evitação ao compartimento associado à droga.

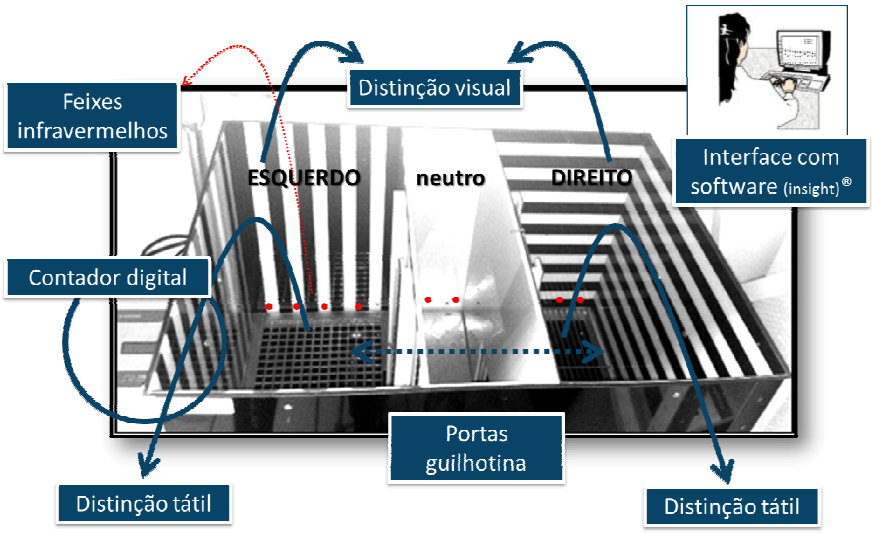

Figura 6. Esquema representativo do equipamento de CPP. CPP = Condicionamento de Preferência de Lugar. Laboratório de Neuropsicopedagogia – UFCSPA.

Este protocolo experimental é amplamente utilizado, em especial, devido às suas numerosas vantagens práticas. Em primeiro lugar, não necessita um equipamento complexo e dispendioso, contrariamente autoadministração. Além disso, a ausência de cirurgia permite manter os animais em boa saúde por longos períodos, muito tempo e assim testá-los muito tempo após a fase de condicionamento, permitindo assim observar se os animais continuam preferindo o compartimento associados à droga vários meses após a sua administração, revelando a intensidade dos efeitos de memória deixados pela droga.

No entanto, este modelo apresenta limitações em sua capacidade de modelar o fenômeno da adição. O modo de administração passivo da droga faz que o animal não seja livre, distanciando-se assim, da fenomenologia humana. Além disso, este teste constitui em certa maneira um teste “do tudo ou nada” na medida em que não permite uma avaliação do consumo bem como mensurar um efeito individual à dose. No entanto, parece eficaz para mostrar um efeito de memória.

3.1.3 Enriquecimento ambiental

A fim de melhorar o modelo de adição em animais, uma das primeiras questões que se levanta é se as condições de vida utilizados no laboratório não são muito diferentes das da vida real. Em outras palavras, o rato de laboratório, sob condições muito restritas, é realmente representante do rato em geral? Será que nossas condições de vida, tão particulares, tanto no que se refere aos aspectos positivos quanto aos negativos da diversidade que nos acompanha em nossas cidades e contextos, conseguem ser consideradas por pesquisadores que buscam justamente explicar e melhorar a condição humana?

Em verdade, a atmosfera utilizada no laboratório é muito limitada. O rato de laboratório é confinado em uma gaiola de tamanho relativamente pequeno, sem distrações disponíveis e, muitas vezes, também socialmente isolados de seus pares, sendo ele um animal muito sociável e explorador. Sob essas condições, não estaria o investigador afastado antes de iniciar a sua experiência do seu objetivo inicial, ou seja, de modelar o fenômeno humano, tanto quanto possível?

Uma forma de mimetizar em laboratório experiências positivas de vida é o estímulo social, cognitivo e físico, usando para tanto manipulações ambientais. A sinergia entre elementos interação social, prática de atividade física e acesso contínuo a elementos que possibilitem exploração e aprendizagem é definida como enriquecimento ambiental (EA) (ROSENZWEIG et al., 1978). Para os roedores, o EA consiste na criação de animais em uma gaiola grande com a disposição de vários objetos (escadas, casas abrigo, canos, rampas) e objetos para interação com diferentes texturas, onde cerca de 6 a 10 animais são alojados em conjunto. Estes objetos devem ser constantemente realocados para possibilitar que a memória dos animais seja estimulada, bem como a curiosidade e exploração (LAVIOLA et al., 2008; NITHIANANTHARAJAH; HANNAN, 2006; VAN PRAAG; KEMPERMANN; GAGE, 2000) (Figura 7). Na maioria dos casos, essas condições ambientais são fornecidas como condições de habitação, ou seja, animais vivem nestes ambientes a maioria ou a totalidade do seu tempo diário (LAVIOLA et al., 2008).

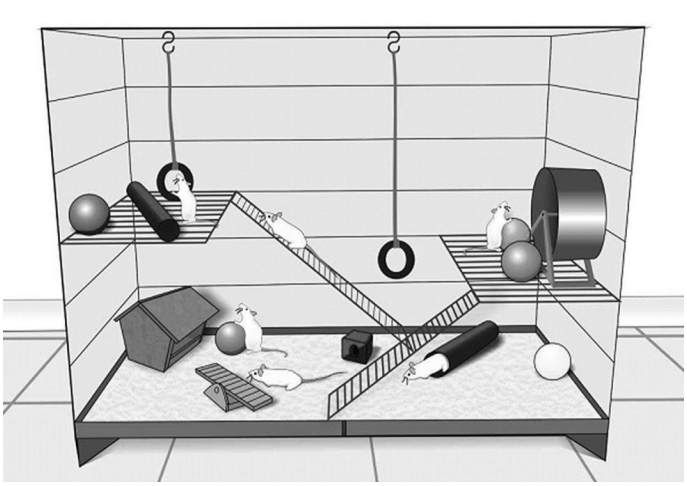

Figura 7. Modelo de enriquecimento ambiental. Condições de moradia dos animais do grupo AE. Caixa de ambiente enriquecido com disposição de objetos para estimulo visual, exploratório e de atividade (adaptado de DOBROSSY & DUNNET, 2001).

Os primeiros trabalhos sobre o efeito do AE nas funções cognitivas de ratos se volta ao início dos anos cinquenta (BINGHAM; GRIFFITHS, 1952; FORGAYS; FORGAYS, 1952). Os resultados favoreciam a ideia de que os ambientes “estimulantes” diferenciavam os animais com relação às capacidades cognitivas daqueles criados em ambiente padrão, com um alto grau de significância estatística. As diferenças eram interpretadas como sendo a oportunidade de aprendizagem durante os primeiros anos de vida determinante para as respostas comportamentais do indivíduo na vida adulta.

Entre outros estudos pioneiros, David Krech, Mark R. Rosenzweig e Edward L. Bennet foram provavelmente os primeiros a estabelecer um protocolo de AE experimental semelhante ao usado hoje, onde os resultados estavam relacionados às mudanças cerebrais induzidas por este modelo (KRECH; ROSENZWEIG; BENNETT, 1960; ROSENZWEIG, 1966; ROSENZWEIG et al., 1962). Nestes estudos, os animais eram divididos em três grandes grupos; um grupo IC (condição de isolamento social) que vivia em condições de empobrecimento e isolamento social e um grupo AE (enriquecimento ambiental). Estes trabalhos levaram à descoberta de que o enriquecimento ambiental era capaz de aumentar o volume do córtex cerebral e, em seguida, verificou-se que este aumento era devido ao maior espessamento do córtex cerebral, maior número de sinapses e de células gliais. Em continuidade aos achados, os autores continuaram demonstrando que os animais criados em

EA apresentavam maior peso, mais atividade enzimática e maior volume em diversas estruturas cerebrais (ROSENZWEIG, 1966).

As sólidas constatações acerca dos benefícios advindos da criação em um ambiente rico em estímulos tiveram um de seus pontos altos no final dos anos de 1970 quando se realizou uma ligação destes benefícios ao o uso de drogas. Um artigo, publicado em 1981 pelo pesquisador Bruce K. Alexander apresentou resultados de experimentos onde os cientistas criaram o que foi chamado de “park rat”, um ambiente com dimensões grandes e com ratos vivendo no que se poderia chamar de uma comunidade complexa. Na idade adulta, ofereceram morfina a estes animais, comparando o consumo ao de animais criados em isolamento. Os ratos criados nas colônias consumiram significativamente menos morfina que os controles. Estes resultados foram vistos com grande entusiasmo e o motivo foi no sentido de que o uso de drogas estaria mais intimamente ligado a questões sociais que individuais (ALEXANDER; COAMBS; HADAWAY, 1978). No entanto, o estudo que foi recusado previamente por revistas como Nature e Neuroscience, pois havia duvidas metodológicas que invalidavam suas descobertas. Os resultados deste trabalho nunca conseguiu ser reproduzido por outros grupos.

Numerosos estudos continuam demonstrando uma série de efeitos positivos do AE sobre várias doenças neurológicas e psiquiátricas (FARES et al., 2013; LAVIOLA et al., 2008; VAN PRAAG et al., 2000). Postula-se que o AE crie uma reserva cognitiva que neutraliza ou compensa os déficits associados a processos neurodegenerativos (NITHIANANTHARAJAH & HANNAN, 2009). O ambiente enriquecido é capaz de induzir mudanças bioquímicas, morfológicas e funcionais no cérebro, fazendo com que haja uma melhor resposta ao estresse na vida adulta (DE CARVALHO et al, 2001; PAMPLONA et al, 2009; VAN PRAAG et al, 2000).

Relativo à adição, um acúmulo considerável de evidências, conforme revisado por SOLINAS e cols. (2010) indica que experiências positivas de vida serviriam como um importante fator de proteção para o uso de drogas. Existe atualmente a concepção de que o AE funcionaria como um estressor funcional, podendo reduzir a força dos efeitos reforçadores das drogas (SOLINAS et al., 2010). Por outro lado, a exposição a formas negativas de estresse predispõe a uma maior vulnerabilidade a dependência química (AMBROGGI et al., 2009; GOEDERS, 2002; MAHONEY et al., 2013; SINHA et al., 2000). Assim, antes de se chegar a resultados mais conclusivos, primeiro foi preciso questionar a comparação com animais em isolamento, que poderia significar um viés importante nos achados, pois o

isolamento em animais sociais pode ser considerado um fator significativo de estresse (VALZELLI, 1973; YORGASON et al., 2016). Com a possibilidade da realização de avaliações neuroendócrinas foi possível constatar que tanto as ondas eletroencefalográficas quanto à secreção de hormônios indicadores de estresse estavam modificados em condições de isolamento (IERACI; MALLEI; POPOLI, 2016; MUCIGNAT-CARETTA et al., 2014; YAMADA et al., 2015). Ainda, Cheeta et al. (2001) realizaram avaliação farmacológica da ação da nicotina em ratos submetidos ao isolamento e verificaram elevação nas respostas de ansiedade, demonstrando potencialização da ação da nicotina nos animais pertencentes aos grupos de privação social.

Uma forma mais eficaz de entender a influência do AE sobre a vulnerabilidade a dependência seria a comparação com um grupo de animais criados em grupo, ou seja, em ambiente padrão, mas sem estímulos. Basicamente, diversos estudos apoiam a ideia de que um AS seria uma situação intermediária entre o isolamento social e o ambiente enriquecido (BARDO et al., 2001; GREEN et al., 2010; MELENDEZ et al., 2004; SOLINAS et al., 2010; THIEL et al., 2009). Em um estudo realizado com ratos criados em ambiente social (AS) e outro em AE verificou que os animais do segundo grupo autoadministravam cocaína significativamente menos que o grupo AS (RANALDI et al., 2011). Em consonância com estas observações, Zakharova et al. demonstraram que, utilizando um ambiente de controle padrão como controle, os ratos criados num ambiente enriquecido eram menos sensíveis aos efeitos psicomotores da cocaína (ie, a sua atividade locomotora após a injeção de cocaína era mais baixa) (ZAKHAROVA et al., 2012). Todos estes resultados indicam que o AE pode ter um efeito preventivo no desenvolvimento da dependência de um fármaco sem o viés do isolamento social.

Além de interferir no processo de aquisição da dependência, também tem sido demonstrado que o AE auxilia nos processos relacionados a comportamentos de busca pela droga, reduzindo os riscos de recaída em estudos para manutenção da abstinência (HAJHEIDARI; MILADI-GORJI; BIGDELI, 2015; PECK et al., 2015; SOLINAS et al., 2008; STAIRS; BARDO, 2009). Estes efeitos preventivos e até benéficos do AE estão associados com importantes mudanças plásticas no cérebro de várias áreas do sistema mesocorticolímbico, tais como o hipocampo, córtex e estriado, que também tem ligação importante com o estabelecimento da dependência a drogas (VAN PRAAG et al., 2000). O AE altera sistemas neurotransmissores, produzindo também alterações na expressão de genes e fatores de transcrição e, por fim, estimulando a neurogênese (KUZUMAKI et al., 2010).

Os efeitos neuroprotetores do AE entendem ainda para a capacidade de melhorar a as respostas frente a processos relacionados ao estresse oxidativo ROS, por exemplo, diminuindo a formação de espécies reativas ao ácido tiobarbitúrico (TBARS) no hipocampo (CECHETTI et al., 2012) e córtex de ratos (MARMOL et al., 2015). Curiosamente, um estudo recente mostrou que quando comparado ao resveratrol, um antioxidante reconhecido, o AE promove uma diminuição mais proeminente na concentração de malondialdeído, outro marcador para o estresse oxidativo (MUHAMMAD et al., 2016). A diminuição nos níveis de ROS sugere que os radicais livres foram removidos com sucesso por antioxidantes endógenos, que são estimulados pela AE (MARMOL et al., 2015). Estudos demonstram que o AE aumenta as células neurais recém-geradas no cérebro adulto, sugerindo que essa estimulação pode aumentar a capacidade de plasticidade e auto-reparação cerebral (MUHAMMAD et al., 2016; VAN PRAAG et al., 2000). A AE também parece possuir propriedades antioxidantes atenuando a peroxidação lipídica (MUHAMMAD et al., 2016) e ativando e regulando enzimas antioxidantes (MATTSON et al., 2001).

3.1.4 Modelos de Escolha

Um dos aspectos mais intrigantes da dependência é que os indivíduos dependentes tendem a se comportar contra os seus maiores interesses e melhores julgamentos anteriores ao uso da droga (BECHARA, 2005; HEYMAN, GENE M., 2009; PAULUS, 2007). Eles procuram usar a droga em detrimento de outras atividades ou ocupações que antes lhes pareciam gratificantes e prazerosas (APA, 2013). Assim, a dependência a cocaína é uma das que mais rapidamente leva a uma negligência progressiva de comportamentos alternativos em favor da busca e consumo da droga, o que resulta em custos altos (por exemplo, problemas escolares/acadêmicos, ocupacionais, nas relações familiares e conjugais, sanções legais, etc.). Normalmente, estes custos deveriam motivar a abstinência do uso. Desta forma, entende-se que este comportamento reflete uma possível perda de capacidade em fazer escolhas livres, racionais e voluntárias. Um dos desafios mais críticos para a o estudo dos aspectos neurobiológicos da dependência à cocaína é identificar estas disfunções que levam a aparente perda de capacidade de julgamento de ações (AHMED, S. H., 2012; AHMED, S. H.; LENOIR; GUILLEM, 2013; KOOB, 2006).

A extensão da validade do modelo de autoadministração não é completamente extrapolada porque o ambiente em que os ratos são testados é desprovido de outras oportunidades, o que contrasta com a riqueza de possibilidades do mundo real (AHMED, S.

H. et al., 2013). Em outras palavras, embora não nulo, o grau de liberdade dos animais em uma caixa de autoadministração é bastante restrito. Diante desse um baixo grau de liberdade, talvez, não seja de se admirar que a maioria dos ratos se autoadministrem as drogas que os seres humanos abusam. O grande desafio para a pesquisa experimental em dependência química é determinar, entre os ratos que se autoadministram cocaína, por exemplo, quais e quantos podem ser considerados dependentes e quais e quantos usam cocaína apenas pela sua disponibilidade num ambiente que não oferece nenhum outro estímulo instigante (AHMED, S. H., 2010).

Assim sendo, existem sérias dúvidas sobre a interpretação de uso de drogas em animais. É sintomático de um estado de dependência subjacente ou meramente uma resposta previsível à falta de escolha? Esta incerteza, por sua vez, lança uma sombra sobre muitas mudanças comportamentais e neurobiológicas documentadas até então. Será que eles refletem disfunções patológicas ou adaptações neurobiológicas normais para experiências e comportamentos gratificantes?

Amplas evidências demonstram que o ajuste de escolha pode influenciar de forma dramática o uso de drogas, tanto em humanos como em animais não humanos (AHMED, S. H., 2010; AHMED, S. H.; KOOB, 2005; ALEXANDER; HADAWAY, 1982; BADIANI et al., 2011). Esta influência é particularmente bem exemplificada em ratos de laboratório que têm acesso à autoadministração intravenosa de cocaína em diferentes contextos. Um série de evidências demonstram que, quando surge uma alternativa interessante "*nondrug*" (por exemplo, água potável adoçada), a grande maioria dos ratos imediatamente cessa o consumo de cocaína (AHMED, S. H.; KOOB, 2005; AHMED, S. H. et al., 2013; LENOIR; AHMED, 2007; LENOIR et al., 2007; VANDAELE et al., 2016). E não importa o quão forte e quão longo é o consumo, apenas uma minoria dos animais (cerca de 10%) continua a se autoadministrar cocaína quando tem a oportunidade de fazer outra escolha valiosa (AHMED, S. H., 2012).

Em uma ordem cronológica de saberes, é notório que quando os ratos têm acesso à cocaína, sem outra escolha, a maioria deles aprende prontamente a autoadministrar a droga e, na grande maioria das vezes, aumentam a sua ingestão com acesso estendido (de 6 horas ou mais dentro da caixa de autoadministração) (AHMED, SERGE H., 2011; OLMSTEAD, 2011). Em contraste, quando estes mesmo animais têm uma escolha entre a cocaína e uma solução de sacarina, por exemplo, a maioria que autoadministrava a droga e aumentava a sua ingestão em um ambiente de não-escolha diminui e até mesmo cessa completamente o uso

droga em favor da opção de *nondrug* (CANTIN et al., 2010; LENOIR et al., 2007; TUNSTALL; KEARNS, 2014). Este comportamento, tipo abstinência, também foi observado com outras drogas de abuso, incluindo metanfetaminas, heroína e nicotina (CAPRIOLI et al., 2015; HUYNH et al., 2015; LENOIR et al., 2013). É geralmente robusto para uma variedade de condições experimentais diferentes (por exemplo, doses da droga) e história prévia de exposição a drogas (AUGIER; VOUILLAC; AHMED, 2012; MADSEN; AHMED, 2015).

Na versão padrão do procedimento de escolha, os ratos devem enfrentar uma escolha diária entre dois comportamentos ou ações gratificantes: pressionar uma alavanca para receber uma dose de cocaína i.v. ou pressionar uma segunda barra para ter acesso a uma alternativa "nondrug" potente (AHMED, 2005; LENOIR et al, 2007) (Figura 8). Embora se possa vislumbrar uma variedade de possíveis recompensas *nondrug* para ratos, optou-se pelo acesso à água doce (ou seja, adoçado com uma concentração ótima de sacarose [0,2 %]) (VENDRUSCOLO et al., 2010).

O açúcar, bem como os alimentos doces, também pode alterar a atividade cerebral através da estimulação de células especializadas, capazes de reconhecer o sabor doce na boca e no intestino e através de mecanismos pós-absortivos cerebrais envolvendo sinalização de glicose (GRAYSON; SEELEY; SANDOVAL, 2013). Evidências demonstram que o açúcar e recompensas doces são muito menos potentes do que a cocaína para aumentar a sinalização de dopamina cerebral. No entanto, esta aparente discrepância com relação À escolha dos animais entre estas duas recompensas também pode sugerir que a dopamina provavelmente não é suficiente para conduzir a preferência e que o açúcar a preferência por açúcar envolve mais do que a elevação da dopamina cerebral (LENOIR et al, 2007; (DILEONE; TAYLOR; PICCIOTTO, 2012) Esta interpretação é apoiada por pesquisas recentes usando métodos optogenéticos em camundongos. Os ratos foram autorizados a escolher entre dois lados: de um lado, lambendo um bebedor onde era entregue água e uma estimulação optogenética de neurônios dopaminérgicos; do outro lado, quando lambiam o outro bebedor era entregue água adoçada com sacarose. Quando as concentrações eram suficientemente altas, os camundongos preferiam a sacarose à estimulação optogenética dos neurônios dopaminérgicos (DOMINGOS et al., 2011). Importantemente, os substratos neurais envolvidos com a recompensa à sacarose envolvem rotas muito mais naturais do que as de drogas de abuso e são claramente não tão comportamental, psicologicamente e / ou neuroquimicamente tóxicas (AHMED et al., 2013; PEREIRA et al., 2015).

Inicialmente, o protocolo de escolha era composto por duas sessões sucessivas: a) Amostragem – quatro ensaios alternando cocaína e sacarose, onde apenas as respectivas

barras eram disponibilizadas, uma de cada vez. b) Escolha – As duas barras ficavam disponíveis. Era realizado no mínimo oito ensaios por sessão (máximo de 90 minutos). Todos os ensaios eram separados por um intervalo inter-ensaio fixo (ITI, do inglês – *inter trial interval*, geralmente 10 minutos). Em geral, as latências de resposta são curtas (menores que 5 minutos) (LENOIR et al., 2007).

As sequencias de estudos que surgiram após estes primeiros trabalhos procuraram demonstrar o efeito da escolha quando o rato já havia sido exposto à cocaína e apresentava tolerância ao efeito, demonstrado pelo escalonamento no número de injeções de cocaína. Surpreendentemente, mesmo após a pré exposição crônica à cocaína, sendo ofertada outra opção, os ratos cessavam o uso da cocaína imediatamente, como explicado anteriormente (AHMED, S. H., 2010; VENDRUSCOLO et al., 2010).

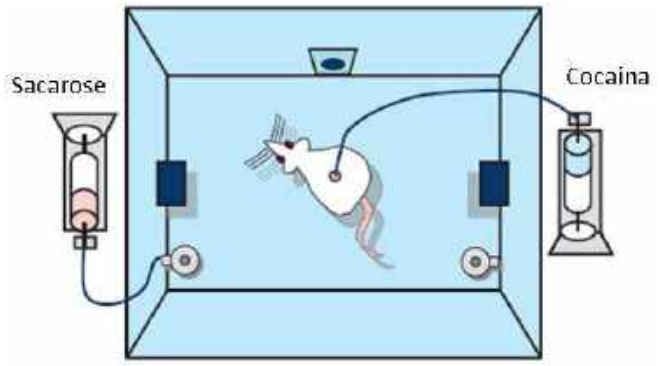

Figura 8: Equipamento de autoadministração e escolha. O desenho experimental de autoadministração por escolha prevê a disponibilidade de duas soluções: uma solução de sacarose para administração oral de um lado da caixa, e do outro, uma solução de cocaína para administração endovenosa. Cada solução é pareada a uma barra ativa. Adaptado de Ahmed et al, 2013.

A pergunta que vem asseguir destas descobertas é em função de que existem algumas exceções neste ambiente de escolha, em que os ratos continuam a usar a droga, pressionando a barra relativa à cocaína, negligenciando a opção de *nondrug*, normalmente altamente preferida (BOZARTH; WISE, 1985). Um estudo bastante recente mostra que isso ocorre em ambientes onde não há ou intervalos de tempo ou este intervalo é muito curto entre escolhas sucessivas (VANDAELE et al., 2016). Por exemplo, apenas o encurtamento do intervalo inter-escolha de 10 para 1 min ou menos foi suficiente para induzir ratos a mudar sua escolha de água doce para cocaína quase que exclusivamente (KANDERSTEIN et al., 2016). Esta mudança ocorreu em uma única sessão e foi reversível após o intervalo inter-escolha ser retornado para 10 min (VANDAELE et al., 2016). Uma característica importante de um ajuste com intervalo curto é que os efeitos farmacológicos diretos de uma escolha pela droga na

escolha anterior podem influenciar as escolhas posteriores (Figura 9). Dito de forma diferente, nesses contextos, a escolha por mais droga é feita sob a influência do efeito da droga. Nos seres humanos também existem evidências de que a intoxicação por cocaína no momento da escolha também pode promover escolhas de cocaína em detrimento de outras opções *nondrug* (DONNY; BIGELOW; WALSH, 2004; VOSBURG et al., 2010).

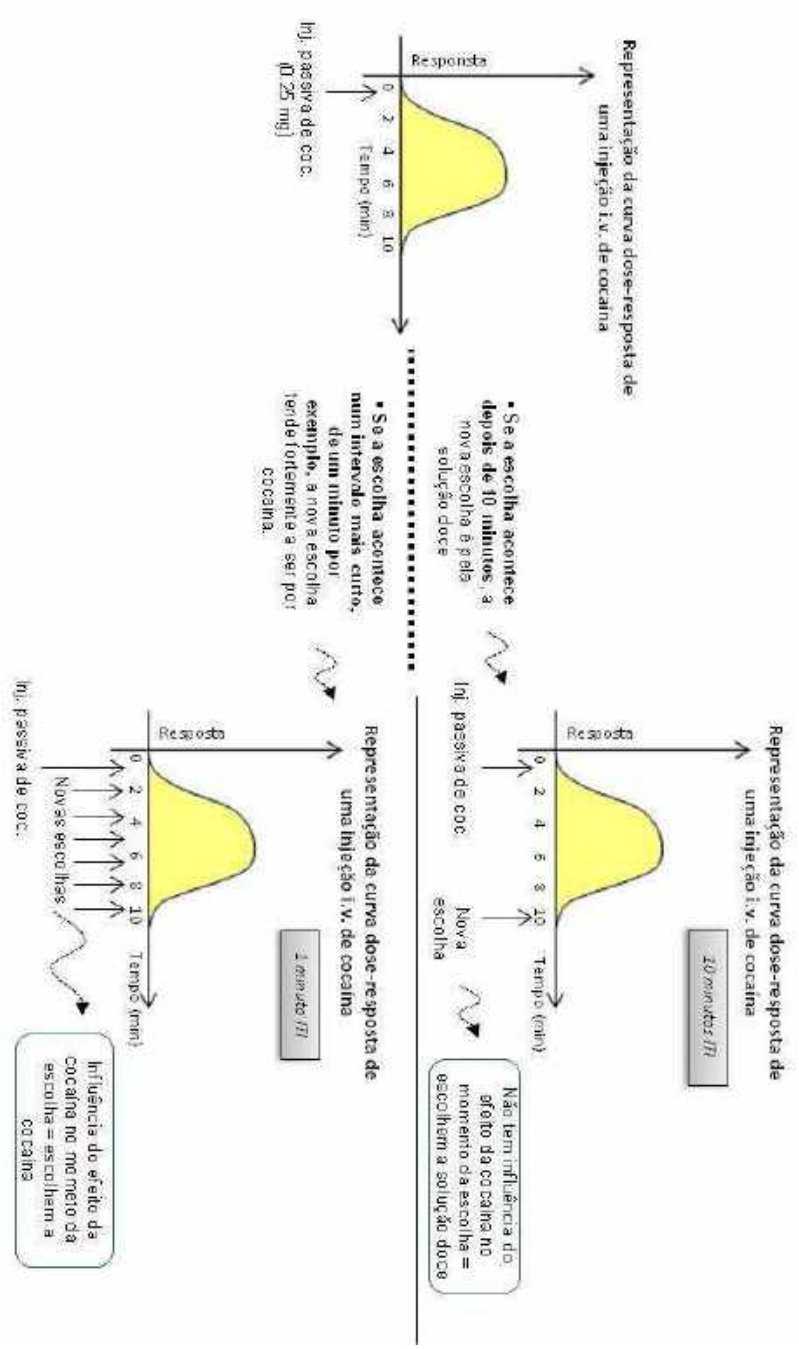

Figura 9: Esquema representativo da curva dose-resposta da cocaína nos intervalos inter-ensaios de 1 ou 10 minutos na autoadministração de cocaína com opção de escolha entre cocaína e sacarina. COC = cocaína.

Em geral, esses resultados indicam que os padrões de escolhas exclusivas de cocaína são mais prováveis de ocorrerem em ambientes onde os efeitos de transição de escolhas anteriores de cocaína podem influenciar escolhas futuras (VANDAELE et al., 2016).

4 JUSTIFICATIVA

O abuso e dependência de cocaína é, cada vez mais, uma das maiores preocupações de saúde pública mundial (UNODOC, 2011). Por seu mecanismo de ação, drogas psicoestimulantes estão comumente associadas a um alto potencial de abuso e dependência (BERRIDGE, 2006; BRADY et al, 2005; BRIGHT, 2008). O desenvolvimento da dependência química, por sua vez, é relacionado a uma complexa interação entre vulnerabilidades individuais e influências sociais e ambientais (PIAZZA & LE MOAL, 1996).

Apesar dos avanços consideráveis no conhecimento, muitas dúvidas sobre o porquê alguns indivíduos tornam-se dependentes, dentre muitos daqueles que experimentam os efeitos da droga, permanecem obscuras. Neste sentido, estudar modelos animais que possam responder melhor à estas questões, ou seja, da relação que existe entre diferentes ambientes e fatores que determinam a escolha em favor do uso ou não, pode contribuir para um melhor entendimento do processo da dependência.

Mais recentemente, uma nova metodologia foi apresentada à comunidade científica, como significativos avanços em relação à estudos de autoadministração i.v. de cocaína. A oferta de escolha entre o consumo de uma solução doce em ratos que já eram submetidos à autoadministração prévia e de longa duração de cocaína; e que diminuíram o consumo após ter duas opções, revolucionou o conhecimento na área. Por outro lado, outros pesquisadores já vinham colocando animais de laboratório em ambientes mais agradáveis / desagradáveis para verificar a influência dos ambientes no uso de drogas.

A hipótese conceitual que se desenvolve no presente trabalho é a de que indivíduos sem alternativas “agradáveis”, pra passar os dias e as noites, tendem a, impulsivamente, usar drogas quando estas são apresentadas. Além disso, o estresse de um ambiente sem ofertas interessantes poderia aumentar a pré-disposição de uma sensibilização aos efeitos psicoestimulantes da cocaína. Ao contrário, um ambiente enriquecido, com opções mais variadas de atividades, bem como a presença de solução adocicada, poderiam favorecer escolhas outras que não o uso de drogas.

5 OBJETIVOS

5.1 Objetivo geral

O objetivo geral deste estudo é verificar se o AE e/ou a presença de sacarina alteram o comportamento de condicionamento à cocaína em ratos submetidos a um protocolo adaptado de CPP e, ainda, determinar o papel neuroprotetor do AE no hipocampo e no córtex pre-frontal dos animais. Também, estimar a duração de efeitos saciantes e anoréxicos da cocaína, medindo as latências de escolha e resposta a cada opção após administração passiva de cocaína em modelo de autoadministração e escolha.

5.2 Objetivos específicos

3.2.1 Testar o modelo adaptado de escolha no CPP tem validade para determinar a preferência dos aninais quando existe uma opção *nondrug*;

3.2.2 Descrever o efeito do AE sobre a atividade das enzimas antioxidantes (catalase e superóxido dismutase) no CPF e hipocampo de ratos após exposição à cocaína;

3.2.3 Investigar o efeito do AE sobre o dano de DNA induzido pela cocaína no CPF e hipocampo de ratos;

3.2.4 Trabalhos anteriores mostraram claramente que a maioria dos animais se afasta da injeção de cocaína em benefício da solução adocicada. Testamos o que acontece com esse comportamento de escolha do rato após uma injeção passiva de cocaína;

3.2.5 Medir as latências de escolha e resposta a cada opção após a administração passiva de cocaína para estimar a influência de seus efeitos saciantes e anoréxicos.

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6 RESULTADOS

6.1 Artigo 1 – Neuroprotection by environmental enrichment in cocaine place preference

- Artigo submetido ao Jornal *Psychopharacology* -

Neuroprotection by environmental enrichment in cocaine-conditioned place preference

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Keywords

Conditioned place preference Saccharine Drug addiction DNA damage ROS Antioxidant enzymes

Acknowledgements

The authors would like to thank Greice Calleti for technical assistance for the dissection of the brain structures. Helena Maria Tannhauser Barros receives a 1C Productivity Grant from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Abstract

Rationale–Chronic cocaine exposure causes neurotoxicity and may result in drug addiction. In rats, enriched environment (EE) has a neuroprotective role and prevents the development of addiction. Furthermore, when rats are allowed to choose between cocaine injection and a nondrug reward (e.g. sweet solution) in and EE, most prefer the nondrug option. Surprisingly, studies exploring the convergence of cocaine toxicity in differentiated behavioral contexts remain uncommon.

Objectives–The aim of this study is to explore the capacity of saccharin (SAC) to change cocaine reward value in conditioned place preference (CPP). It will also investigate whether rats in an EE are better protected from cocaine-induced change in redox profile and DNA damage.

Methods–EE or standard-housed (ST) male Wistar rats were divided into a classical CPP cocaine vs. saline (COC/Saline) group and *CPP choice setting* (COC/SAC) group, where cocaine (15mg/kg; i.p.) was tested along with a saccharin solution [0.2%]. ROS, enzymes and the comet essay were performed on prefrontal cortex (PFC) and hippocampus.

Results–As expected, our EE rats presented less interest in the cocaine-paired chamber. All animals, given the choice, were less prone to show cocaine-place preference. Importantly, EE decreased oxidative stress and cocaine-induced DNA damage in all brain structures.

Conclusions–Altogether, our results demonstrate that EE leads to lower neurotoxicity in important regions linked to addiction. Also, the model of *CPP choice* seems to be effective for evaluating changes in cocaine-conditioning behavior. These results show that the effects of cocaine in rats seem differ based on the environmental context.

Introduction

Studies have been attempting to elucidate the inter-related nature of the compulsive pathological syndrome of dependence, bringing forth the idea that individual biological factors, such as genetic and environmental influences, can interact to have a positive or negative influence on addiction development (Crofton et al. 2015; Ouzir and Errami 2016; Wall et al. 2016). It is known that some people use substances recreationally without ever becoming addicted (Schramm-Sapyta et al. 2007). The evidence indicates that the progression from experimentation to the development of drug use disorder and dependence is the result of a complex interaction between repeated exposure, biological factors and environmental context (Goeders 2002; Kreek et al. 2005; Sinha 2001).

The EE mimics some kinds of positive life experiences and has been used as a model for the study of positive stimuli in humans (Marmol et al. 2015). The evidence suggests that EE facilitates recovery from brain injuries (Will et al. 2004) and reduces the impacts of psychiatric disorders such as drug addiction (Nithianantharajah and

Hannan 2006). Environmental factors directly alter the value of rewards (Simpson and Kelly 2011) and increase resistance to the effects of drugs such as cocaine (Bezard et al. 2003; Solinas et al. 2008) and amphetamines (Bardo et al. 2001).

Until now, rats have been the most frequently used nonhuman animal species in experimental research on drug addiction. They readily learn to self-administer most drugs that lead to addiction in humans, and when given access to cocaine, most readily learn to self-administer the drug and usually escalate their intake with extended access (Ahmed 2011). Previous research has shown that following extended access to cocaine self-administration, rats are more likely to escalate their consumption of cocaine (Ahmed and Koob 1998), working harder (Paterson and Markou 2003) and taking more risk to seek and/or to obtain cocaine (Vanderschuren and Everitt 2004). Surprisingly, when offered a mutually-exclusive choice, most non-food-deprived rats readily give up cocaine use to drink water sweetened with a non-caloric sweetener (i.e., saccharin) – an otherwise biologically inessential rewarding behavior (Cantin et al. 2010; Lenoir and Ahmed 2007; Tunstall and Kearns 2014). In the other words, no matter how large the increase in drug value, it is apparently not sufficient to replace preference for the nondrug option and promote cocaine preference in the animals (Lenoir et al. 2007). However, these results have not been confirmed in other less invasive models such as the place-conditioning paradigm (CPP), which is compatible with classical Pavlovian conditioning and is similar to learned reward associations (Itzhak and Martin 2002).

Cocaine-induced neurochemical alterations in brain areas such as the prefrontal cortex and hippocampus may also contribute to changes in decision-making and memory formation related to drugs of abuse (Pum et al. 2007; Pum et al. 2008). The neurochemical effects of cocaine in the visual cortex (Muller et al. 2007; Muller and Huston 2007) may also be of importance, given the substantial role of cocaine-

associated stimuli in the maintenance and reinstatement of addictive behavior (Di Ciano and Everitt 2004; See 2005). These cocaine effects are strongly linked with excessive concentrations of dopamine, which have in turn been associated with the mechanism that leads to cocaine toxicity (Pereira et al. 2015). Oxidative stress is an imbalance between the reactive oxygen species (ROS) generated and the antioxidant defense system. In brain tissue, the accumulation of ROS leads to persistent neurochemical abnormalities and loss of synaptic integrity (Dietrich et al. 2005; Pereira et al. 2015; Sajja et al. 2016). In contrast, free radicals have been successfully removed by endogenous antioxidants, which are stimulated by EE (Marmol et al. 2015; Mattson et al. 2001). The EE also leads to an increase in newly generated neural cells in the adult brain, suggesting that this stimulation can increase the capacity of the brain for plasticity and self-repair (Muhammad et al. 2016; van Praag et al. 2000).

The current animal models are somewhat limited with respect to construct knowledge about addiction, face validity and predictive validity. Based on observations of the models that can increase this validity, environmental enrichment and choice studies attempt to approximate the laboratory research to the greatest diversity found in human drug addiction. In addition, the protective effects of EE in relation to the neurotoxicity caused by cocaine have not yet been investigated.

Thus, the aim of the present study was to present data on the use of an alternative model to evaluate the rewarding value of cocaine along another non-drug option, saccharine, in an adapted CPP protocol. The study also assesses the protective effect of EE in terms of ROS formation, antioxidant enzyme activity and DNA damage in the prefrontal cortex and hippocampus in rats exposed to cocaine.

Experimental procedures

Subjects

Fifty male Wistar rats were divided into standard (ST) or EE groups from weaning (post-natal day (PND) 21) to PND 50. They were group-housed in accordance with guidelines on a 12-h light/dark cycle, with food and water available *ad libitum*. All experimentation was conducted between 09:00 a.m. and 02:00 p.m, during the light phase of the cycle. The Institutional Animal Care and Use Committee of the UFCSPA approved all animal procedures (Ethics Committee #224/13). All efforts were made to use only the number of animals necessary to produce reliable scientific data.

Housing Conditions

The EE rats were housed 7-10 per cage in a large cage (70×60×80 cm) with 3 floors connected by a ladder, to force them to carry out a physical activity to access the food and water provided on the third floor. Each cage had 5-6 toys of different shapes, a running wheel, a small house, and several cardboard tunnels for the entertainment of the rats. The objects were changed and replaced 3 times per week.

The ST rats were housed (2-3 per cage) in standard polycarbonate cages (40×33×18 cm) under standard conditions.

Solutions

Saccharin (SAC) (Sigma-Aldrich, Brazil) was dissolved in tap water at room temperature (22±2°C). The saccharin solution was prepared fresh each day.

Cocaine hydrochloride (COC) (Merck, Germany) was dissolved in 250- mL sterile bags of 0.9% NaCl to a concentration of 15 mg/ml and stored at 4-8°C. Before use in behavior tests, the solution was kept at room temperature (22±2°C).

Experimental procedure

Conditioned place preference

Six identical CPP equipment setups were used for all behavioral testing (Insight®, Brazil). The boxes (40 × 60 × 38 cm) had three distinct chambers: two larger conditioning chambers (40 × 23 × 38 cm) connected by a smaller neutral chamber (40 × 14 × 38 cm). The chambers were separated by doors and had distinct visual (vertical or horizontal lines on the walls) and tactile (bars, grade or aluminum plate on the floor) characteristics. The boxes were equipped with photobeams and the horizontal movements of the animals were automatically monitored by software coupled to the boxes. The apparatus was cleaned with ethanol solution (70%) and dried immediately to remove the ethanol's smell between trials. The experiments were conducted in an exclusively dimly lit room (red light).

The ST and EE rats were divided into two experimental groups, according to CPP conditions: COC/Saline (n=15) and COC/SAC (n=35). The CPP protocol consisted of an 11-day schedule divided into 3 different phases: pre-conditioning (2 days), conditioning (8 days), and post-conditioning (1 day). During pre-conditioning, the rats were allowed to freely explore the three compartments for 15 minutes each day. The time spent by each animal with all four paws in each of the three compartments was recorded on the second day.

The second conditioning phase consisted of eight 30-min sessions once daily. Immediately after i.p. cocaine administration (15 mg/kg), the animals were confined in the less preferred compartment from the pre-conditioning phase and, on alternate days, received vehicle (saline 1 mL/kg; i.p.) and were confined in the opposite compartment. After the end of the tests, the rats were replaced for their respective home cages (ST or EE).

In the post-conditioning phase, each animal, in a drug-free state, was placed in the neutral compartment and had free access to all three compartments. The time spent in each compartment was recorded for 20 min.

For the COC/SAC group, we developed an adapted CPP protocol, where a sweet drinking solution (saccharine 2%) was offered instead of saline in the conditioning phases. Into the CPP boxes was placed a drinker containing the saccharin solution glued to the middle of the floor. One important note is that the COC/SAC group was first pre-exposed to the saccharine solution one week prior to the CPP experiment to prevent bias from a new factor. During the conditioning phase, on the saccharine day, the animals received an aversive stimulus (injection) with saline (1 mL/kg i.p.).

In CPP, drug is typically paired with the less preferred compartment in the pre-conditioning phase. Since the COC/SAC group were presented two rewards (i.p. cocaine or oral saccharin), the less preferred environments were randomly distributed between the two rewards. Therefore, we had two subgroups, the group of rats whose least preferred compartment was paired with saccharin and another group whose least preferred compartment was paired with cocaine (Figure 1).

Sample collection and preparation

At the end of the CPP test, the animals were decapitated and the PFCs and hippocampi were dissected according to the Paxinos and Watson coordinates (Paxinos and Watson 2007) and frozen in liquid nitrogen. The brain tissue was stored at −80 °C until analysis.

Comet assay

The comet assay for evaluation of genotoxicity was performed as described by Hartmann et al. (Hartmann et al. 2003). Briefly, PFC and hippocampus were placed in separate microtubes with 400 µL PBS cold solution with 20 mmol/L EDTA and 10% dimethylsulphoxide (DMSO) and were mixed with a vortex. The brain regions were allowed to settle and the supernatant containing single cells was collected. The isolated cells were counted in a Countess® (Invitrogen, by Life Technologies) to determine cell concentration and survival by trypan blue exclusion assay. An aliquot of cell suspension (20 µL) was dissolved in 0.75% low-melting point agarose and immediately spread onto a glass microscope slide pre-coated with a layer of 1% normal melting point agarose. The slides were incubated in ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM, 1% Triton X-100 and 10% DMSO, pH 10.0). After lysis, the slides were washed three times for 24 h at 4°C in enzyme buffer (40 mM Hepes, 100 mM KCl, 0.5 Mm Na2EDTA, 0.2 mg/mL BSA, pH 8.0), and incubated with FPG (30 min at 37°C). The slides were then incubated with electrophoresis solution (300 mM NaOH and 1 mM EDTA, pH 13.0) for 20 min to unwind the DNA. Electrophoresis was conducted at 4°C for 20 min at 0.94 V/cm. The slides were then neutralized with Tris buffer (0.4 M Tris, pH 7.5) and stained with silver. For the evaluation of DNA damage, 100 cells per slide were analyzed by optical microscopy. The cells were visually scored by measuring the

DNA migration distance and the amount of DNA in the tail was separated into five classes, from undamaged (0) to maximally damaged (4). The damage index (DI) value was calculated for each sample and ranged from 0 (no tail: 100 cells x 0) to 400 (with maximum migration: 100 cells x 4) (Burlinson et al. 2007).

Protein extract

To determine levels of dichlorofluorescein (DCFH-DA), and activity of superoxide dismutase (SOD) and catalase (CAT), the tissues were homogenized in Tris-HCl buffer (pH 7.4). After 30 min, the tissues were subjected to 6 cycles of 30 seconds at ice cold temperature in the vortex mixer. Each solution was centrifuged for 10 min at 16,000 rpm to separate the tissue debris from the cell extracts. Protein concentration was determined by the Lowry method with minor modifications.

DCFH-DA assay

The DCFH-DA assay, a reliable method for measuring intracellular ROS such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), and hydroperoxides (ROOH), was used to estimate the generation of reactive species in a cell-free assay according to (Ko et al. 2005; Yin et al. 2013). The DCFH-DA assay is. This method is based on the deacetylation of the DCFH-DA probe, and its subsequent oxidation by reactive oxygen species into a highly fluorescent compound (2',7'-dichlorofluorescein; DCF). For the DCHF-DA assay, phosphate-buffered saline (PBS; pH 7.4), 500 µM DCFH-DA and 5 µL of tissue suspension (total volume 200 µL) were incubated in 96-well dark-plates at 37 °C for 30 min. Fluorescence was measured using a SpectraMax M2e Microplate

Reader (Molecular Devices, Sunnyvale, CA, USA). The excitation and emission wavelengths were 490 nm and 525 nm, respectively. All experiments were carrying out in a dark room to prevent oxidation of the DCHF-DA. Results were expressed as fluorescence units normalized to protein content (FU/ mg protein).

Determination of SOD and CAT activity

SOD activity was assayed by measuring the inhibition of superoxide dependent autoxidation of adrenaline, according to the method described by Misra and Fridovich (Misra and Fridovich 1972). Firstly, sample aliquots of protein extract were added into 50 mM glycine buffer (pH 10.2) and 10 mM catalase. Adrenaline was then added and absorbance was immediately recorded every 36 seconds for 15 minutes at 32 °C and 480 nm with a SpectraMax M2e Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The results were calculated as the amount of one unit of SOD required to inhibit 50% of the adrenaline autoxidation, and the specific activity is described in SOD U/mg protein. CAT activity was assessed through the disappearance of H_2O_2 at 240 nm, according to the method described by Aebi (Aebi 1984). Briefly, 50 mM phosphate buffer (pH 7.0; KH_2PO_4 and Na_2HPO_4 in the proportion 1:1.5 (v/v) were added into an aliquot of protein extract. Subsequently, 25 mM H_2O_2 was added and the absorbance was immediately recorded every 36 seconds for 5 min at 240 nm in the SpectraMax M2^e Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Based on the definition of one CAT unit as one µmol of H_2O_2 consumed per minute, the results were calculated as specific activity and are described by CAT U/mg protein.

Statistical analysis

Statistical analyses were performed using Sigma Plot v11 (Stystat Software, CA, U.S.A.). The Kolmogorov-Smirnov test was used to verify the normality of all variables and was followed by an equal variance test. For CPP data, the results were analyzed using two-way repeated measures analysis of variance (2 W-RM-ANOVA) on the percentage of time spent in the drug/reward-paired compartment during the pre and post-conditioning test according to the following formula:

$$\frac{(time\ drug\text{-}paired\ compartment)}{(time\ drug\text{-}paired\ compartment + time\ saline\text{-}paired\ compartment)} \times 100$$ for COC/Saline and

$$\frac{(time\ reward1\text{-}paired\ compartment)}{(time\ reward1\text{-}paired\ compartment + time\ reward2\text{-}paired\ compartment)} \times 100$$ for COC/SAC, where if *reward1* is cocaine, *reward2* is saccharine and vice versa. For DNA damage, the arbitrary units' data were analyzed using two-way ANOVA comparing the housing inside each CPP group (COC/Saline and COC/SAC). The same was performed for oxidative stress. All analyses were followed by Tukey's *post-hoc* test when appropriate. All results are presented as mean ± S.E.M. For all analyses, significance was set at $P \leq 0.05$.

Results

Conditioned place preference

In order to determine the effect of EE, rats reared in a ST or EE environment were subjected to CPP (COC/Saline). Our results confirmed the protective effect of EE. Two-way ANOVA repeated measures performed on the percentage of time spent in the cocaine compartment (% time pre vs. post) showed a significant effect of drug increasing the time only in the ST rats [$F_{(1,8)} = 7.765$, $P = 0.024$] (Fig. 1a). The EE rats

did not show conditioning to CPP [$F_{(1,7)} = 0.439$, $P = 0.532$]. Interestingly, independently of the housing conditions, the rats from the COC/SAC group did not show cocaine conditioning (ST = [$F_{(1,17)} = 1.808$, $P = 0.196$] and EE = [$F_{(1,14)} = 1.704$, $P = 0.213$]) (Fig. 1b). However, when the EE group was divided according the baseline place preference, the data shows different results (Figs. 2a and 2b): a) when saccharine was paired with the less preferred side, the EE rats were conditioned to saccharine [$F_{(1,8)} = 8.268$, $P = 0.024$] (Fig. 2a), while the ST rats were not conditioned [$F_{(1,7)} = 0.147$, $P = 0.714$]; and b) when cocaine was paired with the less preferred side, none of the groups were conditioned, probably because of the presence of the other reward competing with cocaine (ST = [$F_{(1,9)} = 3.497$ $P = 0.094$]; EE = [$F_{(1,7)} = 0.556$, $P = 0.484$]). It is important to note that the ST rats, which are understood by us as the most prone to conditioning to cocaine, also did not develop cocaine-conditioned behavior.

EE housing decreases DNA damage after cocaine-CPP

Using the comet essay technique, it was observed that animals housed in EE displayed much less cocaine-induced cell damage in the PFC [$F_{(1,40)} = 13.403$, $P<0.001$] and hippocampus [$F_{(1,40)} = 22.922$, $P<0.001$] (Fig. 4). The protection from cocaine-induced DNA damage to the PFC and hippocampus was found in both CPP groups, COC/SAC ($P = 0.001$; $P<0.001$) and COC/Saline ($P=0.045$; $P=0.019$).

EE housing decreases ROS production in cocaine versus saccharine CPP

Our results demonstrate decreased formation of DCF in the PFC [$F_{(1,37)} = 4.848$, $P=0.034$] and hippocampus [$F_{(1,38)} = 2.201$, $P=0.009$] of EE-housed rats compared to

ST rats in the cocaine versus saccharine (COC/SAC) group (Fig. 5a). Analysis of the COC/Saline group showed no significant response to housing conditions (P=0.399 and P=0.961). In the PFC, catalase was less expressed in the EE group, with a statistically significant decrease seen in rats in the COC/SAC group [$F_{(1,32)}$ = 6.491, P=0.016] (Fig. 5b) but no statistical difference for the COC/Saline group (P= 0.094). In the hippocampus, EE decreased CAT activity for both the COC/SAC and COC/Saline groups [$F_{(1,34)}$ = 14.656, *P=0.006; P=0.015*, respectively]. Fig. 5c shows the SOD results, with no significant results for PFC [$F_{(1,42)}$ = 1.229, P=0.274] or hippocampus [$F_{(1,42)}$ = 1.971, P=0.168].

Discussion

In our study, EE prevented cocaine CPP in rats. It is already known that environmental stimulation during the early stages of life protects against abuse-related drug reward (Bardo et al. 2001; Cain et al. 2012; Green et al. 2002; Hopfer et al. 2003; Solinas et al. 2009; Stairs and Bardo 2009). Rats reared in an enriched environment show decreased intravenous self-administration of d-amphetamine across repeated sessions compared to rats reared in a socially isolated condition (Bardo et al. 2001; Green et al. 2002). In cocaine sensitization, EE housed rats show less motor activation in response to repeated administration of cocaine injections and reduced responses to cocaine and amphetamine challenges (Bardo et al. 1995; Smith et al. 1997; Solinas et al. 2009). Our results are also in line with most recent studies that showing that mice housed in EE display less acute cocaine effects and less CPP (Nader et al. 2014; Solinas et al. 2008; Solinas et al. 2010; Solinas et al. 2009). Moreover, our results add to our knowledge of EE protective effects, in that they seem to become less relevant when

saccharine is offered as an alternative reward along with cocaine. When one side of the CPP compartment is paired with an alternative reward, the rats become less conditioned to cocaine; this effect is also seen in rats reared in a standard environment, which are clearly affected by cocaine in the COC/Saline CPP group. In the last years, experimental protocols giving the choice between cocaine and a sweet solution have brought a new outlook on animal models of dependence. A long series of experiments were conducted in which rats were given the choice between cocaine self-administration and a nondrug alternative reward (Ahmed 2005; Cantin et al. 2010; Lenoir et al. 2007; Tunstall and Kearns 2014). According to this line of thought, if rats prefer cocaine despite the opportunity to make a different choice, one can speculate that there exists a state of addiction, respecting the idea that there may still be unknown individual differences between animals raised in very similar conditions (Ahmed 2010; Cantin et al. 2010; Lenoir and Ahmed 2007). Unquestionably, when alternative choice is not present, it is difficult to determine whether rats take cocaine by compulsion or for lack of other rewarding options (Ahmed 2010; Cantin et al. 2010). However, if animals have a choice between the drug and another reward, they often prefer rewards such as food over the drug (Ahmed 2010; Cantin et al. 2010; Mello and Negus 1996; Nader and Woolverton 1991). Thus far, no studies have explored other models for assessing the value of the rewarding effect caused by cocaine in rats.

Interestingly, in this study, using an adapted CPP choice protocol, the rats raised in an EE condition preferred the side paired with saccharin. In other words, the rats were more conditioned to the sweet taste over cocaine. Recent findings show that a sweet taste can induce rewards and cravings that are comparable to those induced by drugs (see Ahmed et al. 2013 for a review), but may be considered less toxic. Drugs such as cocaine impair normal brain function and can trigger problematic behaviors.

Sweet foods can also change brain activity via the stimulation of specialized sweet taste cells in the mouth and gut (Brown and Rother 2012; Yarmolinsky et al. 2009) and via post absorptive brain mechanisms involving glucose signaling (Grayson et al. 2013); these are much more natural routes than those of drugs of abuse and are clearly not as behaviorally, psychologically and/or neurochemically toxic (Ahmed et al. 2013; Pereira et al. 2015).

An interesting finding of the present work is that rats raised in EE showed less cocaine-induced DNA damage than the ST group. Previous findings indicate that cocaine induces a potent genotoxic effect and DNA damage, interfering in an important chain of events that ensure genomic stability (see Pereira 2015 for a review). In primary cultures, cocaine causes apoptotic cell death in striatal and mesencephalic cell cultures (Lepsch et al. 2015). A previous study from our laboratory (de Souza et al. 2014) showed that repeated cocaine administration increases DNA damage in distinct brain areas in ovariectomized female rats and the magnitude of cocaine-induced DNA damage in the PFC and hippocampus was very similar to our findings in ST-housed rats (around 300 until 340 DNA damage index units) . However, EE did not reduce the damage to control (without cocaine) levels, which were less than 200 DNA damage index units. In the hippocampus of the COC/SAC group, however, EE did reduce DNA damage to indices very similar to the control group. Thus, depending on the brain region, EE does not seem to be able to completely eliminate the damage caused by repeated exposure to cocaine. Finally, taken together, the previous results and the present data may demonstrate that cocaine toxicity is comparable between genders.

DNA damage is generally caused by an increase in ROS levels in cells (Polidori et al. 2000). Overproduction of ROS induces oxidative stress, which plays a significant role in the harmful effect of cocaine in brain tissue, inducing cell apoptosis (Alvarenga

et al. 2010; Alvarenga et al. 2011; Pereira et al. 2015). Our animals housed in EE presented less DCF fluorescence production, a marker of cellular oxidative stress. This finding is consistent with the results from Cechetti et al. (2012) obtained in an ischemic group of rats, where EE significantly decreased DCF production in the hippocampus. In previous studies, EE also decreased thiobarbituric acid reactive species (TBARS) in the hippocampus (Cechetti et al. 2012) and cortex of rats (Marmol et al. 2015). Interestingly, a recent study showed that when compared to resveratrol, a recognized anti-oxidant, EE promotes a prominent decrease in the concentration of malondialdeyde, another marker for oxidative stress (Muhammad et al. 2016). The decrease in ROS levels suggests that free radicals have been successfully removed by endogenous antioxidants, which are stimulated by EE (Marmol et al. 2015). Is agreement that EE increases newly generated neural cells in the adult brain, suggesting that this stimulation can increase the capacity of the brain for plasticity and self-repair (Muhammad et al. 2016; van Praag et al. 2000). EE seems to also possess antioxidant properties by attenuating lipid peroxidation (Muhammad et al. 2016) and up-regulating and activating antioxidant enzymes (Mattson et al. 2001). In contrast, cocaine might increase oxidative stress via metabolic activation because of the accumulation of dopamine. Dopamine toxicity is believed to be due to the formation of ROS resulting from dopamine metabolism (by auto-oxidation or MAO action), leading to apoptosis of the cells. On the one hand, auto-oxidation may occur in the extracellular medium, leading to the generation of $O_2^{\bullet -}$ and toxic quinones (Banerjee et al. 2014). On the other hand, the dopamine is inactivated by oxidation (catalyzed by the enzyme monoamine oxidase, MAO), converting dopamine to 3,4-dihydroxyphenylacetic acid (DOPAC). H_2O_2 seems to be generated from the metabolism of excess dopamine by MAO (Pereira et al. 2015).

Superoxide dismutase (SOD) and catalase (CAT) are enzymes that protect brain tissue from oxidative stress. Superoxide ($O_2^{\bullet -}$) is a primary ROS formed in cells and is enzymatically converted to H_2O_2 by SOD, which itself can react with free ions to produce highly reactive and toxic hydroxyl radicals. Subsequently, H_2O_2 can be destroyed by catalase or glutathione peroxidase reactions (Yang et al. 2016). Our results show no difference in SOD activity between standard housing and EE. Vitcheva et al. (2015) showed that cocaine increases SOD in the rat brain. Similarly, Dietrish et al. (2005) showed that after chronic cocaine exposure, SOD levels in the prefrontal cortex were increased after 24 hours compared to control. However, after 48 hours the SOD levels returned to normal. In our study, the brain structures were collected 48 hours after the final administration of cocaine (see the CPP protocol). This finding may be due to the transience of the cocaine effect on SOD masking EE's influence. The stimulation of SOD occurred at a time when a significant enhancement of ROS production has also been observed (Dietrich et al. 2005). Taken together, these findings suggest that the activation of antioxidant enzymes represents a mechanism whereby brain cells can get rid of some radicals generated by cocaine.

EE was seen to decrease ROS formation, as described earlier. As the increase in anti-oxidant enzyme activity might be directly related to the ROS level (Benzi and Moretti 1995), the high CAT activity in the present study was paralleled by an increase in total ROS, as evidenced in the standard environment rats. In other words, our EE animals produced less ROS, which would imply a reduced need to produce CAT. These results corroborate those of others, who have reported donw-regulation of antioxidant enzymes by EE in brain tissue (Cechetti et al. 2012) and hippocampus (Muhammad et al. 2016).

In short, two main conclusions can be drawn from this study. First, the current data are consistent with the hypothesis that the reward value of cocaine as well as the cocaine toxicity are linked to environmental influences from the lifestyle. Accordingly, the choice in CPP abolished the cocaine-induced CPP in all groups of rats, even those understood by us as being more susceptible to conditioning, the rats from the impoverished environment. The second conclusion, which is no less important, concerns the DNA damage and ROS formation caused by cocaine, which was consistently shown to be reduced by environmental stimulation, but not completely abolished.

It is possible that we are in accordance with others trains of thought, which state that animal research has not paid sufficient attention to the specific individual aspects of drug addiction, contributing to the present dearth of effective treatments. For the future, focusing on the differences in external influences during the early stages of life could create opportunities for integration of the multifactorial framework of drug addiction into laboratory research.

Conflict of Interests

The authors declare that there is no conflict of interests for any of the authors.

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Figure captions

Fig. 1 Diagram of the design of the conditioned place preference (CPP) protocol implemented on cocaine vs. saccharine (COC/SAC) group of rats. The protocol was constituted of 3 successive phases, performed during 12 days: basal preference (day 1 - 20 minutes); conditioning phase (day 2 to day 10 - 30 minutes per day); followed by test (day 12 - 20 minutes). S=saccharin; C=cocaine

Fig. 2 Percentage of time spent on the cocaine compartment (%). **a**. COC/Saline - Rats from enriched-housing (EE) condition present less interest for the cocaine-paired chamber on cocaine vs. saline (COC/Saline) group. Bars represent the percentage of the time spent in the cocaine-paired compartment during the pre and post-conditioning phase of CPP. Mean ± SEM. *Different from the pre-conditioning ($P \leq 0.05$, Tukey's post hoc analysis following a 2-way RMANOVA). **b.** COC/SAC - The percentage of time spent (%) on cocaine compartment of the rats from cocaine vs. saccharine (COC/SAC) group. Bars represent of the time spent (%) in the cocaine-paired compartment during the pre and post-conditioning phases of CPP. Mean ± SEM

Fig. 3 Represent the break-up of the group COC/SAC (Figure **2.b)** according the less preferred side distribution **a.** Time-spent (%) in the saccharine side when less preferred side of the basal preference was paired with saccharine. The EE rats were conditioned to saccharine. Mean ± SEM. *Different from the pre-conditioning phase ($P \leq 0.05$, Tukey's post hoc analysis following a 2-way RMANOVA). **b.** Time-spent in the cocaine side when less preferred side in the basal preference was paired with cocaine.

Bars represent the time spent (%) in the cocaine-paired compartment during the pre and post-conditioning phases of CPP. Mean ± SEM

Fig. 4. Protective effect of environmental enrichment (EE) on cocaine-induced DNA damage in comparison to standard housing rats (ST). *Different from ST on cocaine vs. saccharine (COC/SAC) and cocaine vs. saline (COC/Saline) groups. Bars represent the means ± SEM. ($P \leq 0.05$, Tukey's post hoc analysis following a two-way ANOVA)

Fig 5. a. DCFH fluorescence production on PFC and hippocampus of rats from the cocaine vs. saccharine (COC/SAC) and cocaine vs. saline (COC/Saline) groups. Comparison of rats housed in enriched environment (EE) and standard (ST). *Different from ST on COC/SAC. **b.** The catalase (CAT) activity on PFC and hippocampus of rats. *Different from ST of COC/SAC and COC/Saline groups; **c.** Superoxide dismutase (SOD) activity on PFC and hippocampus of the rats. Bars represent the means ± SEM. ($P \leq 0.05$, Tukey's post hoc analysis following a two-way ANOVA)

Figure 1

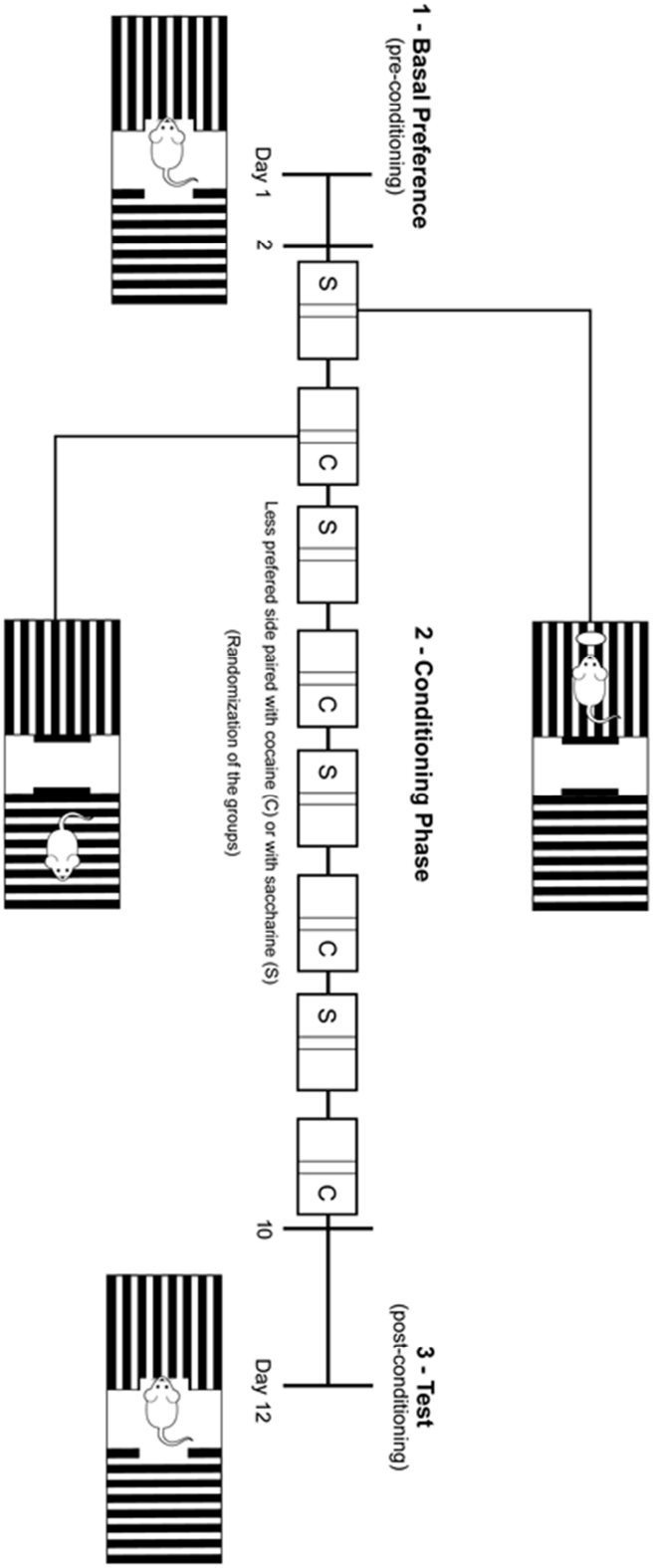

1 - Basal Preference
(pre-conditioning)
Day 1
2
S
C
S
C
S
C
S
C
10
2 - Conditioning Phase
Less prefered side paired with cocaine (C) or with saccharine (S)
(Randomization of the groups)
3 - Test
(post-conditioning)
Day 12

Figure 2a

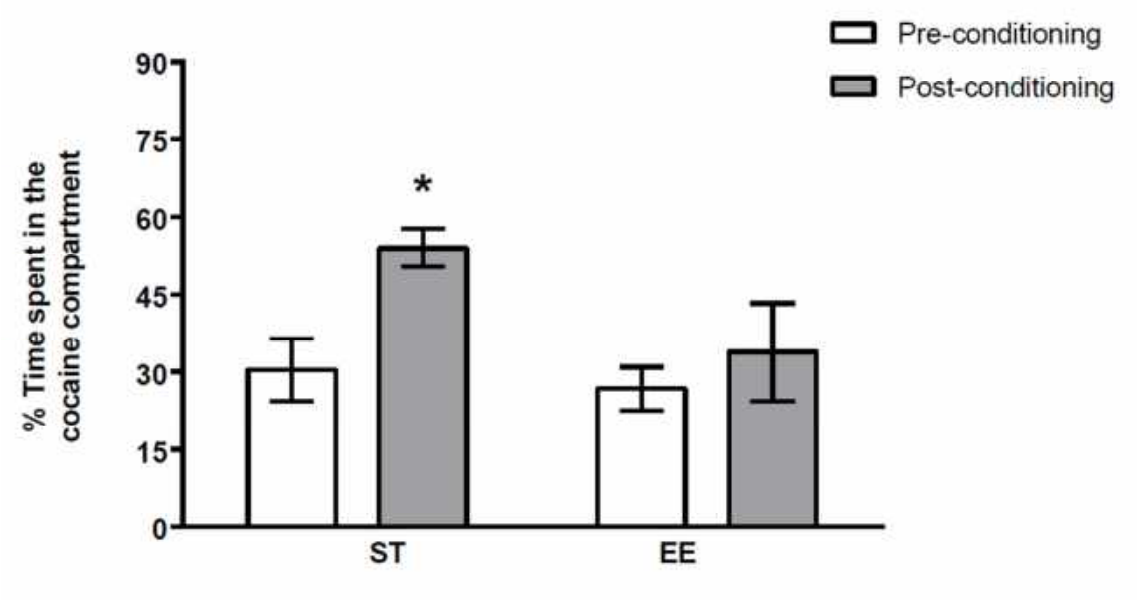


Figure 2b

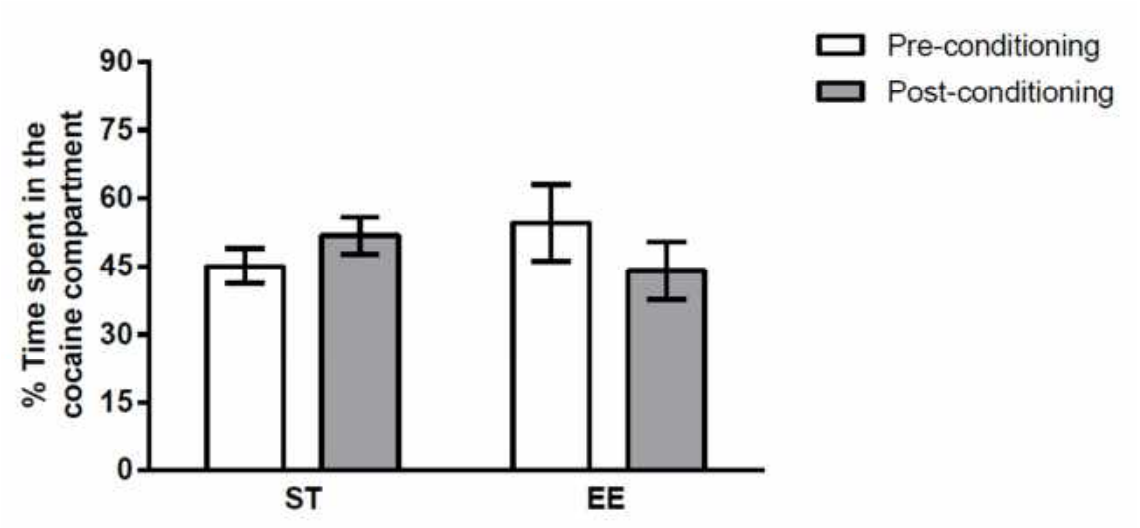


Figure 3a

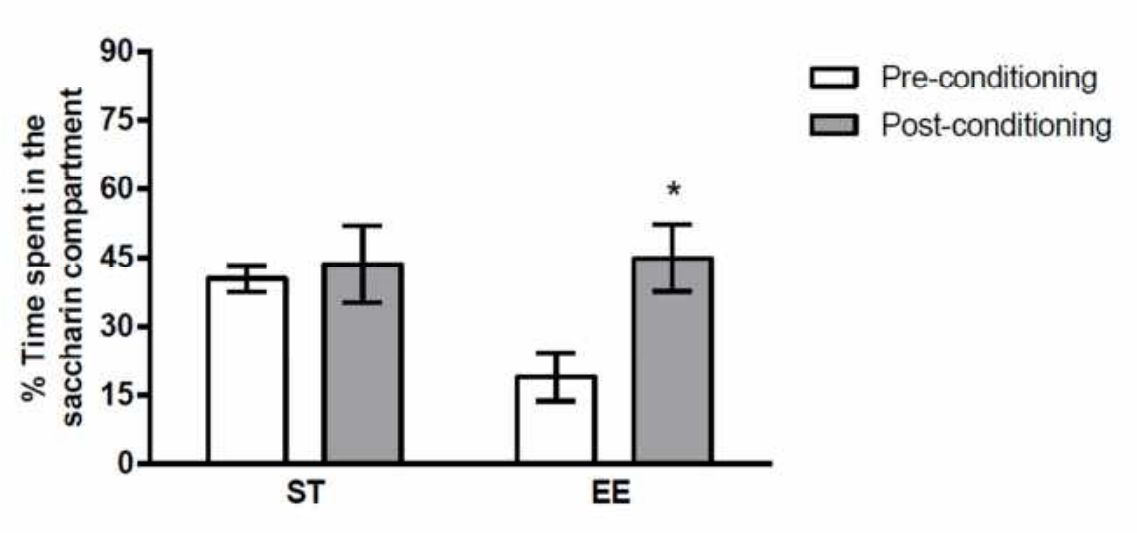


Figure 3b

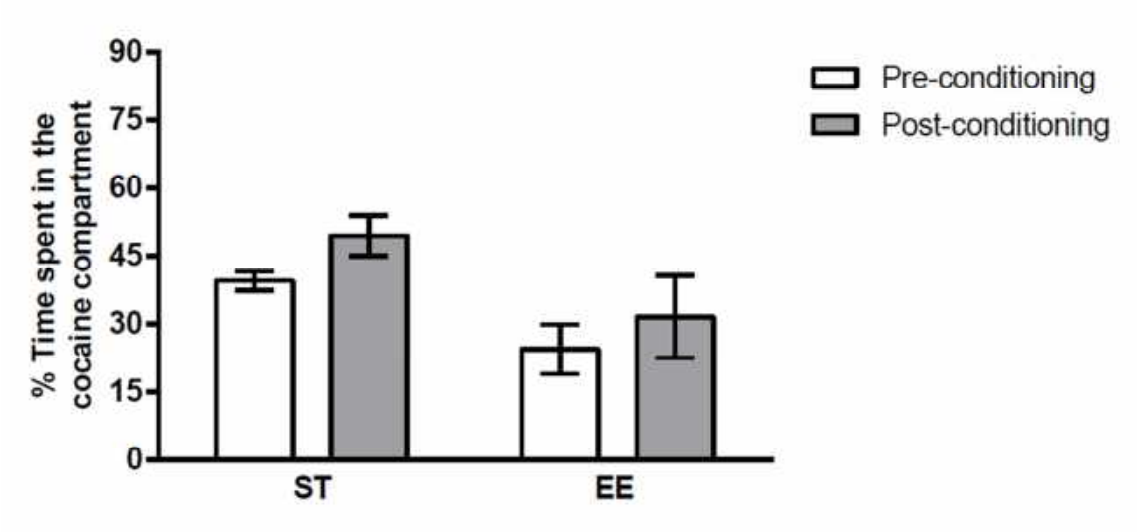


Figure 4

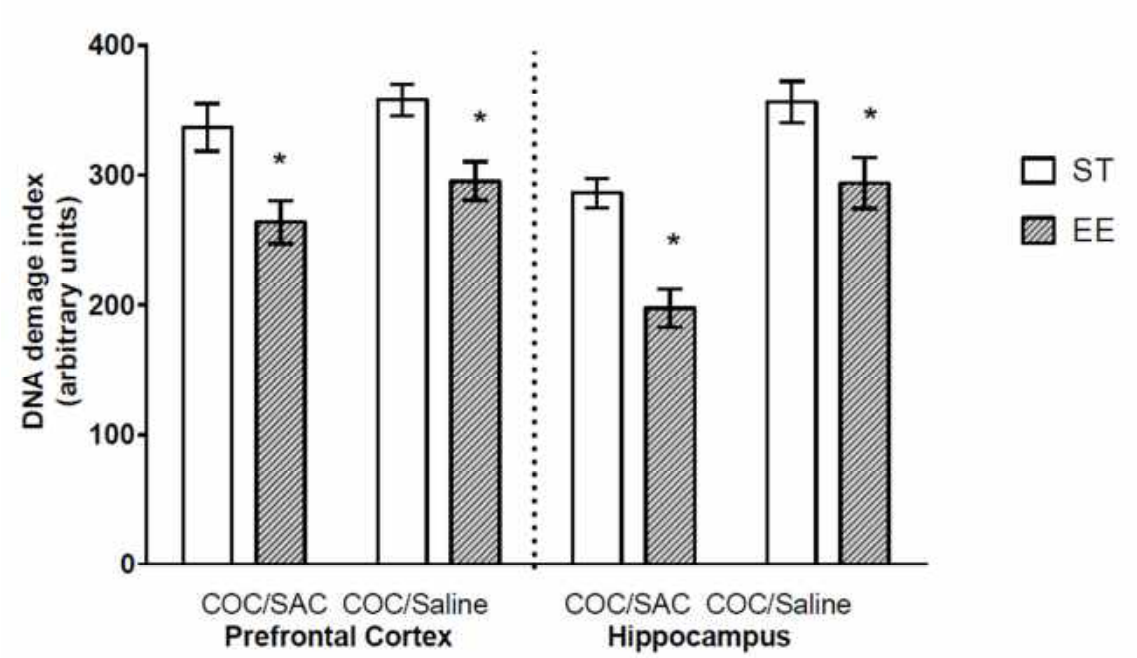


Figure 5a

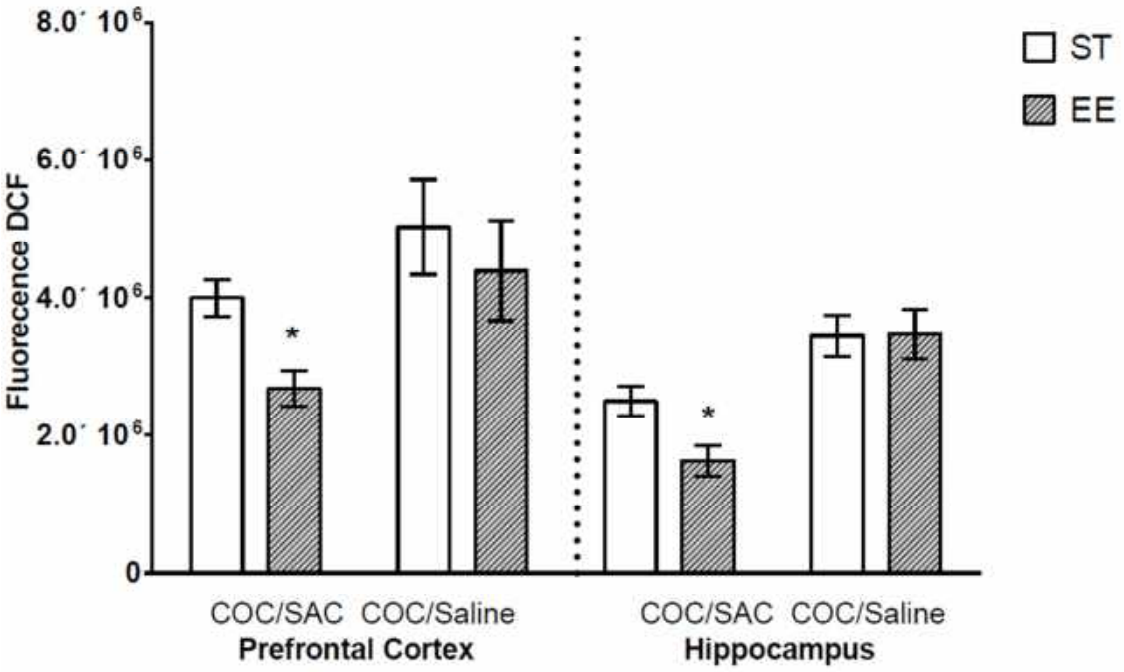


Figure 5b

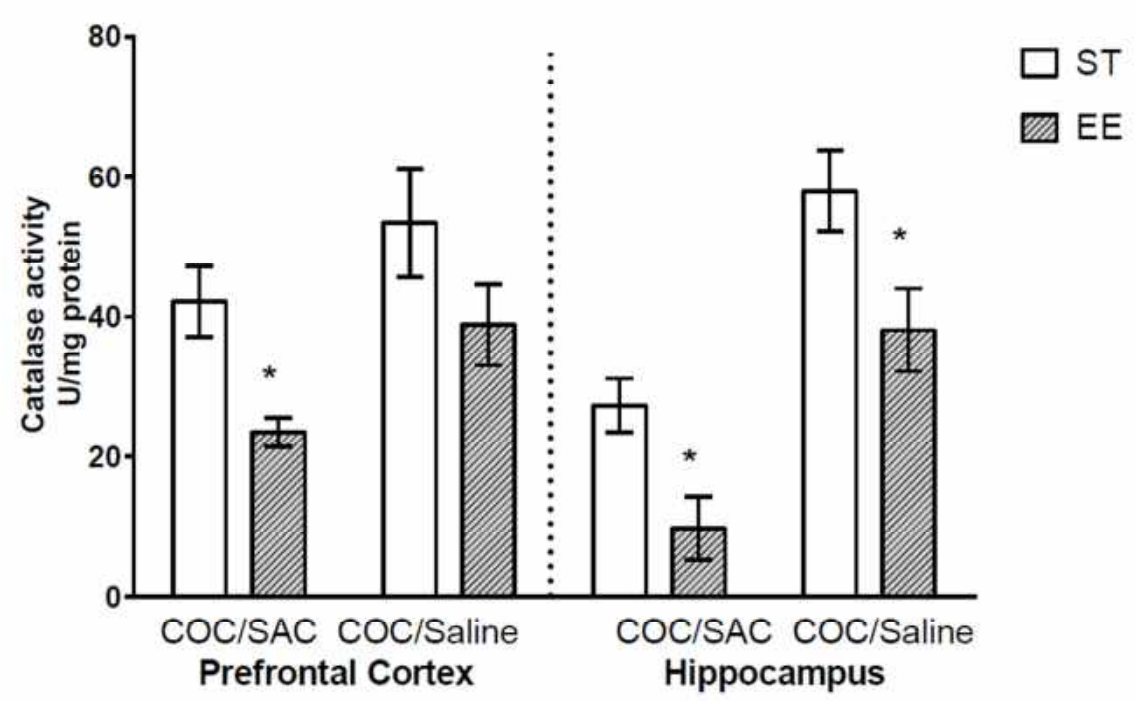
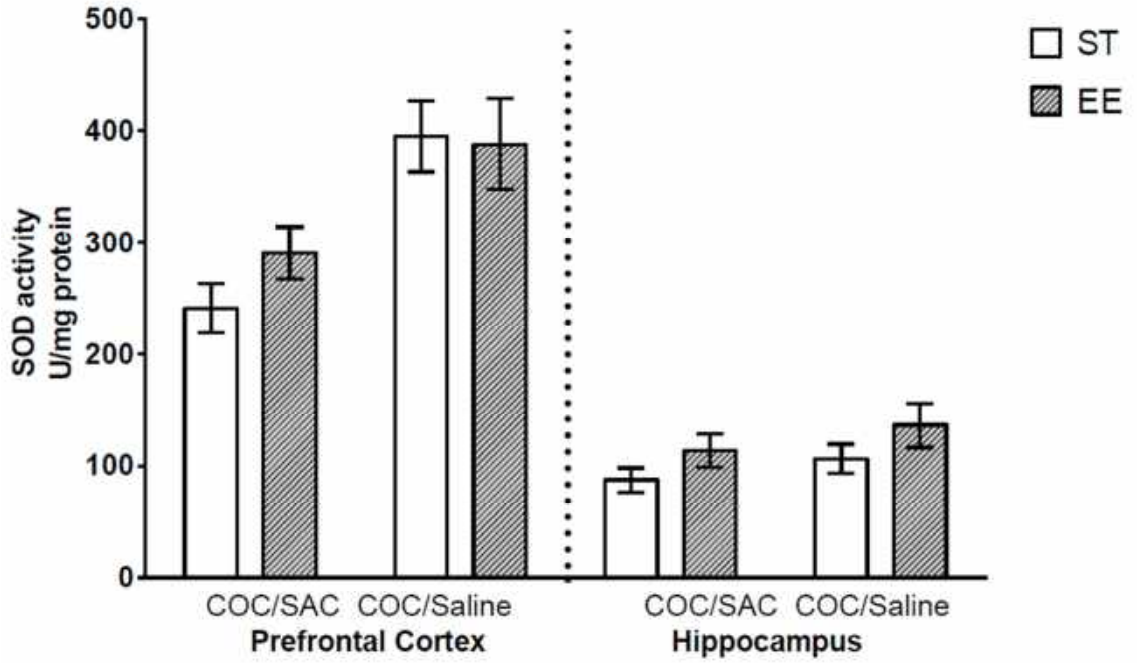


Figure 5c



Artigo 2 – How cocaine choice toward or away from cocaine in different indivuduals

- Artigo Submetido ao Jornal *Addiction Biology* -

Cocaine biases choice toward or away from cocaine in different individuals

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Running title: Cocaine-induced shift of preference

ABSTRACT

Being under the influence during choice between drug and nondrug options can have a dramatic effect on choice outcomes in both animals and humans. We previously showed that when rats are not under the influence, they largely prefer sweet water over intravenous cocaine. In contrast, when they are under the influence of cocaine, they shift their choice to cocaine nearly exclusively. The drug influence was causal because when it was induced before each choice trial by passive administration of cocaine, it caused sweet water-preferring rats to shift their choice to the drug. Here we sought to better characterize the behavioral mechanisms underlying the drug influence on choice. In theory, rats under the influence of cocaine should be in a mixed motivational state, at least temporarily, with both their motivation for cocaine and their motivation for the nondrug option suppressed by the drug satiating and anorexic effects of cocaine, respectively. For this mixed state to shift choice to cocaine, the suppressed motivation for cocaine should recover before that for the preferred nondrug option. The goal of the present study was to test this prediction in rats that expressed a preference for sweet water after extended access to cocaine self-administration. We measured their choice and response latencies to each option after passive administration of cocaine to estimate the duration of its drug satiating and anorexic effects. Overall, we found evidence that partially supports our hypothesis, with some revealing individual variation. Specifically and as predicted, individuals in whom the drug satiating effects of cocaine dissipated before its anorexic effects – representing the majority of rats – shifted

their choice to cocaine when under the influence. Inversely, individuals in whom the drug satiating effects of cocaine dissipated after its anorexic effects – representing a minority – further increased their initial preference for the nondrug option when under the influence.

Key-words: choice; addiction; cocaine; drug influence; anorexic effects.

Introduction

Ample evidence shows that the choice setting can dramatically influence drug use in both humans and nonhuman animals (Ahmed, 2005, 2010; Alexander and Hadaway, 1982; Badiani, 2013; Faupel, 1987; Zinberg, 1984). This influence is particularly well exemplified in laboratory rats that have access to cocaine for intravenous self-administration in different settings. When rats have access to cocaine with no other choice, most of them readily learn to self-administer the drug and eventually escalate their intake with extended access (Ahmed, 2011; Ahmed, 2012). In contrast, when rats are given a choice between cocaine and a potent nondrug reward (e.g., water sweetened with saccharin or sucrose), most that self-administered the drug and escalated their intake in a no-choice setting reduce and even quit drug use in favor of the nondrug option (Cantin *et al*, 2010; Lenoir *et al*, 2007; Tunstall and Kearns, 2013). This abstinence-like behavior was also observed with other drugs of abuse, including methamphetamine, heroin, oxycodone, and nicotine (Caprioli *et al*, 2015b; Huynh *et al*, 2015; Lenoir *et al*, 2013b; Panlilio *et al*, 2015; Secci *et al*, 2016). It is generally robust to a variety of different experimental conditions (e.g., drug doses) and drug exposure histories (Augier *et al*, 2012; Cantin *et al*, 2010; Lenoir *et al*, 2007; Madsen and Ahmed, 2015).

There are, however, some choice settings where rats continue to use the drug to the exclusion of a normally highly preferred nondrug option (Bozarth and Wise, 1985; Thomsen

et al, 2013; Vandaele *et al*, 2016). We and others recently found that this occurs in settings where there is no or short time intervals between successive choices. For instance, merely shortening the inter-choice interval from 10 to 1 min or less was sufficient to induce rats to shift their choice from sweet water to cocaine almost exclusively (Kerstetter *et al*, 2012; Vandaele *et al*, 2016). This shift occurred in one single session and was reversible after the inter-choice interval was returned to 10 min (Vandaele *et al*, 2016). One important feature of a setting with no or short inter-choice interval is that the direct pharmacological effects of a prior drug choice can be carried over to subsequent choices to influence them. Put differently, in such settings, choice can be made under the drug influence. The causal role of this drug influence in rats' preference shift was confirmed in a study where this influence was induced artificially before each choice trial by non-contingent administration of cocaine in a setting where the inter-choice interval was set to 10 min. All else equal, pre-trial administration of cocaine caused rats to shift their choice to cocaine. In humans too, there is some evidence that cocaine intoxication at the moment of choice can also promote cocaine choices over other nondrug options (Donny *et al*, 2004; Vosburg *et al*, 2010). Overall, this research indicates that patterns of exclusive cocaine choices are more likely to occur in settings where the carry-over effects of prior cocaine choices can influence future choices (Vandaele *et al*, 2016).

The mechanisms underlying this drug influence have yet to be fully elucidated. Though several mechanisms are possible, recent evidence suggests that pre-trial administration of cocaine promotes cocaine choices mainly by acutely suppressing responding for the nondrug option rather than by enhancing responding for the drug option itself (Vandaele *et al*, 2016). First, cocaine, like other stimulant drugs, is well known to induce powerful anorexic effects that suppress both intake of and operant responding for sweet food/drink in rats (Balopole *et al*, 1979; Cooper and van der Hoek, 1993; Vandaele *et al*, 2016; Wolgin and Hertz, 1995; Woolverton *et al*, 1978). Second, we observed a strong dose correlation between cocaine-induced suppression of responding for sweet water and cocaine-induced shift of preference

from sweet water to cocaine (Vandaele *et al*, 2016). Finally, no shift of preference between heroin and sweet water was observed when rats were tested with pre-choice administration of heroin. On the contrary, pre-trial heroin further increased sweet water choices (Vandaele *et al*, 2016). Unlike cocaine, heroin does not suppress but instead enhances responding and intake of sweet water (Cooper, 1982; Parker *et al*, 1992; Rideout and Parker, 1996; Vandaele *et al*, 2016).

The goal of the present study was to better understand the effects of pre-trial administration of cocaine on choice between cocaine and sweet water in rats. In our previous experiments, we noted that the shift of choice from sweet water to cocaine induced by pre-trial cocaine was accompanied by a dramatic increase in choice latency, from few seconds to several hundreds. This increased latency indicates that though rats eventually chose cocaine on each trial after pre-trial cocaine, they were apparently not eager to make this choice. This increased choice latency is likely due to the transient reward satiating effects of pre-trial cocaine which is known to suppress transiently responding for the drug (Ahmed and Koob, 1998, 2005; Wise, 1987; Yokel, 1987). Thus, after pre-trial cocaine, rats would be transiently in a mixed motivational state, with both their motivation for cocaine and their motivation for the nondrug option suppressed by the drug satiating and anorexic effects of cocaine, respectively. For this mixed state to shift choice to cocaine, the suppressed motivation for cocaine should recover before that for the preferred nondrug option (Figure 1a), so that the latter remains suppressed when rats are ready to respond again for cocaine. If it was the opposite, pre-trial cocaine would not be expected to cause rats to change their initial preference but instead to maintain it or even to enhance it. The present study was designed to test this prediction.

Materials and Methods

Animals and Housing

A total of 14 adult male Wistar rats (225-250 g at the beginning of experiments, Charles River, Lyon, France) were used. Rats were housed in groups of 2 and were maintained in a light- (reverse light-dark cycle), humidity- (60 ± 20%) and temperature-controlled vivarium (21 ± 2°C). All behavioral testing occurred during the dark phase of the light-dark cycle. Food and water were freely available in the home cages throughout the duration of the experiments. Home cages were enriched with a nylon gnawing bone and a cardboard tunnel (Plexx BV, The Netherlands). One rat did not complete the experiment because of loss of catheter patency, thereby leaving 13 rats for final analysis.

All experiments were carried out in accordance with institutional and international standards of care and use of laboratory animals [UK Animals (Scientific Procedures) Act, 1986; and associated guidelines; the European Communities Council Directive (2010/63/UE, 22 September 2010) and the French Directives concerning the use of laboratory animals (décret 2013-118, 1 February 2013)]. The animal facility has been approved by the Committee of the Veterinary Services Gironde, agreement number A33-063-922.

Surgery

Rats were surgically prepared with an indwelling silastic catheter in the right jugular vein under deep anesthesia. Behavioral testing commenced at least 7 days after surgery. Additional information about surgery and post-operative care can be found elsewhere (Lenoir *et al*, 2013a).

Extended access to cocaine self-administration

One week after intravenous surgery, rats were trained to self-administer intravenous cocaine (0.25 mg per injection over approximately 4.2 s) during 18 daily sessions on a fixed-ratio (FR) 1 time-out 20s schedule of reinforcement, as described elsewhere (Ahmed and Cador, 2006; Ahmed and Koob, 1998). Except for the first 2-h session, each subsequent daily self-administration session lasted 6 h. The aim of the first, short 2-h session was to allow drug naïve rats to have a first experience with cocaine before extended access. This limits the risk of overdose that may occur in naïve rats when tested immediately with extended access to the drug. All self-administration sessions began with extension of one lever (lever C for Cocaine); the other lever (lever S, to be associated with sweet water) remained retracted. Responding on lever C was rewarded by 0.25-mg cocaine and was signaled by illumination of the cue-light above lever C for 20 s. No non-contingent injections of cocaine were given, except on rare occasions when a subject failed to respond within the first 30 min in which case it received two passive injections approximately 20 s apart.

Alternate operant training

After the 17th session of extended access to cocaine self-administration, rats were trained to respond for cocaine (as described above) or sweet water on 6 alternate FR1 sessions. On sweet water sessions, the lever not associated with cocaine (i.e., lever S) was extended to mark the onset of the session and to signal sweet water availability; lever C remained retracted. Responding on lever S was rewarded by a 20-s access to water sweetened with 0.2% saccharin delivered in the adjacent drinking cup and was signaled by illumination of the cue-light above lever S for 20 s. The maximum volume that a rat could drink per 20-s access period was 0.32 ml of sweet water. The session ended after rats had earned a maximum of 20 sweet rewards. We previously showed that an extended operant training with sweet water

is not required to observe a preference for sweet water over cocaine during subsequent choice, even in rats with a history of extended access to cocaine (Cantin *et al*, 2010; Lenoir *et al*, 2007) (see also, (Caprioli *et al*, 2015a; Caprioli *et al*, 2015b)).

Discrete-trials choice procedure

After operant training, rats were tested for choice for a total of 5 daily sessions until stabilization of choice behavior (i.e., 3 consecutive sessions with no increasing or decrease trend). Each choice session consisted of 20 discrete choice trials spaced 10 min apart (Figure 1b). A 10-min inter-trial interval was used to prevent any carry-over effect of prior drug choices (Kerstetter *et al*, 2012; Vandaele *et al*, 2016). Each trial commenced with the simultaneous presentation of both levers S and C. Rats were free to respond to either of these two levers to self-administer the corresponding reward (i.e., 0.25 mg of cocaine or 20-s access to 0.2% saccharin as described above). Reward delivery was signaled by retraction of both levers and illumination of the cue-light above the selected lever. Thus, for the duration of each single trial, choice of one reward excluded the other. If rats failed to respond to either lever within 10 min, both levers were retracted and no reward was delivered. The operant response requirement was set to two consecutive responses to avoid accidental choice. The choice latency was measured as the time elapsed between trial onset and delivery of the chosen reward.

Effects of pre-trial administration of cocaine on choice behavior

After stable baseline choice performance, we insured that, like in our previous study, pre-trial cocaine caused rats to shift their choice to cocaine exclusively and that this shift was also

associated with a large increase in choice latency. This was done in one choice session using a procedure identical to that described above, except that each choice trial was preceded 5 min before by a non-contingent and non-signaled i.v. injection of cocaine (0.75 mg) (Figure 1b). This pre-trial dose of cocaine was shown previously to induce near maximal effects on choice behavior (Vandaele *et al*, 2016). The dose of cocaine available for self-administration during each choice trial remained identical to that used during baseline choice (i.e., 0.25 mg).

Effects of pre-trial administration of cocaine on discrete-trials responding for cocaine or sweet water

As explained in the Introduction, pre-trial cocaine is hypothesized to cause rats to shift their choice to cocaine because the suppressing anorexic effects of cocaine would last longer than its drug satiating effects. To estimate the duration of these two effects, we measured the effects of pre-trial cocaine on the latency to respond for cocaine or sweet water in two no-choice sessions where only one option was available (Figure 1b). These no-choice sessions were strictly identical to a choice session, except that rats had only access to one lever (i.e., lever S or C) and its corresponding reward (i.e., sweet water or cocaine). We assume that the latency to respond for cocaine when it is the sole option available (i.e., no-choice latency for cocaine) should mainly reflect the duration of the drug satiating effects of pre-trial cocaine. Similarly, the latency to respond for sweet water when it is the only option available (i.e., no-choice latency for sweet water) should mainly reflect the duration of the anorexic effects of cocaine. In both cases, the no-choice latency was measured as the time elapsed between trial onset and delivery of the reward available.

Substances

Cocaine hydrochloride (Coopération Pharmaceutique Française, Melun, France) was dissolved in 0.9% NaCl, filtered through a syringe filter (0.22 µm) and stored at room temperature. Sodium saccharin or sucrose (Sigma-Aldrich, St Quentin-Fallavier, France) was dissolved in tap water at room temperature (21 ± 2°C). Sweet solutions were renewed each day.

Statistics

All data were subjected to relevant repeated measures ANOVAs, followed by Tukey post hoc tests where relevant. Comparisons with a fixed theoretical level (e.g., 0 or 50%) were conducted using one sample t-tests. Proportions were compared using the two-proportion z-test. Statistical analyses were run using Statistica, version 7.1 (Statsoft Inc., Maisons-Alfort, France).

Results

As expected, with extended access to cocaine for self-administration, rats gradually increased their drug intake from about 50 to 80 injections per 6-h session ($F_{18,216} = 23.45$, $p < 0.01$) (Figure 2a). Once escalated cocaine intake began to stabilize (i.e., around session 17), rats were trained during 3 sessions that alternated with sessions of cocaine self-administration to respond for saccharin on an alternative lever, lever S. Lever C and cocaine were not available during these sessions. All rats obtained the maximum number of

saccharin rewards (i.e., 20) as early as the first training session. The time required to obtain this maximum number of rewards decreased considerably with repeated session, suggesting rapid learning ($F_{2,24} = 28.22$, $p < 0.01$) (Figure 2b). On the third session, rats drank a volume of 5.6 ± 0.2 ml of sweet water which was close to the maximum allowed (i.e., 20 x 0.32 ml = 6.4 ml). On average, rats received a total of 1411.3 ± 71.8 cocaine rewards and 60.0 ± 0.0 sweet water rewards before choice testing.

During choice testing, all rats completed nearly all choice trials available (maximum = 20) during all sessions (i.e., > 95%). As expected from previous research, they clearly expressed a preference for saccharin over cocaine from the first choice session onward ($F_{1,12} = 35.57$, $p < 0.01$) (Figure 3a,b). This was indicated by a % of cocaine choices systematically lower than the indifference level of 50% ($ts_{12} < -2.64$, $ps < 0.021$). Preference for saccharin tended to further increase with repeated session ($F_{4,48} = 2.42$, $p = 0.060$). During the last 3 sessions, % cocaine choices leveled off at around 20 % (i.e., less than 4 cocaine choices versus about 16 saccharin choices). The volume of sweet water that rats drank per trial was very close to the maximum possible (0.31 ± 00 versus 0.32 ml). The development of saccharin preference was also accompanied by a decrease in the choice latency which baselined over the last 3 choice sessions ($F_{4,48} = 2.47$, $p = 0.056$) (Figure 3c). Note that since rats made very few cocaine choices, baseline choice latency predominantly reflects the choice latency for sweet water. However, in rats (n = 9) that chose cocaine on a sufficient number of trials for analysis (i.e., at least 5 over the last 3 sessions), there was no significant difference in latency between cocaine or sweet water choices (i.e., 17.1 ± 6.7 versus 10.7 ± 3.5 s).

As expected, non-contingent administration of cocaine before each choice trial caused rats to shift their choice from sweet water to cocaine nearly exclusively ($F_{1,12} = 39.45$, $p < 0.01$) (Figure 4a). An analysis of individual behavior revealed, however, that pre-trial administration of cocaine did not induce a shift of preference in 2 rats out of 13 (called thereafter NS rats for Non-Shifters). These rats were temporarily ignored in the following analysis of response

latencies, their small number (< 3) precluding any meaningful comparisons with the remaining majority of rats (called S rats for Shifters, n = 11). As expected, pre-trial cocaine-induced shift to exclusive cocaine choices in S rats ($F_{1,10} = 162.15$, $p < 0.01$) (Figure 4b) was associated with a considerable increase in choice latencies compared to baseline ($F_{1,10} = 124.18$, $p < 0.01$) (Figure 4c). These latencies predominantly reflected cocaine choices. It was not possible to parse the latency data as a function of the chosen reward (i.e., cocaine or saccharin) because many S rats made no more than 2 sweet water choices (n = 9). A within-session, trial-by-trial analysis revealed that pre-trial cocaine caused most S rats to choose cocaine ($Zs_{10} < -2.9$, $ps < 0.05$) with increased latency from the second trial onward (Trial x Pre-trial treatment: $F_{19,190} = 3.95$, $p < 0.01$) (Figure 4d,e).

As previously explained (see Introduction), pre-trial administration of cocaine is hypothesized to cause S rats to shift their choice to cocaine because the devaluation of sweet water by the anorexic effects of cocaine would take more time to dissipate than the devaluation of cocaine by its drug satiating effects (Figure 1a). This difference was assessed in two no-choice sessions in which rats could only respond for cocaine or sweet water after pre-trial cocaine. Overall, behavioral latencies varied as a function of the option available after pre-trial cocaine ($F_{2,20} = 14.82$, $p < 0.01$). Contrary to our prediction, however, the resulting no-choice latency for sweet water was not higher but instead lower than the no-choice latency for cocaine (Figure 5a). By comparison, the latter did not differ from the choice latency, a lack of difference that was expected because the choice latency predominantly reflects cocaine choices. Importantly, though pre-trial administration of cocaine considerably slowed behavior (from about 20 s to more than 200 s), rats nevertheless completed nearly all choice or no-choice trials to obtain the corresponding reward and there was no difference as a function of the available option ($F_{2,20} = 2.18$) (Figure 5b). Finally, though rats completed most trials on the no-choice session where they had only access to sweet water, they nevertheless drank less of it than during baseline sweet water choices (0.31 ± 00 versus 0.23 ± 0.02 ml per rewarded trial; $F_{1,10} = 12.19$, $p < 0.01$). The latter outcome suggests that rats initiated

responding for sweet water while they were still partially under the anorexic effects of pre-trial cocaine.

In the previous experiment, the effects of pre-trial cocaine on choice or no-choice latencies were tested over 3 consecutive sessions in that order: one session of choice followed by one cocaine no-choice session and then by one sweet water no-choice session. It is thus possible that the counterintuitive pattern of behavioral latencies described above is due, at least partly, to a between-session adaption to repeated pre-trial cocaine. To test this possibility, all rats were given additional choice sessions with pre-trial cocaine until evidence for stable choice latency (i.e., 6 in total including the first choice session with pre-trial cocaine). After that, they were retested with pre-trial cocaine during one cocaine no-choice session and one sweet water no-choice session, as described above (see Methods and Figure 1b). With repeated testing, the 2 previously identified NS rats continued to not shift their choice to cocaine in response to pre-trial cocaine (Figure 6a). Similarly, most S rats also continued to shift their choice to cocaine. However, among previously identified S rats, 2 rats eventually stopped to shift their choice to cocaine in response to pre-trial cocaine from the second session onward (Figure 6a). As a result, they were added to the other NS rats to form a separate group of sufficient size for subsequent analysis. Another previously identified S rat also stopped to respond to pre-trial cocaine on session 2 but it began to respond again on session 6. Since its behavior was instable, this rat was not included in subsequent analysis.

The average behavior of S (n = 8) and NS (n = 4) rats is shown in Figure 6b. These two groups were indistinguishable during baseline choice. They both largely preferred sweet water over cocaine and each drank the maximum volume possible of sweet water (i.e., 0.31 ± 0.00 and 0.31 ± 0.01 ml per rewarded trial). However, they responded oppositely to pre-trial cocaine (Group x Session: $F_{6,60} = 14.97$, $p < 0.01$). Pre-trial cocaine shifted choice to cocaine almost exclusively in S rats while it had no effect or even tended to further increase choice of sweet water in NS rats compared to baseline (Figure 6b). After choosing sweet water, NS rats drank the same volume of it per trial than during baseline (0.30 ± 0.01 versus

0.31 ± 0.00 ml). Despite this opposite choice, however, pre-trial cocaine increased choice latencies to the same extent in both groups (Figure 6c). In addition, with repeated testing, there was a similar and parallel decrease in choice latencies in both groups, suggesting a comparable between-session adaptation to the effects of pre-trial cocaine (Session: $F_{6,60} = 30.91$, $p < 0.01$; Group x Session: $F_{6,60} = 0.63$).

Then, both S and NS rats were subsequently retested with pre-trial cocaine during one cocaine no-choice session and one sweet water no-choice session, and their behavior was compared to that recorded during the last 3 choice sessions with pre-trial cocaine. The profile of behavioral latencies varied as a function of the group of rats ($F_{2,20} = 9.66$, $p < 0.01$). Contrary to our prediction, in S rats which shifted their choice to cocaine after pre-trial cocaine, all latencies were virtually indistinguishable (Figure 6d): their no-choice latency for sweet water was not longer than but equal to their no-choice latency for cocaine, suggesting a similar duration between the anorexic and drug satiating effects of cocaine. The lack of difference between their no-choice latency for cocaine and their choice latency was expected because the latter nearly exclusively reflects cocaine choices in S rats (Figure 6b). Interestingly, however, though S rats completed virtually all sweet water trials after pre-trial cocaine, they drank less per trial than during baseline sweet water choices (0.26 ± 0.02 versus 0.031 ± 0.01 ml per rewarded trial; $F_{1,7} = 9.91$, $p < 0.01$). This decreased intake shows that S rats responded for sweet water while they were still partially under the anorexic effects of cocaine.

In contrast, in NS rats, the profile of behavioral latencies was in line with their continued choice of sweet water despite pre-trial cocaine. Their no-choice latency for cocaine was much longer than their no-choice latency for sweet water which was similar to their choice latency (Figure 6d). The lack of difference between their no-choice latency for sweet water and their choice latency was expected because the latter nearly exclusively reflects sweet water choices in NS rats (Figure 6b).

Apart from their opposite choice response to pre-trial cocaine, the only other significant difference found between S and NS rats was in the no-choice latency for cocaine. This latency was much longer in NS rats than in S rats, suggesting longer drug satiating effects in NS rats than in S rats (Figure 6d). This interpretation was consistent with longer mean post-loading inter-injection pauses in NS rats than in S rats (318.1 ± 45.6 versus 237.0 ± 13.6) during the last 5 sessions of cocaine self-administration that preceded choice testing ($F_{1,10} = 4.87$, $p = 0.051$). Inter-injection pauses mainly reflect the duration of cocaine satiation in rats (Ahmed and Koob, 2005; Tsibulsky and Norman, 1999; Wise, 1987). Though both groups had the same initial body weight before behavioral training and testing (300.1 ± 10.1 versus 305.1 ± 2.8 g; $F_{1,10} = 0.39$), NS rats gained slightly less weight over time than S rats (19.6 ± 8.9 versus 41.9 ± 3.9 g; $F_{1,10} = 7.25$, $p < 0.01$). As a result, at the end of the choice experiment, NS rats were slightly lighter than S rats (319.8 ± 6.5 versus 346.9 ± 5.0 g; $F_{1,10} = 10.39$, $p < 0.01$). Though significant, this difference in body weight is nevertheless relatively small and only results in a marginal difference (i.e., less than 9%) in the relative dose of pre-trial cocaine between the two groups (i.e., 2.34 versus 2.16 mg/kg).

Discussion

All rats rapidly developed a preference for sweet water over cocaine when given the choice between the two options after extended access to and escalation of cocaine self-administration, as shown previously (Cantin *et al*, 2010; Caprioli *et al*, 2015a; Caprioli *et al*, 2015b; Lenoir *et al*, 2007). This preference was manifest on the first choice session after only 60 lever-sweet reward pairings compared to more than 1400 lever-cocaine reward pairings. All else being equal, however, non-contingent administration of cocaine before each trial caused most rats to shift their choice away from their preferred nondrug option to cocaine almost exclusively. This drug-induced shift in choice behavior was also accompanied by a large increase in choice latency, confirming that rats were under the drug influence during choice. In addition, this shift occurred very rapidly, as it was already manifest on the 2nd choice trial and maximal on the 3rd one. These findings strongly suggest that cocaine-induced shift in choice is mainly caused by the direct pharmacological effects of cocaine at the time of choice. This interpretation is also consistent with previous research showing that rats quasi-immediately return to their initial preferred option after cessation of pre-trial cocaine (Vandaele *et al*, 2016). Finally, the present study also shows that though pre-trial cocaine induced most rats to shift their choice to cocaine (S rats), few rats apparently resisted this drug influence and, in fact, tended instead to increase their choice of sweet water (NS rats).

S rats did not behave exactly as expected after pre-trial cocaine. The resulting no-choice latencies for sweet water were not longer than those for cocaine. One can interpret this negative finding as suggesting that contrary to our prediction, the suppressing anorexic effects of pre-trial cocaine on responding for sweet water do not last longer than its drug satiating effects (see Figure 1a). However, it is more likely that though strongly indicative, no-choice latencies alone do not completely reflect the durations of the different effects induced

by pre-trial cocaine. In support of this view, we found evidence during no-choice session of sweet water that the anorexic effects of pre-trial cocaine were still present after S rats had responded for sweet water, as they drank less of it once obtained than normally. Why S rats respond for sweet water before complete dissipation of the anorexic effects of cocaine is not clear. One can suggest that once the drug satiating effects of cocaine have dissipated and there is no opportunity to respond for cocaine, S rats would be somehow compelled to respond for the only nondrug option available, even if intake of the latter, once obtained, is still partially suppressed by the anorexic effects of cocaine. There is evidence that under some circumstances, some dopaminergic drugs can indeed cause drug-experienced rats to respond for food when it is the only option available and even if they do not eat the earned food (Collins and Woods, 2008). The relative insensitivity of responding to the current value of the nondrug option may also indicate that after dissipation of the drug satiating effects of cocaine, responding would not be goal-directed but instead habitual or perseverative. This interpretation is generally consistent with other research showing that exposure to cocaine or other stimulant drugs can promote habitual responding for sweet food or liquid in rats (Corbit *et al*, 2014; Nelson and Killcross, 2006; Nordquist *et al*, 2007). Overall, this analysis suggests that after dissipation of the drug satiating effects of pre-trial cocaine, there is a transient period when S rats are both ready to respond for cocaine and still partially under the anorexic effects of cocaine. This mixed behavioral state would explain why responding is biased toward cocaine if this option is available for choice or, if not, toward the uniquely available, albeit partially suppressed, nondrug option.

Paradoxically, the no-choice latencies of NS rats receiving pre-trial cocaine better conformed to our model (Figure 1a). Overall, the latency profile of NS rats was identical to that of S rats, except that they had much longer no-choice latencies for cocaine suggesting longer drug satiating effects. This interpretation was confirmed by longer satiety pauses during escalated levels of cocaine self-administration in NS rats than in S rats. As a result, the drug satiating effects of pre-trial cocaine lasted longer than its anorexic effects in NS rats, thereby

explaining why, unlike S rats, they did not shift their choice to cocaine but instead continued to choose sweet water – their normally preferred option. Of particular note, some NS rats (i.e., 2 rats out of 4) were not resistant to pre-trial cocaine on the first day of testing but acquired their resistance on the second testing day onward. The mechanisms underlying this abrupt change in drug-induced behavior is unknown at present and deserve to be studied in future research.

Overall, our data generally support the view that what a given individual will actually choose between cocaine and a normally preferred nondrug option after pre-trial cocaine is mainly determined by the difference in duration between the drug satiating and anorexic effects of cocaine. Individuals in whom the former effects are shorter than the latter effects will be induced to shift their choice to cocaine nearly exclusively, as was observed here in S rats. Inversely, individuals in whom the former effects are longer than the latter effects will instead continue to choose the preferred nondrug option, as in NS rats. What are the factors that explain this individual variation in the relative duration of the drug satiating and anorexic effects of cocaine remain to be elucidated. However, since the duration of these two effects varied independently across different individuals, this suggests that their underlying mechanisms are dissociable. A possible neurobiological mechanism involves differential cocaine-induced dopamine stimulation of D1R-expressing neurons in different subregions of the nucleus accumbens. There is some evidence that depending on their exact location within the nucleus accumbens, D1R-expressing cells can mediate cocaine reward (Lobo *et al*, 2010) and/or suppress responding for sweet food (Cui and Lutter, 2013; O'Connor *et al*, 2015), perhaps by inducing an aversive state (Al-Hasani *et al*, 2015). Regardless of the underlying neurobiological mechanisms, however, the present study raises the question of how individual variation in response to the effects of passive, pre-trial cocaine will translate in choice settings where these effects result from prior cocaine choices and can thus be self-cumulated. In a recent study, we found that virtually all rats rapidly develop a pattern of exclusive cocaine choices in such settings (Vandaele *et al*, 2016). If this outcome can be

extrapolated to the present study, then even NS rats should eventually develop, albeit perhaps more slowly, a pattern of exclusive cocaine choices in similar settings. If true, this would suggest that despite individual variation in the duration of the different carry-over effects of cocaine, the transition to exclusive cocaine choices would nevertheless remain quasi-inevitable in some settings despite access to a preferred nondrug option. Future research should be devoted to better define this apparent inevitability and its implications for animal models of addiction.

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FIGURE LEGENDS

Figure 1 Schematic representation of the hypothesis and the experimental procedure. (a) The two biphasic curves represent the hypothetical time course of the drug satiating (dark grey) and anorexic effects (light grey) of a pre-trial administration of cocaine (0.75 mg). The vertical dotted lines indicate from left to right: the time of pre-trial cocaine 5 min before the next trial; the time of trial onset (set to 0); and the time of choice per se. Behavioral latencies correspond to the time elapsed between trial onset and choice. It is expected that the latency to respond for cocaine when it is the only option available (white horizontal rectangle) should be shorter than that for sweet water (black horizontal rectangle). (b) Each panel represents a distinctive discrete-trials choice or no-choice procedure with a 10-min inter-trial interval. The two top panels represent the choice procedure without (baseline condition) or with non-contingent administration of cocaine (0.75 mg) before each choice trial (indicated by downward vertical arrows). During choice, rats were presented with levers C and S and could choose between them to obtain the corresponding reward. The bottom panels represent the no-choice procedure with non-contingent administration of cocaine before each trial. Rats were presented with only one lever (S or C) and could obtain the associated reward by pressing on it.

Figure 2 Escalation of cocaine self-administration and initial operant training for sweet water. (a) Mean number of self-administered cocaine injections (± s.e.m.) across 6-h sessions. The last 3 sessions alternated with sessions of access to sweet water. *, different from the first 6-h session ($p<0.05$, Tukey post hoc). (b) Mean time (± s.e.m.) to obtain the maximum possible

number of sweet water rewards (i.e., 20) across sessions. *, different from the first session ($p<0.05$, Tukey post hoc).

Figure 3 Choice between cocaine and sweet water. (a) Mean number (± s.e.m.) of cocaine and sweet water choices across sessions. There was a maximum of 20 choice trials. *, different from cocaine choices ($p<0.05$, Tukey post hoc). (b) Percent cocaine choices (± s.e.m.) across sessions. The horizontal dotted line represents the indifference level. *, different from the indifference level ($p < 0.05$, t-test). (c) Mean choice latency (± s.e.m.) across sessions. *, different from the first session ($p < 0.05$, Tukey post hoc).

Figure 4 Effects of pre-trial administration of cocaine on choice between cocaine and sweet water. (a) Mean % cocaine choices (± s.e.m.) during baseline (BL) or after pre-trial administration of cocaine (PTC). Each circle represents one rat. Pre-trial cocaine caused 11 rats to shift their choice to cocaine (open circle, rats S for Shifters) but had no effect on 2 rats (closed circles). (b) Mean % cocaine choices (± s.e.m.) in rats S during baseline or after pre-trial administration of cocaine. (c) Mean choice latency (± s.e.m.) in rats S during baseline or after pre-trial administration of cocaine. *, different from baseline ($p < 0.01$, Tukey post hoc). (d) % rats that choose cocaine on each successive trial (20 in total) during baseline or after pre-trial administration of cocaine. *, different from baseline ($ps < 0.05$, two-proportion z-test). (e) Mean choice latency (± s.e.m.) on each successive trial. *, different from baseline ($p < 0.01$, Tukey post hoc).

Figure 5 Duration of the drug satiating versus anorexic effects of pre-trial cocaine. (a) Mean latencies (± s.e.m) as a function of the option(s) available after pre-trial cocaine (i.e., cocaine and sweet water [choice]; cocaine only; sweet water only). *, different from the other condition ($p < 0.01$, Tukey post hoc). (b) Mean completed trials (± s.e.m.) as a function of the option(s) available. In each case, there were a maximum of 20 trials available per session.

Figure 6 Individual variation in response to pre-trial administration of cocaine. (a) Individual choice behavior in response to pre-trial cocaine across repeated sessions. Each circle represents one individual. All rats preferred sweet water during baseline (BL). A total of 8 rats were induced after pre-trial cocaine to shift their choice to cocaine during 6 consecutive sessions (S rats for Shifters, open circles). Four rats were not or only transiently influenced by pre-trial cocaine (NS rats for Non-Shifters, closed circles). One rat responded inconsistently to pre-trial cocaine (grey circle). (b) Mean % cocaine choices (± s.e.m.) and (c) mean choice latencies (± s.e.m.) in S and NS rats across repeated sessions with pre-trial cocaine. *, different from baseline ($p < 0.01$, Tukey post hoc); #, different from the first session with pre-trial cocaine. (d) Mean latencies (± s.e.m) in S and NS rats as a function of the option(s) available after pre-trial cocaine (i.e., cocaine and sweet water [choice]; cocaine only; sweet water only). *, different from baseline ($p < 0.01$, Tukey post hoc).

Figure 1

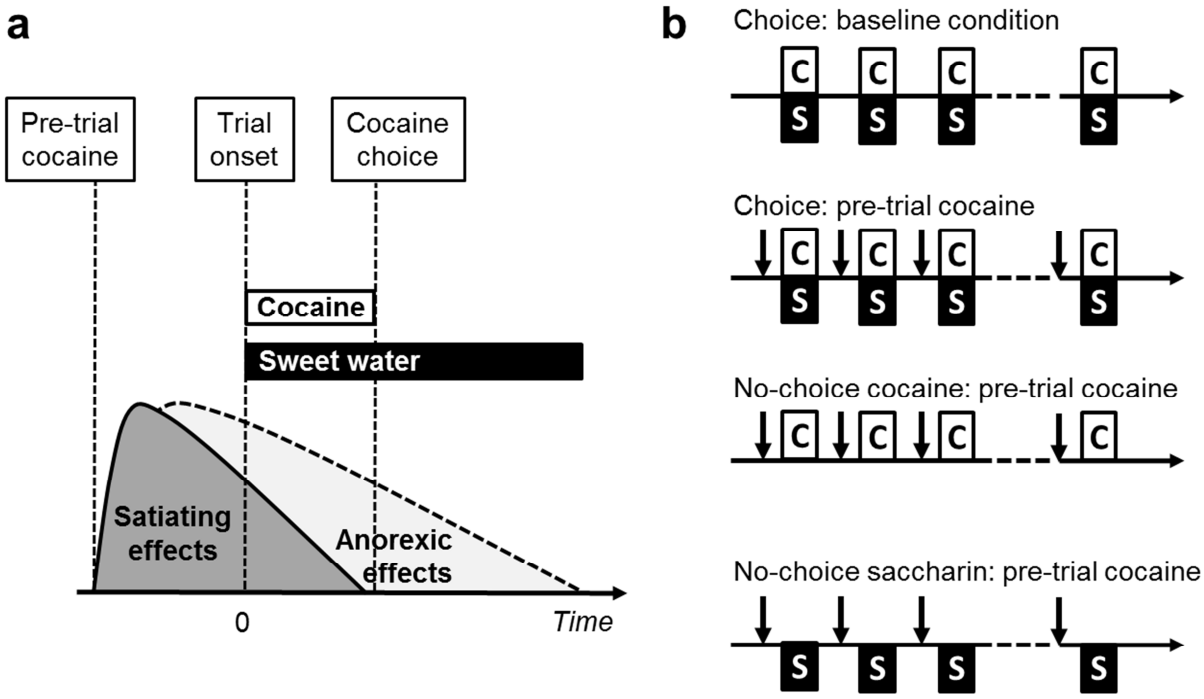

a
Pre-trial cocaine
Trial onset
Cocaine choice
Cocaine
Sweet water
Satiating effects
Anorexic effects
0
Time
b
Choice: baseline condition
Choice: pre-trial cocaine
No-choice cocaine: pre-trial cocaine
No-choice saccharin: pre-trial cocaine
C
S

Figure 2

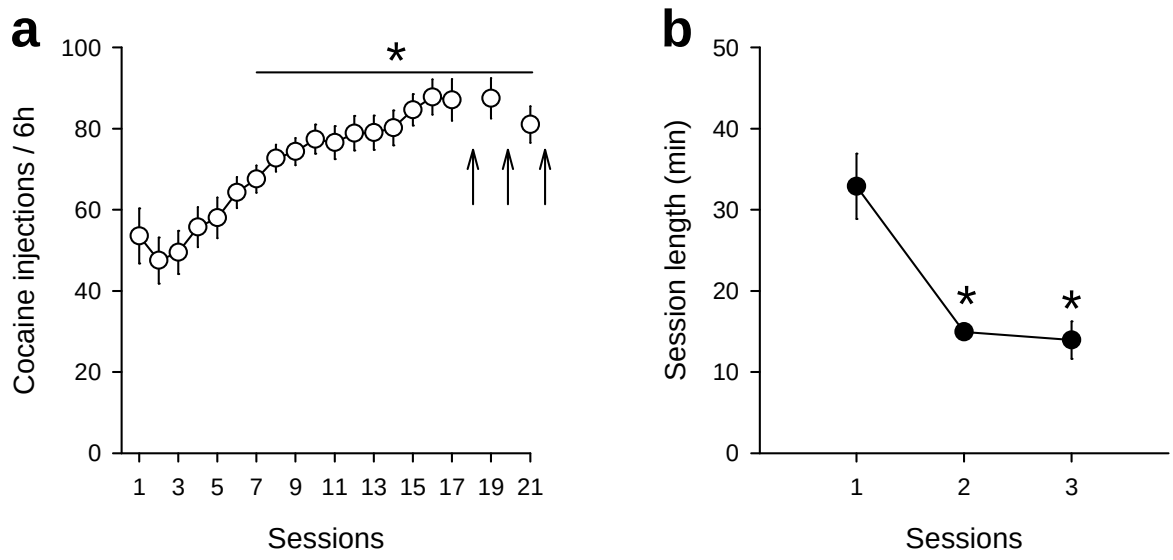

a
Cocaine injections / 6h
100
80
60
40
20
0
*
1 3 5 7 9 11 13 15 17 19 21
Sessions
b
Session length (min)
50
40
30
20
10
0
*
*
1 2 3
Sessions

Figure 3

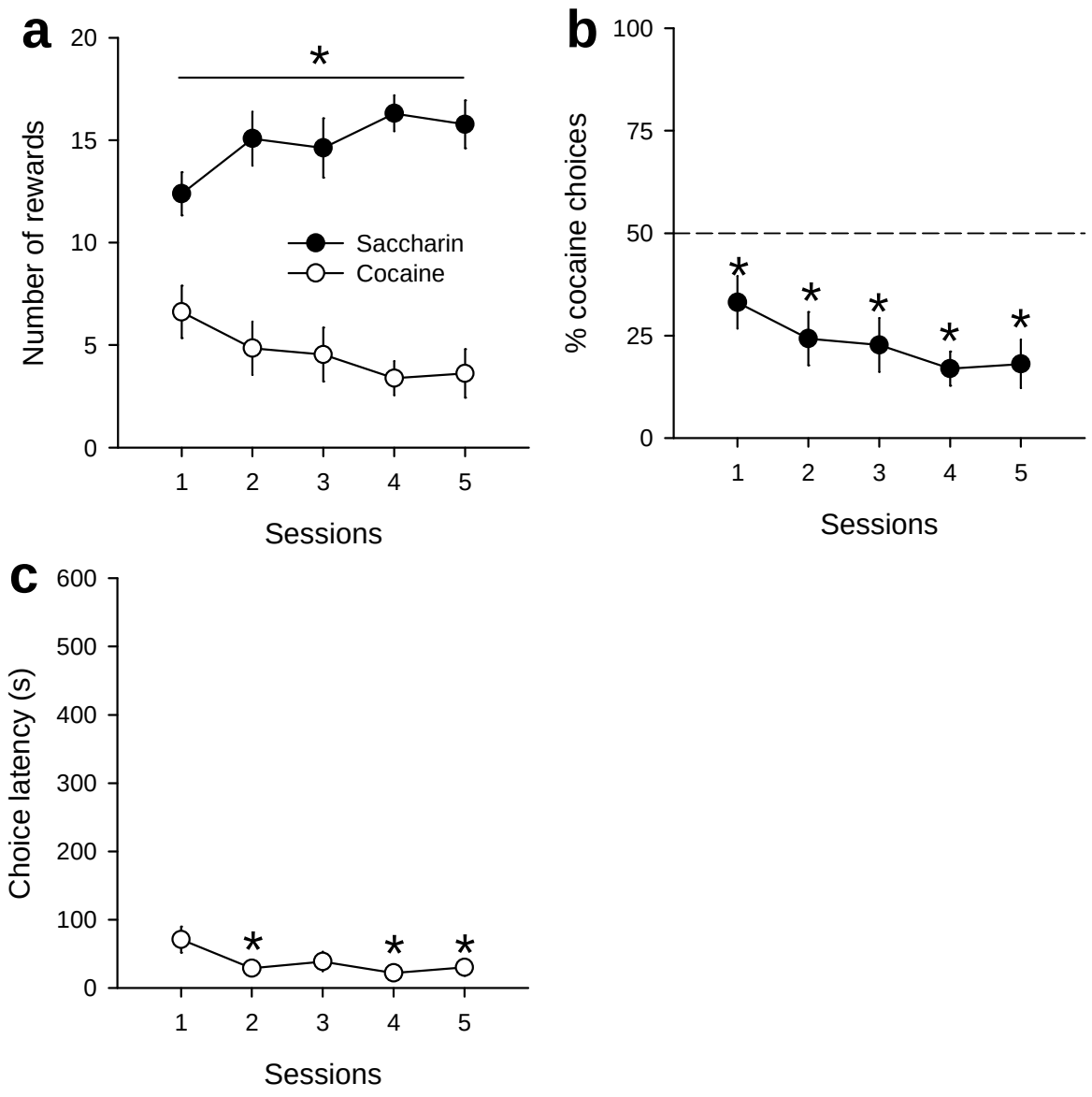

a
Number of rewards
20
15
10
5
0
*
Saccharin
Cocaine
1
2
3
4
5
Sessions
b
% cocaine choices
100
75
50
25
0
*
*
*
*
*
1
2
3
4
5
Sessions
c
Choice latency (s)
600
500
400
300
200
100
0
*
*
*
1
2
3
4
5
Sessions

Figure 4

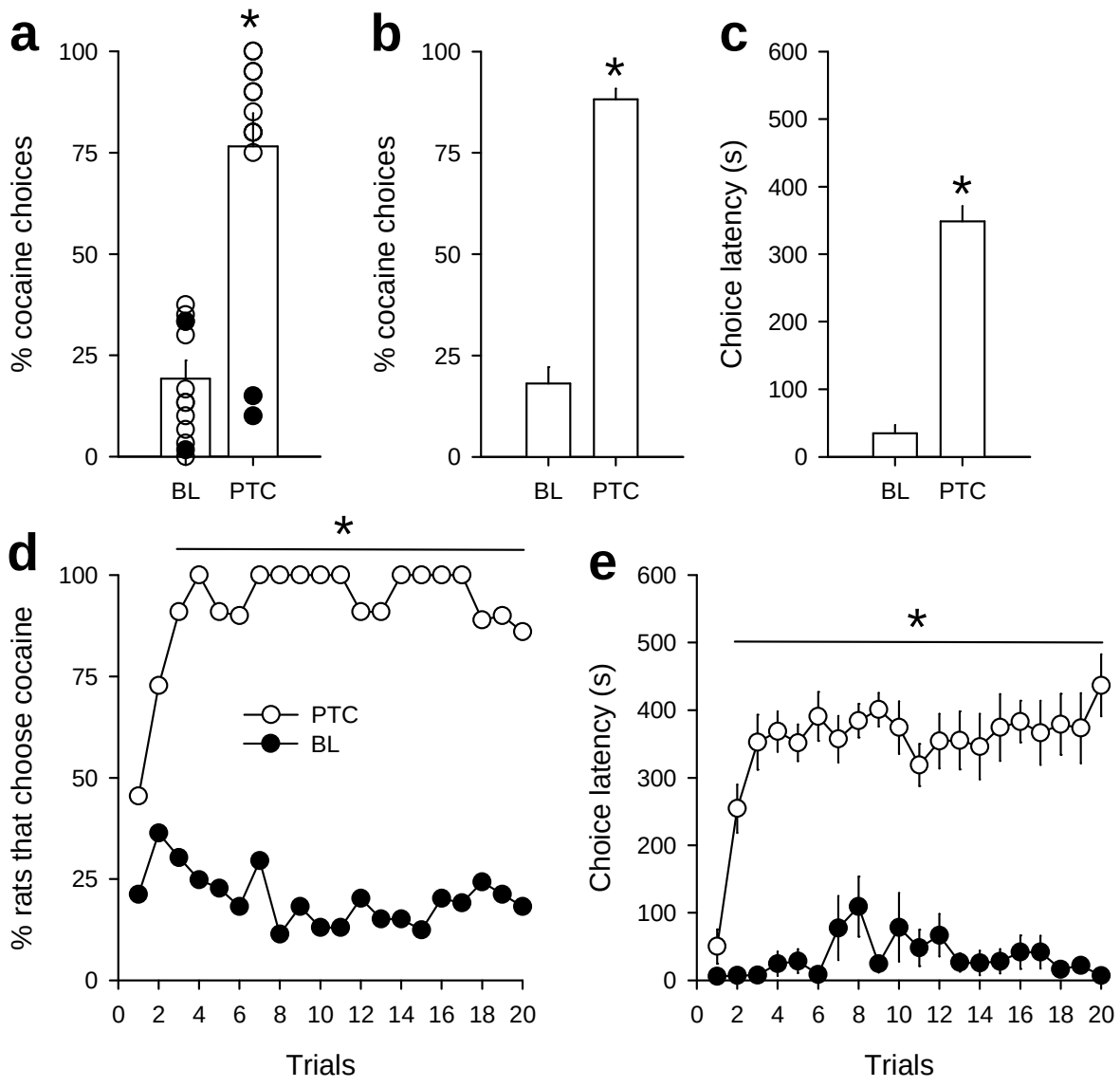

a
b
c
d
e
% cocaine choices
Choice latency (s)
BL
PTC
% rats that choose cocaine
Trials
PTC
BL
*

Figure 5

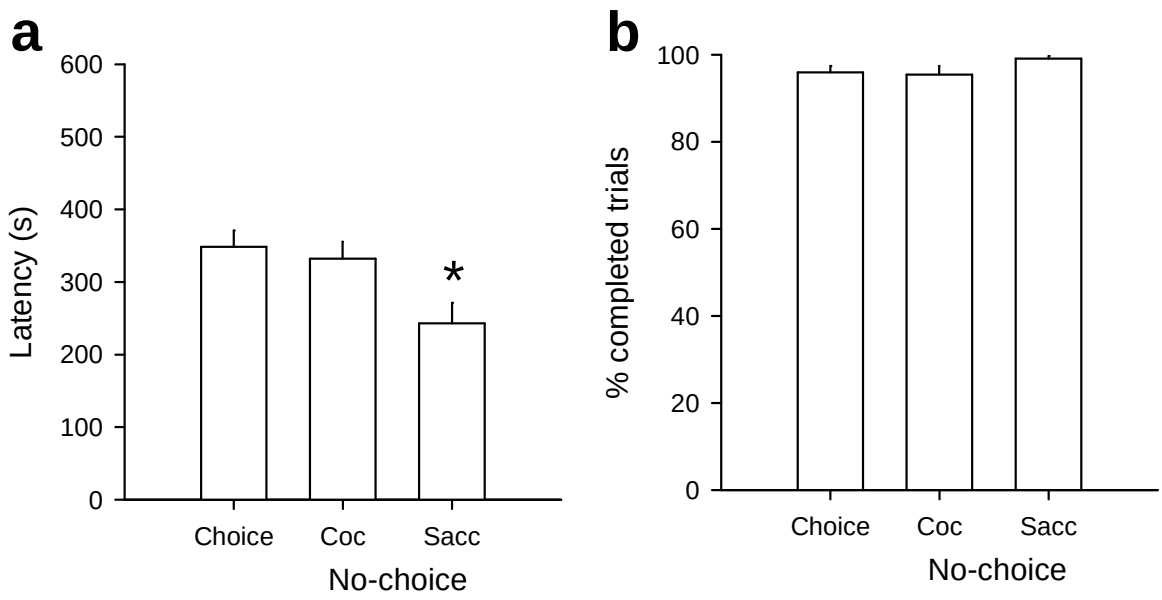
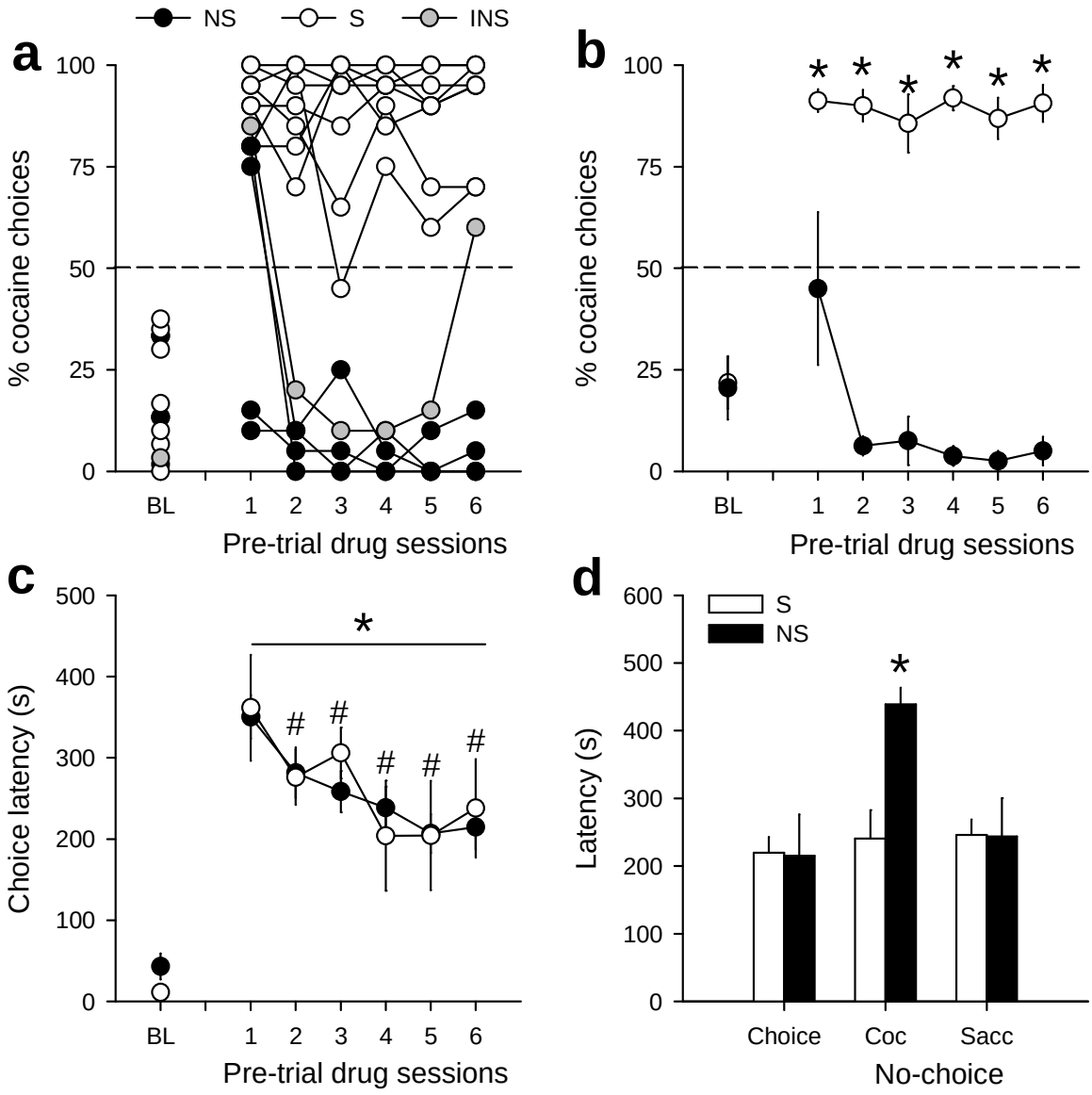

a
600
500
400
300
200
100
0
Latency (s)
*
Choice
Coc
Sacc
No-choice
b
100
80
60
40
20
0
% completed trials
Choice
Coc
Sacc
No-choice

Figure 6


NS
S
INS
a
% cocaine choices
100
75
50
25
0
BL
1
2
3
4
5
6
Pre-trial drug sessions
b
% cocaine choices
Pre-trial drug sessions
c
Choice latency (s)
500
400
300
200
100
0
Pre-trial drug sessions
d
S
NS
Latency (s)
600
Choice
Coc
Sacc
No-choice

8 CONCLUSÕES

Os estudos realizados no presente trabalho foram planejados para obter respostas acerca da influência de fatores como o ambiente e a escolha na busca pelo efeito da cocaína, bem como no comportamento para aquisição de uma nova escolha por obter o efeito. Além disso, avaliou-se também o efeito neuroprotetor do AE em relação ao dano oxidativo sobre o hipocampo e o CPF dos animais. Dessa forma, foi possível concluir que:

- O modelo adaptado de CPP foi eficaz para alterar a mudança do comportamento de condicionamento à cocaína pela sacarina;
- A presença de sacarina diminuiu o condicionamento a cocaína no CPP, mesmo entre os animais mais vulneráveis (criados em ambiente padrão);
- O ambiente enriquecido diminuiu dano de DNA e promoveu uma menor formação de ROS no hipocampo e CPF dos animais expostos à cocaína;
- A escolha entre uma injeção de cocaína e uma opção *nondrug* foi derminada pela duração do efeito da droga, ou seja, a permanência de um estado de intoxicação determina a escolha por mais cocaína;
- Infere-se que a escolha por cocaína ou pela solução doce pode estar condicionada a duração do efeito de saciedade e do efeito anorético da cocaína.

Ante o exposto, é possivel evidenciar que existe uma relação direta entre a oferta de um ambiente diverso e a escolha entre a droga e uma opção *nondrug*. Ainda que os efeitos da cocaína, tanto comportamentais quanto de neurotoxicidade, podem ser diferntes, dependendo dos fatores ambientais implicados.

Ainda, encontramos uma variação individual interessante no tempo de efeito da cocaína, onde os fatores que explicam essa variação ainda precisam ser elucidados. De maneira geral, pesquisas futuras devem ser dedicadas a melhor definir a aparente inevitabilidade da escolha por cocaína em função da duração do seu efeito, bem como criar uma maior diversidade de modelos experimentais para entender as implicações destes fatores em estudos de dependência usando animais de laboratório.

ANEXOS

Anexo A - PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

CEUA –COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

1) PROTOCOLO Nº:126/13 Parecer 224/13

2) DATA DO PARECER:14/07/13

3) TÍTULO DO PROJETO:

Efeitos do enriquecimento ambiental na autoadministração de cocaína em ratos

4) PESQUISADOR RESPONSÁVEL:

Helena Barros

5) RESUMO DO PROJETO:

Serão usados 160 animais. Um grupo criado em ambiente normal e outro em ambiente enriquecido. Aos 21 d ratas fêmeas e machos do grupo testosterona receberão o hormônio.Aos 51 dias animais serão treinados em caixas de autoadministração IV de drogas, onde receberão metilfenidato ou salina. Serão submetidos a cirurgia para colocação de cateter na jugular. Será comparado a quantidade e frequência da autoadministração das duas substancias e posteriormente verificadas alterações sistemas gabaérgicos e dopaminérgicos e níveis BDNF e a influencia do ambiente de criação nesta modulação.

6) OBJETIVOS DO PROJETO:

Verificar se o ambiente enriquecido altera o consumo e o efeito de reforço de psicoestimulantes em ratos submetidos à autoadministração

7) FINALIDADE DO PROJETO: ☐ Ensino ☒ Pesquisa

8) ITENS METODOLÓGICOS E ÉTICOS DO PROJETO:

Título	[x]	Adequado	[ ]	Comentários
Introdução	[x]	Adequada	[ ]	Comentários
Objetivos	[x]	Adequados	[ ]	Comentários
Relevância e Justificativa	[x]	Adequados	[ ]	Comentários
Materiais e Métodos	[x]	Adequados	[ ]	Comentários
Cronograma para execução da pesquisa	[x]	Adequado	[ ]	Comentários
Orçamento e fonte financiadora	[x]	Adequados	[ ]	Comentários
Referências Bibliográficas	[x]	Adequadas	[ ]	Comentários

9) O PROJETO ESTÁ ADEQUADO À LEGISLAÇÃO VIGENTE:

[x] Sim [ ] Não

10) INFORMAÇÕES RELATIVAS AOS ANIMAIS:

Grau de dor/estresse: **B** [ ] **C** [ ] **D** [x] **E** [ ]

Justifique:

Espécie: Wistar **Número Amostral:** 160

Redução Amostral: [ ] Sim [x] Não

Justifique:

Substituição de Metodologia: [ ] Sim [x] Não

Se achar necessário, justifique e sugira uma nova metodologia:

Aprimoramento da Metodologia: ☐ Sim ☒ Não
Se achar necessário, justifique e sugira aprimoramentos da metodologia:

Sugere-se ANOVA 3 vias visto que são 3 variáveis (sexo, MF, AE)

Acomodação e manutenção dos animais: ☒ Adequada ☐ Inadequada
Se achar inadequada cite abaixo as melhorias necessárias:

Manipulação dos animais: ☒ Adequada ☐ Inadequada
Se achar inadequada cite abaixo as melhorias necessárias:

Analgesia dos animais (se aplicável): ☒ Adequada ☐ Inadequada
Se achar inadequada cite abaixo as melhorias necessárias com analgésico substituto:

Anestesia dos animais (se aplicável): ☒ Adequada ☐ Inadequada
Se achar inadequada cite abaixo as melhorias necessárias com anestésico substituto:

Rever no formulário valores de xilasina e cetamina diferentes do corpo do projeto e invertidos.

Eutanásia dos animais (se aplicável): ☒ Adequada ☐ Inadequada
Se achar inadequada cite abaixo as melhorias necessárias com metodologia substituta:

Local de Realização (Biotério/Labotarório): Laboratório Farmacologia

Outra instituição. Qual?

11) CRONOGRAMA DE UTILIZAÇÃO DE ANIMAIS

Data	Espécie	Sexo	Quantidade
Jan 2014	wistar	M e F	80 + 80

12) RECOMENDAÇÃO:

[x] Aprovado

[ ] Com Pendência

[ ] Não aprovado

Comentários gerais sobre o projeto:

Anexo B: - Normas do Jornal Psychopharmacology-

Instructions for Authors

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- Book

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- Online document

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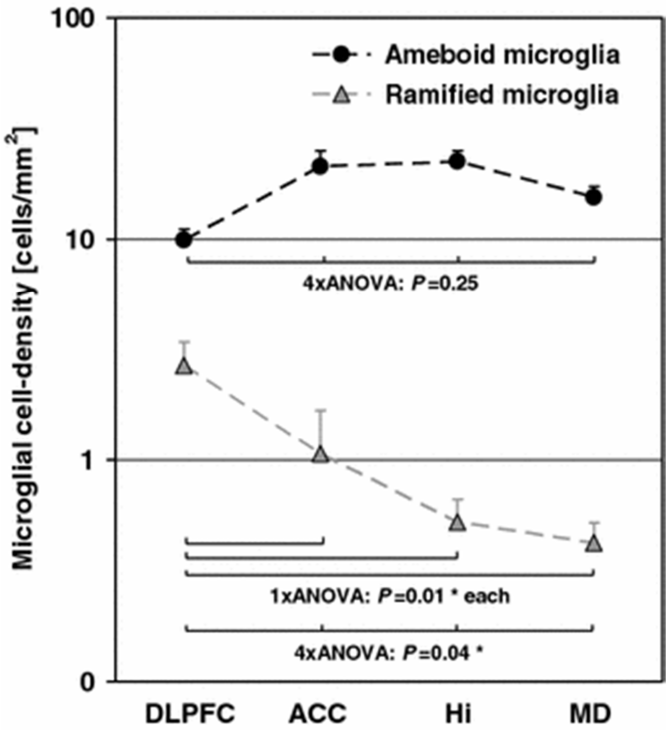

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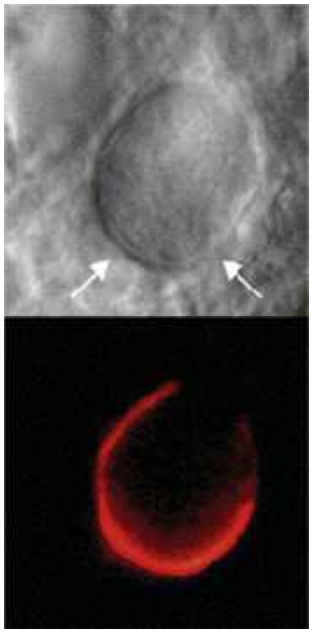

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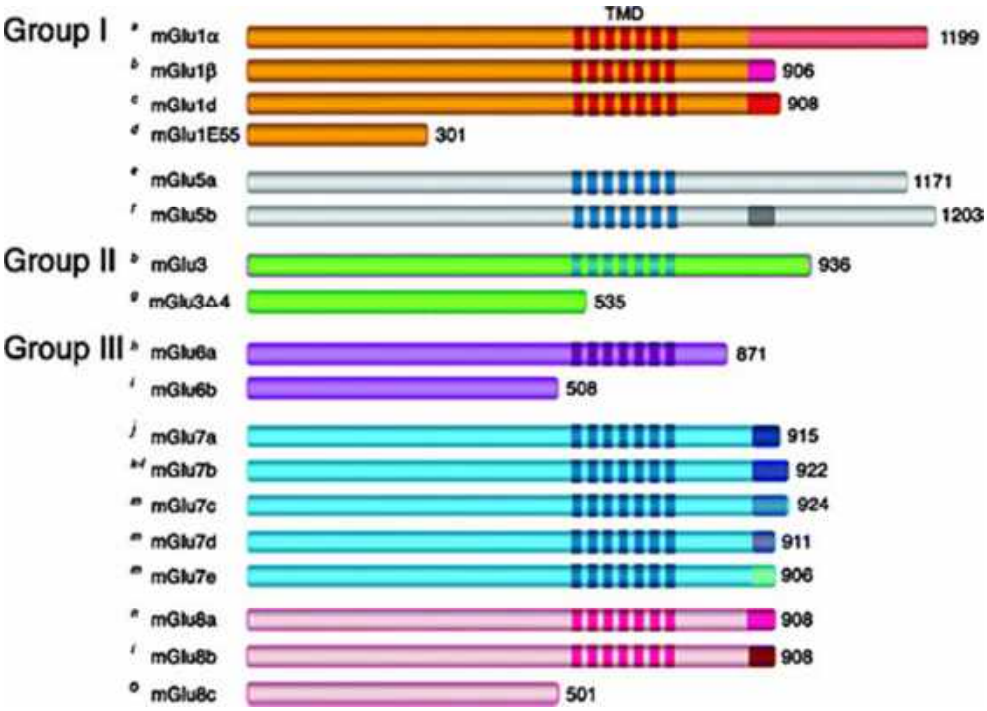

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