UNIVERSIDADE DE SÃO PAULO
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Papel dos mecanismos GABAérgicos do colículo inferior e da substância cinzenta periaquedutal na interface sensoriomotora do medo e ansiedade

VIVIANE MITSUKO NEVES SAITO

Tese apresentada à Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto da Universidade de São Paulo, como parte dos requisitos para a obtenção do título de Doutor em Ciências, Área: Psicobiologia.

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Orientador: Prof. Dr. Marcus Lira Brandão

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AUTORIZO A REPRODUÇÃO E DIVULGAÇÃO TOTAL OU PARCIAL DESTE TRABALHO, POR QUALQUER MEIO CONVENCIONAL OU ELETRÔNICO, PARA FINS DE ESTUDO E PESQUISA, DESDE QUE CITADA A FONTE.

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A meus pais, Takanori Saito e Maria Eva Neves Saito,
e meu irmão Yves Makoto Neves Saito,
razões de minha existência.

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"Whenever I feel really alone, I just sit and stare into the night sky. I've always thought that one of those stars was *my* star, and at moments like this, I know that *my* star will always be there for me. Like a comfortable voice saying, '*Don't give up, kid*.'"

Charlie Brown
The Peanuts Movie (2015)

RESUMO

SAITO, V.M. **Papel dos mecanismos GABAérgicos do colículo inferior e da substância cinzenta periaquedutal na interface sensoriomotora do medo e ansiedade**. 2016. 109 f. Tese (Doutorado) – Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2016.

As reações incondicionadas de defesa observadas em mamíferos são organizadas pelo Sistema Encefálico de Aversão (SEA), composto, entre outras estruturas, pela substância cinzenta periaquedutal dorsal (SCPd) e o colículo inferior (CI). Tem sido proposto que o CI seja parte do circuito sensoriomotor para os estímulos auditivos de natureza aversiva e a SCPd como a principal via de saída (*output*) do SEA para a elaboração de comportamentos defensivos. Ambas as estruturas são reguladas tonicamente pelo neurotransmissor inibitório ácido gama-aminobutírico (GABA). Este trabalho aborda a mediação química GABA/Benzodiazepínica (BZD) do processamento da informação aversiva no CI e das respostas de medo elaboradas pela SCPd. Grupos independentes de animais submetidos ao implante de quimitrodos (eletrodos acoplados a cânulas-guia para injeção de drogas) foram usados para avaliar no CI e SCPd os efeitos de injeções locais de muscimol (agonista de receptores GABA-A), semicarbazida (inibidor da síntese da enzima precursora do GABA – descarboxilase do ácido glutâmico) ou midazolam (agonista BZD). Foram registrados potenciais evocados auditivos (PEA) no CI como medida eletrofisiológica da ativação neuronial, além da determinação dos limiares de congelamento e fuga, com o procedimento de estimulação elétrica (EE), tanto do CI quanto da SCPd. A mesma abordagem farmacológica com injeções de drogas intra-CI foi empregada em animais submetidos ao teste do Labirinto em Cruz Elevado (LCE), um modelo animal tradicional de ansiedade. Adicionalmente, investigou-se a participação de ambas as estruturas na expressão do comportamento de desligar uma luz de intensidade aversiva em um novo teste de medo incondicionado (*Light Switch Off Test*; LSOT) recentemente proposto pelo nosso grupo. Encontramos uma clara segregação funcional entre a porção dorsal e ventral do CI, sendo a última envolvida nos comportamentos defensivos. Mecanismos GABAérgicos em ambas as estruturas influenciam a amplitude do PEA e o congelamento pós-fuga da EE, sugerindo uma relação funcional entre as duas estruturas. Já no LSOT, os resultados indicam o envolvimento de mecanismos GABAérgicos do vCI, mas não da SCPd, na modulação da resposta incondicionada à luz em ratos. Os resultados obtidos permitem ampliar o conhecimento atual sobre a neurobiologia dos estados de medo e ansiedade, em uma abordagem integrada dos mecanismos de processamento das informações sensoriais e da expressão de reações de defesa.

Palavras-chave: Colículo inferior, GABA, Limiares de comportamentos defensivos, Medo, Potenciais evocados auditivos, Substância cinzenta periaquedutal.

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ABSTRACT

SAITO, V.M. **Role of GABAergic mechanisms in the inferior colliculus and periaqueductal gray matter on the sensorimotor gating of fear and anxiety**. 2016. 109 p. Thesis (Doctorate) – Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2016.

Unconditioned defense reactions observed in mammals are organized by the Brain Aversive System, comprising, among other structures, the dorsal periaqueductal gray matter (dPAG) and the inferior colliculus (IC). It has been proposed that the IC is part of the sensorimotor circuitry that processes aversive auditory information and the dPAG is considered the main neural substrate for the expression of defensive behaviors. Both structures are tonically regulated by the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). This work addresses the chemical mediation of GABA/Benzodiazepine (BZD) on aversive information processing in the IC and the elaboration of fear responses by dPAG. Independent groups of animals implanted with chemitrodes (electrodes attached to a guide cannula for drug injection) have been used to evaluate the IC and dPAG regarding the effects of local injections of GABAergic agents (muscimol, semicarbazide, and midazolam). Auditory evoked potentials (AEP) have been recorded in the IC as a measure of electrophysiological neuronal activation, in addition to determining the thresholds of defensive freezing and flight behaviors, using the electrical stimulation (EE) procedure in both IC and dPAG. The same pharmacological regimen of drug injections intra-dPAG and intra-CI have been applied to animals subjected to the elevated plus maze (EPM), a well-known animal model of anxiety, and also to a novel animal test for innate fear (*Light Switch Off Test*, LSOT) that has been developed and proposed by our group. We found a clear functional segregation between the dorsal and ventral portions of the IC, the latter being the specific collicular substrate of defensive behaviors. GABAergic mechanisms in both structures influence the amplitude of the AEP and post-stimulation freezing of EE, suggesting a functional link between the two structures. In the LSOT, our data indicate the involvement of GABAergic mechanisms of the ICv, but not the dPAG, in the modulation of the unconditioned response to light in rats. These original findings presented here contribute to broaden the current knowledge on the neurobiology of fear and anxiety, in an integrative approach of the mechanisms underlying sensory processing and the expression of defensive behaviors.

Keywords: Inferior colliculus, GABA, fear, auditory evoked potentials, periaqueductal gray matter.

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INTRODUÇÃO

A emissão de uma resposta rápida e robusta em face de estímulos visuais e auditivos ameaçadores é uma característica de grande valor evolutivo em espécies sujeitas à predação, como os roedores. Estes animais são capazes de elaborar diferentes estratégias de acordo com as características das ameaças com as quais são confrontados. Dessa forma, a fuga é a reação natural quando um predador está próximo, enquanto que o comportamento de imobilidade defensiva (ou congelamento) é mais comum quando o animal percebe o perigo e este se encontra à distância (Uribe-Marino *et al.*, 2012; Almada *et al.*, 2015). O congelamento pode ser uma estratégia adotada quando o animal necessita adquirir informações sobre o ambiente no qual exista um estímulo aversivo distal ou mesmo para simular a morte (imobilidade tônica) quando deparados com um perigo inescapável (Borelli *et al.*, 2006; Coutinho & Menescal-de-Oliveira, 2010). Tais comportamentos, que servem ao propósito etológico de preservar a integridade do indivíduo, são coletivamente estudados como comportamentos defensivos e estão presentes em toda a escala filogenética animal. Há relatos de que pacientes exibem alterações fisiológicas e relatam sensações extremamente desagradáveis, similares àquelas que ocorrem durante ataques de pânico, devido à estimulação elétrica da substância cinzenta periaquedutal dorsal (Nashold *et al.*, 1969). A estimulação elétrica desta região é utilizada como um modelo de ataques de pânico, pelas similaridades encontradas entre os sintomas manifestados em humanos e as respostas comportamentais em roedores submetidos ao procedimento (Schenberg *et al.*, 1983; Graeff *et al.*, 1986a). Entretanto, ao nos referirmos aos mecanismos neurais que detectam e respondem a ameaças em roedores, entendemos que estes não são os mesmos que dão origem à experiência subjetiva e consciência do medo, acessíveis em humanos. Esta é uma importante observação quando adotamos ao longo deste trabalho o termo geral "medo" para nos referirmos de uma maneira sucinta a um "estado orgânico defensivo" em ratos, gerado pela ativação de um circuito defensivo de sobrevivência (Ledoux, 2014).

Substrato neural dos comportamentos defensivos

A natureza multidimensional da emocionalidade sugere que substratos biológicos distintos possam estar envolvidos no controle da expressão fenotípica da emocionalidade. As reações de defesa são organizadas pelo Sistema Encefálico de Aversão (SEA), composto pelo hipotálamo medial, amígdala, substância cinzenta periaquedutal dorsal (SCPd) e córtex pré-

frontal (Graeff *et al.*, 1986a). Nos últimos anos, têm sido considerados como parte do SEA os colículos superiores e inferiores, áreas neuroanatômicas responsáveis pelo processamento visual e auditivo, respectivamente (Brandão *et al.*, 1999).

O colículo inferior (do lat. *collicullus*; "colinas") é uma estrutura mesencefálica bilateral arredondada que preserva similaridades entre diversas espécies de mamíferos. Descrito em 1911 como parte dos "*tubercules quadrijumeaux*" por Ramón y Cajal, o colículo inferior (CI) é conhecido tradicionalmente como um "relé" obrigatório para vias auditivas (Aitkin, 1986). O CI possui três divisões principais, segundo critérios citoarquitetônicos: o núcleo central (essencial para funções auditivas normais), o córtex externo (multissensorial; integra informações auditivas a somatossensoriais) e o córtex dorsal que recebe a maioria de suas aferências do córtex cerebral e cujo papel na audição ainda não foi totalmente esclarecido (Winer & Schreiner, 2005).

Nos últimos 30 anos, a importância do CI tem sido proposta ir além de processos auditivos, sendo esta estrutura capaz de mediar o também o comportamento emocional (Graeff *et al.*, 1986b; Brandão *et al.*, 1993; Coimbra & Brandão, 1993; Brandão *et al.*, 1994; Cardoso *et al.*, 1994). O CI tem sido envolvido, assim como outras estruturas do SEA, na mediação de respostas defensivas eliciadas por estímulos de perigo – particularmente os de natureza auditiva – em nível de teto mesencefálico (Brandão *et al.*, 1988; Brandão *et al.*, 1999). Sugere-se que o CI possa atuar como um filtro sensoriomotor mesencefálico ("*mesencephalic sensorimotor gating*") por integrar a informação sensorial auditiva de natureza aversiva com a resposta motora para eliciar comportamentos defensivos (Brandão *et al.*, 2005). Isto se baseia em evidências de que o aumento gradual da estimulação elétrica no núcleo central do CI (CIC) elicia um comportamento de defesa progressivamente mais intenso, caracterizado por estados de alerta, congelamento e fuga, seguidos por respostas autonômicas similares àquelas causadas pelo medo eliciado diante de situações ameaçadoras (Melo & Brandão, 1995). Estima-se que, no CI, os potenciais de ação possam reverberar, aumentando a intensidade e duração da resposta induzida pelo estímulo auditivo aversivo (Castellan-Baldan *et al.*, 2006). Adicionalmente, a estimulação química com NMDA nas porções ventral (vCI) ou dorsal (dCI) do colículo inferior induz diferentes níveis de expressão de Fos em outras estruturas cerebrais. Quando o NMDA é injetado intra-dCI, as áreas que apresentam aumento na expressão de Fos são o núcleo geniculado medial, colículos superiores, SCPd e *locus ceruleus*, enquanto que a estimulação intra-vCI causa maior imunorreatividade no córtex pré-límbico, cingulado, amígdala basolateral e medial, substância cinzenta periaquedutal ventral, núcleo cuneiforme e *locus ceruleus*. (Ferreira-Netto *et al.*,

2007). Assim, a porção ventral do CI é considerada parte do circuito neural do medo, enquanto que o dCI possui conexões com estruturas encefálicas que coordenam o processamento da informação auditiva e a integração multissensorial e, portanto, não faz parte do SEA.

Neurotransmissão GABAérgica

O ácido gama-aminobutírico é o principal neurotransmissor inibitório do sistema nervoso central e está presente em grande densidade nas estruturas do teto mesencefálico (Brandão *et al.*, 2005). O interesse específico pelo sistema GABAérgico tem origem na eficácia clínica de drogas utilizadas no tratamento da ansiedade patológica e em pesquisas que utilizam agonistas e antagonistas GABAérgicos (Sandford *et al.*, 2000). Sabe-se que a ativação farmacológica dos receptores do tipo $GABA_A$ resulta em diminuição da ansiedade (Argyropoulos *et al.*, 2000). Outras estratégias de manipulação da transmissão GABAérgica, com o uso de agonistas inversos e ligantes para os demais sítios de ligação alostéricos do receptor $GABA_A$, também tem importância clínica. Os benzodiazepínicos (BDZ) têm sido ferramentas úteis no estudo dos mecanismos neurobiológicos da ansiedade. Seu modo de ação consiste na modulação dos efeitos do GABA através da ligação com um sítio específico para BZD em receptores $GABA_A$.

Componentes sensoriais e motores da expressão das reações de defesa

Já é bem estabelecido que agonistas BZD como o midazolam atenuam a expressão de comportamentos do tipo ansiedade de roedores submetidos ao Labirinto em Cruz Elevado (LCE) (Cruz *et al.*, 1994; Anseloni *et al.*, 1995; Albrechet-Souza *et al.*, 2005), um dos modelos animais mais utilizados para detectar a ação ansiolítica de drogas. O teste é baseado na aversão natural dos roedores a espaços abertos e foi validado para ratos (Pellow *et al.*, 1985) e camundongos (Lister, 1987). As medidas complementares avaliadas no LCE, tais como a exploração da extremidade dos braços abertos, esticamentos e autolimpeza permitem uma análise etofarmacológica mais aprofundada de diferentes aspectos da ansiedade e do modo de ação de drogas ansiolíticas (Cruz *et al.*, 1994; Rodgers & Johnson, 1995; Anseloni & Brandão, 1997; Albrechet-Souza *et al.*, 2008). Outro modelo largamente empregado no estudo

das reações defensivas incondicionadas é o procedimento de estimulação elétrica, ao qual o CI é particularmente responsivo (Cuadra *et al.*, 2000; Lamprea *et al.*, 2002) e que também é sensível à ação antiaversiva dos benzodiazepínicos (Melo *et al.*, 1992; Pandossio & Brandão, 1999). Além disso, a observação do funcionamento basal da resposta eletrofisiológica evocada por estimulação acústica aversiva no colículo inferior é uma medida útil ao se tratar de uma estrutura dedicada à recepção de informações sensoriais auditivas (Meeren *et al.*, 2001; Nobre & Brandão, 2011).

Tendo em vista o estado-da-arte no que se refere à participação do CI na organização de reações de defesa e as evidências obtidas recentemente pelo nosso grupo, esta investigação foi conduzida sob a hipótese de que o CI funciona como um relé para a informação aversiva de origem incondicionada, independente da natureza auditiva do estímulo, apresentando segregação funcional entre suas porções; e que esta função de *input* é regulada por mecanismos GABAérgicos. Ficamos também interessados em investigar neste trabalho em que medida estes mecanismos que regulam o processamento da informação aversiva de passagem pelo CI se associam ao bem conhecido papel dos mecanismos GABAérgicos que exercem uma inibição tônica sobre os substratos neurais do comportamento defensivo organizado na SCPd.

Modelos animais

Modelos animais são amplamente utilizados para estudo de condições clínicas. Para que seja consolidado, um modelo animal deve atender aos critérios de validade de face (analogias fenomenológicas e patofisiológicas com a condição clínica em estudo), validade de construto (etiologia comparável) e validade preditiva (tratamentos usados na clínica devem ser eficazes quando utilizados no modelo). Diferentemente dos modelos animais, que podem ser definidos como um organismo ou estado particular de um organismo que reproduz aspectos de determinada patologia humana, os testes fornecem apenas uma medida comportamental ou fisiológica restrita, criado no intuito de analisar o efeito pontual de manipulações genéticas, farmacológicas ou ambientais. Ambos podem ser classificados como condicionados (quando envolvem algum componente de aprendizado na tarefa) ou incondicionados (quando baseiam-se em comportamentos inatos do organismo).

Fomentada pelas recentes discussões acerca da ética em experimentação animal, esforços têm sido direcionados a aperfeiçoar as metodologias empregadas em pesquisa básica,

sobretudo aquelas relacionadas à Neurobiologia do Comportamento. Nosso grupo no Laboratório de Neuropsicofarmacologia (FFCLRP, USP), dando continuidade ao estudo iniciado por Reis *et al.* (2004), tem investido no desenvolvimento de uma opção aos modelos e testes animais existentes, que potencialmente desencadeiam estados de medo e ansiedade. Baseado em um comportamento naturalmente motivado (cessar um estímulo aversivo), o teste denominado "*light switch-off test*", explora a aversão natural à luz apresentada por roedores. O estímulo luminoso *per se* evoca a resposta de desligar a luz, que é realizada quando o animal cruza de um lado a outro nos compartimentos de uma caixa de esquiva, sem a necessidade de choque nas patas ou condicionamento prévio. É um teste de fácil execução, reprodutível e minimamente estressante ao animal experimental, pois não envolve choques ou outros estímulos demasiadamente dolorosos.

A importância da visão na detecção de estímulos aversivos por roedores vem sendo discutida há muitos anos na literatura (Morato, 2006). Quando os estímulos auditivos e olfativos são controlados, a aversão é deflagrada pela entrada de luz e pela formação de imagens na retina (Morato, 2006). Entre as razões pelas quais a luz intensa é um estímulo aversivo para ratos albinos é a falta de pigmento na íris e coroide, o que reduz a habilidade de se adaptar à luz e predispõe a danos visuais (Stryjek *et al.*, 2013). Embora exista um corpo de evidências que consolida a luz em si como um estímulo aversivo, ainda não há a sistematização de um teste que utilize somente este estímulo incondicionado, sendo que em testes onde se utiliza luz, esta é um estímulo condicionado que precede o evento de interesse (p.ex. choque, recompensa). O procedimento de validação deste teste é discutido no ANEXO 2.

OBJETIVOS

Neste estudo investigamos o papel da neurotransmissão GABA/BZD do colículo inferior e da substância cinzenta periaquedutal dorsal na interface sensoriomotora do medo e ansiedade. Especificamente, analisamos como se dá a mediação química do processamento de informações aversivas (por meio da medida de potenciais evocados auditivos - PEA) e da expressão de reações defensivas (através das medidas dos limiares de fuga e da duração do congelamento pós-fuga determinados pelo procedimento de estimulação elétrica), em uma abordagem integrada com um modelo animal tradicional de ansiedade (Labirinto em Cruz Elevado - LCE). O trabalho foi subdividido em três etapas:

EXPERIMENTO 1: Analisamos os efeitos da droga benzodiazepínica midazolam, em três doses distintas, injetada nas porções dorsal ou ventral do colículo inferior de ratos submetidos ao LCE e ao procedimento de estimulação elétrica do CI. Ao obter resultados consistentes sobre a reatividade farmacológica dessas regiões ao benzodiazepínico, passamos a estudar os efeitos do muscimol e da semicarbazida injetados na porção ventral do CI sobre as respostas defensivas induzidas pela estimulação elétrica do CI ventral.

EXPERIMENTO 2: Investigar a neurotransmissão GABAérgica nesta estrutura com o uso do agonista $GABA_A$ muscimol, do inibidor da síntese da enzima precursora do GABA descarboxilase do ácido glutâmico (semicarbazida) e do midazolam (agonista BZD) diretamente injetados na porção ventral do CI, e analisar seu efeito sobre os potenciais evocados auditivos (registrados no CI) e estimulação elétrica (tanto do CI quanto da SCPd). Nesta etapa, o objetivo foi estudar uma possível relação funcional entre o vCI e a SCPd através da injeção de drogas GABA-BZD em uma área e da estimulação aversiva da outra estrutura. Esta abordagem foi precisamente utilizada em outros estudos quando foi investigado o papel das vias nigrocoliculares GABAérgicas sobre o comportamento defensivo induzido por estimulação aversiva do teto mesencefálico (Coimbra & Brandão, 1993) e Twardowsky & Coimbra (2015) estudaram farmacologicamente a conexão CI - SCPd.

EXPERIMENTO 3: Avaliar como a neurotransmissão GABAérgica nessas estruturas modula a expressão de um comportamento defensivo eliciado por estímulo luminoso, em um novo teste animal de medo inato ("*light switch-off test*"), proposto por este Laboratório de Neuropsicofarmacologia.

EXPERIMENTO 1

Segregação funcional entre as porções dorsal e ventral do CI na expressão e processamento da informação aversiva, observada no LCE e estimulação elétrica, mediante tratamento com benzodiazepínico (midazolam) em distintas doses

Materiais e métodos

Animais e cirurgia

Foram utilizados 186 ratos Wistar machos, com peso entre 250g e 275g, provenientes do biotério central da Universidade de São Paulo, *campus* de Ribeirão Preto. Os animais foram mantidos em biotério setorial por um período mínimo de habituação de 48h, com livre acesso a água e alimento. Estes animais foram submetidos à cirurgia estereotáxica para implantação de cânula-guia direcionada às duas porções do colículo inferior (coordenadas para a porção dorsal: AP: -8,5; ML: -1,6; DV: -3,3; coordenadas para a porção ventral: AP: -8,5; ML: -1,6; DV: -4,3;) ou quimitrodos (coordenadas AP: -8,5; ML: -1,6; DV: -5,3) direcionados especificamente ao vCI (Paxinos & Watson, 2006). Quimitrodos são cânulas-guia anexadas a um eletrodo bipolar, de forma que um dos pólos é formado por um fio de aço inoxidável isolado por PFA (A-M Systems, WA, EUA) exceto pela sua extremidade em contato com o tecido neural e o outro pólo é a extremidade da cânula-guia 1 mm acima do nível do outro pólo.

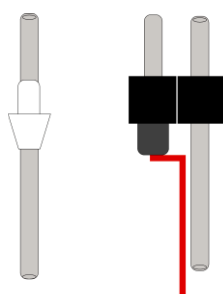

Figura 1. Esquema representativo da cânula-guia (à esq.) e quimitrodo (à dir.) utilizados neste trabalho.

A cirurgia foi realizada sob anestesia com mistura de 0,1 mL de cetamina (90 mg/kg) e 0,1 mL de xilazina (10 mg/kg) e fixados em um aparelho estereotáxico (David Kopf Instruments, CA, USA). Ao término da cirurgia, os animais receberam uma injeção de antibiótico (Pentabiótico, 0,2 mL, i.m.; Fort Dodge, SP, Brasil) e uma injeção de anti-

inflamatório/analgésico flunixin meglumina (Banamine®, 2,5 mg/kg, s.c.; Schering-Plough, SP, Brasil). Os ratos foram mantidos por um período de cinco dias para recuperação cirúrgica antes da realização dos testes comportamentais. Todos os procedimentos e manipulações utilizadas neste trabalho estão de acordo com as normas da Comissão de Ética no Uso de Animais (CEUA) do campus da USP – Ribeirão Preto (Protocolo nº 11.1.308.53.9).

Drogas e microinjeções

Foi utilizado o maleato de midazolam (MDZ; Roche, Brasil) nas doses de 5, 10 ou 20 nmol e o muscimol (MUS; Tocris Bioscience, UK) nas doses de 0,5, 1 ou 2 nmol, microinjetados no CI ou SCPd durante 1 minuto até alcançar o volume total de 0,2 µL. Os animais do grupo controle receberam solução salina (SAL; 0,9% NaCl) em mesmo volume e via de administração. As doses da droga e os tempos de espera foram selecionados com base em estudos prévios (Pandossio & Brandão, 1999; Nobre & Brandão, 2004; 2011) Durante o procedimento de microinjeção da droga, os animais permaneceram em uma caixa de polipropileno medindo 28 × 17 × 13 cm, forrada com papel picado. Uma agulha odontológica (30 G, com 14 mm de comprimento, 0,3 mm de diâmetro externo) foi introduzida na cânula, ultrapassando-a em 1 mm. Esta agulha foi conectada a um tubo de polietileno (PE-10; Becton-Dickinson, NJ, EUA) e a uma seringa Hamilton graduada de 5 µL. As drogas foram injetadas com o auxílio de uma bomba de infusão (Harvard Apparatus, MA, EUA). O deslocamento de uma bolha de ar no interior do tubo de polietileno foi utilizado para monitorar a microinjeção. Após o término da infusão, a agulha foi mantida por mais 1 minuto para evitar refluxo da droga pela cânula-guia.

Equipamentos e procedimentos

Labirinto em cruz elevado. Vinte minutos após a microinjeção da droga, os animais foram submetidos ao Labirinto em Cruz Elevado (LCE). O LCE é feito de madeira e possui dois braços abertos (50 cm x 10 cm), perpendiculares a dois braços fechados de dimensões iguais e cercados por paredes de 40 cm. O aparelho é elevado a 50 cm do chão. Cada rato foi colocado no centro do LCE, de frente para um dos braços fechados, e sua atividade foi registrada em circuito de vídeo por 5 minutos. Analisamos o número de entradas e tempo gasto nos braços abertos e fechados, bem como as medidas etológicas complementares (Cruz

et al., 1994; Anseloni & Brandão, 1997; Albrechet-Souza *et al.*, 2008) tais como descritas: (1) frequência da exploração da extremidade do braço aberto: "*end arm exploration*", na qual o rato atinge o último terço de um dos braços abertos; (2) mergulhos: "*head dipping*", mergulhar a cabeça abaixo do nível do labirinto; (3) levantamentos: "*rearings*", levantamento total ou parcial do rato sobre as patas traseiras; (4) esquadrinhamento: "*scanning*", movimentos horizontais da cabeça em qualquer direção, incluindo farejar o chão ou paredes do LCE; (5) espreitar: "*peeping out*", esticar a cabeça/ombros da área fechada para o centro do LCE; (6) rastejamento: "*flat-back approach*", locomoção quando o animal estica-se completamente e move-se cautelosamente à frente; (7) esticamento: "*stretch-attend posture*", o animal estica-se com as patas dianteiras, mantendo as traseiras no mesmo local, e retrai à posição anterior; (8) auto-limpeza: "*grooming*", sequência típica da espécie, com movimentos iniciados no focinho, prosseguindo às orelhas e tórax, até a região caudal, incluindo coçar-se e lamber-se. Cada animal foi testado no LCE apenas uma vez e este procedimento foi realizado na fase clara do ciclo claro-escuro, entre 9h00 e 12h00. O aparelho foi limpo com etanol a 20% antes de cada teste.

Estimulação elétrica do CI ou SCPd. Cinco dias após a cirurgia, cada animal foi mantido em habituação por 5 minutos em uma caixa quadrada de acrílico (25 × 20 × 20 cm), sob iluminação ambiente de 40 W (80 lux ao nível do assoalho da caixa). A estimulação elétrica mesencefálica (60 Hz, 10 segundos de duração) foi aplicada em intervalos de 1 minuto, por meio de um aparelho estimulador fisiológico (ESF-12, DelVecchio, Brasil), com a intensidade da corrente sendo aumentada em passos de 5 µA até que o animal apresentasse a reação de fuga e o limiar aversivo basal fosse aferido. A corrente elétrica de estimulação foi monitorada através de um osciloscópio (Minipa, São Paulo, Brasil). A mínima intensidade de corrente capaz de produzir galope, fuga e/ou saltos nos animais foi considerada o limiar de fuga basal. Após atingir este limiar, cessou-se a estimulação elétrica e o animal permaneceu por 8 minutos na caixa sob observação. O congelamento pós-estimulação foi avaliado durante este período. O congelamento foi definido operacionalmente como a ausência de movimentos do corpo e vibrissas, exceto aqueles necessários à respiração, por intervalos de 6 segundos no mínimo. Ao final desta sessão, cada animal recebeu o tratamento por meio da injeção intra-CI e foi novamente testado, após um período de espera de 15 minutos, para aferição do limiar de fuga e o tempo congelamento pós-estimulação. Os ratos que não responderam à estimulação até a intensidade de 100 µA foram descartados do estudo. A resposta de congelamento pós-

estimulação com as drogas GABAérgicas/benzodiazepínicas foi medida durante todo o período da sessão.

Desenho experimental

O comportamento de medo incondicionado em ratos tratados com salina ou midazolam (5, 10 ou 20 nmol/0,2 μL) foram avaliados por meio do LCE ou pelo procedimento de estimulação elétrica do CI. Cada um destes testes comportamentais contou com 4 grupos para cada região do CI (dorsal ou ventral): (i) salina, (ii) MDZ 5 nmol, (iii) MDZ 10 nmol, e (iv) MDZ 20 nmol. Aprofundamos o estudo do mecanismo GABAérgico da região ventral do CI injetando muscimol em três doses distintas (0,5 nmol, 1 nmol ou 2 nmol/0,2 μL) especificamente nesta área, e avaliamos as respostas dos animais à estimulação elétrica desta mesma estrutura. O tempo de espera entre o tratamento e a exposição ao teste foi de 15 minutos em todos os casos. O número de ratos por grupo está detalhado na Tabela 1.

Tabela 1 – Número de animais por grupo em cada experimento.

	Região do CI	*Salina*	*MDZ 5nmol*	*MDZ 10nmol*	*MDZ 20nmol*
LCE	Dorsal	6	9	6	9
	Ventral	10	7	9	6
Estimulação elétrica CI	Dorsal	6	10	7	5
	Ventral	9	9	9	10
			MUS 0,5nmol	*MUS 1nmol*	*MUS 2nmol*
Estimulação elétrica CI	Ventral	9	9	8	8
				Total	**161**

Histologia

Ao fim dos experimentos, os animais foram anestesiados com uretano (25%, 5 mL/kg; Sigma). A seguir, os animais foram perfundidos intracardiacamente com solução salina 0,9%, seguida de solução de paraformaldeído a 4%. Os encéfalos foram então removidos e estocados em solução de sacarose (30%). Após um prazo mínimo de 3 dias, os encéfalos foram fatiados (60 μm de espessura) em micrótomo de congelamento nas áreas

correspondentes ao núcleo central do colículo inferior. Foram montadas lâminas que receberam a coloração de Nissl, com a aplicação do corante violeta de cresila, para determinar os sítios das microinjeções.

Análise dos dados

Os dados são apresentados como Média ± Erro Padrão da Média e foram analisados utilizando o *software* GraphPad Prism 5.0 (GraphPad Software, Inc., EUA). Os dados gerados pelo LCE e a diferença entre os limiares de estimulação elétrica (sessão de teste e a linha de base) foram analisados por ANOVA de uma via, seguido pelo teste *post hoc* de Newman-Keuls. Os dados do congelamento pós-estimulação foram analisados utilizando-se ANOVA de duas vias, com as doses de midazolam ou muscimol como um fator e condição (antes ou depois do tratamento) como o outro fator. Em todas as comparações, $p<0,05$ foi utilizado como critério para a significância estatística.

Resultados

A análise histológica revelou o posicionamento da cânula no colículo inferior dos animais utilizados (Figura 1). De acordo com evidências obtidas anteriormente neste laboratório, optamos por fazer uma distinção funcional entre a porção ventral (vCI) e a porção dorsal (dCI) do colículo inferior para refinar a análise da participação desta estrutura na elaboração do comportamento defensivo. Ao compararmos essa divisão funcional com a divisão neuroanatômica de Paxinos & Watson (2006), a porção dorsal corresponde ao núcleo central do dCI enquanto que a porção ventral corresponde ao núcleo central e parte do córtex externo.

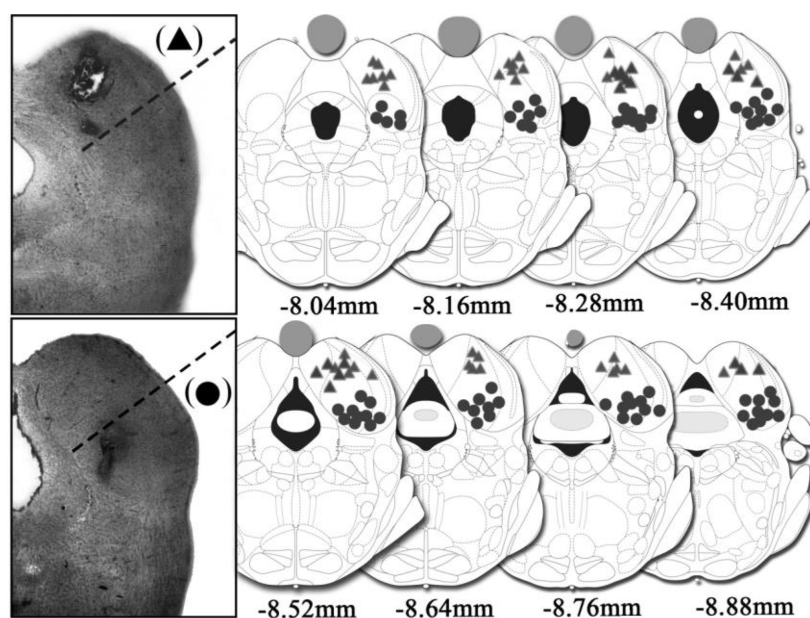

Figura 1.1 - Representação dos sítios de microinjeção no colículo inferior, ilustrando a divisão entre a porção ventral (círculos) e dorsal (triângulos) do colículo inferior utilizada neste trabalho. A distância a partir do bregma está representada em cada seção, de acordo com Paxinos & Watson (2006). O número de sítios de injeção na figura é menor que o número total de animais devido a várias sobreposições.

Efeitos do midazolam no CI de ratos submetidos ao teste do Labirinto em Cruz Elevado. A análise dos dados obtidos no teste LCE revelou efeito do tipo ansiolítico para a dose de 10 nmol do MDZ, quando injetado na porção ventral do CI. Houve aumento da porcentagem de tempo gasto nos braços abertos entre os animais que receberam a dose de 10 nmol de MDZ na porção ventral do CI [$F_{3,28}$=3,81; p<0,05] e também do número de entradas nos braços abertos [$F_{3,28}$=4,686; p<0,05]. Os mesmos tratamentos no dCI não produziram

efeitos significativos na porcentagem do tempo gasto nos braços abertos [$F_{3,26}$=0,75; p>0,05], bem como no número de entradas nestes braços [$F_{3,26}$=0,58; p>0,05].

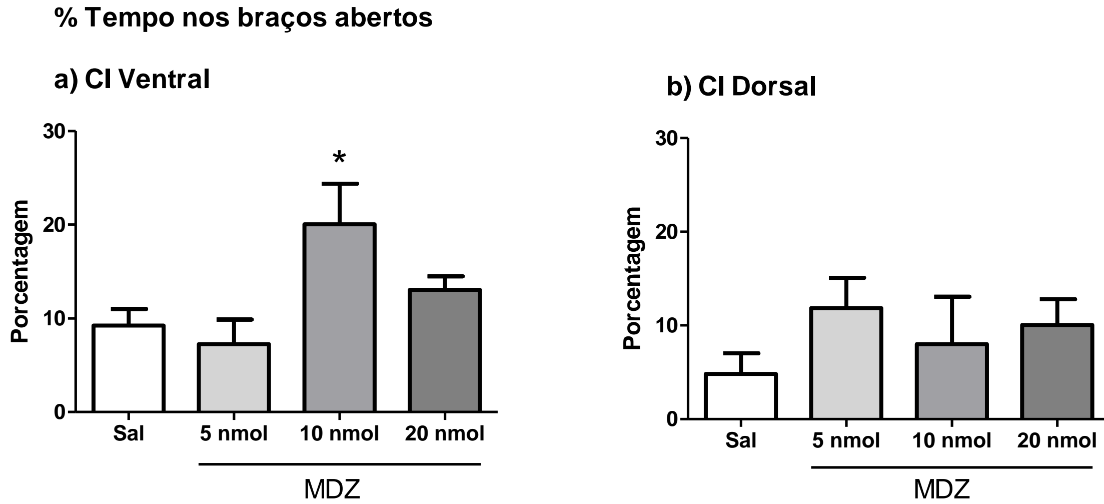

Figura 1.2 - Porcentagem de tempo nos braços abertos. A dose de 10 nmol microinjetada na porção ventral do CI apresentou efeito do tipo ansiolítico no LCE, indicado por aumento na porcentagem de tempo gasto pelos animais nos braços abertos [p=0,02]. Este efeito não foi encontrado em relação aos animais cuja microinjeção foi dirigida ao colículo inferior dorsal (dCI).

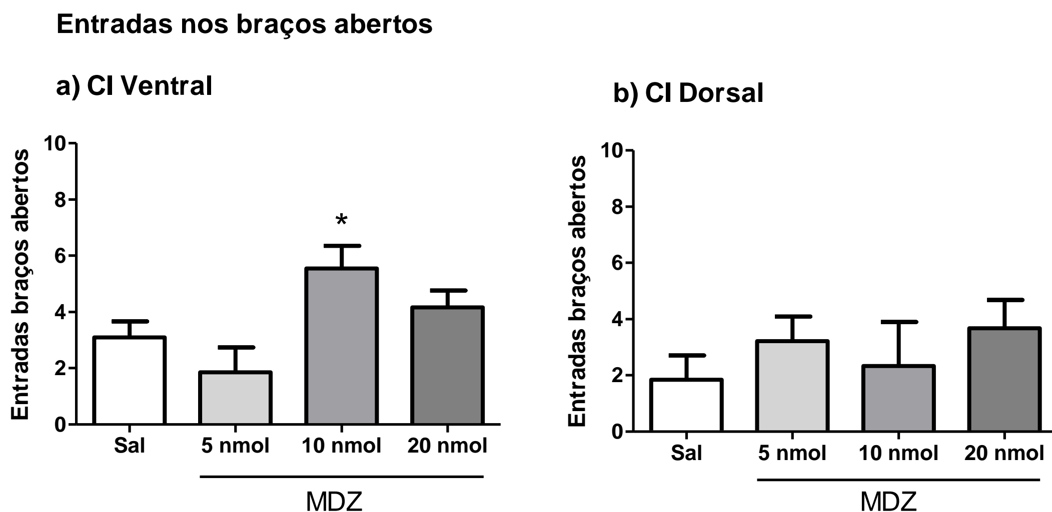

Figura 1.3 - Frequência de entrada nos braços abertos do LCE. A dose de 10 nmol microinjetada na porção ventral do CI resultou em maior número de entradas nos braços abertos do LCE [p=0,0089]. Este efeito não foi encontrado em relação aos animais cuja microinjeção foi dirigida ao dCI.

As medidas complementares relacionadas ao fator ansiedade no LCE apresentaram alterações significativas, tais como a exploração da extremidade dos braços abertos ("*End arm exploration*") [$F_{3,28}$=5,2; p<0,01] e a frequência de mergulhos ("*Head dippings*") [$F_{3,28}$=5,48; p<0,01] quando a dose de 10 nmol de MDZ foi injetada no vCI, sem que este efeito do tipo ansiolítico pudesse ser verificado também para esta mesma dose no dCI [*End arm exploration* $F_{3,26}$=0,8895 e p>0,05; *head dipping* $F_{3,26}$=0,38 e p>0,05].

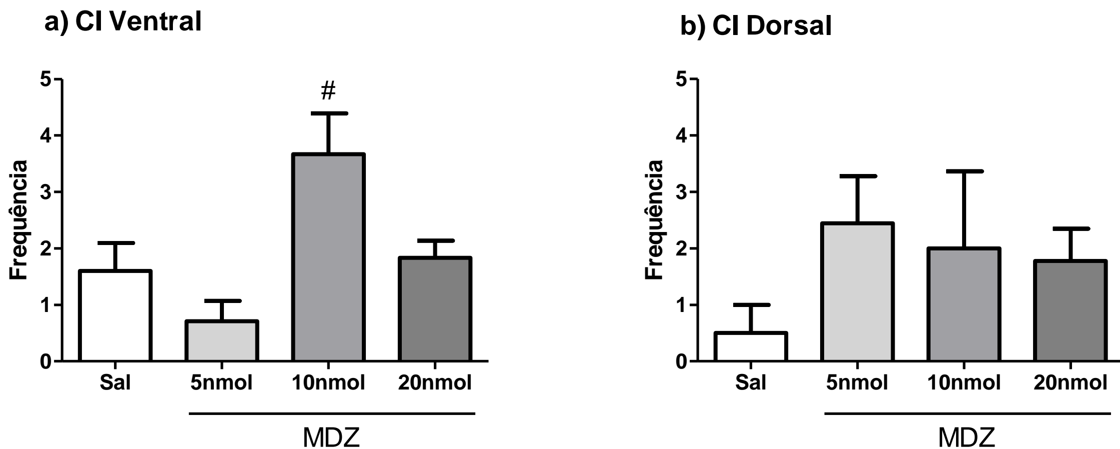

Figura 1.4 - Frequência de atividade exploratória na extremidade dos braços abertos. [p=0,005; # significa diferença estatística em comparação com todos os outros grupos]. Este efeito não foi encontrado em relação aos animais cuja microinjeção foi dirigida ao dCI.

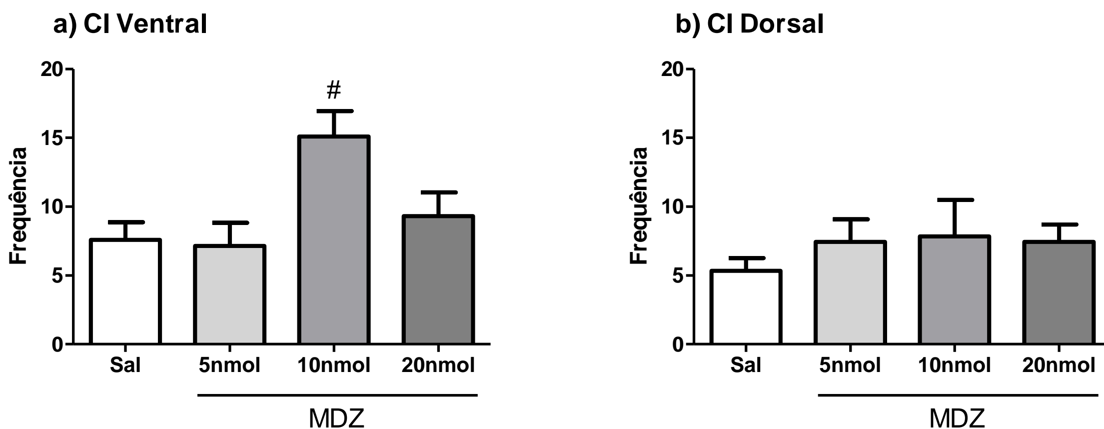

Figura 1.5 - Frequência de mergulhos. [p=0,0043; # diferença estatística em comparação com todos os outros grupos]. Este efeito não foi encontrado em relação aos animais cuja microinjeção foi dirigida ao dCI.

O número de entradas nos braços fechados do labirinto não foi alterado por nenhuma das doses de MDZ empregadas no estudo (Figura 1.6), o que exclui a possibilidade de viés nos resultados por eventual prejuízo na função motora causado pelo benzodiazepínico [vCI $F_{3,28}$=0,39; p>0,05] [dCI $F_{3,26}$= 0,29; p>0,05]. O número de levantamentos, um índice de atividade exploratória vertical no LCE, também não sofreu alterações com nenhuma das doses de MDZ (Figura 1.7) [vCI $F_{3,28}$=0,45; p>0,05] [dCI $F_{3,26}$= 0,207; p>0,05].

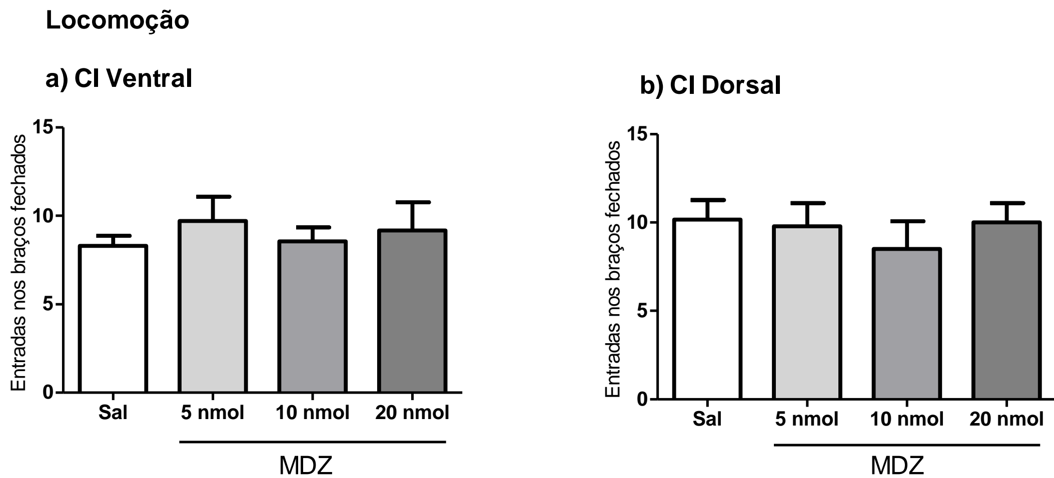

Figura 1.6 - Atividade locomotora dos animais no LCE, representada pelo número de entradas nos braços fechados. Nenhuma das doses de MDZ causou efeito sedativo ou hipolocomoção no LCE [vCI $F_{3,28}$=0,39; p>0,05] [dCI $F_{3,26}$= 0,29; p>0,05].

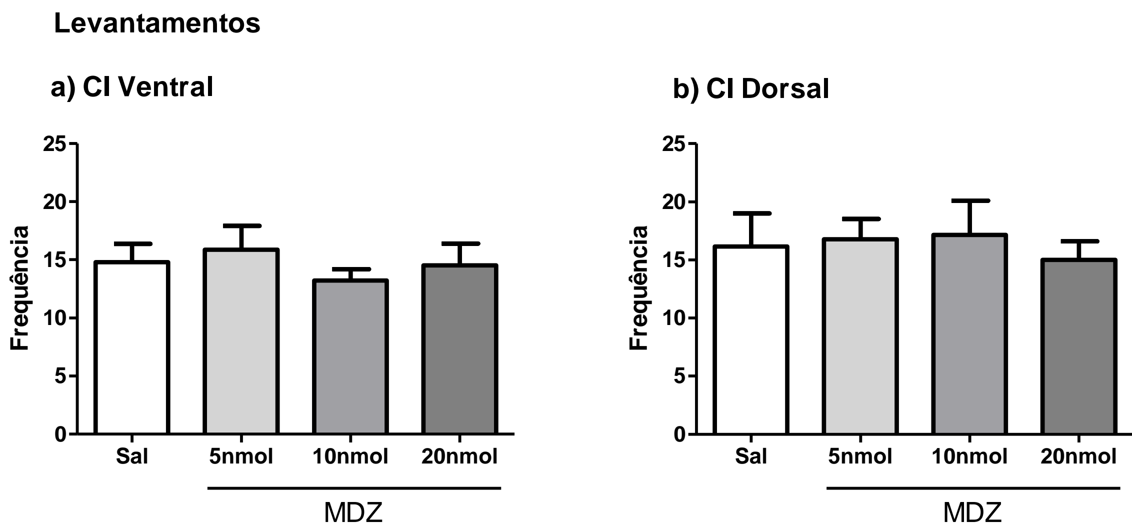

Figura 1.7 - Frequência de levantamentos. Nenhuma das doses de MDZ causou déficits locomotores ou na exploração vertical do LCE [vCI $F_{3,28}$=0,45; p>0,05] [dCI $F_{3,26}$= 0,2; p>0,05].

O comportamento geral exploratório dos animais também não apresentou prejuízos, tal como demonstrado pela frequência de esquadrinhamentos ("*Scanning*") [vCI $F_{3,28}$=1,58; p>0,05] [dCI $F_{3,26}$= 0,48; p>0,05].

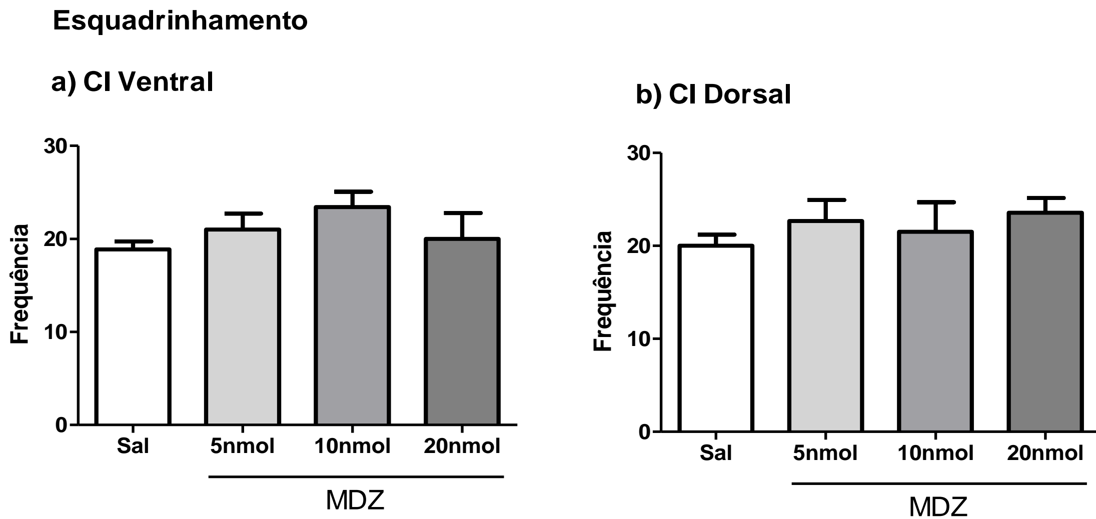

Figura 1.8 - Frequência do comportamento de esquadrinhar. Nenhuma das doses de MDZ causou prejuízos na atividade exploratória dos animais no LCE [p>0,05].

A Figura 1.9 ilustra os efeitos do MDZ sobre comportamentos de avaliação de risco no LCE. As doses de 10 nmol e 20 nmol reduziram significativamente estes comportamentos em relação ao grupo controle [$F_{3,28}$=5,7; p<0,05 e p<0,01] nos animais que receberam a droga intra-vCI. Tais comportamentos são a somatória das medidas de espreitar, rastejar e esticar-se (Figura 1.10), e foram agrupados uma vez que as três categorias carregam positivamente no fator avaliação de risco/decisão na análise fatorial do LCE. O mesmo tratamento no dCI não produziu efeitos significativos [$F_{3,26}$= 1,29; p>0,05].

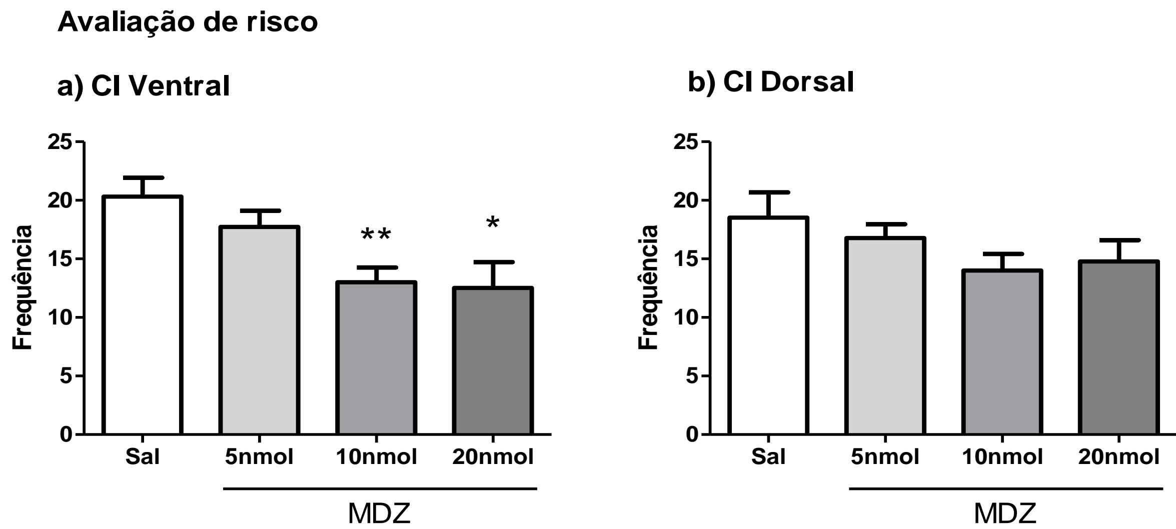

Figura 1.9 - Frequência dos comportamentos de avaliação de risco. As frequências dos comportamentos de esticar-se, rastejar e espreitar foram agrupadas sob uma única categoria. As doses de 10 e 20 nmol, injetadas no vCI, reduziram a frequência de comportamentos de avaliação de risco com relação ao grupo controle, o que não foi encontrado em relação aos animais cuja microinjeção foi dirigida ao dCI [p=0,0035].

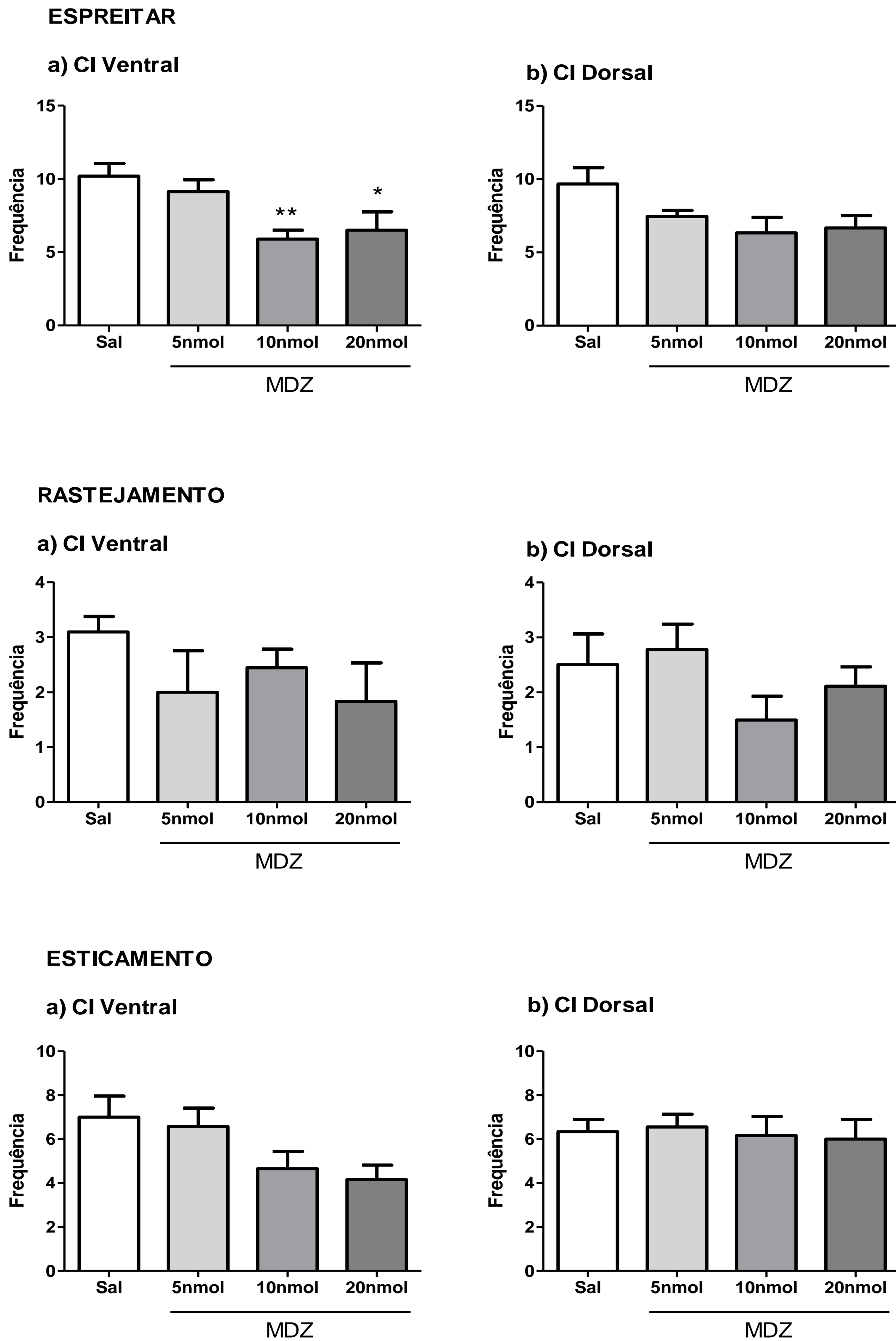

Figura 1.10 – Gráficos dos comportamentos de espreitar (“*Peeping out*”), rastejar (“*Flat-back approach*”) e esticar-se (“*Stretch-attend posture*”), cujos dados foram agrupados sob a categoria de avaliação de risco. Das três medidas, apenas o comportamento de espreitar obteve significância estatística [p=0,0023; #diferença estatística em comparação com todos os outros grupos].

Efeitos do midazolam no teste de estimulação elétrica do CI. Um padrão similar de dissociação da reatividade farmacológica no CI foi obtido ao investigarmos os efeitos do MDZ sobre as respostas incondicionadas geradas pelo procedimento de estimulação elétrica. Curiosamente, a dose mínima eficaz para produzir aumento no limiar de fuga foi maior do que a dose mínima "ansiolítica" observada no LCE. Os ratos que receberam 20 nmol de MDZ no vCI apresentaram aumento nos limiares de fuga após o tratamento, em relação à linha de base [$F_{3,33}$= 5,71; p=0,0029], enquanto que este efeito não foi registrado no dCI [$F_{3,24}$= 1,94; p=0,14] (Figura 1.11).

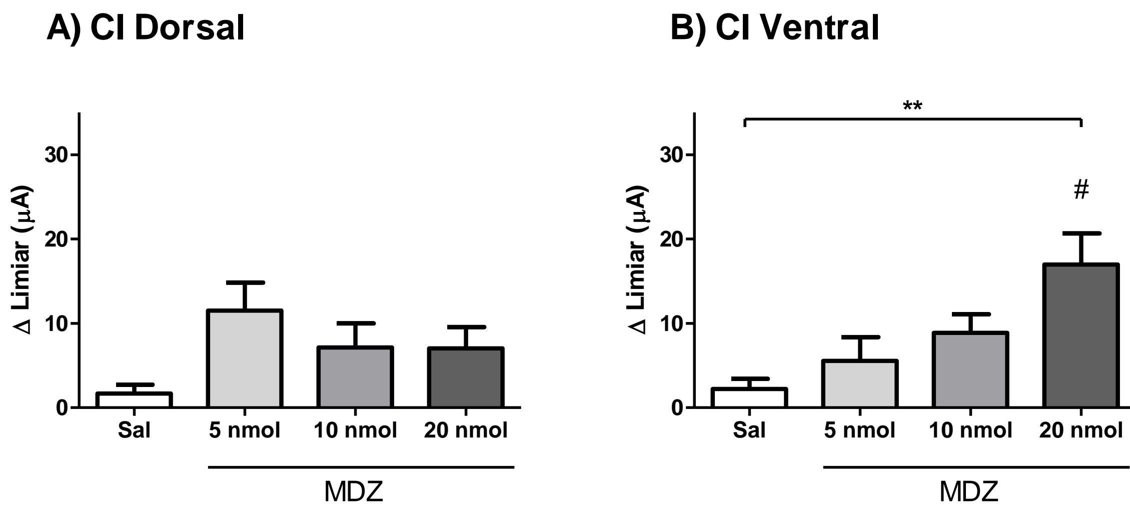

Figura 1.11 - Diferença dos limiares de fuga registrados antes e depois de injeções de solução salina ou midazolam em três doses nas porções ventrais ou dorsais do IC de ratos submetidos à estimulação elétrica do CI. Os animais que receberam 20 nmol no vCI apresentaram um aumento significativo no limiar de fuga em comparação ao grupo controle. p<0,01 e # valor estatisticamente significativo com relação a todos os demais grupos.

Na análise do tempo de congelamento pós-estimulação, a ANOVA de duas vias evidenciou efeito significativo do fator condição para o grupo vCI [$F_{3,33}$=15,4; p=0,0003] mas nenhum efeito do fator doses ou interação condição-doses. O grupo dCI também apresentou o efeito do fator condição (antes ou depois do tratamento) [$F_{3,24}$= 4,78; p=0,038] mas sem efeitos significativos quanto à dose ou à interação condição-doses (Figura 1.12).

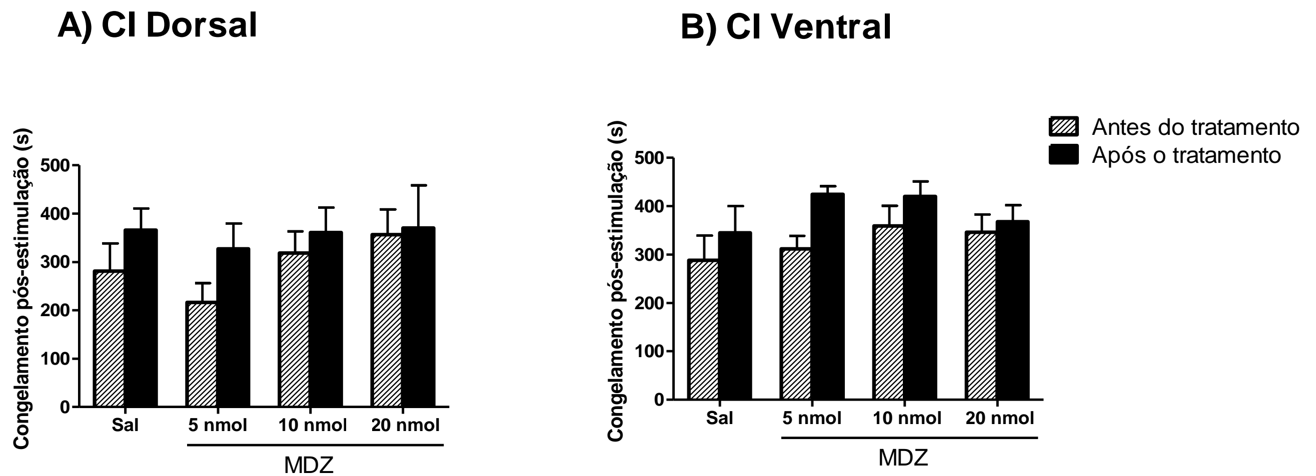

Figura 1.12 - Congelamento pós-estimulação registrado após a injeção de midazolam no IC, em suas divisões dorsal e ventral. Verificou-se efeito significativo da condição (antes ou depois do tratamento) sobre o tempo de congelamento pós-estimulação, mas não houve efeito significativo com relação às doses de midazolam ou à interação entre condição e doses.

Efeitos do muscimol no teste da estimulação elétrica do CI. Tendo por base os dados prévios acerca da divisão funcional entre as porções dorsal e ventral do CI e da distinta reatividade farmacológica do vCI ao midazolam nos dois modelos utilizados neste trabalho, aplicamos o muscimol na porção ventral do CI e submetemos os animais à estimulação elétrica desta estrutura. A dose de 1 nmol causou aumento do limiar de fuga neste modelo [$F_{3,30}$=4,06, p<0,05] (Figura 1.13), enquanto que a menor dose de 0,5 nmol foi suficiente para alterar o tempo de congelamento pós-fuga [$F_{3,30}$=3,906, p<0,05] (Figura 1.14).

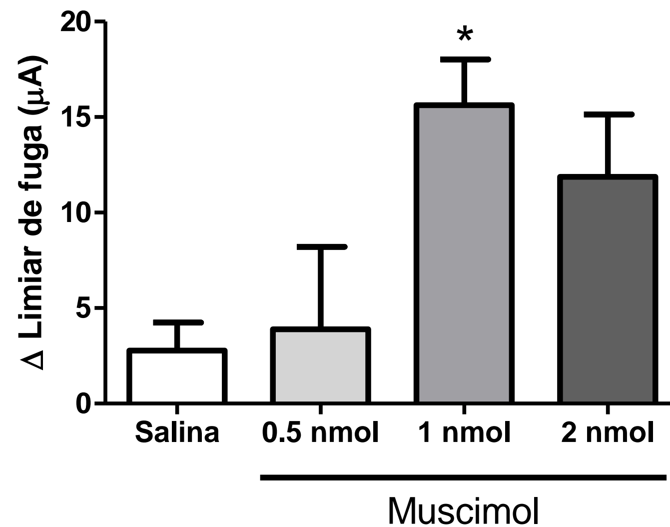

Figura 1.13 - Diferença dos limiares de fuga registrados antes e depois de injeções de solução salina ou muscimol na porção ventral do CI de ratos submetidos à estimulação elétrica. Os animais que receberam 1 nmol no vCI apresentaram aumento significativo no limiar de fuga em comparação ao grupo controle (p=0,01).

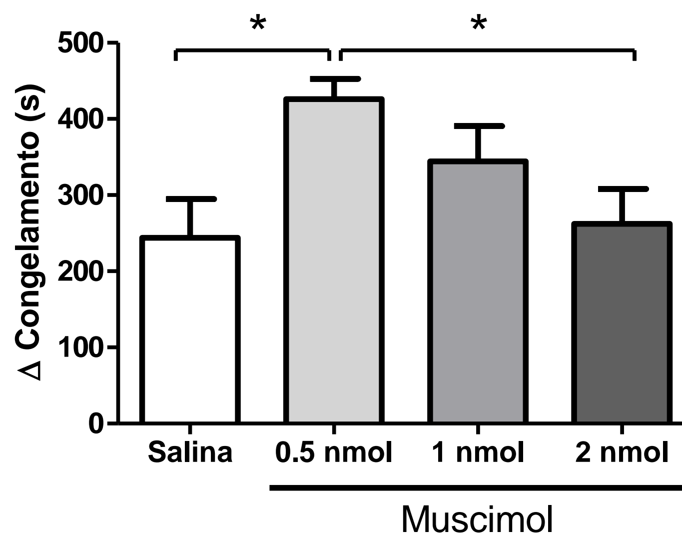

Figura 1.14 - Congelamento pós-estimulação registrado após a injeção de muscimol no vCI. O gráfico representa a diferença entre a sessão teste e a linha de base. A dose de 0,5 nmol de muscimol alterou o tempo de congelamento pós-fuga (p=0,018).

Discussão

A primeira etapa deste trabalho abordou a participação do CI na mediação de comportamento defensivo em ratos submetidos ao LCE, após tratamento com a droga benzodiazepínica midazolam, em três doses distintas. Embora outros estudos tenham explorado o perfil ansiolítico de injeções locais de midazolam no CI em outros modelos animais de ansiedade, pela primeira vez foi realizada uma análise etofarmacológica em que o LCE foi usado para avaliar o envolvimento desta estrutura na geração e elaboração da reação de defesa. Este trabalho mostrou pela primeira vez que o midazolam atua seletivamente no vCI para produzir seus efeitos ansiolíticos.

A curva dose-resposta do midazolam nas doses de 5, 10 e 20 nmol microinjetadas no CI de ratos submetidos ao LCE evidenciou a dose de 10 nmol como a menor dose efetiva para obtenção de efeito do tipo ansiolítico neste modelo, desde que injetada na porção ventral do CI. O efeito ansiolítico detectado nos animais que receberam o tratamento via vCI não foi observado nos animais cujas cânulas estavam posicionadas no dCI, o que confirma a segregação neural que existe entre estas duas áreas. Estas duas regiões do CI atuam em circuitos neurais independentes, responsáveis por crises convulsivas audiogênicas (dCI) e pelas respostas de medo (vCI) (Lamprea *et al.*, 2002; Ferreira-Netto *et al.*, 2007).

O efeito ansiolítico da dose de 10 nmol de midazolam injetado no vCI incluiu o aumento da porcentagem de tempo gasto e número de entradas nos braços abertos, frequência de visitas às extremidades dos braços abertos e mergulhos. A análise fatorial dos comportamentos exibidos no LCE indica que estes índices carregam positivamente em um mesmo fator, que se caracteriza como um fator ligado a estados de ansiedade (Cruz *et al.*, 1994; Albrechet-Souza *et al.*, 2008).

Um dos efeitos indesejados do uso de benzodiazepínicos é a sedação/hipolocomoção, que pode prejudicar a interpretação de dados obtidos de modelos animais; porém, esta medida não foi alterada por nenhuma das doses testadas do midazolam. De fato, o número de entradas nos braços fechados do labirinto, comumente utilizado como índice da atividade locomotora geral (Cruz *et al.*, 1994), não foi significativamente alterado por 10 nmol de midazolam injetado localmente no CI.

O midazolam administrado na porção ventral do CI também foi capaz de reduzir a frequência dos comportamentos de avaliação de risco, um efeito esperado para drogas

ansiolíticas. Esses comportamentos têm sua importância biológica por permitirem ao animal avaliar situações e ambientes potencialmente ameaçadores e definir estratégias de enfrentamento (Rodgers & Johnson, 1995; Anseloni & Brandão, 1997; Albrechet-Souza *et al.*, 2008). Sendo assim, nossos resultados sobre o efeito ansiolítico do midazolam injetado diretamente no CI corroboram outros estudos que obtiveram resultados similares; porém, com administração sistêmica desta droga (Cruz *et al.*, 1994; Anseloni & Brandão, 1997; Albrechet-Souza *et al.*, 2008).

Um dado importante que emergiu deste estudo foi a clara divisão funcional que existe no CI, entre suas porções ventrais e dorsais, na expressão dos comportamentos defensivos. De fato, apenas os animais que receberam o tratamento intra-vCI apresentaram uma resposta substancial à ação antiaversiva do midazolam, tanto no LCE quanto na estimulação elétrica. Desta forma, esperamos contribuir com evidências adicionais para definir conceitualmente a porção ventral do CI como o substrato neural das respostas defensivas elaboradas pelo CI.

Outro avanço importante diz respeito à reatividade farmacológica do vCI a drogas GABAérgicas e benzodiazepínicas. Embora a dose eficaz para reduzir a aversão aos espaços desprotegidos do LCE foi de 10 nmol de midazolam, a dose mais elevada de 20 nmol também produziu a diminuição dos comportamentos de avaliação de riscos nesse modelo. Já o limiar de fuga determinado pela estimulação elétrica também foi sensível apenas à dose de 20 nmol de midazolam, enquanto que duas das três doses de muscimol testadas foram efetivas. Esta diferença na reatividade a drogas GABAérgicas/benzodiazepínicas pode ser explicada pela especificidade da expressão do receptor de GABA em regiões distintas do CI, bem como às diferenças na estrutura molecular e na combinação de subunidades destes receptores, que podem ter mais ou menos sítios de ligação para benzodiazepínicos, resultando assim na heterogeneidade farmacológica (Shiraishi *et al.*, 2001; Jamal *et al.*, 2012). O muscimol é um agonista seletivo para o receptor $GABA_A$ (Savic *et al.*, 2005; Chandra *et al.*, 2010) enquanto que o midazolam age no receptor BZD – que está associado ao receptor GABA e ao canal de cloro, constituindo a unidade supramolecular do complexo GABA-BZD – com diferenças em suas respectivas afinidades, densidade de expressão e modos de ação. Desta forma, não podemos descartar a possibilidade de um efeito não específico destas drogas. Outra hipótese seria a de que cada tarefa comportamental seria sensível a um determinado nível de modulação GABA/BZD. Assim, sugerimos que os aspectos dorsal e ventral do CI possam ter sensibilidades farmacológicas distintas para a ação de drogas GABAérgicas e benzodiazepínicas.

Outro achado interessante deste estudo está relacionado ao componente sensoriomotor da reação de defesa. A estimulação elétrica do CI produz uma sequência de comportamentos que se inicia com um breve período de alerta (protrusão das orelhas, olhos e vibrissas, defecação e micção), seguido de um breve congelamento que rapidamente dá lugar à hiperlocomoção/excitação, até atingir o ápice da reação explosiva de fuga, que inclui corrida circular, saltos e vocalizações. Por conta do padrão explosivo e abrupto da resposta evocada pela estimulação do CI, o congelamento que ocorre antes da resposta de fuga (observado na estimulação de outras estruturas mesencefálicas e que pode ser considerado um comportamento preparatório para uma reação defensiva mais enérgica) não pôde ser determinado. Isto pode ser justificado devido ao fato de que o CI é uma das estruturas mais caudais pertencentes ao Sistema Encefálico Aversivo, o que supostamente faria com que a estimulação elétrica induzisse padrões mais intensos de comportamento defensivo, de acordo com a teoria de McNaughton & Corr (2004). Além disto, ao iniciar a reação de fuga, a maioria dos ratos retraiu a orelha esquerda pouco antes da fuga e/ou correu para o lado esquerdo – que é contralateral ao local de estimulação – da mesma forma como fazem quando recebem informações aversivas do ambiente (Martin *et al.*, 1978; Huston *et al.*, 1980). Curiosamente, o tempo de congelamento pós-estimulação não foi alterado por nenhuma das doses de midazolam, mas sim com a menor dose de muscimol utilizada.

O congelamento pós-estimulação corresponde a um período de intenso processamento da informação aversiva, que tem lugar imediatamente após a exposição a uma situação extremamente ameaçadora. É o momento em que o organismo processa o evento aversivo, reunindo informações restantes do ambiente interno e externo e transferindo-as para estruturas cerebrais superiores (Brandão *et al.*, 2008). Drogas que aumentam a neurotransmissão GABAérgica através do sítio de ligação para benzodiazepínicos, como é o caso do midazolam, não parecem afetar diretamente este comportamento, como mostrado pelos nossos resultados do congelamento pós-estimulação. Mas o aumento da neurotransmissão GABAérgica por um agonista como muscimol alterou esta medida. O fato de o muscimol injetado localmente no CI ter aumentado o congelamento pós-fuga de certa maneira corrobora a ideia de que este comportamento tem na SCPd o seu substrato neural ou a sua porta de saída (*output*). Desta forma, trabalhamos com a hipótese de que o componente sensorial da resposta defensiva seria resistente à ação de drogas benzodiazepínicas, enquanto o substrato neural do medo do vCI seria facilmente modificado por drogas GABAérgicas, inclusive em doses baixas.

Em suma, estas observações apontam para a importância do componente sensorial do comportamento defensivo e sua integração com o componente motor destas respostas. Nosso estudo tem contribuído para o avanço do entendimento sobre o papel do CI como um filtro sensoriomotor das respostas defensivas que atua em conjunto com a SCPd, sendo modulado tonicamente por mecanismos GABAérgicos refinados.

Os resultados deste estudo foram publicados na revista Neuropharmacology em 2015 (ANEXO 1).

EXPERIMENTO 2

Interação funcional entre o CI e a SCPd avaliada por estimulação acústica (potencial evocado auditivo mensurado no CI) e por estimulação elétrica no limiar de fuga (SCPd e CI)

MATERIAIS E MÉTODOS

Animais e cirurgia

Foram utilizados 214 ratos Wistar machos, com peso entre 250g e 275g, provenientes do biotério central da Universidade de São Paulo, *campus* de Ribeirão Preto. Os animais foram mantidos em biotério setorial por um período mínimo de habituação de 48h, com livre acesso a água e alimento. Estes animais foram submetidos à cirurgia estereotáxica para implantação de quimitrodos e cânulas no CI e SCPd, que seguiu o mesmo protocolo utilizado no Experimento 1. Os quimitrodos (eletrodos que possuem também uma cânula-guia para injeção local de drogas) utilizados para registro do potencial evocado são descritos na seção Equipamentos e Procedimentos.

Drogas e microinjeções

Foi utilizado o maleato de midazolam (MDZ; Roche, Brasil) nas doses de 10, 20 ou 40 nmol, muscimol (MUS; Tocris Bioscience, UK) nas doses de 0,5, 1 ou 2 nmol, e semicarbazida (SMC; Vetec, São Paulo, Brasil) nas doses de 6 ou 7 μg, microinjetados no CI ou SCPd durante 1 minuto até alcançar o volume total de 0,2 μL. Os animais do grupo controle (CTRL) receberam solução salina (0,9% NaCl) em mesmo volume e via de administração. As doses da droga e os tempos de espera foram selecionados com base em estudos prévios (Pandossio & Brandão, 1999; Nobre & Brandão, 2004; 2011) e nos resultados do Experimento 1. Durante o procedimento de microinjeção da droga, os animais permaneceram em uma caixa de polipropileno medindo 28 × 17 × 13 cm, forrada com papel picado. Uma agulha odontológica (30 G, com 14 mm de comprimento, 0,3 mm de diâmetro externo) foi introduzida na cânula, ultrapassando-a em 1 mm. Esta agulha foi conectada a um tubo de polietileno (PE-10; Becton-Dickinson, NJ, EUA) e a uma seringa Hamilton graduada de 5 μL. As drogas foram injetadas com o auxílio de uma bomba de infusão (Harvard Apparatus, MA, EUA). O deslocamento de uma bolha de ar no interior do tubo de polietileno foi utilizado para monitorar a microinjeção. Após o término da infusão, a agulha foi mantida por mais 1 minuto para evitar refluxo da droga pela cânula-guia.

Equipamentos e procedimentos

Experimento 2A: Potencial evocado auditivo. O potencial evocado auditivo (PEA), medido na porção ventral do CI de ratos não anestesiados, é uma medida eletrofisiológica da resposta do grupo de neurônios que circundam a área de registro de um eletrodo após um estímulo de natureza auditiva. Estes potenciais se apresentam como pequenas alterações no potencial de campo que podem ser captados por eletrodos, alterações estas que surgem como resposta à apresentação de uma estimulação sonora. Os registros dos PEAs foram conduzidos com os animais mantidos dentro de uma gaiola de contenção em acrílico (19 x 9 x 49 cm) com piso, tampa, frente e fundo compostos de barras de aço inoxidável espaçadas 1,5 cm entre si, e laterais de acrílico com vazamento de 5 x 1 cm, para acomodação do focinho/cauda. Esta gaiola de contenção esteve situada dentro de uma caixa de isolamento acústico (64 x 60 x 40 cm), cujas partes internas (paredes, piso e teto) são compostas por uma malha de metal (*gaiola de Faraday*) com aterramento para isolamento das interferências induzidas pelo ambiente externo, particularmente aquelas na faixa de frequência de 60 Hz, que possam adicionar ruído ao sinal registrado do vCI. Os estímulos sonoros (cliques) foram produzidos por dois *tweeters* (12 Ω, 200 W, LeSon, Brasil) localizados nas laterais da caixa de isolamento acústico, 15 cm acima do piso. Estes estímulos seguiram a seguinte configuração: tom puro de 3000 Hz, com 50 ms de duração e 95 dB de intensidade (SPL). Após 7 dias de recuperação pós-cirúrgica, os animais foram posicionados em uma gaiola de contenção, sem necessidade de habituação prévia, e testados em duas condições: linha de base e tratamento. Dado o tamanho da gaiola de contenção, apesar de não totalmente contidos, os animais ficaram impossibilitados de dar meia-volta ficando, no entanto, com uma pequena margem de espaço para movimentação da cabeça (± 5º direita-esquerda-acima-abaixo). Como esta variação pode induzir alterações de até 5 dB na intensidade sonora, considera-se que os animais foram submetidos à estimulação sonora com intensidade entre 92,5 e 97,5 dB. A apresentação dos estímulos é produzida e controlada por um sistema de aquisição e controle de dados individuais (Sysdin, Lynx, São Paulo, Brasil) coletados ao longo da sessão. Foram apresentados 50 estímulos auditivos, registrados individualmente como a diferença de potencial existente entre a parte não isolada (ponta em contato com o tecido neural) de um eletrodo de aço inoxidável (150 μm o.d.) inserido dentro de uma cânula de aço inox, de maneira que o comprimento final tanto do pólo do eletrodo quanto da microinjeção situaram-se no vCI (Figura 2).

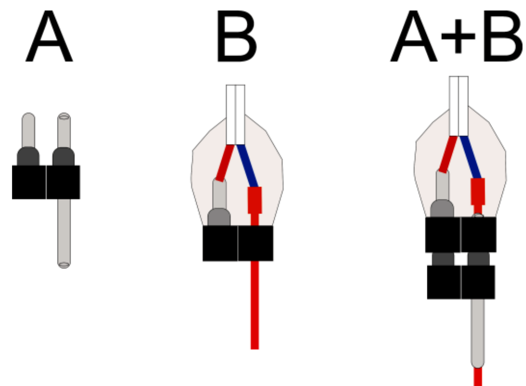

Figura 2. Esquema representativo do quimitrodo implantado intra-CI (A), cabo para registro da amplitude do potencial evocado (B) e a conexão entre cabo e quimitrodo demonstrando a diferença entre pólos (A+B).

A diferença de potencial existente entre estes dois polos é registrada e amplificada (Lynx, TX001, frequências passa alta e baixa 20 e 200 Hz, respectivamente) através de cabos condutores isolados que penetram na caixa experimental por uma passagem no topo de uma gaiola de Faraday. A saída do amplificador de sinais se conecta a um dos 4 canais de um conversor analógico/digital (CAD 12/36) ligado a um PC. Os PEAs são amostrados a uma taxa de 0.33 Hz (1 a cada 3 seg), sendo posteriormente filtrados (20-200 Hz). O software Sysdim foi programado para somar e promediar as amplitudes dos 50 PEAs individuais de cada tentativa (*trials*). A aquisição de dados é iniciada 10 ms antes do início da apresentação do estímulo sonoro, continuando até 200 ms após seu término.

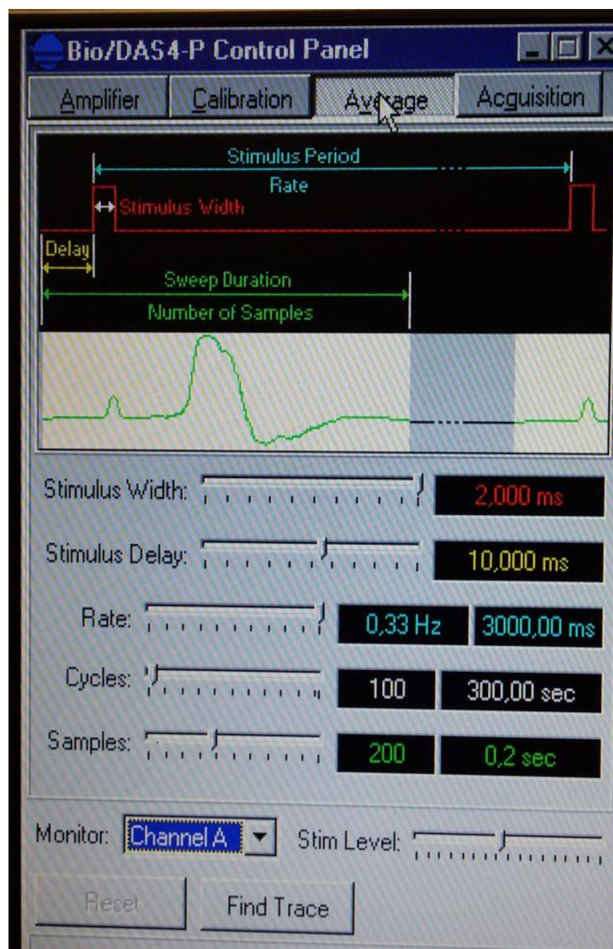

Figura 2.1 – Exemplo da tela de configuração da interface Bio/DAS4-P que controla a emissão e aquisição de sinais.

A primeira onda negativa (N1) assim como a primeira onda positiva (P1) são identificadas visualmente no monitor do PC. A onda P1 é considerada o componente inicial da resposta colicular. Sua amplitude é medida pico-a-pico, sendo a latência para seu pico situada por volta de 5 a 8 ms quando registrada por um eletrodo colocado na calota craniana (Baas *et al.*, 2006). As amplitudes de pico são definidas como as amplitudes máximas (em µV) medida entre N1 e P1, como em outros trabalhos.

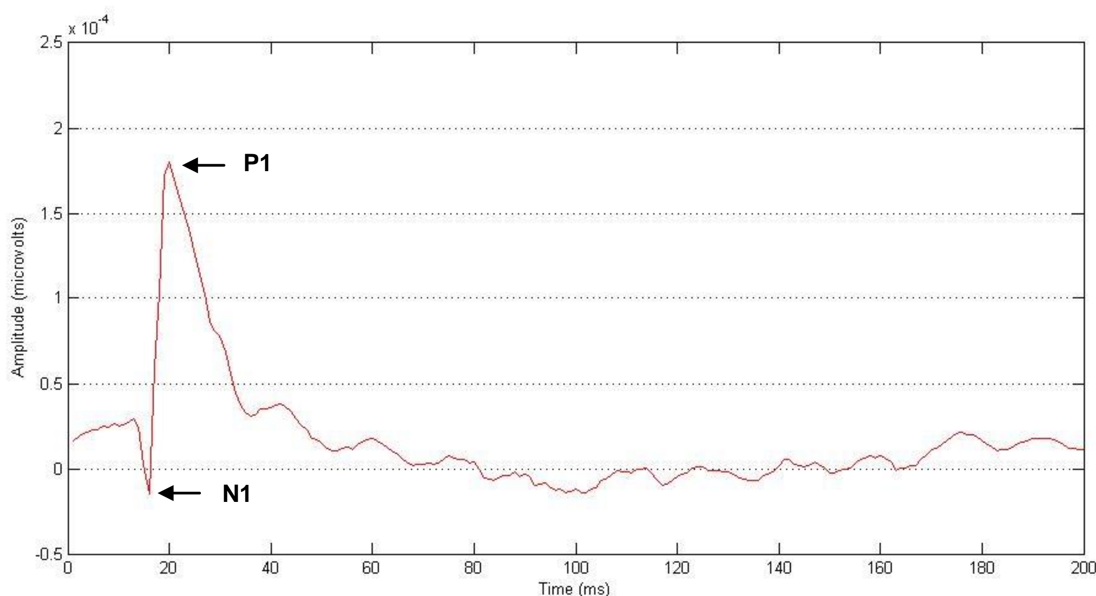

Figura 2.2 – Exemplo de um potencial evocado auditivo adquirido e amplificado. Os pontos utilizados para o cálculo da amplitude são sinalizados com setas.

Experimento 2B: Estimulação elétrica do CI ou SCPd. O equipamento e protocolo utilizados são idênticos ao Experimento 1. Selecionamos as doses para esta etapa do trabalho com base nos resultados anteriores do experimento. Foi utilizado o midazolam na dose de 20 nmol, muscimol na dose de 1 nmol e semicarbazida na dose de 7 µg, todas microinjetadas em um volume total de 0,2 µL.

Desenho experimental

Para o experimento 2A que consistiu na medida do PEA colicular após tratamentos aplicados no vCI ou SCPd, todos animais tiveram um quimitrodo implantado no vCI direito e 7 dias após a cirurgia foram submetidos às sessões de aquisição do PEA. Os animais que tiveram o tratamento intra-vCI receberam apenas um quimitrodo para registro e injeção do tratamento no CI. Os ratos que foram tratados intra-SCPd receberam um quimitrodo de registro direcionado ao vCI e uma cânula-guia na SCPd. Os testes ocorreram durante o período claro do ciclo circadiano, sendo registradas as amplitudes dos PEAs basais (apenas o cabo de registro conectado) e aquelas após tratamento intra-vCI ou intra-SCPd, respeitando-se o tempo de ação das drogas.

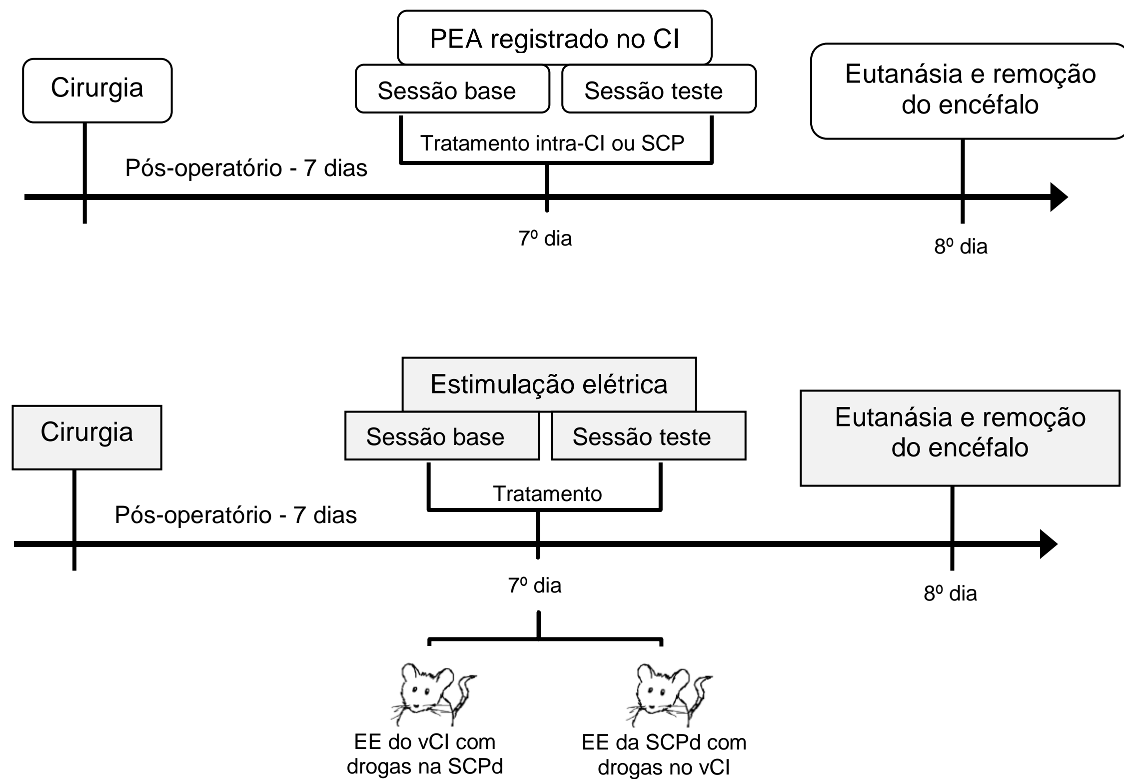

Figura 2.3 - Desenho experimental das etapas A e B do experimento 2.

Histologia

Ver Experimento 1.

Análise dos dados

Os dados do potencial evocado auditivo foram analisados por ANOVA de uma via seguida pelo teste *post-hoc* de Newman-Keuls. Já os dados obtidos a partir da estimulação elétrica foram analisados por meio de ANOVA de duas vias com medidas repetidas, seguida pelo test *post-hoc* de Bonferroni. A condição (linha de base/teste) foi considerada o fator “dentro do grupo” e o tratamento foi o fator “entre grupos”.

RESULTADOS

As Figuras 2.4 e 2.5 representam o posicionamento de quimitrodos e sítios de injeção no vCI e na SCPd no número total de animais utilizados no Experimento 2. Nesta etapa, os sítios de injeção foram concentrados no vCI, primordialmente na região anatômica do núcleo central do IC.

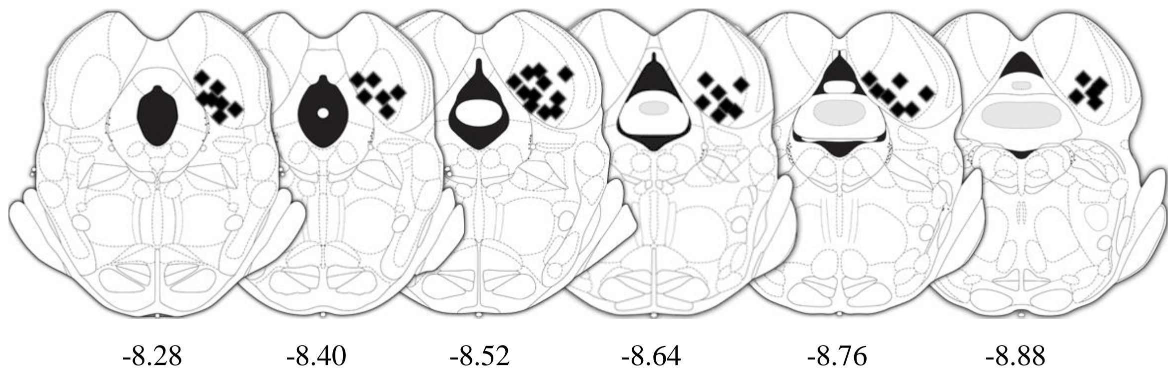

Figura 2.4 - Representação do posicionamento dos sítios de microinjeção na porção ventral do CI. A distância (em mm) a partir do bregma está representada em cada seção, de acordo com Paxinos & Watson (2006). O número de sítios marcados na figura é menor que o número total de animais devido a sobreposições.

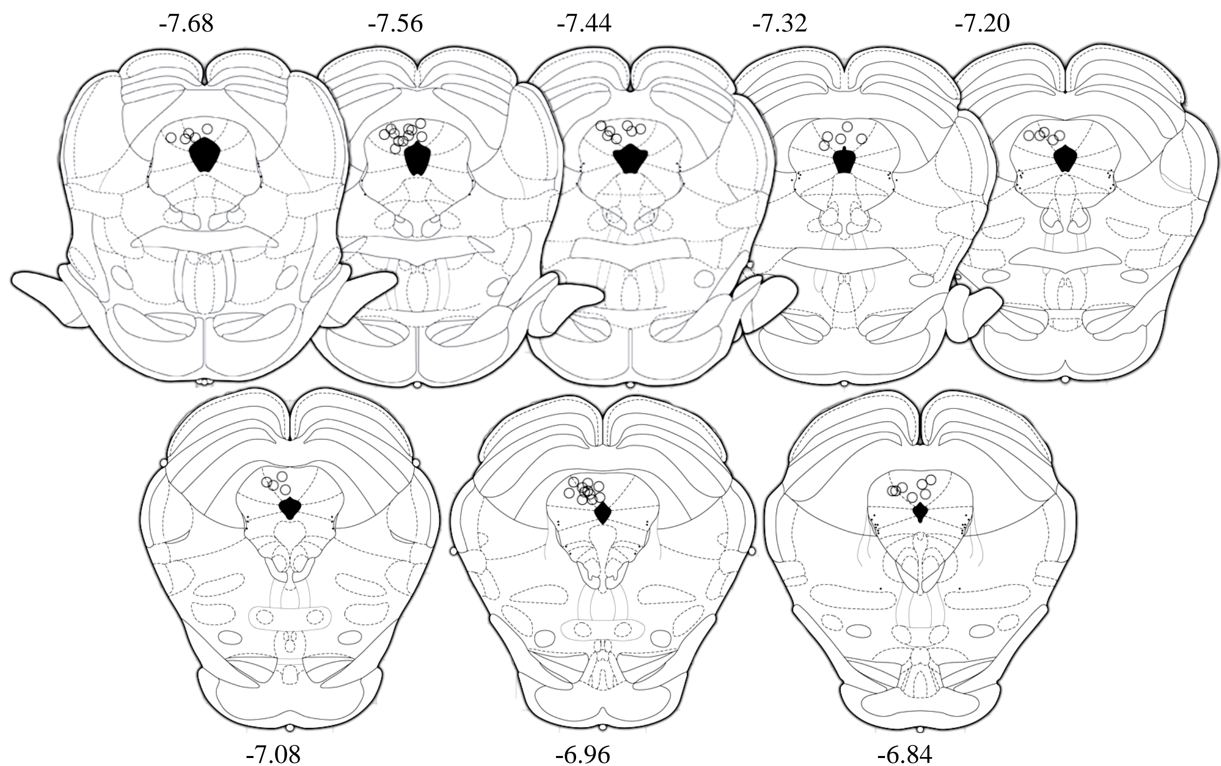

Figura 2.5 - Representação do posicionamento dos sítios de microinjeção na porção dorsal da SCPd. A distância (em mm) a partir do bregma está representada em cada seção, de acordo com Paxinos & Watson (2006). O número de sítios marcados na figura é menor que o número total de animais devido a sobreposições.

Alterações na amplitude do potencial evocado auditivo devido ao tratamento intra-vCI. Os resultados obtidos referem-se à variação da amplitude do PEA mensurado no vCI entre a linha de base e após a injeção local de drogas GABAérgicas e benzodiazepínicas. A análise *post hoc* de Newman-Keuls apontou efeito significativo dos tratamentos ($F_{(8,89)}$= 2,365; p<0,05); entretanto, esta diferença foi evidenciada entre os grupos MUS 2nmol e MDZ 20 nmol (drogas com modos de ação diferentes) e não entre doses de um mesmo fármaco (Figura 2.6).

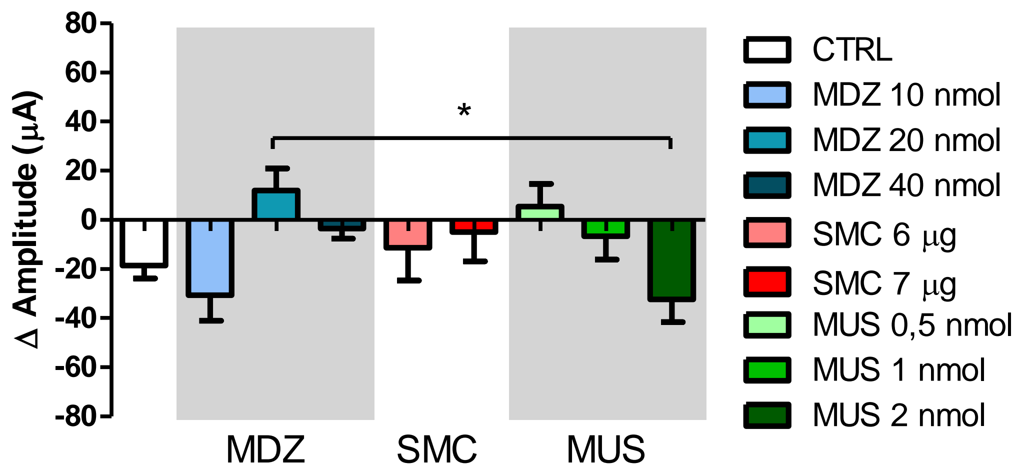

Figura 2.6 - Diferença de amplitude do potencial evocado auditivo mensurado no CI entre a sessão de base e a sessão teste, com as drogas intra-vCI. Na análise de variância com teste post hoc de Newman-Keuls, a dose de 20 nmol de midazolam e a dose de 2 nmol de muscimol apresentaram diferença significativa entre si.

Influência de mecanismos GABAérgicos da SCPd na expressão do PEA registrado no vCI. A Figura 2.7 mostra o efeito do muscimol, midazolam e semicarbazida injetados na SCPd sobre a amplitude do potencial evocado registrado no CI. Não houve diferenças significativas entre os grupos estudados ($F_{(5,45)}$= 1,851; p>0,05). Entretanto, é importante notar que o muscimol injetado na SCPd na dose de 2 nmol/0,2 µL provocou aumento da amplitude do PEA com relação ao grupo controle (Figura 2.7), enquanto que quando esta droga foi injetada diretamente no CI na mesma dose o oposto foi observado (Figura 2.6).

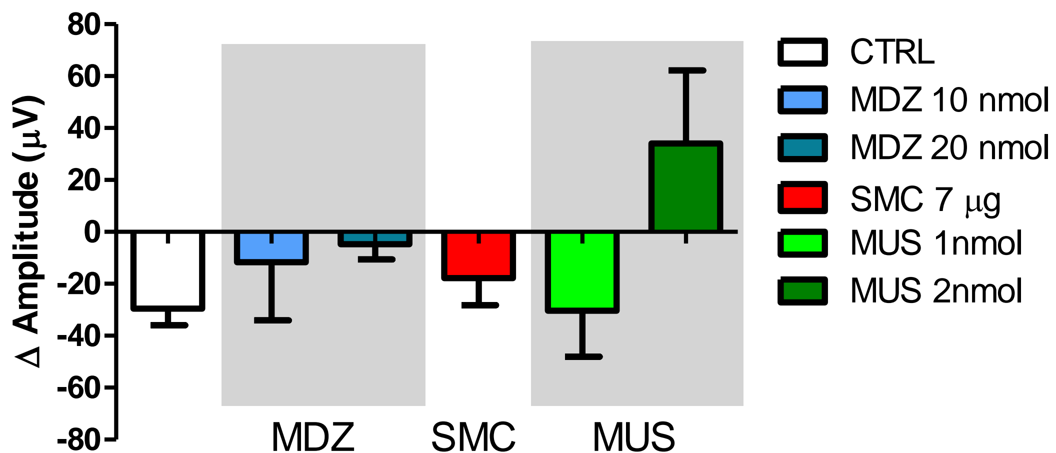

Figura 2.7 – Efeito de injeções intra-SCPd sobre o PEA registrado no vCI. Não houve diferenças significativas entre os grupos estudados ($F_{(5,45)}$= 1,851; p>0,05).

Influência do vCI na expressão de comportamentos defensivos decorrentes da estimulação da SCPd e vice-versa. Estes experimentos foram conduzidos com o intuito de investigar a conexão funcional entre vCI e SCPd na expressão do comportamento defensivo em decorrência da estimulação elétrica de uma ou outra estrutura, injetando-se diferentes drogas na estrutura que não era estimulada. Foram analisadas as diferenças nos limiares de fuga e no congelamento pós-estimulação. No grupo de ratos estimulados eletricamente na SCPd (Figura 2.8b), os tratamentos realizados no vCI causaram aumento significativo nos limiares de fuga ($F_{(3,21)}$= 4,43; p<0,05; interação entre fatores). A análise post-hoc revelou efeito significativo da SMC 7 µg ($F_{(1,21)}$= 8,63; p<0,01). Na Figura 2.9, observa-se que o muscimol injetado na SCPd reduziu o tempo de congelamento pós-fuga em animais cujo CI foi estimulado eletricamente ($F_{(3,25)}$= 4,65; p=0,0102).

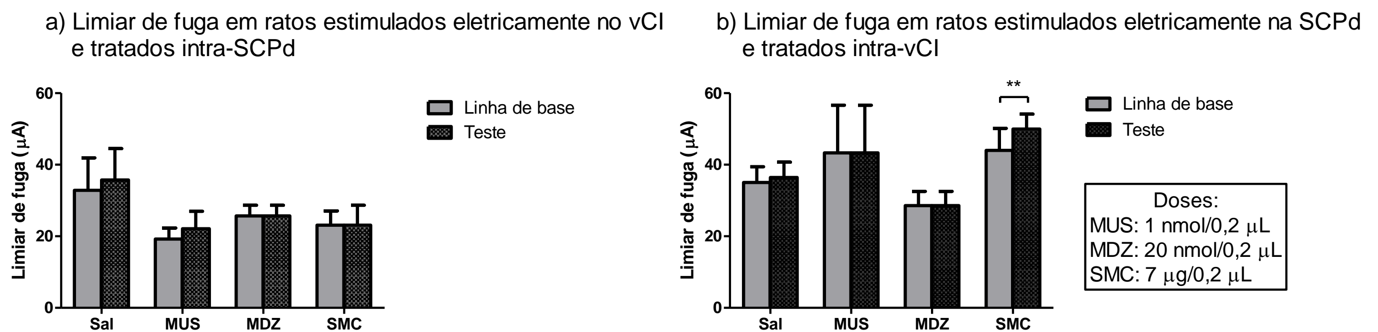

Figura 2.8 - Influência de mecanismos GABAérgicos da SCPd sobre limiares de fuga obtidos pela estimulação elétrica do vCI (a) e efeito do tratamento intra-vCI sobre limiares de fuga obtidos pela estimulação elétrica da SCPd (b). A semicarbazida injetada no vCI aumentou o limiar de fuga obtido pela estimulação elétrica da SCPd ($F_{(3,21)}$= 4,433; p=0,0145).

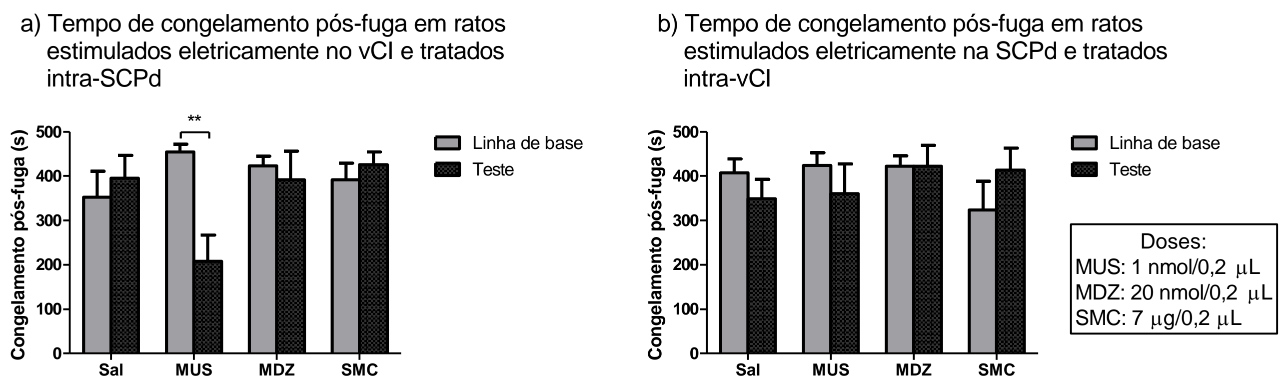

Figura 2.9 - Influência de mecanismos GABAérgicos da SCPd sobre o tempo de congelamento pós-fuga a partir da estimulação elétrica do vCI (a) e vice-versa (b). O muscimol injetado na SCPd reduziu o tempo de congelamento pós-fuga em animais cujo CI foi estimulado eletricamente ($F_{(3,25)}$= 4,65; p=0,0102).

DISCUSSÃO

Esta etapa do projeto permitiu avaliar de maneira conjunta como o vCI e a SCPd influenciam um ao outro, por meio da neurotransmissão GABAérgica, quanto aos dados obtidos no potencial evocado e no procedimento da estimulação elétrica. A utilização do registro dos potenciais evocados auditivos permitiu estudar a natureza das alterações eletrofisiológicas promovidas por drogas GABAérgicas injetadas tanto diretamente no CI quanto na SCPd, enquanto que a estimulação elétrica pôde fornecer alguns dados de como ocorre a expressão e o processamento de informações aversivas que passam por essas estruturas. Dados prévios sugerem que o CI seja regulado por filtros localizados na amígdala (Maisonnette *et al.*, 1996; Macedo *et al.*, 2005) e na substância negra (Castellan-Baldan *et al.*, 2006). Nossos resultados sugerem que não só estas estruturas possam ser filtros para o CI, mas também a SCPd.

Os potenciais evocados auditivos estão entre as respostas sensoriais evocadas mais complexas que se pode registrar no rato. Potenciais evocados de maneira geral representam uma medida geral da atividade do sistema nervoso central e são um método ideal para estudar os efeitos de drogas sobre as funções neurofisiológicas (Shaw, 1988). Sobre o registro do PEA no vCI com drogas injetadas na própria estrutura, encontramos uma diferença significativa entre o efeito do MDZ 20 nmol em relação ao MUS 2 nmol. Isto pode ser atribuído a diferentes formas de interação dessas drogas com o receptor $GABA_A$ e a disponibilidade de GABA endógeno na fenda sináptica (Reves *et al.*, 1985; Chandra *et al.*, 2010). Embora a maioria das correlações não tenha atingido significância estatística, ao compararmos os efeitos das mesmas drogas/doses em ambas estruturas no registro do PEA, notamos que há uma contraposição: enquanto que no CI o MDZ 20 nmol aumenta a amplitude e o MUS 2 nmol a reduz (estatisticamente relevante), o oposto parece ser possível quando as mesmas drogas são aplicadas na SCPd. Um estudo mais robusto seria necessário para confirmar este dado.

Os dados obtidos no experimento 2B também exploram a mediação GABAérgica entre o CI e a SCP e devem ser interpretados com cautela. Assim como o dado apresentado na Figura 1.16, salientamos que a diferença no limiar de fuga dos animais que receberam semicarbazida no vCI enquanto eram estimulados eletricamente na SCPd é de cerca de 6 µA, que apesar de ter relevância estatística, pode significar uma limitação técnica e não um fenômeno biológico. Por outro lado, o congelamento pós-estimulação elétrica da SCPd tem

sido estudado como modelo do transtorno de pânico (Brandão *et al.*, 2008). Esta resposta seria um indicativo de medo extremo, intenso, em que ocorre o processamento da informação aversiva por estruturas rostrais, e portanto resistente à ação de ansiolíticos. Nosso dado reforça a ideia de que o congelamento pós-estimulação seja gerado por mecanismos intrínsecos na própria SCPd (Vianna *et al.*, 2001), pois há redução do tempo gasto neste processamento mesmo que a estimulação tenha ocorrido no vCI. A representação sucinta dos dados obtidos no experimento 2B encontra-se no esquema abaixo:

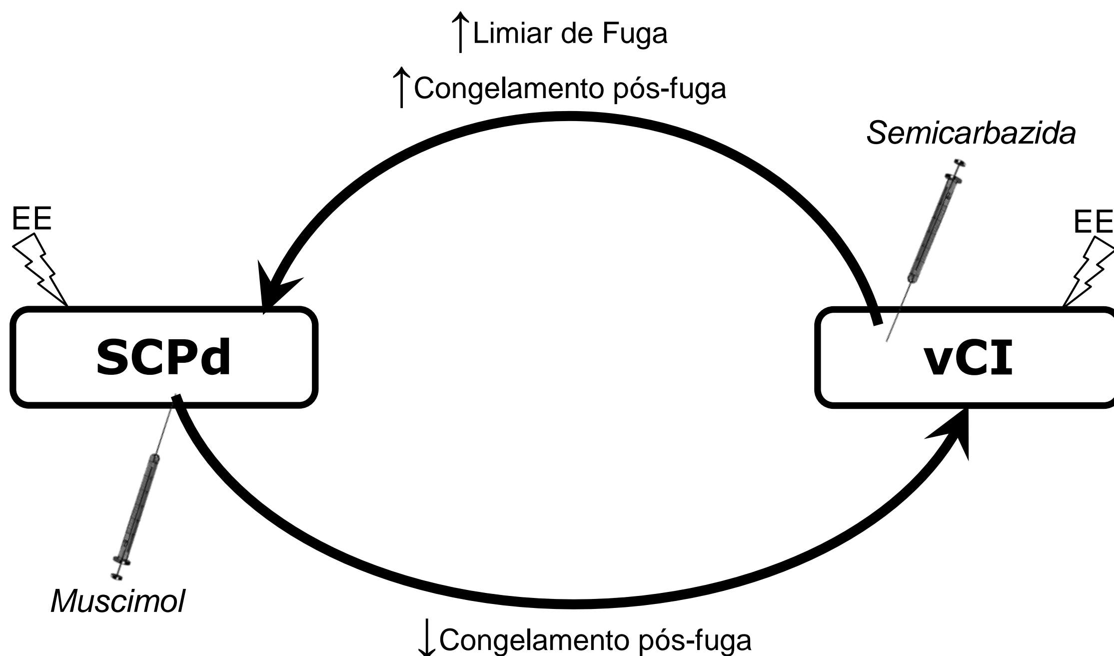

Figura 2.10 – Representação esquemática dos dados obtidos a partir do Experimento 2B.

A relevância dos resultados obtidos nesta etapa da investigação aponta para um aspecto intrigante da interface entre CI e SCP. Assim como uma nova divisão funcional entre as porções dorsal e ventral do CI emergiu do primeiro experimento, esta etapa levantou interessantes questões sobre a ideia de que o CI seja o *input* da informação aversiva e que a SCP seja a simples via de saída comum para o comportamento defensivo (Vianna & Brandão, 2003), com ambas as estruturas participando de um circuito maior que dependa de outras áreas durante o processo. Sabe-se que o núcleo central do CI estabelece conexões recíprocas com as colunas ventrais e laterais da SCP (Osaki *et al.*, 2003). Além disto, estudos neuroanatômicos sugerem uma relação estreita entre os sistemas opioide e GABAérgico, em que um mesmo neurônio pode ser diretamente regulado por GABA e peptídeos opioides endógenos (Kalyuzhny *et al.*, 2000), modulando inclusive comportamentos do tipo pânico

organizado pelo CI e pela SCPd (Twardowschy & Cysne Coimbra, 2015). Xiong e cols. (2015) sugerem que o núcleo central do CI por si só não medeia diretamente o comportamento de fuga induzido por som. Este núcleo participaria essencialmente como relé da informação auditiva ascendente, enviando-a para o núcleo geniculado medial e então para o córtex auditivo. O córtex auditivo por sua vez envia projeções corticocoliculares ao córtex do CI (xCI), que mantém conexões com a SCPd e assim modularia a fuga induzida por som (Xiong *et al.*, 2015). Cabe ressaltar que a via proposta refere-se estritamente à fuga induzida por estimulação sonora aversiva e pode não estar igualmente relacionada ao processamento da informação aversiva de natureza não-auditiva que também ocorre no CI. Assim, não descartamos a hipótese de que haja uma interação direta entre CI e SCP, mediada por mecanismos GABAérgicos, responsável pela aquisição da informação aversiva e modulação da expressão do comportamento defensivo.

EXPERIMENTO 3

Envolvimento de mecanismos GABAérgicos no vCI e na SCPd em um modelo de medo inato a um estímulo luminoso (*Light Switch-Off Test*; LSOT)

MATERIAIS E MÉTODOS

Animais e cirurgia

Foram utilizados 74 ratos Wistar machos, provenientes do Biotério Central da USP-RP, mantidos nas mesmas condições que os sujeitos dos Experimentos 1 e 2 . A cirurgia estereotáxica seguiu o mesmo protocolo utilizado nas etapas prévias deste trabalho, mas somente cânulas-guia foram implantadas no vCI ou SCPd para a realização de microinjeções de fármacos localmente.

Drogas e microinjeções

Foram utilizadas as drogas GABAérgicas muscimol (MUS; 0,5 nmol/0,2 μL), midazolam (MDZ; 5 nmol/0,2 μL), e semicarbazida (SMC; 3,5 μg/0,2 μL.). Para fins de comparação, a sulpirida (SUL; um agonista seletivo dos receptores dopaminérgicos D_2 e D_3) foi utilizada na dose de 3 μg/0,2 μL. O grupo controle recebeu solução salina (CTRL) estéril ou a solução veículo utilizada para preparo da sulpirida (solução fisiológica com Tween 80 a 1%). As doses foram escolhidas a partir dos resultados obtidos nas etapas anteriores do trabalho e em estudos pilotos, de forma a contemplar a menor dose efetiva possível sem que houvesse qualquer alteração na locomoção do animal, o que poderia ocasionar dados falso-positivos.

Equipamentos e procedimentos

Light Switch-Off Test. A caixa experimental possui 2 compartimentos de 30×25×25 cm cada, separadas por um portal de acrílico (Insight, Brazil). As paredes laterais e do fundo são opacas, enquanto a face e o teto são de acrílico transparente. O assoalho possui barras metálicas espaçadas em 1,2 cm. Em cada lado dos fundos da caixa há uma lâmpada incandescente de 40 watts a 12 cm do assoalho. Após 5 minutos de habituação na caixa, 40 estímulos luminosos (duração de 20 s cada) são emitidos em intervalos aleatórios de 15 a 45 s. Ao cruzar de um compartimento a outro durante o período iluminado, o rato desliga o

estímulo (*light switch-off response*). Um software controla a emissão do estímulo luminoso e a aleatoriedade dos intervalos, bem como registra a frequência e latência da resposta e a locomoção entre estímulos (períodos sem luz). Cada animal foi submetido a apenas uma sessão. As sessões experimentais ocorreram no período diurno e a caixa foi limpa previamente com álcool a 20% após cada animal testado. Este estudo foi realizado com base no trabalho anteriormente desenvolvido neste laboratório visando caracterizar o LSOT como um modelo animal de ansiedade (ANEXO 2).

Desenho experimental

Os animais tiveram cânulas-guia implantadas no vCI ou SCPd. Sete dias após a cirurgia, os animais receberam o respectivo tratamento de acordo com o grupo ao qual pertenciam e testados no LSOT. Cada animal foi testado apenas uma vez. Após o teste, os animais foram eutanasiados e seus encéfalos preservados para posterior localização dos sítios de microinjeção.

Histologia

A análise histológica seguiu o mesmo procedimento descrito nos Experimentos 1 e 2. As figuras 3.1 e 3.2 ilustram o local onde as drogas foram injetadas.

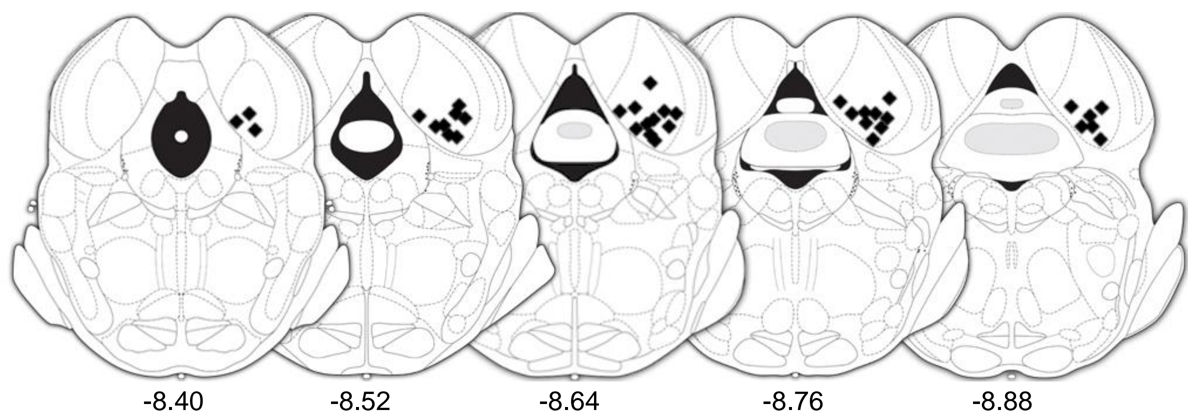

Figura 3.1 - Representação do posicionamento dos sítios de microinjeção na porção ventral do CI. A distância a partir do bregma está representada em cada seção, de acordo com Paxinos & Watson (2006).

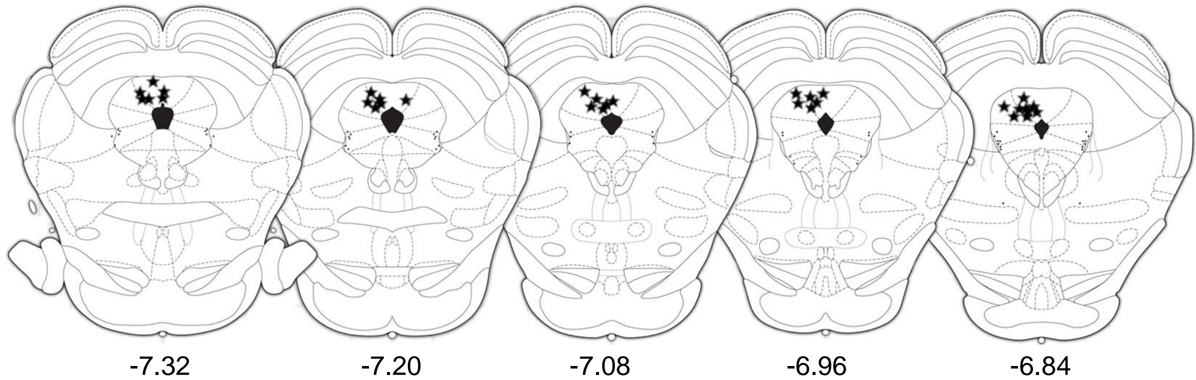

Figura 3.2 - Representação do posicionamento dos sítios de microinjeção na porção dorsal da SCPd. A distância a partir do bregma está representada em cada seção, de acordo com Paxinos & Watson (2006).

Análise dos dados

Para fins de análise, além dos valores totais de cada parâmetro avaliado (frequência e latência de respostas e locomoção entre estímulos), cada sessão foi dividida em 4 blocos de 10 estímulos cada. Os dados foram analisados por ANOVA de uma ou duas vias com medidas repetidas, sendo o tratamento o fator "entre" grupos e os blocos como fator "dentro" (teste *post-hoc* de Newman-Keuls e Bonferroni, respectivamente).

RESULTADOS

O número total de respostas de desligar a luz foi significativamente maior nos animais que receberam sulpirida intra-vCI, com relação ao muscimol e ao midazolam (Figura 3.3a). Tal efeito não ocorreu com as mesmas drogas e doses injetadas intra-SCPd (Figura 3.3b), sugerindo que mecanismos GABAérgicos e o agonista dopaminérgico para receptores D2 e D3 da SCPd não participam diretamente dessa resposta. A sulpirida também torna menor a latência para a emissão da resposta de desligar a luz apenas quando injetada no vCI (Figura 3.4a) sem surtir qualquer alteração quando injetada na SCPd (Figura 3.4b).

Respostas de desligar a luz (*light switch-off responses*)

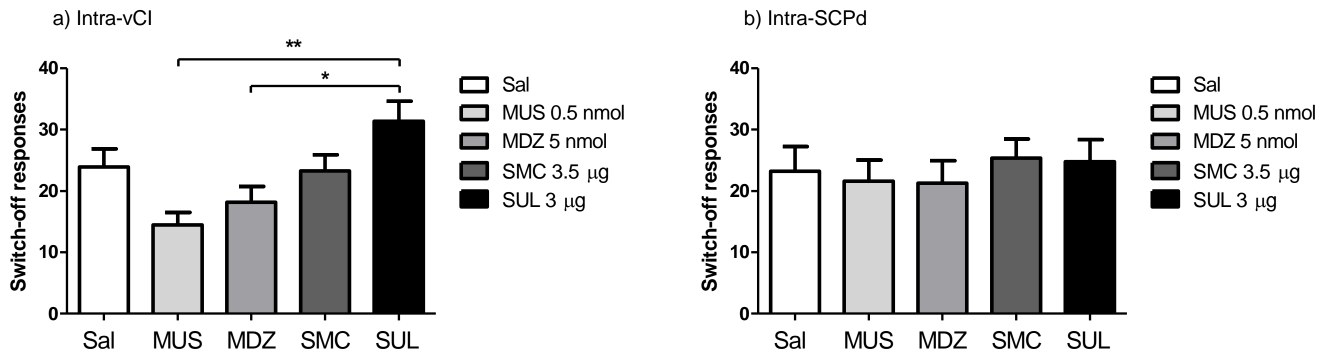

Figura 3.3. A sulpirida, injetada intra-vCI (a), aumenta a frequência de respostas de desligar da luz em relação às drogas GABAérgicas muscimol e midazolam ($F_{(4,32)}$= 5,151; p=0,0026). O mesmo efeito não foi encontrado ao injetar as mesmas drogas e doses na SCPd (b); p=0.8878.

Latência média das respostas

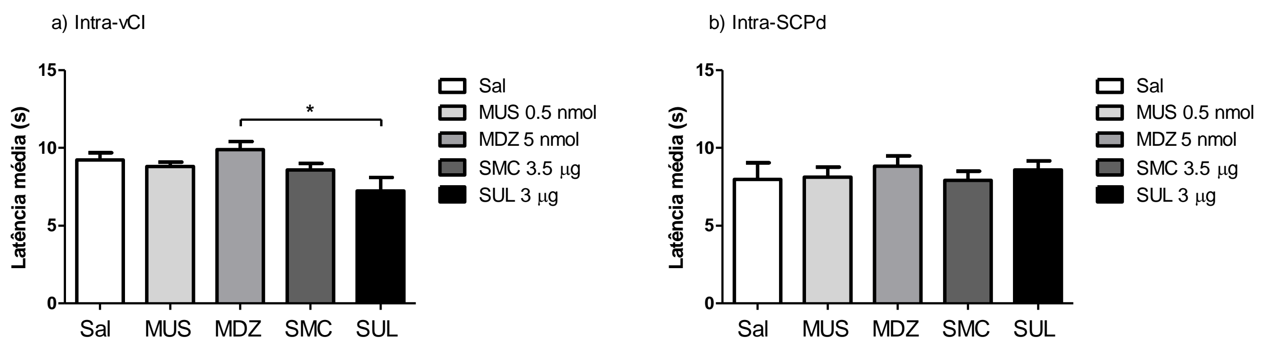

Figura 3.4 - A sulpirida, injetada intra-vCI (a), diminui a latência média de respostas de desligar da luz em relação ao midazolam ($F_{(4,31)}$= 3,251; p=0,0245). O mesmo efeito não foi encontrado ao injetar as mesmas drogas e doses na SCPd (b); p=0.8531.

Com relação número de cruzamentos dos animais nos intervalos entre estímulos, observa-se que nenhuma das drogas interferiu com a atividade locomotora, ou seja, a resposta de desligar a luz não esteve associada à hiperlocomoção e sim ao comportamento defensivo (Figura 3.5).

Locomoção geral

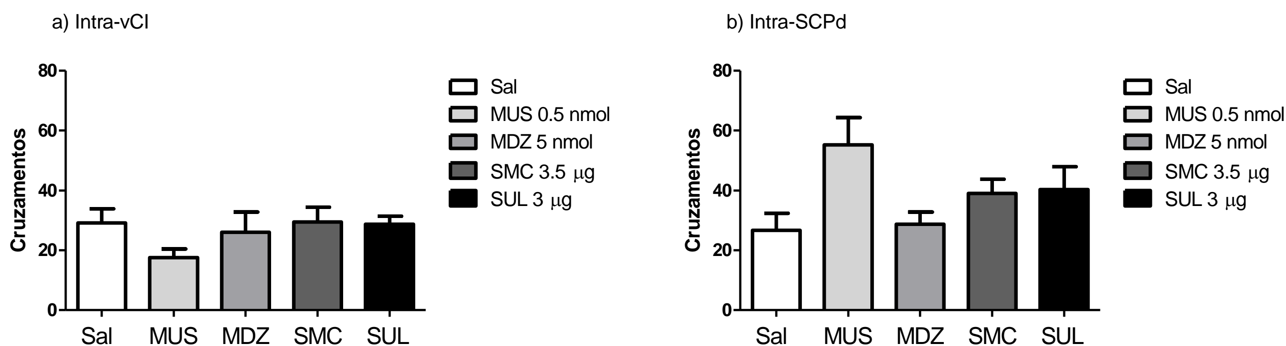

Figura 3.5 - A locomoção dos animais que receberam qualquer uma das drogas no vCI (a) ou SCPd (b) não foi alterada (p=0,2887 e p=0,0596). Entretanto, houve uma tendência do muscimol intra-SCPd em aumentar o número de cruzamentos totais ($F_{(4,32)}$= 2,531; p=0,0596).

A análise dos dados totais sugere que mecanismos GABAérgicos e dopaminérgicos atuam na mesma direção regulando os mecanismos de defesa no teto mesencefálico. A sulpirida é um antagonista dos receptores D_2 e D_3 e produziu efeitos pró-aversivos, logo a dopamina teria um papel inibitório sobre o comportamento defensivo como o faz o muscimol, na expressão dos comportamentos observados no LSOT. Enquanto que a sulpirida possui um perfil pró-aversivo no vCI (aumentando a frequência de respostas de desligar a luz), o midazolam e especialmente o muscimol atenuam a frequência de respostas (Gráfico 3.3a). A latência média para emissão da resposta é menor em ratos tratados com sulpirida no vCI, enquanto que o midazolam aumenta esse tempo (Gráfico 3.4a). Este efeito das drogas citadas não se deve a alterações na locomoção do animal, o que poderia gerar dados falso-positivos (Gráfico 3.5a). Já SCPd parece não ser influenciada pelas drogas utilizadas neste estudo, embora o muscimol tenha apresentado uma tendência (p=0,0596) em aumentar a locomoção dos animais, como visto no gráfico 3.5b.

Em análise detalhada de cada um dos 4 blocos que compõem uma sessão, observamos os mesmos parâmetros em uma perspectiva temporal com relação à duração total do teste. Nos animais tratados intra-vCI, observou-se notável queda no número de respostas no bloco 2 para os que receberam muscimol 0,5 nmol e no bloco 4 para os que receberam midazolam 5 nmol ($F_{(3,93)}$=24,02). O mesmo fenômeno não ocorreu com as drogas agindo sobre a SCP (Figura 3.6). O muscimol também interferiu com a latência média das respostas emitidas no primeiro bloco, diminuindo-a significativamente ($F_{(12,93)}$=1,929) (Figura 3.7). Já a locomoção entre estímulos (índice de atividade motora) passou por diminuição gradual ao longo do tempo do teste, como esperado. Os dados sugerem que nenhuma das drogas causou alterações

no comportamento motor, o que reforça o resultado obtido na frequência de respostas de desligar a luz (Figura 3.8).

Respostas de desligar a luz (*light switch-off responses*).

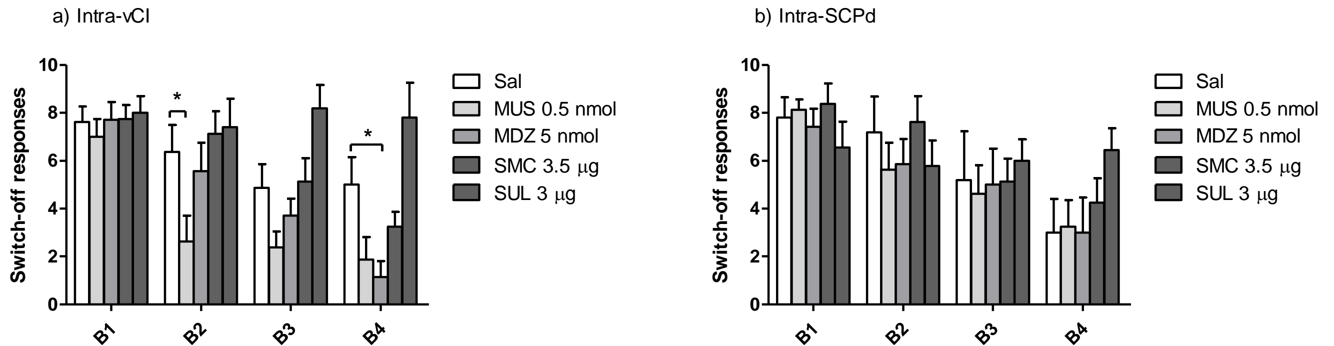

Figura 3.6 - Frequência de respostas de desligar a luz por cada bloco do teste. Nos animais tratados intra-vCI, observou-se notável queda no número de respostas no bloco 2 (para os que receberam muscimol) e no bloco 4 (para os que receberam midazolam) ($F_{(3,93)}$=24,02). O mesmo fenômeno não ocorreu com as drogas agindo sobre a SCP.

Latência média das respostas

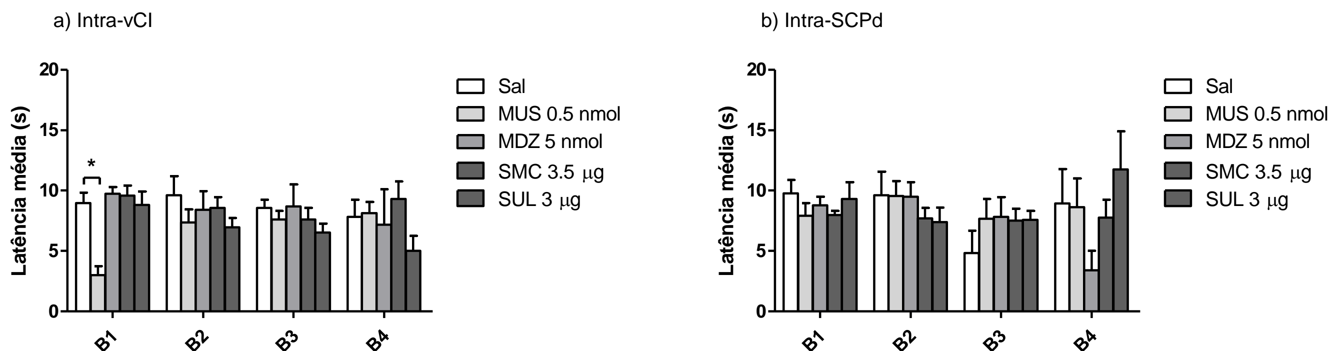

Figura 3.7 - Latência média de respostas por cada bloco do teste. Nos animais tratados intra-vCI, observou-se redução significativa da latência de respostas no bloco 1 (para os que receberam muscimol) ($F_{(12,93)}$=1,929). O mesmo fenômeno não ocorreu com as drogas agindo sobre a SCP.

Locomoção geral

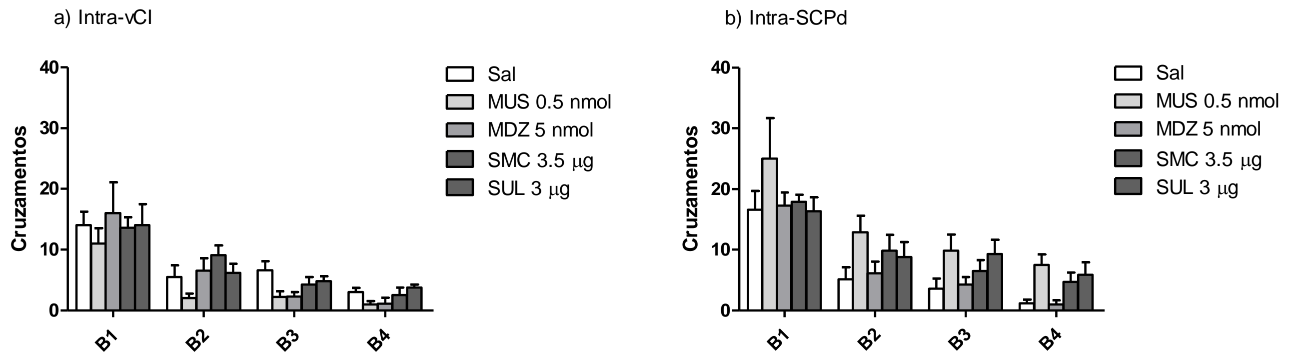

Figura 3.8 - Número de cruzamentos entre estímulos por cada bloco do teste. Nota-se gradual diminuição na exploração da caixa ao longo do tempo, como esperado. Entretanto os dados sugerem que nenhuma das drogas causou alterações no comportamento motor, de forma que os resultados obtidos na frequência de respostas de desligar a luz não podem ser atribuídos a uma interferência da coordenação motora do animal.

DISCUSSÃO

O *light switch-off test* tem sido estudado em nosso laboratório como um teste de medo incondicionado, baseado no comportamento inato de roedores de hábitos noturnos (como é o caso da espécie *Rattus norvergicus*) em escapar de áreas iluminadas, onde eles estariam mais vulneráveis. A primeira publicação que descreve este teste envolveu drogas dopaminérgicas injetadas intraperitonealmente em ratos expostos ao LSOT e seus resultados abriram a possibilidade de consolidar o LSOT como um teste de medo incondicionado (etológico) para ansiedade, assim como o teste claro-escuro ou o labirinto em cruz elevado, nos quais o animal se depara com um ambiente aversivo do qual podem facilmente escapar (Reis *et al.*, 2004).

Em concordância com os dados obtidos anteriormente, a sulpirida atuou aumentando a frequência de SOR e tornando mais rápida a emissão dessa resposta, quando injetada localmente no vCI. Ainda sobre o CI, o muscimol atuou como agente antiaversivo, contrapondo-se ao efeito da sulpirida. E de maneira condizente a sua classe farmacológica, o midazolam aumentou a latência média de respostas de forma oposta à sulpirida. Assim, entendemos que mecanismos GABAérgicos e dopaminérgicos presentes no CI participam ativamente do comportamento de cessar a estimulação aversiva luminosa utilizada no LSOT. Sendo assim, o CI parece ser uma das estruturas diretamente envolvidas nesse teste, que curiosamente não possui qualquer tipo de estimulação auditiva.

A SCPd parece não estar envolvida nos comportamentos observáveis no LSOT, ou pelo menos os mecanismos GABAérgicos atuantes nessa estrutura não fazem parte da saída da resposta a este teste incondicionado. Embora estatisticamente não significativa, encontramos uma tendência do muscimol injetado na SCPd em aumentar o índice de locomoção no LSOT. Se tal resultado fosse efetivamente encontrado em futuros estudos com a mesma droga injetada na mesma estrutura, seria possível fazer referência ao resultado obtido no Experimento 2 descrito anteriormente (Figura 2.9) em que o muscimol intra-SCPd reduziu o tempo de congelamento pós-estimulação elétrica do vCI. O mecanismo proposto para o aumento da locomoção no LSOT seria o mesmo discutido acerca da redução do tempo de congelamento, ou seja, o muscimol nessa dose atuaria sobre interneurônios tonicamente inibidos pelo GABA, desinibindo-os e, desta forma, ocorreria a liberação do comportamento motor.

Um aspecto importante sobre o LSOT é a análise conjunta de resultados totais e divididos bloco a bloco. Esta análise conjunta foi realizada no primeiro estudo envolvendo o LSOT e também nos estudos preliminares envolvendo variações no protocolo, descritos no Anexo 1. Embora a análise dos dados totais ofereça uma perspectiva ampla com relação ao teste como um todo, a análise bloco a bloco pode ser considerada uma análise temporal do efeito da droga testada e evidenciar detalhes não contemplados na análise total. Sendo uma proposta inteiramente inédita na literatura, os resultados obtidos na terceira e última etapa deste trabalho contribuem não só para a fundamentação teórica do novo teste animal proposto, mas também para uma compreensão ampla da participação do CI e da SCP na interface sensoriomotora do medo e ansiedade tal como expressa nos modelos incondicionados empregados neste trabalho.

CONCLUSÕES

CONCLUSÕES

1) Existe uma segregação funcional entre as porções dorsal e ventral do colículo inferior. A porção ventral do colículo inferior (vCI) é o substrato neural das respostas defensivas organizadas nesta estrutura, o que reforça a sua inclusão no Sistema Encefálico de Aversão.

2) A interface sensoriomotora constituída entre o vCI e a SCPd é mediada por mecanismos GABAérgicos tônicos que são capazes de modular a reatividade do substrato neural do medo nessas estruturas.

3) Há indícios de uma conexão funcional de natureza GABAérgica entre o vCI e a SCPd, influenciando o processamento e elaboração do comportamento defensivo. Esta conexão pode, ainda, contemplar outros circuitos neurais de natureza dopaminérgica, opioide, e mesmo glutamatérgica, que abrem perspectivas para estudos futuros.

4) Destaca-se a importância do debate acerca do papel atribuído ao CI de via de entrada da informação aversiva (*input*) e da SCP como via comum de saída do comportamento defensivo (*output*) visto que a comunicação entre estas estruturas pode ser mais estreita do que é reconhecida atualmente, inclusive com relação à forma com que a neurotransmissão em uma estrutura é capaz de interagir funcionalmente com a outra.

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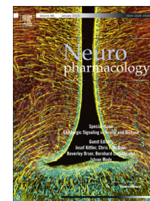

The benzodiazepine midazolam acts on the expression of the defensive behavior, but not on the processing of aversive information, produced by exposure to the elevated plus maze and electrical stimulations applied to the inferior colliculus of rats

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ABSTRACT

Electrical stimulation of midbrain tectum structures, particularly the dorsal periaqueductal gray (dPAG) and inferior colliculus (IC), produces defensive responses such as freezing and escape behavior. Freezing also results after termination of this stimulation (post-stimulation freezing; PSF). Whereas these responses are critically mediated by GABA in the dPAG, it is unclear how GABA-benzodiazepine mechanisms mediate the expression of fear (freezing and escape behaviors) and the processing of aversive information (PSF) produced by electrical stimulation of the IC. Since dorsal (ICd) and ventral regions (ICv) of the IC react differentially to aversive stimulation, we hypothesized that these regions might be sensitive to the action of benzodiazepine drugs when rats are submitted to animal models of anxiety: the elevated plus maze (EPM) and the IC electrical stimulation procedure. Midazolam (5, 10 or 20 nmol) was injected into the ICd or ICv of rats subjected to one of these tests. Intra-ICv, but not intra-ICd injections, of midazolam reduced the aversiveness of the IC electrical stimulation and decreased fear in the EPM, as assessed by its traditional and complementary measures. In contrast, the IC post-stimulation freezing remained unaltered with midazolam treatments. Thus, there is a clear pharmacological dissociation in the reactivity of dorsal and ventral regions of the IC to fear-provoking stimuli of the two animal models of anxiety used in this study. The present results support the proposal that benzodiazepine-mediated mechanisms are only involved in the output mechanisms of defensive behavior and not involved in the processing of ascending aversive information from the IC.

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1. Introduction

The inferior colliculus (IC) is a midbrain tectum structure, known for its pivotal role in acoustic processing. It is similar throughout a variety of species, but in the rat the IC is an ovoid bilateral structure, with the long diameter measuring 3.5 mm and the short diameter 2 mm (Faye-Lund and Osen, 1985). It is a region that has tonotopic and layered topography, morphologically prepared to encode and decipher auditory information in terms of spatial, spectral, and temporal properties (Winer and Schreiner, 2005).

Besides being a relay for auditory information (Aitkin and Phillips, 1984; Faingold et al., 1989), the IC is also capable of organizing responses to aversive stimuli. This notion has been refined over the last 30 years and today it is well accepted that the IC is part of the "Encephalic Aversion System (EAS)" that comprises other structures such as the dorsal periaqueductal gray (dPAG), the medial hypothalamus (MH), and the amygdala (AMYG). Neuroanatomical data establish reciprocal connections between the AMYG, MH and PAG (Bandler and Shipley, 1994; Brandao et al., 1999) but the way IC establishes neural

Abbreviations: BZD, Benzodiazepine; EAS, Encephalic Aversion System; EPM, Elevated plus maze; ICd, dorsal part of inferior colliculus; ICv, ventral part of inferior colliculus; MDZ, Midazolam.

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connectivity with the other structures of the EAS is still under investigation. It is known that electrical or chemical stimulation of the IC, as seen in other EAS areas, produces defensive responses, such as freezing and flight behavior (Pandossio and Brandao, 1999). It is reasonable to think that the IC would be able to elaborate some sort of immediate response after an initial (and rudimentary) processing of sounds that signaled some risk to survival (Casseday and Covey, 1996). However, it is known that this structure may also organize defensive behaviors that do not involve sounds. Indeed, rats submitted to the EPM without any acoustic stimulation present increased *Fos* activity in the IC, among other structures involved in the defense reaction (Silveira et al., 1993). Other *Fos* expression studies have also disclosed neural circuits underlying the freezing behavior elicited by stimulation of distinct regions of the IC (Borelli et al., 2006; Ferreira-Netto et al., 2007; Lamprea et al., 2002). NMDA injections aimed at the dorsal part of the IC (ICd) caused an increase of *Fos* expression in the medial geniculate nucleus, superior colliculus, dPAG and locus coeruleus, while injections of NMDA into the ventral region of IC (ICv) resulted in increased *Fos* expression in the prelimbic and cingulate cortices, basolateral and medial nuclei of AMYG, ventrolateral PAG, cuneiform nucleus and locus coeruleus (Ferreira-Netto et al., 2007). Bagri et al. (1991) found that electrical stimulations of either the dorsal part or ventral part of the IC both elicited wild running, being the ventral part more sensitive than the dorsal part. Indeed, the ICd has been recognized as the neural substrate of audiogenic seizures (Garcia-Cairasco and Sabbatini, 1991), while the ICv may play a key role in the sensorimotor gating of aversive information (Brandao et al., 1993).

The GABA system plays a tonic inhibitory role on the neural substrates of aversion in the inferior colliculus. It is estimated that 20%–40% of IC neurons are GABAergic (Caspary et al., 1990). GABA/benzodiazepine agonists attenuate the aversive outcome of electrical stimulation at this site (Melo et al., 1992; Pandossio and Brandao, 1999) as also shown previously in other related structures of the EAS (Audi and Graeff, 1987; Brandao et al., 1990; Graeff, 1981; Graeff et al., 1986, 1993; Schenberg et al., 1983). Instead of directly enhancing GABAergic neurotransmission with GABA agonists, the use of benzodiazepines, a class of drugs widely used in clinical practice, has proven to be very useful to modulate the GABA system during aversive states generated by an array of threatening conditions. Post-stimulation freezing (PSF) has been related to the processing of aversive information that is relayed to higher structures (Borelli et al., 2006; Brandao et al., 2008; Martinez et al., 2006; Vianna et al., 2001). A key question when we look at the anxiolytic-like actions of benzodiazepines is where, how and when they interfere with the emotional reactivity of the animal facing threatening situations. As for the IC, a recent study from this laboratory showed that there is a clear anatomical specificity regarding the dorsal and ventral regions of this structure as to the reactivity to aversive stimulation; only the ventral regions triggered fear responses to the local injections of the excitatory amino acid NMDA (Ferreira-Netto et al., 2007). From these findings we predicted that these IC regions might be sensitive to anxiolytic-like actions of benzodiazepine drugs when rats are submitted to animal models of anxiety: the elevated plus maze (EPM) and the IC electrical stimulation procedure. Besides, we were also interested in examining the effects of midazolam on the output mechanisms of defensive behavior (freezing and escape behaviors) and in the processing of ascending aversive information from the IC (post-stimulation freezing). A suitable candidate for this experimental purpose is midazolam, a short-acting, non-selective anxiolytic drug that is highly soluble in water, which makes it appropriate for intracerebral injections.

2. Materials and methods

2.1. Animals

Adult male Wistar rats ($n = 127$), weighing 275 ± 25 g, were housed in groups of four per cage, with free access to food and water. The cages were kept in a temperature-controlled room (20–25 °C) and 12-h light/dark cycle (lights on at 7:00 a.m.). All procedures followed the guidelines on the ethical use of animals by the Brazilian Society of Neuroscience and Behavior's (SBNeC) which follows the National Institutes of Health (NIH) guide for the care and use of laboratory animals. This work has also been approved by the Ethics Committee on Animal Use from the University of Sao Paulo (Protocol no. 11.1.308.53.9). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Guide cannulas and chemitrodes

Guide cannulas were made of 23G stainless steel needles (length = 13 mm; Becton–Dickinson, Franklin Lakes, NJ, USA), and were implanted in animals subjected to the EPM. Chemitrodes consisted of a guide cannula attached to a brain electrode. They were made of a stainless steel wire (0.27 mm, o.d.) (A-M Systems, Inc., USA) insulated throughout except at the cross section of the tip. The insulated wire was soldered to one pin of a miniature socket, parallel to a stainless steel guide cannula 1 mm shorter than the tip of wire. A non-insulated stainless steel wire was soldered to the other pin and to one of the anchor screws, thereby serving as the indifferent electrode. Chemitrodes were used in rats that received the drug at the same brain site used for electrical stimulation.

2.3. Stereotaxic surgery

The animals were anesthetized with ketamine/xylazine association (100/7.5 mg/kg respectively, i.p., Agener União, Embu-Guaçu, SP, Brazil) and fixed in a stereotaxic frame (David Kopf, Tujunga, CA). The upper incisor bar was set 3.3 mm below the interaural line to assure horizontality between bregma and lambda. Either a unilateral guide-cannula or chemitrode was implanted over the right IC. According to the atlas of Paxinos and Watson (2006) and with lambda serving as the reference point, the coordinates were a) for guide-cannulas: antero-posterior (AP) −0.9 mm, medio-lateral (ML) −1.7 mm and dorso-ventral (DV) -4.3 ± 0.5 mm (orthogonally) or b) for chemitrodes: AP +1.6 mm, ML -1.7 mm and DV either −4.3 or −5.3 mm (at a 15° angle). For all groups, the cannulas or chemitrodes were fixed to the skull with acrylic resin and two stainless steel anchor screws. Each guide cannula was sealed with a stainless steel wire to protect it from blockage. At the end of surgery, animals received an injection of a polyvalent veterinary antibiotic (Pentabiótico, 0.2 mL, i.m.; Fort Dodge, Campinas, SP, Brazil) and an injection of the anti-inflammatory and analgesic flunixin meglumine (Banamine®, 2.5 mg/kg, s.c.; Schering-Plough, Cotia, SP, Brazil). Afterward, rats were allowed 5 days to recover from the surgical procedure.

2.4. Drugs and microinjection procedure

Midazolam maleate (MDZ; Roche, Brazil) was used in doses of 5, 10 or 20 nmol. Control animals received saline (SAL, 0.9% NaCl) in the same volume and route of administration. The solutions were prepared shortly before use and injected at a rate of 0.2 μL/min by an infusion pump (Harvard Apparatus, Massachussets, USA). By using a volume of 0.2 μL, the drug diffusion was restricted to the target region (Myers, 1966). The doses of the drug and waiting times were selected based on previous studies (Borelli et al., 2006; Ferreira-Netto et al., 2007; Nobre and Brandao, 2004; Pandossio and Brandao, 1999). During the microinjection procedure, the animals remained free in a polypropylene box measuring 28 × 17 × 13 cm, lined with shredded paper. A dental needle (30 G, 14 mm long, 0.3 mm o.d.) was inserted into the cannula, passing it to 1 mm. This needle was connected to a polyethylene tube (PE- 10, Becton Dickinson, NJ, USA) and a 10 μL Hamilton syringe. The displacement of an air bubble inside the PE-10 tube was used to monitor the microinjection. After the end of the infusion, the needle was held in place for an extra minute to avoid reflux of the drug through the guide cannula.

2.5. Behavioral study

2.5.1. Elevated plus maze

A wood apparatus was used, consisting of two open arms (50 cm × 10 cm) crossed at right angles with two closed arms of the same size. The two closed arms were enclosed by walls 50 cm high, with the exception of the central part of the maze (10 cm × 10 cm) where the open and closed arms crossed. The entire apparatus was elevated 50 cm above the floor and it was placed in a room with minimal visual cues visible to the rat. To prevent the rats from falling, a rim of Plexiglas (0.5 cm high) surrounded the perimeter of the open arms. Luminosity at the level of EPM open arms was 30 lux. The experimental sessions were recorded by a video camera interfaced with a monitor and a VCR in an adjacent room. Fifteen minutes after the injections, each rat was gently placed in the central area of the EPM with its nose facing one of the closed arms and was allowed to freely explore the maze for 5 min. Conventional EPM measures (i.e. number of entries and time spent in each arm) were recorded (Pellow et al., 1985). For data analysis purpose, the percent time spent in open arms was used. It was calculated dividing the raw open time by the

total time of the test, then multiplying it by 100. In addition, the frequency of the following 'complementary ethological categories' were measured: (i) head dipping (dipping the head below the level of the maze floor), (ii) stretched-attend postures (the animal stretching to its full length with the forepaws, keeping the hind paws stationary, and returning to its previous position), (iii) peeping out (stretching the head and shoulders from the closed arms to the central platform or open arms), (iv) end-arm exploration (number of entries into the end of an open-arm), and (v) flat-back approach (locomotion when the animal fully stretches and moves forward cautiously). These categories were previously defined for rats and studied in terms of factor analysis (Albrecht-Souza et al., 2008; Anseloni and Brandao, 1997; Cruz et al., 1994). According to factor analysis, the peeping out behavior, flat-back approach and stretch-attend postures were grouped under the "Risk assessment" category (Albrecht-Souza et al., 2008), in which the animal's behavior is directed to evaluate possible threatening cues from the environment. Before the next rat was tested, the maze was cleaned with a 20% ethanol solution and dried with paper towels. Data analysis was performed by a trained researcher using the open source software OBS.

2.5.2. Electrical stimulation of IC

Five days after surgery, each animal was placed in a Plexiglas box (25 × 20 × 20 cm) in an illuminated room with a 40 W fluorescent lamp (80 lux at the box floor level), where it remained undisturbed for 5 min (habituation period). Brain stimulation (60 Hz sine wave for 10 s) was presented at 1 min intervals, by means of a sine wave stimulator (ESF-12, DelVecchio, Brazil), with the current intensity increasing by 5 μA steps to measure the baseline aversive threshold. The stimulation current was monitored by measuring the voltage drop across a 1-kΩ resistor with an oscilloscope (Minipa, Sao Paulo, Brazil). The lowest current intensity that produced running (galloping) or jumping was considered the escape threshold. After reaching the escape threshold, the electrical stimulation of the IC stopped, and the animal remained in the observation chamber without any stimulation. In order to investigate the behavior that persisted after escape, post-stimulation freezing (PSF) was scored by a well-trained observer during the 8 min that followed the electrical stimulation. Freezing was defined as the absence of movement of the body and vibrissa, except that required for respiration, for at least 6 s. At the end of this period, the rat received a microinjection of saline or MDZ into the IC. Fifteen minutes later, the aversive threshold for escape as well as the PSF were again determined. Animals that did not reach an aversive threshold at 100 μA (peak-to-peak) were discarded from the study.

2.6. Experimental design

We assessed anxiety-like behavior and unconditioned fear responses in rats injected with saline or midazolam (5, 10 or 20 nmol/0.2 μL) and exposed to the EPM or electrical stimulation procedures. Therefore, the present study involved two experiments. To analyze the effects of GABA-benzodiazepine mechanisms in the IC on each experiment, animals were randomly allocated to 4 groups for each region (dorsal or ventral part of the IC): (i) saline, (ii) 5 nmol MDZ, (iii) 10 nmol MDZ, and (iv) 20 nmol MDZ. All animals were stimulated or tested in the EPM 15 min after the injections.

2.7. Histology

At the end of experiments, all subjects were deeply anesthetized and then intracardially perfused with saline followed by buffered 10% formaldehyde solution. The brains were removed and maintained in formalin solution for 1 day and then kept in 30% sucrose for another 3 days. Thereafter, serial 60 μm brain sections were cut using a microtome, thaw-mounted on gelatin-coated slides, and subjected to Nissl staining to localize the sites of injection. Stimulation sites were plotted onto coronal diagrams from the rat brain atlas (Paxinos and Watson, 2006).

2.8. Statistics

For the EPM analysis one-way analysis of variance (ANOVA) was used to assess the effects of midazolam and saline injections into the ICv or ICd. Treatment (saline and midazolam) were the independent and the standard and novel ethological measures (EPM) were the dependent variables. The same procedure was conducted for the analysis of the treatment effects on the escape threshold measured with the ICv or ICd electrical stimulation procedure. In this case the difference of escape thresholds measured before and after the injections into the IC was the dependent variable and treatments were the dependent variable. The statistical analysis was performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, California, USA). Data obtained from the post-stimulation freezing was analyzed using two-way ANOVA, with treatments as the between factor and condition (before or after treatment) as the within factor. In all comparisons, Newman–Keuls post hoc comparisons were performed when significant overall F-values were obtained in the ANOVA ($p < 0.05$).

3. Results

Histological analysis revealed the positioning of the cannula and injection sites in the inferior colliculus of animals used (Fig. 1). According to evidence obtained earlier in this laboratory, we chose to consider the ventral (ICv) and dorsal portion (ICd) of the inferior colliculus to refine the analysis of the participation of this structure in the elaboration of defensive behavior.

Elevated plus maze. Analysis of the data obtained from the EPM showed that midazolam when injected into the ventral portion of the IC produced significant effects on the number of entries [$F_{3,28} = 4.686$; $p < 0.05$] and an increase in the percentage of time spent in the open arms [$F_{3,28} = 3.81$; $p < 0.05$], but no changes were found in the locomotor activity [$F_{3,28} = 0.39$; $p > 0.05$]. Post-hoc comparisons revealed that the dose of 10 nmol of the benzodiazepine was responsible for this effect (Fig. 2).

Interesting results were found in the complementary measures related to the anxiety factor in the EPM, such as end arm exploration [$F_{3,28} = 5.207$; $p < 0.01$] and head dippings [$F_{3,28} = 5.486$; $^{**}p < 0.01$] that were significantly increased when the dose of 10 nmol of MDZ was injected into the ICv. The frequency of risk assessment behaviors in the EPM (i.e., the sum of flat-back approach, stretch-attend postures and peeping out behaviors) was decreased by the doses of 10 and 20 nmol intra-ICv injected [$F_{3,28} = 5.718$;,$p < 0.01$] (Fig. 3).

The analysis of data obtained from the injection of midazolam into the ICd did not reveal significant effects on the number of open arms entries [$F_{3,26} = 0.5802$; $p > 0.05$], in the percentage of time spent in the open arms [$F_{3,26} = 0.7539$; $p > 0.05$], or in the number of entries in the closed arms, which is an index of locomotor activity [$F_{3,26} = 0.2902$, $p > 0.05$] (Fig. 4). Similarly, no significant changes were found in complementary categories when the treatment aimed the ICd.

Electrical stimulation of IC. A similar pattern of dissociation in the pharmacological reactivity within the IC was found when evaluating the effects of MDZ in the electrical stimulation procedure. Interestingly, the lowest effective dose to produce an increase in the escape threshold was higher than the anxiolytic dose for the EPM. Rats that received 20 nmol of MDZ in the ICv exhibited greater differences in the escape threshold after the treatment in

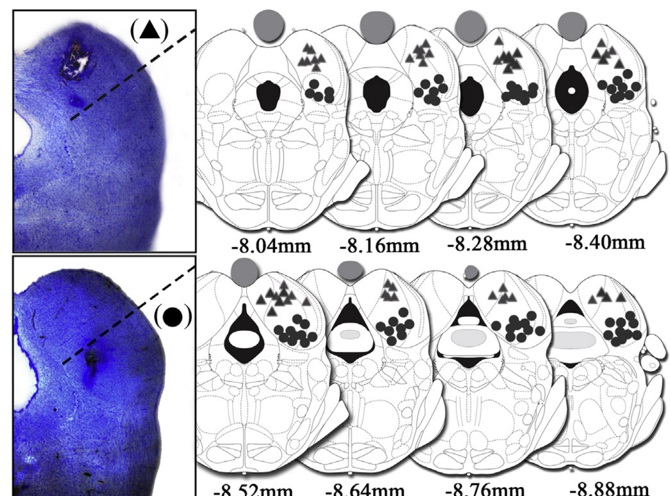

Fig. 1. Location of injection sites in rats that received saline or midazolam into the dorsal (triangles) or ventral parts (circles) of the inferior colliculus. Below each section, the distance from bregma is indicated, according to the atlas of Paxinos and Watson (2006). The number of points in the figure is less than the total number of animals because of overlapping injection sites.

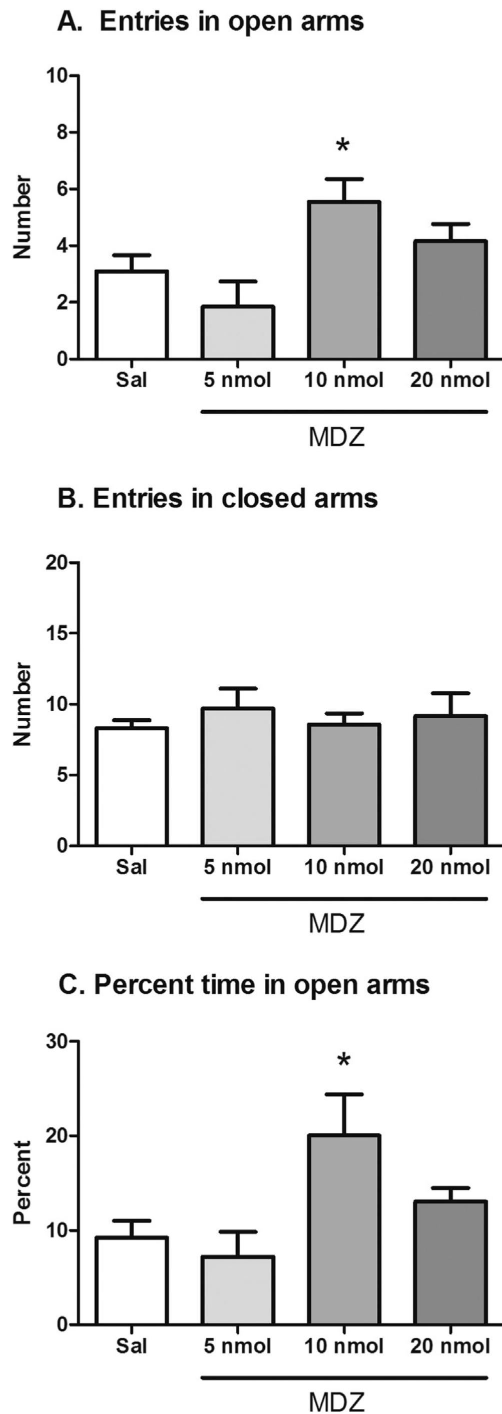

Fig. 2. Traditional ethological categories of the EPM obtained from rats that received midazolam in the ventral portion of IC. The number of entries and percent time spent in open arms were increased by the dose of 10 nmol of midazolam in comparison to the saline and 5 nmol groups ($p < 0.05$). A) Number of entries in the open arms; B) Number of entries in the closed arms; C) Percent time spent in open arms ($n = 6$–9 per group).

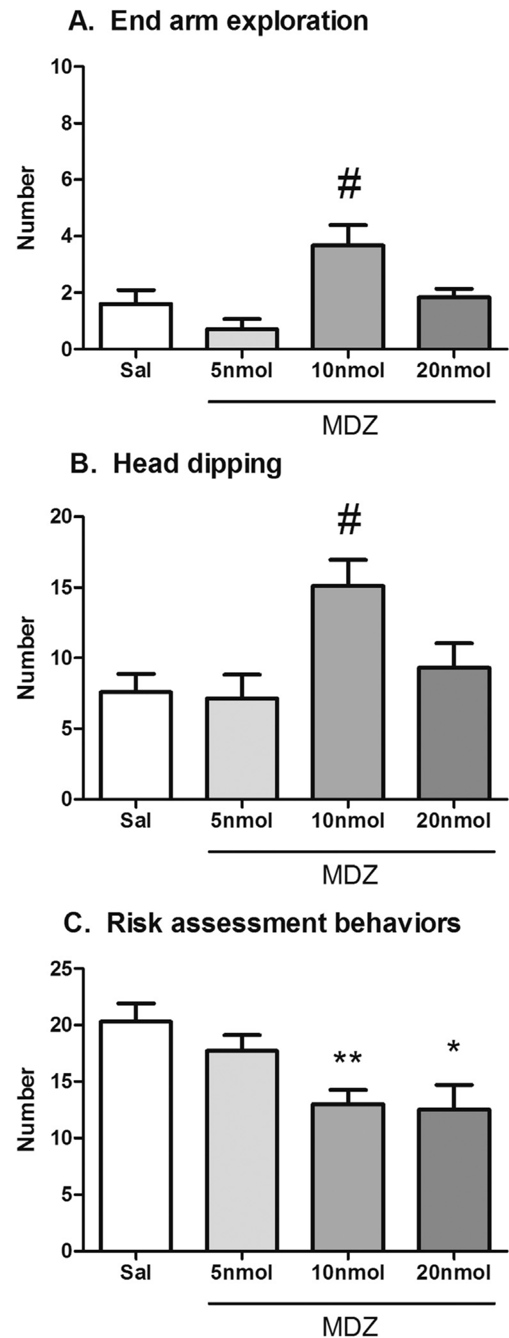

Fig. 3. Complementary measures obtained from animals submitted to the EPM after intra-ICv treatments. While an increase in end arm exploration (A) and head dipping (B) could be seen in rats that received 10 nmol of midazolam intra-ICv, the frequency of risk assessment behaviors (C) decreased with the same dose. (#) compared to all other groups; (*) compared to the control group (Sal) ($n = 6$–9 per group).

comparison to the baseline [$F_{3,33} = 5.71$, $p = 0.0029$], while this outcome was not registered in the ICd [$F_{3,24} = 1.942$, $p = 0.1497$] (Fig. 5).

For the post-stimulation freezing analysis, two-way ANOVA revealed significant effect of condition for the ICv group [$F_{3,33} = 15.4$; $p = 0.0003$] but no condition–doses interaction or dose effect. The ICd group also showed effect of condition [$F_{3,24} = 4.788$; $p = 0.0386$] but no significant dose effect or condition–dose interaction (Fig. 6).

4. Discussion

The present results show that midazolam acts selectively in the ICv to produce its anxiolytic-like effects. Although other studies have reported anxiolytic effects of midazolam injected into the IC (Pandossio and Brandao, 1999), our study focused on detailed ethopharmacological aspects of the fear response. According to

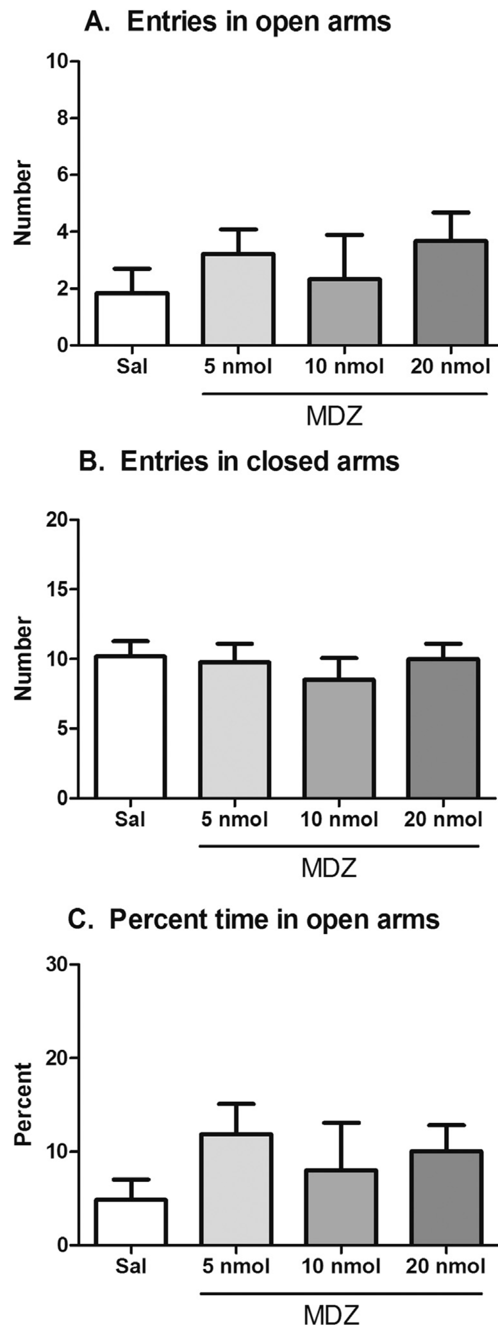

Fig. 4. Traditional ethological categories of the EPM obtained from rats that received midazolam in the dorsal portion of IC. No significant changes were found with any of the doses used. A) Number of entries in the open arms; B) Number of entries in the closed arms; C) Percent time spent in open arms ($n = 6–10$ per group).

evidence obtained previously in our laboratory, we chose to consider the ventral (ICv) and dorsal portion (ICd) of the inferior colliculus to refine the analysis of the participation of this structure in the elaboration of defensive behavior. Differently from the neuroanatomical point of view, the functional segregation between these two areas seems to follow a simple division from the midline drawn across the IC (see Fig. 1). These two regions of the IC are known to participate in independent neural circuits responsible for audiogenic seizures (ICd) and defensive responses (ICv) (Ferreira-Netto et al., 2007; Garcia-Cairasco and Sabbatini, 1991). Indeed, only animals that received intra-ICv treatment showed significant responses to the antiaversive action of midazolam. Therefore, we suggest that the dorsal and ventral aspects of the IC may have a different pharmacological sensitivity to the action of benzodiazepines.

The dose of 10 nmol injected into the ICv resulted in anxiolytic-like responses such as higher number of entries in open arms, end arm exploration, increased head dipping, and percent time spent in open arms, accompanied by decreased risk assessment behaviors. Unlike other reports (Pieri, 1983), midazolam did not cause any significant changes in the locomotor measures of the EPM at the doses used in this study, which excludes the possibility of bias in the results for any motor impairment that could be caused by a benzodiazepine drug. We also showed that 20 nmol of midazolam increased the escape threshold determined by direct electrical stimulation of the IC. This result is supported by previous data obtained with intra-IC injection of midazolam in the switch-off paradigm (Melo et al., 1992).

Our data also point to the differential sensitivity to the minimum effective dose of midazolam of each behavioral category analyzed. While the effective dose for reducing the aversiveness of the traditional ethological measures of the EPM was 10 nmol, higher doses of midazolam produced a decrease in risk assessment behaviors. These behaviors are biologically important to evaluate potentially threatening situations and to define coping strategies (Albrechet-Souza et al., 2008; Anseloni and Brandao, 1997). The electrical stimulation threshold for escape behavior was also only sensitive to the dose of 20 nmol. On the other hand, when an extremely fearful situation has already taken place, an intense processing of the aversive information emerges, that corresponds to the post-stimulation freezing. This behavior can be interpreted as a period when the organism processes the aversive event, gathering remaining information from internal and external environment and transferring them to higher brain structures (Brandao et al., 2008), and it does not seem to be directly affected by benzodiazepines, as shown by our post-stimulation freezing results.

The differences in the sensitivity of the dorsal and ventral regions of the IC to the benzodiazepine action can be explained by the specificity of the GABA receptor expression in distinct regions of the IC, as well as differences in the molecular structure of these receptors, which may have more or fewer binding sites for benzodiazepines. Midazolam is an agonist for the benzodiazepine receptors, which are coupled to the $GABA_A$ receptors and ultimately, has been proven to exert antiaversive effects in the IC by enhancement of GABAergic neurotransmission (Melo et al., 1992; Pandossio and Brandao, 1999). Morphologically, there is a ventralwards decrease in packing density of cells in the central nucleus of IC, and a concomitant increase in cell size and myelin density (Faye-Lund and Osen, 1985). In turn, the dorsomedial part of the IC is characterized by a large number of small neurons, and larger cells are found mainly at the border of the central nucleus (Beyerl, 1978; Faye-Lund and Osen, 1985). Another hypothesis would be that each behavioral task would be sensitive to a specific level of benzodiazepine modulation. The sensory component of the defensive response would be resistant to the action of benzodiazepines, while the neural substrate of fear of the ICv would be easily modified by a lower dose of midazolam. Due to the fact that the IC is one of the most caudal structures belonging to the EAS, its electrical stimulation is supposed to induce the most intense patterns of defensive behavior, according to the theory of McNaughton and Corr (2004).

Electrical stimulation of the IC leads the animal from a brief alertness/arousal period (protrusion of ears, vibrissae and eyeballs, occasional defecation and micturition) to an explosive escape reaction including circular running, jumping, and often vocalizations. Because of this, the species-typical freezing that occurs immediately before the escape response, which has been considered to be a preparatory behavior for the imminent burst of flight behavior was

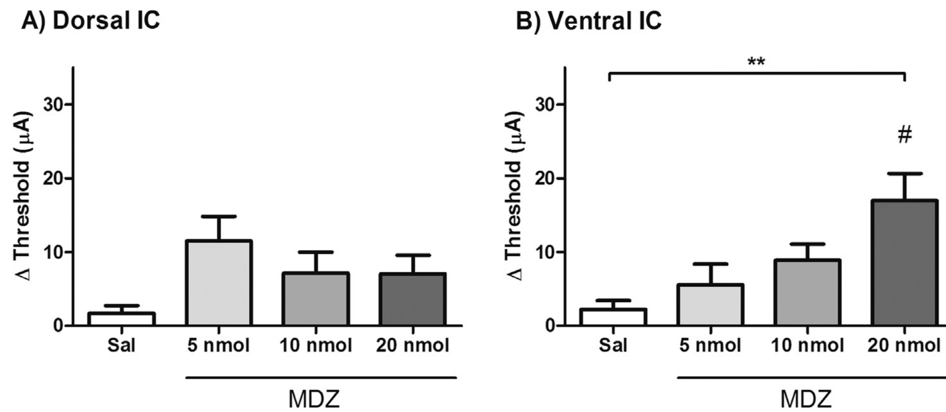

Fig. 5. Difference of escape thresholds recorded before and after injections of saline or midazolam into either dorsal or ventral portions of the IC of rats. Animals that received 20 nmol in the ICv showed a significant increase in the escape threshold compared to the control group. (**) $p < 0.01$ and (#) statistically different from all other groups (ICd: $n = 5–10$ per group; ICv: $n = 6–10$ per group).

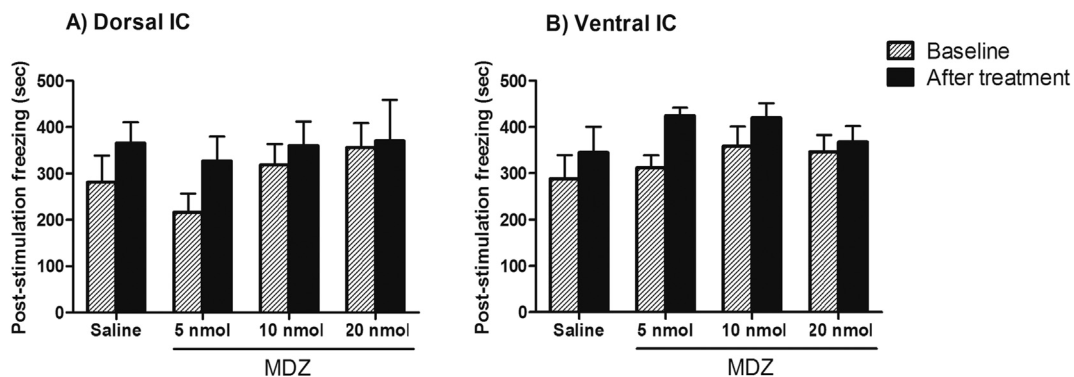

Fig. 6. Post-stimulation freezing recorded after injection of midazolam into the IC. There was only a significant effect of condition (before or after treatment) on the post-stimulation freezing time, but no significant effect was found for the doses of midazolam or interaction between condition and doses of midazolam (ICd: $n = 5–10$ per group; ICv: $n = 6–10$ per group).

not easily determined with the IC electrical stimulation procedure. Another interesting finding of this study is related to the sensory component of the defense reaction. When initiating the escape reaction most rats retracted their left ear just before flight and/or ran towards their left side, which is contralateral to the site of stimulation, in the same way as they do when they receive aversive information from the environment (Huston et al., 1980; Martin et al., 1978). These data point to the importance of the sensory component of the defensive behavior, which has been neglected in the literature. Our study aims to reopen the discussion on this subject.

Our data presented here extends previous research on the pharmacological manipulation of the IC, a phylogenetically old midbrain structure that is part of the Encephalic Aversion System. The expression of defensive responses organized at the level of the IC can be inhibited by benzodiazepines. Besides, there is a clear pharmacological dissociation in the reactivity of the dorsal and ventral regions of the IC to fear-provoking stimuli of the two animal models of anxiety used in this study. Thus, the present results support the proposal that benzodiazepine-mediated mechanisms are only involved in the output mechanisms of defensive behavior and not involved in the processing of ascending aversive information from the IC.

5. Conclusions

Our results suggest that the ventral portions of the IC may generate defense responses that are regulated by GABA-benzodiazepine mechanisms, while the consequences of this stimulation (processing of aversive information in the ICv – post-stimulation freezing) is resistant to this modulation. Thus, there may be different involvement of the GABA-BZD system on the defensive reaction in different regions of the IC, which also depends on the aversive condition.

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Manuscript

The light switch-off response as a putative rodent test of innate fear

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Highlights

- We propose a new animal test of unconditioned fear, based on the rodent innate aversion of bright-lit areas.
- Advantages of this test comprise its easy replication, its ecological and ethological approach, and its minimally stressful protocol.
- To our knowledge, this is the first time that an animal test is proposed based on the task of switching off an unconditioned light stimulus that by itself is aversive.

Abstract

Animal models are widely used to model clinical conditions for basic research. Recent worldwide discussion on the ethical issues in animal experimentation instigates the refinement for methods used in neurobiology of behaviors as fear and anxiety. Our group has developed a rodent test focused on the unconditional fear/anxiety paradigm, continuing the first study by Reis et al. (2004). The light switch-off test is based on the innate motivation to cease an aversive stimulus (bright light). This response is naturally occurring and belongs to the ethological repertoire of rodents in their habitat. 40 male Wistar rats, bred in the animal facilities at USP-RP, were allocated into independent groups: control, Diazepam 1 or 2mg/kg, and meta-Chlorophenylpiperazine (mCPP) 0.5 or 1 mg/kg. The experimental box has 2 compartments of 30×25×25 cm each, separated by an acrylic portal. The back and lateral walls are opaque, while its front and ceiling are made of transparent Plexiglas. The floor is a grid with metal rods spaced 1.2 cm from each other. In each side of the box, there is a 40 watts incandescent light bulb 12 cm high from the floor that can be turned on and off noiselessly. After a 5 min habituation period in the box, 40 light stimuli (lasting up to 20 sec each) are emitted in random intervals ranging from 15 to 45 sec. By crossing from one side to the other during the lighted period, the rat could switch-off the stimulus (*light switch-off response*). A software controls the emission of light and the randomness of intervals, as well as it records the latency of response and the locomotion intertrials. The switch-off response frequency was higher in rats treated with mCPP 1mg/kg, an anxiogenic drug, either as a whole or divided in blocks. Diazepam at the doses used in this study did not produce effects. Animals exposed solely to the box for the length of the test did not respond in a false positive way. Therefore, the *light switch-off response* represents a good index to measure the innate fear of bright-lighten areas displayed by rodents, once they react quickly in order to turn off the stimulus. Further studies will address the aversiveness to increasing amounts of luminosity in this protocol. Among its many advantages, the Light Switch-off Test is a simple procedure, easily replicable, non-invasive and minimally stressful test, since it does not include foot shocks or excessively aversive conditions to be endured by the animals.

Keywords: Animal test, Behavior, Fear, Rat

Introduction

Animal models are widely used to model clinical conditions for basic research (Sousa *et al.*, 2006) though also being considered a controversial issue. The use of experimental animals has been based on factual and moral assumptions, in which it is thought that animal models are reliable to predict results that will be obtained in human studies and that are morally justifiable to avoid atrocities in human subjects, as it happened in the II World War (Ferdowsian & Gluck, 2015). One of the drawbacks pointed is that behavioral tests often present limited explanatory power, since they measure behavior as an indicator, in an attempt to approach the functional role of the phenomena observed.

Recent worldwide discussion on the ethical issues in animal experimentation (Nordgren, 2002; Akhtar, 2015; Ferdowsian & Gluck, 2015) instigates the refinement for methods used in neurobiology of behaviors as fear and anxiety. The concept of fear and anxiety is in itself a matter of debate (Ledoux, 2014; Perusini & Fanselow, 2015), and more so when both concepts are transposed to animal behavior.

One of the most reliable tests used to predict the anxiolytic- and anxiogenic-like effects of drugs in rodents is the light/dark test, a proposed "'exploratory" animal behavior model for the anxiolytic action of benzodiazepines (Crawley & Goodwin, 1980). The main parameters to assess the anxiolytic profile of drug treatment are the number of transitions between the two compartments, the latency time for the first passage from the light compartment to the dark one, the movement in each compartment, and the time spent in each compartment. Transitions in this test are considered an index of activity/exploration whereas the time spent in each compartment reflects aversion/attraction (Pitsikas *et al.*, 2008; Bourin, 2015). Although this test exploits the differences in behavior seen in dark and lit areas, its rationale remains on the natural conflict between exploration and avoidance that arises when rodents are exposed to novel environments (Crawley & Goodwin, 1980).

Our group has developed an animal test focused on the unconditional fear/anxiety paradigm, continuing the first study by Reis et al. (2004). The light switch-off test is based on the innate motivation to cease an aversive stimulus (bright light). The luminous stimulus per se evokes the light switch-off response (SOR), which is made effective when the rat crosses from one side to the other in a shuttle box. There is no need for previous conditioning or foot shocks.

This response is naturally occurring and belongs to the ethological repertoire of rodents in their habitat. The importance of vision is highlighted in the aversion of open spaces that rodents display during the elevated plus maze, a traditional unconditioned model for anxiety- and conflict-like behaviors. In settings where auditory and olfactory stimuli are controlled, aversion is triggered by the light penetration and image formation in the retina (Morato, 2006). One of the arguments that support the intense light as an aversive stimulus to albino rats is the lack of pigment in their iris and choroid, which reduces their vision adaptation and predisposes to visual damage (Stryjek *et al.*, 2013). Although there is a body of evidence indicating that light is an aversive stimulus by itself, no standardized test protocol has been developed using only the light as aversive stimulus. Usually, light is used as conditioned cue that precedes the aversive/rewarding stimulus in other protocols (Cassaday & Thur, 2015; Pezze *et al.*, 2016). Here, we present the protocol that has been used and an initial screening of the effects of two drugs in the test: diazepam (a well-known benzodiazepine used as anxiolytic in clinical practice) and meta-Chlorophenylpiperazine (mCPP), a recreational drug with anxiogenic properties, pharmacologically classified as a non-specific agonist for serotonin receptors.

Experimental procedures

Animals

A total of 46 male adult Wistar rats were used in this study. Animals were housed in collective cages in the colony room (12 h light–dark cycle in a temperature controlled and ventilated room). The animal's weights were ranged between 260 and 280 g at the time of experiments. All procedures were carried out during the light phase of the cycle, between 08:00 a.m. and 17:00 p.m. All animal experimentation reported in the present paper has been conducted in accordance with the guidelines laid down by the Brazilian Neuroscience Society.

Drugs

Diazepam (Roche, Brazil) was dissolved in a sterile saline solution and given i.p. 30 minutes prior the test at the doses of 1 or 2 mg/kg. These doses were used based on

previous studies in the elevated plus maze that did not induce sedation and motor impairment. Meta-Chlorophenylpiperazine (mCPP; RBI, MA, USA) was also dissolved in saline solution and delivered i.p. 15 minutes before the test, at the doses of 0.5 or 1 mg/kg, based on the minimal dose needed for pro-aversive effects obtained by Reimer et al. (2015, unpublished data). The control group received i.p. treatment with saline. A group of animals tested in the chamber without any stimulus (“Time only” group) did not receive any treatment.

Apparatus

The experimental chamber consisted of a shuttle box comprising two compartments measuring 30×25×25 cm (Insight, Brazil). The side and back walls of the chamber were constructed of black Plexiglas and the ceiling and front door were made of transparent Plexiglas. The chamber was divided by an opaque acrylic portal to allow free exploration. A grid floor comprised 15 stainless steel rods with 2.0 mm diameter, spaced 1.2 mm apart. Two 40 watts light bulbs were centered on each side of the rear of the chamber, 12 cm from the floor (Figure 1). The light was turned on and off noiselessly. The software and an appropriate interface connected to a PC provided by the manufacturer of the equipment (Esquiva Ativa; Insight, Brazil) allowed for recording and analysis of the frequencies and latency of escape responses as well as the intertrial locomotor activity.

Figure 1. The experimental box used for the Light Switch-off Test, displaying the light stimulus.

Experimental protocol

Each test session consisted of 40 light stimuli delivered between random intervals (ranging from 15 to 45 seconds). Rats were placed individually into the experimental chamber and acclimatized for 5 minutes before the test started. Once the light stimulus was delivered, the rat was able to turn it off by crossing from one compartment to the other. This escape reaction was recorded as a “switch-off response” (SOR) since it occurred within the 20 sec length of the light stimulus. The number of crossings during the absence of light stimulus was registered as a locomotion index. Each animal was submitted to only one session and the chamber was cleaned with ethanol 20% after each session.

Statistical analysis

Data obtained from the independent groups of animals tested in the light switch-off test were submitted to one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. Frequencies of avoidance responses across the four blocks of trials were subjected to a two-way analysis of variance with repeated measures using the treatment as the between factor and blocks of 10 trials each as the within group repeated measure factor. All values are reported as Mean ± SEM. A level of $p<0.05$ was used to confirm statistically significant differences.

Results

A starting point to the investigation was to find out if light by itself was a stimulus aversive enough to trigger a defensive response. We tested a group of animals in the same protocol except for the absence of any light stimulation. Thus, this group’s total frequency of SOR would be the sum of the random crossings from exploratory behavior during the whole duration of the test. Results showed that the SOR is not an aleatory phenomenon, rats actively cross compartments of the chamber in order to switch the light off (Figure 2). Post hoc analysis indicated significant differences between the two groups (p=0.01; $F_{(1,36)}$=8.3) and along the blocks (p<0.0001; $F_{(3,36)}$=10.2). There is no significant interaction between groups and blocks.

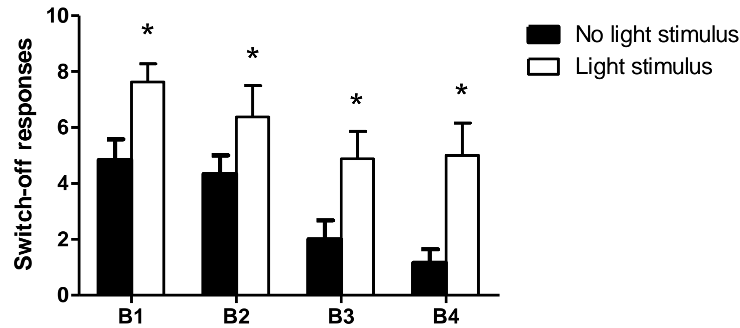

Figure 2. Switch-off responses of rats exposed to the standard protocol of light stimuli compared with a group only exposed to the dark chamber. Rats actively cross compartments of the chamber in order to switch the light off. *Indicates statistical significant difference between groups.

The pro-aversive drug mCPP at a dose of 1 mg/kg caused marked increase in the total number of SOR (p=0.0116; $F_{(5,40)}$=3.415; Figure 3). This effect cannot be attributed to a general boost of motor activity, since the locomotion index did not differ between groups (p=0.392, Figure 4). Regarding the latency to respond, a uniform time window was found across all groups. Once the animal respond to cease the light, it will happen up until the 10th second of stimulation (p=0.4611; Figure 5).

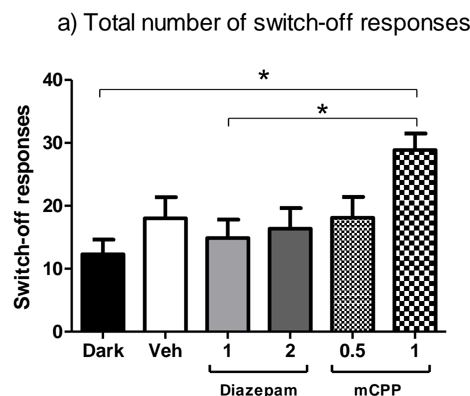

Figure 3. Total frequency of switch-off responses for all groups. Diazepam did not attenuate this response, while the pro-aversive drug mCPP at the dose of 1 mg/kg increased the frequency (p=0.0116; $F_{(5,40)}$=3.415)

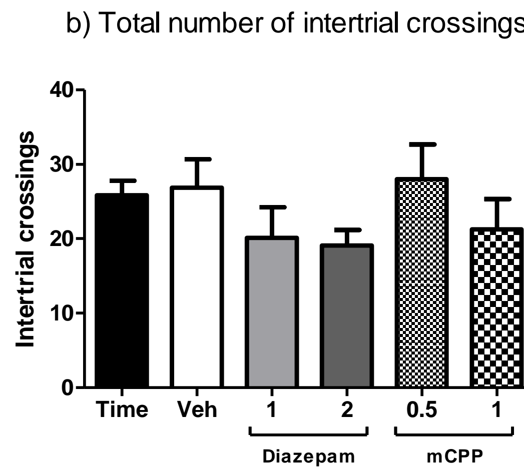

Figure 4. Total number of intertrial crossings, an index of locomotor activity. None of the doses and drugs used caused any motor disturbance (p=0.392).

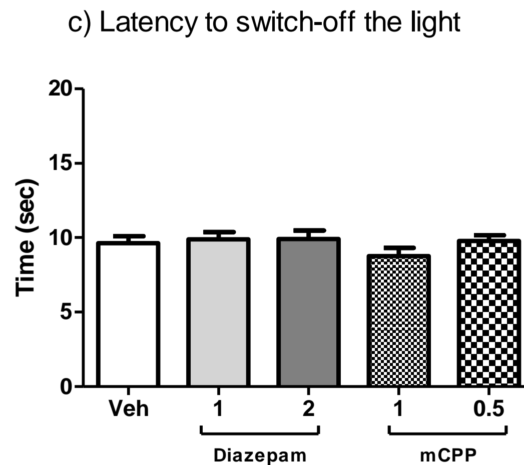

Figure 5. Mean latency to switch-off the light. A uniform latency pattern was found across all groups. Once the response is elicited, it will happen up until the 10th second of the stimulus (p=0.46).

Depending on the frequency of SOR, each test may last around 35 to 40 minutes. Therefore, we clustered 4 blocks of 10 stimuli each, to observe how the parameters are displayed throughout the duration of the test. Figure 5 shows how the SOR is sustained along the time course of the test, while other groups tend to be reduced. Interestingly, both diazepam 2mg/kg and mCPP 1 mg/kg seem to have placated the novelty induced exploration (Figure 6) keeping this index stable along the test. Latency to switch off the light is also a uniform index throughout the test (Figure 7).

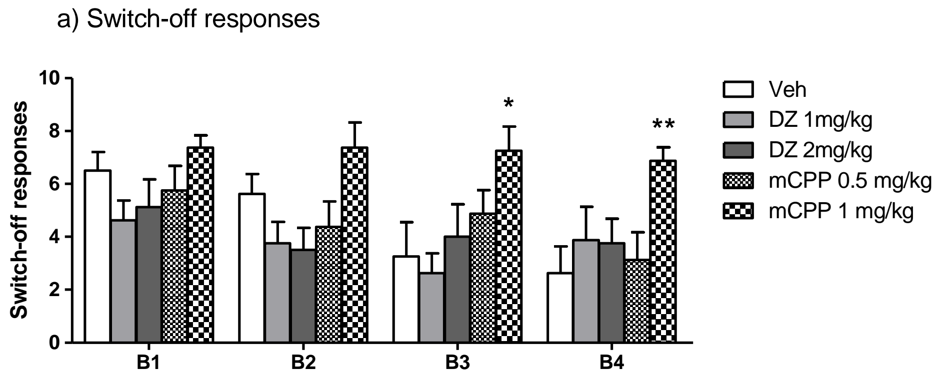

Figure 6. Switch-off responses for all groups, clustered into four blocks of 10 stimuli each. Significant interaction was found ($F_{(12,105)}$=1.993), as well as an effect of the treatment ($F_{(4, 105)}$=3.15) and blocks ($F_{(3, 105)}$= 9.159). The group treated with mCPP 1 mg/kg sustained the high frequency of responses on the third and fourth blocks (compared to the vehicle group).

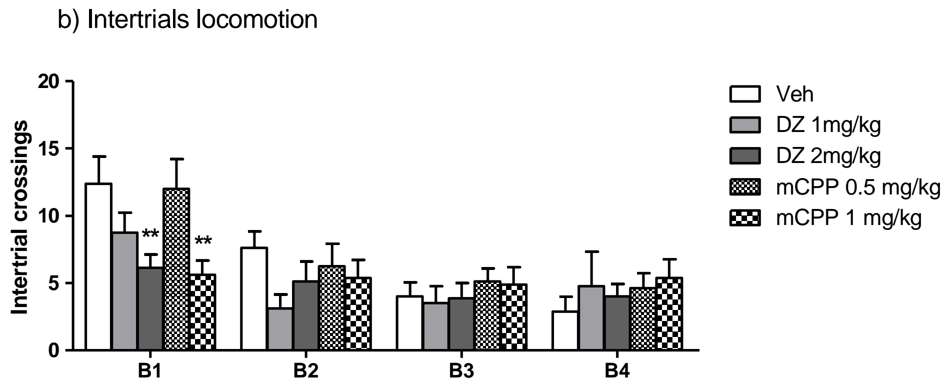

Figure 7. Intertrial crossings as observed in each block. The novelty-induced exploration during the first block was significantly decreased in the Diazepam 2 mg/kg and mCPP 1 mg/kg groups, but this locomotor index remained stable for the remaining blocks. Significant interaction was found ($F_{(12,105)}$=2.206), as well as an effect of the blocks ($F_{(3, 105)}$= 16.22).

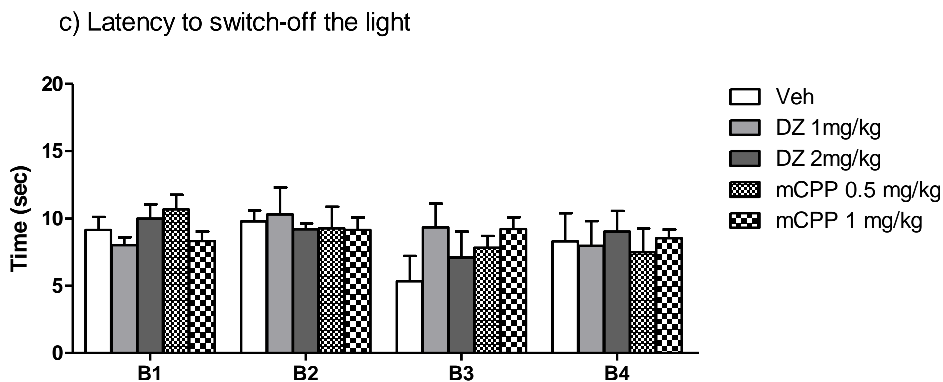

Figure 8. Latency to switch-off the light. No significant difference was found across groups regarding the latency to respond to the light stimulus. Note that the latency is also uniform during the entire time course of the test, which lasts approximately 40 minutes.

Discussion

The Light Switch-Off Test was first designed and used in 2004 when it yielded interesting results on the participation of dopaminergic mechanisms in the SOR expression (Reis *et al.*, 2004). Unlike the light/dark test which is based on the ethological view of shifting relative propensities to explore and to retreat from an unknown space (Crawley & Goodwin, 1980), the LSOT uses an unconditioned luminous stimulus aversive enough to trigger an escape response of the rat.

Recently, our group has been conducting a series of tests to achieve the optimum protocol for reliable and replicable results. One of the important variables is the presence of the acrylic portal that clearly indicates to the rodent the existence of two compartments. The absence of this division has been shown to alter the SOR. So far, the aforementioned protocol described here has proven effective in other studies conducted by our group. Both systemic injections and local microinjection (in the dorsolateral superior colliculus) of the selective dopamine D2 receptor antagonist sulpiride increased the number of SORs (Reis *et al.*, 2004; Muthuraju *et al.*, 2016).

We also addressed the hypothesis that the crossings were a mere product of casualty, by testing animals in the same experimental box and with the same software record system, but with disconnected lamps so no light would be delivered. According to our results, the luminous stimulus is discriminated by the animal, which actively displays a response in order to cease it and this response is not caused by chance. However, as it can be seen in the total number of SOR, the difference between groups “Time only” and “Vehicle” was not statistically significant, but we attribute this fact to the intensity of light delivered by the light bulbs. We are currently working on an increasing scale of light intensities that could reveal the amount of lux necessary to the luminous stimulus to be considered aversive by the rat.

As this test is dependent on a motor behavior, another important aspect to be clarified was if there were any locomotor changes that could invalidate the SOR. None of the drugs at the doses tested were capable of altering the total locomotor index, what strengthens the relevance of the SOR score obtained. An interesting finding was extracted from the block analysis for locomotion of the diazepam 2 mg/kg and mCPP 1 mg/kg groups. Apparently, these drugs curbed the novelty induced locomotor peak in the first block of the test, but did not alter the total count. However, due to its sedative

property, we believe that higher doses of diazepam would impair SOR. To conclude, mCPP at the dose of 1 mg/kg was effective in increasing and keeping the high frequency of SOR especially in the third and fourth blocks, when other groups tend to decrease the response. Compared to other uses of mCPP as an inductor of obsessive-compulsive behavior, this effective dose was relatively low (Papakosta *et al.*, 2013; Tucci *et al.*, 2015).

Therefore, we conclude that the Light Switch-Off Test can be used to measure rodent's aversion to light and their defensive response to it. It constitutes a promising methodology to be further studied and systematized. Among its many advantages, the Light Switch-off Test is a simple procedure, easily replicable, non-invasive and minimally stressful test, since it does not include foot shocks or excessively aversive conditions to be endured by the animals (Beauchamp & Morton, 2015).

Conflict of interest statement

The authors declare no conflicts of interest.

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Review

Dual role of dopamine D_2-like receptors in the mediation of conditioned and unconditioned fear

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ABSTRACT

A reduction of dopamine release or D_2 receptor blockade in the terminal fields of the mesolimbic system, particularly the amygdala, clearly reduces conditioned fear. Similar D_2 receptor antagonism in the neural substrates of fear in the midbrain tectum attenuates the processing of unconditioned aversive information. However, the implications of the interplay between opposing actions of dopamine in the rostral and caudal segments of the dopaminergic system are still unclear. Previous studies from this laboratory have reported the effects of dopaminergic drugs on behavior in rats in the elevated plus maze, auditory-evoked potentials (AEPs) recorded from the midbrain tectum, fear-potentiated startle, and conditioned freezing. These findings led to an interesting framework on the functional roles of dopamine in both anxiety and fear states. Dopamine D_2 receptor inhibition in the terminal fields of the mesolimbic dopamine system generally causes anxiolytic-like effects, whereas the activity of midbrain substrates of unconditioned fear are enhanced by D_2 receptor antagonists, suggesting that D_2 receptor-mediated mechanisms play opposing roles in fear/anxiety processes, depending on the brain region under study. Dopamine appears to mediate conditioned fear by acting at rostral levels of the brain and regulate unconditioned fear at the midbrain level, likely by reducing the sensorimotor gating of aversive events.
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1. Background

Aversive stimuli that underlie aversive or stressful situations can be divided into two categories: unconditioned and conditioned stressors. Unconditioned aversive stimuli, such as pain, asphyxia, and innate fear-inducing stimuli (e.g., snakes and tigers), evoke unconditioned reflexive freezing and escape responses. Conditioned aversive stimuli are neutral stimuli that have acquired aversive properties because they have been previously paired with a noxious or aversive event. A third category, general stressors, can also be included as stimuli of an aversive nature that activate the general alertness system. The latter has been represented by the activating reticular ascending system, and different brain structures subserve each of the defense reactions that are triggered by unconditioned and conditioned stressors [17,28,8,6,7].

When applied to periventricular structures, such as the dorsal periaqueductal gray (dPAG) and medial hypothalamus, electrical stimulation is well known to elicit escape behavior [5]. An important step toward understanding the processes and mechanisms that underlie this defensive response was the demonstration that such periventricular stimulation induced an aversive effect. Animals that received such stimulation readily learned to switch off the stimulation whenever it was given the opportunity to do so [43]. In the mid 1980s, the superior colliculus and inferior colliculus (IC) in the mesencephalon were also shown to be part of this system, which has since been known as the encephalic aversion system (EAS; [11,10,2]. From this time onward, substantial evidence has been obtained to support the notion that defense reactions that are elaborated at the level of the EAS are mediated by multiple mechanisms (Fig. 1). Indeed, γ-aminobutyric acid (GABA), excitatory amino acid, neuropeptide (e.g., neurokinin), opioid, and serotonin systems are now known to act together to neurochemically mediate the neural substrates of aversion in this

Author contributions: Amanda R. Oliveira, Sangu Muthuraju, Ana Caroline Colombo, Viviane M. Saito and Teddy Talbot conducted literature search and data collection and preparation of the figures. Marcus L. Brandão wrote this manuscript and supervised all data collection. All authors contributed to the analysis and interpretation of the data and approved the final version of this paper.

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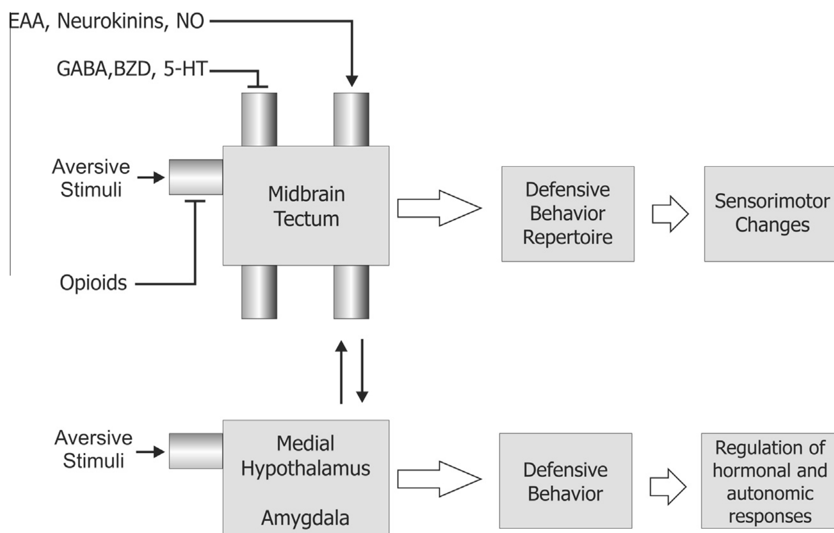

Fig. 1. Schematic diagram that shows the various neurotransmitters that are known to mediate (↑) or regulate (⊤) defensive behavior in the midbrain tectum, medial hypothalamus, and amygdala, structures that belong to the encephalic aversion system (EAS). EAA, excitatory amino acids; GABA, γ-aminobutyric acid; BZD, benzodiazepine; 5-HT, 5-hydroxytryptamine (serotonin); NO, nitric oxide.

system [12,13,6,7]. Endocannabinoids, nitric oxide, and corticotrophins also play a role in mediating the EAS [4,37,38]. An excellent review that was published at the beginning of this century proposed that dopamine can also function as a mediator of conditioned fear in terminal areas of the mesocorticolimbic system [33].

2. Dopamine in conditioned fear

In parallel with studies on the function of the EAS, the last two decades also witnessed a large number of studies that sought to elucidate the neural substrates that elaborate behaviors related to the rewarding and pleasurable effects of stimulating certain brain areas, such as the ventral tegmental area (VTA), nucleus accumbens, and other rostral brain areas [16]. Many of these studies reported the essential involvement of long ascending fiber systems (e.g., dopaminergic fibers). Today, this system is referred to as the reward system. A turning point in the role of dopamine in the modulation of brain function was the postulation that dopaminergic systems that arise from the VTA mediate conditioned fear responses. In fact, many studies have reported the involvement of dopamine in aversive conditioning using diverse conditioned fear paradigms, such as the contextual fear test, fear-potentiated startle with the use of a context or light as conditioned stimuli, and the two-way active avoidance test [39]; for review, see [33].

Despite the widely accepted notion that dopamine is involved in the regulation of anxiety, no agreement has been reached on whether dopamine enhances or diminishes anxiety. The former hypothesis is largely based on earlier experiments in which the effects of drugs that non-selectively act on brain dopamine systems were measured in animals that were subjected to tests of fear conditioning [3,34,40,41,46,31,1,23]. With regard to dopamine's mediation of conditioned fear, an increase in dopamine metabolism in the mesolimbic system is correlated with conditioned fear, and a decrease in dopamine activity in the basolateral amygdala (BLA) reduces the expression of conditioned fear [18,19,20]. In fact, intraperitoneal injections of low doses of the D_2 receptor agonist quinpirole act at autoreceptors on VTA neurons, slowing dopamine release at their terminals and causing a reduction of conditioned fear responses [23,21]. Therefore, the fear response to a light (conditioned stimulus [CS]) appeared to depend on the activation of mesolimbic dopaminergic connections and could be specifically modulated by manipulating these projection neurons. One possibility that cannot be discarded is that the pathway that connects the VTA with the amygdala promotes resistance to the aversive effects of unconditioned stimuli (USs), suggesting that the interplay between neural systems that are responsible for processing conditioned and unconditioned fear stimuli may exist at this level.

3. Dopamine in unconditioned fear

The EAS consists of the dorsal periaqueductal gray (dPAG), superior and inferior colliculi, and the hypothalamus and is responsible for the elaboration and expression of defense reactions to unconditioned or innate aversive stimuli. The behavioral and autonomic changes that are associated with defense reactions are likely to be expressed as an aversive emotional-motivational state. Activation of the dPAG motivates switch-off behavior, inhibits antecedent behavior, facilitates escape from electric footshock, and acts as an aversive US in Pavlovian conditioning and place aversion procedures [17,28,6,7]. Considering that conditioned and unconditioned fear may somehow overlap, one unresolved issue remains. Substantial experimental evidence implicates the mesocorticolimbic dopamine system in anxiety, but the functional role of dopamine in the midbrain tectum associated with fear is still unclear. Some reports associated dopamine in the midbrain tectum with prepulse inhibition, catalepsy, and the modulation of auditory signals from the IC to medial geniculate thalamus [42,32,44,27]. We present evidence that dopamine modulates the neural substrates of fear in the EAS.

Unclear is the role of dopamine in unconditioned fear, particularly in the EAS. No study approached this issue until 2004, when a study conducted in our laboratory showed that dopamine facilitated unconditioned responses in rats in the light switch-off test [36]. One year later, another study showed that systemic administration of the selective dopamine D_2 receptor antagonist sulpiride caused proaversive effects in rats in the elevated plus maze [26].

Given that the EAS does not function only as an output center for defense reactions, certain structures of the midbrain tectum may also be involved in processing aversive information [45,9]. For example, aversive stimulation of the IC at the escape threshold

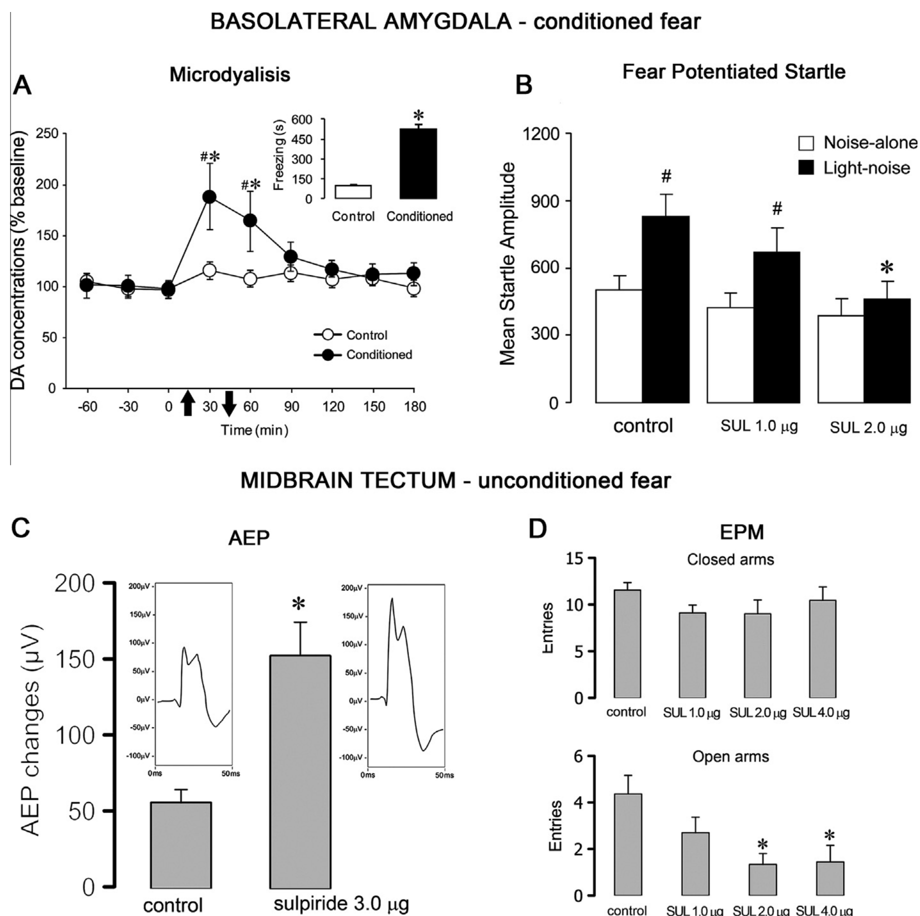

Fig. 2. (A) Dopamine levels in the basolateral amygdala during conditioned fear. Extracellular levels of dopamine (DA) in the BLA in rats that were previously subjected to paired (conditioned) or unpaired (control) exposure to light and footshock and exposed to the light-CS in the fear conditioning test. (Inset) Conditioned freezing behavior during the 20 min period of the light-CS test session without footshock. $^{\#}P < 0.05$, different from baseline (−60, −30, and 0 min); $^{*}P < 0.05$, different from control group (Newman–Keuls *post hoc* test). Arrows indicate the start and end of the conditioned fear test. (B) Effects of injections of the D_2 receptor antagonist sulpiride in the basolateral amygdala on fear conditioning (fear-potentiated startle). The figure shows the mean startle amplitude in rats that were treated with sulpiride (1.0 and 2.0 μg/0.2 μl) before the test session. $^{\#}P < 0.05$, compared with noise-alone; $^{*}P < 0.05$, compared with light-noise in the saline group (Newman–Keuls test). (C) Effects of intra-inferior colliculus (IC) injections of vehicle or 2.0 and 3.0 μg/0.2 μl sulpiride on the amplitude of auditory-evoked potentials recorded in the IC in response to presentations of a loud noise. $^{*}P < 0.05$, compared with control group (Newman–Keuls test). (D) Effects of intra-IC injections of vehicle or 1.0, 2.0, and 4.0 μg/0.2 μl sulpiride/0.2 μl on exploratory behavior in rats in the elevated plus maze. The figure shows the number of entries into the closed and open arms. $^{*}P < 0.05$, compared with control group (Newman–Keuls test). Further details can be found in De Oliveira et al. [20,22] and Muthuraju et al. [35].

enhanced dopamine release in the prefrontal cortex [15,30] and intracollicular injections of the non-selective dopamine receptor antagonist haloperidol enhanced auditory-evoked potentials (AEPs) that were recorded directly from the IC in rats that were subjected to loud sounds as the US [35]. These results support previous studies from our laboratory that showed that systemic injections of sulpiride enhanced escape responses in rats that in the switch-off procedure [36] and increased the frequency of ultrasonic vocalizations during the training sessions in a fear conditioning test [14]. These data are in sharp contrast to other studies on the association between enhanced dopamine transmission and conditioned fear. For example, such studies reported that intra-amygdala injections of sulpiride attenuated the expression of conditioned fear [18,19,20,21,22]. We thus hypothesize a dual role for dopamine in defensive behavior that may explain some of these conflicting results.

4. Integrated view

In an attempt to integrate the above findings, we propose the following. The neurobiological function of dopamine in anxiety has been investigated in several laboratories. The current general idea is that the activation of dopamine pathways that arise from the VTA increases learned anxiety. The functional role of dopamine pathways that connect the VTA to the BLA in the mediation of conditioned fear/anxiety has been suggested by many studies from several laboratories [3,34,40,41,46,31,1,23]. In one of these studies from our laboratory [20], in vivo microdialysis was performed to measure dopamine levels in the BLA in Wistar rats that were subjected to footshock (US) that was associated with a neutral stimulus (CS). When these rats were exposed to the CS alone without footshock, freezing was the most prominent behavioral response, accompanied by the significant release of dopamine in the BLA (Fig. 2A, arrows). In this study, fear-potentiated startle (i.e., a common conditioning model of anxiety in animals) was used to assess the way in which dopamine mechanisms are activated during conditioned fear. The animals were subjected to the pairing of light + footshock. The next day, they were subjected to a session that consisted of 60 trials, half with loud noise and half with loud noise + light (CS). Fear-potentiated startle was evident when startle in response to light + noise was higher than to noise alone. The difference between the magnitude of the startle response under both conditions reflects the state of conditioned fear of the animals and has been utilized as a reliable measure of anxiety in

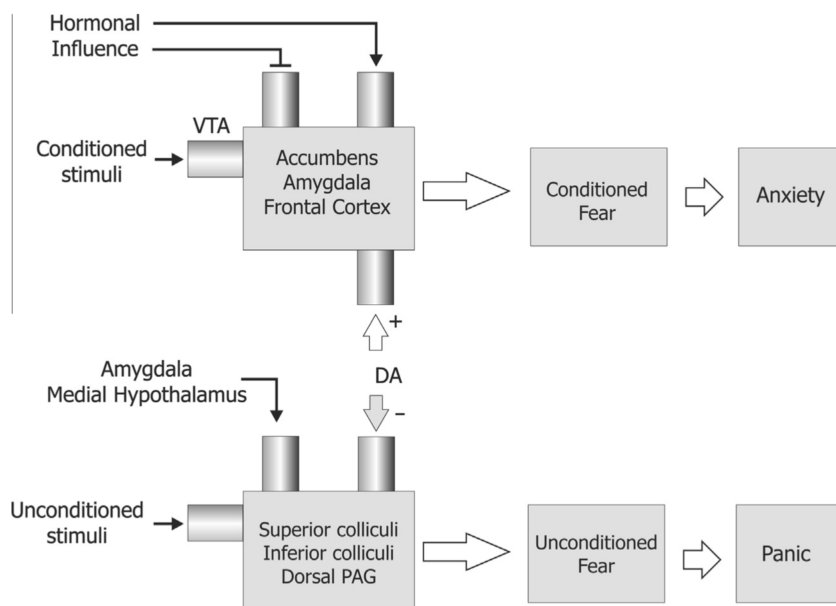

Fig. 3. Schematic diagram that shows the generation and organization of conditioned and unconditioned fear responses in response to respective conditioned and unconditioned stimuli that target the mesocorticolimbic system and encephalic aversion system, respectively. Hormonal factors may inhibit (⊤) or activate (→) defense reactions. VTA, ventral tegmental area. Dopamine has opposite actions on these systems (+, −).

animals. Intra-BLA injections of sulpiride before the test sessions attenuated fear-potentiated startle, indicating that D_2 receptors in this amygdaloid nucleus mediate conditioned fear responses (Fig. 2B). Thus, these data support the hypothesis that dopamine may plan an anxiogenic role in rostral brain structures, such as the hippocampus, amygdala, and prefrontal cortex, which are activated by CSs.

As mentioned above, the EAS is a multifaceted neural circuit that involves several neurotransmitters (GABA, serotonin, excitatory amino acids, and neurokinins, among others) and plays a role in modulating the organization, elaboration, and expression of defense reactions. However, the use of tests of unconditioned fear has generated experimental results that do not always fit the hypothesis of dopamine's anxiogenic role [36,14,22,35]. Studies that used aversive USs that decisively activate brainstem structures, such as the IC, to elicit defensive behavior in rats has led to the suggestion that dopamine exerts an antiaversive action in this brain region [35,22]. Thus, the ascending dopamine pathway that originates in the VTA and innervates the amygdala and frontal cortex has been suggested to facilitate conditioned fear, whereas the periventricular pathway that innervates the dPAG and IC inhibits innate fight/flight reactions to impending danger. These latter studies showed, in contrast to animal models of anxiety, that D_2 receptor antagonism at the level of the midbrain tectum in rats that are exposed to unconditioned aversive stimuli increases defensive reactions in the elevated plus maze and in response to the presentation of repetitive loud noises (100 dB). Indeed, sulpiride injections in the IC increased AEPs that were recorded directly from electrodes implanted in the IC during the presentation of repetitive loud noise (Fig. 2C) and enhanced avoidance of the height and openness of the open arms of the elevated plus maze (Fig. 2D). Supporting these results, a substantial concentration of dopamine has been found in the midbrain tectum, particularly in the IC [29]. We also showed that the same pattern of responses emerged when sulpiride was injected into deep layers of the superior colliculus. Sulpiride injections in the dPAG caused proaversive effects in rats in the elevated plus maze (manuscript in preparation).

Altogether, the extant evidence suggests that the activation of dopamine pathways that arise from the VTA increases learned anxiety, with possibly an opposite action on innate fear. The results support our hypothesis of a dual role of dopamine-mediated mechanisms in unconditioned and conditioned fear and that these different responses to aversive stimuli can be neurally dissociated from each other. This new approach to associating dopaminergic mechanisms with anxiety has led to some intriguing questions. Benzodiazepine anxiolytics supposedly decrease dopamine release in the frontal cortex [24,25], and neuroleptics that are known for their ability to decrease dopamine neurotransmission in limbic structures exert antipsychotic rather than anxiolytic effects. The proposal presented herein opens a window on further discussions on the implications of dopamine's mediation of the multiple facets of anxiety disorders.

5. Concluding remarks

The present review provides evidence of the opposing actions of dopamine-mediated mechanisms in fear/anxiety processes. Depending on the type of threatening condition (i.e., conditioned or unconditioned), dopamine D_2 receptor antagonists may reduce or heighten the aversiveness of the situation. Intra-BLA injections of these compounds clearly reduce conditioned fear in rats that are subjected to animal models of anxiety [20,21,14,23]. To test the hypothesis that dopamine plays a modulatory role in the neural substrates of fear in periventricular structures, we microinjected dopamine receptor antagonists into the IC to inhibit D_2 receptor-mediated mechanisms. This treatment impaired exploratory behavior in rats in the elevated plus maze and enhanced AEPs that were recorded in the IC in response to loud sounds [35,22]. Thus, dopamine appears to mediate conditioned fear by acting at rostral levels of the brain, and dopamine in midbrain areas appears to regulate unconditioned fear, likely by reducing the sensorimotor gating of aversive events. Thus, the reduction of dopamine activity enhances unconditioned fear, likely by acting at the midbrain level, opening sensory gating for aversive events, and reducing conditioned fear by acting at more rostral levels of the brain (Fig. 3). Further studies are needed to clarify the differential roles of dopamine in conditioned and unconditioned fear to shed more light on the neurobiological complexity of mental disorders.

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DISTINCT EFFECTS OF HALOPERIDOL IN THE MEDIATION OF CONDITIONED FEAR IN THE MESOLIMBIC SYSTEM AND PROCESSING OF UNCONDITIONED AVERSIVE INFORMATION IN THE INFERIOR COLLICULUS

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Abstract—Chemical and electrical stimulation of the inferior colliculus (IC) causes defensive behavior. Electrical stimulation of the IC at the escape threshold enhances dopamine (DA) release in the prefrontal cortex. Intra-ventral tegmental area injections of quinpirole at doses that act presynaptically reduce the release of DA in the terminal fields of the mesolimbic system and clearly reduce conditioned fear in several animal models of anxiety. However, little is known about the involvement of DA in the mediation of unconditioned fear, such as the reactivity to acute stressors. The present study investigated the neural substrates mediated by DA transmission associated with emotional changes triggered by the activation or inhibition of D_2 receptors during conditioned and unconditioned fear. We examined the effects of systemic or local injections of the DA-receptor antagonist and agonist haloperidol and quinpirole, respectively, into the IC in rats subjected to fear-potentiated startle, a Pavlovian paradigm that uses loud sounds as the unconditioned stimulus and light previously paired with footshock as the conditioned stimulus. We also assessed auditory-evoked potentials (AEPs) recorded from electrodes implanted in the IC. Intraperitoneal haloperidol administration dose-dependently enhanced AEPs induced by loud tones and inhibited fear-potentiated startle. Intra-IC injections of quinpirole left AEPs unchanged, suggesting that an optimal level of postsynaptic D_2 receptors in the IC may regulate the transmission of aversive information through the midbrain tectum. These findings provide evidence of opposing DA-mediated mechanisms in fear/anxiety processes that depend on the area under study. The activity of the neural substrates of conditioned fear was attenuated by haloperidol, whereas midbrain neural substrates of unconditioned fear were enhanced. Thus, DA appears to regulate unconditioned fear at the midbrain level, likely by reducing the sensory gating of aversive events and reducing conditioned fear by acting at more rostral levels of the brain. **© 2013 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: inferior colliculus, unconditioned fear, auditory-evoked potentials, fear-potentiated startle, conditioned aversion, dopamine.

INTRODUCTION

Studies on the mesencephalic organization of fear processing date back to the beginning of the 1950s, indicating that mesencephalic and bulbospinal preparations show capabilities of expressing defensive reactions compared with hypothalamic animals (Bard and Match, 1951). The inferior colliculus (IC) is a primary acoustic structure of the brainstem that also integrates sensory information of an aversive nature. Indeed, chemical and electrical stimulation of the IC causes unconditioned defensive behavior (Schmitt et al., 1986; Brandao et al., 1988b, 1993, 1994; De Araujo et al., 1999). A circuit that comprises the IC, medial geniculate nucleus, thalamus, and amygdala is involved in the processing of auditory information of an aversive nature, which triggers fear-like behavior (Hoffman and Ison, 1980; Maisonnette et al., 1996; Brandao et al., 1999). With regard to conditioned fear, learning can also be produced in paradigms that use electrical stimulation of the IC as an unconditioned stimulus (UCS). Rats quickly learn to make a shuttling response to avoid or escape from electrical stimulation of the IC (Troncoso et al., 2003). The stimulation of neural circuits in the central nucleus of the IC also supports Pavlovian-conditioned fear using several paradigms (Brandao et al., 1997; Troncoso et al., 1998; Castilho and Brandao, 2001).

Several studies have suggested modulatory roles for GABA, serotonin, opioids, excitatory amino acids, and neuropeptides in the so-called brain aversion system, which includes the dorsal periaqueductal gray (dPAG) and IC (Brandao et al., 1982, 1994, 1999). Recent reports have shown that intracollicular injections of *N*-methyl-D-aspartate (NMDA) enhance haloperidol-induced catalepsy. These effects of NMDA were

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Abbreviations: AEPs, auditory-evoked potentials; ANOVA, analysis of variance; CS, conditioned stimulus; DA, dopamine; IC, inferior colliculus; NMDA, *N*-methyl-D-aspartate; SPL, sound pressure level; UCS, unconditioned stimulus; VTA, ventral tegmental area.

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inhibited by microinjections of MK-801 and AP7 prior to systemic injections of haloperidol (Melo et al., 2010). Local application of the excitatory amino acids glutamate and NMDA into the central nucleus of the IC increased acoustically evoked and spontaneous firing of most neurons in this region (Faingold et al., 1989; Li et al., 1998). We recently showed that fear-evoking stimuli increase the magnitude of the evoked potential directly recorded from the central nucleus of the IC in response to loud sounds (Brandao et al., 2001; Sandner et al., 2002). Interestingly, aversive stimulation of this midbrain structure at the escape threshold enhances dopamine (DA) release in the prefrontal cortex (Cuadra et al., 2000). Other limbic structures such as the nucleus accumbens also respond with increased DA extracellular concentrations to acute stressors (McCullough and Salamone, 1992; Salamone, 1994, 1996; Tidey and Miczek, 1996; Datla et al., 2002; Young, 2004; Marinelli et al., 2005; Anstrom et al., 2009). Studies that investigate the DA-mediated mechanisms that underlie these processes have considerable relevance with regard to the changes in DA transmission that occur following exposure to external acute stressors (Fadda et al., 1978; Biggio et al., 1990; Anisman et al., 1991; Feenstra et al., 1995; Feenstra and Botterblom, 1996). Cortical DA projections are also activated by a wide variety of aversive stimulation (Thierry et al., 1976; Fadda et al., 1978; Deutch et al., 1985; Feenstra and Botterblom, 1996; Goldstein et al., 1996). Relevant to the present study, reductions of DA activity reduce conditioned escape responses in paradigms that use light as the conditioned stimulus (CS) and electrical stimulation of the IC as the UCS (Troncoso et al., 1998, 2003; Castilho et al., 1999; Reis et al., 2004). One surprising finding was that sulpiride, a D_2 receptor antagonist, increased switch-off responses in the light–dark test of unconditioned fear.

The IC appears to be functionally linked to other higher brain structures through dopaminergic mechanisms. The purpose of the present study was to determine whether DA mechanisms are involved in Pavlovian-conditioned and -unconditioned fear using IC stimulation as the UCS. Rats were placed inside an experimental box and subjected to unconditioned tests (loud sounds + auditory-evoked potential [AEP] recordings) and conditioned tests (fear-potentiated startle) using aversive IC stimulation with loud sounds as the UCS and the light previously paired with footshock as the CS. We examined the effects of systemic injections of the classic DA D_2 antagonist haloperidol and classic DA D_2 receptor agonist quinpirole in these paradigms.

EXPERIMENTAL PROCEDURES

Animals

A total of 197 male Wistar rats, weighing 250–300 g, were housed in individual Plexiglas-walled cages under a 12-h/12-h light/dark cycle (lights on at 7:00 AM) at 23 ± 1 °C with free access to food and water throughout the experiment.

Ethical statement

All of the experiments received formal approval (process No. 10.1.595.53.7) from the Committee on Animal Research and Ethics (CEUA) of the University of São Paulo and were performed in compliance with the recommendations of the Brazilian Society for Neuroscience and Behavior, which are in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. The number of animals used was the minimum required to ensure the reliability of the results, and every effort was made to minimize animal suffering.

Surgery

The animals were anesthetized with a mixture of ketamine/xylazine (100/7.5 mg/kg, i.p.) and fixed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). A brain electrode was implanted in the midbrain, aimed at the IC. The electrode was made of stainless-steel wire (160 μm diameter) that was insulated except at the cross-section of the tip. The upper incisor bar was set 3.3 mm below the interaural line so that the skull was horizontal between bregma and lambda. The electrode was introduced vertically using the following coordinates, with lambda as the reference for each plane: anterior/posterior, 1.2 mm; medial/lateral, 1.5 mm; dorsal–ventral, 4.5 mm (Paxinos and Watson, 1997). The electrode was fixed to the skull with acrylic resin and three stainless-steel screws. The electrode wire was connected to a connector so that it could be plugged into an amphenol socket at the end of a flexible electrical cable used for brain stimulation.

Haloperidol-induced catalepsy

The rats were tested for catalepsy 30 min after haloperidol injection (0.1, 0.5, and 1 mg/kg, i.p.) by carefully positioning their forepaws on a horizontal wooden bar set 8 cm above the floor, while their hind paws remained on the floor (Morelli and Di Chiara, 1985). The latency (cut-off time, 5 min) to step down from the horizontal bar was recorded. The experimental session was recorded with a video-recording system and analyzed by two different observers who were not aware of the pharmacological treatment.

Fear-potentiated startle

Matching. The rat's startle reaction put pressure on a response platform that generated an analog signal that was analyzed by Startle Reflex software (Med Associates, St. Albans, VT, USA). The test cage consisted of a wire-grid cage (16.5 × 7.5 × 7.5 cm) that was fixed to the response platform by four thumb screws. The test cage and response platform were located inside a ventilated sound-attenuating plywood-chamber (64 × 60 × 40 cm). A loudspeaker located 10 cm behind the test cage delivered both the startle stimulus (100 dB; 50 ms burst of white noise) and continuous background noise (55 dB). The startle

reaction was recorded within a time window of 100 ms after the onset of the startle stimulus. For the first 2 days, the animals were placed in the test cage for a 5-min habituation period and afterward received a total of 30 startle stimuli with an interstimulus interval of 30 s. Each matching session was 20 min in duration, including the habituation period. To match the animals in the control and drug groups according to the scores obtained in the matching phase, the animals were assigned to different groups such that each group had the same average startle amplitude based on the last matching day.

Training. The animals were conditioned to a light-CS in a cage (20 × 20 × 25 cm) with stainless steel side and back walls, a transparent Plexiglas ceiling, and front door and grid floor that consisted of stainless-steel rods. This training cage was located within a ventilated and sound-attenuated chamber (45 × 45 × 45 cm) and was different from the test cage to avoid conditioning of the context. The animals were placed in the training cage, and each rat received 10 CS–US pairings after a habituation phase of 5 min using a 4-s, 6-W light-CS that co-terminated with a 1-s, 0.6-mA footshock-US. The intertrial interval randomly varied between 60 and 180 s. The duration of each training session was approximately 25 min, including the habituation period.

Testing. Test sessions were conducted without footshock presentation in the same cages used for matching. Twenty-four hours after training, the rats received a drug injection and were placed into the test cage 5 min later. After 5 min of habituation, the animals were exposed to 60 startle stimuli (i.e., noise bursts) with a 30-s interstimulus interval. Half of the startle stimuli were presented in the absence of the CS (noise-alone trials) to provide a baseline, and the other half were presented in the presence of the CS (light-noise trials). In the light-noise trials, the rats were exposed to a 4-s presentation of the light-CS that co-terminated with the startle stimulus. The noise-alone and light-noise trials were intermingled at random. The duration of the test session, including the habituation period, was 37 min.

Table 1 shows the timeline of the procedures for testing the effects of IP or intra-IC injections of haloperidol in the expression of conditioned fear using the fear potentiated startle test.

AEPs

Experimental box. An experimental cage made of Plexiglas (19.0 cm length,13.5 cm height, 9.0 cm width), located inside an insulated Faraday system and surrounded by a ventilated sound-attenuating plywood chamber (64 × 60 × 40 cm), was used. A 7.5-W red bulb at the top of the test box was switched on during the experimental sessions. The floor of the cage consisted of six 4.0-mm diameter stainless-steel bars spaced 10 mm apart. A loudspeaker located 10 cm behind the cage delivered continuous background noise (55-dB sound pressure level [SPL]). Acoustic stimuli (100 clicks, 50-ms duration; 3000-Hz square-wave pulses) presented at a rate of 0.33 Hz (one each 3 s) were delivered via two piezoelectric speakers (12 Ω, 200 W; LeSon, Sao Paulo, Brazil) mounted on the lateral walls of the sound-insulating box, 15 cm from the wire mesh cage. The acoustic stimulus was a pure tone with a 92.5-dB SPL. Software and an appropriate interface (Lynx Electronics, São Paulo, São Paulo, Brazil) controlled the presentation and sequence of the acoustic stimuli. SPLs were measured at the level of the ears of the animals using a 0.125-inch microphone and a type 2636 DK-2580 measuring amplifier (Bruel and Kjaer Sound & Vibration Measurement A/S, DK-2850, Naerum, Denmark). The animals were restrained inside the experimental cage to prevent their movement, with the exception of a small gap. In this condition, the head of the animal was directed to the center of the sound stimulation (i.e., the loudspeaker). The rats were unable to rotate or turn from one side to the other inside the box such that only little variation in the azimuth of sound propagation (5° left–right-top-down; i.e., the space the animals had for head movements) was likely to occur. This variation induced changes in the sound intensity by approximately 2.5 dB. Thus, all of the animals were likely exposed to similar sound levels (92.5 dB). Behavioral restriction is very aversive to rats. However, AEP amplitudes can suffer interference from neck muscles, thus increasing the signal–noise ratio in rats tested during their active period. To avoid both problems in the present study, the animals were previously habituated to the experimental box. In this condition, after a few periods of exposure (i.e., two 30-min periods daily, before the start of the experiments), the rats' attempts to rotate or turn inside the box and bite the stainless steel bars (i.e., the main indicators of stress in this situation) were absent, and the animals often slept. All of the calibration procedures were conducted before the experiments to ensure equivalent sensitivity before each session.

Brainstem AEPs are very small electrical voltage potentials that are recorded from electrodes in response to a repetitive stimulus along a specific brainstem auditory pathway. These potentials reflect neuronal activity in the auditory complex, mainly in the cochlear nucleus, superior olive, and IC (Long and Allen, 1984). Previous studies have shown that AEPs generated in the IC are sensitive to aversive manipulations (Brandao et al., 2001; Nobre et al., 2003; Baas et al., 2006). For example, Nobre et al. (2003) showed that freezing behavior induced by intra-IC microinjections of a low dose of the GABA blocker semicarbazide increased the amplitude of AEPs in laboratory rats. Similarly, some of the electrophysiological brainstem abnormalities observed in anxiety disorders can be replicated in healthy control subjects by inducing a transient state of anxiety (Baas et al., 2006). The stimulus presentation was produced and controlled by a biological data acquisition system (Sysdin, Lynx Electronics, São Paulo, São Paulo, Brazil). The average value was

Table 1. Timeline of the procedures for testing the effects of IP or intra-IC injections of haloperidol on the fear-potentiated startle test

Experimental design						
a. Intraperitoneal injections						
Matching 1	Matching 2	Training	Test			
Day 1	Day 2	Day 3	Day 4 Saline/haloperidol were injected IP. FPS was recorded afterward			
Matching 1	Matching 2	Surgery	Recovery	Training	Test	Histology
b. Intra-IC injections						
Day 1	Day 2	Day 3	Day 8	Day 9	Day 10 saline/haloperidol were locally injected into the IC. FPS was recorded afterward	

obtained at the end of the sessions. AEPs were recorded as the voltage difference between the tips of an insulated wire (150 μm) inserted through the cannula and the tip of the guide-cannula implanted in the IC. This voltage difference was fed into a TX001 amplifier (bandwidth set to 20–200 Hz; Lynx Electronics, São Paulo, São Paulo, Brazil) through two noiseless shielded cables that were passed through a hole in the roof of the Faraday cage. A previous study from our laboratory indicated no hemispheric differences in AEPs recorded under the present experimental conditions (Nobre et al., 2003). The output of the amplifier was connected to one of the four channels on an analog/digital converter (CAD 12/36) that was plugged into a computer. The filtering, amplification, and digitalization of the signals were performed with the Sysdin system (Lynx Electronics, São Paulo, São Paulo, Brazil). The potential signals were filtered (high-pass filter, 20 Hz; low-pass filter, 200 Hz) and sampled at a rate of 0.33 kHz. Sysdin software was programed to sum individual AEP amplitudes. The data acquisition sweep began 10 ms before the onset of the sound stimulus (i.e., the latency to switch on the sound plus sound propagation) and continued for 200 ms after termination of the sound stimulus. During recording, the animals were monitored via a camera system placed in the experimental room. N1 was visually identified as the first negative wave, and P1 was identified at the first positive wave approximately 15 ms after the sound presentation.

The positive peak P1 is considered an early component of the collicular response. Its amplitude was measured peak to peak, with a peak latency between 5 and 8 ms (Hall and Mark, 1967). The AEPs elicited from the IC were recorded from the ventrocaudal portions of the nucleus. This method of analysis is similar to previous studies from our laboratory and other laboratories that used similar protocols (Hall and Mark, 1967; Szczepaniak and Moller, 1995; Cabral et al., 2009). Peak amplitudes were defined as the maximum amplitude measured between N1 and the end of P1, similar to previous studies from our laboratory (Brandao et al., 2001; Nobre et al., 2003; Cabral et al., 2009). This set of data was monitored on the computer screen. The computer output was graphically displayed on an XY plotter (Hewlett-Packard, Palo Alto, CA, USA). The AEP data were stored on a computer and transferred to Microsoft Excel (Microsoft, Mountain View, CA, USA) for off-line visualization and analysis.

Drugs

The drugs, doses, and injection times were based on a previous study from our laboratory (de Oliveira et al., 2009). Haloperidol (Janssen-Cilag Farmaceutica, SP, Brazil) and quinpirole hydrochloride (Sigma, St Louis, MO, USA) were dissolved in 0.9% physiological saline shortly before use. Saline also served as the vehicle control. Haloperidol (0.1, 0.5, and 1 mg/kg) and quinpirole (0.25 and 1 mg/kg) were administered intraperitoneally. The doses of the drugs were administered in a volume of 1 ml/kg, i.p. Haloperidol (0.1 μg/0.5 μl, 0.5 μg/0.5 μl, and 1 μg/0.5 μl) and quinpirole (0.1 μg/0.2 μl, 1 μg/0.2 μl, and 2 μg/0.2 μl) were also administered locally in the IC.

Microinjection procedure

The injection needle was a thin dental needle (0.3-mm outer diameter) connected to a 10 μl Hamilton syringe with a polyethylene-10 tube (Becton–Dickinson, Franklin Lakes, NJ, USA). The displacement of an air bubble inside the polyethylene catheter that connected the syringe needle to the intracerebral needle was used to monitor the microinjection. The needle was held in place for an additional 1 min to maximize diffusion away from the tip of the needle. The needle was introduced into the guide cannula until its lower end reached 1 mm below it. The solutions were injected in a volume of 0.5 or 0.2 μl using an infusion pump (Harvard Apparatus, Holliston, MA, USA).

Histology

Upon completion of the experiments, the rats were transcardially perfused with 0.9% saline followed by 4% formaldehyde. The brains were removed from the skulls and maintained in formaldehyde solution for 2 h, after which the brains were cryoprotected in 30% sucrose until soaked. Serial 60-μm coronal brain sections were cut using a freezing microtome, mounted on gelatin-coated slides, and stained with Cresyl violet (5%; Sigma–Aldrich, St Louis, MO, USA) to localize the positions of the microinjection sites according to the atlas of Paxinos and Watson (1997).

Statistical analysis

The data are expressed as mean ± SEM. The catalepsy and AEP data were subjected to one-way analysis of variance (ANOVA), with drug dose as the factor. The fear-potentiated startle data were subjected two-way repeated-measures ANOVA, with treatment as one factor and trial-type (noise alone and light + noise) as the other factor. *Post hoc* differences between group means were tested using the Newman–Keuls test. Values of $p < 0.05$ were considered significant.

RESULTS

Systemic injections

Catalepsy. Fig. 1 presents the mean step-down latency 30 min after the intraperitoneal administration of haloperidol in the four groups of animals. One-way ANOVA revealed a significant effect of treatment on step-down latency ($F_{3,23} = 238.74$, $p < 0.05$). The Newman–Keuls *post hoc* test revealed that 0.5 and 1 mg/kg haloperidol increased the step-down latency compared with the saline group ($p < 0.05$).

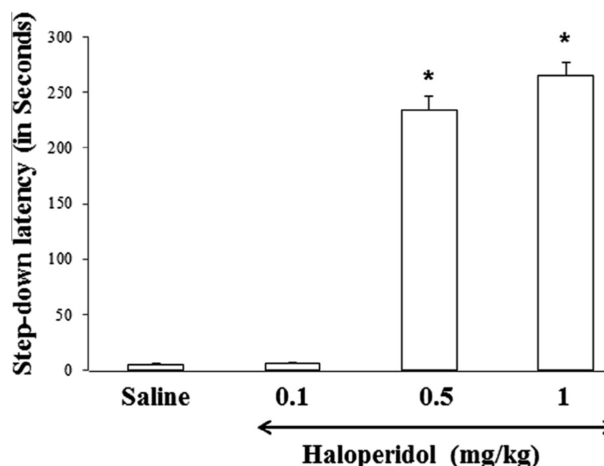

Fig. 1. Haloperidol-induced catalepsy. Step-down latencies were assessed using the horizontal bar test. Bars represent step-down latencies in the Sal (1 ml/kg; $n = 8$), 0.1 ($n = 8$), 0.5 ($n = 8$) and 1.0 mg/kg ($n = 8$) of HAL injected IP. Data are expressed as mean ± SEM. $^{*}p < 0.05$ compared to Sal (saline) group.

Fear-potentiated startle. Table 1 depicts the timeline of the procedures for testing the effects of IP or intra-IC injections of haloperidol in the fear-potentiated startle test. Fig. 2 shows the mean startle amplitude in rats that received an intraperitoneal injection of saline ($n = 18$), 0.1 mg/kg haloperidol ($n = 16$), and 0.5 mg/kg haloperidol ($n = 10$). Two-way repeated-measures ANOVA revealed significant effects of treatment ($F_{2,37} = 7.37$, $p < 0.05$) and condition ($F_{1,79} = 45.07$, $p < 0.05$) and a significant treatment × trial-type interaction ($F_{2,79} = 6.15$, $p < 0.05$). The Student–Newman–Keuls *post hoc* test revealed that 0.1 and 0.5 mg haloperidol decreased the startle amplitude in the light-noise condition compared with the corresponding condition in the saline group ($p < 0.05$).

The effects of systemic injections of quinpirole on fear-potentiated startle were not evaluated in the present study because this treatment has been previously shown to reduce fear-potentiated startle (de Oliveira et al., 2011).

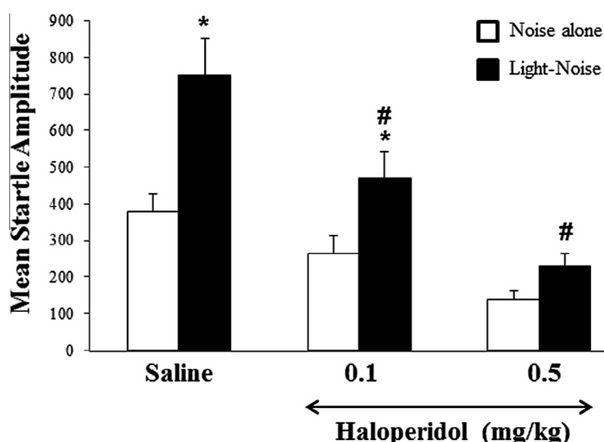

Fig. 2. Effects of intraperitoneal injections of saline ($n = 17$), haloperidol 0.1 ($n = 16$) or 0.5 mg/kg ($n = 10$) on the mean startle amplitude of rats. Results are expressed as mean ± SEM. *Different from noise-alone condition ($p < 0.05$). #Different from light-noise condition in saline group ($p < 0.05$).

AEPs. Table 2 depicts the timeline of the procedures for testing the effects of IP or intra-IC injections of dopaminergic drugs in the processing of acoustic aversive information through the recording of the AEPs. Fig. 3 shows the effects of systemic administration of haloperidol on AEPs recorded from the IC in rats. One-way ANOVA revealed a significant effect of treatment ($F_{3,29} = 5.139$, $p < 0.05$). The Student–Newman–Keuls *post hoc* test revealed that 0.5 mg/kg haloperidol significantly increased AEPs compared with the saline group ($p < 0.05$).

Intra-IC injections

Microinjection sites. Fig. 4 depicts the injection sites in all of the animals used in the present study. The injection sites were located inside the IC, mostly in the ventral portion. A photomicrograph of a representative injection site and histological localization of the injection sites in the IC are shown in the diagrams in the atlas of Paxinos and Watson (1997).

Fear-potentiated startle. Fig. 5 shows the mean startle amplitude in rats that received an injection of saline ($n = 8$) and 0.1 µg/0.5 µl ($n = 9$), 0.5 µg/0.5 µl ($n = 8$), and 1 µg/0.5 µl ($n = 8$) haloperidol into the IC. Two-way repeated-measures ANOVA revealed a significant effect of condition ($F_{1,69} = 64.66$, $p < 0.05$) but no significant effect of treatment ($F_{3,31} = 1.06$, $p > 0.05$) and no treatment × trial-type interaction ($F_{3,69} = 1.72$, $p > 0.05$).

Auditory-evoked potentials. Fig. 6 indicates the mean AEP changes recorded following a local injection of

Table 2. Timeline of the procedures for testing the effects of IP or intra-IC injections of dopaminergic drugs on the auditory-evoked potentials

Experimental design				
Surgery Day 1	Recovery Day 5	Saline Day 6	Test Day 7	Histology
a. Intraperitoneal injections				
		Saline was injected IP. After 25 min AEP was recorded for 5 min	Haloperidol was injected IP. Afte 25 min AEP was recorded for 5 min	
b. Intra-IC injections				
		Saline was locally injected into the IC. After 20 min AEP was recorded for 5 min	Haloperidol or quinpirole were locally injected into the IC. After 20 min AEP was recorded for 5 min	

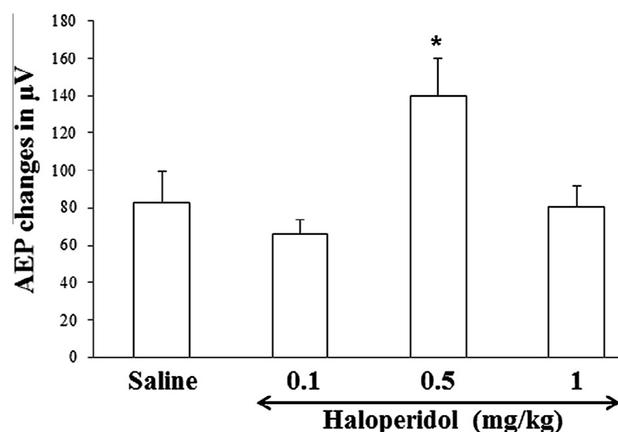

Fig. 3. Mean (±SEM) amplitude of auditory-evoked potentials recorded in rats. Each animal received an intraperitoneal injection of saline ($n = 6$), or haloperidol (0.1 ($n = 9$), 0.5 ($n = 9$) and 1.0 ($n = 6$) mg/kg). $^{*}p < 0.05$, significant difference within saline or haloperidol.

haloperidol into the IC in rats. One-way ANOVA revealed a significant effect of treatment ($F_{3,25} = 4.46$, $p < 0.05$). The Student–Newman–Keuls *post hoc* test revealed that 0.5 μg/0.5 μl haloperidol significantly increased AEPs compared with the saline group ($p < 0.05$).

The effects of intra-IC injections of quinpirole on AEPs at doses that were effective when injected into the ventral tegmental area (VTA) in rats subjected to the fear-potentiated startle test were also evaluated (Fig. 7), but this treatment did not produce any significant effects in the one-way ANOVA ($F_{3,23} = 0.29$, $p > 0.05$).

DISCUSSION

All of the recording sites in the present study were situated throughout the IC. Most of them were located in the central nucleus, a structure traditionally known to process high-pitched sounds (Rose et al., 1963; Merzenich and Reid, 1974). This region of the IC has been associated with the neural substrates of fear because of the aversive properties of its electrical stimulation, and chemical stimulation of the central nucleus of the IC with bicuculline or excitatory amino acids, such as NMDA and glutamate, causes similar aversive responses (De Araujo et al., 1999; Pandossio and Brandao, 1999). Moreover, systemic or local injections of GABA agonists or benzodiazepines in this midbrain structure promptly reduce the aversive consequences of electrical stimulation (Cardoso et al., 1992; Brandao et al., 1999). GABA, serotonin, and excitatory amino acids have been shown to be involved in the mediation of defensive behavior generated and organized at this mesencephalic level (Brandao et al., 1988a, 1999; Bagri et al., 1989; Cardoso et al., 1992, 1994; Pandossio and Brandao, 2000).

Systemic haloperidol administration is widely known to induce catalepsy by blocking postsynaptic striatal DA receptors (Hornykiewicz, 1973; Sanberg, 1980; Wadenberg et al., 2001). Haloperidol injected into the striatum induces catalepsy (Klockgether et al., 1988; Ossowska et al., 1990). However, microinjections of haloperidol into the IC at the same doses (2.5 μg/0.5 μl) did not induce catalepsy, but intracollicular administration of the glutamate receptor antagonists AP7 and MK-801 and glutamate agonist NMDA influences haloperidol-induced catalepsy in rats, with antagonists attenuating catalepsy and agonists potentiating catalepsy (Melo et al., 2010). Our laboratory is currently conducting additional studies to clarify the possible interplay between dopaminergic and glutamatergic mechanisms in the regulation of motor activity and involvement of the neuronal circuits in the IC in emotional reactivity to stressful conditions.

With regard to aversive information filtering processes, accumulating evidence suggests the involvement of dopaminergic mechanisms in the IC in the processing of auditory inputs of an aversive nature (for review, see (Brandao et al., 1999)). Although DA-mediated mechanisms involved in the generation of aversion at the level of the IC have not been studied to date, this region has appreciable dopaminergic activity (Melo et al., 1997). Significant concentrations of DA were found in the IC in a study that demonstrated the presence of D_2 receptor mRNA but not D_1 receptor mRNA in the human IC (Hurdy et al., 2001). In the present study, lower doses of haloperidol (0.5 mg/kg, i.p.) clearly enhanced AEPs associated with the presentation of loud and aversive sounds. Interestingly, intra-IC injections of haloperidol (0.5 μg/0.5 μl) enhanced the amplitude of AEPs recorded from the central nucleus of the IC. This latter effect appeared to

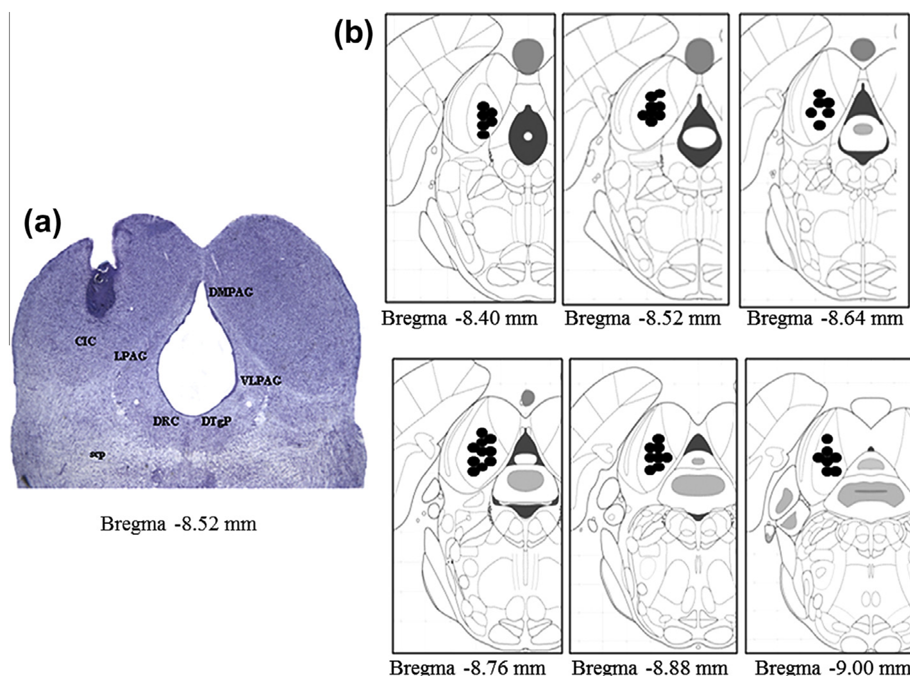

Fig. 4. Target sites for drug injections into the IC. Photomicrograph showing a typical example of an electrode implanted into the central nucleus of the IC (a). Outline of injection sites on coronal sections from the Paxinos and Watson atlas (2007) (b). The number of points in the figure is less than the total number of rats used because several points overlap. CIC, central inferior colliculus; DMPAG, dorsomedial periaqueductal gray; LPAG, lateral periaqueducatal gray; VLPAG, ventrolateral periaqueductal gray; DRC, dorsal raphe nucleus caudal part; SCP, superior cerebellar peduncle; DRC, dorsal tegmental nucleus. Scale bar = 500 μm.

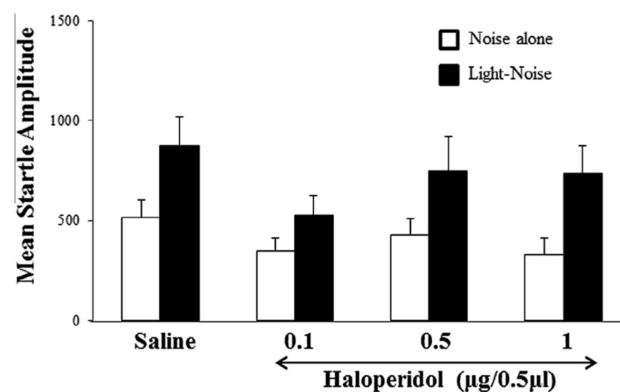

Fig. 5. Effects of local injections of saline ($n = 8$), 0.1 μg ($n = 10$), 0.5 μg ($n = 9$) and 1 μg of haloperidol ($n = 8$) on the startle amplitude of rats. The volume of injections was 0.5 μl. Results are expressed as mean ± SEM. *Different from noise-alone condition ($p < 0.05$).

not be related to catalepsy because local injections of haloperidol in the IC did not cause motor immobility (Melo et al., 2010). Quinpirole injection in the IC left AEPs unchanged. The dopaminergic modulation of the processing of ascending auditory information appears to be tonic because quinpirole has been reported to be a full agonist at D_2 receptor sites (de Oliveira et al., 2011), and the further activation of D_2 receptors in the IC does not result in further dopaminergic modulation of AEPs.

The association between changes in dopaminergic transmission and fear has been previously demonstrated by numerous studies. In fact, alterations in DA transmission always occur after exposure to a wide variety of acute stressors (Anisman et al., 1991), and cortical DA projections are also activated by diverse types of aversive stimulation (Thierry et al., 1976; Fadda et al., 1978; Anisman et al., 1991; Feenstra et al., 1995; Feenstra and Botterblom, 1996; Goldstein et al., 1996). Although the precise neural circuitry of DA transmission involved in aversive states remains unclear, pharmacological and neurochemical studies appear to implicate prefrontal (Espejo and Minano, 1999; Morrow et al., 1999) and nucleus accumbens DA terminals in the response to acute stressors (McCullough and Salamone, 1992; Salamone, 1994, 1996; Tidey and Miczek, 1996; Datla et al., 2002; Young, 2004; Marinelli et al., 2005; Anstrom et al., 2009). Support for a functional link between the activation of these neurons and behavioral responses induced by aversive stimulation of the IC has been recently reported (Cuadra et al., 2000). Dorsal midbrain regions have substantial cortical inputs, and the IC also projects densely to cortical areas, mainly the temporal lobe (Cooper and Young, 1976; Fadda et al., 1978; Adams, 1979; Meininger et al., 1986). In fact, the IC is a key pathway for auditory information, and disturbances at this level may alter transmission to cortical centers. An indirect pathway that connects these structures includes the central nucleus of the IC–medial geniculate nucleus–amygdala–dorsomedial thalamus–prefrontal cortex pathway (Fuster, 1989; Cardoso et al., 1994). This alternate circuit is associated with the processing of auditory information of an aversive nature, which triggers fear-like behavior (LeDoux et al., 1986;

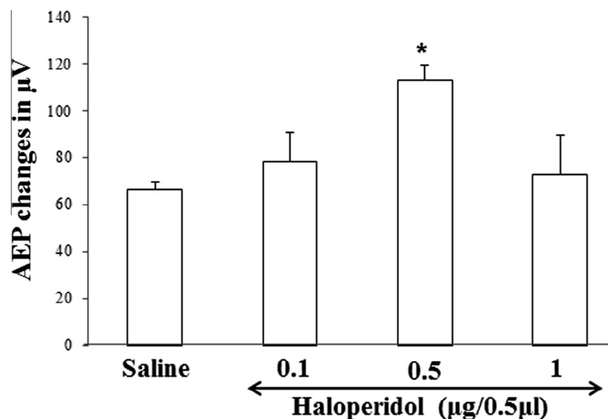

Fig. 6. Mean (±SEM) amplitude of auditory-evoked potentials recorded in rats. Each animal received an intracollicular injection of saline ($n = 6$), or haloperidol (0.1 ($n = 6$), 0.5 ($n = 8$) and 1.0 ($n = 6$) µg/0.5 µl). $^{*}p < 0.05$, significant difference within saline or haloperidol.

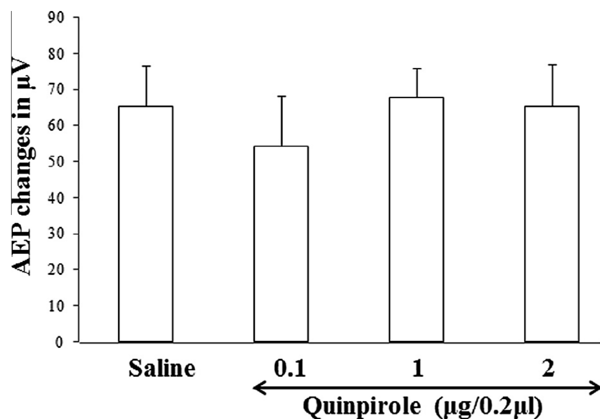

Fig. 7. Effects of intra-IC injections of saline ($n = 6$) or 0.1 ($n = 6$), 1.0 ($n = 6$) and 2 µg/0.2 µl ($n = 6$) of quinpirole on the auditory-evoked potentials recorded from the inferior colliculus of in rats. Results are expressed as mean ± SEM.

Maisonnette et al., 1996). A previous study demonstrated that microinjections of nefazodone, a serotonin receptor antagonist, in the basolateral nucleus of amygdala reduced aversive reactions induced by microinjections of NMDA in the IC (Maisonnette et al., 2000).

Abnormal cortical areas are known to exist in schizophrenia patients and may account for the abnormal processing of auditory information that results in auditory hallucinations and decreased responsiveness to sounds (David et al., 1996). The global control of cortical function involved in sensory processing by the mesolimbic system needs to be considered in this field of study (Melo et al., 1997). In the present study, intraperitoneal injections of the DA antagonist haloperidol dose-dependently inhibited fear-potentiated startle. The observed effects of haloperidol cannot be attributed to nonspecific effects because this effect was observed at a dose of haloperidol (0.1 mg/kg) that did not affect motor performance in the catalepsy test. Moreover, it is well-known that suppression of aversively motivated behavior by haloperidol is context dependent since DA antagonists can block avoidance of an aversive stimulus at doses that do not block escape from the actual shock presentation (Posluns, 1962; Hillegaart and Ahlenius, 1987; Baldessarini, 2000). Depending on the type of threatening condition (i.e., conditioned or unconditioned), the effects observed with haloperidol in this study using the fear-potentiated-startle test appears to be sensorimotor rather than purely motor. Indeed, although catalepsy response predominates in haloperidol-treated rats we know from previous studies of this laboratory that reduction of dopaminergic transmission in the mesolimbic system impairs conditioned fear responses (De Oliveira et al., 2009, 2011). In support of this assumption in a recent study of this laboratory haloperidol caused a strong reduction in the USVs emitted during the testing session of the contextual conditioned fear even at doses that produced catalepsy (Colombo et al., 2013). This latter study made it clear that the effects of haloperidol on catalepsy and conditioned fear were dissociated from each other. As to the higher dose of haloperidol (1.0 mg/kg) it is clear that this dose renders the animal cataleptic, which can prevent rats from startling. This was the reason for showing only lower doses of haloperidol (0.1 and 0.5 mg/kg) in this study.

The present results may indicate that conditioned fear increases DA metabolism in the mesolimbic system. Similar effects were also obtained with intraperitoneal injections of low doses of the D_2 receptor agonist quinpirole (de Oliveira et al., 2006). Under these experimental conditions, this DA agonist preferentially acts at the presynaptic level by reducing the firing of DA neurons. Aversive stimulation causes DA release in DA projection areas, such as the amygdala and frontal cortex, and the reduction of the activity of these DA projection neurons may lead to an anti-aversive action of these drugs. These findings indicate that D_2 receptors contribute to the encephalic aversion system when it is activated by conditioned fear stimuli. Indeed, in the present study, haloperidol exerted an effect that was similar to the effect previously reported for intra-VTA microinjections of quinpirole (de Oliveira et al., 2009) i.e., an attenuation of the expression of conditioned fear in response to a light-CS), supporting the hypothesis that quinpirole's ability to decrease fear-potentiated startle in our previous study resulted from an action on presynaptic D_2 receptors in the VTA that decreased DA levels in the terminal fields of the mesocorticolimbic pathway. Therefore, the fear response to a light-CS appears to depend on the activation of dopaminergic connections and can be specifically modulated by manipulating these projection neurons. One projection area for these neurons could be the nucleus accumbens, which has been shown to have increased DA levels in the presence of an aversive CS (Martinez et al., 2008). We cannot discard the possibility that DA projection neurons in the IC may ascend to more rostral structures instead of descending to output areas that modulate defense reactions. However, the substantia nigra does not appear to be necessary for this dopaminergic activity. Lesions of the substantia nigra

pars compacta did not affect DA levels in the IC (Maisonnette et al., 1998). In contrast, GABAergic neurons from the substantia nigra pars reticulata project to the IC and may modulate motor activity engendered at this level (Castellan-Baldan et al., 2006).

The effects of dopaminergic agents on conditioned fear should be interpreted with caution because pharmacological manipulations of the dopaminergic system on the test day may cause unconditioned changes in behavior. Thus, these changes could indirectly compete with the expression of fear-potentiated startle and freezing. Conditioned freezing has been considered a distinct form of fear response. Depending on the intensity of the threatening stimuli, its time course or magnitude may differ with regard to fear-potentiated startle (for review, see (Albrechet-Souza et al., 2008). In the present study, unconditioned and conditioned responses were differentially affected by the pharmacological manipulations. This may be understood within the framework of the two-dimensional theory proposed by McNaughton and Corr (2004). They stated that the neural substrates of fear (unconditioned) and anxiety (conditioned) are a function of a rostrocaudal gradient in the brain, with the first mapping at more caudal structures, such as the IC, whereas conditioned fear is mapped at more rostral levels, such as amygdala and prefrontal cortex.

In addition to descending projections to the nucleus reticularis pontis (Leitner and Cohen, 1985; Reimer et al., 2008), the IC has ascending connections with the medial geniculate nucleus of the thalamus, which provides major afferents to the striatum. This arrangement suggests that the IC is an important afferent source of sensory and motor information and a recipient of basal ganglia outputs. Thus, in addition to the main loop configuration of the connections with most regions of the cerebral cortex, limbic system, and thalamus, a subcortical loop that consists of the basal ganglia and IC also exists. Projections from basal ganglia output areas (e.g., substantia nigra pars reticulata) back to the IC have also been reported (Castellan-Baldan et al., 2006). The functional significance of these connections has not yet been envisaged. A similar subcortical connection that consists of the superior colliculus, thalamus, and basal ganglia has been studied, providing important insights into its functional significance (Redgrave et al., 2010; Comoli et al., 2012; Stafford et al., 2012).

Sensorimotor filtering is commonly activated in the midbrain tectum when acoustic or visual stimuli are presented to animals. Although some aspects of the acoustic signal (e.g., intensity) are directly relayed to the IC via the cochlea-tectal projection, aversive conditioned stimuli take a more circuitous route to the IC, first being subjected to early processing in limbic structures and the cortex (Borelli et al., 2006; Ferreira-Netto et al., 2007). In fact, DA signaling in the prefrontal cortex has been proposed to support this crucial processing because DA signaling is triggered in the medial prefrontal cortex by inputs from the IC (Cuadra et al., 2000). In the present study, we compared outcomes when the stimuli (loud sounds) were immediately available to the IC for sensory processing and action outcomes when the cortical or limbic processing of the signal was required, such as with fear-conditioned stimuli (i.e., fear-potentiated startle using light as the CS). We found that action acquisition with conditioned stimuli, which recruits the mesocorticolimbic system, was significantly impaired by the DA receptor antagonist haloperidol. In contrast, when the stimulus was eligible for immediate IC processing, the role of DA neurons associated with the aversive conditions that activated afferent sensory pathways via the IC appeared to be primary and possibly instrumental.

Overall, intraperitoneal administration of haloperidol dose-dependently enhanced AEPs induced by loud tones and inhibited fear-potentiated startle. The present results provide evidence of the opposing activity of DA-mediated mechanisms in fear/anxiety processes. The activity of the neural substrates of conditioned fear was attenuated by the DA antagonist haloperidol, whereas midbrain neural substrates of unconditioned fear were enhanced. Thus, reduction of DA activity enhanced unconditioned fear, likely by acting at the midbrain level, opening sensory gating for aversive events, and reducing conditioned fear by acting at more rostral brain levels. Indirect support for these results was provided by studies that demonstrated that the DA receptor antagonist sulpiride increased the switch-off response to light presented as an UCS in the light–dark test and enhanced fear-like behavior in the open arms of the elevated plus maze (Reis et al., 2004; Garcia et al., 2005).

The neuroleptic action of haloperidol has generally been associated to the reduction of neurotransmission in the DA mesolimbic system (Hillegaart and Ahlenius, 1987; Farde et al., 1988; Nyberg et al., 1995). The notion of the mediation of conditioned fear by D2-receptor mechanisms comes from previous studies of this laboratory showing that the D2-receptor agonist quinpirole and the D2-receptor antagonist sulpiride enhances and impairs conditioned fear responses, respectively (De Oliveira et al., 2009, 2011). Interestingly enough, it appears that the density of D2 receptors largely predominates over other DA receptors in the IC (see Hurdy et al., 2001). Nevertheless, further investigations are still needed to consolidate the idea of a differential D2 receptor-mediated regulation of unconditioned and conditioned fear and ongoing experiments are being conducted in this laboratory with this perspective.

In summary, previous studies that investigated the involvement of DA in anxiety have reported anxiolytic-like effects, anxiogenic-like effects, and a lack of effect of DA agonists and antagonists in animal models of anxiety (Rodgers et al., 1994; Garcia et al., 2005). The present findings may shed light on this issue insofar as depending on the type of threatening condition (i.e., conditioned or unconditioned), DA receptor antagonists may reduce or heighten the aversiveness of the situation. They also bring support to several reports in the literature showing that suppression of aversively motivated behavior by haloperidol is context dependent

since DA antagonists can block avoidance of an aversive stimulus at doses that do not block escape from the actual shock presentation (Posluns, 1962; Hillegaart and Ahlenius, 1987; Baldessarini, 2000).

AUTHOR DISCLOSURE STATEMENT

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