UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO” FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS CAMPUS JABOTICABAL

DIFERENÇAS SEXUAIS REFERENTES À PARTICIPAÇÃO DO CANAL CATIÔNICO ASIC1a NO *LOCUS COERULEUS* NA RESPOSTA COMPORTAMENTAL INDUZIDA POR CO_2

Letícia Rodrigues Pinheiro

Bióloga

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UNIVERSIDADE ESTADUAL PAULISTA - UNESP CÂMPUS DE JABOTICABAL

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DADOS CURRICULARES DO AUTOR

Letícia Rodrigues Pinheiro, nascida em 12 de novembro de 1996 na cidade de Tangará da Serra - MT, filha de Lídia Bezerra Rodrigues Pinheiro e Paulo César, ingressou no curso de Ciências Biológicas da Universidade Federal de Uberlândia (UFU), Campus Pontal em março de 2017. Durante o período de graduação, a aluna realizou uma iniciação científica sendo bolsista do Programa de Educação Tutorial-PET BIO. O estágio curricular foi realizado no Laboratório de Fisiologia da Universidade Estadual Paulista "Júlio de Mesquita Filho" Campus Jaboticabal. Colou grau em outubro de 2021 e recebeu o título de Bacharel em Ciências Biológicas. Em janeiro de 2022 iniciou o curso de Mestrado pelo programa de Pós-graduação em Ciência Animal no Departamento de Morfologia e Fisiologia Animal na Universidade Estadual Paulista- UNESP Campus Jaboticabal, onde foi bolsista CAPES.

“Quem quer algo de grande, como diz Goethe, deve saber limitar-se. Quem, pelo contrário, tudo quer, nada, em verdade, quer e nada consegue.” Georg Wilhelm Friedrich Hegel.

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CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Participação do canal catiônico ASIC1a nas alterações cardiorrespiratórias e comportamentais induz.das por CO_2"**, protocolo nº 3451/22, sob a responsabilidade da Profª Drª Luciane Helena Gargaglioni Batalhão, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 15 de junho de 2022.

Vigência do Projeto	01/07/2022 a 03/02/2024
Espécie / Linhagem	C57BL/6
Nº de animais	64
Peso / Idade	25-30g/9 semanas
Sexo	Machos e Fêmeas
Origem	Biotério de Roedores do Departamento de Morfologia e Fisiologia Animal – FCAV/UNESP

Jaboticabal, 15 de junho de 2022.

Fabiana Pilarski
Profª Drª Fabiana Pilarski
Coordenadora – CEUA

DIFERENÇAS SEXUAIS REFERENTES À PARTICIPAÇÃO DO CANAL CATIÔNICO ASIC1a NO *LOCUS COERULEUS* NA RESPOSTA COMPORTAMENTAL INDUZIDA POR CO_2

Resumo: O transtorno de pânico (TP) é uma condição marcada por intensa angústia e ansiedade, na qual o paciente desenvolve rapidamente sintomas respiratórios comofalta de ar, hiperventilação e sensação de sufocamento. Apesar dos mecanismos dessa desordem permanecerem desconhecidos, estudos recentes sugerem provávelconexão entre os desafios respiratórios e o TP, uma vez que episódios de hipóxia ou hipercapnia podem desencadear transtornos relacionados à ansiedade. O locus coeruleus (LC) é uma região quimiossensível capaz de gerar respostas emocionais efísicas durante episódios de estresse. Além disso, o LC possui neurônios noradrenérgicos que participam na resposta ventilatória ao CO_2, e a inalação de ar enriquecido com CO_2 pode desencadear ataques de pânico. Estudos recentes também sugerem a participação do canal iônico sensível a ácido do tipo 1a (ASIC1a)localizados na amígdala na resposta panicogênica induzidas por CO_2. Apesar disso, ainda não se conhece totalmente o envolvimento desse canal no LC na resposta ventilatória e às alterações comportamentais induzidas por concentrações elevadas de CO_2, que é um modelo utilizado para indução de ataques de pânico. O presente estudo teve como objetivo avaliar a participação dos canais ASIC1a do LC nas respostas respiratórias, metabólicas e comportamentais à exposição a 20% de CO_2 em camundongos machos e fêmeas após injeção intra-LC de Psalmotoxina-1 (Pstx- 1, antagonista seletivo de canais ASIC1a). Os experimentos foram realizados em camundongos C57BL/6 machos e fêmeas, que foram submetidos à cirurgia estereotáxica para implante de cân ulas-guia direcionadas ao LC e sensor de temperatura na cavidade abdominal. Sete dias depois, a dose de 50ng/0,1uL de Pstx1 ou salina foi administrada intra-LC e os animais foram expostos ao desafio respiratório. Parâmetros ventilatórios e metabólicos foram medidos por pletismografiade corpo inteiro em sistema fechado. A análise comportamental foi gravada por uma câmera e as respostas de fuga foram analisadas seguindo os parâmetros: número desaltos, *freezing*, corrida e levantamento de patas. Os resultados encontrados em camundongos machos e fêmeas indicam que os canais ASIC1a não parecem estar envolvidos no controle da respiração, mas participam das respostas comportamentais. Em machos, o antagonista promoveu uma diminuição no número de saltos (Salina: 9,33 ± 5,85 vs Pstx-1: 3,40 ± 3,85), já em fêmeas, não foi observado resultado significativo referente ao número de saltos nos animais que receberam Pstx-1 intra-LC (Salina: 1 ± 1 vs Pstx-1: 9 ± 4,51). Nossos dados sugerem que os canais ASIC1a no LC não participam do controle respiratório sob desafio de CO_2, mas estão envolvidos no comportamento de pânico induzido por CO_2, demonstrando uma resposta sexo específica.

Palavras-chave: Locus coeruleus, diferenças sexuais, ventilação, comportamento, ASIC1a

SEXUAL DIFFERENCES REGARDING THE PARTICIPATION OFCATIONIC CHANNEL ASIC1a WITHIN THE *LOCUS COERULEUS* IN BEHAVIORAL RESPONSES INDUCED BY CO_2

ABSTRACT: Panic disorder (PD) is a condition marked by intense anxiety, in which the patient quickly develops respiratory symptoms such as dispnea, hyperventilation and a feeling of suffocation. Although the mechanisms of this disorder remain unknown, recent studies suggest a possible connection between respiratory challenges and PD,since episodes of hypoxia or hypercapnia can trigger anxiety-related disorders. The locus coeruleus (LC) is a chemosensitive region capable of generating emotional andphysical responses during stress episodes. This region has noradrenergic neurons that participate in the ventilatory response to CO_2. The acid-sensitive ion channel type1a (ASIC1a) has been suggested to participate in the panicogenic response induced by CO_2. Despite this, it is not yet fully known the involvement of this channel in the LCin the ventilatory response and behavioral changes induced by hypercapnia. We studied the effects of injection of a ASIC1a antagonist [Psalmotoxin -1 (Pstx-1- 50ng/0.1uL)] into the LC of C57BL/6 male and female mice on respiratory, metabolic and behavioral responses to exposure to 20% CO_2. We observed that Pstx-1 did not change ventilation and metabolism under normocapnic and hypercapnic conditions inboth male and female mice. As to CO_2-behavioral responses, intra-LC Pstx-1 injectiondecreased the number of jumps in males. Therefore, our data suggest that ASIC1a channels in LC do not participate in respiratory control under CO_2 challenge, but are involved in CO_2-induced panic behavior, demonstrating a specific sex-dependent response.

Keywords: Locus coeruleus, sexual differences, ventilation, behavior, ASIC1a

CAPÍTULO 1- CONSIDERAÇÕES GERAIS

Introdução

O Transtorno do Pânico (TP) caracteriza-se por episódios de ataques de pânico súbitos e recorrentes, manifestando sintomas respiratórios, cardiovasculares,gastrointestinais, autonômicos e cognitivos de início agudo (Grant et al., 2006). No TP, o indivíduo vivencia ataques de pânico inesperados e recorrentes, seguidos de momentos de apreensão e/ou medo de sofrer novos ataques. Estes ataques se caracterizam por momentos de medo intenso e desconforto, que alcançam o ápice em poucos minutos, sendo acompanhados de sintomas físicos e/ou cognitivos (American Psychiatry Association, 2013).

Uma ferramenta diagnóstica relevante para o TP é o uso de agentes químicos,denominados de panicogênicos, (Kessler et al., 2006; Esquivel et al., 2010), que estimulam a respiração, sendo esta função fortemente regulada pelo pH. A inalação de CO2 eleva a PCO2 e reduz o pH sanguíneo, resultando em alterações que podem ser percebidas pelo encéfalo. Algumas regiões encefálicas que são sensíveis ao H^+ e ao CO2 estão relacionadas tanto ao controle da ventilação, quanto aos comportamentos defensivos, incluindo os relacionados com o pânico (Esquivel et al.,2010).

Dentre essas estruturas, o Locus coeruleus (LC) desempenha uma importante participação. O desequilíbrio na modulação do sistema LC/noradrenérgico está implicado em inúmeras desordens psiquiátricas, como transtorno de déficit de atenção e hiperatividade, doenças neurodegenerativas, transtorno de estresse pós- traumático, depressão e transtornos de ansiedade, incluindo o TP (Bangasser e Valentino, 2014; Fortress et al., 2015; Isingrini et al., 2016; Weinshenker, 2018). Em estudos clínicos mostraram, que uma regulação noradrenérgica alterada está presente em pacientes com TP e durante ataques de pânico (Charney et al., 1990; Bremner et al., 1996; Balaban, 2002; Dierssen et al., 2002). A elevada atividade noradrenérgica aumenta as respostas comportamentais relacionadas à ansiedade. Além disso, a ativação inadequada do LC pode participar da responsividade exagerada ao estímulo e do aumento da emotividade observada em pacientes com estresse ou transtornos de ansiedade. Por outro lado, o tratamento antidepressivo, que é eficaz em pacientes com transtorno de pânico, diminui o disparo no LC e a expressão da tirosina hidroxilase, assim como as manipulações comportamentais que diminuem o estresse, como o manejo pós-natal, modulam as respostas do sistema noradrenérgico (Escorihuela et al., 1995; Baamonde et al., 2002).

Os mecanismos pelos quais as células do LC detectam as alterações causadas pelo CO_2 ainda não são completamente compreendidos. Dentre as possibilidades, os canais iônicos sensíveis a ácidos (ASIC1a) merecem destaque. Os ASIC1a são particularmente abundantes na amígdala e outras estruturas do circuito de medo, sugerindo uma contribuição para distúrbios comportamentais como ansiedade e depressão (Wemmie et al., 2003; Coryell et al., 2009). De fato, diversos comportamentos induzidos por inalação de CO_2, incluindo congelamento, aversão e evitação, dependem do ASIC1a (Ziemann et al., 2009). Esses canais são controlados por prótons e mediadores-chave das respostas à lesão neuronal. Eles exibem padrões únicos de distribuição no sistema nervoso central (SNC), com alta expressão em neurônios e baixa expressão em células gliais (Cegielski et al., 2022). As correntes provenientes do canal ASIC1a podem sofrer inibição por moduladores seletivos, como a toxina inibitória do nó de cisteína Psalmotoxina-1 (Pstx-1) (Escoubas et al., 2003; Dawson et al., 2012). A Pstx-1 inibe especificamente ASIC1a, não inibindo nenhum outro canal ASIC, ligando-se a esses canais de modo a alterar a sua conformação, fazendo com que sua afinidade ao H^+ se altere (Escoubas et al., 2000; Chen et al., 2006).

É bastante conhecido que o sexo do indivíduo pode afetar o controle da respiração e a suscetibilidade a algumas doenças respiratórias (Gargaglioni et al., 2019; Holley et al., 2012; Jiang et al., 2017). Alguns estudos mostraram que há um dimorfismo sexual em humanos em anormalidades do sistema respiratório, como apnéias repetidas, hipopneias e oscilações respiratórias, que têm uma incidência três vezes maior em homens (Behan e Kinkead, 2011; Gargaglioni et al., 2019). Além disso, a síndrome da morte súbita em neonatos (SIDS) também é mais prevalente em indivíduos do sexo masculino na proporção de 60:4 (Moon et al., 2007), assim como a apneia obstrutiva do sono afeta predominantemente os homens (Wenninger et al., 2009). Por outro lado, as mulheres têm probabilidade duas a três vezes maior de desenvolver transtorno do pânico do que os homens durante a vida (Kessler et al., 2005; Sheikh et al., 2002; Yonkers et al., 1998).

Apesar das evidências apresentadas, pouco se sabe sobre o envolvimento doscanais ASIC1a expressos no LC nas respostas respiratórias e comportamentais relacionadas ao TP, em animais machos e fêmeas, uma vez que diversos distúrbios podem ser prevalentes em um determinado sexo.

Objetivos

Os objetivos deste estudo foram avaliar a participação dos canais ASIC1a do LC nas respostas respiratórias, metabólicas e comportamentais à exposição a 20% CO2 em camundongos machos e fêmeas após a microinjeção intra-LC de Pstx-1.

Revisão de literatura

Transtorno do pânico

O transtorno do pânico (TP) é caracterizado como um transtorno de ansiedade,sendo um dos transtornos psiquiátricos mais comuns, com prevalência média ao longo da vida de 4,5% da população (Kim et al., 2012). O TP é conhecido pela recorrência de ataques de pânico (APs) inesperados, que consistem em episódios de medo intenso acompanhados de alterações autonômicas e respiratórias como taquicardia, sudorese e falta de ar (Grant et al., 2006; American Psychiatry Association, 2013).

De acordo com o DSM-V (American Psychiatry Association, 2013), os APs recorrentes no TP são categorizados como espontâneos (inesperados) ou sinalizados(esperados), e são definidos como um período de intensa angústia ou desconforto caracterizado por pelo menos quatro dos seguintes sintomas típicos: palpitações, tremores, sudorese, asfixia, aperto no peito, dispneia, náusea, tontura, parestesia ou dormência, calafrios ou ondas de calor, descaracterização, enlouquecimento e sensação de morte (Guan e Cao, 2023). A recorrência de APs leva à ansiedade antecipatória, ao medo condicionado e à evitação de contextos de pânico, que levamao comprometimento da vida cotidiana e à incapacidade (Klein, 1993; Smoller e Tsuang, 1998; Craske et al., 2010), sendo sua maior incidência em mulheres do que em homens (Battelan et al., 2007; Carta et al., 2015).

Diversos estudos buscando compreender o comportamento defensivo associados à estratégia de sobrevivência mediante a inúmeras ameaças, principalmente em roedores, têm sido úteis para entender as bases fisiopatológicas dos transtornos de ansiedade, em especial o TP (Guan e Cao, 2023). Desta maneira,é feita a avaliação referente às ameaças ambientais e a partir disso, é gerado respostas apropriadas para mitigar ou evitar danos. Durante este processo, diversas emoções podem ser desencadeadas, entre elas a ansiedade, medo e pânico (Livermore et al., 2021).

Estudos buscaram investigar o circuito neuronal que sustenta o TP para fornecer informações sobre as origens dos estados de ansiedade patológica. Dentre as hipóteses estudadas, tem sido proposto que os APs surgem quando o sensor de sufocamento do encéfalo detecta erroneamente a falta de ar, fazendo com que um sistema evoluído de alarme de sufocamento seja ativado. Um indivíduo com funcionamento incorreto está sujeito a “falsos alarmes de sufocamento” (Klein, 1993;Nardi, 2022). Os desafios respiratórios têm sido úteis no desenvolvimento de teorias sobre o TP, indicando que a sensibilidade ao dióxido de carbono (CO2) seria um componente dos APs (Nardi, 2022).

Como metabólito do corpo humano, o CO2 desempenha um papel insubstituível nos processos fisiológicos. A inalação de concentrações elevadas de CO2, a hiperventilação voluntária e outros métodos para desencadear distúrbios ácido-base, como a infusão de lactato de sódio, podem induzir sintomas panicogênicos semelhantes em alguns pacientes com TP (Nardi et al., 2006; 2007; 2008). Diversos estudos demonstram que desafios respiratórios, como a exposição auma alta concentração de CO2 ou a uma baixa concentração de O2, provocam ataques de pânico (Gorman et al., 1993; Beck et al., 1999; Perna et al., 2004; Fernandes et al., 2019), pois estimulam a respiração, sendo esta função fortemente regulada pelo pH (Esquivel et al., 2010). A inalação de CO2 eleva a pressão parcial de CO2 (PaCO2) e reduz o pH sanguíneo, resultando em alterações no encéfalo. Algumas regiões encefálicas que são sensíveis ao H^+ e ao CO2 estão sendo relacionadas tanto ao controle da ventilação, quanto ao desencadeamento de comportamentos defensivos e respostas autonômicas, relacionadas ao pânico (Esquivel et al., 2010).

Ainda, pode-se evidenciar que a probabilidade de experimentar os efeitos panicogênicos do CO_2 são intensificados empacientes com TP do que em indivíduosque não são acometidos por este transtorno (Coryell et al., 2006; Esquivel et al., 2010;Vollmer et al., 2015). Em camundongos, a inalação de CO_2 provoca comportamento de fuga, evitação e congelamento (Ziemann et al., 2009; D'Amato et al., 2011; Leiboldet al., 2016; Vollmer et al., 2015; Spiacci et al., 2018; Taugheret al., 2020) e mudanças endócrinas, que sugerem que os animais estão passando por uma situação altamente aversiva, que se assemelha ao desencadeamento de um AP (Spiacci et al., 2018).

Quimiorrecepção

Uma das funções mais importantes e essenciais para os seres vivos, é a respiração. No contexto fisiológico, a respiração diz respeito à difusão e transporte demetabólitos entre o organismo e o ambiente externo. As células necessitam constantemente de oxigênio (O_2) para executar a respiração celular, de modo a gerarenergia para o corpo realizar suas funções necessárias. A respiração em mamíferos é contínua, cuja função é adequar a ventilação alveolar dos pulmões para manter dentro de uma faixa constante a pressão parcial de oxigênio (Pa O_2) e PaCO_2, bem como os valores de pH. Apesar de aparentar simplicidade, a respiração é composta por um conjunto de elementos que a tornam de grande complexidade (Del Negro et al., 2018). Dessa maneira, as trocas gasosas são equilibradas emfunção da demandado metabolismo (Gilmour, 2001).

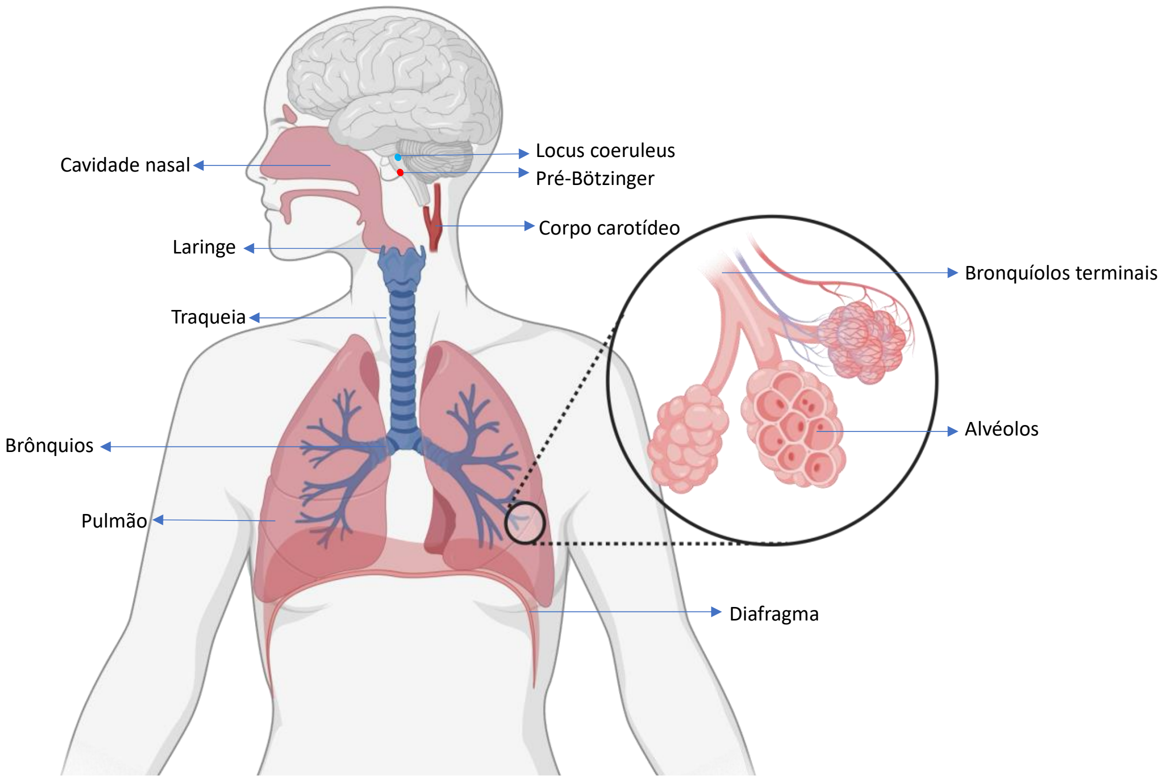

Figura 1: Principais estruturas envolvidas na respiração (Criado com BioRender®).

Uma rede neural no tronco encefálico gera o ritmo respiratório e regula o padrão motor de cada respiração para atender às demandas metabólicas, biomecânicas e comportamentais dos indivíduos. No sentido rostro-caudal, os principais núcleos são o Complexo Bötzinger (BötC), pré-BötC, grupo respiratório ventral rostral (rVRG) e caudal (cVRG), os quais formam a coluna respiratória ventral.Outros núcleos como o complexo pós-inspiratório (PiCo) e o parafacial (pF) e o encontram adjacentes à coluna respiratória ventral e participam do processo respiratório, sendo importantes para geração da fase de pós-inspiração e expiração ativa (Del Negro et al., 2018). O ritmo respiratório é gerado no pré-BötC (Smith et al.,1991). A respiração é dependente de aferências que chegam à essa região referenteàs informações sobre as pressões parciais dos gases sanguíneos, o pH dos líquidoscorporais, os gases inspirados, o estado de estiramento pulmonar e informações detectadas por químiorreceptores e mecanorreceptores. Buscando o pleno funcionamento do organismo, o sistema respiratório necessita estar apto para a geração de um ritmo que permita as trocas gasosas e integre os outros comportamentos relacionados à respiração. O sistema respiratório desempenha grande participação em atividades que requerem um fluxo de ar, como vocalização, deglutição, reflexos das vias aéreas como tossir, espirrar e até mesmo a locomoção (Greer et al., 2006; Klein e Codd, 2010).

Além de garantir a ritmogênese, processo pelo qual há a garantia do ritmo respiratório, outra grande função do sistema de controle respiratório é a quimiorrecepção. Os quimiorreceptores sensíveis a alterações de CO2/pH são fundamentais para o controle da respiração, pelo fato de auxiliarem na geração do ritmo e na modulação do padrão respiratório, visando prevenir grandes variações dasconcentrações de CO2 e pH, uma vez que estas alterações podem ocasionar em danos irreversíveis ao indivíduo. Os quimiorreceptores sensíveis ao CO2/pH são classificados como periféricos (localizados nos corpos carotídeos e no arco aórtico) ecentrais (localizados no SNC), sendo os centrais com predominância sobre a respostaventilatória ao CO2 (Blain et al., 2010; Foster e Smith, 2010; Patrone, 2018). Segundo Guyenet et al. (2010), os quimiorreceptores centrais são células sensíveis a variaçõesna pCO2 e no pH encefálico e contribuem para a estimulação da ventilação causada por hipercapnia ou acidose metabólica. Estes receptores são banhados pelo líquido cefalorraquidiano e isolados do sistema circulatório pela barreira hematoencefálica, que é pouco permeável ao íon H^+, protegendo o encéfalo de variações no pH sistêmico, porém, altamente permeável ao CO2 (Hlastala e Berger, 2001).

Os neurônios intrinsecamente sensíveis ao CO2/pH estão localizados em muitas estruturas no encéfalo (Biancardi etal., 2008; Mulkey et al., 2004; Putnamet al., 2004; Ziemann et al., 2009). Algumas dessas estruturas provavelmente desempenham funções diferentes, como respiração e comportamento defensivo (Esquivel et al., 2009). A contribuição destas estruturas encefálicas na sensibilidade ao CO2, detectando mudanças no pH e provocando uma resposta comportamental relacionada ao medo e ao pânico, ainda é indefinida.

O locus coeruleus (LC) é o maior conjunto de neurônios noradrenérgicos do

SNC, formando um par de núcleos adjacentes ao quarto ventrículo, localizados no tegmento pontino dorsolateral superior. Estima-se que metade de todas as projeçõesnoradrenérgicas no SNC originam-se no LC (Aston-Jones et al., 1985; Berridge e Waterhouse, 2003). É uma estrutura importante para a quimiorrecepção central, possuindo mais de 80% dos neurônios altamente sensíveis às variações de CO_2/pH (Pineda e Aghajanian, 1997; Oyamada et al., 1998; Hilaire et al., 2004; Biancardi et al., 2008; Gargaglioni et al., 2010). A acidificação local do LC por meio de ácidos ou CO_2 resulta em aumentos da atividade do nervo frênico, demonstrando sua participação no controle ventilatório (Elam et al., 1981; Coates et al., 1993). De acordocom alguns estudos, a atividade dos neurônios do LC está relacionada à atividade respiratória (Oyamada et al., 1998; Andrzejewski et al., 2001), e alterações do sistemanoradrenérgico na fase perinatal afetam a atividade dos neurônios respiratórios do tronco encefálico responsáveis pela geração do ritmo respiratório. O LC está relacionado a um grande papel nos processos fisiológicos e comportamentais, entre eles o ciclo sono-vigília, alimentação, nocicepção, termorregulação e aprendizagem (Viemari et al., 2004; Hilaire, 2004).

Ele também está implicado em diversas condições fisiopatológicas, como a síndrome de Rett (RTT) e os transtornos de ansiedade/pânico, apresentando sua atividade aumentada na presença de tais distúrbios. Adicionalmente, a quimiorrecepção central do LC ao CO_2 é um fator que torna essa região de alta relevância no estudo sobre o TP, uma vez que a inalação de ar enriquecido com CO_2 é um dos modelos estabelecidos para indução de ataques de pânico (Klein, 1993; Hasler et al., 2005; Goodwin et al., 2010).

Canais ASICs

A manutenção da homeostase do pH é essencial para as células de indivíduoscomo os mamíferos, pois os íons de hidrogênio desempenham papéis importantes naregulação do metabolismo (Serova et al., 2020). O pH extracelular do encéfalo é um parâmetro essencial que regula muitas variáveis e entre elas comportamento e a respiração. Em um encéfalo *in vivo* o pH intersticial geralmente varia de 7,1 a 7,25, mas esse equilíbrio pode ser comprometido por inúmeras condições, incluindo acidose metabólica, inflamação e hipoventilação, nas quais o pH pode cair para até 6,5 (Kintner et al., 2000).

Os canais iônicos sensíveis a ácidos (ASICs) desempenham um papel crítico na percepção de alterações de pH em condições relacionadas à acidose tecidual. Estes canais catiônicos controlados por prótons, insensíveis à voltagem, são ativadospela diminuição do pH extracelular e estão expressos em todo o SNC e periférico (Figura 2).

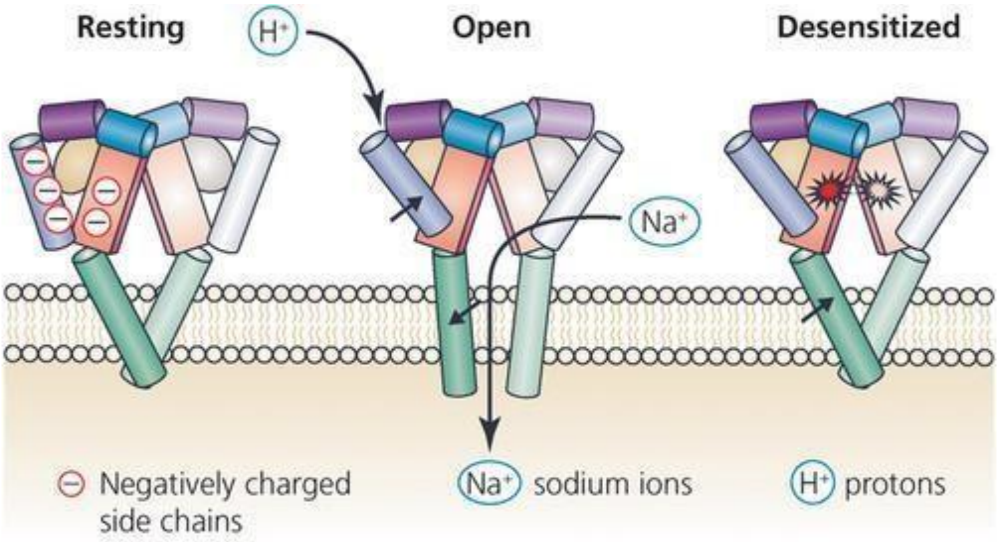

Figura 2: Mudanças conformacionais induzidas por prótons nos canais ASICs (Fonte: Rook et al., 2020).

Os ASICs pertencem à superfamília de canais iônicos ENaC (Canal epitelial para Na^{+} Epitelial)/DEG (Degenerina) e existem 6 subunidades identificadas até o presente momento 1a, 1b, 2a, 2b, 3 e 4, codificadas por 4 genes (ACCN1-4) (Waldmann et al., 1997; Gründer et al., 2000; Ortega-Ramírez et al., 2017). Essas subunidades possuem diferentes sensibilidades de pH, que possibilitam detectar uma ampla gama de pH fisiológico. Os membros da superfamília ASIC compartilham a mesma topologia, consistindo em dois domínios transmembranas hidrofóbicos, uma grande alça extracelular rica em cisteína e terminais N e C intracelulares curtos (Kweon & Suh, 2013). O ASIC1a é um complexo proteico e pode ser ativado em um ambiente extracelular ácido (de la Rosa et al., 2003), o que por sua vez leva a um grande influxo de sódio (Na^{+}) e cálcio (Ca^{2+}), promovendo a liberação de neurotransmissores relacionados (Xiong et al., 2004; Yermolaieva et al., 2004; Zha et al., 2006). Pesquisas recentes mostraram que o ASIC1a desempenha um papel importante em certas condições patológicas, como inflamação, isquemia, tumor e outras doenças com dano tecidual provindo de acidificação (Bychkov et al., 2020; Gaoet al., 2019; Wang et al., 2019; Xiong et al., 2004).

Diferentes subtipos de ASIC têm diferentes sensibilidades à acidose extracelular, na qual ASIC1a e ASIC3 são os subtipos mais sensíveis (Sutherland et al., 2001), e a dessensibilização de ASIC1a ocorre mais rapidamente (Waldmann et al., 1997). Estes canais geralmente são impermeáveis ao Ca^{2+}, no entanto, o ASIC1ahomomérico é o único membro da família relatado como permeável ao Ca^{2+} (Yermolaieva et al., 2004). O aumento na concentração intracelular de Ca^{2+} é crucial para muitos processos fisiológicos e patológicos dos neurônios, como sinalização sináptica e metabolismo energético (Brini et al., 2014). A condutância de Ca^{2+} do ASIC1a homomérico revela as funções específicas que este complexo desempenha em processos fisiológicos e patológicos relacionados ao Ca^{2+}. Os canais ASIC1a são amplamente encontrados no hipocampo, amígdala, córtex cingulado, córtex somatossensorial, estriado e LC (Wemmie et al., 2003; Coryell et al., 2009; Prince et al., 2014). Nos neurônios, o ASIC1a está localizado na região do soma e nas espinhas dendríticas (Zha et al., 2006), sendo expresso principalmente na membrana celular eno citoplasma. No entanto, nos astrócitos, o ASIC1a é distribuído principalmente no núcleo (Huang et al., 2010) e na superfície das células gliais do hipocampo NG2 (Linet al., 2010).

Os canais ASIC1a estão intimamente relacionados à plasticidade sináptica emdiferentes regiões do encéfalo, ou seja, refere-se à eficácia da transmissão sinápticaem resposta à atividade, que inclui duas formas principais: potenciação de longo prazo (LTP) e depressão de longo prazo (LTD) (Mango e Nisticò, 2020). No estriado, o ASIC1a está envolvido na regulação da plasticidade sináptica dos neurônios espinhosos médios, o que é crítico para a memória dependente dessa estrutura e o aprendizado processual (Yu et al., 2018). No circuito hipocampo-pré-frontal, o ASIC1acontribui para a plasticidade cortical relacionada à dor (Wang et al., 2018). No córtex cingulado anterior, o ASIC1a participa na modulação da hipersensibilidade à dor, mediando criticamente a potenciação sináptica (Li et al., 2019).

Uma ferramenta relevante para o estudos dos ASIC1a é a Psalmotoxina-1 (Pstx-1), um polipeptídeo extraído do veneno da toxina da tarântula *Psalmopoeus cambridgei*, que inibe especificamente os ASIC1a homoméricos (Escoubas et al., 2000), não inibindo nenhum outro ASIC, mesmo aqueles contendo a subunidade ASIC1a na forma heterodímera. A Pstx-1 liga-se a esses canais de modo a alterar a sua conformação, fazendo com que sua afinidade ao H^+ se altere (Escoubas et al., 2000; Chen et al., 2006).

Diferenças sexuais

Na história da ciência, a maior parte de todo o conhecimento disponível em fisiologia é centrado nas pesquisas em machos, consequentemente, muitos dados disponíveis são voltados aos machos e há uma tendência em se ignorar as fêmeas (Gargaglioni et al., 2019). É possível observar em diversas estruturas encefálicas diferenças morfológicas entre os sexos, como o LC (Luque et al., 1992; Pendergast et al., 2008; Thanky et al., 2002), área tegmental ventral (Brown et al., 2015), área pré-óptica (Gorski et al., 1980; Raisman e Field, 1971), substância negra (Ma et al., 2007), neocórtex, hipocampo e corpo caloso (Juraska, 1991), sendo necessário investigar as respostas provindas dessa diferenciação.

O sexo pode afetar o controle respiratório e a suscetibilidade a algumas doenças respiratórias (Block et al., 1979; Holley et al., 2012; Jiang et al., 2017; Lin e Winer, 2008; Wenninger et al., 2017). Estudos demonstram que as mulheres apresentam melhor recuperação respiratória do que os homens após um episódio grave de hipóxia (Garcia III et al., 2013). Ainda, sabe-se que existe um dimorfismo sexual em humanos relacionados a anormalidades do sistema respiratório, como apneias, hipopneias e oscilações respiratórias repetidas, no qual a incidência é 3 vezes maior em homens do que em mulheres (Behan et al., 2011).

Diferenças entre machos e fêmeas, podem ser encontradas também nas propriedades morfofisiológicas do LC. A partir de estudos realizados, foi possível evidenciar que em ratas adultas tem o núcleo LC maior que os machos (Busch et al., 1997; Garcia-Falgueras et al., 2005; Guillamón et al., 1988; Hormigo et al., 2015; Luque et al., 1992; Ohm et al., 1997; Pinos et al., 2001). Em mulheres, a região rostraldeste núcleo apresenta maior número de neurônios, enquanto nos homens essa prevalência ocorre na região mais caudal do núcleo (Hormigo et al., 2015). Essa diferença estrutural sexo-dependente compreende um número maior de células noradrenérgicas e dendritos do LC mais longos e mais complexos em ratas adultas doque nos machos, mais especificamente na região peri-LC (Bangasser et al., 2016, Bangasser et al., 2011; Luque et al., 1992; Pinos et al., 2001). Com maior densidade dendrítica, foram encontrados mais contatos sinápticos no peri-LC ventromedial de fêmeas (Guillamón et al., 1988; Luque et al., 1992; Bangasser et al., 2011), e a capacidade de sintetizar e liberar noradrenalina pelos neurônios LC é maior nas fêmeas do que nos machos (Bangasser et al., 2019).

Portanto, em vista das evidências apresentadas, nossa hipótese é que os canais ASIC1a localizados no LC podem participam das respostas respiratórias, metabólicas e comportamentais à altas concentrações de CO_2 e essas resposta diferem de acordo com o sexo.

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CAPÍTULO 2- SEXUAL DIFFERENCES REGARDING THE PARTICIPATION OFCATIONIC CHANNEL ASIC1a WITHIN THE *LOCUS COERULEUS* IN BEHAVIORAL RESPONSES INDUCED BY CO_2

Abstract: Panic disorder (PD) is a condition marked by intense anxiety, in which the patient quickly develops respiratory symptoms such as dispnea, hyperventilation and a feeling of suffocation. Although the mechanisms of this disorder remain unknown, recent studies suggest a possible connection between respiratory challenges and PD,since episodes of hypoxia or hypercapnia can trigger anxiety-related disorders. The locus coeruleus (LC) is a chemosensitive region capable of generating emotional andphysical responses during stress episodes. This region has noradrenergic neurons that participate in the ventilatory response to CO_2. The acid-sensitive ion channel type1a (ASIC1a) has been suggested to participate in the panicogenic response induced by CO_2. Despite this, it is not yet fully known the involvement of this channel in the LC in the ventilatory response and behavioral changes induced by hypercapnia. We studied the effects of injection of a ASIC1a antagonist [Psalmotoxin -1 (Pstx-1 - 50ng/0.1uL)] into the LC of C57BL/6 male and female mice on respiratory, metabolic and behavioral responses to exposure to 20% CO_2. We observed that Pstx-1 did not change ventilation and metabolism under normocapnic and hypercapnic conditions inboth male and female mice. As to CO_2-behavioral responses, intra-LC Pstx-1 injection decreased the number of jumps males, but there was no statistically significant difference in the behavioral parameters of the females. Therefore, our data suggest that ASIC1a channels in LC do not participate in respiratory control under room air and hypercapnia, but are involved in CO_2-induced panic behavior, demonstrating a specific sex-dependent response.

Keywords: Locus coeruleus, sexual differences, ventilation, behavior, ASIC1a

Introduction

Panic disorder (PD) is characterized by panic attacks, with behavioral changes and respiratory symptoms such as shortness of breath, hyperventilation and feeling ofsuffocation (Grant et al., 2006). According to the DSM-V (American Psychiatry Association, 2013), the recurrent PD are categorized as spontaneous (unexpected) orsignaled (expected) and are defined as a period of intense distress or discomfort characterized by typical symptoms such as palpitations, tremors, sweating, asphyxia, chest tightness, dyspnea, nausea and feeling of death.

Some studies suggest a link between changes in chemosensitivity to CO_2/pH and PD, since episodes of hypoxia or hypercapnia can trigger panic attacks (Kessler et al., 2005; Esquivel et al., 2010). In fact, brain regions that are sensitive to H^+ and CO_2 are related to both ventilation control and defensive behaviors, including those related to panic (Esquivel et al., 2010).

Among these structures, the Locus coeruleus (LC) plays an important role in breathing control and anxiety related behaviors (Gargaglioni et al., 2010; Borodovitsyna et al., 2018). Imbalance in the modulation of the LC/noradrenergic system is implicated in numerous psychiatric disorders, such as attention deficit hyperactivity disorder, neurodegenerative diseases, post-traumatic stress disorder, depression and anxiety disorders, including PD (Bangasser and Valentino, 2014; Fortress et al., 2015; Isingrini et al., 2016; Weinshenker, 2018). Clinical studies have shown that altered noradrenergic regulation is present in PD patients and during panicattacks (Charney et al., 1990; Bremner et al., 1996; Balaban, 2002; Dierssen et al., 2002).

The mechanisms by which LC cells detect changes caused by CO_2 are not yet fully understood (Putnam et al., 2004). Among the possibilities, acid-sensitive ion channels (ASIC1a) stand out. ASIC1a are particularly abundant in the amygdala and other structures of the fear circuit, suggesting a contribution to behavioral disorders such as anxiety and depression (Taugher et al., 2017; Zhu et al., 2022). ASIC1a channels respond to decreasing pH, which has been identified as relevant to CO_2- induced panicogenic responses (Wemmie et al., 2003; Coryell et al., 2009). Currents originating from the ASIC1a channel can be inhibited by selective modulators, such as Psalmotoxin-1 (Pstx-1) (Escoubas et al., 2003; Dawson et al., 2012). Pstx-1 specifically inhibits ASIC1a, without inhibiting any other ASIC channel, binding to thesechannels to change their conformation, causing their affinity to H^+ to change (Escoubas et al., 2000; Chen et al., 2006).

It is well known that the sex of the individual can affect breath control and susceptibility to some respiratory diseases (Gargaglioni et al., 2019; Holley et al., 2012;Jiang et al., 2017). Women are two to three times more likely to develop PD than menduring the life (Kessler et al., 2005; Sheikh et al., 2002; Yonkers et al., 1998). However,the role of ASIC1a channels located in LC in CO_2-induced hyperventilation and panic remains unknown, as well as their differences regarding sex. Therefore, we sought to test the hypothesis that LC is an important site of ASIC1A action in CO_2-induced panicbehaviors and breathing control in male and female mice.

Methods

Animals

The experiments were performed with male and female mice C57BL/6, weighting around 20 to 25 g (8 weeks old). All the animals were housed in a temperature-controlled room, maintained at 25 ± 1°C with a 12 h light-dark cycle (lights on at 6:30 a.m.), with water and food provided *ad libitum*.

Experiments were performed between 8:00 a.m. and 1:00 p.m at the Departmentof Animal Morphology and Physiology of the Faculty of Agricultural and Veterinary Sciences – FCAV/UNESP, campus of Jaboticabal. All experimental protocols were approved by the local ethics committee (CEUA - protocol n°3451/22), being in norm with the National Council for the Control of Animal Experimentation (CONCEA-MTC, Brazil).

Surgical procedures

The animals were anesthetized with isoflurane (5% for induction and 1.5% for maintenance). After anesthetic induction, the animals were fixed in a stereotactic apparatus (Kopf Instruments, Kent, England) and a stainless-steel guide cannula wasimplanted (10 mm in length and 0.7 mm in external diameter) aimed at the LC.

The coordinates (Paxinos and Franklin, 2003) used were antero ponterior (AP): -5.2 mm from the bregma; lateral (LL): ±0.8 mm, and dorso ventral (DV): -3.9 mm from the skullfor males and AP: -5.2 mm; LL: ±0.9 mm and DV: -4.0 mm for females. The cannula was fixed to the skull by using dental acrylic and sealed with a stainless-steel wire to prevent occlusion and infection.

At the end of surgery, the animals were placed in boxes with water and food "*adlibitum*" and kept in a room with controlled temperature, humidity, and luminosity. Antibiotic injection (Enrofloxacin, Baytril; 2.5 mg/kg s.c.) and analgesic (Flunixin Meglumine, Banamine; 10 mg/kg s.c.) were administered in a single dose after surgery.

Determination of body temperature

Tc was obtained by implanting a temperature sensor (Biomark, USA) in the abdominal cavity on the same day that the stereotactic surgery was performed.

Drug

Psalmotoxin-1 (Pstx-1) (Sigma-Aldrich) solubilized in sterile saline 0.9% and was applied in the LC in a concentration of 50ng/0.1uL. The dose chosen was based on studies carried out in the laboratory.

Induction of panic attacks

The mice were placed in a round acrylic chamber of 5 liters and exposed to a respiratory challenge composed of a gas mixture of 20% CO_2, 21% of O_2 and N_2 to balance during the time of 15 minutes. Exposure to respiratory challenge was used to induce behavioral and ventilatory panic responses in animals.

Pulmonary ventilation assessment

The pulmonary ventilation (V_E) of mice were acquired by whole body plethysmography – closed system. For V_E measurements, the chamber was fully sealed for approximately 2 min. The animal was placed in a 5L experimental chamberfor a habituation period of 1 hour. Air inlet and outlet flows were 1,800 L/min measuredby a flowmeter. The pressure oscillation due to temperature difference from inhaled and exhaled air were monitored by a differential pressure transducer (TSD 160A, Biopac Systems, Santa Barbara, CA, USA) and fed into a pre-amplifier (DA 100C, Biopac Systems), passed through an analog-to-digital converter and digitized on computer equipped with data acquisition software (MP100ACE, Biopac Systems). A volume calibration was performed by injecting 1 mL of air into the chamber using a graduated syringe. Tidal volume (V_T) was calculated using Drorbaugh and Fenn's formula (1955):

$$V_T = V_K \times (P_T/P_K) \times T_B \times (P_B - P_C) / T_B \times (P_B - P_C) - T_A \times (P_B - P_R)$$

where P_T: pressure deflection associated with each V_T, P_K: pressure deflection associated with the injection of the calibration volume (V_K), T_A: air temperature in the animal chamber, P_B: barometric pressure, P_C: water vapor pressure in the animal chamber, T_B: body temperature (in Kelvin), and P_R: water vapor pressure at Tc.

The V_E was calculated as the product of respiratory frequency (f_R) and V_T and normalized to the animal's body weight. V_E and V_T were presented under ambient barometric pressure conditions at T_B and saturated with water vapor (BTPS). P_C and P_R were calculated indirectly using an appropriate table (Dejours, 1981). The LabChart software (PowerLab System, ADInstruments®/LabChart Software, version 7.3, Sydney, Australia) was used for data analysis.

Determination of oxygen consumption

The indirect calorimetry method by measuring O_2 consumption (VO_2) with flow through push mode configuration was used for metabolic rate inference. The chamber air flow (1.5 L/min) was maintained through a compressor pump that directed the air to the chamber entrance when the animals were exposed to room air and by gas mixture cylinders coupled to a flowmeter when exposed to hypercapnia. The expired gas was dried over a small column of Drierite (W.A. Hammond Drierite Co. Ltd, Xenia, OH, USA)before goes through the gas analyzer. The air was continuously sampled allowing for the determination of VO_2 by a data acquisition program (Power-Lab System, ADInstruments® / Chart Software, version

7.3, Sydney, Australia). The CO_2 was notanalysed or scrubbed, then aiming at a better metabolic rate measurement the VO_2 was calculated using the following equation (Koteja, 1996):

$$VO_2 = [FRe\ (FiO_2 - FeO_2)] / [1 - FiO_2\,(1 - RQ)]$$

where FRe: end flow rate of air through the chamber, FiO_2: inlet O_2 fraction, FeO_2: end O_2 fraction, and RQ: respiratory quotient (considered to be 0.85). The $\dot{V}O_2$ as divided for the body mass and values were presented as STPD (standard conditions of temperature, pressure and dry air).

Behavioral measurements

To analyze the response of the panic attack behavioral, the experiments were recorded by a camera (Logitech, USA), placed to clearly see the animal and its movements. The escape response was analyzed, being considered the number of jumps. The freezing behavioral (total lack of movement except breathing) and the rearing (standing on the hind legs with or without contact on the anterior legs on the side of the chamber) was evaluated by a trained observer.

Central microinjection

For intra-LC microinjection, a needle (10 mm long, 0.3 mm outer diameter) wasintroduced through the guide cannula, using a 5 µL microsyringe (Hamilton, USA). The volume of injections was 0.1 µL for 1 minute using a microinjection machine (model 310, Stoelting Co., Il, EUA). The displacement of an air bubble inside the polyethylene catheter connecting to the syringe was used to monitor the microinjection.

Experimental protocol

All the animals were exposed to the experimental chamber under the conditionof normocapnia (ambient air 21% O_2 and 0,03% CO_2) for a habituation period of approximately 1 hour before the experiment began (Figure 1).

Seven days after stereotactic surgery, the animals underwent the experimental procedure. At first, two control ventilatory and metabolic measurements were performed in ambient air after 10 and 20 minutes of habituation. Escape responses were recorded during all experiment. Subsequently, 0.1 μL of vehicle of saline or Pstx-1 (50ng) was microinjected into the mice LC. After 15 min of the microinjection, respiratory and metabolic measurements were performed under normocapnic conditions. Then, the respiratory challenge was presented (20% CO_2) for 15 minutes and after this period respiratory and metabolic measurements were performed again. Behavioral panic responses were recorded throughout the experiment. However, behavioral data were be presented only during the period of hypercapnia since there was no behavioral change during the period of normocapnia.

Confirmation of the microinjection site

For the determination of the microinjection site the animals were deeply anesthetized and 0.1 μL of Evans blue was injected through the guide cannula. The animals were perfused transcardially with phosphate buffered saline (PBS) 0.1 M, followed by paraformaldehyde 4%. The brain was extracted and maintained in paraformaldehyde 4% for 24 hours. Coronal sections of the brain at 35 μm were made using a cryostat and deposited in slides, for later visualization under a microscope.

Data analyses

The data were evaluated in terms of meeting the assumptions of homogeneity of variances and normality of the errors. Then, they were submitted to analysis of variance by the GLM procedure (General Linear Models) and, in case of significant difference, the means were compared by the Tukey test with probability level of 5% and confidence interval of 95%. For all ventilatory and metabolic analyses, the repeated measures two-way ANOVA test was performed with the following factors: treatment (Pstx-1 or saline) and gas condition (normocapnia and hypercapnia). For behavioral analysis, the T Student test was used to analyze the number of jumps, rearing, running and freezing.

Results

Figure 2 represents the representative section of the brainstem of an animal, demonstrating the confirmation of the microinjection site in the LC. It is possible to observe the presence of Evans blue dye restricted to LC. Only animals with blue colorin this region were incorporated to the data analysis.

Effect of intra-LC microinjection of Pstx-1 on ventilation and metabolism under normocapnia and hypercapnia in males and females

In Figure 3, the values of V_E, V_T and f_R of males (Figure 3A, 3B and 3C) and females (Figure 3D, 3E and 3F) treated with Pstx-1 or saline in normoxia before and pos-injection and hypercapnia condition are represented. The two-way ANOVA revealed significant effects of gas condition in V_E [males: $F(2,35)= 74.227$, $p<0.001$; females: $F(2,35)= 43.107$, $p<0.001$], V_T [males: $F(2,35)= 140.923$, $p<0.001$; females: $F(2,35)= 39.440$, $p<0.001$] and f_R [males: $F(2,35)= 4.943$, $p=0.032$; females: $F(2,35)= 16.934$, $p<0.001$]. As can be seen in the Figure 3C, in males the treatment with Pstx-1 tend to decreasedthe f_R in the animals exposed to CO_2 [$F(1,35) = 6.478$; $p=0.052$]. In females, treatment with Pstx-1 did not change any of the analyzed parameters. For the other parameters, there is no significant statistics difference between saline and Pstx-1 groups.

In Figure 4, the values of VO_2, V_E/VO_2 and Tb of males (Figure 4A, 4B and 4C) and females (Figure 4D, 4E e 4F) treated with Pstx-1 or saline in normoxia before and pos-injection and hypercapnia condition are represented. For males, The two-way ANOVA revealed significant effects of treatment [$F(1,35)= 15.551$, $p=0.011$] and gas condition [$F(2,35)= 104.126$, $p<0.001$] in Tb, but not in VO_2 and V_E/VO_2. For females, there is significant effect of gas condition in V_E/VO_2 [$F(2,35)= 5.800$, $p=0.021$] and Tb [$F(2,35)= 167.175$, $p<0.001$], but not in VO_2. There is no significant statistics difference between salina and Pstx-1 females.

Effect of intra-LC microinjection of Pstx-1 on panic-related responses under normocapnia and hypercapnia in males and females

Figure 5 shows the effect of intra-LC administration of Pstx-1 on jumping, freezing, running, and rearing behaviors expressed by male (Figure 5A, 5B, 5C and 5D) and female (Figure 5E, 5F, 5G and 5H) mice exposed to 20% CO_2.For males, the

Student T test showed that there was a significant difference between Pstx- 1 and saline group in the number of jumps [t(10) 3.412, p=0.006] and rearing [t(12) 2.491, p=0.028], but not in freezing [t(10) 0.264, p=0.797]. As can be seen in the Figure 5C, thetreatment with Pstx-1 tend to decreased the running [t(10) 1.990, p=0.074] in the animals exposed to CO_2.

For females, there was no significant difference between Pstx-1 and saline group in the number of jumps [t(10) 1.542, p=0.154], freezing [t(10) 0.629, p=0.543], running [t(10) 0.859, p=0.410] and rearing [t(10) 0.868, p=0.405].

Discussion

In the present study, we examined a potential role for ASIC1a channels in the LC in the hypercapnic ventilatory, metabolic and behavioral response of male and female C57BL/6 mice. Our results indicate that this channel in the LC do indeed play arole in the hypercapnic behavioral response in males since their blockade with Pstx- 1resulted in a significant reduction in the number of jumps and rearing. However, the behavioral data found in females demonstrated no different response to respiratory challenge. Regarding ventilation data in males and females, ASC1a blockade in the LC did not change ventilation, metabolic rate and respiratory equivalent (V_E/VO_2) during room air and hypercapnic conditions, suggesting that this channel in the LC does not participate in breathing and metabolic control. In relation to Tb, there was a significantattenuation in the drop of Tb under CO_2 exposure only in male animals treated with Pstx-1, indicating that ASIC1a located in LC region plays a role in the hypothermic response induced by hypercapnia.

Under normocapnic conditions, Pstx-1 microinjection into the LC did not alter the ventilatory or metabolic parameters in females suggesting thar ASIC1a in this nucleus does not play a tonic respiratory role. In males, a decrease in f_R was observed, however, this reduction was notenough to change ventilation. Ourfindings support data from previous studies from our laboratory showing that removal of LC noradrenergic neurons does not affect cardiorespiratory control during resting conditions (Biancardi et al., 2008, Carvalho et al., 2010).

Regarding the ventilatory response to 20% CO_2, the ventilatory parameters increased in relation to exposure to normocapnia in all groups independent of sex. However, ASIC1a blockade did not change the hypercapnic ventilatory response in males and females, indicating that this channel located in LC does not participate in the hyperventilatory response induced by CO_2. In relation to the ventilatory response to 20% CO_2, the ventilatory parameters exhibited an increase across all groups, irrespective of sex. However, it is noteworthy that blocking the ASIC1a channel did notalter the hypercapnic ventilatory response in both males and females. Indicating that this acid channel, situated in the LC, does not play a role in the ventilatory response induced by high level of CO_2. A previous study has demonstrated that Pstx-1 injection(10 nM/0.1 μl) in the lateral hypothalamus abolished the increase of phrenic nerve activity induced by acidification of this region, which suggests that ASIC1a is responsible for acid sensing in the hypothalamus of Sprague-Dawley male rats (Song et al., 2012). The same pattern of response was observed in the ventrolateral medullain rats using a higher dose (100 nM/0.1 μl; Song et al., 2016). Therefore, ASIC1a channels seems to be important to the increase in respiration in some CO_2/pH chemosensitive areas such as hypothalamus and medulla, but no in LC. As previouslymentioned, LC acts as a central chemoreceptor in mammals, (Elam et al., 1981; Filosaet al., 2002; Gargaglioni et al., 2010; Imber et al., 2014; Pineda e Aghajanian, 1997) and it is known to send projections to several areas of the central nervous system, including those responsible for respiratory control and for activating behavioral responses related to panic, such as the amygdala (Mccall, et.al., 2017; Giustino et.al.,2020).

In the present study, the observed behavioral pattern exhibited by the animals during exposure to 20% CO_2 aligns with existing literature, illustrating an augmentationin the frequency of jumps and other responses typical of fight-or-flight behaviors in thepresence of elevated CO_2 concentrations (Spiacci et al., 2018, Taugher et al., 2019). Indeed, studies conducted in mice show that inhalation of 20% CO_2 can trigger behavioral responses such as freezing and avoidance (D'Amato et al., 2011; Leibold et al., 2016; Taugher et al., 2014; Vollmer et al., 2016; Ziemann et al., 2009) suggesting that the animals are facing an intense aversive situation, which resembles the triggeringof a panic attack (Spiacci et al., 2018). Although the amygdala may be more importantin the conditioning process that leads to avoidance behavior, a previous study has demonstrated that three patients with focal bilateral amygdala lesions (due to Urbach-Wiethe disease) experienced panic attacks following a CO_2 challenge suggesting that other regions besides amygdala is involved in triggering panic attacks (Goossens et al., 2014). One of the regions suggested by the authors was LC. In fact, we have demonstrated that noradrenaline originating from the LC has an important role in the characterization of jumping and freezing behaviors in both male and female mice, since mutant males lacking or with reduced LC noradrenaline (NA) synthesis had a blunted jumping response and an increased freezing duration compared to wild type animals (Ripamonte et al., in revision).

In the study conducted by Ziemann et. (2009) it was demonstrated that inhaled CO_2 evoked fear behavior in male mice and elimination or inhibition of ASIC1a impairedthe activity of the animals. Interestingly, the localized expression of ASIC1a in the amygdala rescued the fear deficit induced by CO_2 in ASIC1a$^{-/-}$ animals (Ziemann et., 2009). In addition, the nucleus of the stria terminalis (BNST) is considered a key structure in fear-related behaviors evoked by CO_2 (Taugher et al., 2014). BNST mediates CO_2-evoked freezing and conditioned avoidance and the data found in the study also suggest that CO_2 acidifies BNST and that this region can directly detect acidosis through ASIC1a. The interruption of these channels specifically in the BNST reduced the freezing evoked by CO_2, while the expression of ASIC1a mediated by virus vector in the BNST of mice ASIC1a$^{-/-}$ and ASIC1a$^{+/+}$ increased the freezing evoked by CO_2.

The results observed in the study suggest that the inhibition of ASIC1a channels present in the LC has sex-specific effects on the behavioral response of miceexposed to high concentrations of CO_2. We observed that Pstx-1 only reduced the CO_2 behavioral responses in males. Our data add to the scientific literature the important participation of ASIC1a located in the LC are also involved in the expression of flight behavior. However, for females we still find lags in the literature, and this may hinder possible correlations, such as the estrous/menstrual cycle being related to PD.

Interestingly, a study from our laboratory demonstrated that exposure to 20% CO_2 activates LC-NA neurons of male mice while female counterparts do not, highlighting a notable disparity in CO_2 response between the sexes (Ripamonte et al., in revision). Additionally, the release of noradrenaline from the LC is important for the tonic excitatory control of ventilation in male mice, but not in females. One could arguethat the difference in panic-related behavior may be due to β-estradiol. As demonstrated by Zhou et al. (2019), treatment with β-estradiol suppressed the expression of ASIC1a due to increased protein degradation. It has also been shown that the expression level of ASIC1a protein is significantly lower in the cerebral cortex of female mice, which is correlated with a lower degree of brain damage and protectionby ASIC1a inhibitor (Zhou et al., 2019). These differences can, however, be attenuatedby ovariectomy. Therefore, these results indicated that β-estradiol can protect neurons against acidosis-mediated neurotoxicity and ischemic brain injury, reducing ASIC1a protein expression and channel function in females.

In conclusion, our data suggest that ASIC1a channels located in the LC do not participate in respiratory control under room air and hypercapnia, but are involved in CO_2-induced panic behavior in males, demonstrating a specific sex-dependent response. The results of the present study extend to previous studies on the mechanisms of PD and provide evidence regarding behavioral responses between males and females to a CO_2 panic challenge.

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Figure Legends

Fig 1. Illustrative figure of the experimental protocol.

Fig 2. Photomicrographs of cross-sections of the pons of mice showing the site of microinjection of Pstx-1 or saline in the LC with the presence of Evans blue.

Fig 3. Effect of intra-LC microinjection of Pstx-1 or saline on ventilation (V_E) [A, D], tidal volume (V_T) [B, E] and respiratory frequency (f_R) [C, F], during resting conditions in normocapnia or hypercapnia (20% of CO_2) in male [A, B, C] and female [D, E, F] mice (n=6 animals/group).

Fig 4. Effect of intra-LC microinjection of Pstx-1 or saline on ventilation oxygen consumption (VO_2) [A, D], respiratory equivalent (V_E/VO_2) [B, E] and body temperature (Tb) [C, F] during resting conditions in normocapnia or hypercapnia (20% of CO_2) in male [A, B, C] and female [D, E, F] mice (n=6 animals/group).

Fig 5. Effects of intra-LC microinjection with Pstx-1 or saline on the number of jumps [A, E], % freezing time (s) [B, F], running frequency [C, G] and rearing frequency [D, H] of male [A, B, C, D] and female [E, F, G, H] mice submitted to normocapnia and 20% CO_2 (n=6 animals/group). Pstx-1: psalmotoxin-1. *$p<0.05$ compared to the control group.

Figures

Fig. 1

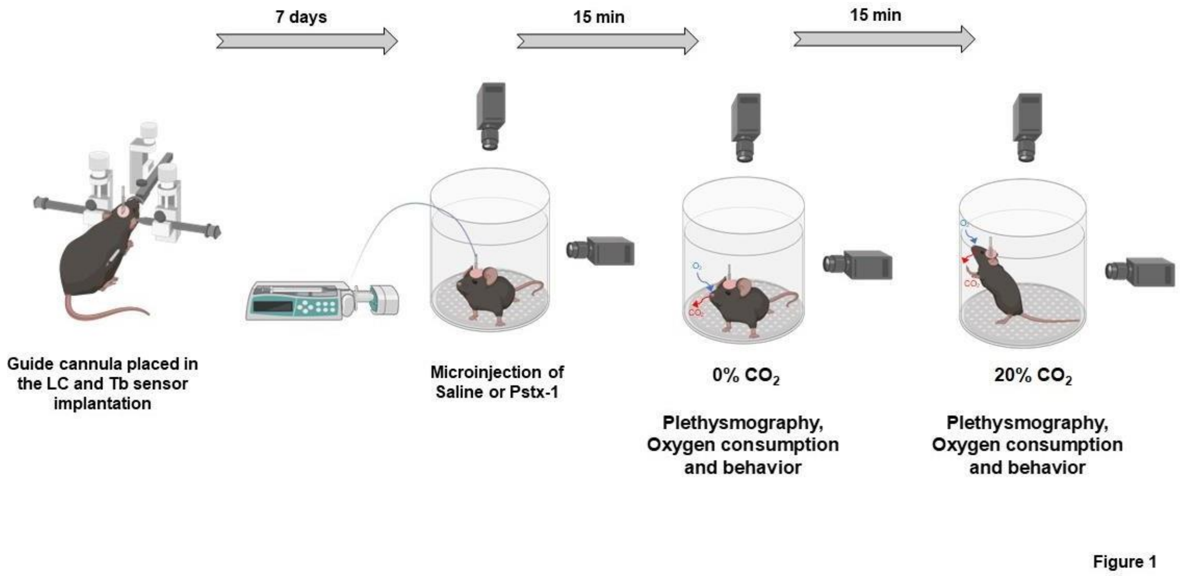


Fig 2.

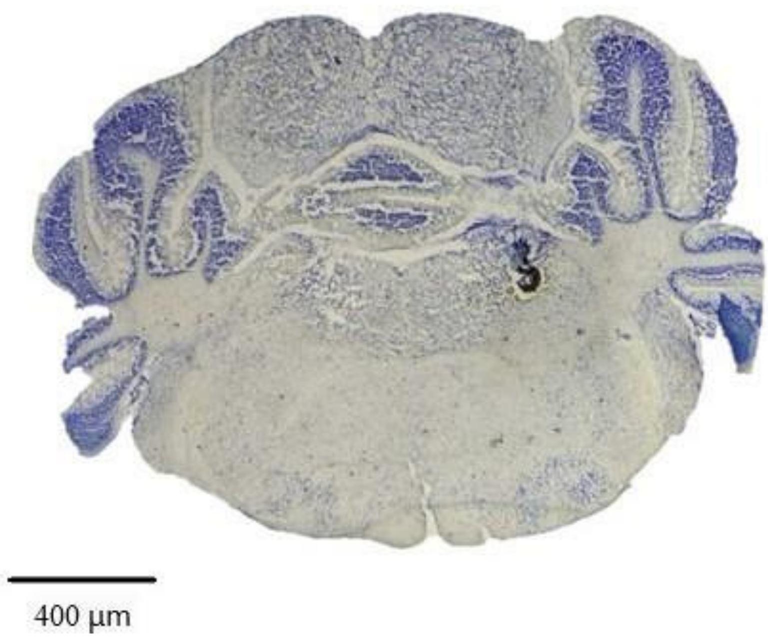


Fig 3.

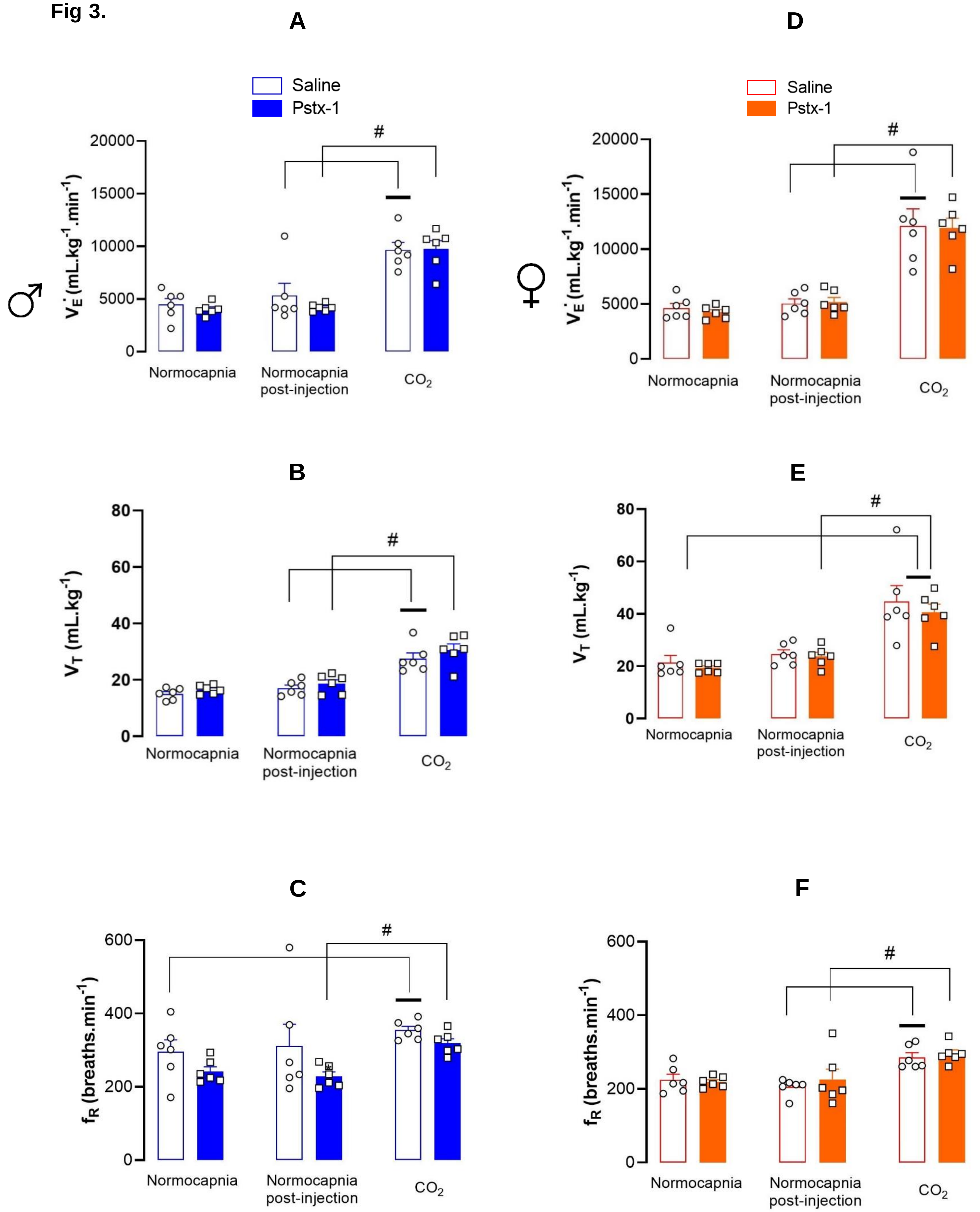

A
Saline
Pstx-1
V̇E (mL.kg-1.min-1)
20000
15000
10000
5000
0
#
Normocapnia
Normocapnia post-injection
CO2
D
Saline
Pstx-1
B
VT (mL.kg-1)
80
60
40
20
0
E
C
fR (breaths.min-1)
600
400
200
0
F

Fig 4.

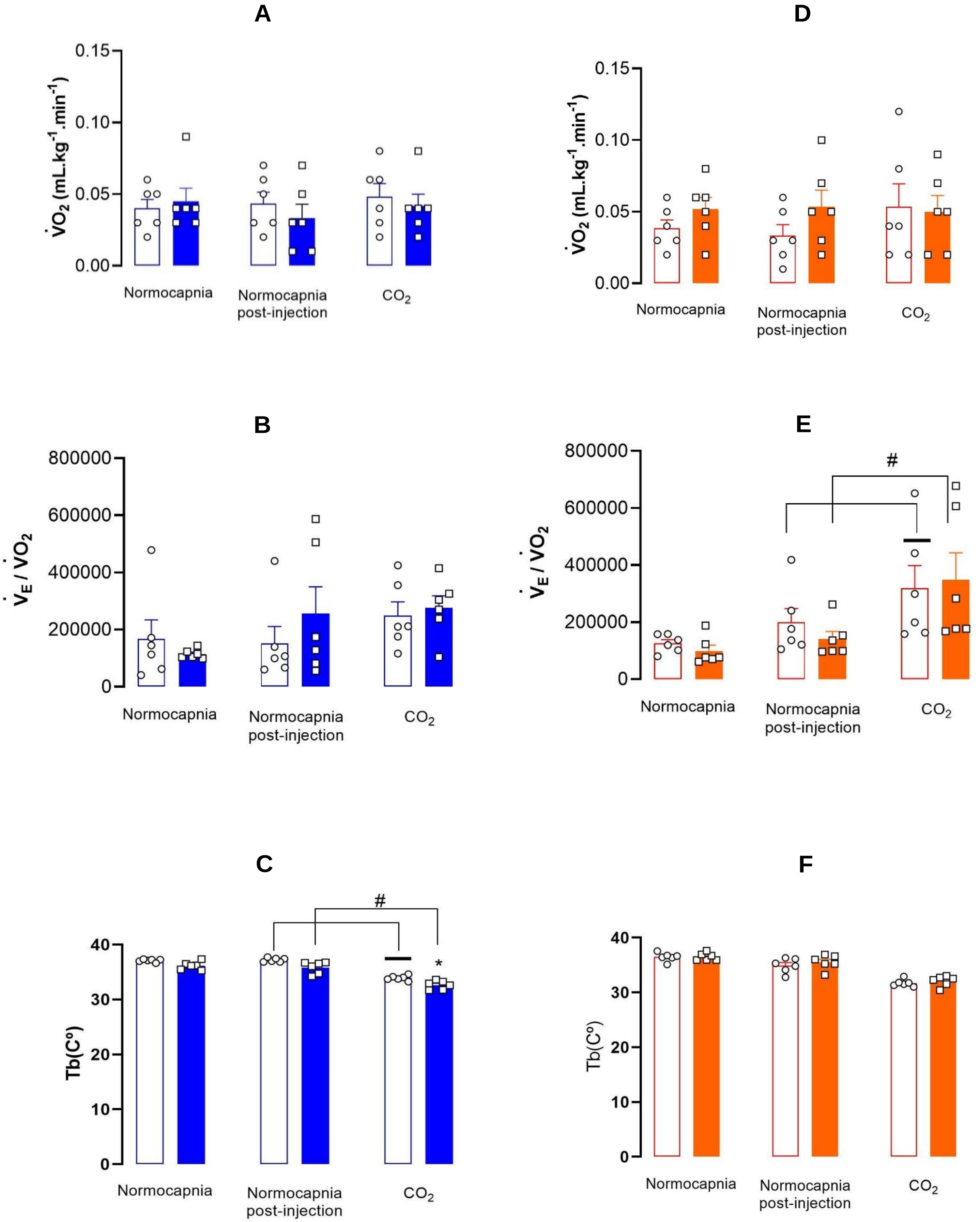
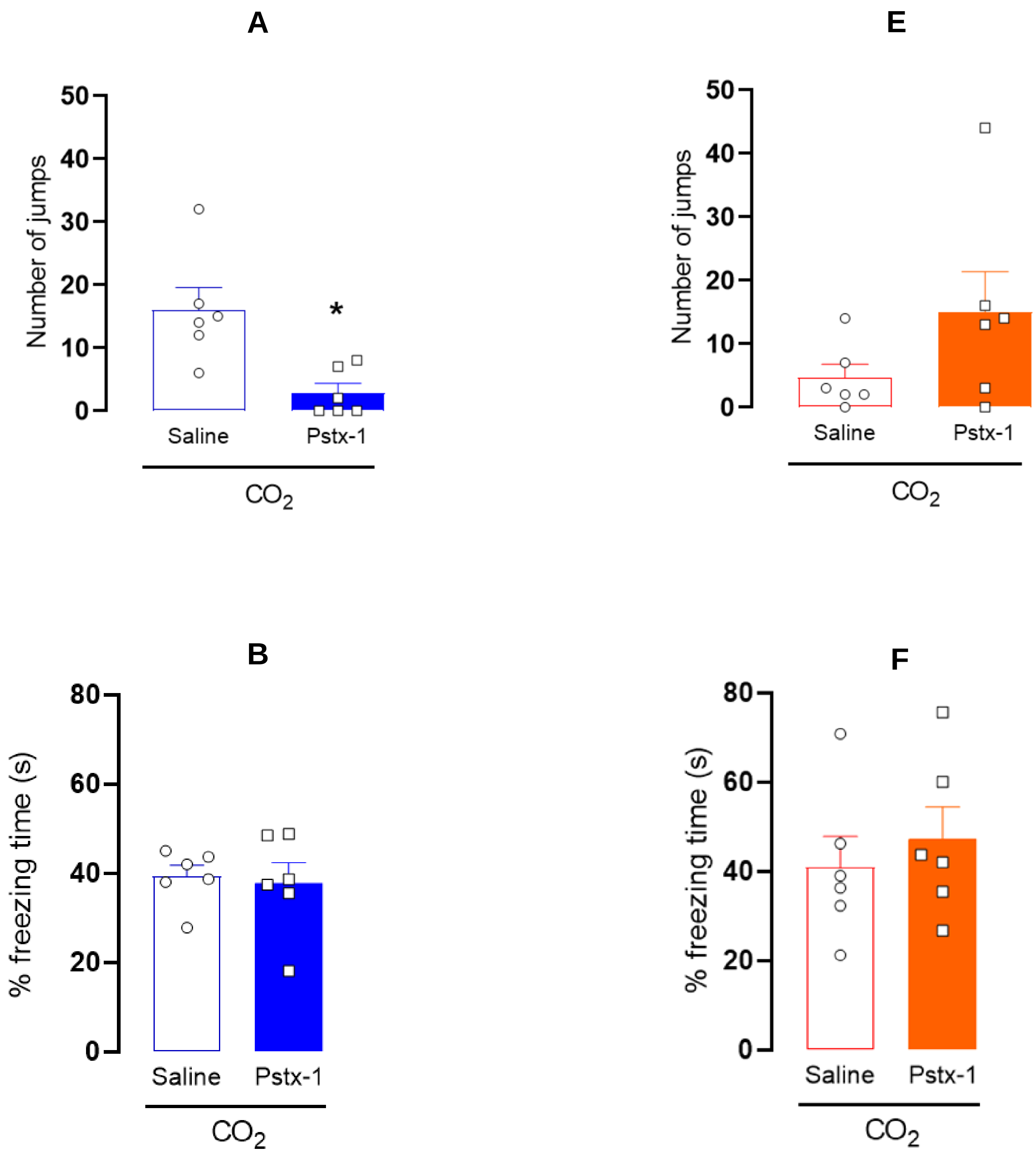
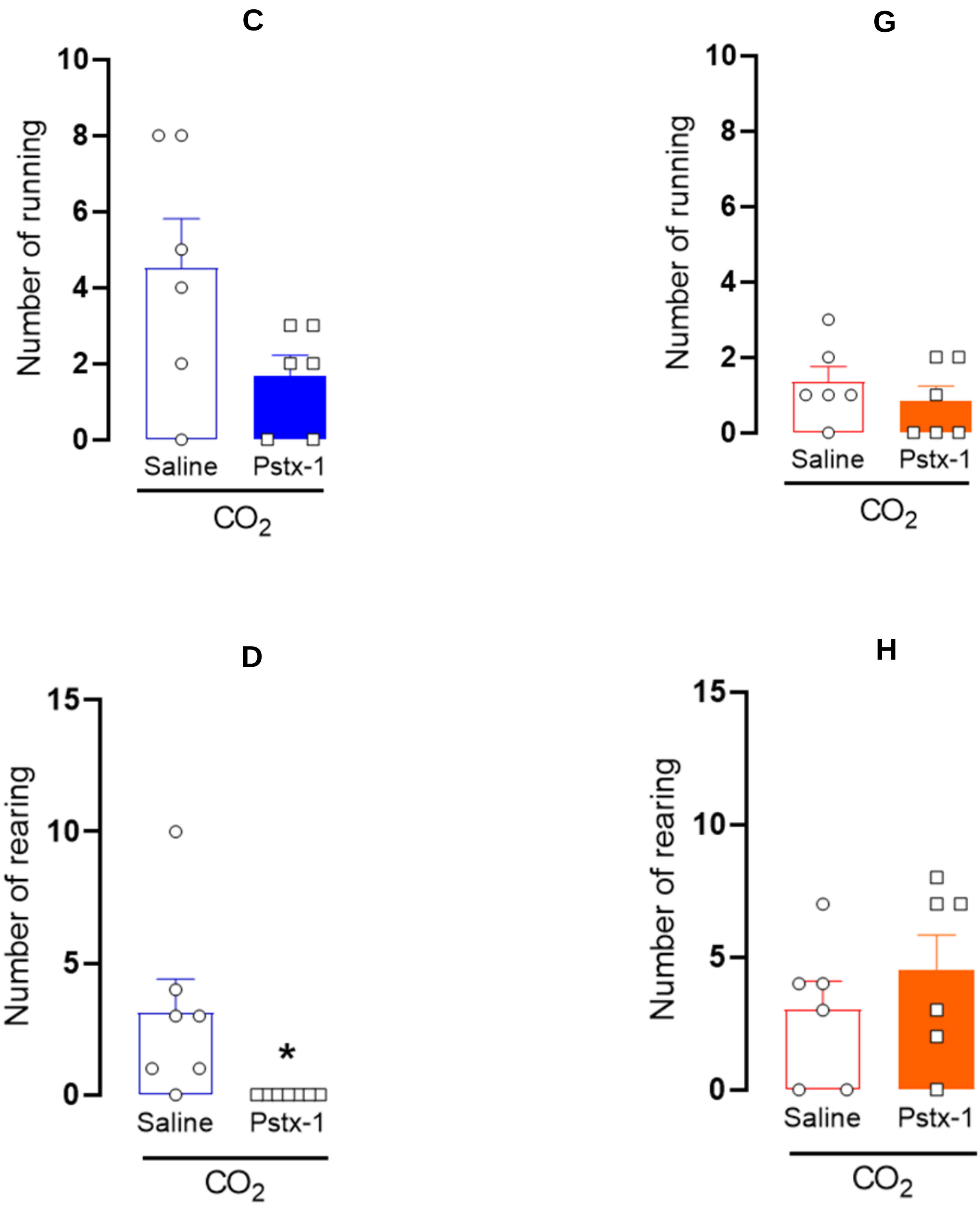

A
D
B
E
C
F
$\dot{V}O_2$ (mL.kg^{-1}.min^{-1})
$\dot{V}_E$ / $\dot{V}O_2$
Tb(C°)
0.15
0.10
0.05
0.00
800000
600000
400000
200000
0
40
30
20
10
Normocapnia
Normocapnia post-injection
CO_2
#
*

Fig 5.


A
E
Number of jumps
50
40
30
20
10
0
*
Saline
Pstx-1
CO2
B
F
% freezing time (s)
80
60
40
20
0
Saline
Pstx-1
CO2


C
Number of running
10
8
6
4
2
0
Saline
Pstx-1
CO_2
G
Number of running
10
8
6
4
2
0
Saline
Pstx-1
CO_2
D
Number of rearing
15
10
5
0
*
Saline
Pstx-1
CO_2
H
Number of rearing
15
10
5
0
Saline
Pstx-1
CO_2