UNIVERSIDADE FEDERAL DE SÃO CARLOS

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

PROGRAMA INTERINSTITUCIONAL DE PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS

Luis Gustavo Alexandre Patrone

Consequências da exposição intra-uterina ao agonista canabinóide WIN 55,212-2: impacto sobre o sistema cardiorrespiratório e ciclo sono-vigília durante o desenvolvimento em ratos

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Tese de doutorado apresentada ao Programa Interinstitucional de Pós-graduação em Ciências Fisiológicas da Universidade Federal de São Carlos/Universidade Estadual Paulista "Júlio de Mesquita Filho" (UFSCar/UNESP) como parte dos requisitos para obtenção do título de Doutor em Ciências Fisiológicas.

Orientadora: Profª. Drª. Luciane Helena Gargaglioni Batalhão

Jaboticabal - SP

2021

UNIVERSIDADE FEDERAL DE SÃO CARLOS

Centro de Ciências Biológicas e da Saúde
Programa Interinstitucional de Pós-Graduação em Ciências Fisiológicas

Folha de Aprovação

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O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.
O Relatório de Defesa assinado pelos membros da Comissão Julgadora encontra-se arquivado junto ao Programa Interinstitucional de Pós-Graduação em Ciências Fisiológicas.

Dedico aos meus pais Luis Carlos e Edna Márcia

Por serem a minha força motriz nessa jornada.

AGRADECIMENTOS

Foram cinco anos e meio de total entrega e dedicação a essa formação que finalmente se concretiza. Com o amadurecimento durante o doutorado consegui enxergar um mundo científico que até então me despertava ansiedade e insegurança, mas que agora me proporciona um sentimento de total satisfação, entusiasmo e de realização pessoal. Foi e continuará sendo um processo de eterna aprendizagem. Desafios e obstáculos sempre vão exister, mas tudo que é feito com amor é superado e se torna gratificante. Eu fui muito feliz nessa jornada, apesar de todas as dificuldades. Inumeras pessoas passaram pela minha convivência nesse tempo e a elas devo meu agradecimento...

Aos meus pais e irmãos que apesar da distância e de nos vermos poucas vezes no ano sempre estiveram presentes no meu dia-a-dia. Agradeço por todos os sacrifícios que já fizeram por mim e por nunca terem medido esforços para me ajudar. Se hoje cheguei até aqui é porque vocês foram a minha maior razão de não desistir!

À minha orientadora Profª. Drª. Luciane Batalhão por mais de uma década de convivência nacional e internacional, *Merci beaucoup*! Eu me sentia mais forte e preparado tendo você por perto e está enraizado no meu ser científico diversos dos seus ensinamentos. É difícil resumir em palavras tudo que já vivemos e toda admiração que tenho por você, mas saiba que sou eternamente grato pelas suas atitudes e considerações para com a minha pessoa. Esses anos todos no seu laborótorio foram os mais marcantes onde eu pude vivênciar as mais diversas experiências, foi onde o menino se transformou em homem, onde a simples curiosidade se transformou em ciência de qualidade. Você abriu portas, foi agente transformadora e é uma referência para mim.

À minha co-orientadora Profª. Drª. Kênia Bícego por toda a colaboração e ajuda no desenvolvimento e progresso desse projeto de pesquisa. Obrigado pela convicência, pelos horários de almoço descontraídos, mas principalmente pelo tempo dedicado e por todo o conhecimento de múltiplas áreas compartilhado fortalecendo o nosso crescimento como cientista. A sua contribuição na minha formação sempre irá ecoar como uma visão multifatorial sobre os eventos fisiológicos.

A todos que estiveram presentes na minha jornada no *Centre National de la Recherche Scientifique* na França, em especial ao pesquisador Gilles Fortin pela oportunidade de experienciar uma nova rotina de laboratório, com técnicas altamente sofisticadas e por agregar conhecimentos à minha bagagem. Uma frase que resume uma

das minhas maiores e mais intensas experiências de vida seria: "*Na Cidade Luz, tudo é sobre sentimento, e ninguém passa indiferente a Paris*".

A todos os colaboradores que foram fundamentais para o êxito desse projeto na sua totalidade. Ao Prof. Dr. Daniel Zoccal e Drª. Marlusa Amarante pelos experimentos *in situ*. Ao Prof. Dr. Wilfried Klein por todo o suporte nos experimentos de mecânica do sistema respiratório. À Profª. Drª. Angelita Stabile pelas análises de *Western Blot*. À Profª. Drª. Luciane Alberici e Dr. Gustavo Ferrari pelos inúmeros experimentos de respiração mitocondrial.

Aos técnicos de laboratório Euclides Secato e Damares Perecim pelo grandioso e incondicional cuidado com os animais, por todo suporte necessário nesses anos todos. Obrigado pela amizade e consideração.

Aos membros da banca por terem aceitado o convite, pela atenção, dedicação e contribuição para a melhoria desse trabalho.

A todos os alunos do laboratório de Fisiologia Animal! Nesses cinco anos diversas pessoas passaram por aqui e deixaram um pouco de si nesse lugar. Eu levo um pouquinho de cada um de vocês comigo. Fica difícil resgistar aqui a importância de cada um de vocês, mas saibam que sem vocês tudo isso teria sido muito mais difícil e sem graça! Nós formamos uma família de convívio diário, com trocas de afeto, conselhos, desabafos, mas o que mais prevaleceu entre nós foi a felicidade. Obrigado por vários momentos em que nossas risadas se sobressaíram ao choro, não importava aonde nos reuníssemos o simples fato de estarmos juntos já era o essencial. Agradeço em especial à Elisa, Danuzia, Carlos, Mariane e Aline pela nossa intensa relação dentro e fora do laboratório, pela amizade recíproca e confidente. Vocês estavam ao meu lado nos melhores momentos e eu tenho um carinho enorme por cada um vocês. À Vivian por todos os anos de convívio, trocas de conhecimento e crescimento profissional mútuo. Foi uma grande companheira que tive nesses anos todos e fico muito feliz que mesmo a distância esse vínculo de carinho se mantém.

Agradeço a todos os meninos que conviveram comigo na república nesse tempo. Em cinco anos muita gente passou por lá, uns com curta temporada outros nem tanto, mas cada um com sua história de vida e com anseios para sua carreira. Ser independente não quer dizer ser solitário, aprendi um pouco com cada um de vocês. Agradeço em especial ao Ivã, uma das pessoas que mais conviveu comigo nesses anos, e se eu pudesse escolher um *roommate* não seria tão bom quanto ele foi! E também ao

Raully que surgiu na reta final desse doutorado, porém foi a pessoa que mais se fez presente no meu dia-a-dia nos últimos tempos, foi um período curto, porém intenso e de grande sintonia. Nesse ano de pandemia, trancados e limitados dentro de casa sua presença foi muito importante e saber que podia contar com você tornou mais fácil, principalmente nessa fase final em que a ansiedade e o nervosismo ficaram a flor da pele. Muito Obrigado pelo companheirismo!

Ao Alexandre e Cristiano, Secretários do Programa de Pós-Graduação em Ciências Fisiológicas da UFSCar/UNESP, obrigado pela atenção e agilidade em sempre solucionar os problemas e esclarecimentos.

Ao Programa de Pós-Graduação em Ciências Fisiológicas da UFSCar/UNESP pela formação e meu obrigado pela compreensão das dificuldades enfrentadas nesse ano difícil.

À UNESP campus de Jaboticabal por todo o suporte e infraestrutura concedida.

À FAPESP, pela bolsa concedida e todo apoio financeiro durante a realização deste trabalho.

Deixo aqui um reconhecimento especial a todos os animais que fizeram parte desse trabalho. Meu imenso respeito e consideração!

APOIO FINANCEIRO:

Agradeço ao apoio financeiro fornecido pela CAPES (Processo n°: 1585677) e pela Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP (Processo n°: 2017/05318-0) os quais viabilizaram a realização deste projeto de pesquisa. Ao programa Ciência sem Fronteiras (Processo n°: 234616/2014-8) pelo período destinado ao doutorado sanduíche no *Centre National de la Recherche Scientifique*, Gif sur Yvette, França.

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Capítulo 2

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Síntese

Capítulo 1

Capítulo 2

2-AG - 2-araquidonoilglicerol

ADP - Adenosina difosfato

AMPc - Monofosfato cíclico de adenosina

ATP – Adenosina trifosfato

BötC – Complexo Bötzinger

BSA – Soro albumina bovina

BTPS - condições de pressão barométrica ambiente, à TC e saturados com vapor d'água

CA – Catecolaminérgico

C_B – Complacência da parede corpórea

CB1/CB2 – Receptor canabinóide tipo 1 / 2

C_L – Complacência pulmonar

CO_2 – Dióxido de carbono

C_T – Complacência total

DMSO – Dimetil sulfóxido

E – Dia embrionário

EEG – Eletroencefalograma

EMG – Eletromiograma

E.P.M / S.E.M – Erro padrão da média

FC / HR – Frequência cardíaca

FeO_2 – Fração de oxigênio expirado

FiO_2 – Fração de oxigênio inspirado

f_R – Frequência respiratória

FRe – Fluxo de ar

GPR – Gerador de padrão respiratório

GRD – Grupo respiratório dorsal

GRP – Grupo respiratório pontino

GRR – Gerador de ritmo respiratório

GRVc – Grupo respiratório ventral caudal

GRVr – Grupo respiratório ventral rostral

HCVR – Resposta ventilatória hipercápnica

HVR – Resposta ventilatória hipóxica

KF – Kölliker-Fuse

LC – Locus coeruleus

N_2 – Nitrogênio

NREM – Sem movimentos rápidos dos olhos

NTS – Núcleo do trato solitário

O_2 – Oxigênio

OXPHOS – Fosforilação oxidativa

P50 – Tubo polietileno

P – Dia pós natal

$PaCO_2$ – Pressão parcial de dióxido de carbono arterial

PaO_2 – Pressão parcial de oxigênio arterial

PAD / DAP – Pressão arterial diastólica

PAM / MAP – Pressão arterial média

PAP – Pressão arterial pulsátil

PAS / SAP – Pressão arterial sistólica

PB – Núcleo Parabraquial

PBS – Solução tampão fosfato

Pico – Complexo pós-inspiratório

PFA – Paraformoldeido

pFRG – Grupo respiratório parafacial

pH – Potêncial hidrogeniônico

Pre-BötC – Complexo pré-Bötzinger

PVN – Núcleo paraventricular

REM – Movimento rápido dos olhos

RTN – Núcleo retrotrapezóide

RVLM – Bulbo ventrolateral rostral

SNC / CNS – Sistema nervoso central

SIDS – Síndrome da morte súbita infantil

T_B – Temperatura corporal

TH – Tirosina hidroxilase

THC - Tetra-hidrocanabinol

T_{TOT} – Tempo total do ciclo respiratório

$\dot{V}_E$ - Ventilação

VEH - Veículo

$\dot{V}_E$ / $\dot{V}O_2$ - Equivalente respiratório

$\dot{V}O_2$ - Consumo de oxigênio

V_T – Volume corrente

WIN – Agonista de receptor canabinóide

SÍNTESE

Políticas de flexibilização e legalização do uso de *Cannabis* em diversos países tem aumentado consideravelmente nos últimos anos e o uso de drogas de abuso durante a gestação pode afetar o desenvolvimento de sistemas fisiológicos incluindo a rede de controle respiratório, principalmente devido a suscetibilidade do período pré-natal a intervenções externas e farmacológicas ocasionando em possíveis consequências na vida pós-natal da prole. Os compostos psicoativos da *Cannabis* podem agir diretamente sobre o sistema endocanabinóide presente no Sistema Nervoso Central (SNC) já nos estágios iniciais de desenvolvimento embrionário sendo um importante elemento para regulação estrutural e funcional da maturação do SNC, incluindo as áreas responsáveis pelo controle cardiorrespiratório. Entretanto, apesar do aumento do consumo dessa substância durante a gestação, o conhecimento sobre a influência de canabinóides exógenos no desenvolvimento do sistema respiratório e as consequências na vida pós-natal é escasso. O presente estudo avaliou os possíveis efeitos da exposição ao agonista de receptor canabinóide (CB) durante a gestação sobre o controle respiratório de ratos e ratas neonatos (P0, P6-7 e P12-13), juvenis (P27-28) e adultos (P80-81) através do implante subcutâneo de bomba osmótica em ratas grávidas para a liberação do veículo ou agonista de receptor canabinóide (WIN 55212-2, 0,5 mg/kg/dia) entre o dia gestacional 0 e 21. A exposição ao WIN interferiu de modo sexo dependente na regulação da ventilação dos filhotes, ocasionando em uma maior sensibilidade ao CO_2 nos machos neonatos, juvenis e adultos. Um quimiorreflexo alterado em resposta à hipóxia foi observado nos machos neonatos P0 e P6-7. Nas fêmeas, o tratamento pré-natal resultou apenas em uma hiperventilação durante hipercapnia durante a fase juvenil e uma reduzida resposta ventilatória ao CO_2 e O_2 quando adulta. Adicionalmente, analises neuroanatômicas do tronco encefálico evidenciaram um aumento do número de

neurônios catecolaminérgicos e expressão de receptores CB1, bem como alterações da respiração tecidual em machos neonatos. Uma expressiva redução da complacência pulmonar também foi observada em machos juvenis tratados. Por fim, alterações cardiovasculares também foram evidenciadas para os animais machos e fêmeas na idade adulta em decorrência da exposição pré-natal ao WIN. Esses achados demonstram que a exposição ao agonista de receptor canabinóide durante a gestação resulta em consequências prolongadas e sexo dependente para o sistema de controle cardiorrespiratório.

Policies for the flexibility and legalization of *Cannabis* use in several countries has increased considerably in recent years and the use of drugs of abuse during pregnancy can affect the development of physiological systems including the respiratory control network, mainly due to the susceptibility of the prenatal period to external and pharmacological interventions resulting in possible consequences in the offspring's postnatal life. *Cannabis* psychoactive compounds can act directly on the endocannabinoid system present in the Central Nervous System (CNS) already in the early stages of embryonic development, being an important element for structural and functional regulation of CNS maturation, including for the areas responsible for cardiorespiratory control. However, despite the increased consumption of this substance during pregnancy, knowledge about the influence of exogenous cannabinoids on the development of the respiratory system and the consequences on postnatal life is limited. The present study evaluated the possible effects of cannabinoid receptor (CB) agonist exposure during pregnancy on the respiratory control system of neonatal (P0, P6-7 and P12-13), juvenil (P27-28) and adult (P80-81) male and female rats through subcutaneous implantation of osmotic pumps in pregnant female rats at embryonic day 0 and delivered vehicle or agonist (WIN 55212-2, 0.5 mg/kg/day) for 21 days. WIN exposure interfered in a sex-specific maner with breathing regulation of offspring, thereby promoting a greater sensitivity to CO_2 in neonatal, juvenile and adult males. An altered chemoreflex in response to hypoxia was observed in P0 and P6-7 newborn males. In females, prenatal treatment resulted only in a hyperventilation during hypercapnia at juvenile age and a reduced ventilatory response to CO_2 and O_2 at adulthood. In addition, brainstem neuroanatomical analysis showed an increase in the number of catecholaminergic neurons and CB1 receptor expression and alteration of

tissue respiration in early stages of males. A significant reduction in lung compliance was also observed in treated juvenile males. Finally, cardiovascular changes were also observed for male and female animals in adulthood due to prenatal WIN exposure. These findings demonstrate that exposure to the cannabinoid receptor agonist during pregnancy results in prolonged and sex-dependent consequences for the cardiorespiratory control system.

Desenvolvimento do sistema de controle respiratório

Em mamíferos, a respiração é um comportamento motor contínuo e vital cuja principal função é adequar a ventilação alveolar dos pulmões para manter dentro de uma faixa constante a pressão parcial arterial de oxigênio (PaO_2) e dióxido de carbono ($PaCO_2$), bem como os valores de pH. Imediatamente após o nascimento, as trocas gasosas, anteriormente realizadas via placenta, passam a ser desempenhadas pelos pulmões. Embora seja funcional ao nascimento, sabe-se que os componentes neurais e mecânicos do sistema respiratório sofrem um processo de amadurecimento na fase pós-natal (Nunez-Abades e Cameron, 1995; Onimaru et al., 1997; Greer et al., 2006; Anju et al., 2013; Imber et al., 2014). Desta forma, o desenvolvimento do sistema respiratório se inicia precocemente, já na vida fetal.

Os movimentos respiratórios fetais são um dos primeiros comportamentos motores a surgirem e a geração de uma respiração fetal mesmo que episódica é essencial para o crescimento e desenvolvimento pulmonar pré e pós-natal (Kotecha, 2000; Thoby-Brisson et al., 2009). Através de registros eletrofisiológicos em preparações tronco encéfalo-medula espinhal, a ritmogênese respiratória foi evidenciada por volta do dia embrionário (E) 17 em fetos de ratos (Greer et al., 1992; Di Pasquale et al., 1992) e E15 em fetos de camundongos (Abadie et al., 2000; Thoby-Brisson et al., 2005). Os movimentos respiratórios episódicos fetais também foram observados por meio de registros ultrassônicos em ratas gravidas (Jansen e Chernick, 1991; Kobayashi et al., 2001), assim como em registros de atividade neuronal do complexo Pré-Bötzinger (pré-BötC) em fatias do bulbo de fetos de ratos (Pagliardini et al., 2003). Em humanos, os movimentos respiratórios fetais intra-uterínos foram detectados por volta da 30° semana de gestação (Florindo et al., 2005), sugerindo que a rede de controle respiratório já possui um papel funcional na fase embrionária. Entretanto, os mecanismos responsáveis

pelo início da atividade respiratória durante a vida fetal são desconhecidos. Especula-se que possa ser uma consequência da característica genética de cada um dos núcleos constituintes da rede de controle respiratório ao atingir certo grau de maturação ou devido a uma possível contribuição de aferências sensoriais recentemente formadas que irão desencadear a atividade oscilatória na rede neural respiratória. Entretanto, a ausência de movimentos respiratórios antes do início da atividade rítmica da rede de controle respiratório no tronco encefálico torna improvável a influência dos mecanorreceptores pulmonares e da caixa torácica no desencadeamento do ritmo respiratório (Beltrán-Castillo et al., 2017).

O pré-BötC é um pequeno núcleo localizado na superfície ventral do bulbo que desempenha um papel essencial e autossuficiente para a geração do ritmo respiratório (Smith et al., 1991; Feldman et al., 2003; Del Negro et al., 2005; Feldman e Kam 2015). Sua atividade rítmica é responsável por gerar os eventos de inspiração do ciclo respiratório (Smith et al., 1991; Rekling e Feldman, 1998; Feldman et al., 2013). Apesar dos esforços científicos em melhor compreender as propriedades ritmogênicas, estruturais e mecanismos celulares a respeito da circuitaria do pré-BötC, algumas lacunas ainda existem, principalmente sobre os seus mecanismos intrínsecos de disparo e a geração do padrão respiratório (Del Negro et al., 2018).

Embora o pré-BötC tenha papel de destaque sobre a geração da inspiração, o gerador de ritmo e padrão respiratório (GRR e GPR) são constituídos por outras estruturas bulbares e pontinas. O GRR inclui populações de neurônios distribuídos ao longo do grupo respiratório ventral (GRV) com propriedades oscilatórias, como o próprio pré-BötC, assim como o complexo pós-inspiratório (PiCo) e o núcleo retrotrapezóide/grupo respiratório parafacial (RTN/pFRG), responsáveis pela atividade pós-inspiratória e pré-inspiratória/expiratória, respectivamente (Onimaru e Homma

2003; Janczewski e Feldman 2006; Anderson et al., 2016). O complexo BötC, apesar de não ser considerado um núcleo oscilador do GRR, também tem participação na geração do ritmo respiratório uma vez que os neurônios dessa região apresentam um papel inibitório sobre a atividade do pré-BötC na fase final da inspiração/início da expiração (Alheid e McCrimmon, 2008; Del Negro et al., 2018). Os neurônios pré-motores do nervo frênico, assim como da musculatura intercostal externa (recrutados na inspiração) estão localizados no grupo respiratório ventral rostral (GRVr) e sua atividade é modulada pela aferência do pré-BötC. O grupo respiratório ventral caudal (GRVc) possui neurônios expiratórios pré-motores abdominais e da musculatura intercostal interna, sendo modulados por projeções de neurônios localizados no BötC e RTN (Del Negro et al., 2018).

A modulação do padrão respiratório (GPR) é realizada por dois grupamentos: o grupo respiratório dorsal (GRD), constituído pelo núcleo do trato solitário (NTS) e pelo grupo respiratório pontinho (GRP), composto pelos núcleos parabraquial e kölliker-fuse. O NTS está localizado bilateralmente na superfície dorsal do bulbo e possui principalmente neurônios inspiratórios os quais são iniciadores da atividade do nervo frênico. A modulação do NTS sobre a atividade dos geradores de ritmo é relevante devido as projeções aferentes dos quimiorreceptores periféricos e dos receptores de estiramento pulmonar que chegam até esse núcleo (Guyenet, 2014; Molkov et al., 2017; Ghali, 2019). Em relação aos núcleos parabraquial e kölliker-fuse, esses estão localizados bilateralmente e na porção rostral da ponte, possuem neurônios pós-inspiratórios e expiratórios fornencendo uma modulação excitatória e inibitória tônica e fásica aos osciladores de ritmo, além de controlarem a resistência das vias áreas superiores (Chamberlin, 2004; Dutschmann e Herbert, 2006; Mörschel e Dutschmann, 2009; Dutschmann e Dick, 2012; Geerling et al. 2017; Barnett et al., 2018).

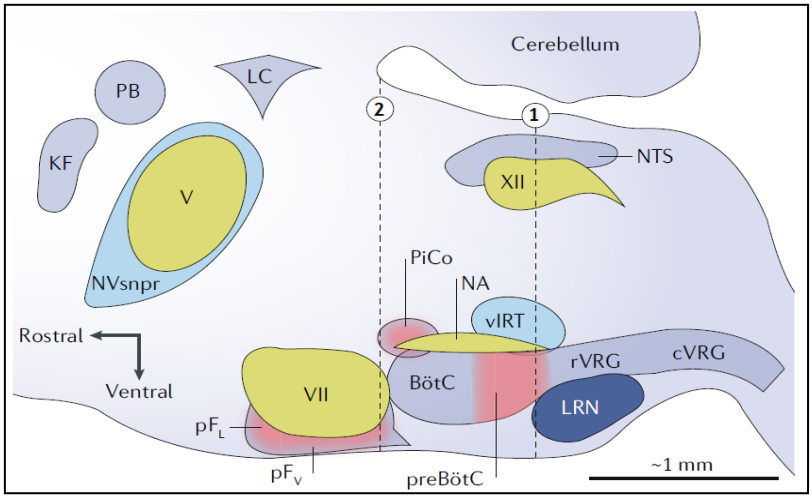


Figura 1. Representação esquemática em corte sagital do tronco encefálico de rato contendo os núcleos geradores de ritmo e padrão respiratório. As regiões ritmogênicas são destacadas em vermelho: pré-BötC (inspiratório); grupo respiratório parafacial lateral (pF_L - expiratório) e ventral (pF_V – expiratório prevalente na fase perinatal) e complexo pós-inspiratório (PiCo). Regiões moduladoras do padrão respiratório: núcleo do trato solitário (NTS), kölliker-fuse (KF) e parabraquial (PB). Núcleos pré-motores: grupo respiratório ventral rostral (VRGr) e caudal (VRGc) (Del Negro et al., 2018).

Em uma visão cronológica da ontogenia do ritmo respiratório em ratos, por volta do dia E12 e 14 ocorre o surgimento dos neurônios do pré-BötC e migração dos axônios do frênico, concomitantente se inicia a formação do músculo diafragma progredindo até o dia E17. A formação do núcleo pré-BötC se concretiza por volta do dia E17, no qual se evidencia o início dos ritmos inspiratórios juntamente com os movimentos respiratórios fetais. Por fim, na fase final do desenvolvimento fetal correspondente entre os dias E18 e 21 ocorre uma maturação da atividade dos motoneurônios do frênico que inervam o diafragma aumentando a frequência e estabilidade dos movimentos respiratórios intra-uterinos (Feldman et al., 1991; Fortin e Thoby-Brisson, 2009; Greer, 2012).

Quimiorrecepção

Além da ritmogênese, uma outra característica essencial do sistema de controle respiratório é a quimiorrecepção. Os quimiorreceptores sensíveis ao CO_2/pH são fundamentais para o controle da respiração, pelo fato de auxiliarem na geração do ritmo e na modulação do padrão respiratório, a fim de prevenir grandes variações das concentrações de CO_2 e pH arterial, visto que pequenas alterações podem resultar em danos irreversíveis ao indivíduo. Os quimiorreceptores sensíveis ao CO_2/pH são classificados como periféricos (localizados nos corpos carotídeos e no arco aórtico) e centrais (localizados no SNC), sendo os centrais com predominância sobre a resposta ventilatória ao CO_2 (Blain et al., 2010; Forster e Smith, 2010).

Em camundongos, a resposta quimiossensível ao CO_2 surge já na fase fetal em torno do dia E14,5 (Eugenin et al., 2006; Thoby-Brisson et al., 2009), contribuindo na coordenação dos movimentos respiratórios fetais, importantes para a estabilização do volume pulmonar e maturação dos pulmões para a respiração aérea contínua pós-parto (Darnall, 2010). Em relação a localização dos quimiorreceptores centrais, Thoby-Brisson e colaboradores (2009) demonstraram através de registros eletrofisiológicos em preparações *in vitro* contendo o bulbo que a região parafacial de fetos de camundongos (homóloga ao RTN de neonatos e adultos) apresenta um aumento de atividade quando exposta a um reduzido pH. Os neurônios serotoninérgicos da rafe bulbar (Richerson, 2004), assim como os neurônios noradrenérgicos do *Locus coeruleus* (LC) (Ritucci et al., 2005; Nichols et al., 2008) são importantes áreas quimiossensíveis na fase neonatal, incluído em animais P0, porém não existem estudos demonstrando a participação dessas regiões na resposta quimiossensível ao CO_2/pH durante a fase fetal.

Nos animais juvenis e adultos, os quimiorreceptores centrais estão amplamente distribuídos pelo SNC, mas principalmente ao longo do tronco encefálico, incluindo o

NTS (Coates et al., 1993; Nattie e Li, 2002; Nichols et al., 2008), núcleo fastigial, RTN (Mulkey et al., 2004; Guyenet et al., 2005), pré-BötC (Solomon et al., 2000; Solomon, 2003), rafe rostral bulbar, LC (Loeschcke, 1982; Coates et al., 1993; Nattie, 1999; Biancardi et al., 2008; Gargaglioni et al., 2010) e os neurônios orexinérgicos do hipotálamo lateral (Williams e Burdakov, 2008; Dias et al., 2010), como demonstrado pela Figura 2.

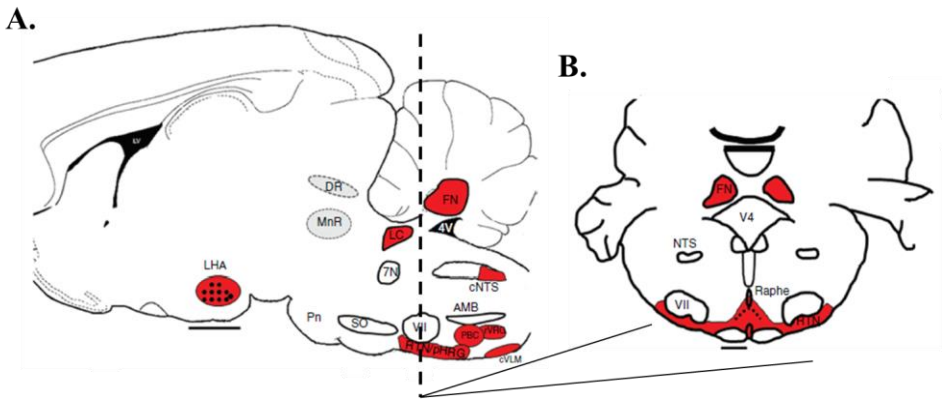


Figura 2. Representação esquemaática da localização dos quimiorreceptores centrais demarcados em vermelho ao longo do SNC em corte sagital (**A**) e em visão coronal no tronco encefálico (**B**). Abreviações: LHA, hipotálamo lateral; FN, núcleo fastigial; LC, locus coeruleus; cNTS, núcleo do trato solitário caudal; PBC, pré-BötC; rVRG, grupo respiratório ventral rostal; cVLM, bulbo ventrol lateral caudal; RTN/pFRG, núcleo retrotrapezóide/ grupo respiratório parafacial (Nattie e Li, 2012).

Em particular sobre o RTN, o mesmo origina-se na transição entre a vida fetal e a pós-natal a partir do grupo parafacial, previamente existente no feto. O RTN é constituído por neurônios glutamatérgicos que expressam um dos marcadores genéticos específicos dentre os neurônios considerados quimiossensíveis, o chamado fator de transcrição *Phox2b* (Stornetta et al., 2006; Guyenet et al., 2009), sendo classificado como uma região com capacidade intrínseca de detecção de alterações relacionadas ao CO_2/pH, mesmo que em reduzidas proporções (Wang et al., 2013; Takakura et al., 2014; Ruffault et al., 2015). Diversos estudos demonstram que o RTN recebe aferências

periféricas provenientes dos quimiorreceptores localizados no corpo carotídeo e dos receptores de estiramento pulmonar, auxiliando na modulação do controle respiratório (Takakura et al., 2006; 2007; Moreira et al., 2007; Guyenet et al., 2008; Guyenet e Mulkey, 2010). Camundongos recém nascidos com mutação para o gene *Phox2b* apresentaram uma resposta atenuada ao CO_2 e uma alta taxa de mortalidade ocasionada pelos eventos apnéicos exacerbados após o nascimento (Dubreuil et al., 2008).

Outra região de destaque na quimiossensibilidade central intríseca é o LC, possuindo mais de 80% dos neurônios altamente sensíveis as variações de CO_2/pH (Pineda e Aghajanian, 1997; Oyamada et al., 1998; Hilaire et al., 2004; Putnam et al., 2004; Biancardi et al., 2008; Gargaglioni et al., 2010). A acidificação local do LC por meio de ácidos ou CO_2 resultou em aumentos da atividade do nervo frênico, demonstrando sua participação no controle ventilatório (Elam et al., 1981; Coates et al., 1993). O LC é maior conjunto de neurônios noradrenérgicos do SNC, formando um par de núcleos adjacentes ao quarto ventrículo, localizados bilateralmente na ponte. Estima-se que metade de todas as projeções noradrenérgicas no SNC originam-se no LC (Aston-Jones et al., 1995; Berridge e Waterhouse, 2003). De acordo com alguns estudos, a atividade dos neurônios do LC está relacionada à atividade respiratória (Oyamada et al., 1998; Andrzejewski et al., 2001), e alterações do sistema noradrenérgico na fase perinatal afetam a atividade dos neurônios respiratórios do tronco encefálico responsáveis pela geração do ritmo respiratório (Viemari et al., 2004; Hilaire, 2006). Fetos de camundongos mutantes para o fator de transcrição *Phox2a*, com ausência dos neurônios noradrenérgicos do LC, apresentam uma frequência respiratória menor e uma variabilidade do ciclo respiratório expressivamente maior (Viemari et al., 2004). Dessa forma, os neurônios do LC parecem ser de grande importância para o desenvolvimento normal do ritmo respiratório em fetos e neonatos.

É importante ressaltar que a contribuição de cada um dos núcleos quimiossensíveis no controle respiratório em mamíferos é condicionado a alguns fatores, como limiar de excitação, sexo, anestesia, bem como o estado de ciclo sono-vigília (Nattie, 2001; Nattie e Li, 2012). As respostas quimiossensíveis variam de acordo com estado de sono-vigília, uma vez que determinados núcleos do tronco encefálico tem maior importância no controle da ventilação durante o estado de vigília e outros durante o sono (Li et al., 1999; Nattie e Li, 2001; da Silva et al., 2010; Dias et al., 2010; Li et al., 2013). Em ratos livres de anestesia, a acidificação local do RTN com 25% CO_2 resultou em um expressivo aumento da ventilação durante a vigília (Li et al., 1999), enquanto que o mesmo estímulo na rafe bulbar ocasionou em um aumento ventilatório proporcional durante o sono (Nattie e Li, 2001). No NTS caudal, o estímulo hipercápnico local resultou em respostas ventilatórias semelhantes independente do estado de sono-vigília (Nattie e Li, 2002). Por outro lado, a acidificação do bulbo ventrolateral caudal através de microdiálise desencadeou aumentos da ventilação apenas durante a vigília (da Silva et al., 2010). Adicionalmente, foi demonstrado que a acidificação dos neurônios orexinérgicos da região do hipotálamo lateral aumentou a ventilação em vigília, o que não foi observado durante o sono (Li et al., 2013). Assim, esses dados sugerem a estreita relação existente entre a quimiorrecepção central e estado de sono-vigília.

Não somente o CO_2, mas também alterações das pressões parciais de oxigênio arterial geram respostas ventilatórias compensatórias. Condições de hipóxia resultam em um aumento da ventilação pulmonar mediado pela ativação de quimiorreceptores periféricos, localizados no corpo carotídeo e no arco da artéria aorta. A informação dos quimiorreceptores periféricos é inicialmente direcionada ao tronco encefálico, chegando aos núcleos respiratórios bulbares, em especial no NTS, para a integração das

informações aferentes periféricas à rede neural de controle respiratório. Essa informação é processada pelos núcleos do SNC e através de vias eferentes motoras uma resposta é desencadeada gerando os ajustes necessários para a modulação da resposta ventilatória compensatória (Gonzalez et al., 1995; Nattie e Li, 2006; Takakura et al., 2006; Guner et al., 2008).

Os quimiorreceptores periféricos não apresentam uma atuação relevante sobre a respiração fetal, embora as pressões parciais de oxigênio sejam baixas no meio uterino, sua atividade não é essencial para o estabelecimento da respiração rítmica pós-natal (Jansen et al., 1981; Blanco et al., 1984). Devido às diferenças de pressões parciais ambientais entre o meio intra-uterino e o ar atmosférico, logo após o nascimento os quimiorreceptores periféricos são relativamente insensíveis à hipóxia. Essa sensibilidade é ajustada à nova condição ambiental do indivíduo alguns dias após o nascimento (Sterni et al., 1999). A resposta ventilatória à hipóxia se estabiliza quando o neonato atinge a idade de P8, mantendo-se constante até P16-21, devido à maturação da rede neural respiratória e dos quimiorreceptores periféricos (Blanco et al., 1984; Kholwadwala e Donnelly, 1992; Bamford et al., 1999; Gauda et al., 2004).

Neuroplasticidade

A plasticidade neural pode ser claramente observada durante o desenvolvimento do SNC e até mesmo na fase adulta. Porém, a plasticidade adaptativa ocorre com maior proeminência no início da fase pós-natal, devido a sucetibilidade do SNC às interferências exógenas e pelo fato de estar em um processo temporário de processos de proliferação, migração, diferenciação, sinaptogênese, mielinização e apoptose celular (Rice e Barone, 2000). A exposição a estímulos físicos e biológicos, especificamente na fase crítica de desenvolvimento, pode resultar em efeitos duradouros

sobre os processos cognitivos, comportamentais, assim como sobre os sistemas de controle (Fleming et al., 2002; Teicher et al., 2012; González-Mariscal e Melo 2013). O desenvolvimento pré-natal consiste em um momento crítico para moldar o SNC e suas interconexões. Assim, interferências nesse processo podem definir um cenário de vulnerabilidade para o acometimento de fisiopatologias a curto, médio e longo prazo. De modo geral, a plasticidade do SNC pode decorrer em resposta à uma demanda fisiológica, como resultado de alterações da atividade neural ou até mesmo em resposta a algum tipo de dano ao tecido nervoso (Caroni et al., 2014). Fatores não genéticos, como estresse, déficit nutricional e exposição a compostos químicos durante a formação do indivíduo podem influenciar negativamente o processo de ontogenia do SNC durante a janela de vulnerabilidade do processo de desenvolvimento e, consequentemente alterar a estruturação e função normal dos sistemas fisiológicos (Maccari et al., 2003; Harris e Seckl, 2011).

Os processos biológicos envolvendo a plasticidade estrutural ou arquitetônica abrangem a neurogênese, migração celular, alterações de excitabilidade neuronal e neurotransmissão, geração de novas conexões e modificações das pré existentes. A remodelação das conexões ocorre através do surgimento de novas sinapses, expansão ou retração da arborização dendrítica, assim como pela ramificação ou poda axonal (Fauth e Tetzlaff, 2016; Von Bernhardi et al., 2017). A eficácia sináptica é normalmente considerada como a capacidade de evocar eventos pós-sinápticos após a liberação de neurotransmissor pelo terminal pré-sináptico. Já a plasticidade sináptica é a capacidade de alterar a eficácia sináptica como consequência de uma intervenção sobre a sinapse. Em determinadas circunstâncias pode existir a dependência de um agente intermediário na interação entre o terminal pré e pós sináptico. Devido as suas características intrínsecas, o sistema endocanabinóide pode atuar nas interações entre os terminais

sinápticos, alterando a eficácia sináptica devido a um processo de plasticidade neural (Freund e Hájos, 2003; Freund et al., 2003). Desta forma, o processo de plasticidade no SNC pode ocorrer sem necessariamente existir uma alteração quantitativa de neurônios e/ou sinapses, de densidade ou área total de sinapse.

Sistema endocanabinóide e controle cardiorrespiratório

O sistema endocanabinóide consiste em receptores acoplados à proteína G (receptores CB1 e CB2), ligantes endógenos (chamados endocanabinóides) e proteínas envolvidas na síntese e inativação dos endocanabinóides (Piomelli, 2003; Di Marzo et al., 2005; Di Marzo e Petrocellis, 2006). Estudos revelaram que o receptor CB1 é o receptor acoplado à proteína G mais abundante no SNC, sendo expresso em áreas que participam do controle cardiorrespiratório e do ciclo sono-vigília (Herkenham et al., 1991; Tsou et al., 1998; Pilowsky e Goodchild, 2002; Calik e Carley, 2017; Méndez-Díaz et al., 2021). O sistema endocanabinóide mostra-se presente no SNC já nos estágios iniciais do desenvolvimento embrionário (Rodriguez de Fonseca et al., 1993; Harkany et al., 2007), uma vez que os receptores CB1 e seus ligantes, assim como os níveis de RNAm, foram detectados em torno do 11-14° dia gestacional em ratos, coincidindo com o tempo de expressão fenotípica da maioria dos neurotransmissores (Mulder et al., 2008; Morozov et al., 2009). Esse sistema parece ser funcional já nos estágios iniciais do desenvolvimento, visto que já estão acoplados a mecanismos de transdução de sinal que envolvem proteínas de ligação ao trifosfato de guanosina (GTP) (Berrendero et al., 1998; Mato et al., 2003). De fato, durante a vida fetal, os endocanabinóides são importantes para o desenvolvimento encefálico, regulando a diferenciação dos progenitores neurais e sinaptogênese, orientando a migração axonal e consolidando comunicações sinápticas (Fernandez-Ruiz et al., 2000; Bernard et al.,

2005; Fride et al., 2009). A densidade de receptores CB1 (Rodriguez de Fonseca et al., 1993) e os níveis de RNAm (McLaughlin e Abood, 1993) aumentam progressivamente durante o desenvolvimento pós-natal, atingindo um pico entre as idades P30-40, pouco antes do início da puberdade, seguido de uma redução dos níveis dos receptores CB1, atingindo valores observados na fase adulta (Rodriguez de Fonseca et al., 1993; Berrendero et al., 1999).

Em relação aos receptores CB2, embora sua presença tenha sido relatada no SNC, por exemplo, no cerebelo, hipocampo e áreas restritas do tronco encefálico (Van Sickle et al., 2005; Onaivi et al., 2006; Chen et al., 2017), estes são principalmente expressos em células do sistema imunológico (Munro et al., 1993). Porém, atualmente está bem estabelecido que ambos os receptores são expressos na periferia e no SNC, como em quimiorreceptores periféricos e na circuitaria neuronal responsável pela ativação e controle cardiorrespiratório (Padley et al., 2003; McLemore et al., 2004). Neste sentido, o RNAm para receptores CB1 é encontrado no corpo carotídeo e em uma grande população de corpos celulares de neurônios nos gânglios sensoriais glossofaríngeo e vagal (jugular, petroso e nodoso) (McLemore et al., 2004).

No que diz respeito aos ligantes de receptores canabinóides, a anandamida (Devane et al., 1992) e 2-araquidonoilglicerol (2-AG) (Mechoulam et al., 1995) foram os primeiros compostos a serem identificados e isolados, e são os mais estudados dentre os endocanabinóides. Devido à natureza lipofílica, os endocanabinóides não são armazenados em vesículas sinápticas, mas sim sintetizados pelos neurônios mediante a demanda, via hidrólise de lipídios precursores da membrana celular, após despolarização da membrana e aumento das concentrações intracelulares de Ca^{2+} (Freund et al., 2003; Piomelli, 2003). Uma vez liberados, os endocanabinóides recentemente sintetizados deslocam-se retrogradamente em direção à fenda sináptica, se

ligando aos receptores canabinóides nos terminais das células pré-sinápticas (Freund et al., 2003). A inibição ou ativação dos canais iônicos são uma das principais consequências da ativação dos receptores canabinóides (Szabo e Schlicker, 2005), que por sua vez podem estimular ou inibir a liberação de neurotransmissores a partir dos terminais axônicos, desempenhando assim um papel importante em várias formas de plasticidade sináptica de curto e longo prazo (Vigano et al., 2005; Chevaleyre et al., 2006; Mackie, 2006). Tal peculiaridade de mecanismo de produção e atuação dos endocanabinóides sugere que esses compostos atuam principalmente como neuromoduladores, ao invés de neurotransmissores clássicos. É importante mencionar que determinados endocanabinóides (Anandamida), assim como fitocanabinóides (Canabidiol e THC) e canabinóides sintéticos (WIN 55,212-2) também podem atuar sobre canais iônicos de potencial receptor transitório, em especial sobre a subfamília vanilóide (TRPV) (De Petrocellis et al., 2011; Patel et al., 2017; Karwad et al., 2019; Muller et al., 2019), assim os efeitos dos canabinóides endógenos e exógenos podem ser em decorrência da ativação sinérgica de outros receptores além dos receptores CB.

De forma interessante, estudos recentes localizaram os receptores CB1 na membrana externa da mitocôndria de neurônios (Bénard et al., 2012; Hebert-Chatelain et al., 2014; Koch et al., 2015). A ativação desses receptores promoveu uma diminuição na concentração de AMPc, da atividade da proteína quinase A e da respiração mitocondrial (Bénard et al., 2012; Fišar et al., 2014; Hebert-Chatelain et al., 2014). Desta forma, os canabinóides exógenos podem ativar os receptores CB1 mitocondriais e alterar o metabolismo energético neuronal, deprimindo a respiração mitocondrial alterando as respostas fisiológicas mediadas pelos endocanabinóides (Whyte et al., 2010; Alger e Tang, 2012; Lipina et al., 2014).

Em relação à regulação respiratória, sabe-se que os canabinóides administrados agudamente deprimem a respiração em animais de laboratório (Graham e Li, 1973; Moss e Friedman, 1976; Doherty et al., 1983; Estrada et al., 1987), uma vez que estudos com fitocanabinóide (THC) e canabinóides sintéticos, administrados sistemicamente, demonstraram o seu efeito depressor respiratório através da ativação de receptores CB1 (Vivian et al., 1998; Schmid et al., 2003). As consequências da intensa diminuição da frequência respiratória em ratos foram quadros de hipoxemia, hipercapnia e acidose sanguínea (Schmid et al., 2003). Neste contexto, Pfitzer et al. (2004) demonstraram que a ativação central de receptores CB1 deprime os neurônios geradores do ritmo respiratório no tronco encefálico, além de causar aumento da pressão arterial, queda na frequência cardíaca e aumento das catecolaminas plasmáticas. Alterações da atividade do nervo frênico, assim como das variáveis cardíacas também foram observadas por Padley et al. (2003) após ativação dos receptores canabinóides do bulbo ventrolateral. Adicionalmente, dados a partir do bloqueio desses receptores em ratos recém-nascidos reforçam a participação dos endocanabinóides no controle ventilatório durante a normoxia, bem como durante a hipóxia, uma vez que esse bloqueio resultou em um aumento da ventilação e redução dos eventos de apneia (Tree et al., 2014). Os mesmos autores também relataram que alterações no sistema endocanabinóide durante a gestação resultaram em uma hiperventilação em condições basais, alteração do quimiorreflexo periférico e apnéias prolongadas em neonatos de ratos (P0-2 e P10-12). Acredita-se que a ação dos canabinóides na ventilação ocorra principalmente sobre as vias centrais, embora estudos tenham comprovado que os canabinóides exógenos podem afetar a função das vias periféricas envolvidas na regulação respiratória, como quimio e barorreceptores, receptores de estiramento pulmonar, além de influenciar a

resistência das vias aéreas agindo diretamente sobre os brônquios (Calignano et al., 2000; Schmid et al., 2003).

Diversos estudos têm demonstrado a participação direta do sistema endocanabinóide no controle das funções cardiovasculares (Lake et al., 1997; Sierra et al., 2017). Em humanos, a administração aguda intravenosa de THC induziu uma forte taquicardica, juntamente com um aumento da pressão arterial média (Perez-Reyes et al., 1972; Weiss et al., 1972; Roth et al., 1973), enquanto uma hipotensão e bradicardia foram prevalecentes em condições de uso crônico (Benowitz e Jones, 1975; Benowitz et al., 1979; Mathew et al., 1992). Estudos realizados em ratos hipertensivos, a estimulação dos receptores CB1 com agonista no núcleo paraventricular resultou em reduções da pressão arterial e da frequência cardíaca, provavelmente relacionado a diminuição da atividade simpática (Grzęda et al., 2017). Curiosamente, as alterações cardiovasculares em resposta à estimulação do sistema endocanabinóide variam de acordo com a região encefálica. Pfitzer et al. (2004) demonstraram que a ativação dos receptores CB1 por meio de injeções intra cisterna magna de canabinóide sintético ocasionou em aumentos da pressão arterial, porém a frequência cardíaca reduziu. Entretanto, a ativação local dos receptores CB1 localizados no bulbo ventrolateral rostral resultou em respostas opostas (Padley et al., 2003; Wang et al., 2017). Acredita-se que o mecanismo de ação e modulação do sistema endocanabinóide na função cardiovascular ocorra por influência direta sobre a liberação de neurotransmissores no SNC e também localmente sobre os receptores β adrenérgicos da musculature lisa dos vasos sanguíneos (Beaconsfield et al., 1972; Martz et al., 1972; Hillard, 2000).

Exposição fetal aos canabinóides exógenos

O desenvolvimento dos componentes neurais e sua rede altamente complexa de conexões não segue um modelo genético rigidamente predeterminado (Carroll e Agarwal, 2010). Pelo contrário, o desenvolvimento estrutural dos sistemas fisiológicos e a diferenciação dos neurônios em fenótipos funcionais específicos são resultados de interações extremamente complexas entre genes, fatores transcricionais e neurotróficos. Estes fatores operam sob restrições estruturais e temporais variáveis à medida em que o desenvolvimento ocorre, sendo que todos esses processos dinâmicos estão sujeitos a alterações por influências externas durante o desenvolvimento (Carroll e Agarwal, 2010). Desta forma, o desenvolvimento dos sistemas fisiológicos, incluindo o sistema de controle cardiorrespiratório, é um processo altamente dinâmico que pode ser influenciado durante a maturação por fatores ambientais (por exemplo, hipóxia ou hiperóxia), químicos (por exemplo, nicotina, medicamentos ou drogas de abuso) e até psicológicos (depressão materna) (Bavis e Mitchell, 2008; Campos et al., 2009; Cayetanot et al., 2009; Bairam et al., 2015). A exposição a condições adiversas *in utero* também pode ser responsável por remodelar o desenvolvimento ontogenético ocasionando em alterações na formação e maturação do SNC, incluindo dos núcleos responsáveis pela função cardiovascular e respiratória, e assim resultar em desregulações epigenéticas no controle cardiorrespiratório na vida pós-natal do indivíduo (Burggren, 2014; Burggren e Crews, 2014; Koos e Rajaee, 2014). Cada vez mais estudos epigenêticos lancam mão de ferramentas para demonstrar como o ambiente de desenvolvimento do indivíduo modula a transcrição de genes, produzindo efeitos a longo prazo, mais especificamente na vida pós-natal, sobre a expressão desses genes, assim como sobre os fenótipos (Silveira et al., 2007; Gluckman et al., 2016; O'Donnell e Glover, 2016).

Particularmente em relação às intervenções químicas que podem ocorrer na gestação, a manipulação perinatal do sistema endocanabinóide, seja pela administração de canabinóides ou pelo consumo de *Cannabis* materna, altera a neutransmissão e as funções comportamentais da prole (Correa et al., 2016). O principal componente psicoativo da *Cannabis sativa*, o Δ9-tetrahidrocanabinol (THC), afeta profundamente as funções neurais e fisiológicas, incluindo os processos motores, cognitivos, nociceptivos, termorregulatórios e cardiorrespiratórios, através da ligação à receptores canabinóides (Onaivi et al., 2002).

De acordo com estudo da Fiocruz (2015), no Brasil a *Cannabis* é a droga ilícita com maior prevalência de consumo entre pessoas de 12 a 65 anos, e consistem em uma das drogas recreativas mais usadas em idades altamente correlacionadas com uma gravidez e estão entre as drogas de abuso mais utilizadas pelas mulheres grávidas na sociedade ocidental (Fried e Smith, 2001; Fried, 2002; Nida, 2005), principalmente no primeiro trimestre de gestação devido as características antieméticas dessas substâncias (Volkow et al., 2017). Nos últimos anos, o consumo de compostos canabinomiméticos entre mulheres gravidas tem aumentado expressivamente, motivado pelas políticas de flexibilização e legalização do uso de compostos a base de *Cannabis* (Young-Wolff et al., 2019; Bérard, 2020). A concentração de THC na maconha aumentou de 4% no ano de 1995 para 12% em 2014 (Volkow et al., 2017). Acredita-se que aproximadamente 50% do THC e outros canabinóides presentes nas preparações de *Cannabis* são inalados e entram na corrente sanguínea (fase aguda). A alta solubilidade pelos lipídios dos canabinóides leva a um rápido acúmulo no tecido adiposo, a partir do qual são liberados lentamente para o organismo (fase crônica). Os canabinóides são capazes de atravessar a barreira placentária durante a gestação (Vardaris et al., 1976; Hutchings et al., 1989; Little e VanBeveren, 1996), podendo também ser transferidos através do leite materno

durante a lactação. Portanto, essas substâncias podem atingir o encéfalo fetal e neonatal em quantidades substanciais durante o período de desenvolvimento e resultar em consequências a curto, médio e longo prazo para a prole no período pós-natal.

Um estudo clínico sobre a influência do uso de psicotrópicos maternos em recém-nascidos, relatou que a exposição à *Cannabis* pode aumentar o risco de comprometimentos respiratórios em neonatos (Lacroix et al., 2007). Scragg et al. (2001) mostraram que um fator de risco para Síndrome da Morte Súbita Infantil (SIDS) poderia estar associado ao uso frequente de maconha durante a gestação. Os endocanabinóides ao nascimento parecem modular a respiração e proteger o recém-nascido contra as apnéias. No entanto, quando exposta pré-natalmente a estes componentes, a rede respiratória em desenvolvimento parece ser modificada, provavelmente tornando o recém-nascido mais vulnerável frente a ambientes instáveis, como meios hipóxicos (Gonzalez et al., 2005; Desai et al., 2013). Dados de preparações *in vitro*, contendo o bulbo de neonatos de camundongos com exposição pré-natal ao canabinóide sintético, demonstraram uma depressão exagerada da atividade do nervo C4 durante hipóxia, sugerindo um impacto negativo da exposição pré-natal ao composto químico sobre a rede respiratória rítmica bulbar (Tree et al., 2014).

Diversos estudos têm relatado que a exposição pré-natal aos canabinóides exógenos pode afetar o desenvolvimento de vários sistemas de neurotransmissores. Em particular, estudos demonstraram os efeitos dos canabinóides sobre a maturação do sistema catecolaminérgico (Fernandez-Ruiz et al., 1999; 2004). Tree et al. (2010) demonstraram que os receptores CB1 estão localizados em uma densa rede de fibras ao redor do soma de neurônios catecolaminérgicos bulbares, relatando uma influência dos endocanabinóides sobre a neurotransmissão catecolaminérgica. Os efeitos dos canabinóides no desenvolvimento das vias catecolaminérgicas aparecem sobre a

diferenciação e maturação das projeções desses neurônios à suas regiões-alvo. Em particular, na fase final da gestação, os canabinóides são capazes de afetar a expressão de genes-chave para a transmissão catecolaminérgica como o da tirosina hidroxilase (TH) (Bonnin et al., 1996). Neurônios de ratos em cultura, obtidos a partir de fetos expostos diariamente a partir do 5° dia gestacional ao THC, exibiram uma atividade da TH mais elevada em comparação com células controles (Hernandez et al., 2000). Assim, esses dados sugerem que a interferência de canabinóides externos sobre os eventos envolvendo a expressão do gene para TH, durante o desenvolvimento embrionário, pode contribuir para uma maturação pré e pós-natal anormal dos neurônios catecolaminérgicos, bem como dos seus núcleos alvos. De fato, estudos prévios sugerem que alterações do sistema catecolaminérgico durante o período neonatal seja responsável pelo surgimento de distúrbios clínicos respiratórios (Viemari et al., 2005).

Apesar de todas as informações aqui apresentadas, os estudos relacionados à manipulação da sinalização do sistema endocanabinóide durante a vida fetal e os possíveis efeitos sobre o controle cardiorrespiratório a curto, médio e longo prazo na vida pós-natal da prole ainda são escassos. Esses dados são importantes para uma melhor compreensão e avaliar a existência de adaptações e/ou plasticidade neural que acarrete em mudanças no padrão respiratório, assim como nas respostas de controle respiratório às condições ambientais adversas.

Tendo elucidado todos esses elementos e buscando melhor compreender os efeitos da exposição fetal a canabinóides no controle cardiorrespiratório durante a vida pós-natal de ratos, o presente trabalho é apresentado em dois capítulos cujos objetivos gerais foram:

Capítulo 1

Avaliar os efeitos da exposição pré-natal a canabinóide sintético sobre o controle respiratório e metabólico, a complacência do sistema respiratório, a expressão de receptores CB1, neurônios catecolaminérgicos e respiração tecidual do tronco encefálico na vida pós-natal de ratos e ratas neonatos (P0, P6-7 e P12-13) e juvenis (P27-28).

Capítulo 2

Investigar as consequências a longo prazo da exposição intra-uterina a canabinóide sintético sobre a modulação do controle cardiorrespiratório e metabólico durante estado de sono-vigília, complacência pulmonar, temperatura corporal, expressão de receptores CB1 e neurônios catecolaminérgicos do tronco encefálico de ratos e ratas adultos (P80-81).

O protocolo de exposição pré-natal a canabinóide foi realizado através de implante de bomba osmótica no dorso de ratas grávidas para infusão do veículo (50% DMSO) ou do fármaco (WIN 55212-2; 0,5 mg/kg/dia) entre os dias 0 e 21° de gestação. De acordo com Bara et al. (2018), a dose diaria de 0,5 mg/kg possui característica clínica relevante uma vez que corresponde a uma exposição moderada à *Cannabis* em mulheres grávidas, visto que o tratamento nas ratas gravidas não ocasionou em partos prematuros, malformação congênita ou até mesmo redução da massa corpórea dos filhotes.

Capítulo 1

A abordagem experimental utilizada no Capítulo 1 foi a realização de medidas de ventilação "*in vivo*" em condições normais, hipercápnicas e hipóxicas por meio de pletismografia de pressão para os animais neonatos e por pletismografia de corpo inteiro para os juvenis. A taxa metabólica foi mensurada através de medidas de calorimetria indireta. A mecânica respiratória dos animais foi avaliada por meio da determinação da complacência pulmonar. A quantificação de receptores CB1 no tronco encefálico foi determinada por técnicas de *Western Blot*. Também foi realizada imunohistoquímica para quantificar os neurônios catecolaminérgicos do tronco encefálico. Análises de respiração tecidual do tronco encefálico foram feitas a partir da mensuração do consumo de oxigênio em respirômetro.

Capítulo 2

No Capítulo 2 abordamos as medidas de ventilação "*in vivo"* em animais adultos (P80-81) por meio da plestismografia de corpo inteiro em condições normais e

frente a hipercapnia e hipóxia. O consumo de oxigênio foi utilizado para inferência da taxa metabólica. Através de implantes de eletrodos de eletroencefalograma e eletromiograma foi possível determinar os estados de sono e vigília do animal. Os paramentos cardiovasculares foram aferidos através de canulação da artéria femoral. O componente mecânico do sistema respiratório foi avaliado pela complacência pulmonar. Um sensor foi inserido na cavidade abdominal dos animais para medidas de temperatura corporal. Os receptores CB1 no tronco encefálico foram quantificados por técnicas de *Western Blot*. A avaliação quantitativa dos neurônios catecolaminérgicos do tronco encefálico foi realizada através de imunohistoquimica para TH.

Capítulo 1

Os resultados obtidos demonstram que a exposição crônica a canabinóide exógeno (WIN) durante a gestação promoveu uma excitação tônica do *drive* respiratório em machos neonatos (P0) e juvenis (P27-28), acompanhado de uma queda na eficiência respiratória tecidual nos animais P0, e significativas reduções da complacência pulmonar nos juvenis. As consequências do tratamento pré-natal para os machos também se caracterizaram por um aumento da resposta quimiossensível ao CO_2 em todas as idades pós-natais, com exceção dos neonatos P6-7 os quais apresentaram uma hipoventilação. Nas fêmeas, foram observadas alterações na resposta ventilatória à hipercapnia apenas na idade juvenil. Na hipóxia, os machos neonatos P0 tiveram uma hiperventilação, enquanto os P6-7 uma hipoventilação. Paralelamente, um aumento no número de neurônios catecolaminérgicos em determinadas regiões do tronco encefálico foi observado para os machos recém-nascidos, assim como um significativo aumento na expressão de receptores CB1 no tronco encefálico.

Capítulo 2

O tratamento intra-uterino com canabinóide sintético (WIN) desencadeiou mudanças robustas no controle ventilatório relacionadas à quimiossensibilidade ao CO_2 e O_2 de uma maneira sexo dependente para os animais adultos, e algumas dessas mudanças foram dependentes do ciclo sono-vigília. Os machos tratados apresentaram um aumento da resposta ventilatória à hipercapnia e hipóxia durante o sono, enquanto as fêmeas uma redução, seja em estado de sono ou vigília. A exposição pré-natal ocasiou em importantes alterações no controle dos parâmetros cardiovasculares para ambos os sexos. Apesar das alterações cardiorrespiratórias, não foram relatadas alterações

neuroanatômicas quanto à quantificação dos neurônios catecolaminérgicos do tronco encefálico e nem à expressão dos receptores CB1.

Os principais resultados do presente estudo demonstram que a exposição ao agonista de receptor canabinóide (WIN) *in utero* promove mudanças no sistema de controle respiratório de uma forma sexo específica, tanto na vida pós-natal de neonatos quanto na fase adulta. Também foram evidenciadas alterações neuroanatômicas nas idades iniciais do desenvolvimento pós-natal, no qual uma maior expressão de receptores CB1 no tronco encefálico e um aumento quantitativo de neurônios em alguns núcleos catecolaminérgicos nos machos pré-natalmente tratados reforçam os efeitos adversos da exposição ao agonista durante a gestação.

O tratamento pré-natal com WIN não promoveu alterações no tempo de gestação, no ganho de massa corpórea das ratas durante a gravidez, no número de filhotes por ninhadas e nem alterou o peso corporal dos neonatos (P0, P6-7 e P12-13), juvenis (P27-28) e adultos (P80-81) de ambos os sexos. No entanto, a exposição pré-natal ao WIN afetou a mortalidade, resultando em um aumento de mortes de recém-nascidos logo após o nascimento. Sabe-se que o consumo moderado de *Cannabis* durante a gravidez não está associado a um aumento nas taxas de aborto, partos prematuros, anomalias físicas ou quaisquer outras complicações durante a gravidez (Fried, 2002; Gray et al., 2005). No entanto, alguns estudos em modelos animais e estudos de casos em humanos, com utilização dos princípios ativos da *Cannabis* em doses mais elevadas, reportaram uma diminuição na massa corporal (Zuckerman et al., 1989; Fergusson et al., 2002; El Marroun et al., 2009), e uma correlação com aumento nos casos de morte neonatal (Abel et al., 1980; Howard et al., 2019; Grzeskowiak et al., 2020).

Em relação ao controle ventilatório, em condições basais um aumento da ventilação ($\dot{V}_E$) foi evidenciado para os machos P0 tratados, similar aos dados prévios

de Tree et al. (2014). Acreditamos que esse aumento da $\dot{V}_E$ possa estar relacionado aos sintomas de abstinência da droga, uma vez que a administração aguda de canabinóides resulta em uma diminuição da função ventilatória (Padley et al., 2003; Schmid et al., 2003). Adicionalmente, a ativação crônica e excessiva da sinalização endocanabinóide durante a gravidez promoveu alterações no sistema de controle respiratório que afetam principalmente a quimiossensibilidade ao CO_2 durante o desenvolvimento pós-natal inicial, bem como na fase juvenil e adulta. Interessantemente, os machos expostos ao tratamento apresentaram uma hiperventilação frente a hipercapnia nas idades P0, P12-13, P27-28, e nos adultos durante o estado de sono. Para as fêmeas, um padrão de resposta diferente foi encontrado, visto que a hiperventilação ocorreu apenas na idade juvenil e na fase adulta uma hipoventilação ficou evidenciada. Curiosamente, os efeitos do tratamento pré-natal foram evidenciados nas fêmeas apenas a partir da idade juvenil, período em que ocorre o aumento das concentrações dos hormônios sexuais circulantes (Ojeda et al., 1980). Sabe-se que os hormônios sexuais podem atuar sobre sistemas neuromoduladores que influenciam a rede integradora do padrão e geradora do ritmo respiratório no tronco encefálico, *input* sensorial periférico e atividade motora dos músculos respiratórios ocasionando em diferentes respostas ventilatórias entre machos e fêmeas (Regensteiner et al., 1989; Jensen et al., 2008; Gargaglioni et al., 2019).

Os dados do presente estudo sugerem uma maior suscetibilidade dos machos à influência do canabinóide exógeno WIN durante a fase de desenvolvimento intra-uterino e na formação da rede respiratória. Em consonância com os dados ventilatórios, a expressão de receptores CB1 no tronco encefálico foi singnificativamente maior para os machos nas idades iniciais do desenvolvimento. Adicionalmente, a expressão de neurônios catecolaminérgicos nas regiões A1/C1, A2 e C3 foi maior para os machos expostos ao canabinóide sintético durante a gestação. Sabe-se que os receptors CB1

estão localizados em diversas áreas centrais relevantes para a geração e modulação dos padrões respiratórios e integração da atividade motora (Haji et al., 2000), incluindo o gânglio nodoso, o núcleo do trato solitário (Rohof et al., 2012) e o núcleo motor do hipoglosso (Mukhtarov et al., 2005). Sinergicamente, a ativação de núcleos catecolaminérgicos como A1/C1, A2 e C3 é responsável por aumentos acentuados da ventilação (Burke et al., 2014; Menuet et al. 2014; Yamamoto et al., 2015), assim a exacerbada resposta ventilatória ao CO_2 pode ser em decorrência de uma plasticidade da rede de controle respiratório, principalmente pelo fato de que as análises de complacênica do sistema respiratório apenas identifiou o efeito da exposição pré-natal ao WIN nos machos juvenis.

Na fase adulta, a exposição pré-natal ao canabinóide sintético resultou em uma resposta ventilatória quimissensível distinta, no qual os machos apresentaram hiperventilação, enquanto as fêmeas hipoventilação frente aos estímulos hipercápnico e hipóxico. Acreditamos que essas alterações se devam principalmente as modificações na rede de controle respiratório, uma vez que o componente mecânico do sistema respiratório não foi afetado pela exposição pré-natal ao WIN, embora não tenha sido observado alterações neuroanatômicas, em específico sobre a expressão de receptores CB1 e neurônios catecolaminérgicos do tronco encefálico. Tanto os machos quanto as fêmeas podem ser afetados pela ação do canabinóide exógeno na idade embrionária e, assim, o desenvolvimento da rede de controle respiratório sofre alterações, mas as consequências na vida pós-natal podem ser distintas, uma vez que existem diferenças na fisiologia respiratória em que o sexo é um fator importante (Gargaglioni et al., 2019). Alguns núcleos que integram a rede respiratória são sexualmente dimórficos, como o LC (Luque et al., 1992; Hormigo et al., 2015; Bangasser et al., 2016; Gargaglioni et al., 2019) e rafe bulbar (Cordero et al., 1999), ou possuem uma quimiossensibilidade

diferente entre os sexos como o RTN (Niblock et al., 2010; 2012), portanto, a estimulação crônica do sistema endocanabinóide durante a ontogenia do SNC na fase pré-natal pode ter influenciado de forma desigual o desenvolvimento e maturação desses núcleos de controle respiratório resultando em respostas antagônicas entre machos e fêmeas na idade adulta.

Em relação aos parâmetros cardiovasculares em ratos machos juvenis, o único efeito observado nos animais tratados com WIN foi um ligeiro aumento da pressão arterial sistólica (PAS) em repouso, sem afetar a pressão arterial média (PAM) e uma pequena redução da frequência cardíaca (FC) durante a hipercapnia. Portanto, parece que a superestimulação do sistema endocanabinóide não teve grandes impactos no controle cardiovascular, pelo menos em ratos jovens. Já nos animais adultos, em relação a FC, tanto os machos quanto as fêmeas expostos ao WIN tiveram uma FC aumentada em condição de hipóxia. A PAM basal das ratas tratadas foi significativamente maior, e a hipotensão induzida pela hipóxia foi menor para ambos os sexos. Adicionalmente, foi observado uma bradicardia para as fêmeas tratadas em condições de hipercapnia.

Estudos têm demonstrado a ocorrência de hipotensão reflexa induzida por hipóxia (Biancardi et al., 2010; Perim et al., 2020). Essa resposta pode ser observada nos animais adultos controles do presente estudo, entretanto a exposição pré-natal ao agonista atenuou a queda reflexa da PAM durante hipóxia em ambos sexos. Sabe-se que o sistema endocanabinóide atua em regiões de controle cardiovascular como o NTS (Mailleux e Vanderhaeghen, 1992), bulbo ventrolateral rostral (RVLM) (Wang et al., 2017) e núcleo paraventricular do hipotálamo (PVN) (Grzęda et al., 2017). Além disso, os receptores CB1 também são localizados no coração e nos vasos sanguíneos (Liu et al., 2000; Bonz et al., 2003), dessa forma a exposição *in utero* ao agonista também pode ter afetado a função vasomotora desses animais, dificultando mecanismos de

vasocontrição/vasodilatação. Estudos têm demonstrado a participação direta dos endocanabinóides na pressão arterial, contratilidade e modulação da frequência cardíaca (Lake et al., 1997; Sierra et al., 2017). A hipertensão e a taquicardia foram os efeitos cardiovasculares mais marcantes causados pelo uso agudo de *Cannabis* (Weiss et al., 1972) ou administração intravenosa de THC em humanos (Perez-Reyes et al., 1972; Roth et al., 1973). Desta forma, o uso de canabinóides durante a gestação pode promover alterações a longo prazo na rede de controle cardiovascular, acarretando em alterações de repostas pressoras na vida adulta em machos e fêmeas. Interessantemente, a respota bradicardica observada nas fêmeas tratadas em condição de hipercapnia pode ser um evento de causa consequência em decorrência da redução da temperatura coporal que também foi observada nesses animais. Estudos prévios reportaram a influência da temperatura corporal sobre a FC, no qual uma queda da temperatura coporal pode resultar em diminuição da atividade das células marca-passo do coração e dessa forma reduzir a FC (LeBlanc et al., 1976; Davies e Maconochie, 2009). Assim, os efeitos cardiovasculares obtidos para as fêmeas tratadas durante hipercapnia pode ser uma resposta reflexa a redução da temperatura corporal das femeas tratadas, visto que o sistema endocanabinóide, em determinada circunstâncias, pode desempenhar papel regulatório na termogênese (Silvestri e Marzo, 2013; Krott et al., 2016).

Em conjunto, nossos dados demonstraram que a exposição ao agonista de receptor canabinóide durante a gestação tem consequências duradouras e sexo-específicas para o sistema de controle respiratório, afetando o número de neurônios catecolaminérgicos, a expressão de receptores CB1 do tronco encefálico em neonatos de P0 a P12-13, a respiração mitocondrial em neonatos e a complacência pulmonar de animais juvenis. Já na idade adulta, foi observada uma maior quimiossensibilidade ao CO_2 e O_2 para ratos e diminuída para as fêmeas. O controle cardiovascular também foi

alterado em ambos os sexos, onde animais tratados com canabinóide sintético no período pré-natal apresentam maior probabilidade de apresentar hipertensão e taquicardia durante condições ambientais adversas. Porém, não foram observadas alterações no componente mecânico do sistema respiratório, bem como não ocorreram alterações neuroanatômicas, como aumento da expressão de receptores CB1 no tronco encefálico, nem aumento do número de neurônios nas regiões catecolaminérgicas. Esses achados destacam que a interferência externa na sinalização dos endocanabinóides durante o desenvolvimento embrionário causa efeitos de longa duração específicos do sexo para o sistema cardiorrespiratório na idade adulta. Essas descobertas são particularmente relevantes, uma vez que o uso global de *Cannabis* tem aumentado e políticas mais liberais do uso recreativo foram adotadas. Além disso, existe uma grande falta de compreensão entre a população em geral sobre os riscos potenciais do uso de *Cannabis* durante a gravidez. Nossas observações pré-clínicas apóiam que embora as preparações de *Cannabis* possam ser utilizadas de forma medicinal e ter efeitos benéficos em alguns casos, elas também podem causar alterações no sistema cardiorrespiratório em desenvolvimento, e podem ter efeito até na vida adulta da prole. Desta forma, cautela deve ser considerada quando potenciais usos terapêuticos de medicamentos à base de canabinóides forem definidos e regulamentados para mulheres grávidas.

Tabela 1. A - Resumo dos principais resultados obtidos no Capítulo 1 para ratos (esquerda) e ratas (direira) neonatos (P0, P6-7 e P12-13) e juvenis (P27-28). **B** - Resumo dos resultados obtidos no Capítulo 2 para ratos (esquerda) e ratas (direira) adultos (P80-81), durante sono (S) e vigília (V).

A.

CAPÍTULO 1: Neonatos e Juvenis

Parâmetros corporais	-		
Comportamento reflexo	-		
Mecânica respiratória	↓ P27-28		
Respiração tecidual	↓ P0		
Receptores CB1	↑ P0, P6-7 e P12-13		
Neurônios CA	↑ P0 e P6-7		
	Basal	Hipercapnia	Hipóxia
Ventilação	↑ P0 e P27-28	↑ P0, P12-13 e P27-28 ↓ P6-7	↑ P0, ↓ P6-7
Metabolismo	↑ P0	-	-

Parâmetros corporais	-		
Comportamento reflexo	-		
Mecânica respiratória	-		
Respiração tecidual	-		
Receptores CB1	-		
Neurônios CA	-		
	Basal	Hipercapnia	Hipóxia
Ventilação	-	↑ P27-28	-
Metabolismo	-	-	-

B.

CAPÍTULO 2: Adultos

Massa Corporal	-		
Mecânica respiratória	-		
Receptores CB1	-		
Neurônios CA	-		
	Basal	Hipercapnia	Hipóxia
Ventilação	-	↑ S	↑ S
Metabolismo	-	-	-
PAM	-	-	↑ V/S
FC	-	-	↑ S

Massa Corporal	-		
Mecânica respiratória	-		
Receptores CB1	-		
Neurônios CA	-		
	Basal	Hipercapnia	Hipóxia
Ventilação	-	↓ S/V	↓ S/V
Metabolismo	-	-	-
PAM	↑V/S	-	↑ V/S
FC	-	↓ V	↑ V/S

CAPÍTULO 1

Prenatal chronic stimulation of endocannabinoid signaling affects the respiratory control system in neonatal and juvenile rats

ABSTRACT

Cannabis legalization has risen in many countries and its use during pregnancy has increased. The psychoactive compounds of this substance act directly on the endocannabinoid system that is already present in the central nervous system (CNS) at early stages of embryonic development and shows to be an important element for regulating structural and functional brain maturation, including in areas responsible for respiratory control. Regardless of increasing reports of *Cannabis* use during pregnancy, data on the influence of external cannabinoids on the respiratory system development and the possible resulting consequences during postnatal life is limited. We evaluated the possible effects of exposure to a synthetic cannabinoid during the gestational phase on the respiratory control system in neonates (P0, P6-7 and P12-13) and juvenile (P27-28) male and female rats by implanting subcutaneously osmotic pumps in pregnant female rats at embryonic day 0 and delivered vehicle or cannabinoid (WIN 55212-2, 0.5 mg/kg/day) for 21 days. WIN administration to pregnant rats interfered in a sex-specific manner with breathing regulation of offspring, thereby promoting a greater sensitivity to CO_2 at all ages in males (except P6-7) and in juvenile females. An altered chemoreflex in response to hypoxia was observed in P0 (hyperventilation) and P6-7 (hypoventilation) males, without any effects on females. In line with breathing alterations, brainstem analysis showed an increase in the number of catecholaminergic neurons and CB1 receptors and alteration of tissue respiration in early stages of males. A reduction in pulmonary compliance was also observed in juvenile male rats. These findings demonstrate that excess stimulation of the endocannabinoid system during gestation has prolonged and sex-specific consequences for the respiratory control system.

Cannabis is the most common drug of abuse used during pregnancy, and its consumption has been rising among pregnant women in recent years (Brown et al., 2016; Young-Wolff et al., 2019; Bérard, 2020), mainly during the first trimester of gestation due its antiemetic properties (Volkow et al., 2017). Indeed, global legalization and increasing accessibility highlight the critical need for generating more studies concerning possible risks and benefits (e.g. pain reduction) of *Cannabis* use. Cannabinoids easily cross the placenta, thus may interfere with fetal endocannabinoid signaling pathways during neurodevelopment, causing long-lasting effects (Bara et al., 2018). Studies have shown that the psychoactive component of *Cannabis sativa* profoundly affects neural and physiological functions, including motor, cognitive, nociceptive, thermoregulatory and cardiorespiratory processes, through the connection to cannabinoid receptors (Devane et al., 1988; Onaivi et al., 2002). Furthermore, during fetal life, endocannabinoids show to be important for brain development, regulating the differentiation of neural progenitors and synaptogenesis, guiding axonal migration and consolidating synaptic communications (Fernandez-Ruiz et al., 2000; Bernard et al., 2005; Fride et al., 2009).

The development of the ventilatory control system begins early in pregnancy and is a highly dynamic process that can be influenced by environmental factors (e.g. hypoxia or hyperoxia) and chemicals substances (e.g. nicotine, prescription drugs or drugs of abuse) (Bavis and Mitchell, 2008; Campos et al., 2009; Cayetanot et al., 2009; Bairam et al., 2015; Bravo et al., 2016). Particularly about chemical interventions that may occur during the prenatal phase, perinatal manipulation of the endocannabinoid system, either by administration of cannabinoids or by maternal consumption of *Cannabis* may alter the respiratory control system since it is well known that CB1

receptors are expressed in brainstem areas involved in respiratory control and also in the alveolar Type II cells in the lung (Pilowsky et al., 1990; Herkenham et al., 1991; Rice et al., 1997; Tsou et al., 1998; Padley et al., 2003). On the other hand, CB2 receptors are less present in the CNS, e.g., in the cerebellum and some restricted areas of the brainstem (Van Sickle et al., 2005; Onaivi et al., 2006), and mainly expressed in the periphery, such as in cells immune system (Munro et al., 1993). In addition, both receptors are expressed as well in peripheral chemoreceptors (McLemore et al., 2004). Remarkably, central CB1 receptors are already present in the early stages of embryonic development, around 11^{th} to 14^{th} gestational day in rodent (Harkany et al., 2007), coinciding with the time of phenotypic expression of most neurotransmitters (Mulder et al., 2008; Morozov et al., 2009), and seem to be functional already at this phase (Berrendero et al., 1999; Mato et al., 2003), with a progressively increase in the density of CB1 receptors during postnatal development (Rodriguez de Fonseca et al., 1993; Correa et al., 2016).

Studies in animal models show that acute activation of CB1 receptors depress ventilation by acting on the brainstem respiratory rhythm-generating neurons (Graham and Li, 1973; Moss and Friedman, 1976; Doherty et al., 1983; Estrada et al., 1987; Vivian et al., 1998, Schmid et al., 2003; Pfitzer et al., 2004). More importantly, exposure to *Cannabis in utero* is associated with increases in respiratory disease, hypotonia, hypotrophy, neonatal withdrawal and is considered a risk factor for Sudden Infant Death Syndrome (SIDS) (Scragg et al., 2001; Lacroix et al., 2007; Desai et al., 2013). In fact, prenatal exposure to the synthetic cannabinoid WIN 55,212-2 (WIN) in rats increases apnea duration in room air and shows a tendency for the occurrence of increased apneas during hypoxia, reinforcing the participation of endocannabinoids in ventilatory control of neonates (Tree et al., 2014).

Here, we examined the effects of a chronic stimulation of the endocannabinoid system during prenatal phase by using preexposure to a synthetic cannabinoid (WIN) during the entire gestation and by evaluating the postnatal consequences on ventilatory control of neonatal (P0, P6-7 and P12-13) and juvenile (27-28) male and female rats. We observed that fetal exposure to synthetic cannabinoid caused marked sex-specific alterations of breathing regulation of offspring, and chemosensitivity to CO_2 and hypoxia, with a greater sensitivity in males. We also found that embryonic WIN exposure induced an increase in brainstem catecholaminergic (CA) neurons and CB1 receptors and alterations of tissue respiration in early stages of males. Also, a reduction in pulmonary compliance was observed in juvenile male rats. Hence, these findings demonstrate that over stimulation of endocannabinoid system during gestation has long-lasting and sex-specific consequences for the offspring's respiratory control system.

Animals and ethical approval

The male and female arrays were acquired from UNESP – Botucatu, SP, Brazil. Pregnant female Wistar rats and their litters (first generation) were individually placed in cages 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*. The offspring were born in our animal care facility and stayed in the same cage with the mother until they were weaned (P21). Experiments were performed between 7:00 a.m. and 6:00 p.m., during the light phase, on unanesthetized neonatal (P0, P6-7 and P12-13) and juvenile (P27-28) male and female rats obtained randomly from different litters. An accurate animal sexing was done at the day of birth and confirmed at the experimental day.

All the experiments were done in compliance with the guidelines of the National Council of Control in Animal Experimentation (CONCEA-MCT-Brazil), and with the approval of the local College of Agricultural and Veterinary Sciences Animal Care and Use Committee (CEUA-FCAV-UNESP-Jaboticabal; Protocol: 011284/17). All efforts were made to minimize the number of animals and their suffering throughout the experiments.

Drug and gas mixture

The synthetic cannabinoid (WIN 55,212-2 mesylate salt) was purchased from Sigma Chemical CO. (St. Luis, MO, USA) and dissolved in DMSO 50%. The hypercapnic (7% CO_2, 21% O_2, balance N_2) and hypoxic (10% O_2, balance N_2) gas mixture was purchased from White Martins Gases Industrials Ltda (Osasco, SP, Brazil).

Cannabinoid treatment protocol

The pregnant female rats were treated constantly from the 0 to the 21st day of gestation and divided in two groups: 1) treated with vehicle (DMSO 50%, diluted in sterile water); 2) treated with synthetic cannabinoid WIN 55,212-2 (WIN) (Sigma Aldrich, USA), dose of 0.5 mg/kg/day (based on Mereu et al., 2003; Tree et al., 2014; Bara et al., 2018). The vehicle or drug were delivered to the pregnant female rats through osmotic pumps (Alzet Osmotic Pumps, Cupertino, CA, USA; model 2ML4; 2.5 µL/hour/28 days) implanted subcutaneously into the back of the animals between the scapulars, after confirmation of sperm via vaginal smear, under inhalation anesthesia with 5% of isoflurane (Cristália, Sao Paulo, Brazil) for induction and 1% for maintenance. After giving birth, the mothers were anesthetized with isoflurane and the osmotic pump removed.

Straightening reflex and mastication

The straightening and mastication reflexes were evaluated in the male and female newborn rats 4-6 hours after birth. To investigate mastication behavior, the number of jaw opening elicited by an oral stimulation with a P50 tubing (Chatonnet et al., 2007) were counted during 30 s. For the straightening reflex, the rats were placed in the supine position and the time (in sec) until get the prone position was measured (Kroeze et al., 2016).

Respiratory measurements

Neonates (P0, P6-7 and P12-13)

Ventilation ($\dot{V}_E$) of neonates was measured using the pressure-plethysmography method as previously described (Mortola, 1984; Mortola and Frappell,

2013; Patrone et al., 2018; 2020). For each age group a different animal was used. One chamber was used to house the animal's body (50 mL for P0 and P6-7; 80 mL for P12-13) and another chamber was used to allocate the head (15 mL and 30 mL, respectively). The chambers were connected and sealed by each other with a pliable neck collar made of plastic film, and the room air, hypercapnic (7% CO_2, 21% O_2, balance N_2) or hypoxic (10% O_2, balanced with N_2) gas mixture was delivered to the face mask.

The two chambers were placed inside a water bath with a heater maintained at 35°C for P0, 33°C for P6-7 and 30°C for P12-13 (PolyScience, Model 9112 - Serial G48325, IL, USA), keeping the animal's body temperature constant (~36°C), which is the recommended ambient temperature for thermal comfort at these ages (Julien et al., 2008). During the measurements, airflow was not interrupted. The pressure signal obtained from the body chamber during breathing (animal´s ribcage movement) was directly proportional to tidal volume (V_T) of the animal's breath. Volume calibration (0.2 mL of air for P0 and P6-7, and 0.4 mL for P12-13) was performed during each experiment using a graduated syringe attached to the body chamber, which allowed for the calibration of pressure signals (volts) to V_T (mL). The signals 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 a computer equipped with data acquisition software (MP100ACE, Biopac Systems). The sampling frequency was 200 Hz. The LabChart software (PowerLab System, ADInstruments®/ LabChart Software, version 7.3, Sydney, Australia) was used for data analysis. $\dot{V}_E$ was obtained from the multiplication of respiratory frequency (f_R) and V_T, and was normalized to the animal's body mass.

Juveniles (P27-28)

The barometric method by whole body plethysmography – closed system was used to measure ventilation of juvenile rats (P27-28) (Drorbaugh and Fenn, 1955; Patrone et al., 2014; 2018). The animal was placed in a Plexiglas experimental chamber (700 mL) for at least 30 min before initiating measurements at an ambient temperature of 25°C. During $\dot{V}_E$ measurements, airflow was interrupted and the chamber persisted fully sealed for approximately 1 min. The airflow was maintained at 0.7 $L.min^{-1}$ using a flow meter coupled to a suction pump (MFS, Sable Systems International, Inc, Las Vegas, USA) at the air outlet of the chamber. A volume calibration was performed for each experiment by injecting 0.6 mL of air into the chamber using a graduated syringe. V_T was calculated with the appropriate formula from Drorbaugh and Fenn (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 is the pressure deflection associated with each V_T, P_K is the pressure deflection associated with the injection of the calibration volume (V_K), T_A is the air temperature in the animal chamber, P_B is the barometric pressure, P_C is the water vapor pressure in the animal chamber, T_B is the body temperature (in Kelvin), and P_R is the water vapor pressure at T_C. $\dot{V}_E$ and V_T were presented under ambient barometric pressure conditions, at T_C and saturated with water vapor (BTPS). P_C and P_R were calculated indirectly using an appropriate table (Dejours, 1981).

O_2 consumption measurements

Metabolic rate was inferred by indirect calorimetry, measuring O_2 consumption ($\dot{V}O_2$), which was recorded using a flow-through *Pull mode* configuration by an open respirometry system (Mortola, 1984; Cummings et al., 2011; Patrone et al., 2018;

2020). A pump inside the oxygen analyzer (model ML206, ADInstuments®, Australia), connected to the outlet port of the head chamber, controlled the inflow gas rate (100 $mL.min^{-1}$ for P0 and P6-7, and 150 $mL.min^{-1}$ for P12-13). For juvenile (P27-28) animals (150 $mL.min^{-1}$), a MFS (Mass Flow System, Sable Systems International, Las Vegas, IL, USA) was coupled to the animal's chamber outlet to control the airflow inside the chamber (700 $mL.min^{-1}$).

For all experiments, the expired gas was dried over a small column of Drierite (W.A. Hammond Drierite Co. Ltd, Xenia, OH, USA) before passing through the analyzer. The air was continuously sampled by the O_2 analyzer (model ML206, ADInstuments®, Australia), allowing for the determination of $\dot{V}O_2$ by a data acquisition program (Power-Lab System, ADInstruments® / Chart Software, version 7.3, Sydney, Australia).

As CO_2 was neither analyzed nor scrubbed, the $\dot{V}O_2$ was calculated using the following equation (Koteja, 1996):

$$\dot{V}O_2 = [FR_e (F_iO_2 – F_eO_2)] / [1 – F_iO_2 (1 – RQ)]$$

where FR_e is the flow rate of air through the chamber, F_iO_2 is the inlet O_2 fraction, F_eO_2 is the end O_2 fraction, and RQ is the respiratory quotient (considered to be 0.85). The $\dot{V}O_2$ was corrected for the body mass.

Cardiovascular and body temperature measurements

One day before the experiment, the juvenile P27-28 animals were anesthetized with isoflurane (Cristália, Sao Paulo, Brazil) 5% for induction and 1% for maintenance and underwent two surgeries. Through the femoral artery, a catheter [PE-10 connected to PE-50 (Clay Adams, Parsippany, NJ, USA)] was inserted into the abdominal aorta to

measure pulsatile arterial pressure (PAP). The catheter was taken subcutaneously to the animal's dorse until the neck region and externalized. In the next day, in a way that allowed free movement of the rat, this catheter was connected to the pressure transducer (TSD 104A, Biopac systems), the signal was amplifier (DA 100C, Biopac systems) and digitized on a computer equipped with data acquisition software (MP100ACE; Biopac Systems). The cardiovascular parameters, systolic (SAP) and diastolic (DAP) arterial pressure, mean arterial pressure (MAP) and heart rate (HR) were quantified from the PAP records using the LabChart program (Power-Lab System, ADInstruments® / Chart Software, version 7.3, Sydney, Australia).

At the same surgical procedure, a temperature datalogger (SubCue Dataloggers, Calgary, Canada) was inserted into the abdominal cavity through a midline laparotomy for body temperature (T_B) measurements. The datalogger was programmed to acquire data every 5 min. At the end of the surgery, the animals were treated with antibiotic (enrofloxacin, 10 $mg.kg^{-1}$, I.M.; Bayer SA, Sao Paulo, Brazil) and analgesic (flunixin meglumine, 2.5 $mg.kg^{-1}$, S.C.; Schering-Plough Santé Animale, Segré, France) agents.

Experimental protocol

Control (VEH) and WIN-treated P0, P6-7 and P12-13 male and female animals were placed individually into the body and head chambers as previously described, and P27-28 rats were allowed to move freely inside a Plexiglas chamber at room temperature of 25ºC. Initially, the chambers were flushed with room air (21% O_2) for 30 min during the acclimation phase. Posteriorly, $\dot{V}_E$ and $\dot{V}O_2$ were recorded in room air conditions for 10 min. The animals were then exposed to hypercapnic condition (7% CO_2 gas mixture) for 20 min. After CO_2 exposure, the chamber was ventilated again

with room air for 40 min to allow recovery baseline values. Subsequently, the animals were submitted to hypoxia (10% O_2 gas mixture) for 20 min, followed by a recovery period of 20 min with room air. For neonatal animals, $\dot{V}_E$ and $\dot{V}O_2$ were recorded throughout the experiment. For juveniles, the $\dot{V}O_2$ was constantly measured, and the ventilatory parameter measurements were performed at the end of room air condition, and at 5, 10 and 20 min during each gas mixture exposure. The cardiovascular parameters and T_B of the juveniles were also recorded during the entire experiment.

Determination of respiratory mechanics

Measurements of respiratory system mechanics were performed based on previous studies (Frappell et al., 1998; Hedrick et al., 2011). Neonatal (P0, P6-7 and P12-13) and juvenile (P27-28) male and female rats were euthanized by isoflurane inhalation. Next, through a tracheostomy, a cannula was inserted into the animal's trachea. A pressure transducer (TSD 104A, Biopac systems), coupled to the tracheal cannula by a three-way connector, was used to measure intra-tracheal pressure. The signals were amplified (DA 100C, Biopac systems), filtered and recorded in a data acquisition system (Biopac Systems Inc., Santa Barbara, CA, USA). A graduated syringe was also connected to the tracheal cannula to allow the injection and removal of air volumes into the lungs. To obtain the inflation and deflation pressure-volume curves, to assess the static respiratory mechanics, the animals were placed in the supine position and the lungs were gradually inflated and deflated. The volumes of air injected were established according to the animals' age (P0: 0.05 mL; P6-7 and P12-13: 0.1 mL; and P27-28: 0.5 mL). Inflation was stopped when intra-tracheal pressure reached approximately 30 cmH_2O. The lungs were then emptied in the same gradual manner, until the pressure reached -20 cmH_2O, and finally inflated again to the resting lung

pressure (0 cmH_2O). At the end, the trachea was opened to the atmosphere to balance intra-tracheal pressure. To prevent the lungs from collapsing, before starting a new curve, the lungs were fully inflated and then spontaneously allowed to return to resting volume by opening the system to the atmosphere. The static volume–pressure curves allowed determining residual lung volumes as well as the maximum lung volume of each animal. Following the determination of the static pressure-volume curves, maximum volume was injected steadily over an interval of 20 s to determine the dynamic respiratory mechanics. These entire procedures were repeated three times for each animal. After the static and dynamic measurements were carried out on intact animals (C_T), the body cavity and rib cage were opened, the ribs, muscles, diaphragm and abdominal organs were removed leaving the lungs completely exposed. Subsequently, the static and dynamic experimental protocols were repeated with the lungs exposed (C_L). At the end of the experiments, the animals' heart and lungs were removed and weighed.

Quantitative analysis of CB1 receptors

The Western Blot technique was used to assess the expression of CB1 receptor in the brainstem of neonatal (P0, P6-7 and P12-13) and juvenile (P27-28) male and female animals. To this end, the animals were deeply anesthetized with isoflurane and the brainstem quickly removed and frozen in 2-methylbutane at -20°C. The samples were homogenized in RIPA buffer (50 mM tris, 150 mM NaCl, 0.1% triton, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). Previous to Western Blot, quantification of proteins in the tissue was performed by Lowry's method. Samples with 15 μg of protein were mixed with the sample buffer (1.25 M tris pH 6.8, 2% SDS, 0.01% blue bromophenol, 10% glycerol, and 250 mM 2-ME) and heated to 95ºC for 5

minutes. The samples were submitted to SDS-PAGE in bis-acrylamide gel with 10% gradient, in buffer (192 mM glycine, 25 mM tris, and 0.1% SDS, pH 8.3). The proteins that migrated in the gel were transferred to the nitrocellulose membrane (Bio-Rad Laboratories, ON, Canada) by wet electrotransfer (15 mM tris, 120 mM glycine, and 20% methanol, pH 8.3). After transfer, non-specific sites on the membrane were blocked with T-TBS buffer (20 mM tris, 150 mM NaCl, and 0.1% tween 20, pH 7.4) with 5% bovine serum albumin (BSA) for 1 hour at room temperature, under constant agitation. After washing the blocking solution with T-TBS, the membranes were incubated overnight at 4ºC with the rabbit polyclonal anti-CB1 antibody (1:1000; Sigma) in T-TBS solution with 3% BSA. The membranes were washed five times for 5 minutes with T-TBS, and then incubed with anti-rabbit peroxidase-labeled secondary antibody (1:5000, Santa Cruz Biotechonology) diluted in T-TBS with 5% BSA for 1 h at room temperature. Posteriorly, the membranes were washed again five times for 5 minutes with T-TBS and added the chemiluminescence enhancer (ECL) and the film exposed. The bands were quantified using the ImageJ program (available for free download at https://imagej.nih.gov/ij/download.html). The bands of the CB1 protein were normalized by β-actin using mouse monoclonal anti-βActin (1:15000, Sigma), followed by anti-mouse peroxidase-labeled secondary antibody (1:20000, Santa Cruz Biotechonology).

Assessment of catecholaminergic (CA) neurons

At the end of the plethysmography experiments, neonatal (P0, P6-7 and P12-13) and juvenile (P27-28) male and female rats were deeply anesthetized with isoflurane and perfused intracardially using a pump machine (Masterflex; Cole-Parmer Instrument Company, Vernon Hills, IL, USA) with phosphate buffered saline (PBS, 0.01 M, pH

7.4), followed by 4% paraformaldehyde (PFA) in 0.2 M phosphate buffer (PB). The brain was removed from the skull, postfixed with 4% PFA at 4°C for 12 h, and then immersed in 30% sucrose solution for at least 48 h at 4°C. The brain was dipped in 2-methylbutane at -20°C, frozen and fixed in Tissue-Plus (Fisher Healthcare™ O.C.T. Compound, CA, USA). Serial sections (40 μm) of the brainstem were made in triplicates using a cryostat microtome (CM1860 – Ag Protect; Leica, Wetzlar, Germany).

Immunohistochemistry for tyrosine hydroxylase (TH) was performed to quantify the CA neurons in the brainstem (Xu et al., 2003). Initially, the slices were washed 3 times with PBS for 5 min, followed by an antigenic recovery process, where slices were incubated for 30 min in a target retrieval solution (Dako, Glostrup, Denmark) at 70°C, then cooled to room temperature and washed 3 times with PBS for 5 min. The slices were incubated in 1% hydrogen peroxide solution for 3 min, washed and followed by 1 h in a 10% horse serum solution (Life Technologies, USA) at room temperature to prevent non-specific binding. After rinse, slices were incubated for 24 h with a mouse monoclonal anti-TH antibody (1:10000; Sigma) in T-PBS (0.3% Triton-PBS, pH 7.4) solution with 5% horse serum at room temperature with constant agitation. Then, the slices were washed and incubated for 3 hours with secondary goat anti-mouse IgG antibody (h&l), conjugated to dy light 488 (1:300, Immunoreagents, NC, USA,) at room temperature, also on a shaker. After that, the slices were washed 3 times with PBS. Finally, the slices were mounted on gelatinized sheets, dried and covered with coverslip.

Tissue respiration measurement

To obtain fresh biological material for brainstem tissue respiration analysis, neonatal (P0, P6-7 and P12-13) and juvenile (P27-28) male and female rats were profoundly anesthetized with isoflurane and the brain was removed from the skull and the brainstem dissected. A longitudinal cut in the midline of the brainstem was performed to obtain approximately 30 mg of wet tissue, then the sample was homogenized in 5 mL of MiR05 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1 g/L albumin, pH 7.1) of which 2.1 mL were inserted into each respirometric chamber. Respiration was maintained by 10 mM glutamate and 4 mM malate.

Respiratory rates were determined monitoring oxygen consumption in an Oxygraph-2k respirometer (Oroboros, Innsbruk, Austria) containing 2.1 mL of air saturated respiration medium. The respiratory states were determined as follows: NADH-linked, after substrate (9 mM glutamate and 5 mM malate) addition; OXPHOS (phosphorylation), in the presence of adenosine diphosphate (ADP, 1 mM); LEAK (non-phosphorylating), after ATP synthase inhibition by oligomycin (1 μg/mL); ETS (non-coupled), in the presence of the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP, 1 μM); Rox (residual), after complex III inhibition by antimycin A (AA, 3 μM). The value of Rox was subtracted from the other states.

Data and statistical analysis

The body mass was acquired from animals of different experimental protocols throughout the study and grouped according to treatment and age, and for heart and lungs weight, animals were obtained from respiratory mechanic protocol.

The cardiorespiratory and metabolic parameters were collected at the end of room air conditions and at 10 min during exposure to hypercapnia and hypoxia, when the effects were most evident. Approximately 2 min of recording were used to respiratory (f_R and V_T) and metabolic ($\dot{V}O_2$) measurements for P0, P6-7 and P12-13 animals, and 1 min for P27-28. Additionally, the variability of breath duration (T_{TOT}) was analyzed, as described by Patrone et al. (2018). $\dot{V}O_2$ was calculated with the suitable formula and also normalized by animal's body mass. The air convection requirement ($\dot{V}_E / \dot{V}O_2$) was obtained by dividing the values of $\dot{V}_E$ by the $\dot{V}O_2$. The respiratory and metabolic variables are represented as percentile and median with the graphs presented as boxplots, whereby the mean, 25%, and 75% quartiles are used, except for variability data, which is reported as mean ± SEM. The cardiovascular data are presented as mean ± SEM. All the analysis and interpretation in the present study were performed based on the means.

The evaluation of the mechanical component of the respiratory system was assessed through the static and dynamic compliance of the total system (C_T, intact animal), lungs (C_L, lungs exposed) and body wall (C_B, $C_T - C_L$). The static compliance was measured at the steepest stretch of both inflation and deflation curve around 0-15 cmH_2O, which is the normal range experienced *in vivo* intrapulmonary pressures over a ventilatory cycle. This curve was constructed using the values of intra-tracheal pressure (cmH_20) for each volume of air injected (mL). The highest and lowest point on the steep inflation and deflation curve were applied to the formula: V2-V1 / P2-P1 to obtain inspiratory and expiratory compliance. Dynamic compliance was calculated using the same formula for intra-tracheal pressure values of 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 26, 31 and their corresponding injected air volume, following Hedrick et al., (2011). For both static and dynamic measurements, body wall (C_B) compliance was calculated by

the differences between C_T and C_L ($1/C_T = 1/C_L + 1/C_B$). Static compliance data are given as percentile and median with the graphs presented as boxplots, while dynamic compliance is shown as interconnected mean.

Brainstem immunohistochemistry photomicrographs were captured using a fluorescence microscope (Zeiss, Axio Image Z2, Baden-Württemberg, Germany) using the LAS image acquisition program. The analysis was based in the quantification of immunoreactive cells for TH (TH-ir) within brainstem CA nuclei using a computerized image analysis system (ImageJ). Using one of the triplicates, the cell bilaterally counting was performed over the entire length of the brainstem in which it contained the CA nuclei A1/C1, A2, C3, A5 and A7 (based on anatomical landmarks from Paxinos and Watson, 1998). Specifically, for the A6 region, due to the large number of noradrenergic neurons, the comparative analysis between the groups was done through the density of TH expression per fixed sampled area. The intensity of fluorescent light emitted in the tissue, as well as the adjustment of contrast and brightness were the same among all animals. To avoid background interference, the density of the sampled A6 region was subtracted by the density region without TH labelling. Representative sections from the control and WIN-treated groups were acquired at the same coordinate. The quantification data are represented as percentile and median with the graphs presented as boxplots.

The parameters evaluated in tissue respiration were oxygen consumption during phosphorylation (OXPHOS), oxygen consumption with ATP synthase inhibition (LEAK), oxygen consumption during maximum performance, stimulated by chemical uncoupler CCCP (ETS), ratio of normalized phosphorylation by uncoupling (P/L), ratio of uncoupling with phosphorylation as reference (L/P), ratio of phosphorylation with maximum capacity as a reference (P/E) and ratio of uncoupling with the maximum

respiratory capacity as a reference (L/E). The data are reported as percentile and median with the graphs presented as boxplots.

Sigma-Stat version 11 software was used for statistical analyses. The variables of the present study were compared between groups by two-way ANOVA, with repeated measures when appropriated. *Post-hoc* multiple comparisons were performed using Tukey's test. The results of the statistical analysis are detailed in the Supplementary Figure 2. Statistical results with $P < 0.05$ were considered significant.

The exposure during pregnancy to synthetic cannabinoid (WIN 55,212-2; 0.5 mg/kg/day) did not promote premature or late births, since control and WIN-treated females gave birth between the 21 and 22st day of gestation in the same proportion. The treatment also did not alter the body mass gain of females during pregnancy, as well as did not change the average number of newborns per litter. However, prenatal WIN exposure affected neonatal mortality, since an increase of 29% of death at birth was observed in the treated group litters ($P < 0.001$, Chi-square). Regarding the offspring, Table 1 shows the body mass and weight of heart and lungs of neonatal and juvenile rats. As expected, the body mass significantly increased with age, and in the opposite way, the ratio of heart and lungs' weight to body mass decreased, but none of these parameters were altered by prenatal WIN-treatment.

Table 1. Body mass and weight of heart and lungs for P0, P6-7, P12-13 and P27-28 control and WIN-treated male and female rats.

			Body mass (g)	Heart (g.kg^{-1})	Lungs (g.kg^{-1})
P0	**Male**	VEHICLE	7.0 ± 0.8	8.6 ± 0.5	20.9 ± 0.8
		WIN	6.7 ± 0.5	8.5 ± 0.4	21.7 ± 1.0
	Female	VEHICLE	6.8 ± 0.3	9.0 ± 0.4	22.8 ± 1.2
		WIN	6.6 ± 0.2	9.9 ± 0.4	22.7 ± 0.9
P6-7	**Male**	VEHICLE	18.7 ± 0.8	8.3 ± 0.2	18.1 ± 0.6
		WIN	16.7 ± 0.5	9.2 ± 0.8	20.5 ± 0.5
	Female	VEHICLE	17.1 ± 0.9	7.6 ± 0.3	19.9 ± 0.4
		WIN	16.3 ± 0.5	8.5 ± 0.3	20.8 ± 0.9
P12-13	**Male**	VEHICLE	32.6 ± 1.6	7.4 ± 0.3	16.1 ± 0.8
		WIN	32.0 ± 0.7	7.6 ± 0.3	17.6 ± 0.9
	Female	VEHICLE	30.8 ± 1.3	7.7 ± 0.4	14.9 ± 0.8
		WIN	31.1 ± 0.9	7.0 ± 0.3	16.9 ± 1.0
P27-28	**Male**	VEHICLE	85.7 ± 2.7	6.2 ± 0.4	7.9 ± 0.4
		WIN	86.8 ± 3.7	6.0 ± 0.3	8.5 ± 0.3
	Female	VEHICLE	82.6 ± 2.7	6.2 ± 0.3	8.5 ± 0.2
		WIN	79.4 ± 2.7	5.7 ± 0.3	8.7 ± 0.3

Values are expressed as mean ± S.E.M. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 2A.

Reflex behavior

Figure 1 shows the results for straightening (A) and mastication (B) reflexes for P0 control and WIN-treated male and female animals. As evidenced, the prenatal treatment with synthetic cannabinoid did not cause significant differences in these reflex behaviors for both sexes.

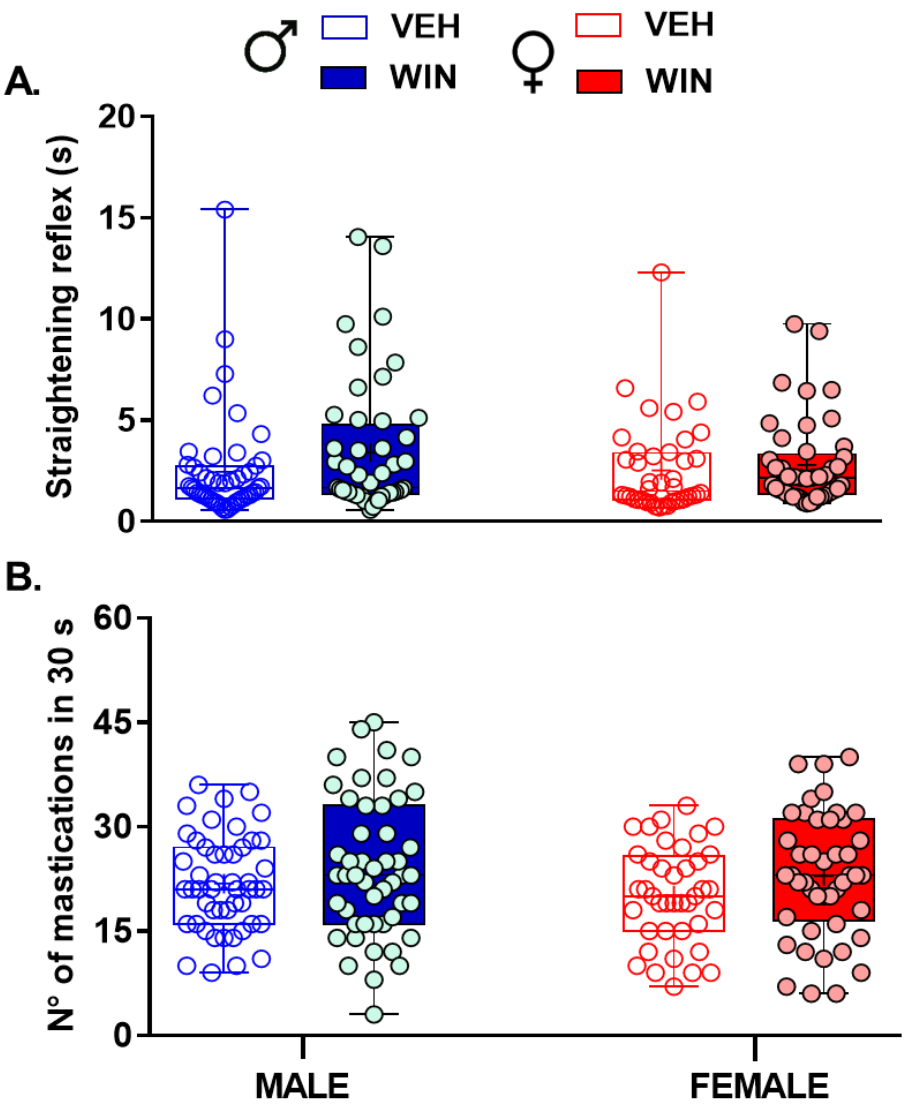


Figure 1: Effect of prenatal WIN exposure on straightening (**A**) and mastication reflexes (**B**) in P0 male and female rats. The graphs are presented as boxplots. Values are expressed as percentile and median. $^{+}$indicates the mean.

Breathing pattern and Metabolism

The intra-uterine synthetic cannabinoid WIN exposure resulted in significant alterations in breathing control during postnatal development. Table 2 shows the effect of WIN-treatment on ventilation ($\dot{V}_E$), tidal volume (V_T), respiratory frequency (f_R), oxygen consumption ($\dot{V}O_2$) and air convection requirement ($\dot{V}_E / \dot{V}O_2$) for P0, P6-7, P12-13 and P27-28 control and WIN-treated male and female rats, under resting condition. For P0 male, prenatal treatment resulted in a significant increase of 45% in resting $\dot{V}_E$ compared with control group, as well as an increase in basal $\dot{V}O_2$. The intra-uterine exposure also caused changes in the baseline $\dot{V}_E$ of juvenile male rats with an increase of 25%, due to a significant increase in V_T. Additionally, an increase in $\dot{V}_E / \dot{V}O_2$ for the P27-28 treated male was evidenced. There were no effects of treatment for other males' age, as well as for females.

Table 2. Ventilation ($\dot{V}_E$), tidal volume (V_T), respiratory frequency (f_R), oxygen consumption ($\dot{V}O_2$) and air convection requirement ($\dot{V}_E / \dot{V}O_2$) for P0, P6-7, P12-13 and P27-28 control and WIN-treated male and female rats, under resting condition.

			$\dot{V}_E$ (mL.kg^{-1}.min^{-1})	V_T (mL.kg^{-1})	f_R (breaths.min^{-1})	$\dot{V}O_2$ (mL.kg^{-1}.min^{-1})	$\dot{V}_E / \dot{V}O_2$
P0	**Male**	VEHICLE	1216.4 ± 73.8	12.7 ± 0.9	99.0 ± 9.1	41.6 ± 5.6	32.0 ± 3.6
		WIN	1762.7 ± 197.4*	14.9 ± 1.5	118.5 ± 6.4	61.3 ± 7.7*	29.0 ± 0.8
	Female	VEHICLE	1426.7 ± 160.6	12.4 ± 1.4	115.6 ± 4.1	56.2 ± 5.4	25.6 ± 2.4
		WIN	1827.0 ± 137.8	15.9 ± 1.1	114.8 ± 6.8	66.8 ± 5.5	27.7 ± 1.5
P6-7	**Male**	VEHICLE	1927.6 ± 188.8	11.7 ± 0.6	163.9 ± 10.9	45.3 ± 4.0	42.7 ± 2.0
		WIN	1625.7 ± 94.4	10.3 ± 0.4	156.5 ± 4.6	45.7 ± 3.3	36.4 ± 2.5
	Female	VEHICLE	1654.3 ± 112.3	11.1 ± 0.9	152.2 ± 9.9	42.4 ± 2.9	39.2 ± 1.5
		WIN	1539.0 ± 104.3	10.4 ± 0.6	147.4 ± 4.9	41.4 ± 3.6	37.7 ± 1.8
P12-13	**Male**	VEHICLE	971.4 ± 86.0	7.5 ± 0.4	129.8 ± 9.1	42.9 ± 1.9	22.7 ± 1.9
		WIN	1156.0 ± 129.3	8.5 ± 0.7	133.9 ± 4.7	43.5 ± 2.1	26.9 ± 2.7
	Female	VEHICLE	958.9 ± 19.3	7.3 ± 0.3	131.1 ± 4.2	44.1 ± 1.7	21.8 ± 0.9
		WIN	1025.4 ± 77.9	8.7 ± 0.6	118.5 ± 4.2	43.7 ± 1.8	22.1 ± 1.0
P27-28	**Male**	VEHICLE	1868.1 ± 87.7	12.7 ± 0.5	148.3 ± 5.6	36.4 ± 1.9	52.3 ± 3.1
		WIN	2371.7 ± 196.8*	15.9 ± 1.1*	148.7 ± 5.2	38.0 ± 3.5	65.0 ± 5.6*
	Female	VEHICLE	1635.8 ± 54.2	11.6 ± 0.5	141.3 ± 4.6	30.5 ± 3.0	57.7 ± 5.8
		WIN	1960.8 ± 94.0	13.3 ± 0.7	148.3 ± 6.3	36.9 ± 4.2	58.0 ± 7.8

Values are expressed as mean ± S.E.M. * Indicates significant difference between control and WIN-treated groups at the same age. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 2B.

With regard to the hypercapnic ventilatory response (HCVR), high levels of CO_2 caused an increase in $\dot{V}_E$ in all male and female groups, because of increases in V_T and f_R in control and WIN-treated male and female animals. The values related to ventilatory (A) and metabolic (B) parameters during 7% CO_2 exposure for neonatal and juvenile males and females are shown in the Figure 2 and 3, respectively. As can be seen, the ventilatory response to CO_2 of all postnatal male ages was affected by intra-uterine exposure to synthetic cannabinoid WIN (Figure 2A), since P0, P12-13 and P27-28 treated males had an increased $\dot{V}_E$, mainly due to a significant increase in V_T, and also in the f_R for P0 WIN-treated male; however, P6-7 treated group had a reduced $\dot{V}_E$ and V_T compared with the control group. Prenatal exposure to WIN did not result in

metabolic changes at any male age (Figure 2B), but $\dot{V}_E / \dot{V}O_2$ of P0, P12-13 and P27-28 treated males was higher, and for P6-7 treated group significantly lower.

For female offspring, prenatal exposure to WIN resulted in an increased $\dot{V}_E$ during CO_2 challenge only for P12-13 and P27-28 treated rats (Figure 3A), also followed by a higher V_T. The use of synthetic cannabinoid WIN during pregnancy did not result in ventilatory control changes at other postnatal age for females, nor did it alter the metabolic demand and the $\dot{V}_E / \dot{V}O_2$ of these animals, except for juvenile WIN-treated group, which showed a significant increase in $\dot{V}_E / \dot{V}O_2$ (Figure 3B).

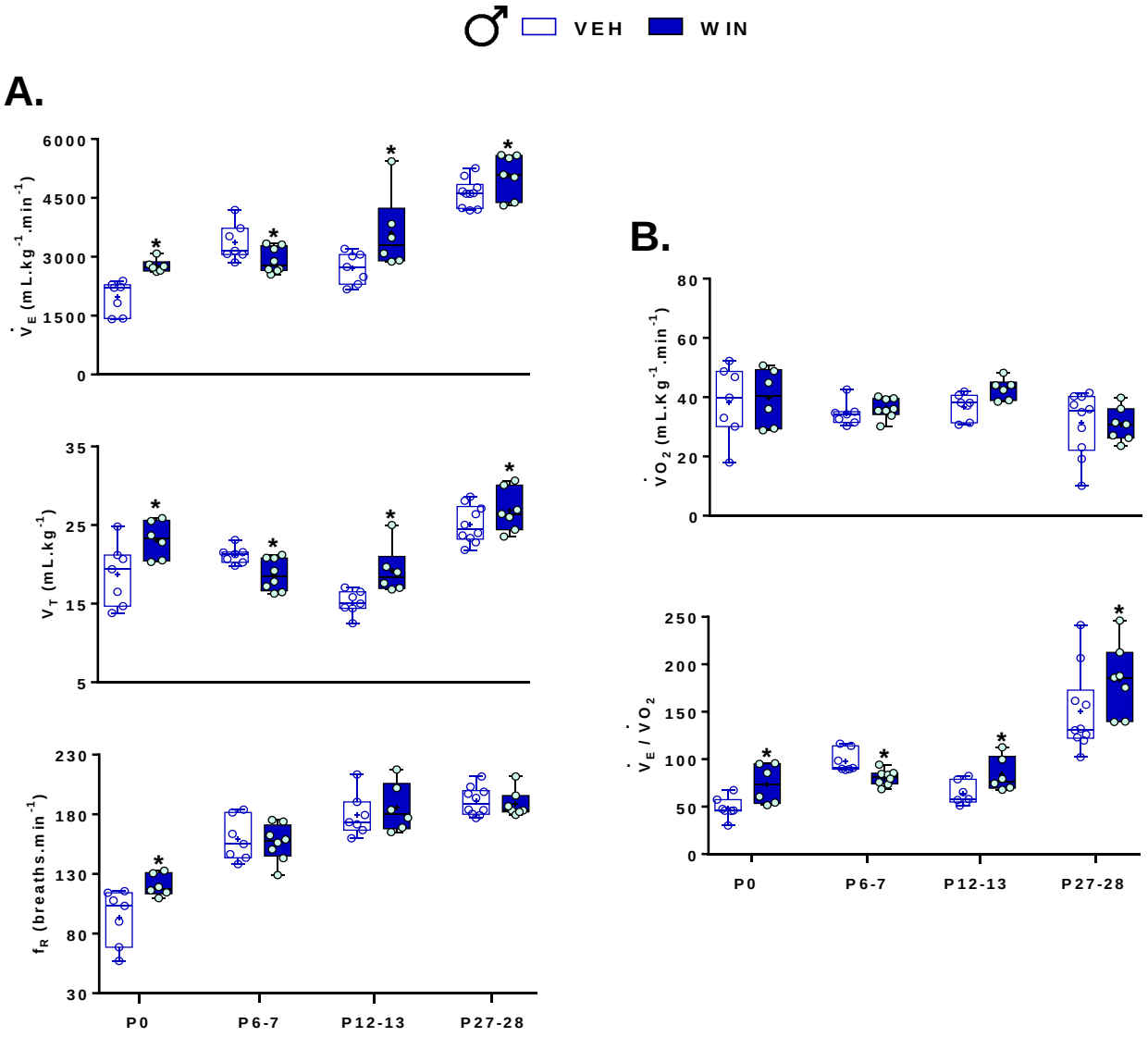


Figure 2: Effect of prenatal WIN exposure on **A**: ventilation ($\dot{V}_E$), tidal volume (V_T), respiratory frequency (f_R); **B**: oxygen consumption ($\dot{V}O_2$) and air convection requirement ($\dot{V}_E / \dot{V}O_2$) for P0, P6-7, P12-13 and P27-28 control and WIN-treated male rats during hypercapnia (7% CO_2) condition. The graphs are presented as boxplots. Values are expressed as percentile and median. $^+$ indicates mean. * indicates a significant difference between control and WIN-treated groups at the same age. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 2C.

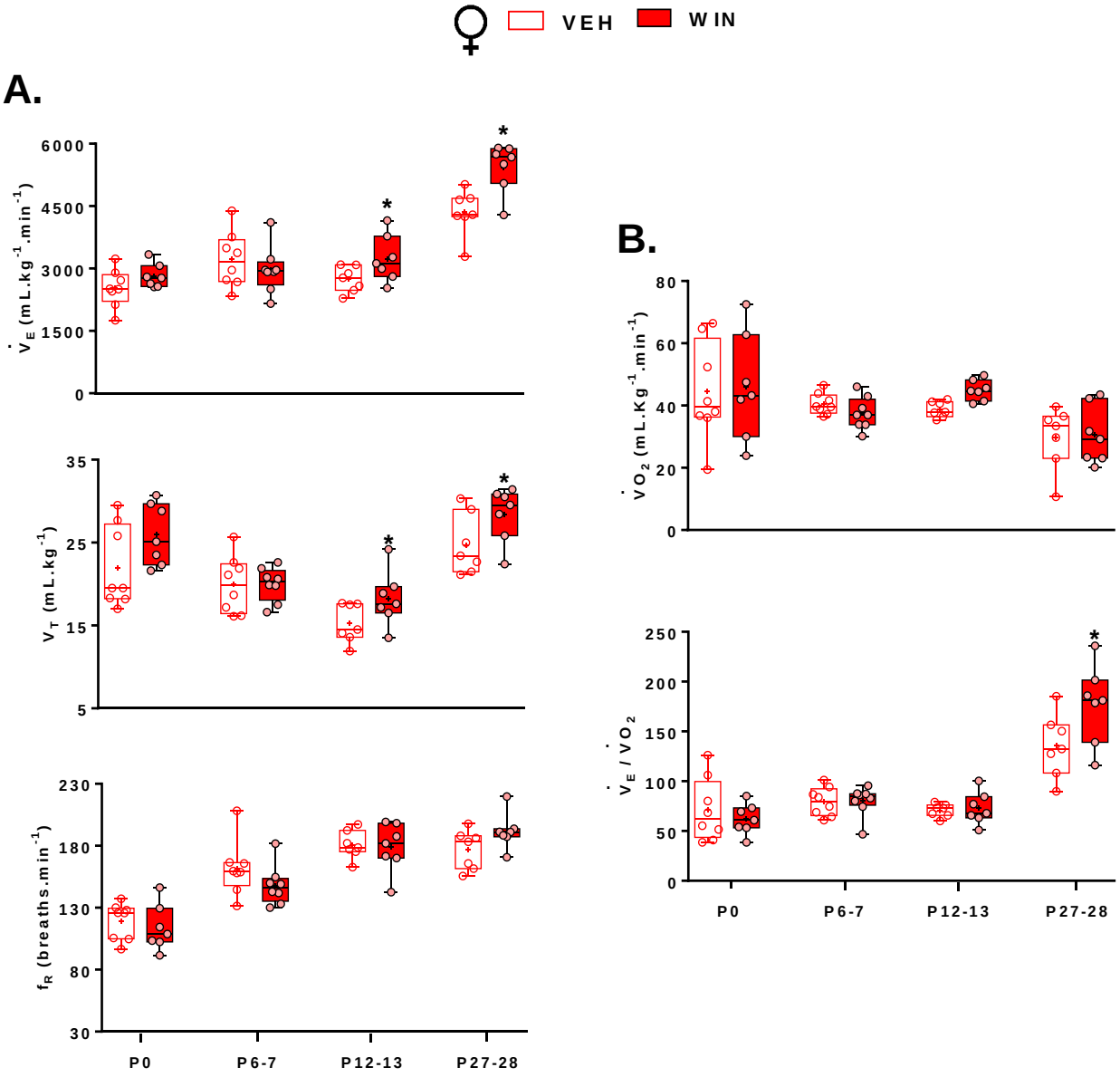


Figure 3: Effect of prenatal WIN exposure on **A**: ventilation ($\dot{V}_E$), tidal volume (V_T), respiratory frequency (f_R); **B**: oxygen consumption ($\dot{V}O_2$) and air convection requirement ($\dot{V}_E / \dot{V}O_2$) for P0, P6-7, P12-13 and P27-28 control and WIN-treated female rats during hypercapnia (7% CO_2) condition. The graphs are presented as boxplots. Values are expressed as percentile and median. $^+$ indicates mean. * indicates a significant difference between control and WIN-treated groups at the same age. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 2D.

The $\dot{V}_E$ of all postnatal ages of both sexes was significantly increased not only by high levels of CO_2, but also by lower environmental O_2 concentration, with sustained higher V_T and f_R. Further, $\dot{V}O_2$ of all groups was reduced by exposure to hypoxia, with

the exception of P6-7 male and female groups, which had no change in the $\dot{V}O_2$ compared to the corresponding control groups. Figures 4 and 5 show the consequences of prenatal WIN exposure on respiratory (A) and metabolic (B) variables during hypoxic condition for male and female rats, respectively. For males, the prenatal WIN-treatment resulted in a higher hypoxic ventilatory response (HVR) at P0 age, due to an increase in f_R; however, at P6-7 age, the HVR of treated newborns was lower compared with control group, although there were no significant changes in both V_T and f_R parameters, as evidenced in Figure 4A. In addition, no change in $\dot{V}O_2$ was observed for both ages. Thus, a significant increase in $\dot{V}_E / \dot{V}O_2$ for P0 treated newborns, as well as a reduction for P6-7 treated rats were observed (Figure 4B). For older males, no ventilatory and metabolic changes were observed in WIN-treated animals during hypoxic condition.

Regarding females, WIN exposure during gestational period did not affect the postnatal ventilatory response to hypoxia. As shown in the Figure 5A, $\dot{V}_E$ of neonatal and juvenile WIN-treated females was not changed during hypoxia exposure, although alterations in the ventilatory pattern was observed, since an increase in V_T, concomitant with a reduction of f_R was found in P0 treated newborns, and a reduction in f_R for P12-13. Regarding the metabolic measurement for female group, the intra-uterine exposure to WIN did not affect the $\dot{V}O_2$ at any postnatal age, in the same way that $\dot{V}_E / \dot{V}O_2$ was not altered by WIN treatment (Figure 5B).

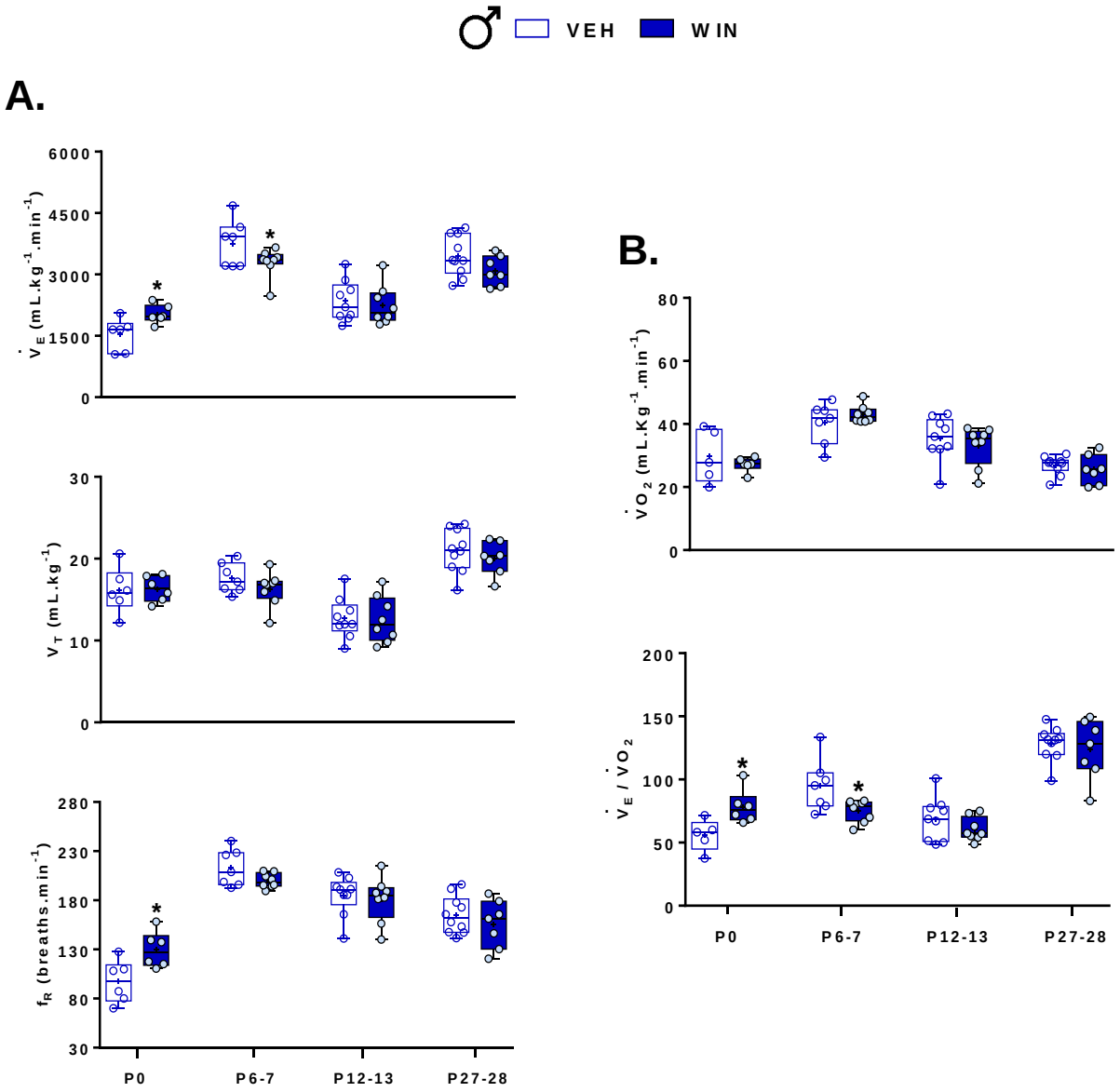


Figure 4: Effect of prenatal WIN exposure on **A**: ventilation ($\dot{V}_E$), tidal volume (V_T), respiratory frequency (f_R); **B**: oxygen consumption ($\dot{V}O_2$) and air convection requirement ($\dot{V}_E / \dot{V}O_2$) for P0, P6-7, P12-13 and P27-28 control and WIN-treated male rats during hypoxic (10% O_2) condition. The graphs are presented as boxplots. Values are expressed as percentile and median. $^+$ indicates mean. * indicates a significant difference between control and WIN-treated groups at the same age. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 2E.

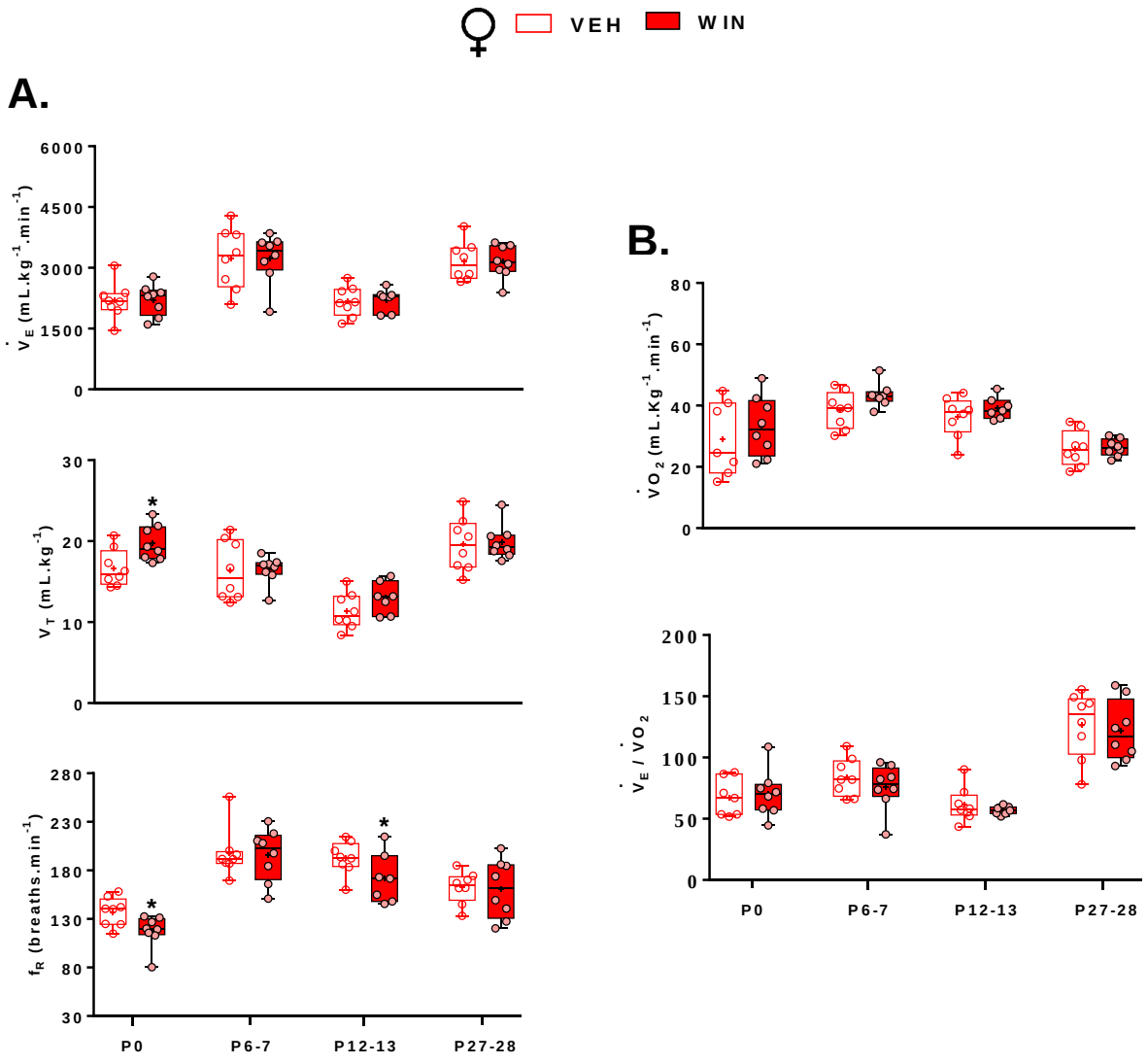


Figure 5: Effect of prenatal WIN exposure on **A**: ventilation ($\dot{V}_E$), tidal volume (V_T), respiratory frequency (f_R); **B**: oxygen consumption ($\dot{V}O_2$) and air convection requirement ($\dot{V}_E / \dot{V}O_2$) for P0, P6-7, P12-13 and P27-28 control and WIN-treated female rats during hypoxic (10% O_2) condition. The graphs are presented as boxplots. Values are expressed as percentile and median. $^+$ indicates mean. * indicates a significant difference between control and WIN-treated groups at the same age. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 2F.

The breathing variability was analyzed by quantification of cycle duration (T_{TOT}) distribution points (SD1 and SD2) during basal, hypercapnic and hypoxic conditions for neonatal and juvenile control and WIN-treated male and female rats,

which is shown in Table 3. The main findings for synthetic cannabinoid prenatal treatment on T_{TOT} variability was at baseline, with P6-7 treated males presenting a lower variance, P12-13 treated males a reduction in SD1 and SD2 variables, while P12-13 female WIN-treated group showed a significant increase. Additionally, P27-28 prenatal treated animals of both sexes exhibit an increase in breathing variability during resting condition. There was no evidence of WIN treatment effects in other conditions. In general, exposure to high levels of CO_2, as well as reduced oxygen content, resulted in significant reduction respiratory variability as described in the Table 3 and Supplementary Table 2G.

Table 3. The variability of breath duration (mean ± S.E.M.) at basal, hypercapnia (7% CO_2) or hypoxia (10% O_2) of P0, P6-7, P12-13 and P27-28 control (VEH) and WIN-treated male and female rats.

AGE	VEH			WIN		
P0	**Basal**	**7% CO_2**	**10% O_2**	**Basal**	**7% CO_2**	**10% O_2**
Male						
SD1	533.0 ± 201.1	270.0 ± 113.0	211.3 ± 59.1^{+}	339.4 ± 146.3	123.5 ± 26.2	90.7 ± 12.2
SD2	598.5 ± 229.2	272.0 ± 98.1	207.7 ± 51.0^{+}	433.8 ± 190.8	146.9 ± 29.9	96.1 ± 10.4^{+}
Female						
SD1	242.5 ± 113.0	96.9 ± 26.8	110.9 ± 39.5	412.6 ± 142.0	203.6 ± 60.4^{+}	143.7 ± 42.5^{+}
SD2	287.9 ± 116.2	131.3 ± 19.8	116.8 ± 29.8	433.1 ± 134.0	199.7 ± 46.9^{+}	170.4 ± 54.0^{+}
P6-7						
Male						
SD1	91.9 ± 19.6	65.6 ± 7.3	34.8 ± 6.4^{+}	58.2 ± 9.9*	48.9 ± 5.6	40.1 ± 6.2
SD2	128.3 ± 21.7	108.7 ± 10.2	48.8 ± 6.9^{++}	86.2 ± 7.6*	83.9 ± 3.3	55.3 ± 7.1
Female						
SD1	73.2 ± 10.0	49.2 ± 5.9^{+}	49.7 ± 5.5^{+}	84.4 ± 9.3	58.7 ± 5.5^{+}	49.2 ± 3.0^{+}
SD2	111.4 ± 12.4	86.2 ± 7.9	70.3 ± 6.2^{+}	121.7 ± 11.5	100.1 ± 9.3	63.2 ± 2.7^{++}
P12-13						
Male						
SD1	105.8 ± 21.6	34.6 ± 6.0^{+}	23.7 ± 1.9^{+}	60.4 ± 8.2*	46.2 ± 11.1	29.4 ± 3.1
SD2	138.5 ± 29.7	54.4 ± 9.1^{+}	35.2 ± 3.1^{+}	78.3 ± 12.0*	63.1 ± 12.6	43.1 ± 4.5
Female						
SD1	47.7 ± 7.4	41.5 ± 7.1	27.5 ± 4.6	85.8 ± 15.7*	22.1 ± 3.1^{+}	33.3 ± 6.7^{+}
SD2	73.5 ± 10.0	62.4 ± 7.9	41.7 ± 4.9	117.4 ± 21.7*	35.7 ± 4.3^{+}	50.6 ± 8.4^{+}
P27-28						
Male						
SD1	23.5 ± 1.2	9.7 ± 1.7	51.9 ± 2.8^{++}	53.3 ± 19.8*	14.0 ± 2.0^{++}	82.3 ± 6.8*
SD2	28.5 ± 2.6	12.3 ± 2.1	79.8 ± 4.5^{++}	82.7 ± 36.6*	17.5 ± 2.4^{++}	88.0 ± 12.2
Female						
SD1	24.2 ± 3.0	12.8 ± 2.3	84.6 ± 13.7^{++}	55.9 ± 18.8*	9.9 ± 1.7^{++}	85.2 ± 7.9
SD2	35.9 ± 5.4	16.6 ± 3.5	90.7 ± 10.8^{++}	98.0 ± 34.1*	12.1 ± 2.0^{++}	91.7 ± 6.0

Both SD1 and SD2 presented in ms. $^{+}$ Indicates significant difference compared with basal condition. $^{++}$ Indicates significant difference compared with the other two environmental conditions. * Indicates significant difference between control and WIN-treated groups in the same condition. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 2G.

Mechanical component of the respiratory system

Chronic exposure to synthetic cannabinoid WIN during pregnancy also affected the mechanical component of the respiratory system in the postnatal offspring's life. Figure 6 shows the static mechanical properties as total (C_T – A and B), lung (C_L – C and D) and body wall (C_B – E and F) compliance during inflation and deflation for P0, P6-7, P12-13 and P27-28 control and WIN-treated males. Prenatal WIN-treatment mostly caused mid-term postnatal changes in all static compliance parameters, since

WIN-treated juvenile male rats had a lower C_T and C_L during inflation and deflation, as well as a reduced C_B at deflation. A significant, but punctual reduction in C_L at deflation was observed for P0 treated newborns, however not sustained for other mechanical variables, as well as any change was observed for P6-7 and P12-13 male ages.

The same static mechanical components were evaluated in females, and as can be seen in Figure 7, intra-uterine exposure to synthetic cannabinoid WIN did not result in respiratory system compliance changes, with the exception of a reduction in C_B at deflation for juvenile treated group.

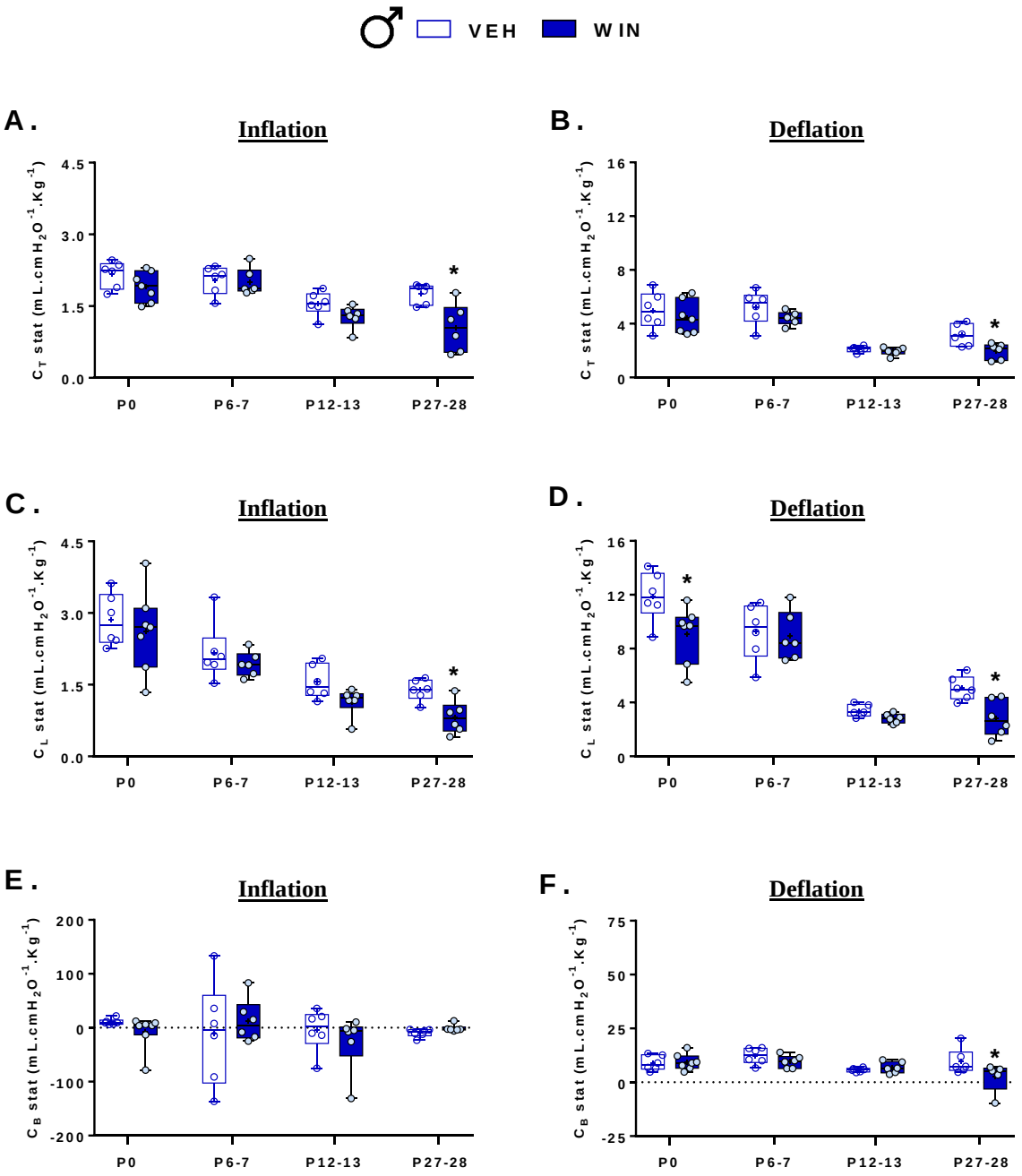


Figure 6: Effect of prenatal WIN exposure on total (C_T – **A** and **B**), lung (C_L – **C** and **D**) and body wall (C_B – **E** and **F**) static compliance during inflation and deflation for P0, P6-7, P12-13 and P27-28 control and WIN-treated male rats. The graphs are presented as boxplots. Values are expressed as percentile and median. $^+$ indicates mean. * indicates a significant difference between control and WIN-treated groups at the same age. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 2H.

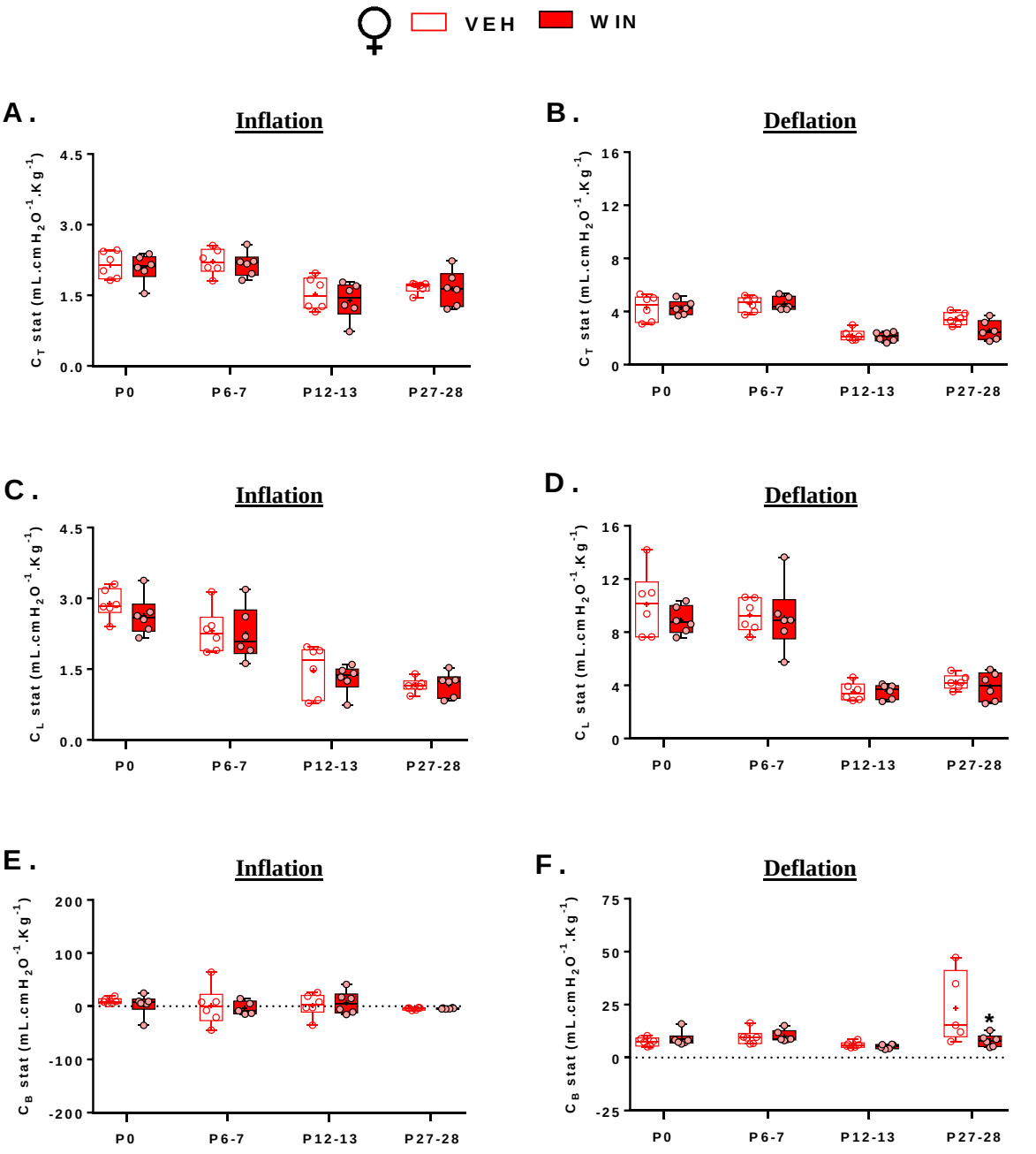


Figure 7: Effect of prenatal WIN exposure on total (C_T – **A** and **B**), lung (C_L – **C** and **D**) and body wall (C_B – **E** and **F**) static compliance during inflation and deflation for P0, P6-7, P12-13 and P27-28 control and WIN-treated female rats. The graphs are presented as boxplots. Values are expressed as percentile and median. $^+$ indicates mean. * indicates a significant difference between control and WIN-treated groups at the same age. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 2H.

Regarding the dynamic component of the respiratory mechanics, Figure 8 shows the data for dynamic total (C_T – center panel), lung (C_L – upper panel) and body

wall (C_B – bottom panel) compliance of control and WIN-treated male and female rats at P0 (A), P6-7 (B), P12-13 (C) and P27-28 (D) ages. According to the results, the effects of prenatal WIN treatment on dynamic compliance during postnatal development were mostly observed at P27-28 age, more specifically for males (Figure 8D). Juvenile treated male rats presented a robust reduction in dynamic C_T between pressures of 5 to 15, and at 21, which was in line with a decreased dynamic C_L at pressures of 9 to 17, and 21. Juvenile female WIN-treated group showed subtle changes as higher dynamic C_T only at pressures of 11 and 15, and lower at 26, and for dynamic C_L female treated group had a significant reduction at pressures 9 to 13.

Occasional changes were observed for younger animals, such as a punctual increase in dynamic C_T at pressure 25 for P0 treated females, and for P0 treated males a higher dynamic C_L at 9 cmH_2O, followed by a significant decrease at 13 cmH_2O (Figure 8A). Although small, P6-7 neonatal rats had an increased dynamic C_T at 1 cmH_2O for treated males, and also an increase at 26 cmH_2O for treated females, as well as a higher C_L at 13 and 15 cmH_2O for WIN-treated female. The C_B was lower only at 1 cmH_2O for P6-7 treated females (Figure 8B). At P12-13 age, only an increase in C_L at 26 cmH_2O for males and reduced C_B at 3 cmH_2O of pressure for females treated groups (Figure 8C).

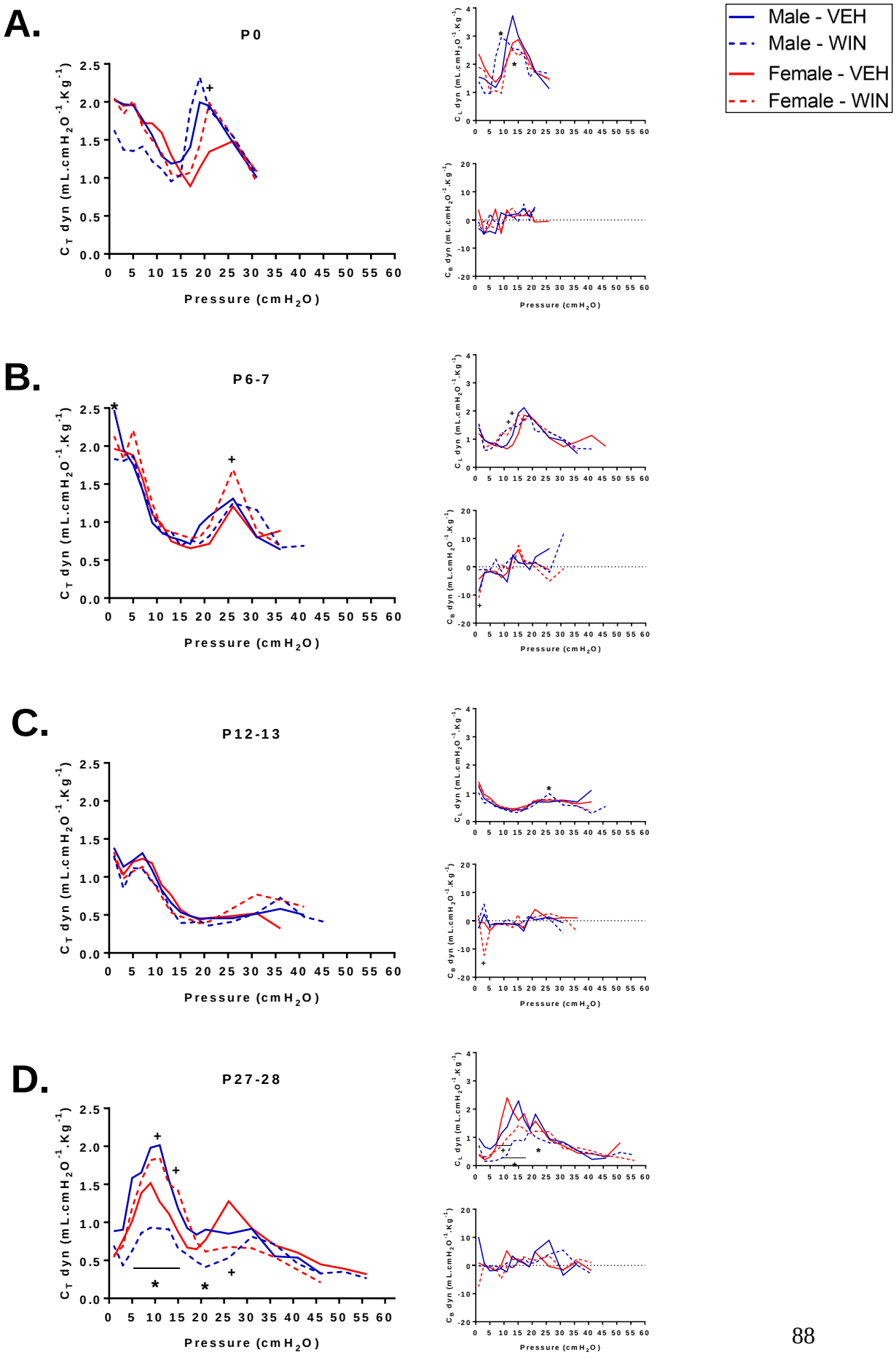

A.
P0
B.
P6-7
C.
P12-13
D.
P27-28
Male - VEH
Male - WIN
Female - VEH
Female - WIN
Pressure (cmH2O)

Figure 8: Effect of prenatal WIN exposure on total (C_T – center panel), lung (C_L – upper panel) and body wall (C_B – bottom panel) dynamic compliance for P0 (**A**), P6-7 (**B**), P12-13 (**C**) and P27-28 (**D**) control and WIN-treated male and female rats. Values are expressed as interconnected means over the intra-tracheal pressure. * indicates a significant difference between control and WIN-treated males at the same age. $^{+}$ indicates a significant difference between control and WIN-treated females at the same age. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 2I.

Brainstem CB1 receptor expression

The effect of WIN exposure during the prenatal period for CB1 receptor expression in the brainstem of neonatal and juvenile male (A) and female (B) animals is provided by the Figure 9. Cannabinoid type 1 receptor expression levels were higher in WIN-treated P0, P6-7 and P12-13 newborn males when compared to control groups. Juvenile males, as well as all postnatal female ages, did not have CB1 receptor expression affected by prenatal treatment with synthetic cannabinoid WIN.

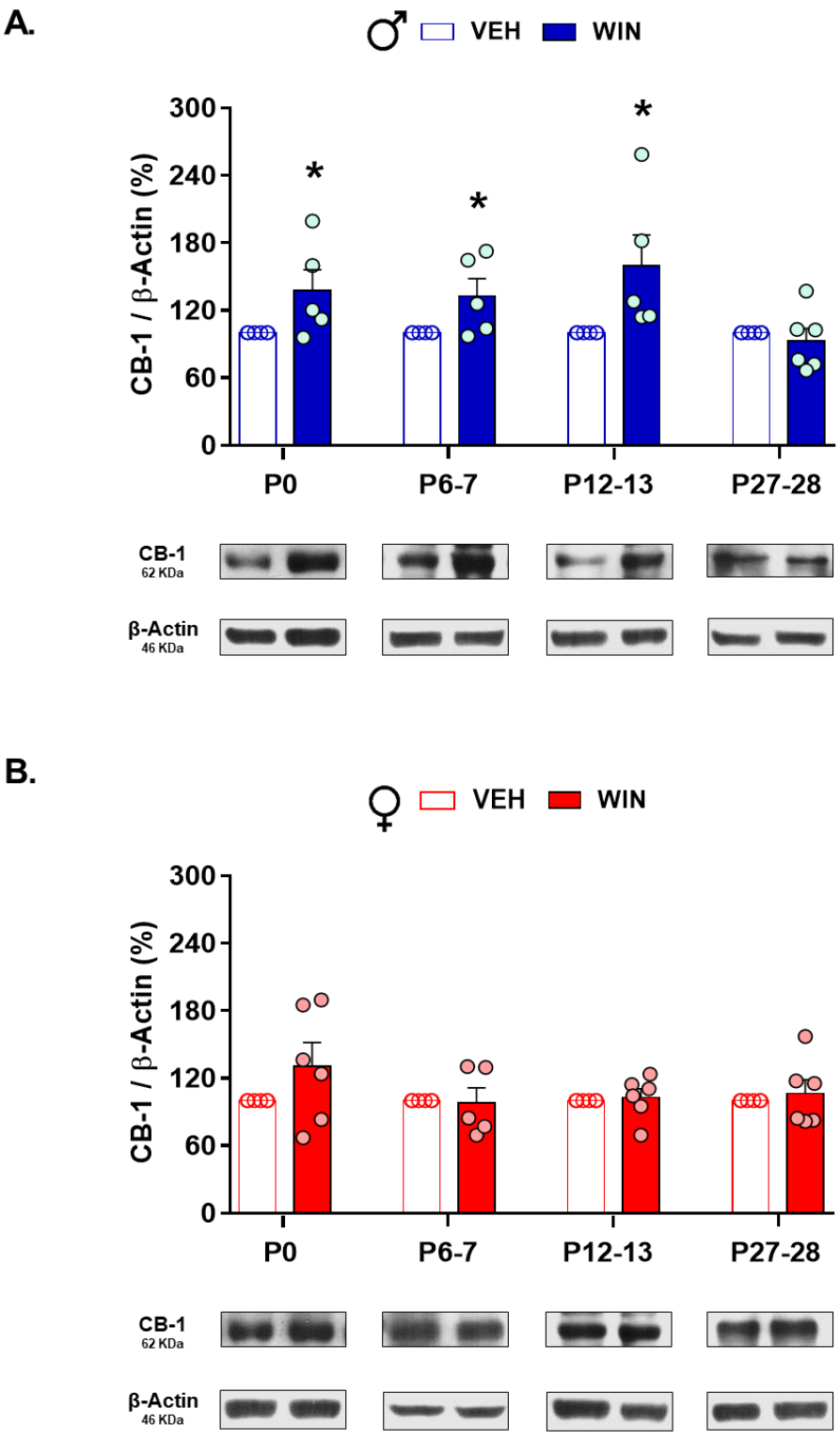


Figure 9: Effect of prenatal WIN exposure on expression of CB1 receptor protein in the brainstem of P0, P6-7, P12-13 and P27-28 control and WIN-treated male (**A**) and female (**B**) rats. Values are expressed as percentage ± S.E.M. * indicates a significant difference between control and WIN-treated groups at the same age. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 2J.

Catecholaminergic neurons

The quantification of TH-positive neurons per section in the brainstem CA groups A1/C1, A2, C3, A5 and A7, as well as the reflected light intensity of the A6 region for P0 (A), P6-7 (B), P12-13 (C) and P27-28 (D) control and WIN-treated male and female animals are shown in Figures 10 and 11, respectively. *In utero* exposure to synthetic cannabinoid WIN during the pregnancy caused a significant increase in the number of CA neurons in the A1/C1, A2, and C3 region for P0 and P6-7 males, as well as a higher intensity of light reflected in the A6 for P6-7 males (Figure 10B and 12). For P12-13 and P27-28 males, and for all female ages, intrauterine exposure to WIN did not result in significant changes of TH neurons expression.

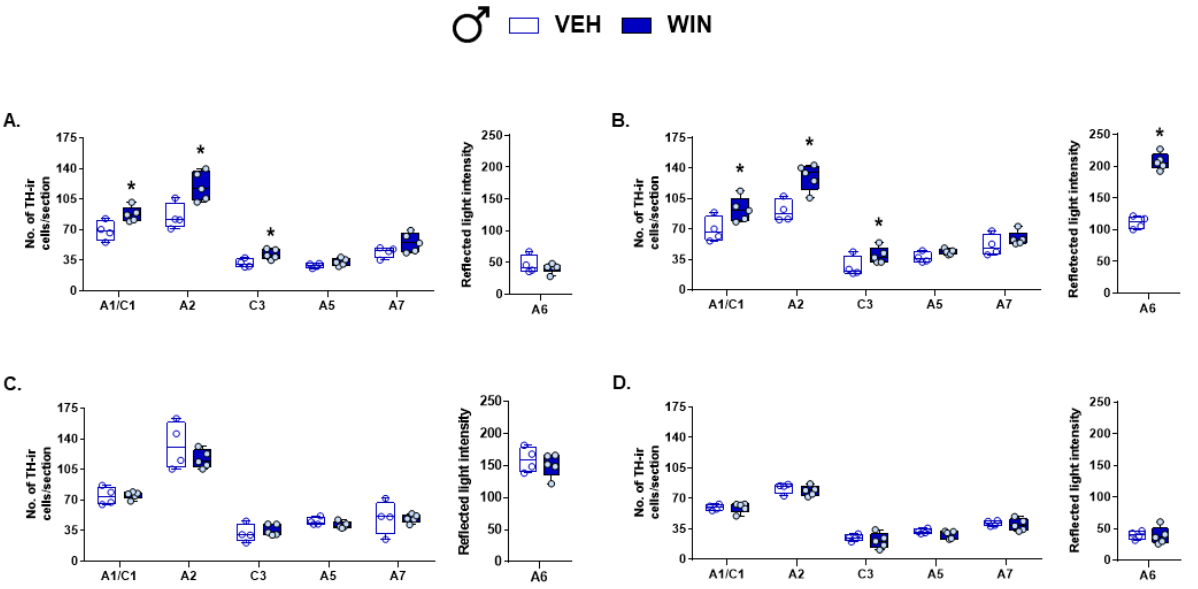


Figure 10: Effect of prenatal WIN exposure on quantification of CA neurons in A1/C1, A2, C3, A5 and A7 regions, as well as light intensity reflected in A6 for P0 (**A**), P6-7 (**B**), P12-13 (**C**) and P27-28 (**D**) control and WIN-treated male rats. The graphs are presented as boxplots. Values are expressed as percentile and median. + indicates mean. * indicates a significant difference between control and WIN-treated groups at the same age. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 2K.

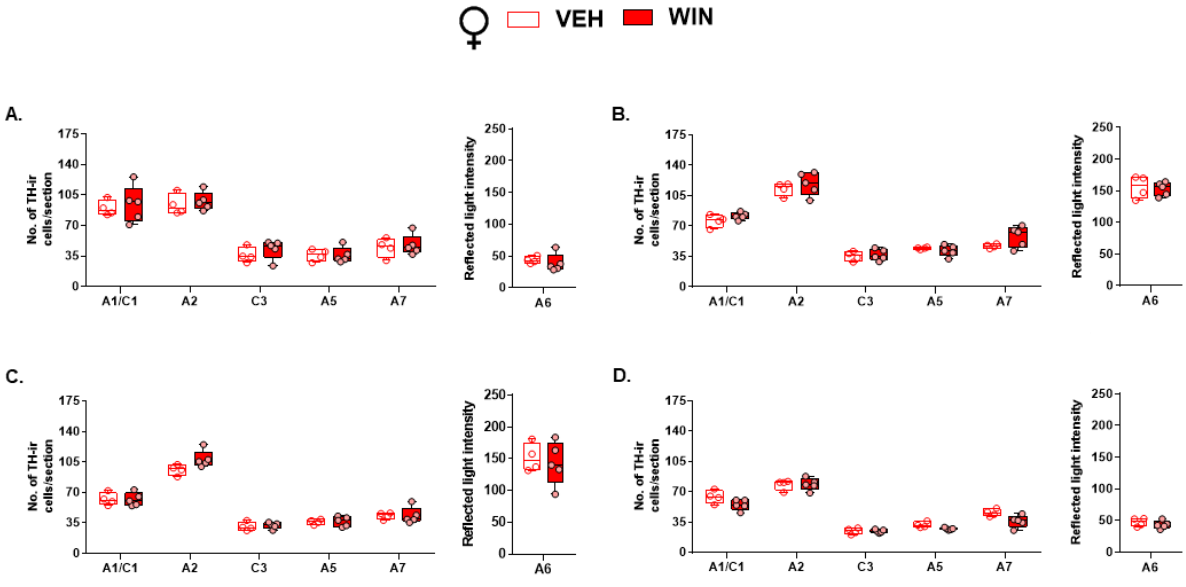


Figure 11: Effect of prenatal WIN exposure on quantification of CA neurons in A1/C1, A2, C3, A5 and A7 regions, as well as light intensity reflected in A6 for P0 (**A**), P6-7 (**B**), P12-13 (**C**) and P27-28 (**D**) control and WIN-treated female rats. The graphs are presented as boxplots. Values are expressed as percentile and median. + indicates mean. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 2K.

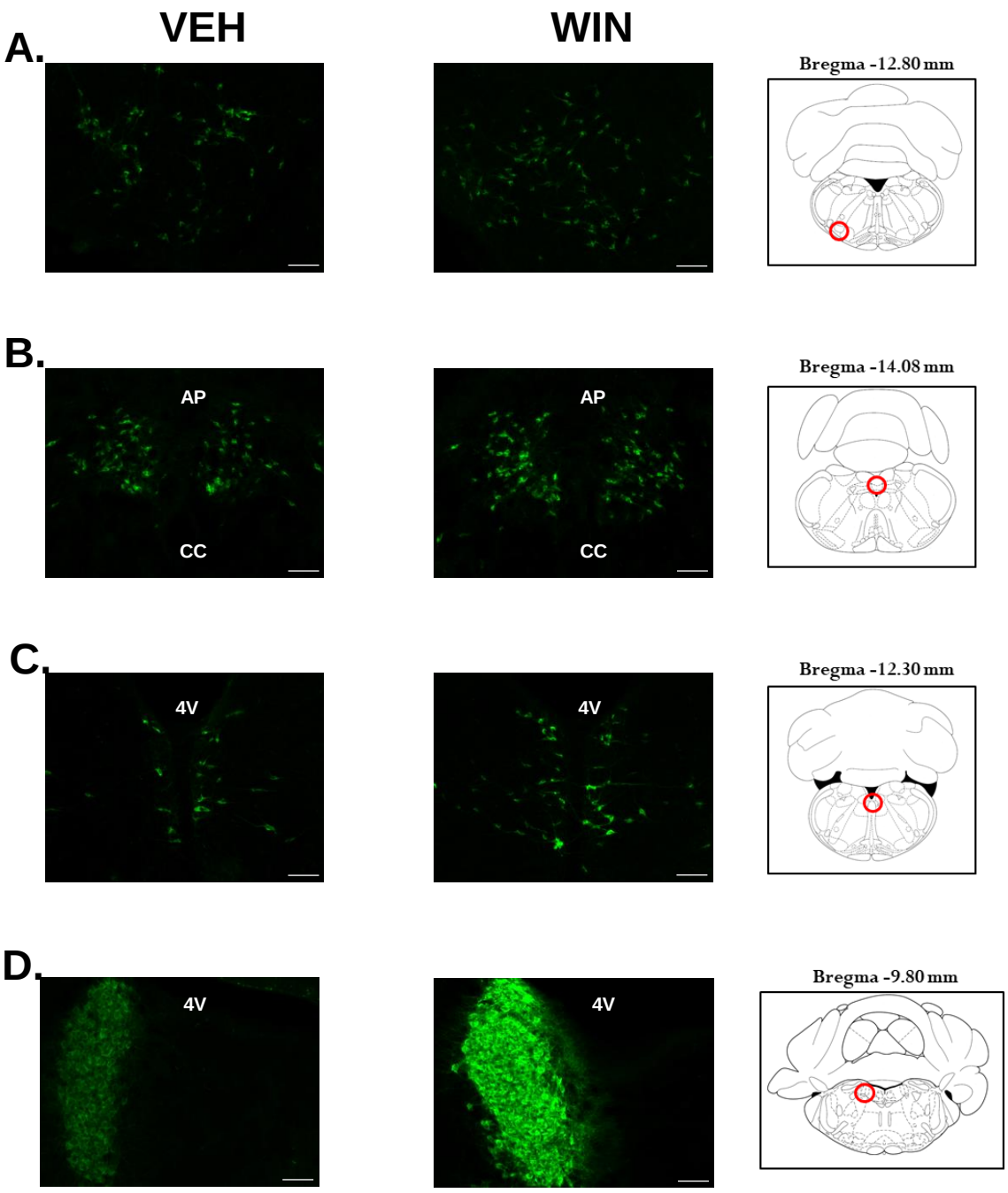


Figure 12: Representative photomicrographs in a coronal plane of brainstem A1/C1 (**A**), A2 (**B**) and C3 (**C**) region of P0, and A6 (**D**) region of P6-7 control (left side) and WIN-treated (middle) animals, under a 10× objective. Schematic drawing of the location of the nuclei (red circle) with an overview of the slice with the specific coordinates for each photomicrograph (right side). Scale bar = 100 μm. AP: area postrema, CC: central canal, 4V: fourth ventricle.

Tissue respiratory performance

Figure 13 shows the effect of WIN exposure during prenatal period on brainstem tissue respiration throughout offspring's postnatal development. The mitochondrial variables assessed of neonatal and juvenile control and WIN-treated males were oxygen consumption during phosphorylation (A), oxygen consumption with inhibition of ATP synthase (B), maximum oxygen consumption, stimulated by chemical uncoupler CCCP (C), normalized phosphorylation ratio by uncoupling (D), ratio of phosphorylation with maximum capacity as reference (E), ratio of uncoupling with phosphorylation as a reference (F), and uncoupling ration with maximum respiratory capacity as a reference (G).

Prenatal WIN exposure induced postnatal changes in tissue respiration function of P0 newborn males, such as an increased LEAK (Figure 13B), an reduction in the P/L ratio (Figure D), and a higher L/P and L/E ratio (Figure F and G, respectively), compared to the control group. Additionally, P12-13 WIN-treated animals had a lower ETS value (Figure 13C), with increased P/E and L/E ratio (Figure E and G, respectively).

The same tissue respiration parameters were also evaluated for neonatal and juvenile females (Figure 14). Only two differences were detected for females, in which the P6-7 treated newborns had a raised P/E ratio (Figure 14E) and P27-28 treated group showed a higher oxygen consumption during phosphorylation (Figure 14A).

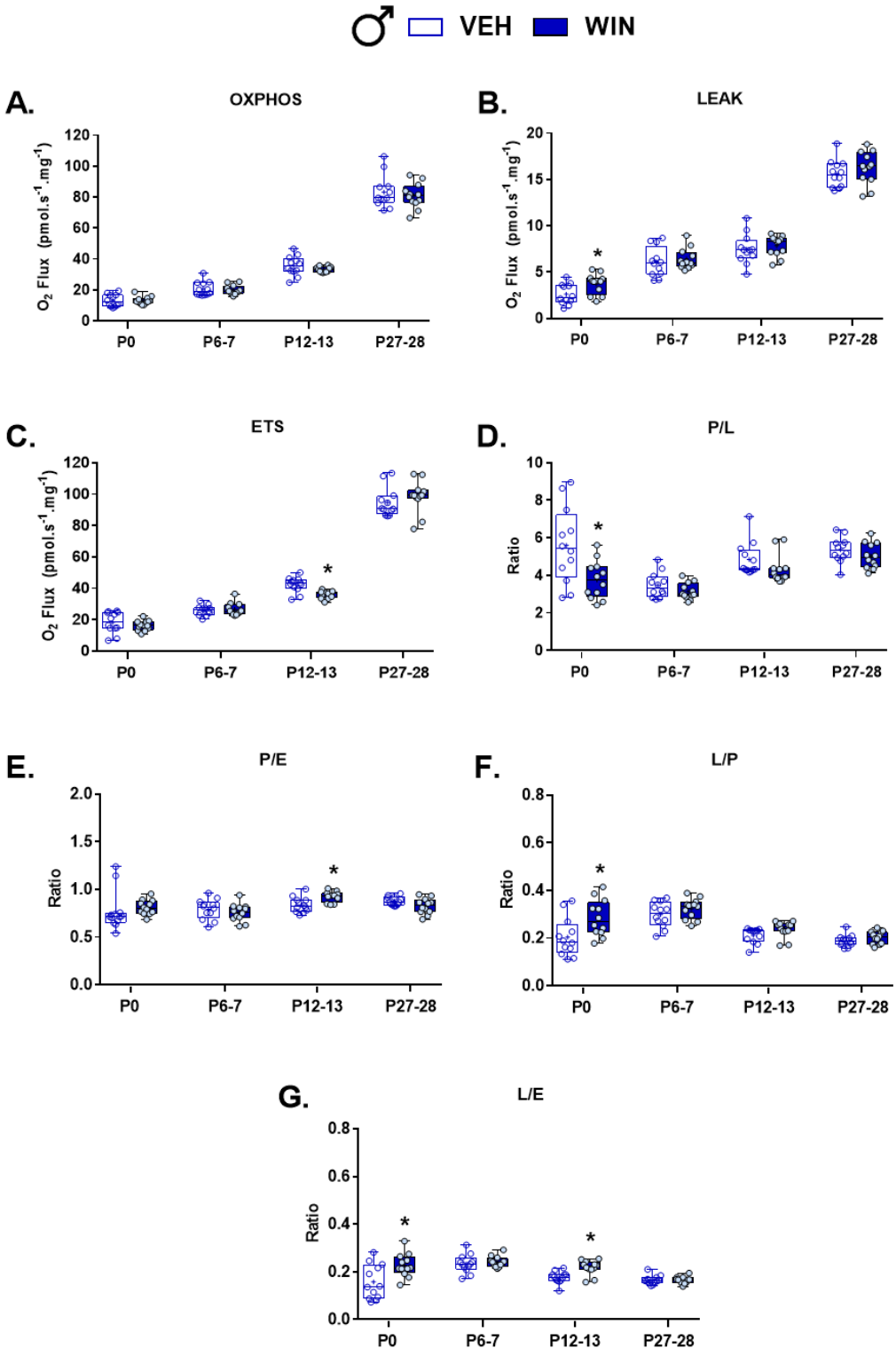


Figure 13: Effect of prenatal WIN exposure on OXPHOS (**A**), LEAK (**B**), ETS (**C**), P/L ratio (**D**), P/E ratio (**E**), L/P ratio (**F**), and L/E ratio (**G**) for P0, P6-7, P12-13 and P27-28 control and WIN-treated male rats. The graphs are presented as boxplots. Values are expressed as percentile and median. $^{+}$ indicates mean. * indicates a significant difference between control and WIN-treated groups at the same age. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 2L.

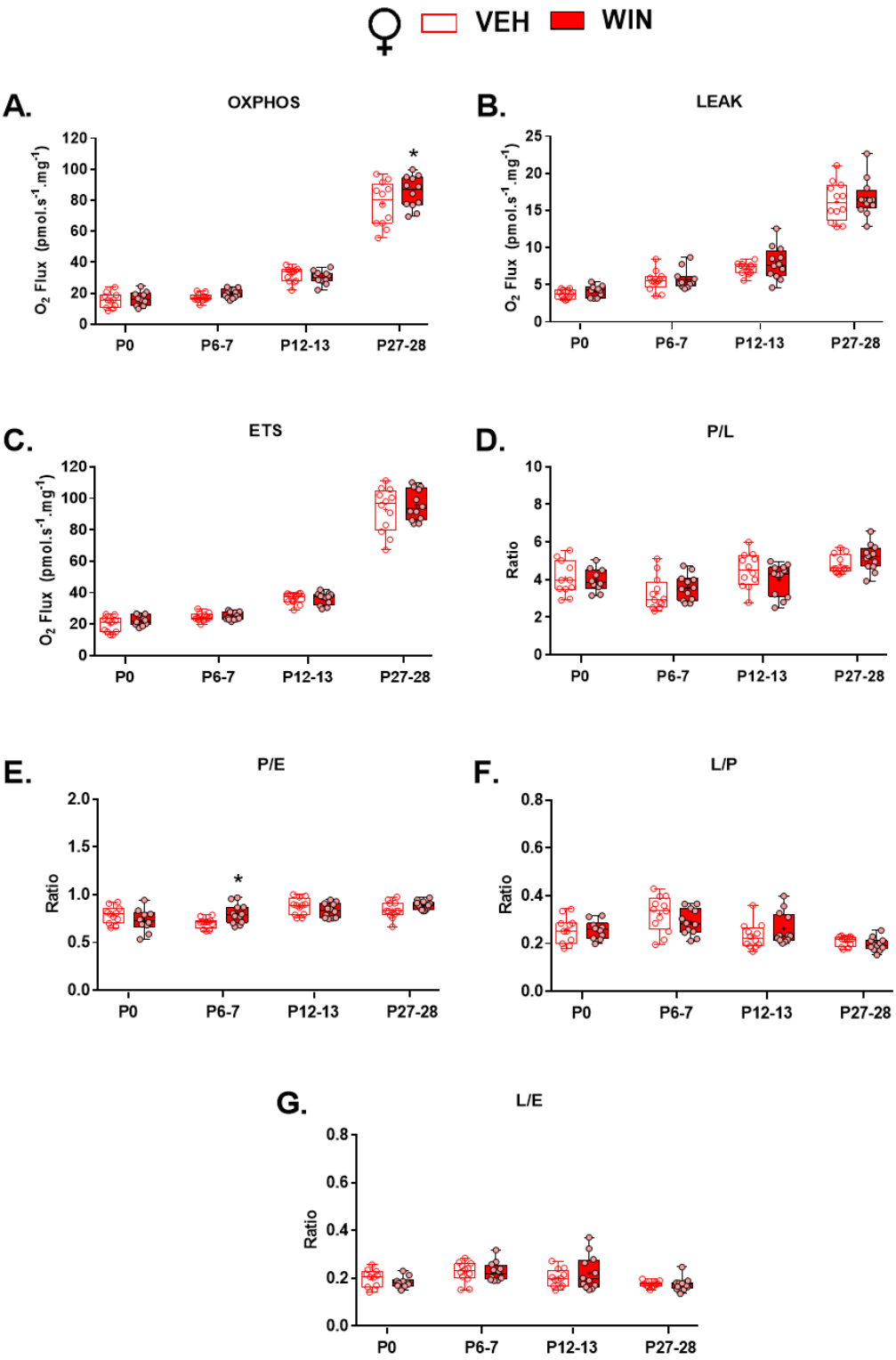


Figure 14: Effect of prenatal WIN exposure on OXPHOS (**A**), LEAK (**B**), ETS (**C**), P/L ratio (**D**), P/E ratio (**E**), L/P ratio (**F**), and L/E ratio (**G**) for P0, P6-7, P12-13 and P27-28 control and WIN-treated female rats. The graphs are presented as boxplots. Values are expressed as percentile and median. $^{+}$ indicates mean. * indicates a significant difference between control and WIN-treated groups at the same age. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 2L.

Cardiovascular and body temperature measurements

Systolic arterial pressure (SAP), diastolic arterial pressure (DAP), mean arterial pressure (MAP) and heart rate (HR) assessment for juvenile control and WIN-treated male and female rats showed the prenatal treatment effects on cardiovascular variables at resting, hypercapnic or hypoxic condition (Supplementary Figure 1A). Some changes were observed such as an increase in basal SAP, and a bradycardia during hypercapnia for WIN-treated males. Additionally, an effect of hypoxic condition on cardiovascular parameters were observed and best demonstrated in the Supplementary Figure 1A.

The T_B for control and prenatal WIN treatment juvenile rats of both sexes was monitored during resting, hypercapnic and hypoxic condition. Intra-uterine exposure to synthetic cannabinoid did not influence the body temperature control of male and female animals at rest or during ventilatory challenge, and as expected, exposure to hypoxia resulted in a significant drop in T_B in all groups, without any effect of WIN treatment (Supplementary Figure 1B).

Although *Cannabis* usage during pregnancy is rising, little is known concerning how prenatal chronic stimulation of endocannabinoid signaling alters newborns' ventilatory control system. Here, we investigated the short and medium-term consequences of exposure to synthetic cannabinoid (WIN) during the gestational phase on the respiratory control in neonates and juvenile male and female rats. We show that exposure to WIN during the prenatal period promotes a tonic excitatory drive on $\dot{V}_E$ in newborn (P0) and juvenile males accompanied by a reduction in brainstem tissue respiratory efficiency and decrease in pulmonary compliance, respectively. Notably, these changes were sex-specific, since only male rats presented these basal alterations. WIN-exposed male offspring were also characterized by increased CO_2 chemoresponse, except P6-7 neonates, that presented a hypoventilation. A greater HCVR was observed only in juvenile females. As to hypoxia, only P0 and P6-7 males presented a higher hyperventilation and hypoventilation, respectively. In parallel, an increase in the number of neurons from some brainstem CA regions and CB1 expression in early postnatal ages were observed in males, but not in females. Thus, *in utero* manipulation of cannabinoid signaling promotes short and medium-lasting changes in the respiratory control system in a sex-specific way.

The WIN prenatal treatment did not cause changes in premature or late births, neither altered body mass gain of the female during pregnancy, as well as the size of the litters and the body weight of neonates (P0, P6-7 and P12-13) and juvenile (P27-28) of both sexes. However, prenatal WIN exposure affected neonatal mortality, resulting in an increase of death at birth. It is known that moderate consumption of *Cannabis* during pregnancy is not associated with an increase in abortion rates, premature births, physical anomalies, or any other complications during pregnancy (Fried, 2002; Gray et al.,

2005). The dose of WIN used in the present study (0.5 mg/kg/day) was the same as in previous studies and it is considered clinically relevant, since it corresponds to a moderate exposure of *Cannabis* in humans, correcting for the differences in route of administration and body weight surface area (Garcia et al., 1998; Mereu et al., 2003; Bara et al., 2018). However, some studies in animal models using the active principles present in *Cannabis* at higher doses, and some prospective cohort studies with humans have reported a decrease in body weight (Zuckerman et al., 1989; Fergusson et al., 2002; El Marroun et al., 2009), lower body size of the newborns (Zuckerman et al., 1989; Day and Richardson, 1991), and a correlation with increases in neonatal death cases (Abel et al., 1980; Howard et al., 2019; Grzeskowiak et al., 2020). Also, no differences in chewing and righting reflexes were observed in P0 animals, indicating that *in utero* WIN exposure did not impact the early maturation reflexes of rats.

During room air conditions, an increase in resting $\dot{V}_E$ was observed in the P0 WIN-treated males similar to previous data by Tree et al. (2014). This is the opposite to WIN acute administration, which depresses ventilation (Padley et al., 2003; Schmid et al., 2003). Therefore, we believe that this increase in $\dot{V}_E$ might be related to drug withdrawal symptoms. Likewise, *in utero* morphine exposed neonatal guinea pig hyperventilated during withdrawal (Nettleton et al., 2008). In addition, neonate rats head shaking, paw tremors or wet-dog shakes were observed in the first hours after birth characterizing withdrawal syndrome (data not shown). Similarly, motor hyperactivity has been reported to be caused postnatally by WIN (Mereu et al., 2003), which can contribute to higher metabolic rate and ventilation. In humans, increased tremors and prolonged and exaggerated startle reflexes are observed in the first week and persisted for 9 and 30 days of life in those infants who were exposed to marijuana *in utero* (Fried et al., 1987).

CB1 receptors are located in many peripheral and central areas relevant for the generation of respiratory patterns and integration of motor activity (Haji et al., 2000), including the nodosum ganglion, the NTS (Rohof et al., 2012), and the hypoglossal motor nucleus (Mukhtarov et al., 2005). We indeed observed an upregulation of CB1 receptors located in the brainstem in all neonate males but not in juveniles, although, through the technique used is not possible to determine whether these receptors are in the active form in the cell membrane, or internalized in the cell. Overall, these findings support that embryonic WIN administration transiently increases CB1 function in the developing brain. In contrast, previous study that evaluate embryonic THC exposure (E12.5 to E16.5) observed a down regulation in THC-treated embryonic brains at E17.5 and a return to normal levels at P2.5 (de Salas-Quiroga et al., 2015). However, the authors evaluated the whole brain, whereas our analysis was restricted to the brainstem. Also, the synthetic agonist used and the period of *in utero* exposure were different, which might contribute to the discrepancies between the two studies.

In any case, syntethic cannabinoid (WIN) overexposure causes an increase in $\dot{V}_E$ and $\dot{V}O_2$ in P0 and a hyperventilation in juvenile males. Hence, the increase in $\dot{V}_E$, observed in P0 WIN-treated males, may be due to the action of the agonist in the areas responsible for respiratory control. Additionally, the effect on resting $\dot{V}_E$ may indicate the effect of the circulating drug and not properly a plasticity of the system as previously discussed. However, at this age we have already observed an increase in the number of catecholaminergic neurons in A1/C1, A2 and C3 cell groups. The stimulation of these groups activates breathing (Burke et al., 2014; Menuet et al. 2014; Yamamoto et al., 2015), therefore, the higher ventilation observed in P0 animals might be related to the higher respiratory drive mediated by these cell groups. Interestingly, the ratio of normalized phosphorylation by uncoupling (P/L - liquid phosphorylation) was lower for

P0 treated males, that is, the efficiency of mitochondria in the production of ATP is negatively affected by exposure to WIN. According to these results, the percentage of uncoupling with phosphorylation as reference (L/P), as well as the percentage of uncoupling with maximum respiratory capacity as reference (L/E), were significantly higher for WIN-treated P0 males, corroborating the lower brainstem mitochondrial yield in the production of ATP, indicating a possibly disfunction. Recent studies have located CB1 receptors on the outer membrane of neuronal mitochondria (Bénard et al., 2012; Hebert-Chatelain et al., 2014; Koch et al., 2015). Activation of these receptors leads to a decrease in cAMP concentration, protein kinase A activity and mitochondrial respiration (Bénard et al., 2012; Hebert-Chatelain et al., 2014). In this way, exogenous and endogenous cannabinoids can activate mitochondrial CB1 receptors and regulate neuronal energy metabolism, depressing mitochondrial breathing and altering the physiological responses mediated by endocannabinoids (Alger and Tang, 2012).

Regarding juvenile males, hyperventilation was caused by an increase in $\dot{V}_E$ due to a greater V_T, with no change in $\dot{V}O_2$. A reduction of total and lung static and dynamic compliance were also observed for these juvenile treated males. Therefore, it is possible that a more rigid respiratory system with reduced compliance, the animal prioritizes deeper inspirations (higher V_T), instead of ventilating the system more often, which would be more energetically costly. According to Vitalis and Milsom (1986), an increase in V_T keeps the compliance equal, but an increase in frequency reduces compliance even more. Therefore, the animal that ventilates with greater V_T and lower frequency, takes advantage of a more compliant system, thus compensating for the reduced compliance due to WIN treatment. In addition, some studies have reported the presence of CB1 receptors in lung tissue (Galieque et al., 1995; Rice et al., 1997), and that CB1 receptor activation inhibits airway contraction by inhibiting cholinergic-

induced contractions (Wang et al., 2016). It was also demonstrated that CB-mediated effect is associated with the improvement of static lung elastance and reduced collagen fiber content (Vuolo et al., 2019).

The breathing pattern in neonates is highly erratic and unstable, mainly in the first hours after birth, but stabilizes with increasing age as the animal transitions to postnatal life (Hoppenbrouwers et al., 1978; Mortola, 1984; Barrett et al., 2012). In the present study, prenatal WIN treatment promoted sex and age-specific effects on breathing variability under room air conditions. We found that P6-7 and P12/13 WIN-treated males displayed a reduced breathing variability compared to their controls, whereas in P12/13 WIN-treated females, the breathing variability was greatest. The opposite response occurred in WIN-treated juvenile males that presented a greater breathing variability, similar to juvenile females. These changes may be related to an imbalance of excitatory and inhibitory modulation of respiratory network excitability.

It is important to note that, during room air conditions, P0 WIN-treated females had no alterations in breathing, which indicates a greater sensitivity of P0 males to the cannabinoid agonist. Sex differences in the endocannabinoid system and the behavioral effects of synthetic cannabinoids have been previously recognized (Fattore and Fratta, 2010). In this regard, a recent study by Bara et al. (2018) demonstrated that fetal exposure to cannabinoids causes sex-specific changes in behavioral and synaptic functions. Specifically, the authors showed that prenatal exposure to the cannabinoid reduces social interactions in males, but not in females. At the same time, prenatal exposure to THC specifically altered neuronal excitability and synaptic plasticity in the prefrontal cortex of male rats rather than females. In agreement, the affinity of the cannabinoids for the CB1 receptor is greater in males, while the receptor density is similar in both sexes in the limbic forebrain; however, in the midbrain both parameters

are higher in males (Rodríguez de Fonseca et al., 1994; Bara et al., 2018). In agreement, we also found an increased expression of brainstem CB1 receptors in males. Thus, the differences found in $\dot{V}_E$ at P0 age may be due to the fact that the receptors are more sensitive in males.

The data from the current study show that chronic and excessive activation of the endocannabinoid signaling during pregnancy promotes changes in the respiratory control system that affects CO_2 and O_2 chemosensitivity during the early postnatal development, as well as in the juvenile phase. As evidenced in the P0 animals, males that were exposed to the synthetic cannabinoid during prenatal period showed a greater HCVR, and HVR. Since no significant differences were observed in $\dot{V}O_2$ under these conditions, we can suggest that these individuals presented a hyperventilation possibly due to changes in the respiratory control network during the intra-uterine development phase, that affected the chemosensitivity of CO_2 and O_2. Interestingly, P0 females prenatally exposed to WIN did not present such ventilatory changes, either in hypercapnia or hypoxia, except for a change in the ventilatory pattern with a decrease in f_R and an increased V_T during hypoxia, without significant effects on $\dot{V}_E$. These data suggest a greater susceptibility of P0 males to the influence of exogenous cannabinoid during the intra-uterine development phase and the formation of the respiratory network, as well as in the ventilatory control in the first days of life.

In contrast, in P6-7 males we found that WIN exposure resulted in a reduced hypoxic and hypercapnic chemoreflex response. According to previous studies, there is a developmental window during the first two weeks of life in rats through which the respiratory control system undergoes significant changes as it completes its development (Putnam et al., 2005; Davis et al., 2006; Liu and Wong-Riley, 2008; Greer, 2012; Bavis and MacFarlane, 2017; Wong-Riley et al., 2019, Sprenger and Milsom,

2021). Comparing P0, P6-7 and P12-13 control animals, we can observe that hypercapnic and hypoxic ventilatory response in rats changed markedly post-natally, with a higher response in P6-7 animals. Therefore, a critical period of potential vulnerability at P6-7 WIN-treated males when pups hypoventilate under hypercapnia and hypoxia presenting a lower chemosensitivity compared to control animals might be related to abnormal maturation of the respiratory control system. Intriguingly, we found a greater TH expression in A1/C1, A2 and C3 neurons, an upregulation of CB1 receptors in the brainstem similar to P0 WIN-treated males. Different from P0 WIN-treated males, P6-7 animals also presented a higher expression of TH at A6 neurons. However, previous studies have demonstrated that this area is excitatory for hypercapnic response (Biancardi et al., 2008; De Carvalho et al, 2010; Gargaglioni et al., 2010) and has no role in hypoxic drive. Therefore, it is possible that the lower chemosensitivity at this age in the WIN-treated group must be due to an effect in the peripheral chemoreceptors. In fact, CB1 mRNA is expressed within the peripheral arterial chemoreceptors, with the greatest expression in the nodose-petrosal-jugular ganglia complex, moderate expression in the superior cervical ganglia, and minimal expression in the carotid body, and the level of expression in these tissues increases with postnatal age (McLemore et al., 2004). Also, in this study, the authors observed that CB1 receptors mRNA levels significantly increased in the nodose-petrosal-jugular ganglia complex from P5 to P7, with no further change at P14. Therefore, P7 seems to be the peak of receptor expression.

The data from the present study also showed that chronic intra-uterine exposure to the synthetic cannabinoid promotes ventilatory changes in the neonatal (P12-13) and juvenile (P27-28) animals under CO_2 challenge. Males and females of both ages had a higher ventilatory response to CO_2, with increased V_T without changes

in the metabolic rate, indicating a higher sensitivity to CO_2. In this case, as the response persisted up to P27-28, probably a plasticity of central CO_2/pH chemosensitive areas may be occurring. Many studies support the idea of humans with anxiety and panic disorders showing a hypersensibility of the CO_2 chemoreception system (Perna et al., 1996; Bellodi et al., 1998; Coryell et al., 2001; Battaglia et al., 2007). Therefore, it is possible that prenatal cannabis may increase the vulnerability to panic disorders, since these animals are more sensitive to CO_2. In fact, prenatal exposure to *Cannabis* is associated with children self-reported anxiety symptoms (Goldschmidt et al., 2004; Gray et al., 2005; Leech et al., 2006; Nashed et al., 2021). In this regard, P12 rat pups prenatally exposed to THC displayed an increase in the frequency of ultrasonic vocalizations when removed from the nest, a behavior that is possibly analogous to human infant crying and that may suggest long-term neuro-behavioral changes (Trezza et al., 2008). When tested during adolescence and adulthood, these rats exhibited a decrease in play behavior and social interaction and an increase in anxiety-like behavior on the elevate plus-maze test, respectively.

During hypoxic condition, we observed the same pattern of normoxic conditions, P0 showing a greater HVR whereas P6-7 a lower hypoxic chemosensitivity. In *in vitro* preparations containing neonatal medulla from mice (P0-2) exposed prenatally to WIN, an exaggerated depression of C4 nerve activity during hypoxia was observed, suggesting an impact of WIN prenatal exposure on the medullar rhythmic respiratory network (Tree et al., 2014). The same authors also reported that this prenatal exposure to cannabinoid was responsible for changes in $\dot{V}_E$ in intact P0-2 and P10-12 animals, such as hyperventilation at baseline, an altered chemoreflex response to hypoxia and longer apneas. The excitatory effects were only observed in P0 males, which may indicate the direct effect of the circulating drug on sensitivity to hypoxia. In

fact, previous studies from Kobayashi and Yamamoto (2010) showed that anandamide, a potent endocannabinoid, has an excitatory effect on the carotid body, which can increase the sensitivity of this sensor to hypoxia. As pointed out before, the opposite effects observed in P0 and P6-7 might be related to different developmental events in CB1 receptors expression and peripheral chemoreceptor sensitivity.

Cannabinoid receptors type 1 are present in regions outside the CNS, such as in the heart and blood vessels (Liu et al., 2000; Bonz et al., 2003). Despite this knowledge, the literature lacks information on the use of cannabinoids during pregnancy and its postnatal effects on cardiovascular control. There is evidence demonstrating that the endocannabinoid system is highly related to cardiovascular physiology, modulating blood pressure, contractility and heart rate (Lake et al., 1997; Sierra et al., 2017). In addition, acute use of external cannabinoids by humans, such as *Cannabis* promotes an increase in mean arterial pressure and heart rate (Weiss et al., 1972), acting on the release of neurotransmitters in the CNS and sympathetic nerve terminals, or even locally by modulating the smooth muscle of the vessels (Hillard, 2000). Regarding cardiovascular parameters in juvenile male rats, the only effect that was observed WIN-treated animals was a slight increase in SAP under resting conditions, without affect MAP and a small reduction in HR during hypercapnia. Therefore, it seems that overstimulation of the endocannabinoid system did not have a great impact the cardiovascular control, at least in juvenile rats.

In summary, this preclinical study has major implications for understanding the impact of prenatal exposure of cannabinoid agonist on neonatal and juvenile ventilatory control system. Taken together, our data demonstrated that excess stimulation of the endocannabinoid system during gestation has long-lasting and sex-specific consequences for the respiratory control system, affecting the number of catecholaminergic neurons, brainstem CB1 expression, tissue respiration and pulmonary compliance. These findings are particularly relevant since global cannabis use has been rising and more liberal recreational cannabis policies have been adopted. In addition, there is a major lack of understanding among the general population regarding the potential risks of *Cannabis* use during pregnancy. Therefore, our study raises a note of caution that might be considered when the potential therapeutic or recreational uses of cannabinoid-based medicines are defined and regulated for pregnant women.

Supplementary Table 1. A - Systolic arterial pressure (SAP), diastolic arterial pressure (DAP), mean arterial pressure (MAP) and heart rate (HR); **B** - Body temperature (T_B - °C) for P27-28 control and WIN-treated male and female rats, under resting, hypercapnic and hypoxic conditions.

A.

Basal			SAP (mmHg)	DAP (mmHg)	MAP (mmHg)	HR (bpm)
	Male	VEHICLE	92.7 ± 3.6	69.0 ± 1.8	80.7 ± 2.1	425.6 ± 17.1
		WIN	104.7 ± 4.7*	72.8 ± 4.0	87.7 ± 2.8	426.3 ± 22.0
	Female	VEHICLE	102.2 ± 6.5	81.7 ± 2.3	91.5 ± 3.5	394.7 ± 18.4
		WIN	112.4 ± 5.5	82.4 ± 2.5	96.7 ± 2.7	438.2 ± 5.8
Hypercapnia			SAP (mmHg)	DAP (mmHg)	MAP (mmHg)	HR (bpm)
	Male	VEHICLE	96.8 ± 3.1	70.9 ± 1.4	83.5 ± 0.8	404.0 ± 13.8
		WIN	103.4 ± 4.0	75.1 ± 3.3	88.2 ± 2.2	347.7 ± 11.77*
	Female	VEHICLE	99.9 ± 7.0	80.5 ± 2.4	89.6 ± 3.5	361.6 ± 20.2
		WIN	114.4 ± 6.1	82.8 ± 2.6	97.3 ± 3.6	388.4 ± 10.3
Hypoxia			SAP (mmHg)	DAP (mmHg)	MAP (mmHg)	HR (bpm)
	Male	VEHICLE	88.1 ± 3.3	72.0 ± 4.9	80.1 ± 3.5	544.0 ± 12.7^{++}
		WIN	99.5 ± 4.3^{++}	71.7 ± 3.1	85.3 ± 2.0	509.2 ± 24.5^{++}
	Female	VEHICLE	91.3 ± 3.1^{++}	76.6 ± 3.5	83.9 ± 2.4^{++}	528.5 ± 13.3^{++}
		WIN	104.8 ± 4.7	79.6 ± 3.4	92.2 ± 3.8	544.8 ± 33.1^{++}

B.

$T_{B.}$			**Basal**	**Hypercapnia**	**Hypoxia**
	Male	VEHICLE	37.6 ± 0.3	37.6 ± 0.2	36.4 ± 0.3^{++}
		WIN	37.8 ± 0.1	37.7 ± 0.2	36.7 ± 0.3^{++}
	Female	VEHICLE	37.8 ± 0.3	37.4 ± 0.2	36.7 ± 0.3^{++}
		WIN	38.1 ± 0.3	37.6 ± 0.4	36.5 ± 0.5^{++}

Values are expressed as mean ± S.E.M. $^{++}$ Indicates significant difference compared with the other two conditions.* Indicates significant difference between control and treated (WIN) groups. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 2M.

Supplementary Table 2. Results of two-way ANOVA statistical analyzes for body mass, and heart and lungs weight (**A**), respiratory and metabolic measurements during baseline (**B**) and during hypercapnia for males (**C**) and females (**D**), ventilation and oxygen consumption during hypoxia for males (**E**) and females (**F**), breathing variability (**G**), static (**H**) and dynamic (**I**) compliance, brainstem CB1 receptor expression (**J**), CA neurons quantification (**K**), brainstem mitochondrial respiration (**L**), and cardiovascular and body temperature data (**M**).

A.

	Two-way ANOVA		
	Treatment effect	Age effect	Factorial Interaction
Body Mass			
Male	n.s	$P < 0.001$ $F_{(3, 115)} = 682.73$	n.s
Female	n.s	$P < 0.001$ $F_{(3, 112)} = 977.03$	n.s
Heart			
Male	n.s	$P < 0.001$ $F_{(3, 83)} = 17.43$	n.s
Female	n.s	$P < 0.001$ $F_{(3, 93)} = 33.13$	n.s
Lungs			
Male	n.s	$P < 0.001$ $F_{(3, 81)} = 116.86$	n.s
Female	n.s	$P < 0.001$ $F_{(3, 93)} = 127.88$	n.s

B.

	Two-way ANOVA repeated measures		
	Treatment effect	Gas effect	Factorial Interaction
Basaline MALE P0			
V_E	$P < 0.01$ $F_{(1, 11)} = 24.59$	n/a	n.s
VO_2	$P < 0.03$ $F_{(1, 11)} = 2.31$	n/a	n.s
P27-28			
V_E	$P < 0.03$ $F_{(1, 15)} = 7.55$	n/a	n.s
V_T	$P < 0.01$ $F_{(1, 15)} = 6.54$	n/a	n.s
V_E/VO_2	$P < 0.05$ $F_{(1, 15)} = 4.64$	n/a	n.s

C.

	Two-way ANOVA repeated measures		
	Treatment effect	Gas effect	Factorial Interaction
Hypercapnia MALE			
P0			
V_E	$P < 0.001$ $F_{(1, 11)} = 24.59$	$P < 0.001$ $F_{(1, 11)} = 44.09$	n.s
V_T	$P < 0.03$ $F_{(1, 11)} = 12.32$	$P < 0.001$ $F_{(1, 11)} = 22.94$	n.s
f_R	$P < 0.03$ $F_{(1, 11)} = 6.61$	n.s	n.s
VO_2	n.s	$P < 0.007$ $F_{(1, 11)} = 10.88$	$P < 0.03$ $F_{(1, 11)} = 6.01$
V_E/VO_2	$P < 0.002$ $F_{(1, 11)} = 6.21$	$P < 0.001$ $F_{(1, 11)} = 32.19$	$P < 0.03$ $F_{(1, 11)} = 6.83$
P6-7			
V_E	$P < 0.04$ $F_{(1, 13)} = 3.66$	$P < 0.001$ $F_{(1, 13)} = 794.74$	n.s
V_T	$P < 0.005$ $F_{(1, 13)} = 8.09$	$P < 0.001$ $F_{(1, 13)} = 503.78$	n.s
VO_2	n.s	$P < 0.001$ $F_{(1, 13)} = 21.53$	n.s
V_E/VO_2	$P < 0.001$ $F_{(1, 13)} = 11.05$	$P < 0.001$ $F_{(1, 13)} = 405.28$	$P < 0.04$ $F_{(1, 13)} = 5.20$
P12-13			
V_E	$P < 0.008$ $F_{(1, 11)} = 4.40$	$P < 0.001$ $F_{(1, 11)} = 174.24$	$P < 0.05$ $F_{(1, 11)} = 4.97$
V_T	$P < 0.002$ $F_{(1, 11)} = 7.08$	$P < 0.001$ $F_{(1, 11)} = 358.72$	$P < 0.01$ $F_{(1, 11)} = 9.63$
f_R	n.s	$P < 0.001$ $F_{(1, 11)} = 55.27$	n.s
V_E/VO_2	$P < 0.008$ $F_{(1, 11)} = 4.03$	$P < 0.001$ $F_{(1, 11)} = 355.11$	$P < 0.01$ $F_{(1, 11)} = 9.16$
P27-28			
V_E	$P < 0.04$ $F_{(1, 15)} = 7.55$	$P < 0.001$ $F_{(1, 15)} = 601.25$	n.s
V_T	$P < 0.05$ $F_{(1, 15)} = 6.54$	$P < 0.001$ $F_{(1, 15)} = 353.05$	n.s
f_R	n.s	$P < 0.001$ $F_{(1, 15)} = 177.75$	n.s
V_E/VO_2	$P < 0.04$ $F_{(1, 15)} = 7.64$	$P < 0.001$ $F_{(1, 15)} = 113.11$	n.s

D.

	Two-way ANOVA repeated measures		
	Treatment effect	Gas effect	Factorial Interaction
Hypercapnia FEMALE			
P0			
V_E	n.s	$P < 0.001$ $F_{(1, 13)} = 205.01$	n.s
V_T	n.s	$P < 0.001$ $F_{(1, 13)} = 347.05$	n.s
VO_2	n.s	$P < 0.001$ $F_{(1, 13)} = 16.88$	n.s
V_E/VO_2	n.s	$P < 0.001$ $F_{(1, 13)} = 40.00$	n.s
P6-7			
V_E	n.s	$P < 0.001$ $F_{(1, 14)} = 155.04$	n.s
V_T	n.s	$P < 0.001$ $F_{(1, 14)} = 566.80$	n.s
V_E/VO_2	n.s	$P < 0.001$ $F_{(1, 14)} = 171.28$	n.s
P12-13			
V_E	$P < 0.01$ $F_{(1, 12)} = 4.77$	$P < 0.001$ $F_{(1, 12)} = 257.55$	n.s
V_T	$P < 0.03$ $F_{(1, 12)} = 4.58$	$P < 0.001$ $F_{(1, 12)} = 180.34$	n.s
f_R	n.s	$P < 0.001$ $F_{(1, 12)} = 141.46$	n.s
V_E/VO_2	n.s	$P < 0.001$ $F_{(1, 12)} = 201.64$	n.s
P27-28			
V_E	$P < 0.001$ $F_{(1, 12)} = 21.46$	$P < 0.001$ $F_{(1, 12)} = 334.62$	$P < 0.04$ $F_{(1, 12)} = 5.03$
V_T	$P < 0.02$ $F_{(1, 12)} = 7.01$	$P < 0.001$ $F_{(1, 12)} = 177.96$	n.s
f_R	n.s	$P < 0.001$ $F_{(1, 12)} = 93.29$	n.s
V_E/VO_2	$P < 0.02$ $F_{(1, 12)} = 2.66$	$P < 0.001$ $F_{(1, 12)} = 157.41$	$P < 0.03$ $F_{(1, 12)} = 6.38$

E.

	Two-way ANOVA repeated measures		
	Treatment effect	Gas effect	Factorial Interaction
Hypoxia MALE P0			
V_E	$P < 0.03$ $F_{(1, 10)} = 13.53$	$P < 0.02$ $F_{(1, 10)} = 32.61$	n.s
V_T	n.s	$P < 0.02$ $F_{(1, 10)} = 8.76$	n.s
f_R	$P < 0.02$ $F_{(1, 10)} = 8.20$	n.s	n.s
VO_2	n.s	$P < 0.001$ $F_{(1, 10)} = 23.62$	$P < 0.02$ $F_{(1, 10)} = 7.69$
V_E/VO_2	$P < 0.002$ $F_{(1, 10)} = 3.19$	$P < 0.001$ $F_{(1, 10)} = 125.19$	$P < 0.002$ $F_{(1, 10)} = 16.17$
P6-7			
V_E	$P < 0.05$ $F_{(1, 13)} = 3.81$	$P < 0.001$ $F_{(1, 13)} = 275.88$	n.s
V_T	n.s	$P < 0.001$ $F_{(1, 13)} = 114.70$	n.s
f_R	n.s	$P < 0.001$ $F_{(1, 13)} = 169.38$	n.s
V_E/VO_2	$P < 0.003$ $F_{(1, 13)} = 6.59$	$P < 0.001$ $F_{(1, 13)} = 201.91$	$P < 0.05$ $F_{(1, 13)} = 4.62$
P12-13			
V_E	n.s	$P < 0.001$ $F_{(1, 15)} = 156.04$	n.s
V_T	n.s	$P < 0.001$ $F_{(1, 15)} = 60.33$	n.s
f_R	n.s	$P < 0.001$ $F_{(1, 15)} = 73.52$	n.s
VO_2	n.s	$P < 0.001$ $F_{(1, 15)} = 18.59$	n.s
V_E/VO_2	n.s	$P < 0.001$ $F_{(1, 15)} = 113.36$	n.s
P27-28			
V_E	n.s	$P < 0.001$ $F_{(1, 15)} = 83.68$	$P < 0.004$ $F_{(1, 15)} = 11.82$
V_T	n.s	$P < 0.001$ $F_{(1, 15)} = 65.77$	$P < 0.01$ $F_{(1, 15)} = 7.66$
f_R	n.s	$P < 0.03$ $F_{(1, 15)} = 5.48$	n.s
VO_2	n.s	$P < 0.001$ $F_{(1, 15)} = 56.19$	n.s
V_E/VO_2	n.s	$P < 0.001$ $F_{(1, 15)} = 386.30$	$P < 0.02$ $F_{(1, 15)} = 6.18$

F.

	Two-way ANOVA repeated measures		
	Treatment effect	Gas effect	Factorial Interaction
Hypoxia FEMALE P0			
V_E	n.s	$P < 0.001$ $F_{(1, 14)} = 28.51$	n.s
V_T	$P < 0.05$ $F_{(1, 14)} = 6.27$	$P < 0.001$ $F_{(1, 14)} = 35.10$	n.s
f_R	$P < 0.03$ $F_{(1, 14)} = 3.59$	$P < 0.01$ $F_{(1, 14)} = 8.96$	n.s
VO_2	n.s	$P < 0.001$ $F_{(1, 14)} = 24.54$	n.s
V_E/VO_2	n.s	$P < 0.001$ $F_{(1, 14)} = 72.71$	n.s
P6-7			
V_E	n.s	$P < 0.001$ $F_{(1, 14)} = 128.32$	n.s
V_T	n.s	$P < 0.001$ $F_{(1, 14)} = 124.66$	n.s
f_R	n.s	$P < 0.001$ $F_{(1, 14)} = 50.62$	n.s
V_E/VO_2	n.s	$P < 0.001$ $F_{(1, 14)} = 114.64$	n.s
P12-13			
V_E	n.s	$P < 0.001$ $F_{(1, 13)} = 181.88$	n.s
V_T	n.s	$P < 0.001$ $F_{(1, 13)} = 99.81$	n.s
f_R	$P < 0.03$ $F_{(1, 13)} = 4.20$	$P < 0.001$ $F_{(1, 13)} = 155.21$	n.s
VO_2	n.s	$P < 0.001$ $F_{(1, 13)} = 7.04$	n.s
V_E/VO_2	n.s	$P < 0.001$ $F_{(1, 13)} = 148.87$	n.s
P27-28			
V_E	n.s	$P < 0.001$ $F_{(1, 13)} = 157.03$	n.s
V_T	n.s	$P < 0.001$ $F_{(1, 13)} = 119.75$	n.s
f_R	n.s	$P < 0.04$ $F_{(1, 13)} = 5.40$	n.s
VO_2	n.s	$P < 0.001$ $F_{(1, 13)} = 16.09$	n.s
V_E/VO_2	n.s	$P < 0.001$ $F_{(1, 13)} = 124.17$	n.s

G.

		Two-way ANOVA		
		Treatment effect	Gas effect	Factorial Interaction
P0				
Male				
	SD1	n.s	$P < 0.03$ $F_{(2, 22)} = 6.86$	n.s
	SD2	n.s	$P < 0.003$ $F_{(2, 22)} = 7.43$	n.s
Female				
	SD1	n.s	$P < 0.001$ $F_{(2, 28)} = 8.71$	n.s
	SD2	n.s	$P < 0.001$ $F_{(2, 28)} = 9.81$	n.s
P6-7				
Male				
	SD1	$P < 0.03$ $F_{(1, 26)} = 6.36$	$P < 0.002$ $F_{(2, 26)} = 7.69$	n.s
	SD2	$P < 0.008$ $F_{(1, 26)} = 4.52$	$P < 0.001$ $F_{(2, 26)} = 17.58$	n.s
Female				
	SD1	n.s	$P < 0.001$ $F_{(2, 28)} = 10.71$	n.s
	SD2	n.s	$P < 0.001$ $F_{(2, 28)} = 18.07$	n.s
P12-13				
Male				
	SD1	$P < 0.007$ $F_{(1, 26)} = 8.12$	$P < 0.001$ $F_{(2, 26)} = 14.85$	$P < 0.04$ $F_{(2, 26)} = 3.61$
	SD2	$P < 0.009$ $F_{(1, 26)} = 7.80$	$P < 0.001$ $F_{(2, 26)} = 11.90$	n.s
Female				
	SD1	$P < 0.003$ $F_{(1, 26)} = 8.96$	$P < 0.001$ $F_{(2, 26)} = 14.19$	$P < 0.004$ $F_{(2, 26)} = 6.71$
	SD2	$P < 0.009$ $F_{(1, 26)} = 7.78$	$P < 0.001$ $F_{(2, 26)} = 14.18$	$P < 0.008$ $F_{(2, 26)} = 5.88$
P27-28				
Male				
	SD1	$P < 0.002$ $F_{(1, 32)} = 13.57$	$P < 0.001$ $F_{(2, 32)} = 23.26$	n.s
	SD2	$P < 0.01$ $F_{(1, 32)} = 5.99$	$P < 0.001$ $F_{(2, 32)} = 10.00$	n.s
Female				
	SD1	$P < 0.03$ $F_{(1, 27)} = 6.16$	$P < 0.001$ $F_{(2, 27)} = 26.09$	n.s
	SD2	$P < 0.006$ $F_{(1, 27)} = 8.14$	$P < 0.001$ $F_{(2, 27)} = 12.49$	n.s

H.

	Two-way ANOVA		
	Treatment effect	Age effect	Factorial Interaction
Male			
P0			
C_L deflation	$P < 0.02$ $F_{(1, 41)} = 11.29$	$P < 0.001$ $F_{(3, 41)} = 70.16$	n.s
P27-28			
C_T inflation	$P < 0.001$ $F_{(1, 41)} = 13.50$	$P < 0.001$ $F_{(3, 41)} = 16.47$	n.s
C_T deflation	$P < 0.03$ $F_{(1, 41)} = 6.74$	$P < 0.001$ $F_{(3, 41)} = 31.59$	n.s
C_L inflation	$P < 0.05$ $F_{(1, 41)} = 6.63$	$P < 0.001$ $F_{(3, 41)} = 27.45$	n.s
C_L deflation	$P < 0.02$ $F_{(1, 41)} = 11.29$	$P < 0.001$ $F_{(3, 41)} = 70.16$	n.s
C_B deflation	$P < 0.006$ $F_{(1, 40)} = 8.20$	$P < 0.01$ $F_{(3, 40)} = 4.01$	n.s
Female			
All Ages			
C_T inflation	n.s	$P < 0.001$ $F_{(3, 41)} = 15.99$	n.s
C_T deflation	n.s	$P < 0.001$ $F_{(3, 41)} = 41.69$	n.s
C_L inflation	n.s	$P < 0.001$ $F_{(3, 41)} = 41.97$	n.s
C_L deflation	n.s	$P < 0.001$ $F_{(3, 41)} = 56.10$	n.s
P27-28			
C_B deflation	$P < 0.001$ $F_{(1, 39)} = 4.22$	$P < 0.002$ $F_{(3, 39)} = 5.83$	$P < 0.005$ $F_{(3, 39)} = 4.90$

I.

		Two-way ANOVA repeated measures		
		Treatment effect	Pressure effect	Factorial Interaction
P0				
Male				
	C_L	$P < 0.05$ $F_{(1, 108)} = 5.72$	n/a	n.s
Female				
	C_T	$P < 0.01$ $F_{(1, 115)} = 7.34$	n/a	n.s
P6-7				
Male				
	C_T	$P < 0.03$ $F_{(1, 118)} = 8.82$	n/a	$P < 0.03$ $F_{(1, 118)} = 1.93$
Female				
	C_T	$P < 0.03$ $F_{(1, 118)} = 6.20$	n/a	n.s
	C_L	$P < 0.02$ $F_{(1, 117)} = 6.84$	n/a	n.s
	C_B	$P < 0.03$ $F_{(1, 110)} = 6.04$	n/a	n.s
P12-13				
Male				
	C_L	$P < 0.01$ $F_{(1, 116)} = 7.14$	n/a	n.s
Female				
	C_B	$P < 0.001$ $F_{(1, 118)} = 10.46$	n/a	n.s
P27-28				
Male				
	C_T	$P < 0.003$ $F_{(1, 120)} = 14.88$	n/a	$P < 0.003$ $F_{(1, 120)} = 2.70$
	C_L	$P < 0.008$ $F_{(1, 112)} = 11.55$	n/a	$P < 0.04$ $F_{(1, 112)} = 1.85$
Female				
	C_T	$P < 0.01$ $F_{(1, 120)} = 7.86$	n/a	$P < 0.002$ $F_{(1, 120)} = 2.76$
	C_L	$P < 0.04$ $F_{(1, 124)} = 3.93$	n/a	$P < 0.04$ $F_{(1, 124)} = 1.85$

J.

	Two-way ANOVA		
	Treatment effect	Age effect	Factorial Interaction
MALE			
P0	$P < 0.05$ $F_{(1, 36)} = 12.07$	n.s	n.s
P6-7	$P < 0.05$ $F_{(1, 36)} = 12.07$	n.s	n.s
P12-13	$P < 0.002$ $F_{(1, 36)} = 12.07$	n.s	n.s

K.

	Two-way ANOVA		
	Treatment effect	Age effect	Factorial Interaction
MALE			
P0 and P6-7			
A1/C1	$P < 0.007$ $F_{(1, 28)} = 10.73$	$P < 0.001$ $F_{(3, 28)} = 8.56$	$P < 0.03$ $F_{(3, 28)} = 3.37$
A2	$P < 0.002$ $F_{(1, 28)} = 7.62$	$P < 0.001$ $F_{(3, 28)} = 14.11$	$P < 0.001$ $F_{(3, 28)} = 7.68$
C3	$P < 0.04$ $F_{(1, 28)} = 4.90$	$P < 0.008$ $F_{(3, 28)} = 4.79$	n.s
P6-7			
A6	$P < 0.001$ $F_{(1, 28)} = 19.94$	$P < 0.001$ $F_{(3, 28)} = 218.27$	$P < 0.001$ $F_{(3, 28)} = 31.44$

L.

	Two-way ANOVA		
	Treatment effect	Age effect	Factorial Interaction
MALE			
P0			
LEAK	$P < 0.04$ $F_{(1, 88)} = 4.40$	$P < 0.001$ $F_{(3, 88)} = 370.27$	n.s
P/L	$P < 0.001$ $F_{(1, 88)} = 13.43$	$P < 0.001$ $F_{(3, 88)} = 15.35$	$P < 0.02$ $F_{(3, 88)} = 3.18$
L/P	$P < 0.001$ $F_{(1, 88)} = 11.99$	$P < 0.001$ $F_{(3, 88)} = 22.31$	n.s
L/E	$P < 0.001$ $F_{(1, 88)} = 14.58$	$P < 0.001$ $F_{(3, 88)} = 14.55$	$P < 0.008$ $F_{(3, 88)} = 4.23$
P12-13			
ETS	$P < 0.01$ $F_{(1, 88)} = 3.57$	$P < 0.001$ $F_{(3, 88)} = 817.20$	$P < 0.04$ $F_{(3, 88)} = 2.95$
P/E	$P < 0.05$ $F_{(1, 88)} = 2.83$	$P < 0.001$ $F_{(3, 88)} = 5.62$	n.s
L/E	$P < 0.001$ $F_{(1, 88)} = 14.58$	$P < 0.001$ $F_{(3, 88)} = 14.55$	$P < 0.008$ $F_{(3, 88)} = 4.23$
FEMALE			
P6-7			
P/E	$P < 0.008$ $F_{(1, 85)} = 3.87$	$P < 0.001$ $F_{(3, 85)} = 12.12$	$P < 0.008$ $F_{(3, 85)} = 4.20$
P27-28			
OXPHOS	$P < 0.02$ $F_{(1, 85)} = 3.58$	$P < 0.001$ $F_{(3, 85)} = 431.04$	n.s

M.

	Two-way ANOVA		
	Treatment effect	Gas effect	Factorial Interaction
MALE			
Basal			
SAP	$P < 0.03$ $F_{(1, 22)} = 6.52$	n/a	n.s
Hypercapnia			
HR	$P < 0.02$ $F_{(1, 22)} = 7.32$	$P < 0.004$ $F_{(2, 22)} = 15.33$	n.s
Hypoxia			
SAP	n.s	$P < 0.007$ $F_{(2, 22)} = 14.46$	n.s
HR	n.s	$P < 0.001$ $F_{(2, 22)} = 33.60$	n.s
FEMALE			
Hypoxia			
SAP	n.s	$P < 0.001$ $F_{(2, 23)} = 11.05$	n.s
MAP	n.s	$P < 0.002$ $F_{(2, 23)} = 8.28$	n.s
HR	n.s	$P < 0.001$ $F_{(2, 23)} = 53.87$	n.s
T_B			
Male	n.s	$P < 0.001$ $F_{(2, 32)} = 43.01$	n.s
Female	n.s	$P < 0.001$ $F_{(2, 28)} = 15.18$	n.s

CAPÍTULO 2

Long-term effects on cardiorespiratory control of male and female rats prenatally exposed to cannabinoid agonist

The development of the respiratory network can be affected by the use of drugs of abuse during pregnancy, since the prenatal period is highly sensitive to pharmacological interventions, leading to long-term consequences. The psychoactive compounds of *Cannabis* act directly on the endocannabinoid system, and the deleterious effects of external cannabinoids during gestation may be related to negative interference in the central nervous system (CNS) formation, structuring and functioning of the respiratory and cardiovascular system. Nevertheless, the influence of external cannabinoids on the cardiorespiratory network development, as well as in the chemosensitivity and its future consequences at adulthood is still unclear. We aimed to evaluate the effects of prenatal exposure to cannabinoid agonist on the cardiorespiratory control system of male and female adult rats by osmotic pump implantation in pregnant female rats delivering vehicle or synthetic cannabinoid (WIN 55212-2, 0.5 mg/kg/day) during the entire gestation. Exogenous cannabinoid exposure during pregnancy resulted in a sex-dependent difference in breathing control, specifically with a higher chemosensitivity to CO_2 and O_2 for males and decreased for females. An altered cardiovascular control was also found, where prenatally treated male and female show to be more likely to have hypertension and tachycardia during adverse environmental conditions. However, changes in the mechanical component of the respiratory system were not observed, as well as there were no neuroanatomical alterations such as increased expression of CB1 receptors in the brainstem, nor increase in the number of neurones in the catecholaminergic regions. These findings highlight that external interference in the cannabinoid signaling during embryonic development cause sex-specific long-lasting effects for the cardiorespiratory system at adulthood.

Disorders caused by use of drugs during pregnancy continue to be a current major public health issue, representing a risk to the child's development and imposing socioeconomic charges, with increased need for medical and social services. *Cannabis* is one of the most widely used drug of abuse at ages highly correlated with a possible pregnancy, and its use during gestation has considerably increased in recent years driven by government flexibilization policies (Corsi et al., 2019; Volkow et al., 2019). Worldwide legalization initiatives emphasize the crucial need to better understand the risks and consequences of *Cannabis* use during pregnancy in the mothers' health and their offspring, especially because cannabinomic substances has been prescribed for pregnant women due to its antiemetic properties (Volkow et al., 2017).

The exogenous, phyto or synthetic cannabinoid, readily cross the placenta and can enter the fetal bloodstream (Richardson et al., 2016), affecting prenatal and postnatal biological processes, including central nervous system (CNS) ontogeny (Fergusson et al., 2002). The endocannabinoid system plays a key role in neurodevelopmental processes such as proliferation, migration and cell differentiation, as well as neural connectivity and synaptic function (Mulder et al., 2008; Lubman et al., 2015). Thus, *in utero* exposure to synthetic (WIN 55212-2) or phyto (Δ^9-tetrahydrocannabinol, THC) cannabinoid can disturb the fetal endocannabinoid signaling and cause long-lasting deficits (Wu et al., 2011; Bara et al., 2018). Studies performed in humans and animals strongly suggest that disruption of endogenous cannabinoid system may lead to deficiencies in physiological functions, such as in cognitive, motor, thermoregulatory and cardiorespiratory control (Devane et al., 1988; Onaivi et al., 2002; Corsi et al., 2020).

The development of the cardiorespiratory control system starts early in pregnancy, with fetal breathing movements as one of the first motor behaviors to emerge and appears to be essential for pre and postnatal lung growth (Kotecha, 2000; Thoby-Brisson et al., 2009). Respiratory and cardiovascular system development is a highly dynamic process that is subject to external influences (Carroll & Agarwal, 2010), whether physical, psychological or chemical (Bavis & Mitchell, 2008; Cayetanot et al., 2009; Bairam et al., 2015). Regarding the pharmacological intervention during pregnancy, the endocannabinoid signaling interference by maternal consumption of *Cannabis*, has been shown to be an important element for altering the newborn's cardiorespiratory control network (Tree et al., 2010; 2014). The exogenous cannabinoids acts in the CNS mainly via cannabinoid type 1 (CB1) receptors, which was already found throughout the brainstem regions highly related to cardiorespiratory (Pilowsky et al., 1990; Mailleux & Vanderhaeghen, 1992; Rooney et al., 1994; Haji et al., 2000; Harkany et al., 2007), as well as in the lungs (Galieque et al., 1995) and heart (Liu et al., 2000; Bonz et al., 2003).

Studies carried out in human and animal models shows the inhibitory role of the endocannabinoid system on ventilation, and stimulatory effect on blood pressure (Bellville et al., 1975; Malit et al., 1975; Doherty et al., 1983; Padley et al., 2003; Tree et al., 2010). Particularly for breathing maintenance, the contribution of each respiratory nucleus is dependent on the animal's sleep-wake state (Dias et al., 2010; Vicente et al., 2016), and the endocannabinoid system participates in the sleep architecture (Méndez-Díaz et al., 2021; Petrunich-Rutherford & Calik, 2021), being used in treatment of respiratory disorders sleep-related (Carley & Radulovacki, 2008; Prasad et al., 2013). Respiratory control is also influenced by sex (Gargaglioni et al., 2019), in which certain respiratory nuclei have a sexual dimorphism, as retrotrapezoid nucleus (Niblock et al.,

2010) and locus coeruleus (Hormigo et al., 2015; Bangasser et al., 2016), and interestingly, studies have reported a sex-dependent long-term effects of exposure to cannabinoids (for review see Viveros et al., 2012).

Despite the knowledge already established in the literature, long-term studies on the effects of prenatal exposure to cannabinoid on cardiorespiratory control are currently limited. Therefore, in the present study we investigated the long-term consequences of intra-uterine synthetic cannabinoid (WIN) exposure on respiratory and cardiovascular central control in male and female rats at adulthood. The results obtained demonstrate a sex-dependent effect in control of breathing, specifically in chemosensitivity. Additionally, significant changes in the cardiovascular control of prenatally WIN treated male and female animals.

Animal model

The Wistar rats matrices were obtained from UNESP – Botucatu, SP, Brazil. In our animal care facilities, the crossings were performed and pregnant female (first generation) and their newborns were individually placed in cages until weaning (P21). The offspring were then separated from their mothers and allocated in groups of up to 5 individuals per cage according to sex until reach experimental age. 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 7:00 a.m. and 6:00 p.m., during the light phase, on unanesthetized adult (P80-81) male and female rats obtained randomly from different litters.

All the experimental procedures were performed in compliance with the guidelines of the National Council of Control in Animal Experimentation (CONCEA-MCT-Brazil), with the approval of the local College of Agricultural and Veterinary Sciences Animal Care and Use Committee (CEUA-FCAV-UNESP-Jaboticabal; Protocol: 011284/17).

Cannabinoid treatment and gas mixture exposure

Pregnant female rats received constantly from the 0 to the 21st day of gestation vehicle (DMSO 50%, diluted in sterile water) or synthetic cannabinoid (WIN 55,212-2 mesylate salt, Sigma Chemical CO., St. Luis, MO, USA), in a daily dose of 0.5 mg/kg (based on Mereu *et al*., 2003; Tree *et al*., 2014; Bara *et al*., 2018). After confirmation of sperm via vaginal smear, females underwent a surgical procedure under inhalation anesthesia with 5% of isoflurane (Cristália, Sao Paulo, Brazil) for induction and 1% for

maintenance, for subcutaneous osmotic pump (Alzet Osmotic Pumps, Cupertino, CA, USA; model 2ML4; 2.5 µL/hour/28 days) implantation in the back of the animal close to the scapula to delivery the vehicle or drug to the pregnant females. Under anesthesia, the osmotic pump was removed from the female right after giving birth.

The ventilatory challenges were accomplished using gas mixture of hypercapnia (7% CO_2, 21% O_2, balance N_2) and hypoxia (7% CO_2, 21% O_2, balance N_2) purchased from White Martins Gases Industrials Ltda (Osasco, SP, Brazil).

Electromyogram (EMG) and electroencephalogram (EEG) implantation

One week prior to the experimental day, adult (P73-74) male and female rats were anesthetized with isoflurane 5% induction and 1% maintenance and fixed in a stereotaxic apparatus (Kopf Instruments, Kent, England) to allow electrodes implantation for EEG and EMG for sleep/wake cycle recordings. Three EEG electrodes were screwed into the skullcap: frontal electrode at 2 mm anterior to bregma and 2 mm lateral to the midline; parietal electrode at 4 mm anterior to the lambda and 2 mm lateral to the midline; and the third one, laterally between the frontal and parietal electrodes forming a triangle. For EMG recordings, two electrodes were inserted deep into the neck musculature of the rats. All the electrodes were fixed to the animal's head using a mini connector soaked in acrylic cement. At the end, the animals were treated with antibiotic (enrofloxacin, 10 mg/kg, subcutaneous) and analgesic (flunixin meglumine, 2.5 mg/kg, subcutaneous) agents followed by the next 3 days and kept in cages up to four animals.

The signals from the EEG and EMG electrodes were sampled at 150 Hz, filtered at 0.3–50 and 0.1–100 Hz, respectively, and recorded on a computer with a data analysis software (AcqKnowledge MP150, BioPac Systems, Inc., Santa Barbara, CA,

USA). Wakefulness, rapid eye movement (REM) or non-rapid eye movement (NREM) sleep states were registered constantly throughout the experiments. REM sleep state were short and occurred irregularly between the experiments; thus, REM sleep periods were excluded from analysis. The sleep/wake state was determined by analyzing the EEG and EMG records as previously described (Nattie & Li, 2002; Vicente *et al*., 2016; Leirão *et al*., 2018), allowed cardiorespiratory and metabolic parameters analyses during different phases of the sleep/wake cycle.

Cardiovascular and body temperature measures

One day before the experiment, male and female adult rats received inhalation anesthesia (isoflurane, 5% for induction and 1% for maintenance) for a catheter [PE-10 connected to PE-50 (Clay Adams, Parsippany, NJ, USA)] insertion into the abdominal aorta through the femoral artery to allow pulsatile arterial pressure (PAP) measures. The catheter was externalized in the animal's dorse close to the neck region. On the experimental day, the catheter was connected to the pressure transducer (TSD 104A, Biopac systems), the signal was amplifier (DA 100C, Biopac systems) and digitized on a computer equipped with data acquisition software (MP100ACE; Biopac Systems). Systolic (SAP) and diastolic (DAP) arterial pressure, mean arterial pressure (MAP) and heart rate (HR) were quantified from the PAP records using the LabChart program (Power-Lab System, ADInstruments®/Chart Software, version 7.3, Sydney, Australia).

For body temperature (T_B) measurements, a temperature datalogger (SubCue Dataloggers, Calgary, Canada) was inserted into the abdominal cavity through a midline laparotomy, at the same surgical procedure. The datalogger was programmed to acquire data every 5 min.

Pulmonary ventilation assessment

The ventilation ($\dot{V}_E$) of male and female adult rats were acquired by whole body plethysmography – closed system (Drorbaugh & Fenn, 1955; Patrone *et al*., 2018). The airflow was maintained at 1.8 $L.min^{-1}$ using a flow meter coupled to a suction pump (MFS, Sable Systems International, Inc, Las Vegas, USA), and interrupted for $\dot{V}_E$ measurements, with the chamber fully sealed for approximately 2 min. 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 a computer equipped with data acquisition software (MP100ACE, Biopac Systems). The sampling frequency was 200 Hz. A volume calibration was performed by injecting 1 mL of air into the chamber using a graduated syringe. Tidal volume (V_T) was calculated applying Drorbaugh & 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 T_C. $\dot{V}_E$ was calculated as the product of respiratory frequency (f_R) and V_T and normalized to the animal's body weight. $\dot{V}_E$ and V_T were presented under ambient barometric pressure conditions at T_C 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 ($\dot{V}O_2$) with flow-through *Pull mode* configuration in an open respirometry system was used for metabolic rate determination (Mortola, 1984; Cummings *et al*., 2011; Patrone *et al*., 2018). An O_2 analyzer (model ML206, ADInstuments®, Australia) was connected to a MFS pump ("Mass Flow System", Sable Systems International, USA) that was coupled to the outlet of the plethysmography chamber, keeping the airflow at 1.8 $L.min^{-1}$, with subsampling of 150 $mL.min^{-1}$ by the O_2 analyzer. The expired gas was dried over a small column of Drierite (W.A. Hammond Drierite Co. Ltd, Xenia, OH, USA) before goes through the analyzer. The air was continuously sampled allowing for the determination of $\dot{V}O_2$ by a data acquisition program (Power-Lab System, ADInstruments® / Chart Software, version 7.3, Sydney, Australia).

The CO_2 was not analyzed or scrubbed, then aiming at a better metabolic rate measurement the $\dot{V}O_2$ was calculated using the following equation (Koteja, 1996):

$$\dot{V}O_2 = [FR_e (F_iO_2 - F_eO_2)] / [1 - F_iO_2 (1 - RQ)]$$

where FR_e: end flow rate of air through the chamber, F_iO_2: inlet O_2 fraction, F_eO_2: end O_2 fraction, and RQ: respiratory quotient (considered to be 0.85). The $\dot{V}O_2$ was corrected for the body mass.

Experimental protocol

Control and WIN-treated male and female rats were individually placed inside a plethysmography chamber allowing to move freely at room temperature of 25ºC. Firstly, the chamber was flushed with room air (21% O_2) for 40 min during the acclimation phase. Then, $\dot{V}_E$ and $\dot{V}O_2$ were recorded in normoxic normocapnic conditions for 1 h. Subsequently, the animals were exposed to hypercapnia (7% CO_2 gas

mixture) for 1 h min. After CO_2 exposure, the chamber was ventilated again with room air for 1 h to allow for recovery baseline values. Posteriorly, the animals were submitted to hypoxia (10% O_2 gas mixture) for 1 h. The $\dot{V}O_2$, PAP, T_B measurements and sleep/wake cycle signals were recorded throughout the experiment. The ventilatory parameter measurements were performed every 10 min during each environmental condition.

Respiratory mechanics evaluation

The mechanical component of the respiratory system was evaluated according to previous studies (Frappell et al., 1998; Hedrick et al., 2011). Male and female adult rats were euthanized by isoflurane inhalation, and a cannula was inserted into the animal's trachea. A pressure transducer (TSD 104A, Biopac systems), was connected to the cannula for intra-tracheal pressure measurements. The signals were amplified (DA 100C, Biopac systems), filtered and recorded in a data acquisition system (Biopac Systems Inc., Santa Barbara, CA, USA). To allow injections of known air volumes (2 mL) into the lungs a graduated syringe was connected to the tracheal cannula by a three-way connector. In a supine position, the lungs were gradually inflated and deflated to build the pressure-volume static curve. Inflation ended when intra-tracheal pressure reached approximately 30 cmH_2O. Posteriorly, the lungs were deflated in the same gradual manner, until -20 cmH_2O of pressure, and finally inflated again to the resting lung pressure (0 cmH_2O), and the trachea opened to the atmosphere to balance intra-tracheal pressure. Before starting another curve, the lungs were fully inflated and then spontaneously allowed to return to resting volume by opening the system to the atmosphere, to prevent collapsing. The maximum lung volume determined by the static

curve protocol was injected steadily over an interval of 20 s for dynamic respiratory mechanics measurements.

For each animal, these procedures were repeated three times with on intact animal (C_T), then the body cavity and rib cage opened and the muscles and organs removed leaving the lungs free. Next, the static and dynamic experimental protocols were repeated with the lungs exposed (C_L). At the end of the experiments, the animals' heart and lungs were removed and weighed.

Western blotting analysis

To assess the CB1 receptor protein expression in the brainstem of male and female adult rats, Western Blot technique was applied. Control and WIN-treated animals were deeply anesthetized with isoflurane and the brainstem removed and frozen in 2-methylbutane at -20°C. RIPA buffer (50 mM tris, 150 mM NaCl, 0.1% triton, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) was used to homogenize the samples, and Lowry's method for protein quantification. Samples containing 15 μg of protein were mixed with buffer (1.25 M tris pH 6.8, 2% SDS, 0.01% blue bromophenol, 10% glycerol, and 250 mM 2-ME) and heated to 95ºC for 5 minutes, then submitted to SDS-PAGE in bis-acrylamide gel with 10% gradiente, in buffer (192 mM glycine, 25 mM tris, and 0.1% SDS, pH 8.3). Proteins migrated in the gel were transferred to the nitrocellulose membrane (Bio-Rad Laboratories, ON, Canada) by wet electrotransfer (15 mM tris, 120 mM glycine, and 20% methanol, pH 8.3). Next, non-specific sites on the membrane were blocked with T-TBS buffer (20 mM tris, 150 mM NaCl, and 0.1% tween 20, pH 7.4) with 5% bovine serum albumin (BSA) for 1 hour at room temperature, under constant agitation. The blocking solution was washed with T-TBS and the membranes incubated overnight at 4ºC with the rabbit polyclonal anti-CB1

antibody (1:1000; Sigma) in T-TBS solution with 3% BSA. The membranes were washed five times for 5 minutes with T-TBS, and then incubed with anti-rabbit peroxidase-labeled secondary antibody (1:5000, Santa Cruz Biotechonology) diluted in T-TBS with 5% BSA for 1 h at room temperature. Posteriorly, the membranes were washed again with T-TBS, chemiluminescence enhancer (ECL) added and the film exposed. The bands were quantified using the ImageJ program (available for free download at https://imagej.nih.gov/ij/download.html). The bands of the CB1 protein were normalized by β-actin using mouse monoclonal anti-βActin (1:15000, Sigma), followed by anti-mouse peroxidase-labeled secondary antibody (1:20000, Santa Cruz Biotechonology).

Catecholaminergic (CA) neurones labeling

Adult male and female rats were deeply anesthetized with isoflurane at the end of the plethysmography experiments. The animals were perfused intracardially using a pump machine (Masterflex; Cole-Parmer Instrument Company, Vernon Hills, IL, USA) with phosphate buffered saline (PBS, 0.01 M, pH 7.4), followed by 4% paraformaldehyde (PFA) in 0.2 M phosphate buffer (PB). Quickly, the brain was removed, postfixed with 4% PFA at 4°C for 12 h, and then immersed in 30% sucrose solution for at least 48 h at 4°C. The brain was dipped in 2-methylbutane at -20°C, frozen and fixed in Tissue-Plus (Fisher Healthcare™ O.C.T. Compound, CA, USA). Serial sections (40 μm) of the brainstem were made in triplicates using a cryostat microtome (CM1860 – Ag Protect; Leica, Wetzlar, Germany).

To labeling and quantify brainstem catecholaminergic (CA) neurones, immunohistochemistry for tyrosine hydroxylase (TH) was performed (Xu et al., 2003). First, the slices were washed with PBS, followed by an antigenic recovery with 30 min

incubation in a target retrieval solution (Dako, Glostrup, Denmark) at 70°C, then cooled to room temperature and washed with PBS. To avoid non-specific binding, the slices were incubated in 1% hydrogen peroxide solution for 3 min, washed and followed by 1 h in a 10% horse serum solution (Life Technologies, USA) at room temperature. After rinse, slices were incubated for 24 h with a mouse monoclonal anti-TH antibody (1:10000; Sigma) in T-PBS (0.3% Triton-PBS, pH 7.4) solution with 5% horse serum at room temperature with constant agitation. Then, slices were washed and incubated for 3 hours with secondary goat anti-mouse IgG antibody (h&l), conjugated to dy light 488 (1:300, Immunoreagents, NC, USA,) at room temperature with constant agitation. Finally, the slices were washed 3 times with PBS, and mounted on gelatinized sheets, dried and covered with coverslip.

Data analyses

The cardiorespiratory and metabolic parameters were analyzed based on 2 min of records at intervals of every 10 min throughout the time recording during room air, hypercapnia or hypoxia. The results obtained for all variable for each of these conditions were grouped according to sleep and wakefulness state. The $\dot{V}_E$ and $\dot{V}O_2$ was calculated as previous described and normalized by animal's body mass. The air convection requirement ($\dot{V}_E / \dot{V}O_2$) was obtained by dividing the values of $\dot{V}_E$ by the $\dot{V}O_2$. The cardiorespiratory and metabolic variables are represented as mean ± SEM.

The mechanical component of the respiratory system was evaluated through the static and dynamic compliance of the total system (C_T, intact animal), lungs (C_L, lungs exposed) and body wall (C_B, $C_T - C_L$). The inflation and deflation curve was built using the volume of air injected (mL) *versus* the corresponding intra-tracheal pressure (cmH_20). The highest and lowest point of the curves were applied to the formula: V2-

V1 / P2-P1 to obtain inspiratory and expiratory compliance. The static compliance was calculated around the pressures 0-15 cmH_2O of the inflation and deflation curve, which correspond to the normal range that occurs during *in vivo*. Dynamic compliance was calculated using the same formula, but for pre-established intra-tracheal pressure: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 26, 31 and their corresponding injected air volume, as described by Hedrick et al., (2011). The static compliance is represented as mean ± SEM, while dynamic compliance as interconnected means.

The TH-immunoreactive (TH-ir) cells were analyzed by a fluorescence microscope (Zeiss, Axio Image Z2, Baden-Württemberg, Germany) using the LAS image acquisition program, that was consisted in the quantification of TH-ir cells within brainstem CA nuclei using a computerized image analysis system (ImageJ). One of the triplicates was used for bilaterally counting over the entire length of the brainstem in which it contained the CA nuclei A1/C1, A2, C3, A5 and A7 (based on anatomical landmarks from Paxinos & Watson, 1998). For A6 region, since it has a high amount of clustered CA neurones, the analysis between the groups was done through the density of TH expression per fixed sampled area. To avoid background interference, the A6 region sampled density was subtracted by the density region without TH labelling. The quantification data are represented as mean ± SEM.

All the statistical analyzes were performed between groups by two-way ANOVA, with repeated measures when appropriated. *Post-hoc* multiple comparisons were performed using Tukey's test. The results of the statistical analysis are detailed in Supplementary Figure 1. Statistical results with $P < 0.05$ were considered significant.

The exposure to synthetic cannabinoid (WIN) during embryonic development did not cause changes in body mass gain when offspring reaches the adulthood (Table 1), although an expected effect of sex has been evident, with a lower body mass in female groups. The heart and lungs' weight of adult animals also was not affected by prenatal WIN treatment. A significant sex-difference in heart weight was observed for females but may be due to a direct influence by female's body weight, since the variable was normalized by body mass.

Table 1. Body mass, weight of heart and lungs of control and WIN-treated adult male and female rats.

		Body mass (g)	**Heart (g.kg^{-1})**	**Lungs (g.kg^{-1})**
Male	VEHICLE	339.2 ± 6.4	3.6 ± 0.1	5.1 ± 0.2
	WIN	322.6 ± 12.3	3.7 ± 0.1	5.0 ± 0.1
Female	VEHICLE	215.4 ± 5.7$^{\#}$	4.6 ± 0.2$^{\#}$	5.9 ± 0.1
	WIN	213.5 ± 3.7$^{\#}$	4.2 ± 0.1$^{\#}$	5.5 ± 0.5

Values are expressed as mean ± S.E.M. $^{\#}$ Indicates significant difference between sex in the same group of treatment. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 1A.

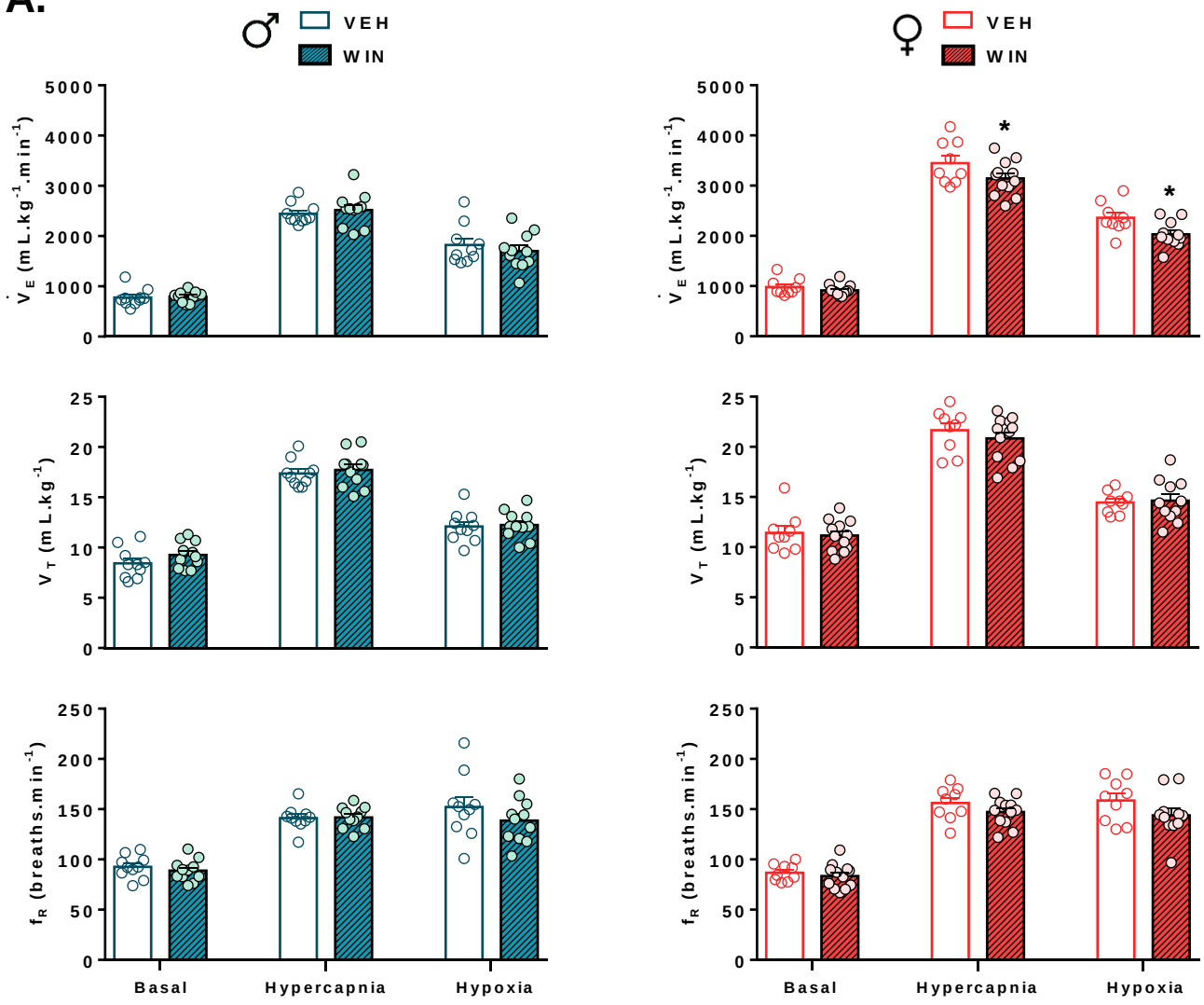
Respiratory control and metabolism

Regardless of sleep or wakefulness state, exposure to high levels of CO_2, as well as low concentrations of oxygen caused a robust increase in $\dot{V}_E$ for all groups of both sexes, due to increased V_T and f_R. The $\dot{V}O_2$ was altered only by the hypoxia, decreasing during this condition. On the other hand, the excess stimulation of the endocannabinoid system by fetal exposure to synthetic cannabinoid seems to have a long-term effect on breathing control of adult rats in a sex-specific manner. The $\dot{V}_E$,

V_T, f_R, $\dot{V}O_2$ and the $\dot{V}_E / \dot{V}O_2$ for control and WIN-treated male and female rats during basal condition, hypercapnic or hypoxic exposure are demonstrated in the Figure 1 (awake state) and Figure 2 (sleep state). As evidenced, WIN-treatment at gestational phase did not result in respiratory (Figure 1A) or metabolic (Figure 1B) changes in any environmental conditions during awake state for males at adulthood. In contrast, adult females prenatally WIN exposed showed no resting $\dot{V}_E$ and $\dot{V}O_2$ alteration; however, the hypercapnic (HCVR) and hypoxic (HRV) ventilatory response was significantly reduced by WIN treatment, although there was no significant alteration in the V_T and f_R. No treatment effects were found for $\dot{V}O_2$ during both ventilatory challenges, but a smaller increase in $\dot{V}_E / \dot{V}O_2$ was evidenced for hypercapnia and hypoxia exposure during awake state.

AWAKE

A.


♂
VEH
WIN
♀
VEH
WIN
$\dot{V}_E$ (mL.kg^{-1}.min^{-1})
V_T (mL.kg^{-1})
f_R (breaths.min^{-1})
0
1000
2000
3000
4000
5000
0
5
10
15
20
25
0
50
100
150
200
250
*
*
Basal
Hypercapnia
Hypoxia

B.

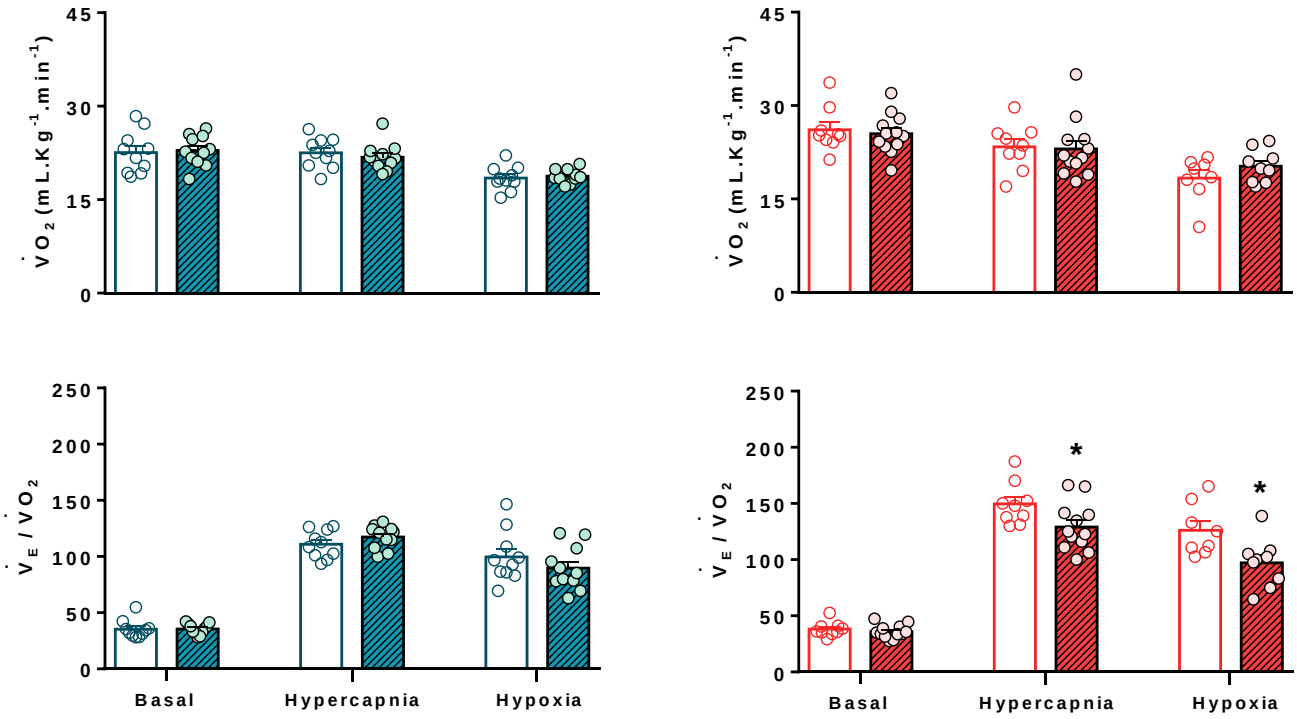
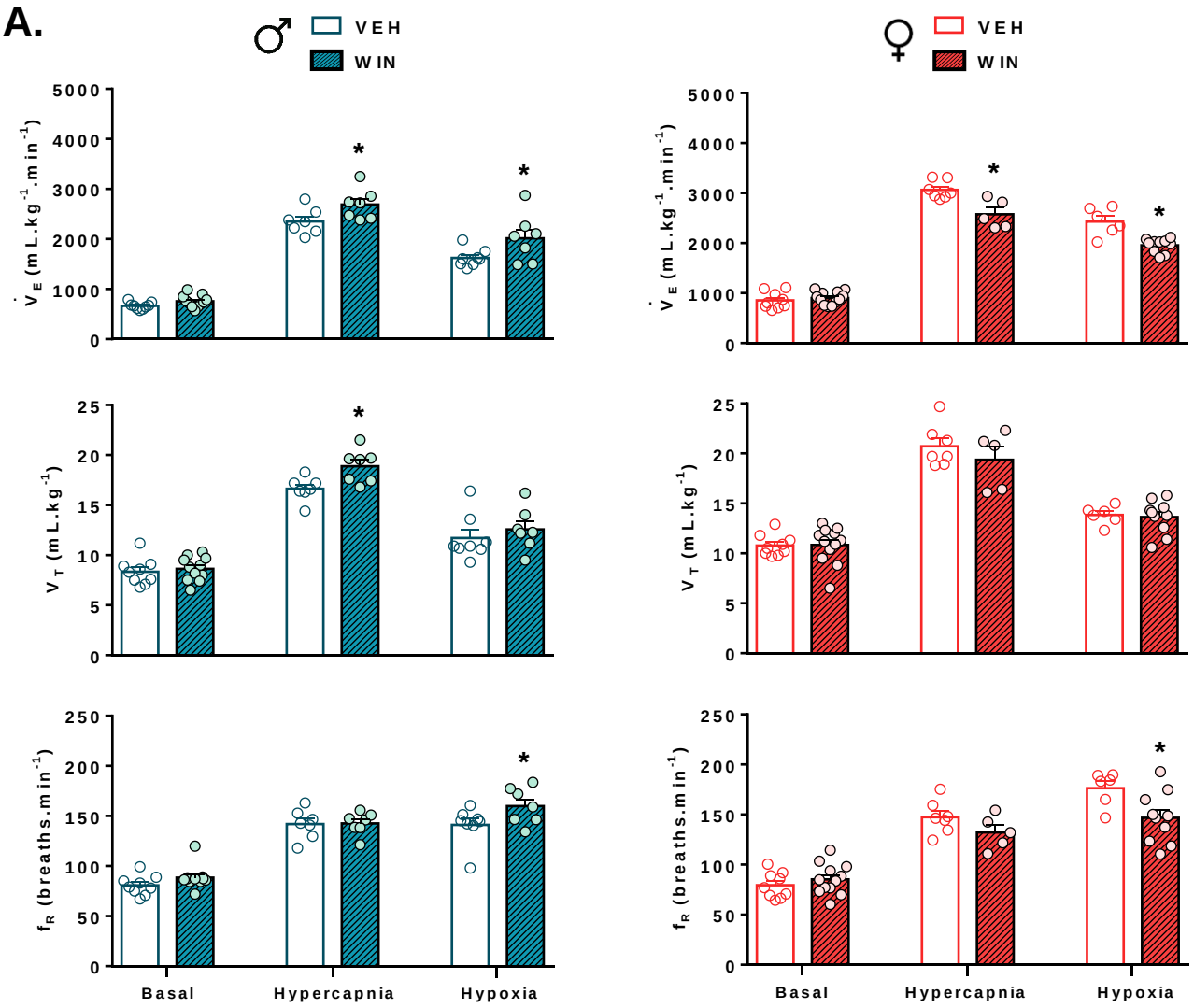


Figure 1: Effect of prenatal WIN exposure on **A**: ventilation ($\dot{V}_E$), tidal volume (V_T), respiratory frequency (f_R); **B**: oxygen consumption ($\dot{V}O_2$) and air convection requirement ($\dot{V}_E / \dot{V}O_2$) for control and WIN-treated male and female adult rats during resting condition (Basal), hypercapnia (7% CO_2) and hypoxia (10% O_2) at awake state. Values are expressed as mean ± S.E.M. * indicates a significant difference between control and WIN-treated groups. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 1B.

With regard the ventilatory control and metabolic demand in sleep state, all the same parameters were evaluated when animals were sleeping, and it is shown in Figure 2. In this context, prenatal exposure to synthetic cannabinoid seems to result in a higher HCVR and HVR for adult male rats, mainly due to an increase in V_T for CO_2 exposure and in the f_R for hypoxia, without any effect on resting ventilation (Figure 2A). In adult female rats, WIN-treated group presented a reduced ventilatory response to CO_2 and O_2 at sleep state compared to control group, and the effect during hypoxic challenge was mainly caused by a reduced f_R. There was no influence of prenatal WIN treatment on

the metabolic rate in adulthood for both sexes, since $\dot{V}O_2$ was not different between control and treated animals (Figure 2B). Nevertheless, the $\dot{V}_E / \dot{V}O_2$ equivalent was significantly higher for treated rats in both environmental challenges, and significant reduced in females, in line with ventilatory response.

SLEEP


A.
♂
VEH
WIN
♀
VEH
WIN
$\dot{V}_E$ (mL.kg^{-1}.min^{-1})
V_T (mL.kg^{-1})
f_R (breaths.min^{-1})
Basal
Hypercapnia
Hypoxia

B.

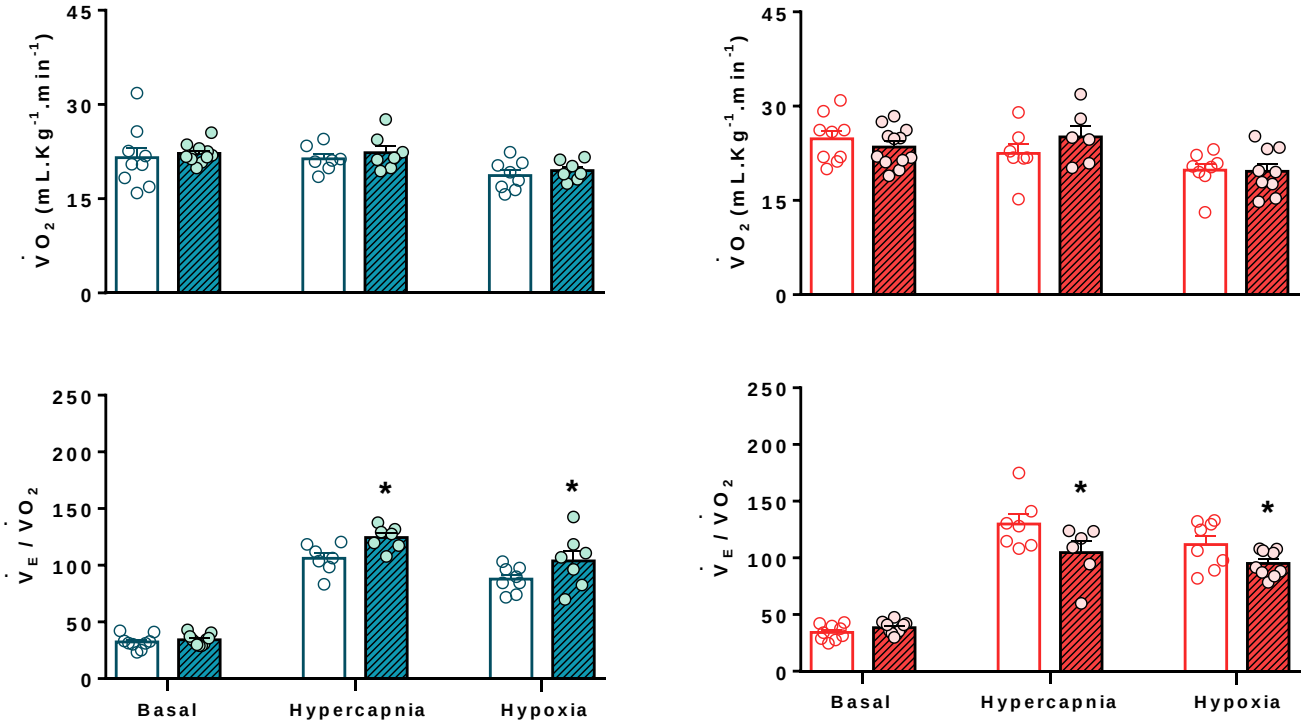


Figure 2: Effect of prenatal WIN exposure on **A**: ventilation ($\dot{V}_E$), tidal volume (V_T), respiratory frequency (f_R); **B**: oxygen consumption ($\dot{V}O_2$) and air convection requirement ($\dot{V}_E / \dot{V}O_2$) for control and WIN-treated male and female adult rats during resting condition (Basal), hypercapnia (7% CO_2) and hypoxia (10% O_2) at sleep state. Values are expressed as mean ± S.E.M. * indicates a significant difference between control and WIN-treated groups. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 1C.

Respiratory system compliance

The experimental protocols of the ventilatory system mechanical component of male and female adult rats demonstrate only a sex-difference between the total (C_T – A and B) and lung (C_L – C and D) compliance parameters during inflation and deflation, without WIN treatment effects as can be observed in Figure 3. Thereby, control and prenatal WIN exposed female adult rats had an increased compliance when compared with respective male group.

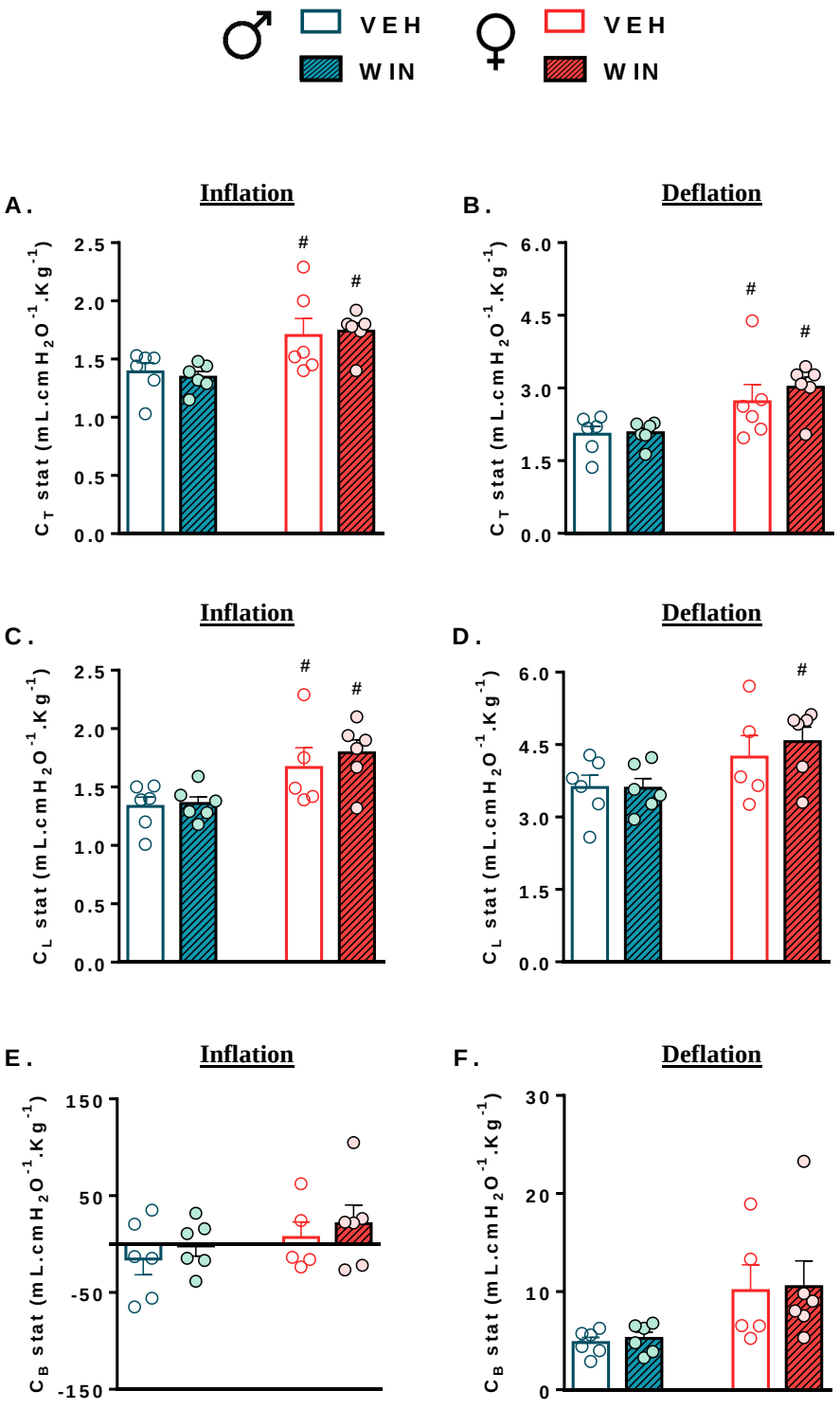


Figure 3: Effect of prenatal WIN exposure on total (C_T – **A** and **B**), lung (C_L – **C** and **D**) and body wall (C_B – **E** and **F**) static compliance during inflation and deflation for control and WIN-treated male and female adult rats. Values are expressed as mean ± S.E.M. # Indicates significant difference between sex in the same group of treatment. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 1D.

The dynamic compliance of the respiratory system of male and female adult animals, exposed to external synthetic cannabinoid during the embryonic phase, was not remarkable altered by treatment, since only occasional changes were observed (Figure 4). For C_T, just at the pressure 5 cmH_2O on the curve was significantly higher for the treated male group. Not differently, the C_L compliance was affected by WIN-treatment only at 15 cmH_2O of pressure for female group, without no effects on C_B for both sexes.

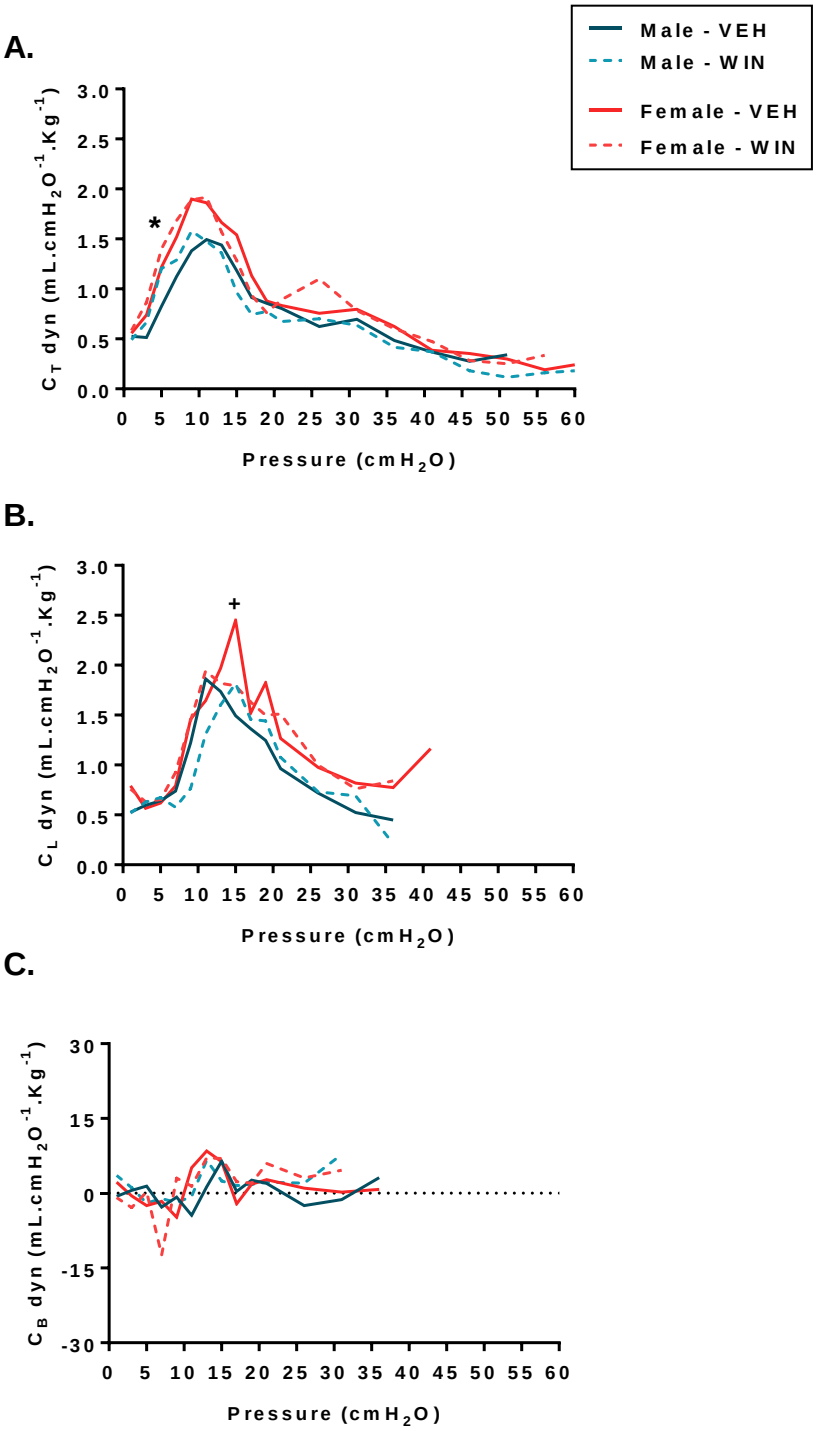


Figure 4: Effect of prenatal WIN exposure on total (C_T – **A** and **B**), lung (C_L – **C** and **D**) and body wall (C_B – **E** and **F**) dynamic compliance for control and WIN-treated male and female adult rats. Values are expressed as mean ± S.E.M. * indicates a significant difference between control and WIN-treated male groups. # indicates a significant difference between control and WIN-treated female groups. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 1D.

Cardiovascular and body temperature data

The systolic arterial pressure (SAP), diastolic arterial pressure (DAP), mean arterial pressure (MAP) and heart rate (HR) parameters of male and female adult rats were measured to assess the possible long-term effect of prenatal WIN exposure on cardiovascular control during hypercapnic and hypoxic condition during wakefulness (Figure 5) and sleep (Figure 6). Regarding males at awake state (Figure 5A), the MAP during hypoxic condition was significantly affected by prenatal treatment, since WIN-treated animals did not present hypoxia-induced MAP decrease, mainly caused by a higher SAP and DAP. No other changes were observed for males in cardiovascular control during awake state. As to females (Figure 5B), exposure to synthetic cannabinoid in the prenatal phase caused significant increases in basal MAP, as a consequence of an increased SAP. Also, the treatment attenuated the MAP reduction induced by hypoxia, due to changes in both SAP and DAP. Additionally, treatment with WIN caused a dichotomous changes in the HR, since a bradycardia was observed under conditions of hypercapnia in the treated females, while a tachycardia during hypoxic exposure.

It is worth mentioning that MAP, SAP and DAP of both sexes had a significantly decrease as an effect of hypoxic environmental condition, as well as HR for hypercapnic gas mixture.

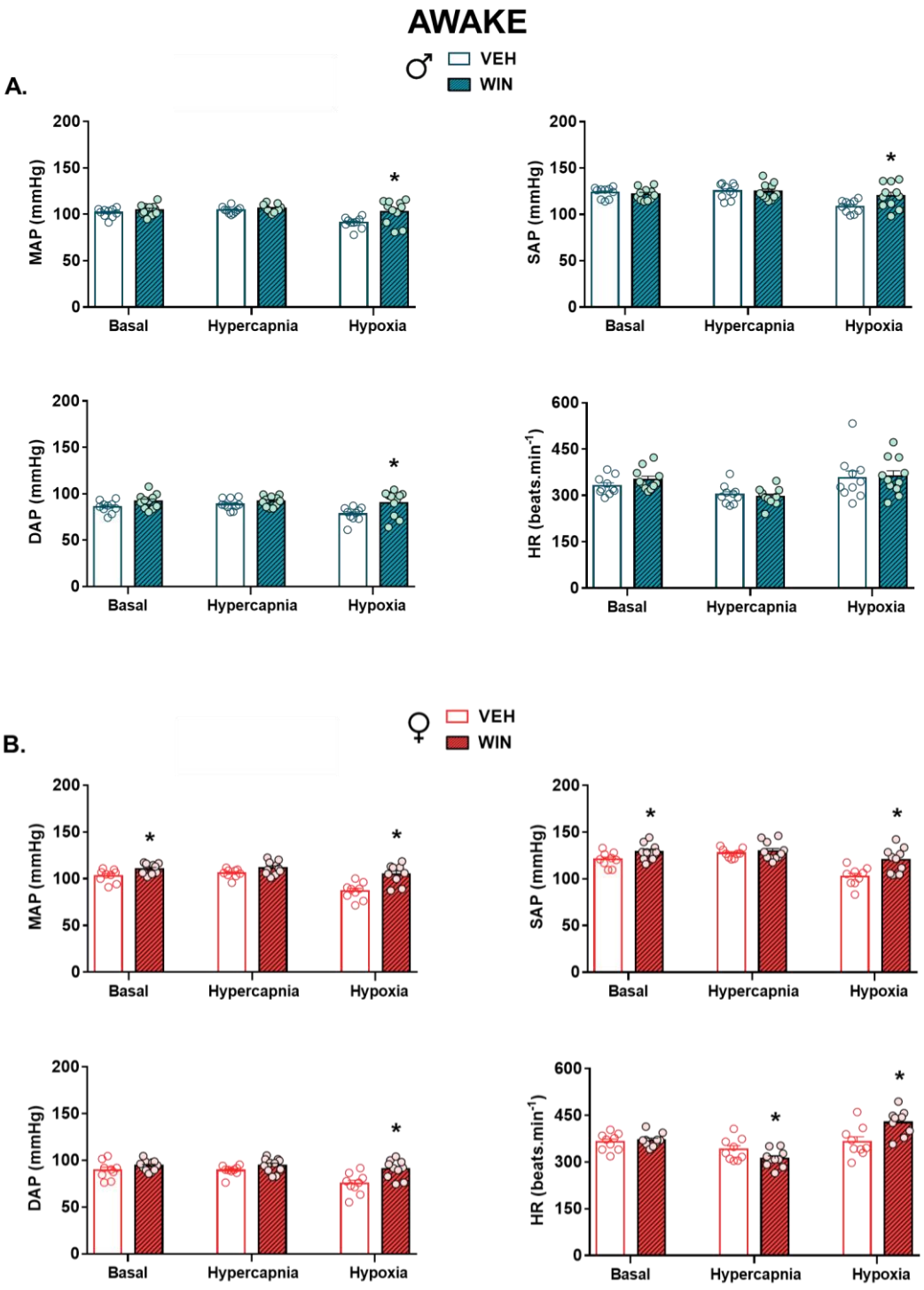


Figure 5: Effect of prenatal WIN exposure on mean arterial pressure (MAP), systolic arterial pressure (SAP), diastolic arterial pressure (DAP), and heart rate (HR) for control and WIN-treated male (**A**) and female (**B**) adult rats under resting, hypercapnic (7% CO_2) and hypoxic (10% O_2) conditions at awake state. Values are expressed as mean ± S.E.M. * indicates a significant difference between control and WIN-treated groups. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 1E.

In the Figure 6, the cardiovascular data are displayed during sleep state. As noted for wakefulness, the effect of intra-uterine WIN exposure on MAP and DAP during hypoxia was maintained in sleep condition for adult males (Figure 6A). Additionally, an effect on HR was evidenced during hypoxic condition, since treated males showed a tachycardia. For female group (Figure 6B), fetal exposure to WIN resulted in a hypertensive MAP at resting and prevented the hypoxia-induced reflex reduction of MAP, supported by an increase in DAP at rest, and SAP and DPA during hypoxia, respectively. The HR also was affected by cannabinoid treated, with the occurrence of tachycardia in WIN-exposed females.

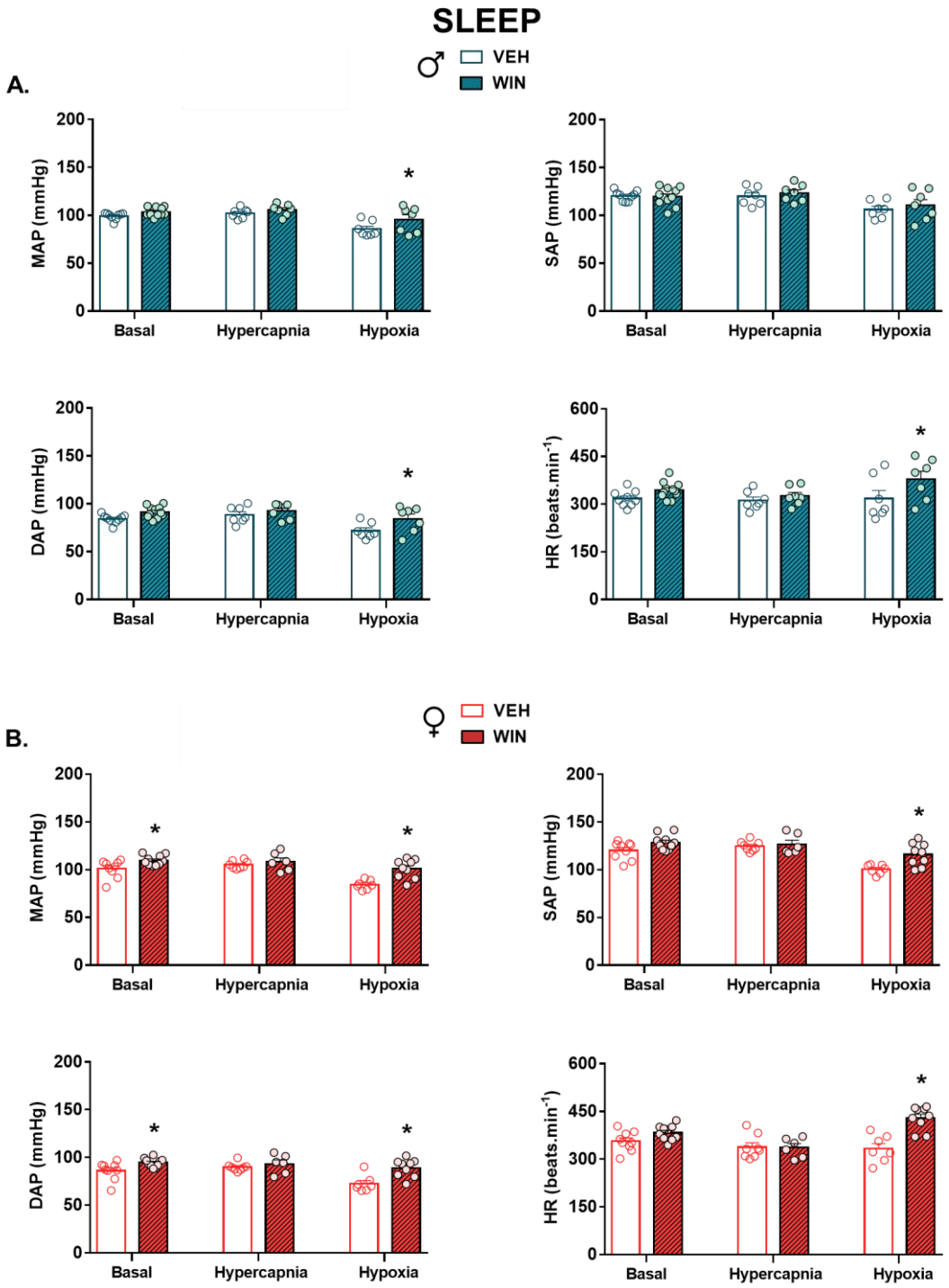


Figure 6: Effect of prenatal WIN exposure on mean arterial pressure (MAP), systolic arterial pressure (SAP), diastolic arterial pressure (DAP), and heart rate (HR) for control and WIN-treated male (**A**) and female (**B**) adult rats under resting, hypercapnic (7% CO_2) and hypoxic (10% O_2) conditions at sleep state. Values are expressed as mean ± S.E.M. * indicates a significant difference between control and WIN-treated groups. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 1E.

The T_B of control and WIN-treated male (A) and female (B) adult rats were accompanied during room air, hypercapnia and hypoxia (Figure 7). As expected, low O_2 levels resulted in a significant drop in T_B for all groups evaluated. In addition, female prenatally WIN exposed had an expressive reduction in T_B during 7% CO_2 condition.

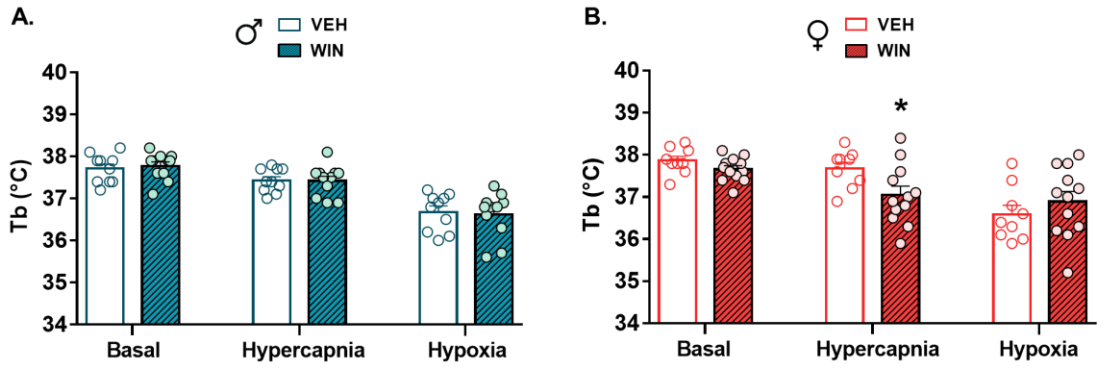


Figure 7: Effect of prenatal WIN exposure on body temperature (T_B) for control and WIN-treated male (**A**) and female (**B**) adult rats under resting, hypercapnic (7% CO_2) and hypoxic (10% O_2). Values are expressed as mean ± S.E.M. * indicates a significant difference between control and WIN-treated groups. The results of two-way ANOVA statistical analyzes are represented in Supplementary Table 1F.

Brainstem CB1 receptor and CA neurones assessment

The chronic and over exposure to synthetic cannabinoid during fetal life did not result in long-term consequences related to brainstem CB1 receptor expression levels, since no significant differences were found in Western Blot analyzes for control and WIN-treated male and female adult animals (Figure 8).

In an aligned way, the exposure to synthetic cannabinoid during embryonic phase, also did not trigger changes in brainstem CA neurones quantification throughout the respiratory control regions, either for males (A) or females (B) in the adulthood (Figure 9).

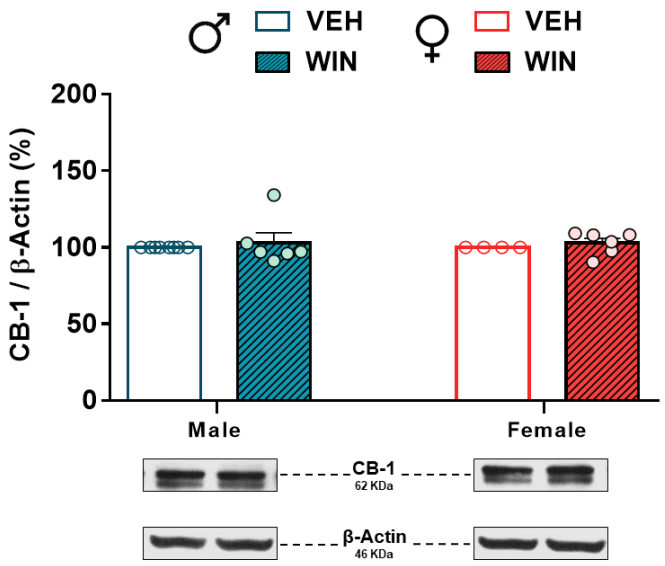


Figure 8: Effect of prenatal WIN exposure on expression of CB1 receptor protein in the brainstem of control and WIN-treated male (**A**) and female (**B**) adult rats. Values are expressed as percentage ± S.E.M.

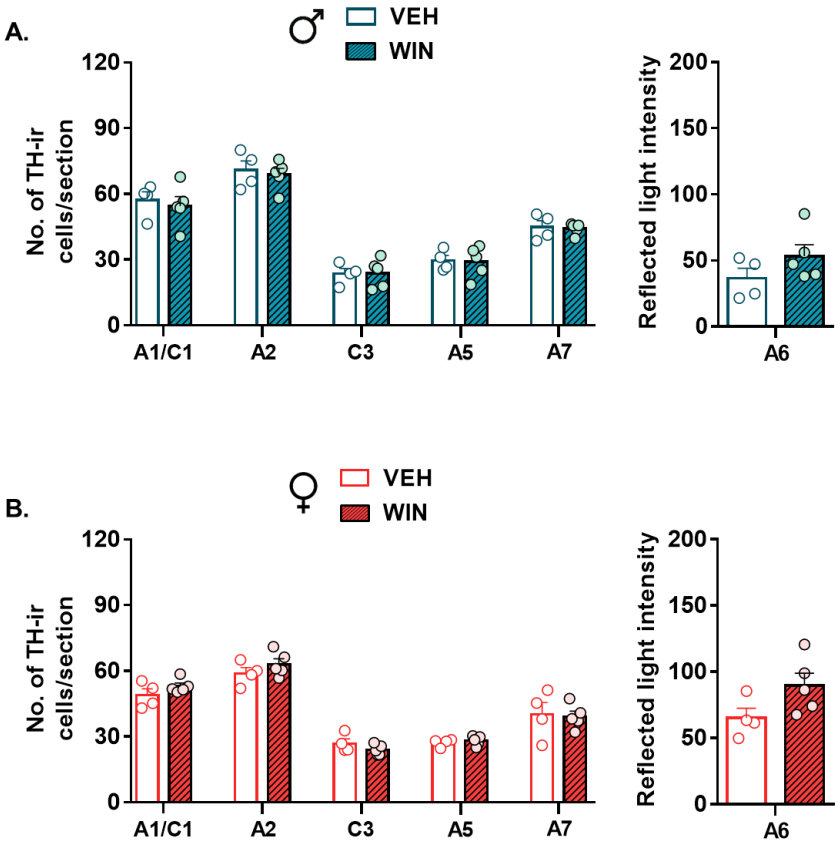


Figure 9: Effect of prenatal WIN exposure on quantification of CA neurones in A1/C1, A2, C3, A5 and A7 regions, as well as light intensity reflected in A6 for control and WIN-treated male (**A**) and female (**B**) adult rats. Values are expressed as mean ± S.E.M.

Cannabis use during pregnancy and its possible long-term effects on offspring, specifically on respiratory and cardiovascular control has been little investigated, while the use of this substance has worldwide steadily increased, including by pregnant women (Volkow et al., 2019; Corsi et al., 2020). Seeking for a better understanding of how exogenous cannabinoids may interfere in the physiological systems development during embryonic phase, particularly in the cardiorespiratory control network, and its postnatal life's assignments, we investigated the long-term consequences of intra-uterine synthetic cannabinoid WIN exposure on respiratory and cardiovascular central control in male and female rats at adulthood. We found that prenatal chronic exposure to external cannabinoid (WIN) triggers robust changes in ventilatory control related to chemosensitivity to CO_2 and O_2 in a sex-dependent manner. Interestingly, some of these changes are sleep-wake cycle dependent. In consonance, *in utero* cannabinoid signaling disturbance imply important changes in the control of cardiovascular parameters in adulthood of male and female rats. Despite the cardiorespiratory alterations, no neuroanatomical changes were reported regarding to brainstem CA neurones quantification, nor CB1 receptor expression.

Fetal exposure to exogenous cannabinoid WIN did not result in changes of animals' body mass when they reached adulthood. The association between *Cannabis* use during pregnancy and the body mass of offspring it is still controversial. Studies in animal models, but mainly humans, present distinct results in which the effect of prenatal exposure to *Cannabis* on body mass of the newborn is evidenced with an expressive reduction (Quinlivan & Evans, 2002; El Marroun et al., 2009; Gunn et al., 2016; Howard et al., 2019), while other studies do not show this alteration (Shankaran et al., 2004; Lozano et al., 2007; Shabani et al., 2011; Gargari et al., 2012). In fact, these

inconsistent results are possibly due to a dose-dependent effect, active principle used and time of exposure of each study.

Intra-uterine chronic exposure to WIN did not result in basal ventilatory changes when offspring reaches the adulthood. Although several studies reported the potential inhibitory role of cannabinoids on respiratory function after THC or synthetic cannabinoid agonist were acutely administrated in humans and animals (Bellville et al., 1975; Doherty et al., 1983; Estrada et al., 1987; Padley et al., 2003; Pfitzer et al., 2004), the prenatal WIN exposure does not appear to result long-lasting changes in respiratory control system under resting conditions. However, a long-term effect in the chemosensitivity of adult rats was found, since the ventilatory response to hypercapnia and hypoxia was increased in adult males prenatally WIN-exposed, specifically during sleep state and unrelated to metabolic changes. Interestingly, the ventilatory responses to CO_2 and O_2 were sex-dependent, since WIN-treated females had an opposite response with decreased HCVR and HVR, without any metabolic alteration, at both sleep and wakefulness state.

It is known that the endocannabinoid system plays an essential role in the ontogeny of the CNS during the pre and postnatal life, with notably differences in the expression and activity of its components during the fetal and postnatal development (Romero et al., 1997; Berrendero et al., 1999; Fernández-Ruiz et al., 2000; Harkany et al., 2007; Fride et al., 2009). Hence, early gestational exposure to cannabinoid could be able to entail lasting neurodevelopmental alterations, since endocannabinoid system is highly correlated with processes of migration, proliferation and differentiation of neurones and glial cells, besides neuronal connectivity and synaptic function (Lubman et al., 2015; El Marroun et al., 2016; Fogaça et al., 2018). Therefore, the changes in ventilatory control, especially caused by chemosensitivity alteration found in the present

study, may be due to a long-term consequence of the respiratory system structuring during embryonic phase in which a CNS plasticity during postnatal development did not occur or not sufficient to annul the effects of prenatal exposure to cannabinoid.

We believe that these ventilatory changes are mainly due to modifications in the respiratory central network because the mechanical component of the respiratory system were not affected by prenatally WIN exposure, since total or lung compliance, as well as the size of the heart and lungs were not different between the WIN and control groups. Although CB1 receptors are mainly found in the CNS, studies have reported its presence in peripheral tissues, including the lungs (Galieque et al., 1995). An effect of THC in pulmonary function was observed in humans (Graham, 1986), and in certain circumstances in a dose-dependent manner, where moderate doses of THC increased airway conductance (Tashkin et al., 1983), while heavy *Cannabis* smoking resulted in decreased airway conductance (Tashkin et al., 1976). According to Rice et al. (1997), CB1 receptors were found in alveolar Type II cell of the lungs that are responsible for synthesizes and secretes surfactant phospholipids and proteins in a highly regulated manner important for successful adaptation to air-breathing at birth in the perinatal period (Rooney et al., 1994). Thus, the role of CB1 receptors in the lungs may is more related to early lifetime. In this sense, corroborating our findings, the intravenous administration of anandamide did not result in dynamic pulmonary compliance changes in adult guinea pigs (Stengel et al., 1998). Regarding the sex difference related to the mechanical component of the respiratory system, we believe that this significant difference in compliance between male and female is due to a direct effect of body mass on the mechanical variable, since the gross data, which is not corrected by the body mass, do not show such statistical differences depending on sex.

In agreement, studies performed in humans (Gibson et al., 1976) or mice (Schulz et al., 2002), did not show effect of sex on the pulmonary static compliance.

The modulation of the ventilatory control in face to adverse conditions is carried out by several nuclei along the CNS, especially in the brainstem, and the contribution of each nucleus to the ventilatory pattern maintenance alters depending on the animal's sleep-wake state (Dias et al., 2010; Vicente et al., 2016), which can also affect the chemosensitive responses of these nuclei (Nattie & Li, 2010). Among nuclei with prominence role in the ventilatory modulation during sleep, the medullary raphe shows to be one of the important regions that is also chemosensitive to CO_2 (Taylor et al., 2005; Dias et al. , 2010; da Silva et al., 2011; Ray et al., 2013; dos Santos et al., 2015). Additionally, CB1 receptors are located in several brainstem regions, relevant to the respiratory pattern generation and integration of the motor activity (Haji et al., 2000), including in serotonergic neurones of the medullary raphe (Häring et al., 2007; Mendiguren et al., 2018), and the activation of these receptors possibly can modulate the activity of these neurones, and consequently the breathing pattern. Hence, the increased $\dot{V}_E$ found in prenatal WIN-exposed adult males during sleep state, may be due to changes in the respiratory network structuring during fetal life responsible for ventilatory control maintenance during sleep, also shifting the chemosensitivity of these neurones during respiratory adverse condition, as high levels of CO_2 and hypoxia.

Interestingly, exogenous cannabinoid can be used to treat respiratory disorders sleep-related (Carley & Radulovacki, 2008). In this context, the use of dronabinol, a CB1 and CB2 receptor agonist, has been shown to stabilize breathing in rats with spontaneous central apneas during sleep (Carley et al., 2002). The clinical relevance of this finding is highlighted by the fact that dronabinol demonstrates improvements on the respiratory disorder in patients with obstructive sleep apnea syndrome (Prasad et al.,

2013), a result that may reflect the stabilization of the breathing pattern generation and increased muscle activity in the upper airways. In line with this view, recent experiments using an apnea model in anesthetized rats have shown that activation of the nodose ganglia CB receptors suppress apneas induced by serotonin and increased the phasic activity of the genioglossus muscle (Calik et al., 2014). In addition, the systemic antagonism of CB1 or CB2, individually or in combination, prevented dronabinol from suppressing serotonin-induced apneas (Calik & Caley, 2014). It is known that cannabinoids can allosterically modulate many ionotropic receptors, including serotonergic, glutamatergic and cholinergic receptors (Demuth & Molleman, 2006). However, according to more recent studies, the suppression of apneas by dronabinol is given mainly by the activation of CB1 receptors (Calik & Caley, 2014).

Regarding the sex-dependent difference in the HCVR and HVR found in the present study, we observed opposite effect of WIN exposure with a greater chemosensitivity to CO_2 and O_2 for males and decreased for females. It is worth mentioning that both male and female animals can be affected by the action of the exogenous cannabinoid at the embryonic age and thus the development of the respiratory control network may be impacted, but the consequences in postnatal life can be distinct since there are differences in respiratory physiology in which the sex is an important factor (Gargaglioni et al., 2019). We must emphasize that some nuclei that integrate the respiratory network are dimorphic, such as RTN (Niblock et al., 2010; 2012), locus coeruleus (Luque et al., 1992; Hormigo et al., 2015; Bangasser et al., 2016; Gargaglioni et al., 2019), and medullary raphe (Cordero et al., 1999), thereby the chronic stimulation of the endocannabinoid system during CNS ontogeny in the prenatal phase may have unevenly influenced the development and maturation of these

respiratory control nuclei resulting in antagonistic responses between male and female rats at the adulthood.

The sex differences in brain regions can create an unequal neural condition for the action of *Cannabis*, especially during fetal development, with the brain more vulnerable to structural and/or functional changes, such as during reducing the number of synapses (*synaptic prunning)*. Interestingly, a study by McQueeny et al. (2011) found greater volumes of the amygdala in teenage girl users of *Cannabis* than in non-users, which was not observed in boys. Several other sex-dependent morphoneurological alterations correlated with exogenous cannabinoids are described in the literature (for review see Viveros et al., 2012). Interestingly, we did not find changes in the brainstem CA groups of male and female adult rats, since the quantification of TH-ir neurones did not differ between the control group and those exposed to WIN during pregnancy. In the same line, we also did not observe long-term modification in the expression of CB1 receptors located in the brainstem. Despite the fact that no difference in CB1 expression be found, another point that must be considered is that there is a difference in the affinity of the cannabinoids for the CB1 receptor being higher in males in the limbic forebrain and midbrain (Rodríguez de Fonseca et al., 1994). Corroborating this study, prenatal exposure to THC altered neuronal excitability and synaptic plasticity in the prefrontal cortex of male rats rather than females (Bara et al., 2018). Therefore, this increase in HCVR and HVR in males and decrease in females may be due to the fact that the receptors are more sensitive in males. Additionally, although there are still controversies, the influence of sex hormones on the ventilatory responses to hypercapnia and hypoxia in adult animals should be a factor to be considered, since the modulation of the hypercapnic response by β progesterone receptors appears to be different between males and females (Boukari et al., 2016), and the performance of

these receptors on nuclei responsible for respiratory control might be altered due to prenatal exposure to the synthetic cannabinoid WIN.

Intra-uterine synthetic cannabinoid exposure also affects the cardiovascular control of the offspring when reached the adulthood. The present study found a long-lasting effect in cardiovascular parameters, where WIN exposed males had an increased MAP and HR in hypoxic condition, whereas treated females had higher MAP baseline and hypoxic condition, bradycardia at CO_2 exposure and an opposite effect on HR during hypoxia.

It is known that the endocannabinoid system is present in cardiovascular control regions like NTS (Mailleux & Vanderhaeghen, 1992), rostral ventrolateral medulla (RVLM) (Wang et al., 2017), and paraventricular nucleus of the hypothalamus (PVN) (Grzęda et al., 2017). In addition, CB receptors are present in regions outside of the CNS, such as the heart and blood vessels (Liu et al., 2000; Bonz et al., 2003). Studies have shown the direct participation of the endocannabinoids in the blood pressure, contractility and heart rate modulation (Lake et al., 1997; Sierra et al., 2017). A hypertension and tachycardia were the most marked cardiovascular effect caused by acute use of *Cannabis* (Weiss et al., 1972) or intravenous administration of THC in humans (Perez-Reyes et al., 1972; Roth et al., 1973). According to Pfitzer et al. (2004), central activation of CB1 receptors by WIN administration was also responsible for increased MAP, but in a drop in HR. However, the local activation of CB1 receptors in different nuclei, as in the RVLM, the observed responses were opposed with a decreased MAP (Padley et al., 2003; Wang et al., 2017).

Studies have shown that knockout mice for CB1 receptors exhibited an absence of cannabinoid-induced hypotension and bradycardia (Ledent et al., 1999), and this response was mainly due to the participation of CB1 receptors located in the nuclei that

control the activity of sympathetic pre-motor neurons (Vollmer et al., 1974; Niederhofer & Szabo, 2000). Accordingly, the stimulation of CB1 receptors in the PVN was responsible for a drop in MAP, reduced HR and sympathetic activity in hypertensive rats (Grzęda et al., 2017), as well as CB1 receptors activation in the cardiac muscle resulted in hypotension and bradycardia (Kaschina, 2016). The cannabinoid mechanism action in the cardiac function may can occur by direct influence on the release of neurotransmitters in the CNS and sympathetic nerve terminals, or even locally by β adrenergic receptors modulating the smooth muscle of the vessels (Beaconsfield et al., 1972; Martz et al., 1972; Hillard, 2000). Thus, despite the lack in the literature about the use of cannabinoid during pregnancy and its long-term postnatal effects on cardiovascular control, the endocannabinoid system shows to be part of cardiovascular control network, especially because disorders such as hypertension, heart disease and atherosclerosis have been linked to dysfunction of the endocannabinoid system (Randall & Kendall, 1997; Hiley & Ford, 2003; Duerr et al., 2015).

The role of the endocannabinoid system in thermoregulation has been described by several studies. The expression of CB1 receptor was found in critical regions for the control of body temperature (Tsou et al., 1998). The systemic administration or focused injections in the preoptic area of exogenous cannabinoid (THC, WIN and CP 55,940) resulted in a hypothermic condition (Fitton & Pertwee, 1982; Martin, 1986; Little et al., 1988; Fan et al., 1994), mediated mainly by CB1 receptors, a primary mediator of cannabinoid-induced hypothermia (Rawls et al., 2002; Valiveti et al., 2004; Ripamonte et al., 2020); however, the endocannabinoid system does not participate in the tonic control of body temperature (Compton et al., 1996; McGregor et al., 1996; Boctor et al., 2007). Despite reports of thermoregulation control by the endocannabinoid system, it was not observed major changes in body temperature

control in adulthood of male and female prenatally WIN exposure rats, except for the treated females during hypercapnic condition in which a lower T_B was observed in WIN treated females. The effect on body temperature possibly involves a smaller increase of $\dot{V}_E$ and a lower HR during the hypercapnic challenge.

In conclusion, this study provides results of great relevance for a better understanding about cannabinoids use in pregnancy and its impacts in offspring's adulthood. A long-term and sex divergent alteration in breathing control was observed, in which an opposite chemosensitive response to adverse respiratory conditions occurred between male and females. Also, important cardiovascular changes were observed in adult rats prenatally exposed to WIN. The indiscriminate use of *Cannabis* has increased worldwide, including among pregnant women, driven by increasingly liberal government policies without established scientific knowledge about the medium and long-term future consequences. Therefore, the existence of studies of this subject is becoming even more necessary, and in this sense our study raises a precautionary note that must be taken into account when it comes to medicinal or recreational use of *Cannabis* during pregnancy.

Supplementary Table 1: Results of two-way ANOVA statistical analyzes for body mass and heart weight (**A**), respiratory and metabolic parameters during baseline, hypercapnia and hypoxia during awake (**B**) and sleep state (**C**) for male and female rats, static and dynamic compliance (**D**), cardiovascular data at awake and sleep state (**E**), and body temperature (**F**) for adult males and females.

A.

	Two-way ANOVA		
	Treatment effect	Sex effect	Factorial Interaction
Body Mass			
Male	n.s	$P < 0.001$ $F_{(1, 38)} = 217.92$	n.s
Female	n.s		
Heart			
Male	n.s	$P < 0.001$ $F_{(1, 62)} = 25.48$	n.s
Female	n.s		

B.

	Two-way ANOVA repeated measures		
	Treatment effect	Gas effect	Factorial Interaction
AWAKE			
Male			
V_E	n.s	$P < 0.001$ $F_{(2, 38)} = 388.13$	n.s
V_T	n.s	$P < 0.001$ $F_{(2, 38)} = 347.89$	n.s
f_R	n.s	$P < 0.001$ $F_{(2, 38)} = 91.13$	n.s
VO_2	n.s	$P < 0.001$ $F_{(2, 38)} = 26.53$	n.s
V_E/VO_2	n.s	$P < 0.001$ $F_{(2, 38)} = 262.27$	n.s
Female			
V_E	$P < 0.02$ $F_{(1, 37)} = 6.03$	$P < 0.001$ $F_{(2, 37)} = 458.50$	n.s
V_T	n.s	$P < 0.001$ $F_{(2, 37)} = 265.40$	n.s
f_R	n.s	$P < 0.001$ $F_{(2, 37)} = 150.56$	n.s
VO_2	n.s	$P < 0.001$ $F_{(2, 34)} = 14.53$	n.s
V_E/VO_2	$P < 0.03$ $F_{(1, 34)} = 4.98$	$P < 0.001$ $F_{(2, 34)} = 147.83$	n.s

C.

	Two-way ANOVA repeated measures		
	Treatment effect	Gas effect	Factorial Interaction
SLEEP			
Male			
V_E	$P < 0.02$ $F_{(1, 23)} = 5.66$	n/a	n.s
V_T	$P < 0.04$ $F_{(1, 23)} = 7.43$	n/a	n.s
f_R	$P < 0.05$ $F_{(1, 23)} = 8.67$	n/a	n.s
V_E/VO_2	$P < 0.01$ $F_{(1, 23)} = 6.52$	n/a	n.s
Female			
V_E	$P < 0.001$ $F_{(1, 24)} = 14.95$	n/a	$P < 0.001$ $F_{(2, 24)} = 12.59$
f_R	$P < 0.03$ $F_{(1, 24)} = 6.41$	n/a	$P < 0.006$ $F_{(2, 24)} = 6.39$
V_E/VO_2	$P < 0.03$ $F_{(1, 24)} = 5.19$	n/a	n.s

D.

	Two-way ANOVA		
	Treatment effect	Sex effect	Factorial Interaction
STATIC			
C_T inflation	n.s	$P < 0.001$ $F_{(1, 20)} = 14.25$	n.s
C_T deflation	n.s	$P < 0.002$ $F_{(1, 20)} = 12.68$	n.s
C_L inflation	n.s	$P < 0.002$ $F_{(1, 20)} = 13.09$	n.s
C_L deflation	n.s	$P < 0.01$ $F_{(1, 20)} = 7.13$	n.s
DYNAMIC			
Male			
C_T	$P < 0.01$ $F_{(1, 120)} = 7.32$	n.s	n.s
Female			
C_L	$P < 0.03$ $F_{(1, 105)} = 6.12$	n.s	n.s

E.

	Two-way ANOVA repeated measures		
	Treatment effect	Gas effect	Factorial Interaction
AWAKE			
Male			
MAP	$P < 0.001$ $F_{(1, 38)} = 5.54$	$P < 0.001$ $F_{(2, 38)} = 13.74$	$P < 0.007$ $F_{(2, 38)} = 5.73$
SAP	$P < 0.004$ $F_{(1, 38)} = 8.56$	$P < 0.001$ $F_{(2, 38)} = 16.28$	$P < 0.002$ $F_{(2, 38)} = 7.05$
DAP	$P < 0.002$ $F_{(1, 38)} = 5.32$	$P < 0.001$ $F_{(2, 38)} = 6.84$	$P < 0.04$ $F_{(2, 38)} = 3.53$
HR	n.s	$P < 0.001$ $F_{(2, 38)} = 14.25$	n.s
Female			
MAP	$P < 0.003$ $F_{(1, 33)} = 12.37$	$P < 0.001$ $F_{(2, 33)} = 32.88$	$P < 0.003$ $F_{(2, 33)} = 7.01$
SAP	$P < 0.02$ $F_{(1, 33)} = 7.60$	$P < 0.001$ $F_{(2, 33)} = 45.63$	$P < 0.001$ $F_{(2, 33)} = 45.63$
DAP	$P < 0.007$ $F_{(1, 33)} = 9.42$	$P < 0.001$ $F_{(2, 33)} = 13.58$	$P < 0.02$ $F_{(2, 33)} = 4.39$
HR	$P < 0.01$ $F_{(1, 33)} = 10.46$	$P < 0.001$ $F_{(2, 33)} = 20.71$	$P < 0.001$ $F_{(2, 33)} = 8.50$
SLEEP			
Male			
MAP	$P < 0.05$ $F_{(1, 23)} = 5.80$	n/a	n.s
DAP	$P < 0.02$ $F_{(1, 23)} = 6.05$	n/a	n.s
HR	$P < 0.02$ $F_{(1, 23)} = 5.69$	n/a	$P < 0.02$ $F_{(2, 23)} = 4.82$
Female			
MAP	$P < 0.002$ $F_{(1, 25)} = 12.63$	n/a	$P < 0.001$ $F_{(2, 25)} = 10.56$
SAP	$P < 0.01$ $F_{(1, 25)} = 7.11$	n/a	$P < 0.01$ $F_{(2, 25)} = 5.59$
DAP	$P < 0.002$ $F_{(1, 25)} = 12.75$	n/a	$P < 0.003$ $F_{(2, 25)} = 7.52$
HR	$P < 0.004$ $F_{(1, 25)} = 10.45$	n/a	$P < 0.001$ $F_{(2, 25)} = 13.12$

F.

	Two-way ANOVA repeated measures		
	Treatment effect	Gas effect	Factorial Interaction
MALE	n.s	$P < 0.001$ $F_{(2, 38)} = 36.03$	n.s
FEMALE	$P < 0.03$ $F_{(1, 38)} = 6.20$	$P < 0.001$ $F_{(2, 38)} = 38.09$	$P < 0.008$ $F_{(2, 38)} = 5.50$

Abadie V, Champagnat J, Fortin G. Branchiomotor activities in mouse embryo. *Neuroreport*, v. 11(1), p. 141-145, 2000.

Abel EL, Dintcheff BA, Day N. Effects of Marihuana on Pregnant Rats and Their Offspring. *Psychopharmacol.*, v. 71, p. 71-74, 1980.

Alger BE, Tang AH. Do cannabinoids reduce brain power? *Nat. Neurosci.*, v. 15(4), p. 499-501, 2012.

Alheid GF, McCrimmon DR. The chemical neuroanatomy of breathing. *Respir. Physiol. Neurobiol.*, v. 164(1-2), p. 3-11, 2008.

Anderson TM, Garcia 3rd AJ, Baertsch NA, Pollak J, Bloom JC, Wei AD, Rai KG, Ramirez JM. A novel excitatory network for the control of breathing. *Nature*, v. 536, p. 76-80, 2016.

Andrzejewski M, Muckenhoff K, Scheid P, Ballantyne D. Synchronized rhythms in chemosensitive neurones of the locus coeruleus in the absence of chemical synaptic transmission. *Respir. Physiol*., v. 129, p. 123-140, 2001.

Anju TR, Naijil G, Shilpa J, Roshni T, Paulose CS. Neonatal hypoxic insult-mediated cholinergic disturbances in the brain stem: effect of glucose, oxygen and epinephrine resuscitation. *Neurol. Sci.*, v. 34(3), p. 287-296, 2013.

Aston-Jones G, Shipley MT, Grzanna R. The Locus coeruleus, A5 and A7 noradrenergic cell groups In: *The rat nervous system"*, ed. G. Paxinos (Boca Raton: Academic Press), p. 183-213, 1995.

Bairam A, Uppari N, Mubayed S, Joseph V. An overview on the respiratory stimulant effects of caffeine and progesterone on response to hypoxia and apnea frequency in developing Rats. *Adv. Exp. Med. Biol.*, v. 860, p. 211-220, 2015.

Bamford OS, Sterni LM, Wasicko MJ, Montrose MH, Carroll JL. Postnatal maturation of carotid body and type I cell chemoreception in the rat. *Am. J. of Physiol. Lung Cell. and Molec. Physiol.*, v. 276(5), p. L875-884, 1999.

Bangasser DA, Wiersielis KR, Khantsis S. Sex differences in the locus coeruleus norepinephrine system and its regulation by stress. *Brain Res*., v. 1641, p. 177-188, 2016.

Bara A, Manduca A, Bernabeu A, Borsoi M, Serviado M, Lassalle O, Murphy MN, Wager-Miller J, Mackie K, Pelissier-Alicot AL, Trezza V, Manzoni OJ. Sex-dependent effects of in utero cannabinoid exposure on cortical function. *Elife*, v. 11(7), p. e36234, 2018.

Barnett WH, Jenkin SEM, Milsom WK, Paton JFR, Abdala AP, Molkov IY, Zoccal DB. The Kölliker-Fuse nucleus orchestrates the timing of expiratory abdominal nerve bursting. *J. Neurophysiol*., v. 119, p. 401-412, 2018.

Barrett KT, Kinney HC, Li A, Daubenspeck JA, Leiter JC, Nattie EE. Subtle alterations in breathing and heart rate control in the 5-HT1A receptor knockout mouse in early postnatal development. *J. Appl. Physiol. (1985)*, v. 113, p. 1585-1593, 2012.

Battaglia M, Ogliari A, Harris J, Spatola CAM, Pesenti-Gritti P, Reichborn-Kjennerud T, Torgersen S, Kringlen E, Tambs K. A genetic study of the acute anxious response to carbon dioxide stimulation in man. *J. Psychiatr. Res.*, v. 41(11), p. 906-917, 2007.

Bavis RW, MacFarlane PM. Developmental plasticity in the neural control of breathing. *Exp. Neurol*., v. 287(Pt 2), p. 176-191, 2017.

Bavis RW, Mitchell GS. Long-term effects of the perinatal environment on respiratory control. *J. Appl. Physiol*., v. 104, p. 1220–1229, 2008.

Beaconsfield P, Ginsburg J, Rainsbury R. Marihuana smoking. Cardiovascular effects in man and possible mechanisms. *N. Engl. J. Med.*, v. 287, p. 209-212, 1972.

Bellville JW, Swanson GD, Agleh KA. Respiratory effects of delta-9-tetrahydrocannabinol. *Clin. Pharmacol. Ther.*, v. 17(5), p. 541-548, 1975.

Bellodi L, Perna G, Caldirola D, Arancio C, Bertani A, Di Bella D. CO_2-induced panic attacks: a twin study. *Am. J. Psychiatry.*, v. 155, p. 1184-1188, 1998.

Beltrán-Castillo S, Morgado-Valle C, Eugenín J. The Onset of the Fetal Respiratory Rhythm: An Emergent Property Triggered by Chemosensory Drive? *Adv. Exp. Med. Biol.*, v. 1015, p. 163-192, 2017.

Bénard G, Massa F, Puente N, Lourenço J, Bellocchio L, Soria-Gómez E, Matias I, Delamarre A, Metna-Laurent M, Cannich A, et al. Mitochondrial CB_1 receptors regulate neuronal energy metabolism. *Nat. Neurosci.*, v. 15(4), p. 558-64, 2012.

Benowitz NL, Rosenberg J, Rogers W, Bachman J, Jones RT. Cardiovascular effects of intravenous delta-9- tetrahydrocannabinol: autonomic nervous mechanisms. *Clin. Pharmacol. Ther*., v. 25, p. 440-446, 1979.

Benowitz NL, Jones RT. Cardiovascular effects of prolonged delta-9-tetrahydrocannabinol ingestion. *Clin. Pharmacol*. *Ther*., v. 18, p. 287-297, 1975.

Bérard A. The importance of generating more data on cannabis use in pregnancy. *Nat. Med.*, v. 26(10), p. 1515-1516, 2020.

Bernard C, Milh M, Morozov YM, Ben-Ari Y, Freund TF, Gozlan H. Altering cannabinoid signaling during development disrupts neuronal activity. *Proc. Natl. Acad. Sci. USA*, v. 102, p. 9388–9393, 2005.

Berrendero F, Sepe N, Ramos JA, Di Marzo V, Fernandez-Ruiz JJ. Analysis of cannabinoid receptor binding and mRNA expression and endogenous cannabinoid contents in the developing rat brain during late gestation and early postnatal period. *Synapse*, v. 33, p. 181–191, 1999.

Berrendero F, Garcia-Gil L, Hernandez ML, Romero J, Cebeira M, de Miguel R, Ramos JA, Fernandez-Ruiz JJ. Localization of mRNA expression and activation of signal transduction mechanisms for cannabinoid receptor in rat brain during fetal development. *Development*, v. 125, p. 3179–3188, 1998.

Berridge CW, Waterhouse BD. The locus coeruleus-noradrenergic system: modulation of behavioral state and state-dependent cognitive processes. *Brain Res. Brain Res. Rev*., v. 42(1), p. 33-84, 2003.

Biancardi V, da Silva LT, Bícego KC, Gargaglioni LH. Role of locus coeruleus noradrenergic neurons in cardiorespiratory and thermal control during hypoxia. *Respir. Physiol. Neurobiol.*, v. 170(2), p. 150-156, 2010.

Biancardi V, Bícego KC, Almeida MC, Gargaglioni LH. Locus coeruleus noradrenergic neurons and CO_2 drive to breathing. *Pflugers Arch*., v. 455(6), p. 1119-1128, 2008.

Blain GM, Smith CA, Henderson KS, Dempsey JA. Peripheral chemoreceptors determine the respiratory sensitivity of central chemoreceptors to CO(2). *J. Physiol.*, v. 588(Pt 13), p. 2455-2471, 2010.

Blanco CE, Dawes GS, Hanson MA, McCooke HB. The response to hypoxia of arterial chemoreceptors in fetal sheep and new-born lambs. *The J. of Physiol.*, v. 351, p. 25, 1984.

Boctor SY, Martinez Jr JL, Koek W, France CP. The cannabinoid CB1 receptor antagonist AM251 does not modify methamphetamine reinstatement of responding. *Eur. J. Pharmacol.*, v. 571, p. 39-43, 2007.

Bonnin A, de Miguel R, Castro JG, Ramos JA, Fernandez-Ruiz JJ. Effects of perinatal exposure to delta9-tetrahydrocannabinol on the fetal and early postnatal development of tyrosine hydroxylase-containing neurons in rat brain. *J. Mol. Neurosci.*, v. 7, p. 291–308, 1996.

Bonz A, Laser M, Küllmer S. Cannabinoids acting on CB1 receptors decrease contractile performance in human atrial muscle. *J. Cardiovasc. Pharmacol*., v. 41, p. 657-664, 2003.

Boukari R, Rossignol O, Marcouiller F, Bairam A, Joseph V. Membrane progestrone receptors α and β contribute to regulation of breathing in adult male and female mice. *Am. Thorac. Soc*., p. A2558-A2558, 2016.

Bravo K, Eugenín JL, Llona I. Perinatal fluoxetine exposure impairs the CO_2 chemoreflex. Implications for Sudden Infant Death Syndrome. *Am. J. Respir. Cell. Mol. Biol*., v. 55(3), p. 368-376, 2016.

Brown QL, Sarvet AL, Shmulewitz D, Martins SS, Wall MM, Hasin DS. Trends in marijuana use among pregnant and nonpregnant reproductive-aged women, 2002–2014. *JAMA*, v. 72(12), 1235–1242, 2016.

Burke PG, Stephen B, Coates MB, Viar KE, Stornetta RL, Guyenet PG. Optogenetic stimulation of adrenergic C1 neurons causes sleep state-dependent cardiorespiratory

stimulation and arousal with sighs in rats. *Am. J. Respir. Crit. Care. Med*., v. 190, p. 1301-1310, 2014.

Campos M, Bravo E, Eugenin J. Respiratory dysfunctions induced by prenatal nicotine exposure. *Clin. Exp. Pharmacol. Physiol*., v. 36, p. 1205–1217, 2009.

Calignano A, Katona I, Désarnaud F, Giuffrida A, La Rana G, Mackie K, Freund TF, Piomelli D. Bidirectional control of airway responsiveness by endogenous cannabinoids. *Nature*, v. 408, p. 96–101, 2000.

Calik MW, Carley DW. Effects of Cannabinoid Agonists and Antagonists on Sleep and Breathing in Sprague-Dawley Rats. *Sleep*, v. 40(9), p. zsx112, 2017.

Calik MW, Carley DW. Cannabinoid type 1 and type 2 receptor antagonists prevent attenuation of serotonin-induced reflex apneas by dronabinol in Sprague-Dawley rats. *PLoS One*, v. 9(10), p. e111412, 2014.

Calik MW, Radulovacki M, Carley DW. Intranodose ganglion injections of dronabinol attenuate serotonin-induced apnea in Sprague-Dawley rat. *Respir. Physiol. Neurobiol*., v. 190, p. 20–24, 2014.

Carley DW, Radulovacki M. Pharmacology of vagal afferent influences on disordered breathing during sleep. *Respir. Physiol. Neurobiol*., v. 164(1-2), p. 197-203, 2008.

Carley DW, Paviovic S, Janelidze M, Radulovacki M. Functional role for cannabinoids in respiratory stability during sleep. *Sleep*, v. 25(4), p. 391-398, 2002.

Caroni P, Chowdhury A, Lahr M. Synapse rearrangements upon learning: from divergent-sparse connectivity to dedicated sub-circuits. *Trends Neurosci*., v. 37(10), p. 604-614, 2014.

Carroll JL, Agarwal A. Development of ventilatory control in infants. *Paediatr. Respir. Rev.*, v. 11, p. 199-207, 2010.

Cayetanot F, Larnicol N, Peyronnet J. Antenatal environmental stress and maturation of the breathing control, experimental data. *Respir. Physiol. Neurobiol.*, v. 168, p. 92–100, 2009.

Chamberlin NL. Functional organization of the parabrachial complex and intertrigeminal region in the control of breathing. *Respir. Physiol. Neurobiol*., v. 143, p. 115-125, 2004.

Chatonnet F, Wrobel LJ, Mézières V, Pasqualetti M, Ducret S, Taillebourg E, Charnay P, Rijli FM, Champagnat J. Distinct roles of Hoxa2 and Krox20 in the development of rhythmic neural networks controlling inspiratory depth, respiratory frequency, and jaw opening. *Neural. Dev.*, v. 2(19), p. 1-13, 2007.

Chen D-J, Gao M, Gao FF, Su QX, Wu J. Brain cannabinoid receptor 2: expression, function and modulation. *Acta. Pharmacol. Sin.*, v. 38, p. 312-316, 2017.

Chevaleyre V, Takahashi KA, Castillo PE. Endocannabinoid-mediated synaptic plasticity in the CNS. *Annu. Rev. Neurosci*., v. 29, p. 37–76, 2006.

Coates EL, Li A, Nattie EE. Widespread sites of brainstem ventilatory chemoreceptors. *J. of Appl. Physiol*., v. 75, p. 5-14, 1993.

Compton DR, Aceto MD, Lowe J, Martin BR. In vivo characterization of a specific cannabinoid receptor antagonist (SR141716A): inhibition of delta 9-tetrahydrocannabinol-induced responses and apparent agonist activity. *J. Pharmacol. Exp. Ther.*, v. 277, p. 586-594, 1996.

Cordero ME, Valenzuela CY, Torres R. Sexual dimorphism in human medial raphe nuclei. Preliminary study with the Golgi cox method. *Rev. Med. Chil*., v. 127(5), p. 532-538, 1999.

Correa F, Wolfson ML, Valchi P, Aisemberg J, Franchi AM. Endocannabinoid system and pregnancy. *Reproduction*, v. 152, p. R191–R200, 2016.

Corsi DJ, Donelle J, Sucha E, Hawken S, Hsu H, El-Chaâr D, Bisnaire L, Fell D, Wen SW, Walker M. Maternal cannabis use in pregnancy and child neurodevelopmental outcomes. *Nat. Med.*, v. 26(10), p. 1536-1540, 2020.

Corsi DJ, Hsu H, Weiss D, Fell D B, Walker M. Trends and correlates of cannabis use in pregnancy: a population-based study in Ontario, Canada from 2012 to 2017. *Can. J. Public Health*, v. 110, p. 76-84, 2019.

Coryell W, Fyer A, Pine D, Martinez J, Arndt S. Aberrant respiratory sensitivity to CO(2) as a trait of familial panic disorder. *Biol. Psych.*, v. 49, p. 582-587, 2001.

Cummings KJ, Hewitt JC, Li A, Daubenspeck JA, Nattie EE. Postnatal loss of brainstem serotonin neurons compromises the ability of neonatal rats to survive episodic severe hypoxia. *J. Physiol.*, v. 589, p. 5247-5256, 2011.

da Silva GS, Giusti H, Benedetti M, Dias MB, Gargaglioni LH, Branco LG, Glass ML. Serotonergic neurons in the nucleus raphe obscurus contribute to interaction between central and peripheral ventilatory responses to hypercapnia. *Pflugers Arch*., v. 462(3), p. 407-418, 2011.

da Silva GS, Li A, Nattie E. High CO_2/H^+ dialysis in the caudal ventrolateral medulla (Loeschcke's area) increases ventilation in wakefulness. *Respir. Physiol. Neurobiol.*, v. 171(1), p. 46-53, 2010.

Darnall RA. The role of CO(2) and central chemoreception in the control of breathing in the fetus and the neonate. *Respir. Physiol. Neurobiol*., v. 173(3), p. 201-212, 2010.

Davies P, Maconochie I. The relationship between body temperature, heart rate and respiratory rate in children. *Emerg. Med. J.*, v. 26(9), p. 641-643, 2009.

Davis SE, Solhied G, Castillo M, Dwinell M, Brozoski D, Forste HV. Postnatal developmental changes in CO_2 sensitivity in rats. *J. Appl. Physiol. (1985)*, v. 101, n. 1097-1103, 2006.

Day NL, Richardson GA. Prenatal cannabis use: epidemiology, methodologic issues, and infant outcome. *Clin. Perinatol.*, v. 18, p. 77–91, 1991.

De Carvalho D, Bícego KC, de Castro OW, da Silva GS, Garcia-Cairasco N, Gargaglioni LH. Role of neurokinin-1 expressing neurons in the locus coeruleus on ventilatory and cardiovascular responses to hypercapnia. *Respir. Physiol. Neurobiol*., v. 172(1-2), p. 24-31, 2010.

De Petrocellis L, Ligresti A, Moriello AS, Allarà M, Bisogno T, Petrosino S, Stott CG, Di Marzo V. Effects of cannabinoids and cannabinoid-enriched Cannabis extracts on TRP channels and endocannabinoid metabolic enzymes. *Br. J. Pharmacol*., v. 163(7), p. 1479-1494, 2011.

De Salas-Quiroga A, Díaz-Alonso J, García-Rincón D, Remmers F, Vega D, Gómez-Cañas M, Lutz B, Guzmán M, Galve-Roperh I. Prenatal exposure to cannabinoids evokes long-lasting functional alterations by targeting CB1 receptors on developing cortical neurons. *Proc. Natl. Acad. Sci. USA*, v. 112(44), p. 13693-13698, 2015.

Dejours, P. Principles of comparative respiratory physiology, 2nd ed. Elsevier, New York, 1981.

Del Negro CA, Funk GD, Feldman JL. Breathing matters. *Nat. Rev. Neurosci.*, v. 19(6), p. 351-367, 2018.

Del Negro CA, Morgado-Valle C, Hayes JA, Mackay DD, Pace RW, Crowder EA, Feldman JL. Sodium and calcium current-mediated pacemaker neurons and respiratoryrhythm generation. *J. Neurosci*., v. 25(2), p. 446-53, 2005.

Demuth DG, Molleman A. Cannabinoid signalling. *Life Sci*. v. 78(6), p. 549-563, 2006.

Desai RI, Thakur GA, Vemuri VK, Bajaj S, Makriyannis A, Bergman J. Analysis of tolerance and behavioral/physical dependence during chronic CB1 agonist treatment: effects of CB1 agonists, antagonists, and noncannabinoid drugs. *J. Pharmacol. Exp. Ther*., v. 344, p. 319–328, 2013.

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science*, v. 258, p. 1946–1949, 1992.

Devane WA, Dysarz FA, Johnson MR, Melvin LS, Howlett AC. Determination and characterization of a cannabinoid receptor in rat brain. *Mol. Pharmacol.*, v. 34, p. 605–613, 1988.

Di Marzo V, Petrocellis LD. Plant, synthetic, and endogenous cannabinoids in medicine. *Annu. Rev. Med.*, v. 57, p. 553–574, 2006.

Di Marzo V, De Petrocellis L, Bisogno T. The biosynthesis, fate and pharmacological properties of endocannabinoids. *Handb. Exp. Pharmacol.*, p. 147–185, 2005.

Di Pasquale E, Monteau R, Hilaire G. In vitro study of central respiratory-like activity of the fetal rat. *Exp. Brain Res*., v. 89, p. 459-464, 1992.

Dias MB, Li A, Nattie E. The orexin receptor 1 (OX1R) in the rostral medullary raphe contributes to the hypercapnic chemoreflex in wakefulness, during the active period of the diurnal cycle. *Respir. Physiol. Neurobiol*., v. 170(1), p. 96-102, 2010.

Doherty PA, McCarthy LE, Borison HL. Respiratory and cardiovascular depressant effects of nabilone, N-methyllevonantradol and Δ9-tetrahydrocannabinol in anesthetized cats. *J. Pharmacol. Exp. Ther.*, v. 227, p. 508–516, 1983.

Dos Santos TS, Krüger J, Melleu FF, Herold C, Zilles K, Poli A, Güntürkün O, Marino-Neto J. Distribution of serotonin 5-HT1A-binding sites in the brainstem and the hypothalamus, and their roles in 5-HT-induced sleep and ingestive behaviors in rock pigeons (Columba livia). *Behav. Brain. Res*., v. 295, p. 45-63, 2015.

Drorbaugh JE, Fenn WO. A barometric method for measuring ventilation in newborn infants. *Pediatrics*, v. 16, p. 81-87, 1955.

Dubreuil V, Ramanantsoa N, Trochet D, Vaubourg V, Amiel J, Gallego J, Brunet JF, Goridis C. A human mutation in Phox2b causes lack of CO_2 chemosensitivity, fatal central apnoea and specific loss of parafacial neurons. *Proc. Natl. Acad. Sci. USA*, v. 105, p. 1067-1072, 2008.

Duerr GD, Heinemann JC, Gestrich C. Impaired border zone formation and adverse remodeling after reperfused myocardial infarction in cannabinoid CB2 receptor defcient mice. *Life Sci*., v. 138, p. 8–17, 2015.

Dutschmann M, Dick TE. Pontine mechanisms of respiratory control. *Compr. Physiol*., v. 2(4), p. 2443-2469, 2012.

Dutschmann M, Herbert H. The Kölliker-Fuse nucleus gates the postinspiratory phase of the respiratory cycle to control inspiratory off-switch and upper airway resistance in rat. *Eur. J. Neurosci.*, v. 24, p. 1071-1084, 2006.

El Marroun H, Tiemeier H, Franken IH, Jaddoe VW, van der Lugt A, Verhulst FC, Lahey BB, White T. Prenatal cannabis and tobacco exposure in relation to brain morphology: a prospective neuroimaging study in young children. *Biol. Psychiatry*, v. 79, p. 971-979, 2016.

El Marroun H, Tiemeier H, Steegers EA. Intrauterine cannabis exposure affects fetal growth trajectories: the Generation R Study. *J. Am. Acad. Child. Adolesc. Psych.*, v. 48, p. 1173-1181, 2009.

Elam M, Yao T, Thoren P, Svensson TH. Hypercapnia and hypoxia: chemoreceptor-mediated control of locus coeruleus neurons and splanchnic, sympathetic nerves. *Brain Res*., v. 222, p. 373-81, 1981.

Estrada U, Brase DA, Martin BR, Dewey WL. Cardiovascular effects of Δ9- and Δ9(11)-tetrahydrocannabinol and their interaction with epinephrine. *Life Sci.*, v. 41, p. 79–87, 1987.

Eugenin J, Von Bernhardi R, Muller KJ, Llona I. Development and pH sensitivity of the respiratory rhythm of fetal mice in vitro. *Neuroscience*, v. 141, p. 223-231, 2006.

Fan F, Compton DR, Ward S, Melvin L, Martin BR. Development of cross-tolerance between delta 9-tetrahydrocannabinol, CP 55,940 and WIN 55,212. *J. Pharmacol. Exp. Ther.*, v. 271(3), p. 1383-1390, 1994.

Fattore L, Fratta W. How important are sex differences in cannabinoid action? *Br. J. Pharmacol.*, v. 160, p. 544-548, 2010.

Fauth M, Tetzlaff C. Opposing Effects of Neuronal Activity on Structural Plasticity. *Front. Neuroanat.*, v. 10:75, 2016.

Feldman JL, Kam K. Facing the challenge of mammalian neural microcircuits: taking a few breaths may help. *J. Physiol.*, v. 593(1), p. 3-23, 2015.

Feldman JL, Del Negro CA, Gray PA. Understanding the rhythm of breathing: so near, yet so far. *Annu. Rev. Physiol.*, v. 75, p. 423-452, 2013.

Feldman JL, Mitchell GS, Nattie EE. Breathing: rhythmicity, plasticity, chemosensitivity. *Annu. Rev. Neurosci.*, v. 26, p. 239-266, 2003.

Feldman JD, Bazzy AR, Cummins TR, Haddad GG. Developmental changes in neuromuscular transmission in the rat diaphragm. *J. Appl. Physiol.*, v. 71, p. 280-286, 1991.

Fergusson DM, Horwood LJ, Swain-Campbell N. Cannabis use and psychosocial adjustment in adolescence and young adulthood. *Addiction*, v. 97, p. 1123–1135, 2002.

Fernandez-Ruiz J, Gomez M, Hernandez M, de Miguel R, Ramos JA. Cannabinoids and gene expression during brain development. *Neurotox. Res.*, v. 6, p. 389–401, 2004.

Fernandez-Ruiz J, Berrendero F, Hernandez ML, Ramos JA. The endogenous cannabinoid system and brain development. *Trends Neurosci.*, v. 23, p. 14–20, 2000.

Fernandez-Ruiz J, Berrendero F, Hernandez ML, Romero J, Ramos JA. Role of endocannabinoids in brain development. *Life Sci.*, v. 65, p. 725–736, 1999.

Fisar Z, Singh N, Hroudová J. Cannabinoid-induced changes in respiration of brain mitochondria. *Toxicol. Letters*, v. 231, p. 62-71, 2014.

Fitton AC, Pertwee RG. Changes in body temperature and oxygen consumption rate of conscious mice produced by intrahypothalamic and intracerebroventricular injections of A9-tetrahydrocannabinol. *Br. J. Pharmacol.*, v. 75, p. 409-414, 1982.

Fleming AS, Kraemer GW, Gonzalez A, Lovic V, Ress A, Melo AI. Mothering begets mothering: the transmission of behavior and its neurobiology across generation. *Pharmacol. Biochem. Behav.*, v. 73, p. 61-75, 2002.

Florido J, Cortés E, Gutiérrez M, Soto VM, Miranda MT, Navarrete L. Analysis of fetal breathing movements at 30-38 weeks of gestation. *J. Perinat. Med.*, v. 33, p. 38-41, 2005.

Fogaça MV, Campos AC, Coelho LD, Duman RS, Guimarães FS. The anxiolytic effects of cannabidiol in chronically stressed mice are mediated by the endocannabinoid system: Role of neurogenesis and dendritic remodeling. *Neuropharmacol*., v. 135, p. 22-33, 2018.

Forster HV, Smith CA. Contributions of central and peripheral chemoreceptors to the ventilatory response to CO_2/H^+. *J. Appl. Physiol.* (1985), v. 108(4), p. 989-994, 2010.

Fortin G, Thoby-Brisson M. Embryonic emergence of the respiratory rhythm generator. *Respir. Physiol. Neurobiol*., v. 168, p. 86-91, 2009.

Frappell PB, Boggs DF, Kilgore Jr DL. How stiff is the armadillo? A comparison with the allometrics of mammalian respiratory mechanics. *Respir. Physiol.*, v. 113, p. 111-122, 1998.

Freund TF, Hájos N. Excitement reduces inhibition via endocannabinoids. *Neuron*, v. 38, p. 362-365, 2003.

Freund TF, Katona I, Piomelli D. Role of endogenous cannabinoids in synaptic signaling. *Physiol. Rev.*, v. 83, p. 1017–1066, 2003.

Fride E, Gobshtis N, Dahan H, Weller A, Giuffrida A, Ben-Shabat S. The endocannabinoid system during development: emphasis on perinatal events and delayed effects. *Vitam. Horm.*, v. 81, p. 139–158, 2009.

Fried PA. Conceptual issues in behavioral teratology and their application in determining long-term sequelae of prenatal marihuana exposure. *J. Child Psychol. Psychiatry,* v. 43, p. 81-102, 2002.

Fried PA, Smith AM. A literature review of the consequences of prenatal marihuana exposure. An emerging theme of a deficiency in aspects of executive function. *Neurotoxicol Teratol.*, v. 23, p. 1-11, 2001.

Fried PA, Watkinson B, Dillon RF, Dulberg CS. Neonatal neurological status in a low-risk population after prenatal exposure to cigarettes, marijuana, and alcohol. *J. Dev. Behav. Pediatr.*, v. 8(6), p. 318-326, 1987.

Galieque S, Mary S, Marchand J, Dussossoy D, Carriere D, Carayon P, Bouaboula M, Shire D, LeFur G, Casellas P. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur. J. Biochem*., v. 232, p. 54-61, 1995.

Garcia DE, Brown S, Hille B, Mackie K. Protein kinase C disrupts cannabinoid actions by phosphorylation of the CB1 cannabinoid receptor. *J. of Neurosci.*, v. 18, p. 2834-2841, 1998.

Gargaglioni LH, Marques DA, Patrone LGA. Sex differences in breathing. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.*, v. 238, p. 1105-1143, 2019.

Gargaglioni LH, Hartzler LK, Putnam RW. The Locus coeruleus and central chemosensitivity. *Respir. Physiol. Neurobiol*., v. 173(3), p. 264-273, 2010.

Gargari SS, Fallahian M, Haghighi L, Hosseinnezhad-Yazdi M, Dashti E, Dolan K. Maternal and neonatal complications of substance abuse in Iranian pregnant women. *Acta. Med. Iran.*, v. 50, p. 411-416, 2012.

Gauda EB, McLemore GL, Tolosa J, Marston-Nelson J, Kwak D. Maturation of peripheral arterial chemoreceptors in relation to neonatal apnoea. In: Seminars in neonatology, WB Saunders., v. 9(3), p. 181-194, 2004.

Geerling JC, Yokota S, Rukhadze I, Roe D, Chamberlin NL. Kölliker-Fuse GABAergic and glutamatergic neurons project to distinct targets. *J. Comp. Neurol.*, v. 525, p. 1844-1860, 2017.

Ghali MGZ. Respiratory rhythm generation and pattern formation: oscillators and network mechanisms. *J. Integr. Neurosci.*, v. 18(4), p. 481-517, 2019.

Gibson GJ, Pride NB, O'cain C, Quagliato R. Sex and age differences in pulmonary mechanics in normal nonsmoking subjects. *J. Appl. Physiol.*, v. 41(1), p. 20-25, 1976.

Gluckman PD, Buklijas T, Hanson MA. The Developmental Origins of Health and Disease (DOHaD) Concept: Past, Present, and Future. The Epigenome and Developmental Origins of Health and Disease, ed. Elsevier p. 1-15, 2016.

Goldschmidt L, Richardson GA, Cornelius MD, Day NL. Prenatal marijuana and alcohol exposure and academic achievement at age 10. *Neurotoxicol. Teratol.*, v. 26, p. 521-532, 2004.

Gonzalez C, Vicario I, Almaraz L, Rigual R. Oxygen sensing in the carotid body. *Biol. Signals.*, v. 4(5), p. 245-256, 1995.

González-Mariscal G, Melo AI. Parental behavior. In: Pfaff DW (ed) Neuroscience in the 21st century. Springer Science Business Media, New York, p. 2069-2100, 2013.

González S, Cebeira M, Fernández-Ruiz J. Cannabinoid tolerance and dependence: a review of studies in laboratory animals. *Pharmacol. Biochem. Behav.*, v. 81, p. 300-318, 2005.

Graham JDP. The bronchodilator action of cannabinoids. In: Mechoulam, R. Ed, Cannabinoids as Therapeutic Agents. CRC Press, Boca Raton, FL, pp. 147-158, 1986.

Graham JDP, Li DMF. Cardiovascular and respiratory effects of cannabis in cat and rat. *Br. J. Pharmacol.*, v. 49, p. 1–10, 1973.

Gray KA, Day NL, Leech S, Richardson GA. Prenatal marijuana exposure: effect on child depressive symptoms at ten years of age. *Neurotoxicol. Teratol.*, v. 27, p. 439-448, 2005.

Greer JJ. Control of breathing activity in the fetus and newborn. *Compr. Physiol*., v. 2(3), p. 1873-1888, 2012.

Greer JJ, Funk GD, Ballanyi K. Preparing for the first breath: prenatal maturation of respiratory neural control. *J. Physiol.*, v. 570(Pt 3), p. 437-444, 2006.

Greer JJ, Smith JC, Feldman JL. Respiratory and locomotor patterns generated in the fetal rat brain stem-spinal cord in vitro. *J. Neurophysiol*., v. 67(4), p. 996-999, 1992.

Grzęda E, Schlicker E, Toczek M. CB1 receptor activation in the rat paraventricular nucleus induces bi-directional cardiovascular efects via modifcation of glutamatergic and GABAergic neurotransmission. *Naunyn. Schmied. Arch. Pharmacol*., v. 390, p. 25-35, 2017.

Grzeskowiak LE, Grieger JA, Andraweera P, Knight EJ, Leemaqz S, Poston L, McCowan L, Kenny L, Myers J, Walker JJ, Dekker GA, Roberts CT. The deleterious effects of cannabis during pregnancy on neonatal outcomes. *Med. J. Aust*., v. 212(11), p. 519-524, 2020.

Gunn JKL, Rosales CB, Center KE, Nuñez A, Gibson SJ, Christ C, Ehiri JE. Prenatal exposure to cannabis and maternal and child health outcomes: a systematic review and meta-analysis. *BMJ Open*, v. 6(4), e009986, 2016.

Guner I, Sahin G, Yelmen NK, Aksu U, Oruc T, Yildirim Z. Intracerebroventricular serotonin reduces the degree of acute hypoxic ventilatory depression in peripherally chemodenervated rabbits. Chin J Physiol., 51, 136-145, 2008.

Guyenet PG. Regulation of breathing and autonomic outflows by chemoreceptors. *Compr. Physiol.*, v. 4, p. 1511-1562, 2014.

Guyenet PG, Mulkey DK. Retrotrapezoid nucleus and parafacial respiratory group. *Respir. Physiol. Neurobiol*., v. 173(3), p. 244-255, 2010.

Guyenet PG, Bayliss DA, Stornetta RL, Fortuna MG, Abbott SB, DePuy SD. Retrotrapezoid nucleus, respiratory chemosensitivity and breathing automaticity. *Respir. Physiol. Neurobiol*., v. 168(1-2), p. 59-68, 2009.

Guyenet PG, Stornetta RL, Bayliss DA. Retrotrapezoid nucleus and central chemoreception. *J. of Physiol.*, v. 586, p. 2043-2048, 2008.

Guyenet PG, Stornetta RL, Bayliss DA, Mulkey DK. Retrotrapezoid nucleus: a litmus test for the identification of central chemoreceptors. *Exp. Physiol.*, v. 90, p. 247-253, 2005.

Haji A, Takeda R, Okazaki M. Neuropharmacology of control of respiratory rhythm and pattern in mature mammals. *Pharmacol. Ther.*, v. 86(3), p. 277-304, 2000.

Häring M, Marsicano G, Lutz B, Monory K. Identification of the cannabinoid receptor type 1 in serotonergic cells of raphe nuclei in mice. *Neuroscience*, v. 146(3), p. 1212-1219, 2007.

Harkany T, Guzman M, Galve-Roperh I, Berghuis P, Devi LA, Mackie K. The emerging functions of endocannabinoid signaling during CNS development. *Trends Pharmacol. Sci.*, v. 28, p. 83-92, 2007.

Harris A, Seckl J. Glucocorticoids, prenatal stress and the programming of disease. *Horm. Behav.*, v. 59, p. 279-289, 2011.

Hebert-Chatelain E, Reguero L, Puente N, Lutz B, Chaouloff F, Rossignol R, Piazza PV, Benard G, Grandes P, Marsicano G. Cannabinoid control of brain bioenergetics: Exploring the subcellular localization of the CB1 receptor. *Mol. Metab.*, v. 3(4), p. 495-504, 2014.

Hedrick MS, Hillman SS, Drewes RC, Withers PC. Pulmonary compliance and lung volume varies with ecomorphology in anuran amphibians: implications for ventilatory-assisted lymph flux. *J. Exp. Biol.*, v. 214(19), p. 3279-3285, 2011.

Herkenham M, Lynn AB, Johnson MR, Melvin LS, De Costa BR, Rice KC. Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. *J. Neurosci*., v. 11, p. 563-583, 1991.

Hernandez M, Berrendero F, Suarez I, Garcia-Gil L, Cebeira M, Mackie K, Ramos JA, Fernandez-Ruiz J. Cannabinoid CB(1) receptors colocalize with tyrosine hydroxylase in cultured fetal mesencephalic neurons and their activation increases the levels of this enzyme. *Brain Res.*, v. 857, p. 56-65, 2000.

Hilaire G. Endogenous noradrenaline affects the maturation and function of the respiratory network: possible implication for SIDS. *Auton. Neurosci*., v. 126-127, p. 320-31, 2006.

Hilaire G, Viemari JC, Coulon P, Simonneau M, Bévengut M. Modulation of the respiratory rhythm generator by the pontine noradrenergic A5 and A6 groups in rodents. *Respir. Physiol. Neurobiol.*, v. 143, p. 187-197, 2004.

Hiley CR, Ford WR. Endocannabinoids as mediators in the heart: a potential target for therapy of remodelling after myocardial infarction? *Br. J. Pharmacol*., v. 138, p. 1183-1184, 2003.

Hillard CJ. Endocannabinoids and vascular function. *J. Pharmacol. Exp. Ther*., v. 294, p. 27-32, 2000.

Hoppenbrouwers T, Harper RM, Hodgman JE, Sterman MB, McGinty DJ. Polygraphic studies on normal infants during the first six months of life. II. Respiratory rate and variability as a function of state. *Pediatr. Res.*, v. 12, p. 120-125, 1978.

Hormigo S, Gómez-Nieto R, Castellano O, Herrero-Turrión MJ, López DE, de Anchieta de Castro e Horta-Júnior J. The noradrenergic projection from the locus coeruleus to the cochlear root neurons in rats. *Brain Struct. Funct*., v. 220, p. 1477-1496, 2015.

Howard DS, Dhanraj DN, Devaiah CG, Lambers DS. Cannabis use based on urine drug screens in pregnancy and its association with infant birth weight. *J. Addict. Med*., v. 13(16), p. 436-441, 2019.

Hutchings DE, Martin BR, Gamagaris Z, Miller N, Fico T. Plasma concentrations of delta-9-tetrahydrocannabinol in dams and fetuses following acute or multiple prenatal dosing in rats. *Life Sci.*, v. 44, p. 697-701, 1989.

ICICT, Fiocruz. III levantamento Nacional sobre o Uso de Drogas pela População Brasileira 2015.

Imber AN, Santin JM, Grahama CD, Putnam RW. A HCO_3^--dependent mechanism involving soluble adenylyl cyclase for the activation of Ca^{2+} currents in locus coeruleus neurons. *Bioch. et Bioph. Acta.*, v. 1842, p. 2569-2578, 2014.

Janczewski WA, Feldman JL. Novel data supporting the two respiratory rhythm oscillator hypothesis. Focus on "respiration-related rhythmic activity in the rostral medulla of newborn rats". *J. Neurophysiol*., v. 96(1), p. 1-2, 2006.

Jansen AH, Chernick V. Fetal breathing and development of control of breathing. *J. Appl. Physiol.*, v. 70, p. 1431–1446, 1991.

Jansen AH, Ioffe S, Russell BJ, Chernick V. Effect of carotid chemoreceptor denervation on breathing in utero and after birth. *J. of Appl. Physiol.*, v. 51(3), p. 630-633, 1981.

Jensen D, Duffin J, Lam Y-M, Webb KA, Simpson JA, Davies GAL, Wolfe LA, O'Donnell DE. Physiological mechanisms of hyperventilation during human pregnancy. *Respir. Physiol. Neurobiol.*, v. 161(1), p. 76-86, 2008.

Julien C, Bairam A, Joseph V. Chronic intermittent hypoxia reduces ventilatory long-term facilitation and enhances apnea frequency in newborn rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, v. 294(4), p. R1356-1366, 2008.

Karwad MA, Couch DG, Wright KL, Tufarelli C, Larvin M, Lund J, O'Sullivan SE. Endocannabinoids and endocannabinoid-like compounds modulate hypoxia-induced permeability in CaCo-2 cells via CB1, TRPV1, and PPARα. *Biochem Pharmacol*, v. 168, p. 465-472, 2019.

Kaschina E. Cannabinoid CB1/CB2 receptors in the heart: expression, regulation, and function. In: Cannabinoids in Health and Disease: InTech; p. 169-85, 2016.

Kholwadwala D, Donnelly DF. Maturation of carotid chemoreceptor sensitivity to hypoxia: in vitro studies in the newborn rat. *J. Physiol*., v. 453, p. 461-473, 1992.

Kobayashi K, Lemke RP, Greer JJ. Development of fetal breathing movements in the rat. *J. Appl. Physiol.*, v. 91, p. 316-320, 2001.

Kobayashi N, Yamamoto Y. Hypoxic responses of arterial chemoreceptors in rabbits are primarily mediated by leak K channels. *Adv. Exp. Med. Biol.*, v. 669, p. 195-199, 2010.

Koch M, Varela L, Kim JG, Kim JD, Hernández-Nuño F, Simonds SE, Castorena CM, Vianna CR, Elmquist JK, Morozov YM, Rakic P, Bechmann I, Cowley MA, Szigeti-Buck K, Dietrich MO, Gao XB, Diano S, Horvath TL. Hypothalamic POMC neurons promote cannabinoid-induced feeding. *Nature,* v. 519(7541), p. 45-50, 2015.

Koos BJ, Rajaee A. Fetal Breathing Movements and Changes at Birth. *Advances in Experimental Medicine and Biology*, p. 89-101, 2014.

Kotecha S. Lung growth for beginners. *Paediatr. Respir. Rev.*, v. 1, p. 308–313, 2000.

Koteja, P. Measuring Energy Metabolism with Open-Flow Respirometric Systems: Which Design to Choose? *Funct. Ecol*., v. 10, p. 675, 1996.

Kroeze Y, Dirven B, Janssen S, Kröhnke M, Barte RM, Middelman A, van Bokhoven H, Zhou H, Homberg JR. Perinatal reduction of functional serotonin transporters results in developmental delay. *Neuropharmacology*, v. 109, p. 96-111, 2016.

Krott LM, Piscitelli F, Heine M. Endocannabinoid regulation in white and brown adipose tissue following thermogenic activation. *J. Lipid. Res.*, v. 57, p. 464-473, 2016.

Lacroix I, Hurault C, Saivin S, Raoul V, Berrebi A, Souchet E, Desboeuf K, Montastruc JL, Damase-Michel C. Exposition in utero à des substances psychoactives: résultats de l'étude "NENUPHAR". *Thérapie* v. 62, p. 177–183, 2007.

Lake KD, Compton DR, Varga K. Cannabinoidinduced hypotension and bradycardia in rats mediated by CB1-like cannabinoid receptors. *J. Pharmacol. Exp. Ther*., v. 281, p. 1030-1037, 1997.

LeBlanc J, Blais B, Barabe B, Cote J. Effects of temperature and wind on facial temperature, heart rate, and sensation. *J. Appl. Physiol.*, v. 40, p. 127-131, 1976.

Ledent C, Valverde O, Cossu G. Unresponsiveness to cannabinoids and reduced addictive efects of opiates in CB1 receptor knockout mice. *Science*, v. 283, p. 401-404, 1999.

Leech SL, Larkby CA, Day R, Day NL. Predictors and correlates of high levels of depression and anxiety symptoms among children at age 10. *J. Am. Acad. Child. Adolesc. Psych.*, v. 45, p. 223-230, 2006.

Leirão IP, Silva Jr CA, Gargaglioni LH, da Silva GSF. Hypercapnia-induced active expiration increases in sleep and enhances ventilation in unanaesthetized rats. *J. Physiol.*, v. 596, p. 3271-3283, 2018.

Li N, Li A, Nattie E. Focal microdialysis of CO_2 in the perifornical-hypothalamic area increases ventilation during wakefulness but not NREM sleep. *Respir. Physiol. Neurobiol.*, v. 185, p. 349-355, 2013.

Li A, Randall M, Nattie EE. CO(2) microdialysis in retrotrapezoid nucleus of the rat increases breathing in wakefulness but not in sleep. *J. Appl. Physiol.*, v. 87, p. 910-919, 1999.

Lipina C, Irving AJ, Hundal HS. Mitochondria: a possible nexus for the regulation of energy homeostasis by the endocannabinoid system? *Am. J. Physiol. Endocrinol. Metab*., v. 307, p. E1–E13, 2014.

Little BB, VanBeveren TT. Placental transfer of selected substances of abuse. *Semin. Perinatol., v.* 20, p. 147–53, 1996.

Little PJ, Compton DR, Johnson MR, Melvin LS, Martin BR. Pharmacology and stereoselectivity of structurally novel cannabinoids in mice. *J. Pharmacol. Exp. Ther.*, v. 247(3), p. 1046-1051, 1988.

Liu J, Gao B, Mirshahi F. Functional CB1 cannabinoid receptors in human vascular endothelial cells. *Biochem. J*., v. 346(Pt 3), p. 835-840, 2000.

Liu Q, Wong-Riley MT. Postnatal changes in the expression of serotonin 2A receptors in various brain stem nuclei of the rat. *J. Appl. Physiol.*, v. 104, p. 1801-1808, 2008.

Loeschcke HH. Central chemosensitivity and the reaction theory. *J. Physiol.*, v. 332, p. 1-24, 1982.

Lozano J, García-Algar O, Marchei E, Vall O, Monleon T, Di Giovannandrea R, Pichini S. Prevalence of gestational exposure to cannabis in a Mediterranean city by meconium analysis. *Acta Paediatr.*, v. 96, p. 1734–1737, 2007.

Lubman DI, Cheetham A, Yucel M. Cannabis and adolescent brain development. *Pharmacol. Ther*. v. 148, p. 1–16, 2015.

Luque JM, de Blas MR, Segovia S, Guillamón A. Sexual dimorphism of the dopamine-beta-hydroxylase-immunoreactive neurons in the rat locus ceruleus. *Brain Res. Dev. Brain Res*. v. 67, p. 211-215, 1992.

Maccari S, Darnaudery M, Morley-Fletcher S, Zuena AR, Cinque C, Van Reeth O. Prenatal stress and long-term consequences: implications of glucocorticoid hormones. *Neurosci. Biobehav. Rev.*, v. 27, p. 119-127, 2003.

Mackie K. Mechanisms of CB1 receptor signaling: endocannabinoid modulation of synaptic strength. *Int. J. Obes. (Lond)*, v. 30 (Suppl. 1), p. S19–S23, 2006.

Mailleux P, Vanderhaeghen JJ. Distribution of neuronal cannabinoid receptor in the adult rat brain: a comparative receptor binding radioautography and in situ hybridization histochemistry. *Neuroscience*, v. 48, p. 655-668, 1992.

Malit LA, Johnstone RE, Bourke DI, Kulp RA, Klein V, Smith TC. Intravenous delta9-Tetrahydrocannabinol: Effects of ventilatory control and cardiovascular dynamics. *Anesthesiology*, v. 42(6), p. 666-673, 1975.

Martin BR. Cellular effects of cannabinoids. *Pharmacol. Rev.*, v. 38, p. 45-74, 1986.

Martz R, Brown DJ, Forney RB, Bright TP, Kiplinger GF, Rodda BE. Propranolol antagonism of marihuana induced tachycardia. *Life Sci.*, v. 11, p. 999-1005, 1972.

Mathew RJ, Wilson WH, Humphreys D, Lowe JV, Wiethe KE. Middle cerebral artery velocity during upright posture after marijuana smoking. *Acta Psychiatr. Scand.*, v. 86, p. 173-178, 1992.

Mato S, Del Olmo E, Pazos A. Ontogenetic development of cannabinoid receptor expression and signal transduction functionality in the human brain. *Eur. J. Neurosci.*, v. 17, p. 1747–1754, 2003.

McGregor IS, Issakidis CN, Prior G. Aversive effects of the synthetic cannabinoid CP 55,940 in rats. *Pharmacol. Biochem. Behav.*, v. 53, p. 657-664, 1996.

McLaughlin CR, Abood ME. Developmental expression of cannabinoid receptor mRNA. *Brain Res. Dev. Brain Res.*, v. 76, p. 75–78, 1993.

McLemore GL, Cooper RZ, Richardson KA, Mason AV, Marshall C, Northington FJ, Gauda EB. Cannabinoid receptor expression in peripheral arterial chemoreceptors during postnatal development. *J. Appl. Physiol.*, v. 97, p. 1486–1495, 2004.

McQueeny T, Padula CB, Price J, Medina KL, Longan P, Tapert SF. Gender effects on amygdala morphometry in adolescent marijuana users. *Behav. Brain Res.*, v. 224, p. 128-134, 2011.

Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S, Martin BR, Compton DR. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem. Pharmacol.*, v. 50, p. 83–90, 1995.

Méndez-Díaz M, Ruiz-Contreras AE, Cortés-Morelos J, Prospéro-García O. Cannabinoids and Sleep/Wake Control. *Adv. Exp. Med. Biol*., v. 1297, p. 83-95, 2021.

Mendiguren A, Aostri E, Pineda J. Regulation of noradrenergic and serotonergic systems by cannabinoids: relevance to cannabinoid-induced effects. *Life Sci.*, v. 192, p. 115-127, 2018.

Menuet C, Sevigny CP, Connelly AA, Bassi JK, Jancovski N, Williams DA, Anderson CR, Llewellyn-Smith IJ, Fong AY, Allen AM. Catecholaminergic C3 neurons are sympathoexcitatory and involved in glucose homeostasis. *J. Neurosci.*, v. 34, p. 15110-15122, 2014.

Mereu G, Fa M, Ferraro L, Cagiano R, Antonelli T, Tattoli M, Ghiglieri V, Tanganelli S, Gessa GL, Cuomo V. Prenatal exposure to a cannabinoid agonist produces memory deficits linked to dysfunction in hippocampal long-term potentiation and glutamate release. *Proc. Natl. Acad. Sci. U. S. A.*, v. 100, p. 4915–4920, 2003.

Molkov YI, Rubin JE, Rybak IA, Smith JC. Computational models of the neural control of breathing. *Wiley Interdisc. Rev. Syst. Biol. Med.*, v. 9(2), p. 1-33, 2017.

Moreira TS, Takakura AC, Colombari E, Guyenet PG. Activation of 5-hydroxytryptamine type 3 receptor-expressing C-fiber vagal afferents inhibits retrotrapezoid nucleus chemoreceptors in rats. *J. Neurophysiol*., v. 98(6), p. 3627-3637, 2007.

Morozov YM, Masaaki TM, Rakic P. Origin, early commitment, migratory routes, and destination of cannabinoid type 1 receptorcontaining interneurons. *Cereb. Cortex*, v. 19, p. i78–i89, 2009.

Mörschel M, Dutschmann M. Pontine respiratory activity involved in inspiratory/expiratory phase transition. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, v. 364, p. 2517-2526, 2009.

Mortola JP, Frappell PB. Measurements of air ventilation in small vertebrates. *Respir. Physiol. Neurobiol.*, v. 186(2), p. 197-205, 2013.

Mortola JP. Breathing pattern in newborns. *J. of Appl. Physiol.*, v. 56, p. 1533-1540, 1984.

Moss IR, Friedman E. Δ9-tetrahydrocannabinol: depression of ventilatory regulation; other respiratory and cardiovascular effects. *Life Sci.*, v. 19, p. 99–104, 1976.

Mukhtarov M, Ragozzino D, Bregestovski P. Dual Ca^{2+} modulation of glycinergic synaptic currents in rodent hypoglossal motoneurones. *J. Physiol.*, v. 569(3), p. 817-831, 2005.

Mulder J, Aguado T, Keimpema E, Barabas K, Ballester Rosado CJ, Nguyen L, Monory K, Marsicano G, Di Marzo V, Hurd YL, Guillemot F, Mackie K, Lutz B, Guzman M, Lu HC, Galve-Roperh I, Harkany T. Endocannabinoid signaling controls pyramidal cell specification and long-range axon patterning. *Proc. Natl. Acad. Sci. USA*, v. 105, p. 8760–8765, 2008.

Mulkey DK, Stornetta RL, Weston MC, Simmons JR, Parker A, Bayliss DA, Guyenet PG. Respiratory control by ventral surface chemoreceptor neurons in rats. *Nat. Neurosci.*, v. 7, p. 1360-1369, 2004.

Muller C, Morales P, Reggio PH. Cannabinoid Ligands Targeting TRP Channels. Front. Mol. Neurosci, v. 11, p. 487, 2019.

Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. *Nature, v.* 365, p. 61–65, 1993.

Nashed MG, Hardy DB, Laviolette SR. Prenatal Cannabinoid Exposure: Emerging Evidence of Physiological and Neuropsychiatric Abnormalities. *Front. Psychi.*, v. 11:624275, 2021.

Nattie E, Li A. Central chemoreceptors: locations and functions. *Compr. Physiol.*, v. 2(1), p. 221-54, 2012.

Nattie E, Li A. Central chemoreception in wakefulness and sleep: evidence for a distributed network and a role for orexin. *J. Appl. Physiol*., v. 108(5), p. 1417-1424, 2010.

Nattie E, Li A. Central chemoreception 2005: a brief review. *Auton. Neurosci*., v. 126-127, p. 332-338, 2006.

Nattie EE, Li A. CO_2 dialysis in nucleus tractus solitarius region of rat increases ventilation in sleep and wakefulness. *J. Appl. Physiol*., v. 92, p. 2119-2130, 2002.

Nattie EE, Li A. CO_2 dialysis in the medullary raphe of the rat increases ventilation in sleep. *J. Appl. Physiol*., v. 90, p. 1247-1257, 2001.

Nattie E. Central chemosensitivity, sleep, and wakefulness. *Resp. Physiol.*, v. 129, p. 257-268, 2001.

Nattie E. CO_2, brainstem chemoreceptors and breathing. *Progr. in Neurobiol. Progr. in Neurosci*., v. 59, p. 299-331, 1999.

Nettleton RT, Wallisch M, Olsen GD. Respiratory effects of chronic in utero methadone or morphine exposure in the neonatal guinea pig. *Neurotoxicol. Teratol.*, v. 30(5), p. 448-454, 2008.

Niblock MM, Lohr KM, Nixon M, Barnes C, Schaudies M, Murphy M. Cells in the female retrotrapezoid region upregulate c-fos in response to 10%, but not 5%, carbon dioxide. *Brain Res*., v. 1433, p. 62-68, 2012.

Niblock MM, Gao H, Li A, Jeffress EC, Murphy M, Nattie EE. Fos-Tau-LacZ mice reveal sex differences in brainstem c-fos activation in response to mild carbon dioxide exposure. *Brain Res*., v. 1311, p. 51-63, 2010.

Nichols NL, Hartzler LK, Conrad SC, Dean JB, Putnam RW. Intrinsic chemosensitivity of individual nucleus tractus solitarius (NTS) and locus coeruleus (LC) neurons from neonatal rats. *Adv. Exp. Med. Biol*., v. 605, p. 348-352, 2008.

Nida. NIDA Research Monograph Series: Marijuana Abuse. NIH, 2005.

Niederhofer N, Szabo B. Cannabinoids cause central sympathoexcitation and bradycardia in rabbits. *J. Pharmacol. Exp. Ther*., v. 294, p. 707-713, 2000.

Nunez-Abades P, Cameron W. Morphology of developing rat genioglossal motoneurons studied in vitro: relative changes in diameter and surface area of somata and dendrites. *J. Comp. Neurol.*, v. 353, p. 129-142, 1995.

O'Donnell KJ, Glover V. Maternal Prenatal Stress and the Developmental Origins of Mental Health: The Role of Epigenetics. The Epigenome and Developmental Origins of Health and Disease. Ed, Elsevier, p. 103-126, 2016

Ojeda SR, Andrews WW, Advis JP, White SS. Recent advances in the endocrinology of puberty. *Endocr Rev*., v. 1(3), p. 228-257, 1980.

Onaivi ES, Ishiguro H, Gong JP, Patel S, Perchuk A, Meozzi PA, Myers L, Mora Z, Tagliaferro P, Gardner E, Brusco A, Akinshola BE, Liu QR, Hope B, Iwasaki S, Arinami T, Teasenfitz L, Uhl GR. Discovery of the presence and functional expression of cannabinoid CB2 receptors in brain. *Ann. N. Y. Acad. Sci.*, v. 1074, p. 514–536, 2006.

Onaivi ES, Leonard CM, Ishiguro H, Zang PW, Lin Z, Akinshola BE, Uhl GR. Endocannabinoid and receptor genetics. *Prog. Neurobiol.*, v. 567, p. 1–38, 2002.

Onimaru H, Homma I. A novel functional neuron group for respiratory rhythm generation in the ventral medulla. *J. Neurosci.*, v. 23, p. 1478-1486, 2003.

Onimaru H, Arata A, Homma I. Neuronal mechanisms of respiratory rhythm generation: an approach using in vitro preparation. *Jpn. J. Physiol*., v. 47, p. 385-403, 1997.

Oyamada Y, Ballantyne D, Mückenhoff K, Scheid P. Respiration modulated membrane potential and chemosensitivity of locus coeruleus neurones in the in vitro brainstemspinal cord of the neonatal rat. *J. Physiol*., v. 513, p. 381-398, 1998.

Padley JR, Li Q, Pilowsky PM, Goodchild AK. Cannabinoid receptor activation in the rostral ventrolateral medulla oblongata evokes cardiorespiratory effects in anaesthetized rats. *Br. J. of Pharmacol.*, v. 140, p. 384–394, 2003.

Pagliardini S, Ren J, Greer JJ. Ontogeny of the pre-Botzinger complex in perinatal rats. *J. Neurosci.*, v. 23, p. 9575-9584, 2003.

Patel S, Hill MN, Cheer JF, Wotjak CT, Holmes A. The endocannabinoid system as a target for novel anxiolytic drugs. *Neurosci. Biobehav. Rev.*, v. 76, p. 56-66, 2017.

Patrone LGA, Capalbo AC, Marques DA, Bícego KC, Gargaglioni LH. An age- and sex-dependent role of catecholaminergic neurons in the control of breathing and hypoxic chemoreflex during postnatal development. *Brain Res.*, v. 1726, 146508, 2020

Patrone LGA, Biancardi V, Marques DA, Bícego KC, Gargaglioni LH. Brainstem catecholaminergic neurones and breathing control during postnatal development in male and female rats. *J. Physiol*., v. 596, p. 3299-3325, 2018.

Patrone LGA, Bicego KC, Hartzler LK, Putnam RW, Gargaglioni LH. Cardiorespiratory effects of gap junction blockade in the locus coeruleus in unanesthetized adult rats. *Respir. Physiol. Neurobiol*., v. 190, 86-95, 2014.

Paxinos G, Watson C. The rat brain in stereotaxic coordinates, 3ª ed., San Diego, CA: Academic, 1998.

Perez-Reyes M, Timmons MC, Lipton MA, Davis KH, Wall ME. Intravenous injection in man of 9-tetrahydrocannabinol and 11-OH- 9 –tetrahydrocannabinol. *Science*, v. 188, p. 633-635, 1972.

Perim RR, Kubilis PS, Seven YB, Mitchell GS. Hypoxia-induced hypotension elicits adenosine-dependent phrenic long-term facilitation after carotid denervation. *Exp. Neurol.*, v. 333, p. 113429, 2020.

Perna G, Bertani A, Caldirola D, Bellodi L. Family history of panic disorder and hypersensitivity to CO_2 in patients with panic disorder. *Am. J. Psychiat.*, v. 153, p. 1060-1064, 1996.

Petrunich-Rutherford ML, Calik MW. Effects of Cannabinoid Agonists and Antagonists on Sleep in Laboratory Animals. *Adv. Exp. Med. Biol.*, v. 1297, p. 97-109, 2021.

Pfitzer T, Niederhoffer N, Szabo B. Central effects of the cannabinoid receptor agonist WIN55212-2 on respiratory and cardiovascular regulation in anaesthetised rats. *Br. J. Pharmacol.*, v. 142(6), p. 943-952, 2004.

Pilowsky PM, Goodchild AK. Baroreceptor reflex pathways and neurotransmitters: 10 years on. *J. Hypertens*., v. 20, p. 1675–1688, 2002.

Pilowsky PM, Jiang C, Lipski J. An intracellular study of respiratory neurons in the rostral ventrolateral medulla of the rat and their relationship to catecholamine-containing neurons. *J. Comp. Neurol*., v. 301, p. 604-617, 1990.

Pineda J, Aghajanian GK. Carbon dioxide regulates the tonic activity of locus coeruleus neurons by modulating a proton- and polyamine-sensitive inward rectifier potassium current. *Neuroscience*, v. 77(3), p. 723-743, 1997.

Piomelli D. The molecular logic of endocannabinoid signalling. *Nat. Rev. Neurosci.*, v. 4, p. 873–884, 2003.

Prasad B, Radulovacki MG, Carley DW. Proof of concept trial of dronabinol in obstructive sleep apnea. *Front. Psychiatry*, v. 4, p. 1, 2013.

Putnam RW, Conrad SC, Gdovin MJ, Erlichman JS, Leiter JC. Neonatal maturation of the hypercapnic ventilatory response and central neural CO_2 chemosensitivity. *Respir. Physiol. Neurobiol*., v. 149(1-3), p. 165-179, 2005.

Putnam RW, Filosa JA, Ritucci NA. Cellular mechanisms involved in CO_2 and acid signalling in chemosensitive neurons. *Am. J. Physiol. Cell. Physiol.*, v. 287, p. C1493-C1526, 2004.

Quinlivan JA, Evans SF. The impact of continuing illegal drug use on teenage pregnancy outcomes a prospective cohort study. *BJOG*, v. 109, p. 1148-1153, 2002.

Randall MD, Kendall DA. Involvement of a cannabinoid in endothelium-derived hyperpolarizing factor-mediated coronary vasorelaxation. *Eur. J. Pharmacol*., v. 335, p. 205-209, 1997.

Rawls SM, Cabassa J, Geller EB, Adler MW. CB1 receptors in the preoptic anterior hypothalamus regulate WIN 55212-2 [(4,5-dihydro-2- methyl-4(4-morpholinylmethyl)-1-(1-naphthalenyl-carbonyl)-6Hpyrrolo[3,2,1ij]quinolin-6-one]-induced hypothermia. *J. Pharmacol. Exp. Ther*., v. 301, p. 963-968, 2002.

Ray RS, Corcoran AE, Brust RD, Soriano LP, Nattie EE, Dymecki SM. Egr2-neurons control the adult respiratory response to hypercapnia. *Brain Res*., v. 1511, p. 115-125, 2013.

Regensteiner JG, Woodard WD, Hagerman DD, Weil JV, Pickett CK, Bender PR, Moore LG. Combined effects of female hormones and metabolic rate on ventilatory drives in women. *J. Appl. Physiol. (1985)*, v. 66(2), p. 808-813, 1989.

Rekling JC, Feldman JL. Pre-Bötzinger complex and pacemaker neurons: hypothesized site and kernel for respiratory rhythm generation. *Ann. Rev. Physiol*., v. 60, p. 385-405, 1998.

Rice D, Barone Jr S. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environ. Health Perspect.*, v. 108, p. 511-533, 2000.

Rice W, Shannon JM, Burton F, Fiedeldey D. Expression of a brain-type cannabinoid receptor (CB1) in alveolar Type II cells in the lung: regulation by hydrocortisone. *Eur. J. Pharmacol.*, v. 327(2-3), p. 227-232, 1997.

Richardson KA, Hester AK, McLemore GL. Prenatal cannabis exposure the "first hit" to the endocannabinoid system. *Neurotoxicol. Teratol.*, v. 58, p. 5–14, 2016.

Richerson GB. Serotonergic neurons as carbon dioxide sensors that maintain pH homeostasis. *Nat. Rev. Neurosci*., v. 5, p. 449-461, 2004.

Ripamonte GC, Bernardes-Ribeiro M, Patrone LGA, Vicente MC, Bícego KC, Gargaglioni LH. Functional role for preoptic CB1 receptors in breathing and thermal control. *Neurosci. Lett.*, v. 732, 135021, 2020.

Ritucci NA, Dean JB, Putnam RW. Somatic vs dendritic responses to hypercapnia in chemosensitive locus coeruleus neurons from neonatal rats. *Am. J. Physiol. Cell. Physiol.*, v. 289, p. C1094-C1104, 2005.

Rodríguez de Fonseca F, Gorriti MA, Fernández-Ruiz JJ, Palomo T, Ramos JA. Downregulation of rat brain cannabinoid binding sites after chronic delta 9-tetrahydrocannabinol treatment. *Pharmacol. Biochem. Behav.*, v. 47, p. 33-40, 1994.

Rodriguez de Fonseca F, Ramos JA, Bonnin A, Fernandez-Ruiz JJ. Presence of cannabinoid binding sites in the brain fromearly postnatal ages. *NeuroReport*, v. 4, p. 135–138, 1993.

Rohof WO, Aronica E, Beaumont H, Troost D, Boeckxstaens GE. Localization of mGluR5, GABAB, GABAA, and cannabinoid receptors on the vago-vagal reflex pathway responsible for transient lower esophageal sphincter relaxation in humans: an immunohistochemical study. *Neurogastroen. Motil*, v. 24(4), p. 383-e173, 2012.

Romero J, Garcia-Palomero E, Berrendero F, Garcia-Gil L, Hernandez ML, Ramos JA, Fernandez-Ruiz JJ. Atypical location of cannabinoid receptors in white matter areas during rat brain development. *Synapse*, v. 26, p. 317-323, 1997.

Rooney SA, Young SL, Mendelson CR. Molecular and cellular processing of lung surfactant. *FASEB J*. v. 8, p. 957-967, 1994.

Roth WT, Tinkleinberg JR, Kopell BS, Hollister LE. Continuous electrocardiographic monitoring during marihuana intoxication. *Clin. Pharmacol. Ther.*, v. 14(4), p. 533-540, 1973.

Ruffault PL, D'Autreaux F, Hayes JA, Nomaksteinsky M, Autran S, Fujiyama T, Hoshino M, Hagglund M, Kiehn O, Brunet JF, Fortin G, Goridis C. The retrotrapezoid nucleus neurons expressing Atoh1 and Phox2b are essential for the respiratory response to CO_2. *Elife*, v. 4, e07051, 2015.

Schmid K, Niederhoffer N, Szabo B. Analysis of the respiratory effects of cannabinoids in rats. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, v. 368, p. 301-308, 2003.

Schulz H, Johner C, Eder G, Ziesenis A, Reitmeier P, Heyder J, Balling R. Respiratory mechanics in mice: strain and sex specific differences. *Acta. Physiol. Scand.*, v. 174(4), p. 367-375, 2002.

Scragg RK, Mitchell EA, Ford RP, Thompson JM, Taylor BJ, Stewart AW. Maternal cannabis use in the sudden death syndrome. *Acta Paediatr*., v. 90, p. 57-60, 2001.

Shabani M, Hosseinmardi N, Haghani M, Shaibani V, Janahmadi M. Maternal exposure to the CB1 cannabinoid agonist WIN 55212-2 produces robust changes in motor function and intrinsic electrophysiological properties of cerebellar Purkinje neurons in rat offspring. *Neuroscience*, v. 172, p. 139-152, 2011.

Shankaran S, Das A, Bauer CR, Bada HS, Lester B, Wright LL, Smeriglio V. Association between patterns of maternal substance use and infant birth weight, length, and head circumference. *Pediatrics*, v. 114, p. 226-234, 2004.

Sierra S, Luquin N, Navarro-Otano J. The endocannabinoid system in cardiovascular function: novel insights and clinical implications. *Clin. Auton. Res.*, v. 28(1), p. 35-52, 2017.

Silveira PP, Portella AK, Goldani MZ, Barbieri MA. Origens desenvolvimentistas da saúde e da doença (DOHaD). *J. Pediatr*, v. 83, p. 494-504, 2007.

Silvestri C, Di Marzo V. The endocannabinoid system in energy homeostasis and the etiopathology of metabolic disorders. *Cell Metab*., v. 17, p. 475-490, 2013.

Smith JC, Ellenberger HH, Ballanyi K, Richter DW, Feldman JL. Pre-Botzinger complex: a brainstem region that may generate respiratory rhythm in mammals. *Science*, v. 254, p. 726-729, 1991.

Solomon IC. Focal CO_2/H^+ alters phrenic motor output response to chemical stimulation of cat pre-Botzinger complex in vivo. *J. Appl. Physiol. (1985)*, v. 94(6), p. 2151-2157, 2003.

Solomon IC, Edelman NH, O'Neal 3rd MH. CO(2)/H(+) chemoreception in the cat pre-Bötzinger complex in vivo. *J. Appl. Physiol. (1985)*, v. 88(6), p. 1996-2007, 2000.

Sprenger RJ, Milsom WK. Respiratory development in burrowing rodents: Effect of perinatal hypercapnia. *Respir. Physiol. Neurobiol.*, v. 288, 103640, 2021.

Stengel PW, Rippy MK, Cockerham SL, Devane WA, Silbaugh SA. Pulmonary actions of anandamide, an endogenous cannabinoid receptor agonist, in guinea pigs. *Eur. J. of Pharmacol.*, v. 355, p. 57-66, 1998.

Sterni LM, Bamford OS, Wasicko MJ, Carroll JL. Chronic hypoxia abolished the postnatal increase in carotid body type I cell sensitivity to hypoxia. *Am. J. of Physiol. Lung Cell. and Mol. Physiol.*, v. 277(3), p. L645-652, 1999.

Stornetta RL, Moreira TS, Takakura AC, Kang BJ, Chang DA. Expression of Phox2b by brainstem neurons involved in chemosensory integration in the adult rat. *J. Neurosci*., v. 26, p. 10305-10314, 2006.

Szabo B, Schlicker E. Effects of cannabinoids on neurotransmission. *Handb. Exp. Pharmacol.*, p. 327–365, 2005.

Takakura AC, Barna BF, Cruz JC, Colombari E, Moreira TS. Phox2b expressing retrotrapezoid neurons and the integration of central and peripheral chemosensory control of breathing in conscious rats. *Exp. Physiol*., v. 99, p. 571-585, 2014.

Takakura AC, Moreira TS, West GH, Gwilt JM, Colombari E, Stornetta RL, Guyenet PG. GABAergic pump cells of solitary tract nucleus innervate retrotrapezoid nucleus chemoreceptors. *J. Neurophysiol*., v. 98, p. 374-81, 2007.

Takakura AC, Moreira TS, Colombari E, West GH, Stornetta RL, Guyenet PG. Peripheral chemoreceptor inputs to retrotrapezoid nucleus (RTN) CO_2 sensitive neurons in rats. *J. Physiol. (Lond).*, v. 572, p. 503-523, 2006.

Tashkin DP, Shapiro BJ, Frank IM. Acute pulmonary physiologic effects of smoked marijuana and oral delta-9-tetrahydrocannabinol in healthy young men. *New Engl. J. Med*., v. 289, p. 336-341, 1983.

Tashkin DP, Shapiar BJ, Lee YE, Harper CE. Subacute effects of heavy marihuana smoking on pulmonary function in healthy men. *New Engl. J. Med*., v. 294, p. 125-129, 1976.

Taylor NC, Li A, Nattie EE. Medullary serotonergic neurones modulate the ventilatory response to hypercapnia, but not hypoxia in conscious rats. *J. Physiol.*, v. 566(2), p. 543-557, 2005.

Teicher MH, Anderson CM, Polcari A. Childhood maltreatment is associated with reduced volume in the hippocampal subfields CA3, dentate gyrus, and subiculum. *Proc. Natl. Acad. Sci. USA*, v. 109(9), p. 563-572, 2012.

Thoby-Brisson M, Karlen M, Wu N, Charnay P, Champagnat J, Fortin G. Genetic identification of an embryonic parafacial oscillator coupling to the preBotzinger complex. *Nat. Neurosci.*, v. 12, p. 1028-1035, 2009.

Thoby-Brisson M, Trinh JB, Champagnat J, Fortin G. Emergence of the pre-Bötzinger respiratory rhythm generator in the mouse embryo. *J. Neurosci.*, v. 25, p. 4307-4318, 2005.

Tree K, Di Perretolo MS, Peyronnet J, Cayetanot F. In utero cannabinoid exposure alters breathing and the response to hypoxia in newborn mice. *Eur. J. of Neurosci.*, v. 40, p. 2196-2204, 2014.

Tree K, Caravagna C, Hilaire G, Peyronnet J, Cayetanot F. Anandamide centrally depresses the respiratory rhythm generator of neonatal mice. *Neuroscience*, v. 170, p. 1098-1109, 2010.

Trezza V, Campolongo P, Cassano T, Macheda T, Dipasquale P, Carratù MR, Gaetani S, Cuomo V. Effects of perinatal exposure to delta-9-tetrahydrocannabinol on the emotional reactivity of the offspring: a longitudinal behavioral study in Wistar rats. *Psychopharmacol. (Berl)*, v. 198(4), p. 529-537, 2008.

Tsou K, Brown S, Sanudo-Pena MC, Mackie K, Walker JM. Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. *Neuroscience*, v. 83, p. 393- 411, 1998.

Valiveti S, Hammell DC, Earles DC, Stinchcomb AL. Transdermal delivery of the synthetic cannabinoid win 55,212-2: in vitro/in vivo correlation. *Pharm. Res.*, v. 21, p. 1137–1145, 2004.

Van Sickle MD, Duncan M, Kingsley PJ, Mouihate A, Urbani P, Mackie K, Stella N, Makriyannis A, Piomelli D, Davison JS, Marnett LJ, Di Marzo V, Pittman QJ, Patel KD, Sharkey KA. Identification and functional characterization of brainstem cannabinoid CB2 receptors. *Science*, v. 310, p. 329–332, 2005.

Vardaris RM, Weisz DJ, Fazel A, Rawitch AB. Chronic administration of delta-9-tetrahydrocannabinol to pregnant rats: studies of pup behavior and placental transfer. *Pharmacol. Biochem. Behav.*, v. 4, p. 249–254, 1976.

Vicente MC, Dias MB, Fonseca EM, Bícego KC, Gargaglioni LH. Orexinergic system in the locus coeruleus modulates the CO_2 ventilatory response. *Pflugers Arch.*, v. 468(5), p. 763-774, 2016.

Viemari JC, Maussion G, Bévengut M, Burnet H, Pequignot JM, Népote V, Pachnis V, Simonneau M, Hilaire G. Ret deficiency in mice impairs the development of A5 and A6 neurons and the functional maturation of the respiratory rhythm. *Eur. J. Neurosci.*, v. 22(10), p. 2403-12, 2005.

Viemari JC, Bévengut M, Burnet H, Coulon P, Pequignot JM, Tiveron MC, Hilaire G. Phox2a gene, A6 neurons, and noradrenaline are essential for development of normal respiratory rhythm in mice. *J. Neurosci.*, v. 24, p. 928-937, 2004.

Vigano D, Rubino T, Parolaro D. Molecular and cellular basis of cannabinoid and opioid interactions. *Pharmacol. Biochem. Behav.*, v. 81, p. 360–368, 2005.

Vitalis TA, Milsom WK. Pulmonary mechanics and the work of breathing in the semi-aquatic turtle Pseudemys scripta. *J. Exp. Biol.*, v. 125, p. 137-155, 1986.

Viveros MP, Llorente R, Suarez J, Llorente-Berzal A, López-Gallardo M, Rodriguez de Fonseca F. The endocannabinoid system in critical neurodevelopmental periods: sex differences and neuropsychiatric implications. *J. Psychopharmacol.*, v. 26(1), p. 164-176, 2012.

Vivian JA, Kishioka S, Butelman ER, Broadbear J, O'Lee K, Woods JH. Analgesic, respiratory and heart rate effects of cannabinoid and opioid agonists in Rhesus monkeys: antagonist effects of SR141716A. *J. Pharmacol. Exp. Ther.*, v. 286, p. 697–703, 1998.

Volkow ND, Han B, Compton WM, McCance-Katz EF. Self-reported medical and nonmedical cannabis use among pregnant women in the United States. *JAMA*, v. 322, p. 167-169 2019.

Volkow ND, Compton WM, Wargo EM. The risk of marijuana use during pregnancy. *JAMA*, v. 317, p. 129-130, 2017.

Vollmer RR, Cavero I, Ertel RJ. Role of the central autonomic nervous system in the hypotension and bradycardia induced by (-)-delta 9-trans-tetrahydrocannabinol. *J. Pharm. Pharmacol*., v. 26, p. 186-192, 1974.

Von Bernhardi R, Von Bernhardi LE, Eugenín J. What Is Neural Plasticity? *Adv. Exp. Med. Biol.*, v. 1015, 1-15, 2017.

Vuolo F, Abreu SC, Michels M, Xisto DG, Blanco NG, Hallak JE, Zuardi AW, Crippa JA, Reis C, Bahl M, Pizzichinni E, Maurici R, Pizzichinni MMM, Rocco PRM, Dal-Pizzol F. Cannabidiol reduces airway inflammation and fibrosis in experimental allergic asthma. *Eur. J. Pharmacol.*, v. 843, p. 251-259, 2019.

Wang LL, Zhao R, Li JY, Li SS, Liu M, Wang M, Zhang MZ, Dong WW, Jiang SK, Zhang M, Tian ZL, Liu CS, Guan DW. Pharmacological activation of cannabinoid 2 receptor attenuates inflammation, fibrogenesis, and promotes re-epithelialization during skin wound healing. *Eur. J. Pharmacol.*, v. 786, p. 128-136, 2016.

Wang S, Shi Y, Shu S, Guyenet PG, Bayliss DA. Phox2b-expressing retrotrapezoid neurons are intrinsically responsive to H^+ and CO_2. *J. Neurosci*., v. 33, p. 7756-7761, 2013.

Wang T, Li GQ, Zhang HP. Overactivation of cannabinoid receptor type 1 in rostral ventrolateral medulla promotes cardiovascular responses in spontaneously hypertensive rats. *J. Hypertens.*, v. 35, p. 538-545, 2017.

Weiss JL, Watanabe AM, Lemberger L. Cardiovascular efects of delta-9-tetrahydrocannabinol in man. *Clin. Pharmacol. Ther.*, v. 13, p. 671-684, 1972.

Whyte DA, Al-Hammadi S, Balhaj G, Brown OM, Penefsky HS, Souid AK. Cannabinoids inhibit cellular respiration of human oral cancer cells. *Pharmacology*, v. 85, p. 328-335, 2010.

Williams RH, Burdakov D. Hypothalamic orexins/hypocretins as regulators of breathing. *Expert. Rev. Mol. Med.*, v. 10:e28, 2008.

Wong-Riley MTT, Liu Q, Gao X. Mechanisms underlying a critical period of respiratory development in the rat. *Respir. Physiol. Neurobiol.*, v. 264, p. 40-50, 2019.

Wu CS, Jew CP, Lu HC. Lasting impacts of prenatal cannabis exposure and the role of endogenous cannabinoids in the developing brain. *Future Neurol*. v. 6, p. 459–480, 2011.

Xu S, Guo S, Jiang X, Yin Q, Umezawa T, Hisamitsu T. Effect of indomethacin on the c-fos expression in AVP and TH neurons in rat brain induced by lipopolysaccharide. *Brain Res.*, v. 966(1), p. 13-18, 2003.

Yamamoto K, Lalley P, Mifflin S. Acute intermittent optogenetic stimulation of nucleus tractus solitarius neurons induces sympathetic long-term facilitation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, v. 308, p. 266-275, 2015.

Young-Wolff KC, Sarovar V, Tucker LY, Avalos LA, Alexeeff S, Conway A, Armstrong MA, Weisner C, Campbell CI, Goler N. Trends in marijuana use among pregnant women with and without nausea and vomiting in pregnancy, 2009-2016. *Drug Alcohol Depend*., v. 196, p. 66-70, 2019.

Zuckerman B, Frank DA, Hingson R, Amaro H, Levenson SM, Kayne H, Parker S, Vinci R, Aboagye K, Fried LE. Effects of maternal marijuana and cocaine use on fetal growth. *N. Engl. J. Med*., v. 320, p. 762-768, 1989.