UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CÂMPUS DE JABOTICABAL

PARTICIPAÇÃO DOS CANAIS PARA POTÁSSIO ATIVADOS POR CÁLCIO NAS RESPOSTAS RESPIRATÓRIAS E METABÓLICAS AO CO_2/pH DURANTE O DESENVOLVIMENTO EM CAMUNDONGOS

Bianca de Avila Martins

Médica Veterinária

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Bianca de Avila Martins

Orientadora: Profa. Dra. Luciane Helena Gargaglioni Batalhão

Coorientador: Dr. Luis Gustavo Alexandre Patrone

Dissertação apresentada à Faculdade de Ciências Agrárias e Veterinárias – UNESP, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Mestre em Ciência Animal

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DADOS CURRICULARES DO AUTOR

Bianca de Avila Martins – nascida em 28 de junho a 1997, natural de Arujá – SP, filha de Elisangela Ribeiro de Avila Martins e de João Carlos Martins, ingressou no curso de Medicina Veterinária da Universidade Estadual Paulista “Júlio de Mesquita Filho” – UNESP, Campus de Araçatuba – FMVA, em fevereiro de 2015. Durante o período de graduação a aluna realizou estágios nas disciplinas de anatomia, radiografia e fisiologia, além da participação de 2 anos em Empresa Jr. de prestação de serviços à comunidade da cidade de Araçatuba – SP. O estágio curricular foi realizado na Faculdade de Medicina Veterinária da Universidade Estadual Paulista “Júlio de Mesquita Filho” – UNESP, Campus de Araçatuba – FMVA, no Laboratório de Fisiologia, coordenado pela Prof.(a) Dr.(a) Gisele Zoccal Mingoti, sob orientação do Prof. Dr. Guilherme de Paula Nogueira. Colou grau em janeiro de 2022 e recebeu o título de Médica Veterinária. Em junho de 2022 iniciou o curso de Mestrado pelo Programa de Pós-Graduação em Ciência Animal no Departamento de Morfologia e Fisiologia Animal na Universidade Estadual Paulista – UNESP Campus Jaboticabal – FCAV, onde foi bolsista CNPq, realizando seu Exame Geral de Qualificação em 12 de julho de 2023.

Dedico todo e qualquer sucesso meu aos meus pais, que, sob muito sol,
me fizeram chegar aqui pela sombra e com água fresca.

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Acima de tudo, agradeço a quem me lê e me oferece seu tempo, pois denota a importância do meu trabalho, validando meu esforço.

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À Faculdade de Ciências Agrárias e Veterinárias - UNESP e o Programa de Pós-Graduação em Ciência Animal pelo ensino e estrutura necessários para a execução do meu trabalho.

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CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

C E R T I F I C A D O

Certificamos que o projeto de pesquisa intitulado **"Participação dos canais para potássio ativados por cálcio nas respostas respiratórias ao CO_2/pH e à hipóxia durante o desenvolvimento em camundongos"**, protocolo nº 9486/22, sob a responsabilidade da Profa. Dra. Luciane Helena Gargaglione Batalhão, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 15 de fevereiro 2023.

Vigência do Projeto	16/02/2023 a 01/12/2024
Espécie / Linhagem	*Mus musculus* – C57BL/6
Nº de animais	120
Peso / Idade	0-1 dia (2g); 6-7 dias (6g); 12-13 dias (10g); 27-28 dias(15g); 56-57 dias (25g)
Sexo	Machos e Fêmeas
Origem	Biotério Departamento de Morfologia e Fisiologia Animal - FCAV/Unesp

Jaboticabal, 15 de fevereiro de 2023.

Profª Drª Fabiana Pilarski
Coordenadora – CEUA

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PARTICIPAÇÃO DOS CANAIS PARA POTÁSSIO ATIVADOS POR CÁLCIO NAS RESPOSTAS RESPIRATÓRIAS AO CO_2/pH DURANTE O DESENVOLVIMENTO EM CAMUNDONGOS

RESUMO

Os canais para potássio ativados por cálcio (BK) são fundamentais para a regulação da excitabilidade celular e estão amplamente presentes nos tecidos de mamíferos, incluindo o sistema nervoso central (SNC). No SNC, esses canais estão localizados nos neurônios quimiossensíveis do locus coeruleus (LC), região importante para a detecção de CO_2/pH e para o controle da respiração. A ativação dos canais BK, promovida por um aumento na concentração de cálcio intracelular, resulta na redução da excitabilidade neuronal em resposta à hipercapnia, funcionando como um mecanismo de "freio" nas respostas ventilatórias frente a níveis elevados de CO_2. Durante o desenvolvimento pós-natal, a resposta ventilatória ao CO_2 passa por fases críticas, com destaque para o período entre os dias pós-natais 12 e 13 (P12-P13), um período chave no desenvolvimento respiratório. Nessa fase, há mudanças significativas na neurotransmissão, incluindo uma redução da atividade excitatória e um aumento na inibitória, além de alterações na expressão de canais iônicos, como os BK. Com o passar do tempo, a densidade desses canais nos neurônios do LC aumenta, o que diminui a sensibilidade desses neurônios ao CO_2 e ao pH, impactando o controle ventilatório em resposta a condições de hipercapnia e reduzindo a capacidade de resposta ao aumento de CO_2 no organismo. Estudos indicam que hormônios sexuais, como o estradiol, podem modular a função dos canais BK, o que sugere que as respostas ventilatórias podem variar entre machos e fêmeas. Essa modulação hormonal pode contribuir para diferenças sexuais no controle ventilatório, como observado em fêmeas knockout para os canais BK, que demonstram uma resposta exacerbada à hipercapnia durante o período neonatal. Esse fato sugere uma possível influência hormonal sobre a função desses canais, particularmente no desenvolvimento das respostas respiratórias ao CO_2/pH. Com base nessas observações, este estudo investiga o papel dos canais BK na modulação das respostas respiratórias e metabólicas ao CO_2/pH ao longo do desenvolvimento em camundongos, explorando as variações relacionadas ao sexo e ao genótipo. Ao investigar essas interações, o trabalho visa aprofundar o conhecimento sobre os mecanismos de regulação ventilatória e suas possíveis implicações em distúrbios respiratórios associados à quimiossensibilidade.

Palavras-chave: Canalopatia, desenvolvimento, quimiorrecepção, ventilação

ABSTRACT

Calcium-activated potassium (BK) channels are essential for the regulation of cell excitability and are widely present in mammalian tissues, including the central nervous system (CNS). In the CNS, these channels are located in the chemosensitive neurons of the locus coeruleus (LC), a important region for detecting CO_2/pH and controlling respiration. Activation of BK channels, promoted by an increase in intracellular calcium concentration, results in a reduction in neuronal excitability in response to hypercapnia, acting as a “brake” mechanism on ventilatory responses to high levels of CO_2. During postnatal development, the ventilatory response to CO_2 goes through critical phases, especially the period between postnatal days 12 and 13 (P12-P13), a key point in respiratory development. During this phase, there are significant changes in neurotransmission, including a reduction in excitatory activity and an increase in inhibitory activity, as well as changes in the expression of ion channels, such as BK. Over time, the density of these channels in LC neurons increases, which decreases the sensitivity of these neurons to CO_2 and pH, impacting ventilatory control in response to hypercapnia and reducing the ability to respond to increased CO_2 in the body. Studies indicate that sex hormones, such as estradiol, can modulate the function of BK channels, which suggests that ventilatory responses may vary between males and females. This hormonal modulation may contribute to sexual differences in ventilatory control, as observed in BK channel knockout females, which show an exacerbated response to hypercapnia during the neonatal period. This suggests a possible hormonal influence on the function of these channels, particularly in the development of respiratory responses to CO_2/pH. Based on these observations, this study investigates the role of BK channels in modulating respiratory and metabolic responses to CO_2/pH throughout development in mice, exploring variations related to sex and genotype. By investigating these interactions, the work aims to deepen knowledge about the mechanisms of ventilatory regulation and their possible implications in respiratory disorders associated with chemosensitivity.

Keywords: Channelopathy, development, chemoreception, ventilation

CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

INTRODUÇÃO

O desenvolvimento do sistema nervoso central (SNC) é um processo complexo que envolve uma série de mecanismos celulares e moleculares, sistematicamente organizados em diferentes etapas temporais. Esse desenvolvimento começa na fase embrionária e continua até a vida adulta. Os canais para potássio ativados por cálcio (BK) estão presentes em quase todos os tecidos de mamíferos (Bailey et al., 2019), e são fundamentais para a homeostase elétrica do organismo e manutenção das funções fisiológicas ideais. Distúrbios nesses canais podem levar a uma série de desordens em vários sistemas, como ataxia, epilepsia, hipertensão, acidente vascular cerebral, disfunção erétil, alterações hormonais, dentre outras diversas (Bailey et al., 2019; Halm et al., 2017; Meredith et al., 2004; Rüttiger et al., 2004; Sausbier et al., 2004; Tomás et al., 2008).

O genoma dos mamíferos possui mais de 70 genes responsáveis pela formação dos canais para íons de potássio (K^+), o que tem como consequência uma grande variedade de canais para esse íon, dentre eles os ativados por cálcio, os quais podem ser divididos em grupos de acordo com o seu nível de condutância: pequena (SK), intermediária (IK) e grande (BK). Os canais BK são ativados por aumentos na concentração de cálcio intracelular (Miller et al., 2000; Putnam et al., 2004; Putnam, 2010). A inibição farmacológica ou genética dos canais BK em neurônios do SNC pode modular a taxa de disparo, aumentando-a ou diminuindo-a, tanto em condições de disparo evocado quanto espontâneo. (Brenner et al., 2005; Meredith et al., 2006; Pitts, Ohta, & McMahon, 2006; Li et al., 2007; Matthews, Weible, Shah, & Disterhoft, 2008). A ativação desses canais reduz a taxa de disparo neuronal em resposta a concentrações elevadas de dióxido de carbono e íons de hidrogênio (CO_2/H^+), funcionando como um mecanismo de limitação na resposta quimiossensível neuronal (Imber et al., 2014). Quando esses canais são inibidos, ocorre um aumento na taxa de disparo dos neurônios do Locus coeruleus (LC) em resposta ao aumento de CO_2 (Imber & Putnam, 2012; Imber et al., 2014).

Estudo prévio demonstrou a ocorrência de uma maior ativação dos canais para íons de cálcio (canal para Ca^{2+} tipo L) em ratos neonatos com idade pós-natal superior a 10 dias (>P10), ao passo que a inibição desses canais em neonatos com idade inferior a P10 reduz a taxa de disparo dos neurônios do LC em resposta à hipercapnia. Além disso, o bloqueio dos canais para Ca^{2+} durante o estímulo hipercápnico aumenta a frequência de disparo dos neurônios do LC em ratos neonatos com idade superior a 10 dias, o que sugere que essa resposta de disparo apresente uma saturação, podendo representar uma via limitadora da resposta quimiossensível ao CO_2 pela ativação dos canais BK nesses neurônios (Imber; Putnam, 2012). O mecanismo proposto para sensibilidade ao CO_2 proposto por Imber e Putnam (2012) está apresentado na Figura 1.

Esse mecanismo de limitação tem sido considerado como um potencial meio de controle da resposta quimiossensível dos neurônios do LC à hipercapnia, contribuindo para um melhor entendimento de determinadas condições patofisiológicas como a apneia do sono e o transtorno do pânico (TP), uma vez que esse núcleo participa ativamente dessas condições devido à alta sensibilidade a alterações das concentrações de CO_2 e pH (Biancardi et al., 2010; Imber et al., 2018). Contudo, ainda não se sabe o papel dos canais BK na resposta quimiossensível em camundongos durante o desenvolvimento, e se essa resposta é sexo-específica.

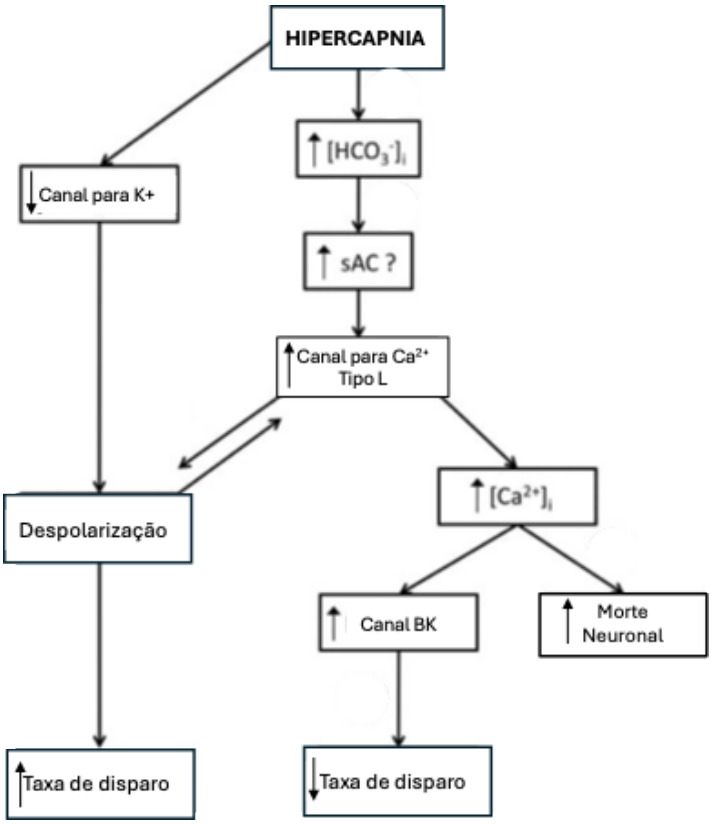

Figura 1: Modelo das vias quimiossensíveis sensíveis ao K^+ e ao Ca^{2+} em neurônios do LC. À esquerda, é apresentado um resumo do papel proposto dos canais para K+ sensíveis a H^+ na despolarização hipercápnica e no aumento da taxa de disparo em neurônios quimiossensíveis do LC. À direita, é ilustrada uma possível via para a ativação hipercápnica de canais para Ca^{2+} e potenciais funções do Ca^{2+} na sinalização quimiossensível em neurônios do LC. Adaptado de Imber e Putnam (2012). Abreviações: sAC – adenilato ciclase solúvel.

Revisão da Literatura

Desenvolvimento do sistema respiratório

Durante o período pré-natal nos mamíferos, ocorre uma fase crucial marcada por mudanças essenciais em vários sistemas fisiológicos, incluindo o sistema respiratório. A principal função desse sistema é manter constantes as pressões parciais de oxigênio (PaO_2) e dióxido de carbono ($PaCO_2$), além de estabilizar os valores de pH. Assim, as trocas gasosas, antes realizadas através

da placenta, precisam ser imediatamente assumidas pelos pulmões após o nascimento. Embora seja amplamente reconhecido que os componentes neurais e musculares do sistema respiratório continuam se desenvolvendo após o nascimento, eles devem estar plenamente funcionais ao nascimento, permitindo a geração de um ritmo que viabilize as trocas gasosas de forma eficiente nos alvéolos, além de integrar a deglutição e outras ações associadas à respiração (Greer et al., 2006; Darnal et al., 2010; Anju et al., 2013).

A respiração envolve uma interação complexa entre o encéfalo, medula espinhal, nervos cranianos e espinhais, músculos específicos, pulmões, neurotransmissores e receptores. Embora a função respiratória esteja presente logo após o nascimento, vários estudos apontam que a rede neural responsável pela respiração passa por um processo de maturação significativa durante o período pós-natal. Esse processo abrange mudanças nas propriedades eletrofisiológicas (Nunez-Abades e Cameron, 1995; Parkins e Berger, 1997; Imber et al., 2014), ajustes na geração do ritmo respiratório (Onimaru et al., 1997) e alterações funcionais e estruturais em neurotransmissores, neuromoduladores e receptores (Greer et al., 2006; Anju et al., 2013; Wong-Riley et al., 2013).

Além disso, componentes distintos do circuito neuronal são essenciais para a maturação dessa rede, como o sistema catecolaminérgico, que desempenha um papel importante no desenvolvimento do sistema respiratório (Guyenet et al., 1993; Erickson e Milhorn, 1994; Carson e Robertson, 2002). O desenvolvimento do sistema catecolaminérgico nos primeiros dias de vida é fundamental para o controle e modulação do ritmo respiratório, contribuindo para a irregularidade ventilatória observada em ratos neonatos até a idade P12 (Viemari et al., 2004; 2005). Esse sistema também é um dos primeiros neuromoduladores a ter um papel crítico no desenvolvimento do sistema nervoso central como um todo (Thomas et al., 1995; Carson e Robertson, 2002; Hilaire et al., 2004), além de influenciar o desenvolvimento de outros sistemas neuronais (Guyenet et al., 1993; Erickson e Milhorn, 1994; Oyamada et al., 1998).

O processo de desenvolvimento de cada componente do sistema respiratório ainda não é totalmente compreendido e pode apresentar variações entre diferentes espécies. No que se refere ao desenvolvimento do ritmo respiratório, estudos baseados em registros eletrofisiológicos *in vitro*, em preparações de tronco encefálico e medula espinhal de fetos de ratos,

mostraram que esse processo começa já na fase embrionária, especificamente no 17º dia embrionário (E17) (Greer et al., 1992; DiPasquale et al., 1992). De fato, registros ultrassônicos de movimentos respiratórios intermitentes em fetos de ratas grávidas anestesiadas (Jansen e Chernick, 1991; Kobayashi et al., 2001) e registros eletrofisiológicos de neurônios do complexo Pré-Bötzinger (pré-BötC) em fatias do bulbo de fetos de ratos (Pagliardini et al., 2003) corroboram esses achados.

O pré-BötC, localizado na superfície ventral do bulbo, é considerado essencial e autossuficiente para a geração do ritmo respiratório (Smith et al., 1991; Rekling e Feldman, 1998; Feldman et al., 2003). Desse modo, os circuitos do pré-BötC produzem e transmitem atividade rítmica que resulta no evento respiratório conhecido como inspiração (Smith et al., 1991; Rekling e Feldman, 1998). No entanto, apesar do grande interesse nas propriedades ritmogênicas dos circuitos do pré-BötC nas últimas décadas, as propriedades estruturais, funcionais e os mecanismos celulares envolvidos nessa função ainda não são totalmente consensuais (Richter e Spyer, 2001; Feldman et al., 2003; Duffin, 2004; Ezure, 2004; Del Negro et al., 2005; Koizumi et al., 2013; Del Negro et al., 2018). Embora o pré-BötC tenha uma grande influência na geração do ritmo respiratório, outras áreas da superfície ventral do bulbo também participam da modulação da ritmogênese respiratória, como a porção anterior do grupo respiratório ventral (GRV), incluindo o núcleo retrotrapezóide (RTN), o grupo respiratório parafacial (GRP, na fase embrionária) e o BötC (Figura 2; Alheid e McCrimmon, 2008).

Especificamente sobre o RTN, ele se origina no período neonatal a partir do grupo parafacial previamente existente na fase embrionária. O RTN é constituído por agrupamentos glutamatérgicos que expressam o fator de transcrição Phox2b, sendo classificados como quimiossensíveis devido à sua capacidade intrínseca de detectar alterações no CO_2/pH (Wang et al., 2013; Takakura et al., 2014; Ruffault et al., 2015). Além disso, diversos estudos demonstraram que o RTN recebe informações periféricas através de projeções dos quimiorreceptores localizados no corpo carotídeo e dos receptores de estiramento pulmonar, auxiliando no controle respiratório (Takakura et al., 2006; 2007; Moreira et al., 2007; Guyenet et al., 2008; Guyenet e Mulkey, 2010).

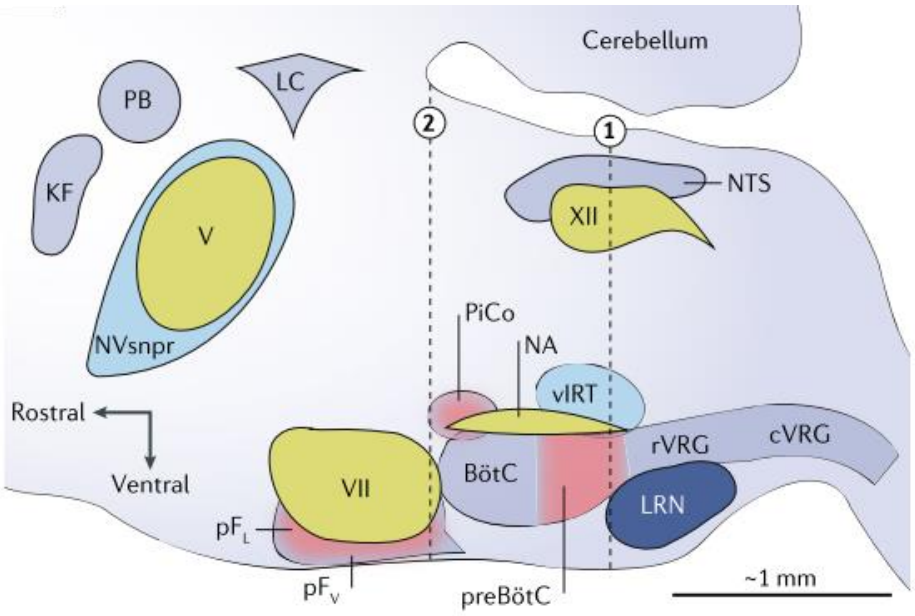

Figura 2. Vista parasagital do tronco encefálico com os locais geradores de ritmo respiratório em vermelho: Complexo pré-Bötzinger (preBötC; inspiratório), pFL (expiratório), grupo respiratório ventral parafacial (pFV; rítmico apenas no período perinatal) e complexo pós-inspiratório (PiCo). Locais relacionados ao ritmo, como a formação reticular ventral intermediária (vIRT) e o núcleo sensorial trigeminal mastigatório (NVsnpr), estão em azul. Núcleos motores cranianos em verde: núcleo motor hipoglosso (XII), núcleo ambíguo (NA), núcleo motor facial (VII) e núcleo motor trigeminal (V). Áreas associadas ao padrão motor respiratório e integração sensório-motora estão em cinza: grupo respiratório ventral rostral (rVRG; pré-motores inspiratórios) e caudal (cVRG; pré-motores expiratórios), núcleo de Kölliker-Fuse (KF), núcleo parabranquial (PB), núcleo do trato solitário (NTS) e BötC expiratório. Outros locais incluem locus coeruleus (LC), que recebe projeções do préBötC, cerebelo e núcleo reticular lateral (LRN). As inserções mostram secções ao nível do preBötC e pF, incluindo o trato trigeminal espinhal (Sp5), os núcleos trigeminais espinhais (Sp5O, Sp5I), a oliva inferior (IO) e o trato piramidal (pyr). Fonte: Del Negro et al. (2018).

Quimiossensibilidade

As flutuações na demanda de oxigênio (O_2) e na produção de dióxido de carbono (CO_2) fazem com que o sistema respiratório desempenhe uma de suas principais funções: ajustar o processo de trocas gasosas para manter, relativamente estáveis, as pressões parciais de O_2 e CO_2 no sangue arterial. Esses ajustes são realizados por meio de quimiorreceptores periféricos e

centrais, que detectam variações nas pressões parciais de O_2 e CO_2 e no pH, garantindo a manutenção desses gases e a homeostase do equilíbrio ácido-básico (Haldane e Priestley, 1905).

Os quimiorreceptores que respondem ao CO_2/pH são cruciais para o controle respiratório, pois ajudam a gerar e modular o ritmo respiratório, evitando grandes oscilações nos níveis de CO_2 e pH, uma vez que alterações significativas podem causar danos fisiológicos irreversíveis. Esses quimiorreceptores podem ser divididos em periféricos (localizados nos corpos carotídeos e no arco aórtico) e centrais (situados no SNC). O estudo de Blain et al. (2010) mostrou que as informações dos quimiorreceptores periféricos têm um efeito sinérgico e hiperaditivo na sensibilidade dos quimiorreceptores centrais, indicando uma interação entre esses sensores, com a resposta ventilatória à hipercapnia sendo mediada por ambos. No entanto, os quimiorreceptores centrais, anteriormente considerados localizados predominantemente na superfície ventrolateral do bulbo (Mitchell et al., 1963; Loeschcke, 1982), desempenham um papel principal na resposta ventilatória ao CO_2 em comparação com os quimiorreceptores periféricos. Pesquisas sugerem que o corpo carotídeo é responsável por cerca de 30% da resposta ventilatória à hipercapnia sistêmica (Dempsey e Forster, 1982; Pan et al., 1998; Forster et al., 2008), com o restante sendo mediado pelos quimiorreceptores centrais (Forster e Smith, 2010).

Alguns estudos sugerem que nos primeiros cinco dias após o nascimento (P1-P5), a resposta ventilatória ao CO_2 é extremamente intensa, mas diminui posteriormente, atingindo seu ponto mais baixo em animais entre P8-P10 (Stunden et al., 2001; Greer, 2012). Depois, essa resposta aumenta novamente e se estabiliza a partir de P16, mantendo-se nos níveis observados em adultos (Stunden et al., 2001; Greer, 2012). Putnam et al. (2001) também descreveram pelo menos duas ou três fases no desenvolvimento da resposta ventilatória ao CO_2. Diferente dos estudos de mencionados anteriormente, os autores demonstraram que em filhotes de ratos, a resposta ventilatória diminui na primeira semana pós-natal (fase I). Contudo, em outras espécies, essa redução não é evidente, mas em todas as espécies estudadas, a resposta ao CO_2 é baixa em recém-nascidos durante a segunda semana de vida (fase II). Na terceira

semana, a resposta aumenta novamente, atingindo níveis semelhantes aos de animais adultos (fase III).

Diversas pesquisas reforçam a ideia de que os quimiorreceptores centrais estão amplamente distribuídos pelo SNC, em diferentes regiões do tronco encefálico, incluindo núcleos como o NTS, o núcleo fastigial do cerebelo, o RTN (Mulkey et al., 2004; Guyenet et al., 2005), a rafe rostral bulbar e o LC (Loeschcke, 1982; Coates et al., 1993; Nattie, 1999; Solomon et al., 2000; Nattie e Li, 2002; Biancardi et al., 2008; Gargaglioni et al., 2010; Patrone et al., 2014). A localização exata dos quimiorreceptores centrais ativos durante a fase fetal ainda não é totalmente compreendida, mas alguns estudos indicam a região parafacial (Onimaru et al., 2008), precursora do RTN, os neurônios serotoninérgicos da rafe (Richerson, 2004) e os neurônios noradrenérgicos do LC (Nichols et al., 2008; Gargaglioni et al., 2010), uma vez que a quimiorrecepção central já está funcional durante a fase fetal, auxiliando na coordenação dos movimentos respiratórios fetais, essenciais para a manutenção do volume pulmonar e preparação para a respiração contínua após o nascimento (Darnall, 2010).

No desenvolvimento pós-natal, um desequilíbrio marcante e transitório entre um aumento da inibição e uma redução da excitação é evidente tanto neuroquimicamente quanto eletrofisiologicamente nos neurônios respiratórios (Wong-Riley e Liu, 2008). Essas mudanças ocorrem especialmente por volta de P12-P13, período em que há uma queda abrupta nos níveis de neurotransmissores excitatórios, como o glutamato e seus receptores, um aumento na neurotransmissão inibitória via GABA e glicina, bem como seus receptores, e uma diminuição repentina na atividade da enzima citocromo oxidase em vários núcleos respiratórios do tronco encefálico (Liu e Wong-Riley, 2002; 2003; 2005; Wong-Riley e Liu, 2008). Nesse contexto, Wong-Riley e Liu (2005) sugerem que o período crítico na resposta ventilatória ao CO_2 (P12-P13) pode estar relacionado à redução na neurotransmissão glutamatérgica e ao aumento da GABAérgica nas regiões do tronco encefálico envolvidas na quimiorrecepção central. Essa alteração pode ser uma possível explicação para a incidência da Síndrome da Morte Súbita Infantil (SIDS) em bebês entre 2 e 3 meses de vida, devido a anormalidades no controle respiratório e autonômico. Alterações no sistema noradrenérgico durante esse período também podem

estar associadas ao desenvolvimento de desordens como a SIDS e a Síndrome de Rett, em que os pacientes apresentam comprometimento significativo do sistema respiratório, sendo mais comum em meninas (Viemari et al., 2005).

Para uma região ser considerada intrinsecamente quimiossensível, ela deve apresentar algumas respostas, como aumento da ventilação (V_E) como consequência de acidificação de apenas aquela área, por exemplo o LC ou NTS, também chamada de resposta ventilatória à hipercapnia (HCVR), outra característica que deve possuir consiste em, quando a área é lesionada (tem a maioria dos seus neurônios lesionada, há diminuição da HCVR, visto pela diminuição da resposta ventilatória ao CO_2 quando os neurônios do LC são consideravelmente lesionados (Li and Nattie, 2006; Biancardi et al., 2008). Além desses dois fatores, a área deve se projetar para a rede respiratória (Putnam et al., 2001).

Em relação ao LC, há uma mudança significativa no desenvolvimento da quimiossensibilidade intrínseca dos neurônios desta região que pode estar relacionada aos canais BK (Imber et al., 2014). Em neonatos de ratos com menos de 10 dias pós-natais (P10), existe uma alta porcentagem (70-80%) de neurônios do LC que são quimiossensíveis (Fig. 3A) e a magnitude da resposta, medida pelo índice de quimiossensibilidade (CI; Wang e Richerson, 1999), foi muito alta (cerca de 235%) (Fig. 3B), similar a alguns dos neurônios mais quimiossensíveis medidos *in vitro* na rafe bulbar (Wang et al., 1998). Nem a porcentagem de neurônios, nem o índice de quimiossensibilidade foram afetados pelo meio de bloqueio de sinapses elétricas (carbenoxolona) em neurônios LC de neonatos jovens (<P10), indicando que essas respostas neuronais à hipercapnia são intrínsecas (Hartzler et al., 2007; Nichols et al., 2008). Em contraste, nos neurônios do LC de neonatos mais velhos (>P10), uma alta porcentagem de neurônios também foi ativada pela hipercapnia, mas a magnitude da resposta foi significativamente menor (CI de 125%; um CI de 100% indica um neurônio não quimiossensível). Assim como nos neonatos mais jovens, o meio de bloqueio sináptico teve pouco efeito nesses valores, mas, de forma notável, a carbenoxolona reduziu drasticamente a porcentagem de neurônios intrinsecamente quimiossensíveis (cerca de 20%) (Fig. 3A) sem afetar o CI (Fig. 3B) (Hartzler et al., 2007; Nichols et al., 2008).

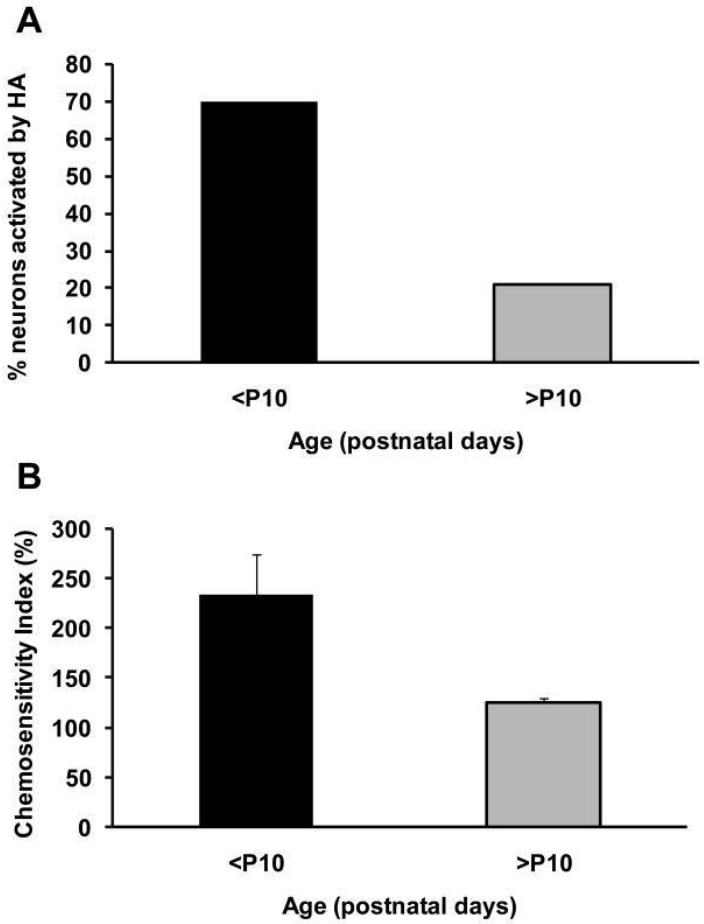

Figura 3. (A) Percentagem de neurônios ativados (aumento da taxa de disparo) por acidose hipercápnica (15% CO_2). (B) Índice de quimiossensibilidade (%) (magnitude relativa da resposta quimiossensível) para os neurônios ativados por acidose hipercápnica. Fonte: Gargaglioni et al., 2010.

Canais para potássio ativados por cálcio

Os canais iônicos são expressos de forma ubíqua em todo o corpo e realizam processos-chave de transporte de membrana cruciais para a função fisiológica normal. Alterações na atividade dos canais iônicos podem interromper as funções homeostáticas, levando a distúrbios como enxaquecas hemiplégicas, epilepsia ou arritmias cardíacas, coletivamente chamadas de "canalopatias" (Meredith, 2015). Os canais para potássio contribuem para as propriedades elétricas das membranas, controlando a orientação e magnitude dos gradientes de concentração do íon K^+. As concentrações de K^+ intracelular normalmente altas e seu vazamento para o meio extracelular levam a diferenças de potencial elétrico da membrana celular (Vm), sendo negativa no interior celular em

comparação ao meio exterior. O genoma dos mamíferos contém uma família de mais de 70 genes que se expressam para a formação de canais para K^+, subdivididos em 5 famílias diferentes, como demonstrado na figura 4 (González et al., 2012). Essa variedade de proteínas permite uma ampla gama de controles sutilmente distintos que fazem com que as células aumentem ou diminuam a condutância de K^+ pela membrana celular e, assim, ajustem a magnitude de Vm.

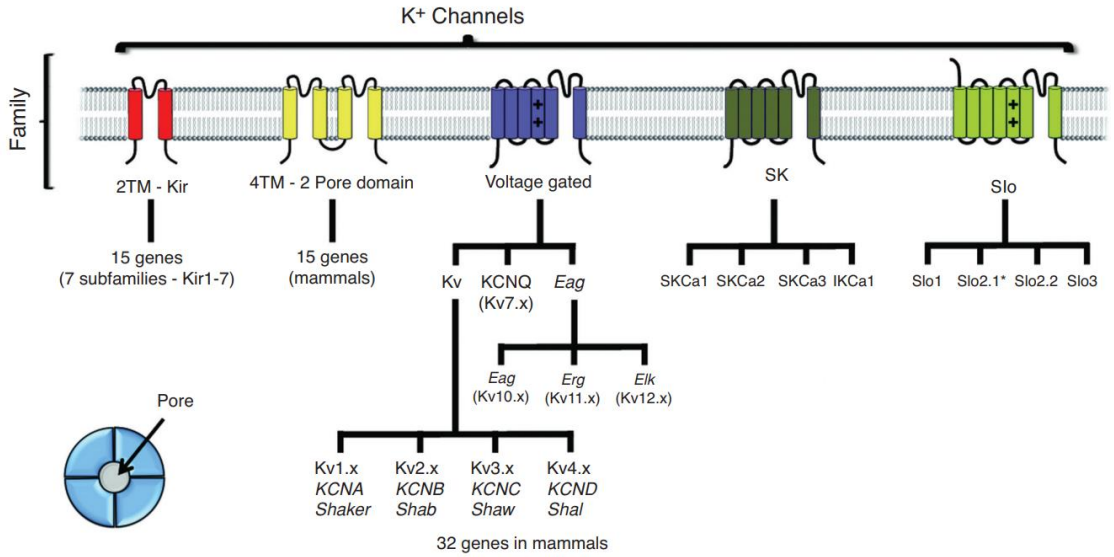

Figura 4. Famílias de canais para potássio organizados de acordo com a estrutura de sua subunidade. Famílias de canais para potássio podem ser agrupadas por ter dois segmentos transmembranares (2TM; Kir), 4TM (domínio de 2 poros), 6 TM (dependente de voltagem e SK), e 7TM (*Slo*). Note que pela simplificação, a família de canais de grande condutância *Slo* inclui os canais *Slo2.x*, que possuem apenas seis domínios transmembranares. A classe de domínio 6TM pode ser dividida em quatro famílias: voltagem-dependente Vk, voltagem-dependente tipo KCNQ (KCNQ), éter-a-go-go (Eag), e canais ativados por Ca^{2+} (SK). Subdivisões dos canais de voltagem-dependentes Kv em quatro subfamílias e Eag em três subfamílias também são nomeados de acordo com genes de *Drosophila melanogaster*. Na família SK, IKCa1 representa o canal para potássio ativado por cálcio de condutância intermediária. Adaptado de González et al, 2012.

O canal BK (Kcnma1, KCa1.1, *maxi*-K, *slo*1) é encontrado em vários tipos de células (Contreras et al., 2013), e sua atividade suprime a excitabilidade do potencial de ação e/ou liberação vesicular de moléculas sinalizadoras. As inúmeras expressões do canal BK o tornam um componente crucial da

membrana, permitindo um ajuste de desempenho celular ideal. Em humanos, os canais BK são amplamente expressos em diversos tecidos, incluindo os sistemas nervoso, muscular esquelético, endócrino, cardiovascular, digestivo, urinário e reprodutivo (Adams et al., 1982; Becker; Brenner; Atkinson, 1995; Contet et al., 2016; Cui; Yang; Lee, 2009; Fagerberg et al., 2014; Hirukawa et al., 2008; Latorre et al., 2017; McCobb et al., 1995). Antes da identificação de pacientes com canalopatia ligada a Kcnma1, alterações na expressão ou atividade do canal BK foram implicadas em estudos humanos de disfunção erétil, bexiga hiperativa, hipertensão e contração uterina prematura durante a gravidez (Christ et al., 2001, 2009; Grimm; Sansom, 2010; Li et al., 2014; Tomás et al., 2008; Yang et al., 2013). Contudo, apesar desses estudos sugerirem a função do canal BK em várias desordens, a ligação genética e a análise de tecidos humanos não demonstraram os papéis do canal BK *in vivo* (Bailey et al., 2019). Estudos em modelos animais forneceram uma avaliação mais completa dos vários papéis dos canais BK na fisiologia e fisiopatologia (Bailey et al., 2019). No entanto, embora os fenótipos neurológicos e musculares estejam bem representados, o estudo da função do canal BK em roedores avaliando outros sistemas e suas vias de regulação faz-se mais amplamente necessário.

De forma interessante, os canais BK são amplamente distribuídos no SNC e desempenham um papel essencial na regulação da atividade dos circuitos neurais do encéfalo, fornecendo um mecanismo de *feedback* negativo à excitabilidade da membrana celular frente aos aumentos locais nas concentrações de Ca^{2+} intracelular (Contet et al., 2016). Nos neurônios, os canais BK regulam o tempo e a duração do efluxo de K^+, de modo que podem aumentar ou diminuir a taxa de disparo neuronal, dependendo das condições celulares, podendo suprimir a liberação de neurotransmissores dos terminais pré-sinápticos. Além disso, os canais BK localizados em astrócitos e miócitos arteriais modulam o fluxo sanguíneo encefálico. Não surpreendentemente, tanto a perda quanto o ganho da função do canal BK têm sido associados a distúrbios do SNC, como epilepsia, ataxia, deficiência intelectual e dor crônica (Bailey et al., 2019, Vidal et al., 2019). Por outro lado, o papel neuroprotetor desempenhado pelos canais BK em várias situações patológicas poderia ser potencializado para corrigir a disfunção neurológica (Contet et al., 2016).

De forma interessante, a atividade do canal BK em neurônios respiratórios afeta a duração dos padrões de disparo de potenciais de ação que regulam a frequência respiratória em camundongos (Bissonnette, 2002). O canal BK também regula função do músculo liso nas vias aéreas. Nas células do músculo liso das vias aéreas, os canais BK são fundamentais para regular o tônus e a contratilidade muscular. Sua ativação produz correntes espontâneas transientes externas de potássio (STOCs), resultando em hiperpolarização da membrana, o que reduz o influxo de cálcio. Esse processo diminui os níveis intracelulares de cálcio e relaxa o músculo liso das vias aéreas, facilitando o fluxo de ar (Bolton e Imaizumi, 1996; Hull et al., 2013; Gao et al., 2023).

Os canais BK são expressos nos neurônios do LC, região noradrenérgica localizada na ponte com importante função de detecção de CO_2/pH e modulação do controle ventilatório (Gargaglioni; Hartzler; Putnam, 2010). Recentemente, nosso laboratório, juntamente com colaboradores, demonstrou que a acidose hipercápnica promove aumento do Ca^{2+} intracelular nos neurônios do LC, por meio da abertura de canais para Ca^{2+} do tipo L em neonatos com idade maiores que P10 (Imber et al., 2014). O influxo de Ca^{2+} causa aumento da taxa de disparo dos neurônios quimiossensíveis e, possivelmente, um aumento da liberação de noradrenalina. Contudo, em ratos recém-nascidos com mais de 10 dias, o aumento da taxa de disparo é menor quando comparado aos animais mais novos. Essa redução da sensibilidade ao CO_2/pH nestes neurônios em neonatos maiores que P10 parece ocorrer devido a ativação dos canais BK no LC, que aumentam sua densidade acordo com a idade do animal (Figura 5; Imber et al., 2014). De acordo com nossos dados, os canais BK dos neurônios do LC apresentam um papel limitador (*Braking pathway*) na resposta quimiossensível neuronal (Imber et al., 2014), sendo que a acidose hipercápnica ativa os canais BK que causam o efluxo de K^+ do neurônio, hiperpolarizando os neurônios do LC, diminuindo assim a resposta da taxa de disparo neuronal à acidose hipercápnica. Contudo, ainda não é conhecido como esses canais poderiam influenciar a reposta ventilatória à hipercapnia ou se a resposta é similar em machos e fêmeas.

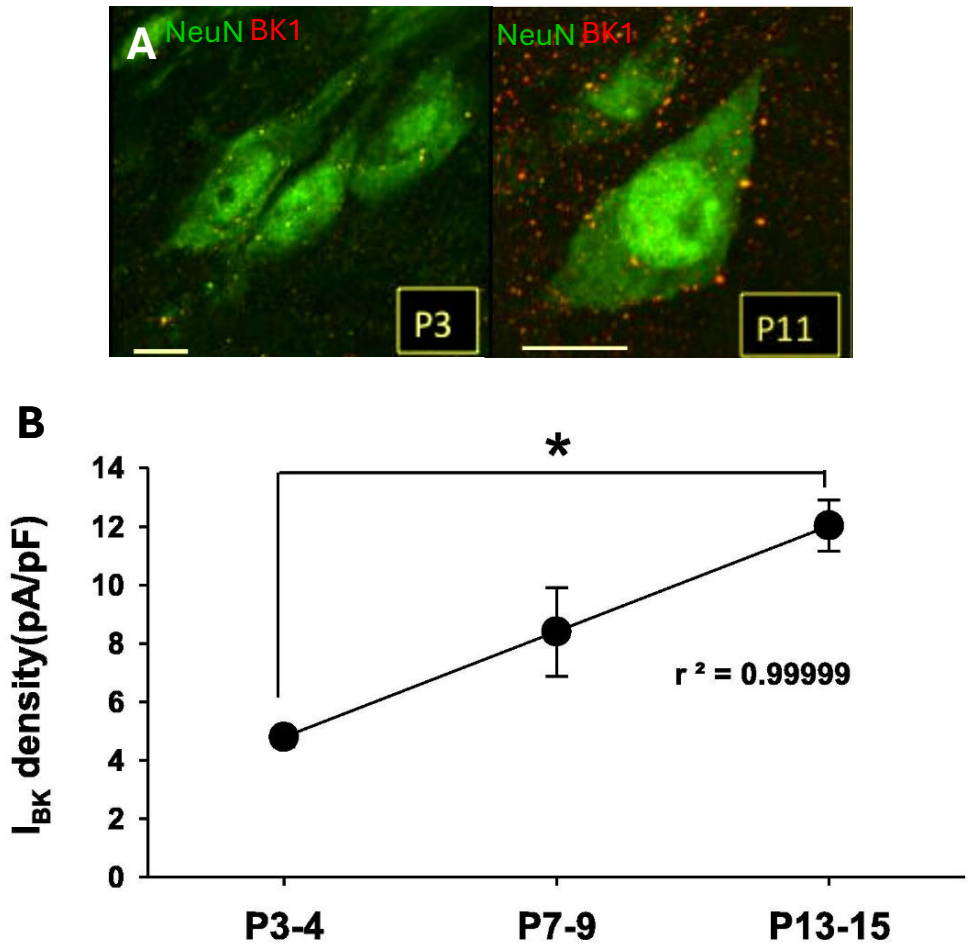

Figura 5. (A). Imuno-histoquímica para canais BK em neurônios do LC em ratos Sprague-Dawley P3 e P11. (Putnam *et al.,* dados não publicados) (B). Gráfico das densidades médias da corrente de pico Pax DI em três grupos etários: P3-P4 (n= 4), P7-P9 (n= 3) e P13-P15 (n= 3), conforme medido na pinça de tensão com um pré-pulso para +10 mV e um passo de tensão para +80 mV. A densidade de Pax DI (corrente BK) aumentou significativamente do grupo etário mais jovem para o mais velho. *P< 0.05. (Fonte: Imber et al., 2018).

O estudo de Patrone et al. (2014) demonstrou que a inibição dos canais BK resulta em alterações da resposta ventilatória à hipercapnia. Os autores mostraram que ao inibir os canais BK no LC, pela injeção bilateral de Paxilina no LC de ratos Wistar, foi observado um aumento na ventilação decorrente da hipercapnia.

Um ponto importante em relação aos canais BK é que podem ser modulados por hormônios sexuais. O estudo de Valverde et al. (2000) testou o

efeito da aplicação de estradiol em oócitos de *Xenopus* que expressavam os canais BK de humanos. Eles descobriram que o 17β-estradiol aumentou a atividade dos canais BK compostos pelas subunidades α e β, mas não teve efeito nos canais compostos apenas pelas subunidades α. Quando o estrogênio foi acoplado à albumina sérica bovina (impedindo o hormônio de atravessar a membrana plasmática), o canal BK α/β ainda apresentou uma atividade aumentada, demonstrando que o estrogênio atua extracelularmente. Esses experimentos indicam que a ligação direta do estradiol ocorre em um sítio externo nos canais BK, disponível apenas quando a subunidade β está presente, resultando em uma atividade aumentada do canal.

Para os estudos envolvendo especificamente os canais BK, foram produzidos camundongos com silenciamento gênico inibindo a expressão desses canais, os chamados animais *knockout* para canais BK ou BK^{KO}. Nesse processo, o gene *mSlo1* para a subunidade formadora do poros do canal BK é excluído (Meredith et al., 2004). Em um estudo conduzido por Meredith et al., (2004), camundongos $Slo^{-/-}$ foram gerados para investigar as funções do gene *Slo*1. Um fragmento genômico contendo o exon 1 foi clonado em um vetor e foram adicionadas sequências loxP para permitir a deleção do exon via Cre recombinase. Além disso, um gene de proteína fluorescente verde (EGFP) foi inserido em uma sequência intrônica do mSlo1 para fins de rastreamento.

Após a criação do vetor de direcionamento, ele foi transfectado em células-tronco embrionárias e os clones corretamente integrados foram identificados. Esses clones foram injetados em embriões de camundongos, gerando fundadores que transmitiram a mutação de forma estável. Para obter camundongos $Slo^{-/-}$ completos, os fundadores foram cruzados com uma linha que expressa a enzima Cre de forma ubíqua, resultando na deleção do exon 1. Posteriormente, esses camundongos foram retrocruzados por várias gerações para obter homozigotos, que foram confirmados por genotipagem via PCR.

Esse animal desenvolvido pela Dra. Andrea Meredith da *Stanford University* - EUA forneceu um modelo interessante de estudo que pode ser utilizado para avaliar a influência dos canais BK no controle de diversos sistemas fisiológicos *in vivo* e *in vitro* (Halm et al., 2017; Meredith et al., 2004; Sausbier et

al., 2004). O gene *Slo* de camundongos codifica o canal BK expresso nos neurônios, o qual é ativado (aberto) por aumento nas concentrações de Ca^{2+} intracelular. A abertura de canais BK permite o K^+ fluir passivamente para o meio externo através do canal, reduzindo o gradiente eletroquímico. Em condições fisiológicas normais, o efluxo de K^+ da célula resulta na hiperpolarização da membrana celular limitando o potencial da célula neural

Em vista do exposto, fica evidente o envolvimento dos canais BK na regulação da excitabilidade neuronal e na resposta ao CO_2/pH. Disfunções nesses canais podem contribuir para anormalidades na resposta respiratória potencialmente exacerbando ou precipitando fisiopatologias, uma delas, o transtorno do pânico (TP), por exemplo. Em pacientes, disfunções nessa resposta quimiossensível podem exacerbar sintomas, como falta de ar e taquicardia, que são comuns durante os ataques de pânico. Os canais BK desempenham um papel importante nessa modulação da resposta ao CO_2, e alterações em seu funcionamento podem estar ligadas à maior vulnerabilidade a episódios de pânico.

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CAPÍTULO 2 – ROLE OF BK CHANNEL IN THE RESPIRATORY AND METABOLIC RESPONSES TO CO_2/pH DURING DEVELOPMENT IN MALE AND FEMALE MICE

Abstract

Calcium-activated potassium (BK) channels are found in almost all mammalian tissues and play a crucial role in suppressing action potential excitability and the release of signaling molecules contained in vesicles. In the central nervous system (CNS), BK channels also provide a negative feedback mechanism to regulate the local increase in intracellular Ca^{2+} concentrations. In neurons, these channels regulate the timing and duration of Ca^{2+}, thus influencing the firing rate (F_R). However, the influence of these channels on the respiratory response to hypercapnic is not yet known. To this end, thirty-nine mice were used, 15 males and 24 females, divided into three different genotypes: knockout (BK^{KO}; n=13), intermediate (HET; n=16) and wildtype (WT; n=10). Each animal was consistently monitored throughout its development, from postnatal day (P)0 to adulthood. Ventilation (V_E), tidal volume (V_T) and respiratory rate (f_R), O_2 consumption (VO_2) and air convection requirement (V_E/VO_2) were recorded in the animal, which underwent a plethysmography test in closed whole-body pull-mode system to obtain these parameters. The respiratory and metabolic variables were compared among the groups by two-way ANOVA, comparing treatments (genotypes) and exposure condition (normocapnia or hypercapnia). BK^{KO} litters showed a higher mortality rate, both on the day of birth and during subsequent development and a lower body mass compared to WT and HET groups. No change in chewing and righting reflex was observed among groups. The data obtained showed that the absence of BK channels did not alter ventilatory and metabolic parameters during normocapnic conditions. CO_2 challenge increased V_E in all groups with no change in VO_2. BK^{KO} female mice presented an increased hypercapnic ventilatory response at P6-7, P12-13 and juvenile stage, and an increase in VO at adult stage. The only effect observed in males was an augmented V_T at P12-13 and a higher VO_2 at juvenile stage under CO_2 challenge. The results obtained suggest that BK channel play an important role in the ventilatory and metabolic responses elicited by CO_2 in females and may possibly have a sex and age dependency.

Keywords: channelopathy, development, chemoreception, ventilation

Introduction

The main function of breathing is to keep the partial pressures of oxygen (PaO_2) and carbon dioxide ($PaCO_2$) in the arterial blood, as well as the pH values, relatively consistent. This complex process involves a highly integrated network of interaction between the brain, spinal cord, cranial and spinal nerves, specific muscles and the lungs. In addition, it depends on structural and functional changes in neurotransmitter, neuromodulators, as well as ion channels and receptors (Anju et al., 2011; Wong-Riley et al., 2013; Del Negro et al., 2018).

Research suggests that ion channels, such as calcium channels, play a crucial role in regulating rhythmic oscillations in neuronal membrane potential. These oscillations are modulated through the dynamic opening and closing of these channels, which influence the membrane's resistance and contribute to neuronal excitability and signaling (Williams e Marshall, 1987; Oyamada et al., 1998). The large-conductance calcium- and voltage-activated potassium (BK) channel, also known as MaxiK, KCa1.1, or Slo1, is encoded by the *KCNMA1* gene and functions as a highly selective, high-conductance potassium channel and it is widely distributed in excitable cells of the central nervous system (CNS) (Adams et al., 1982; Becker; Brenner; Atkinson, 1995; Contet et al., 2016; Cui; Yang; Lee, 2009; Fagerberg et al., 2014; Hirukawa et al., 2008; Latorre et al., 2017; McCobb et al., 1995).

BK channel activity influences the shape, frequency, and propagation of action potentials and plays a pivotal role in synaptic function and neurotransmitter release (Contet et al., 2016; Latorre et al., 2017; Ancatén-González et al., 2023). BK channels function as feedback regulators of neuronal excitability. Upon activation by depolarization, they allow a large efflux of K+ ions, leading to cell membrane hyperpolarization and thereby reducing excitability (Echeverría et al., 2024). Interestingly, BK channel expression patterns change significantly during early development, suggesting that their role in regulating excitability may be developmentally dependent. In rodents, total BK mRNA levels increase several-fold from late embryonic stages to the first postnatal week (MacDonald et al., 2006).

It was also demonstrated by Valverde et al. (2000) that BK channels can be modulated by 17β-estradiol. Their study showed that this hormone increased the activity of BK channels formed by α (intracellular side) and β (extracellular side) subunits but had no effect on channels composed solely of α subunits. When estradiol was conjugated to bovine serum albumin, preventing it from crossing the plasma membrane, the α/β BK channel still exhibited increased activity. This finding indicates that estradiol acts extracellularly, binding directly to a site accessible only when the β subunit is present, thereby enhancing channel activity.

In the respiratory system, the involvement of the BK channel is particularly significant in the airways, where its ability to promote muscle relaxation is crucial for individuals with bronchial obstruction and remodeling. By facilitating airflow, the BK channel supports more efficient gas exchange in the lungs (Manzanares et al., 2011). BK channel activity in respiratory neurons influences the duration of action potential firing patterns that regulate respiratory rate in mice (Bissonnette, 2002). Under conditions of hypercapnia, the rhythmic oscillations in neuronal membrane potential may increase due to acidosis, which activates the calcium channels (Filosa and Putnam, 2003; Imber and Putnam, 2012), resulting in a reduction in membrane resistance and facilitating the depolarization process. Previous studies have shown that CO_2/pH chemosensitive neurons in the locus coeruleus (LC) express BK channels on their membrane (Sausbier et al., 2006; Williams et al., 1984), which play a significant role in ventilatory control in response to hypercapnia by acting as a brake on the chemoreflex response in the LC (Imber et al., 2018). In vitro and in vivo protocols have demonstrated that hypercapnic acidosis (7% CO_2) activates BK channels in the LC, and this activation limits neuronal firing rate due to hyperpolarization, leading to an attenuated ventilatory response to CO_2 (Imber et al., 2018). In this context, the braking pathway of BK channels serves as an important modulatory mechanism of respiratory neural network activity in response to increased CO_2.

The development of mice lacking BK channel expression (global knockout, BK^{KO}) has created a valuable model for investigating the influence of this K^+ channel type in whole-organism physiology (Meredith et al., 2004). Although BK^{KO} mice exhibit numerous deficits, these are generally not severe enough to

preclude survival in a reasonable proportion of individuals. $Slo^{-/-}$ mice, genetically modified to lack the *Slo* gene encoding the BK channel, provide unique insights into the physiological roles of BK channels. Therefore, based on the evidence provided of a possible protecting role of BK channels, and its modulation by sexual hormones, the aim of our study was to verify the participation of calcium-activated potassium channels in ventilatory and metabolic responses under normocapnic and hypercapnia conditions in wildtype, heterozygous and knockout mice male and female mice during postnatal development, searching whether they do in fact participate in, or modulate those responses to hypercapnic acidosis (HA), comparing the animals with or without the BK channel expression, and least, if there's any sex differences in their role in HA.

Methods

Animals

The study was carried out at the Department of animal Morphology and Physiology of the Faculty of Agricultural and Veterinary Sciences (FCAV) of São Paulo State University (UNESP), in Jaboticabal campus. The experiments were carried out with mice of the C57BL/6 strain, from colonies with different genetic profiles, from the Rodent Animal Facility of Department of animal Morphology and Physiology – FCAV/UNESP. The animals were housed in mini-isolator with clean wood shavings and environmental enrichment (tubes, paper, nets) in ventilated chambers at a controlled temperature of 25 ± 1°C. they were subjected to a 12/12h light/dark cycle (lights on at 6:30 a.m. and off at 6:30 p.m.). They had free access to food and water and were handled weekly.

All experiments were carried out from 8 a.m. to 6 p.m., with adult females undergoing experimental protocols in the morning due to hormonal fluctuations. All the protocols were approved by the local ethics committee (CEUA – protocol n°9486/22), in accordance with the National Council for the Control of Animal Experimentation (CONCEA).

The experiments were conducted using male and female C57BL/6 mice at postnatal ages P0-1, P6-7, P12-13, juveniles (P30) and adults (P80), of three different genetic profiles, being wildtype animals (WT), heterozygous (HET) and

knockout animals (BK^{KO}) for the Slo1 gene, the one responsible for encoding the pore formation of the BK channels. Eleven parental animals (6 females and 5 males) were donated by dr. Danh Halm from Wright State University, Ohio (US), and the breeding were carried out in our animal facility to establish the colony prior to the start of the experiments.

After the litters were born, biological material was collected to obtain genetic mapping and animal identification by excising one of the digits in the P0. In the litters destined to experimental protocols, this sample collection was made at the end of the protocol.

Genotyping

The genotype of the animal was determined by PCR using DNA extracted from the tissue of the animals' tails and/or phalanges, previously collected for identification on the day of birth. The biological material was subjected to the DNA extraction process using a solution of lysis buffer (a solution composed of Tris, EDTA, "Sodium Dodecyl Sulfate" and NaCl) and proteinase K, incubated in this solution for approximately 12 hours in a water bath, and then subjected to a sequence of cycles in a refrigerated centrifuge to obtain the final DNA strands. The primers used for the $Slo^{-/-}$ mouse were, for the KO band: Neo 5' – 5' ATA GCC TGA AGA ACG AGA TCA GC 3', e RA 14025 3' – 5' CCT CAA GAA GGG GAC TCT AAA 3', and for the WT band: Exon1 5'-3 – 5' TTC ATC ATC TTG CTC TGG CGG ACG 3', e WT 3'-2 – 5' CCA TAG TCA CCA ATA GCC C 3'. The thermal cycle amplification consists of 5 cycles of 2 minutes at 94°C, 30 seconds at 94°C, and 30 cycles of 30 seconds at 55°C-50°C, 2 minutes at 68°C, 30 seconds at 94°C, and last 30 seconds 50°C, 2 minutes at 68°C, e 5 minutes at 72°C, and maintained at 4°C. The PCR products were analyzed on a 2% agarose gel.

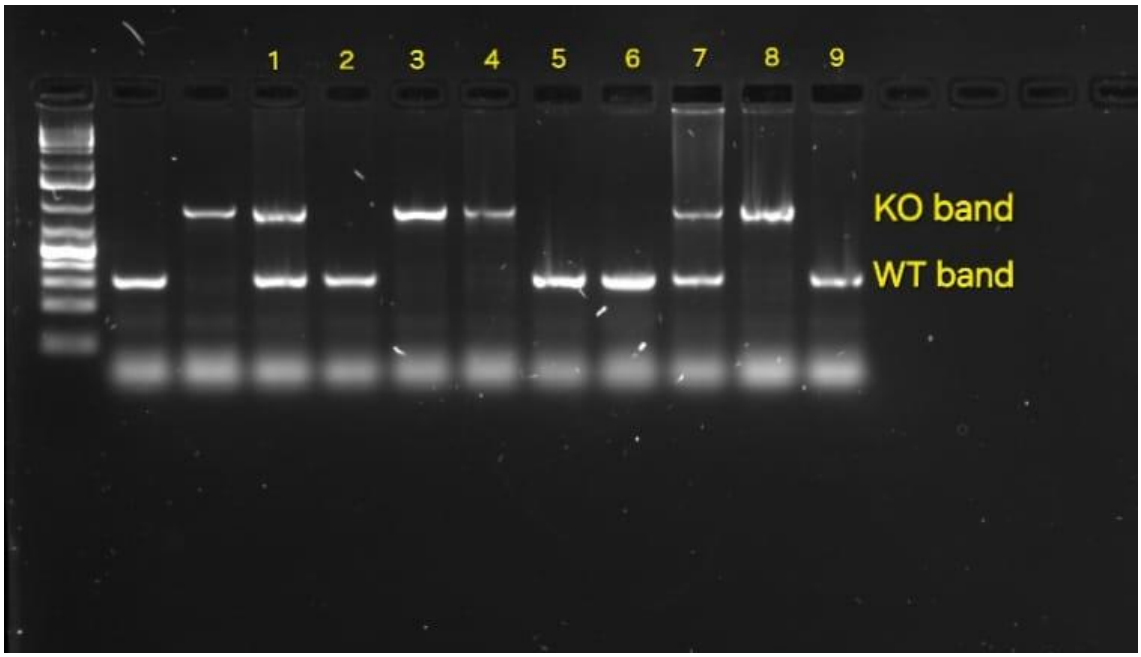

Figure 1. Photo of agarose gel electrophoresis for genetic identification of the animals, showing the WT and BK^{KO} bands, corresponding to the molecular weight of 1 kb.

Chewing and righting reflexes

Chewing and righting reflexes were assessed in neonates (P0) four hours after birth to evaluate perinatal motor control. To measure the chewing reflex, a polyethylene tube (PE-50) was gently inserted into the newborn's mouth for 30 seconds, during which the number of jaw openings was recorded. For the righting reflex assessment, each pup was placed on its back and observed until it naturally assumed an upright position with all four paws on the table. To ensure consistency and comparability of the data, all reflex analyses were conducted by the same researcher throughout the study.

Determination of ventilation in neonates

Pulmonary ventilation ($\dot{V}_E$) of the animals at ages P0-1, P6-7 and P12-13 was determined using the nest plethysmography method for neonates (Bícego e Mortola, 2017). In order to record ventilation using the closed-system plethysmography (Bartlett e Tenney, 1970), the neonate was kept inside the

chamber in a heated water bath, called a nest, ate the ideal temperature for each age (P0-1 at 37ºC; P6-7 at 35ºC; P12-13 at 32ºC). The oscillations in volume generated by the animal's breathing were detected by another chamber outside the nest (reference chamber) at a lower temperature and without humidity (P0-1 at 27ºC; P6-7 at 25ºC; P12-13 at 22ºC), thus generating an oscillation in the pressure inside the chamber, making it possible to acquire the signal from the pressure transducer which was connected to the reference chamber. A temperature sensor was placed in the animal's chamber, as well as in the reference chamber for the ambient temperature. The pressure oscillation due to respiration was monitored by a pressure differential transducer (TSD 160A, Biopac Systems, Santa Barbara, CA - EUA), whose signal was sent to an amplifier amplificador (DA 100C, Biopac Systems), passing through an analog-digital converter, and digitalized on a computer equipped with data acquisition software (AcqKnowledge MP 100, Biopac Sytems, Inc., Santa Barbara, CA - EUA). Air flow was kept constant throughout the experiment (40 $mL.min^{-1}$ for P0-1, 70 $mL.min^{-1}$ for P6-7 and 85 $mL.min^{-1}$ for P12-13) using an SS-4 pump (Sable Systems Internatinal, USA) coupled to a gas analyzer (ADInstruments®, Sidnei, Austrália). Two respiratory variables were measured: respiratory rate (f_R) and V_T. The $\dot{V}_E$ was calculated by the product of f_R and V_T. The calibration volume (0.1 mL of air) was measured for each experiment using a graduated syringe connected to the animal's body chamber, making it possible to calibrate the pressure signal in volts to V_T in mL. The hypercapnic stimulus was based on gas mixtures purchased from White Martins Gases Industriais Ltda (Osasco, SP) of 7% CO_2, 21% O_2 in N_2 balance.

Determination of ventilation in juveniles (P30) and adults (P80)

The $\dot{V}_E$ in juvenile animals (P30) and adults (P80) was measured by whole-body plethysmography in a closed system (Bartlett e Tenney, 1970; Biancardi et al., 2008; De Carvalho et al., 2017). The experimental chamber had a volume of 900mL for juveniles, and 5L for the adults, and underwent a habituation period of at least 40 min. During the measurements of $\dot{V}_E$, taken at minutes 10 and 20 after the start of each gas condition, the air flow was interrupted, and the animal chamber remained sealed for around 2 min. The

incoming air flow was approximately 700 mL.min^{-1} for the juvenile animals and 1.8L.min^{-1} for the adults, measured by a digital flow meter, and the outgoing flow determined by the pump attached to the gas analyzer (ADInstruments®, Sidnei, Austrália). Signals from a pressure differential transducer (TSD 160ª, Biopac Systems, Santa Barbara, CA – EUA), connected to the animal chamber, were collected by an amplifier (DA 100C, Biopac Systems), passed through and analog-digital converter, digitalized on a computer equipped with an acquisition from a datalogger (SubCue Dataloggers, Calgary, Canada), and body temperature was measured by a sensor attached to the analyzer (ADInstruments®, Sidnei, Austrália), rectally. The f_R and V_T, were measured, the latter being calculated using a formula (Biancardi et al., 2008; De Carvalho et al., 2017; Patrone et al., 2023).

$$\mathbf{V_T = VK \times (PT/PK) \times TC \times (PB - PC) / TC \times (PB - PC) – TA \times (PB – PR)}$$

V_T: Volume of flowing air.

VK: Volume of air injected into the animal's chamber for calibration.

PT: Pressure deflection associated with each volume of flowing air.

PK: Pressure deflection associated with the volume injected for calibration.

TC: Body temperature (in Kelvin).

TA: Air temperature inside the animal's chamber.

PR: Vapor pressure of water at TC.

PB: Barometric pressure.

PC: Vapor pressure of the water vapor in the animal's chamber.

The calibration volume (0.2 mL of air) was measured for each experiment using a graduated syringe connected to the animal's chamber, making it possible to calibrate the pressure signal in volts to V_T in mL. Rectal temperature was measured as a representative body temperature (Tc) using a sensor (Thermistor Pod ML 309, ADInstrumensts®, Austrália) inserted into the animal's colon.

Determination of metabolic rate

The indirect calorimetry method (oxygen consumption, $\dot{V}O_2$) using de Pull-mode configuration was used to determine metabolism using the open respirometry system (Mortola, 1984; Patrone et al., 2023). A pump (SS-4 Sub-Sampler Flow Meter and Pump), connected to the O_2 analyzer allowing $\dot{V}O_2$ to be determined by the data acquisition program (Power-Lab System, ADInstruments® Sidnei, Austrália). The $\dot{V}O_2$ was calculated using the appropriate formula (Patrone et al., 2023):

$$\mathbf{VO_2 = FI \times (F'iO_2 - F'eO_2) / 1 - F'eO_2 (1-RQ)}$$

FI: air flow rate into the chamber.
F'iO_2: fraction of O_2 inspired.
F'eO_2: fraction of O_2 expired.
RQ: value referring to the energy source used by the animal.

Experimental protocols

Protocol 1: Involvement of BK channels in ventilatory and metabolic control Against hypercapnia in mice during development: Animals aged P0 to P13 were placed in whole-body chambers which were initially ventilated with atmospheric air (21% O_2) for an acclimatization period of at least 10 min. Measurements of V_E and VO_2 were taken during all the experiments with the control and transgenic animals. First, two measurements of V_E during normocapnia (at minutes 10 and 20 after the start). At the end of the second measurement, the injection of 0,1 mL was made for the calibration. After a 5-minute break, the animals were subjected to hypercapnia for 20 minutes (V_E measurements at 10 and 20 minutes after the start) where the chamber was ventilated with a gas mixture containing 7% CO_2, 21% O_2 and balanced with N_2. Tc was continuously measured using the temperature sensor inserted into the animal's chamber and the reference chamber, and VO_2 was also continuously measured. On the first day of the experiment, P0-1, a straightening and chewing test was conducted. For the former, the neonate was placed in the dorsal decubitus position and the

measurement consisted of the time in seconds it took for the animal to turn completely to the ventral decubitus position. In the second, the animal was restrained and then a PE-10 polyethylene tube (Clay Adams, Parsippany, NJ) was introduced, and the measurement consisted of how many chews the animal reproduced during the 30-second period. These tests were carried out before the experiment to expose the animals to normocapnia and hypercapnia, in order to avoid any behavioral changes, and after the protocol, the phalanges were weighed and collected for identification and also to obtain a sample for genotyping.

Protocol 2: Assessment of ventilation and metabolism during normocapnia and hypercapnia in juvenile and adults, male and female control and knockout mice for BK channels: In the juvenile animals, the animals were previously placed in a whole-body plethysmography chamber, of 900 mL for the juveniles and 5L for the adults and underwent a period of habituation of around 40 min to one hour in room air. After this, two measurements of V_E were taken during normocapnia (at minutes 10 and 20). After this procedure, the chamber was ventilated with a mixture of 7% CO_2 and V_E measurements were also made at minutes 10 and 20. The chamber temperature was measured continuously, and rectal Tc measurements were taken on the animal after the V_E measurements.

Data analysis

The data was evaluated to ensure that the assumptions of variance and normality of the studied errors were met. They were then submitted to analysis of variance using the GLM (General Linear Models) procedure in the SAS® program and, in the event of significant difference, the means were compared using the Tukey test with a 5% probability level and a 95% confidence interval. In the event of significant violations of the homoscedasticity prerequisite, the Kruskal-Wallis non-parametric analysis was used, followed by the Mann-Whitney test in the event of statistical significance. Body mass data were analyzed using the Kruskal-Wallis test followed by Dunn's post hoc test. The level of statistical significance used was 95% ($p<0.05$). For all the other analysis, a two-way ANOVA test was carried out with the factors being treatment (genotype) and gas exposure (normocapnia and hypercapnia). The respiratory variables measured were V_T, fR

and V_E. The f_R was quantified by analyzing the number of respiratory events per minute. Ventilation was obtained by multiplying these two variables = V_T x f_R, and corrected for body mass. The values obtained for V_E and VO_2 at the same age were analyzed as measurements over time and the data was analyzed using two-way ANOVA to compare the treatments over time with the two factors, with Tukey's post-test.

Results

Mortality rate

Table 1 shows the mortality rates of transgenic Slo1 mice. Among the litters bred for these study's protocols, as well as those used to maintain the colony, we observed a higher mortality rate in the BK^{KO} animals, both on the day of birth and during subsequent development.

In addition to the significant mortality of the knockout animals, the litters of this strain also showed a higher proportion of females compared to males in most litters.

Table 1. Mortality rate of male and female animals, comparing wildtype (WT), heterozygous (HET) and knockout (BK^{KO}) genotypes.

	Mortality rate					
	MALE			**FEMALE**		
	WT	HET	BK^{KO}	WT	HET	BK^{KO}
Born	5	8	3	5	9	7
Dead	1	0	1	0	0	4
Rate (%)	20%	0	33%	0	0	57%

Body mass gain

For ventilatory and metabolic measurements, animals were weighed at each experimental session in Protocols 1 and 2, tracking the development of each individual over time. Over the course of the experiments, BK^{KO} mice possess a lower body mass compared to WT group, with statistic difference at P30 ($p=0.002$), even though they were kept under the same conditions in the animal housing, accompanied by their mother during the neonatal period (Table 2).

Table 2. Body mass (g) of wildtype (WT) heterozygous (HET) and knockout (BK^{KO}) animals, measured throughout development at ages P0-1, P6-7, P12-13, P30 and P80.

Values ae presented as mean ± standard deviation. Different letters indicate difference among groups.

Age	Genotype		
	WT	HET	BK^{KO}
P0-1	1.4 ±0.1	1.4 ± 0.2	1.3 ± 0.2
P6-7	3.1 ±0.3	3.1 ± 0.5	2.7 ± 1.1
P12-13	5.5 ±0.9	5.3 ± 1.1	4.2 ±0.3
P30	14.2 ± 2.7[a]	13.0 ± 2.3[ab]	8.7 ± 1.4[b]
P80	21.7 ±2.7	21 ± 2.3	19.8 ± 2.2

Motor reflexes

Figures 2 and 3 show the righting and chewing motor reflexes, respectively. No difference was observed among the groups.

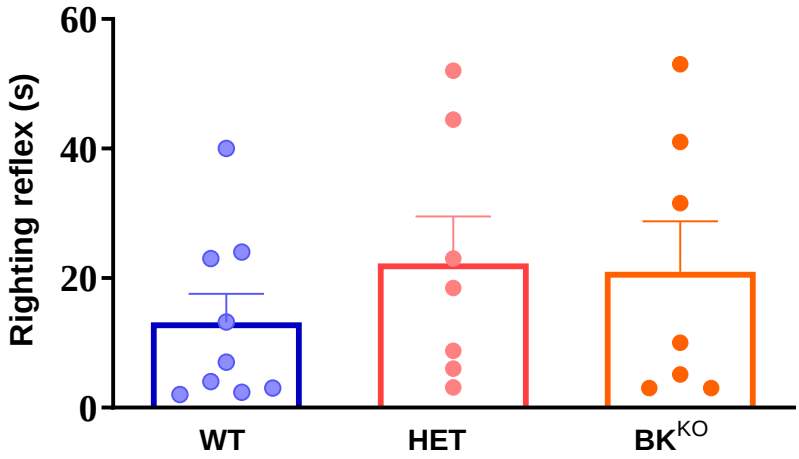

Figure 2. Measurements of the righting reflex in seconds, taken at age P0-1, with animals submitted to Protocol 1, WT (n=9), HET (n=7) and BK^{KO} (n=7). Values are expressed as mean ± SEM. Graphs are presented as bars, together with individual values.

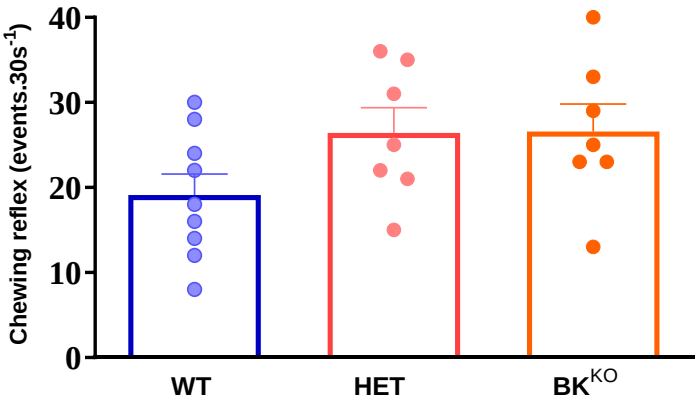

Figure 3. Measurements of the chewing reflex at events.30^{-1}, taken at age P0-1, with animals submitted to Protocol 1, WT (n=9), HET (n=7) and BK^{KO} (n=7). Values are expressed as mean ± SEM. Graphs are presented as bars, together with individual values.

Evaluation of ventilation and metabolism during normocapnia and hypercapnia in neonatal (P0-1, P6-7 and P12-13) wildtype, heterozygous and knockout mice for BK channels

Experiments were carried out with 39 animals, 15males and 24 females, in the three different genotypes: wildtype (WT), heterozygous (HET) and knockout (BK^{KO}), which were subjected to the aforementioned experimental protocols throughout their development.

Figures 4 to 13 show the ventilation values ($\dot{V}_E$), tidal volume (V_T), respiratory rate (f_R), oxygen consumption ($\dot{V}O_2$) and air convection requirement ($\dot{V}_E / \dot{V}O_2$) of WT, HET and BK^{KO} animals at ages P0-1, P6-7, P12-13, P30 and P80, respectively, and categorized by sex, comprising a total of 19 females and 13 males, distributed across various genotype groups. Measurements were taken under both room air conditions and hypercapnia (7% CO_2). The two measurements obtained during hypercapnia were plotted using box plots, which display mean values alongside individual data points.

Figure 4 shows $\dot{V}_E$, f_R, V_T, $\dot{V}O_2$ E $\dot{V}_E / \dot{V}O_2$ in females from the three groups, comparing gas conditions and genotypes at age P0. During room air conditions, no difference was observed among the groups for any of the ventilatory or metabolic variables. Exposure to CO_2 caused an increase in $\dot{V}_E$ due to an increase in both V_T and f_R. The increase in $\dot{V}_E$ during hypercapnia was greater in the HET group compared to the BK^{KO} group (p=0.044) at 10 min of 7% CO_2, possibly due to a higher V_T, in HET group during hypercapnia, comparing to BK^{KO} group. Neither CO_2 exposure nor the genotype promote changes in $\dot{V}O_2$. CO_2 exposure caused an increase in $\dot{V}_E / \dot{V}O_2$ with no difference among groups.

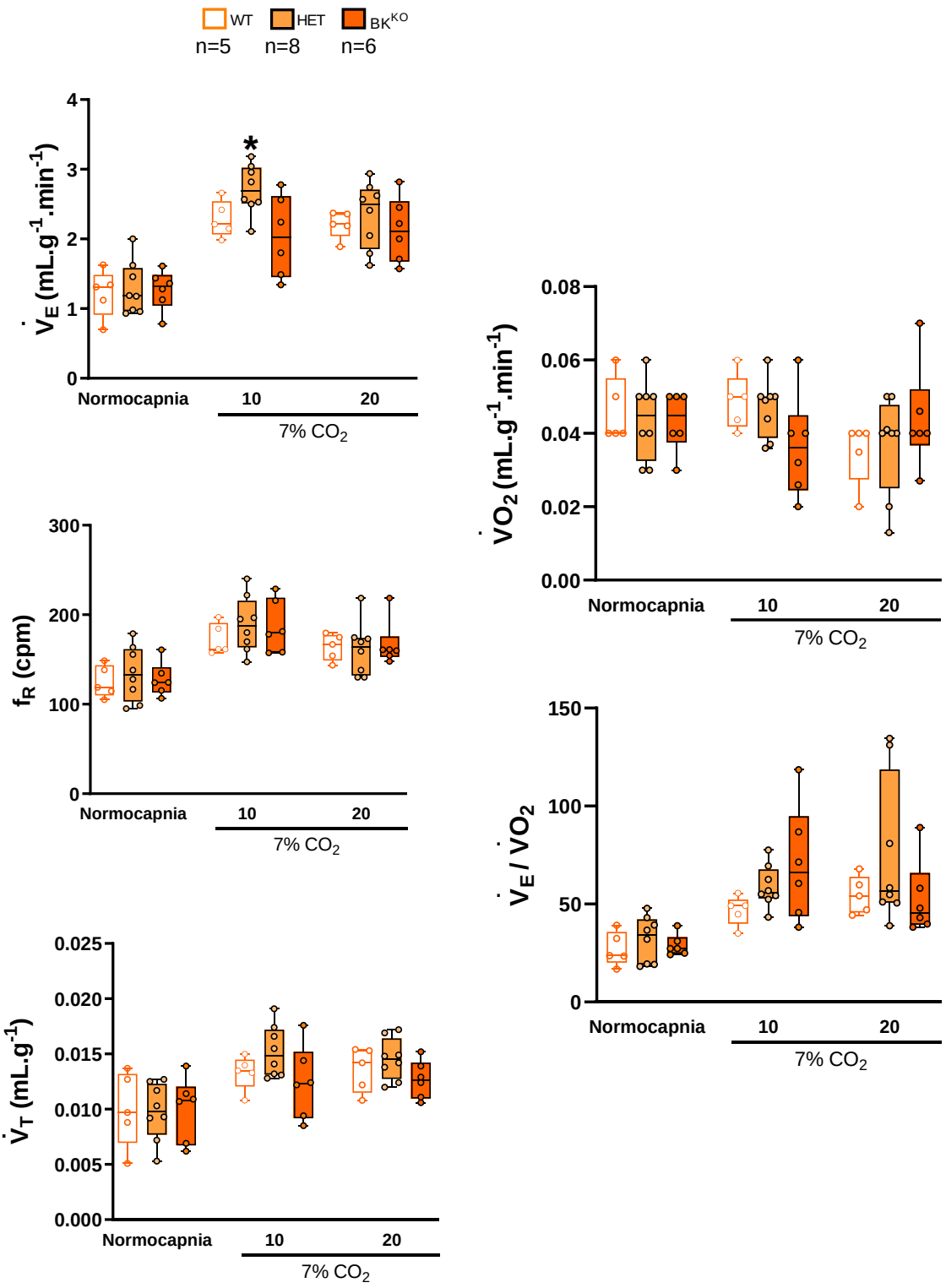

Figure 4. Ventilation ($\dot{V}_E$), tidal volume (V_T), respiratory rate (f_R), oxygen consumption ($\dot{V}O_2$) and air convection requirement ($\dot{V}_E / \dot{V}O_2$) of WT (n=5), HET (n=8) and BK^{KO} (n=6) P0-1 females under room air condition and under hypercapnic exposure (7% CO_2). Values are expressed as median with individual data points. *Indicates difference from BK^{KO} group.

Regarding P0-1 males, Figure 5 shows the respiratory and metabolic variables of WT, HET and BK^{KO} mice. Under room air conditions, no significant differences were observed among the groups for any of the ventilatory or metabolic variables. Hypercapnia caused an increase in all ventilatory parameters and also in $\dot{V}_E / \dot{V}O_2$, with no change in $\dot{V}O_2$. There were no statistical differences in the ventilatory and metabolic responses to CO_2 among the groups.

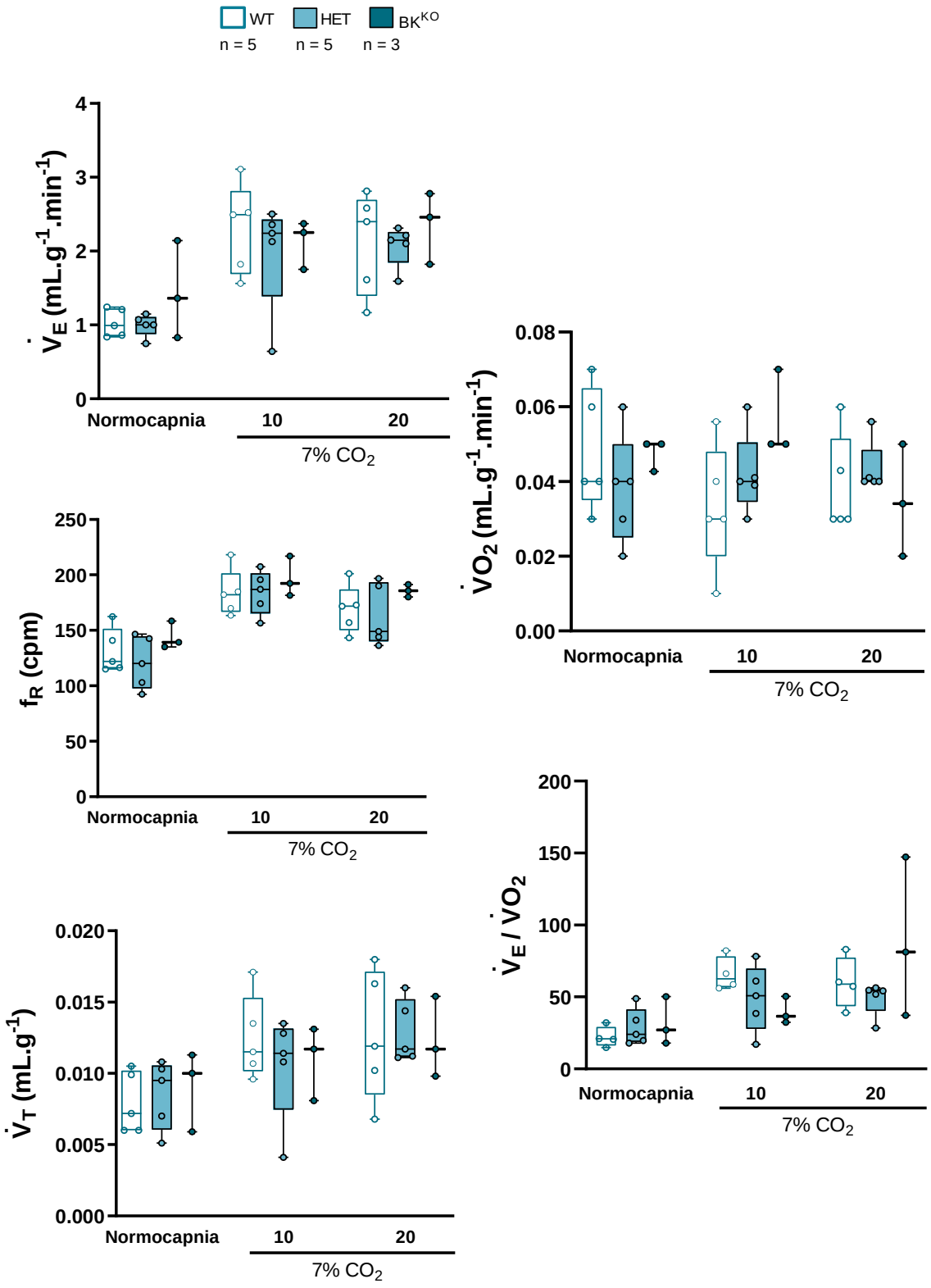

Figure 5. Ventilation ($\dot{V}_E$), tidal volume (V_T), respiratory rate (f_R), oxygen consumption ($\dot{V}O_2$) and air convection requirement ($\dot{V}_E / \dot{V}O_2$) of WT (n=5), HET (n=5) and BK^{KO} (n=3) P0-1 males under room air condition and hypercapnia (7% CO_2). Values are expressed as median with individual data points.

Figure 6 shows the results of the respiratory and metabolic variables of P6-7 females. Under room air conditions, no significant differences were observed among the groups for any of the ventilatory or metabolic variables. Hypercapnia increased $\dot{V}_E$, f_R, V_T and $\dot{V}_E / \dot{V}O_2$ compared to room air exposure. In addition, BK^{KO} group had a greater increase in $\dot{V}_E$ compared to WT (p=0.0154) and HET mice (p=0.0003) in the first measurement of hypercapnic exposure, with no statistical difference in other variables.

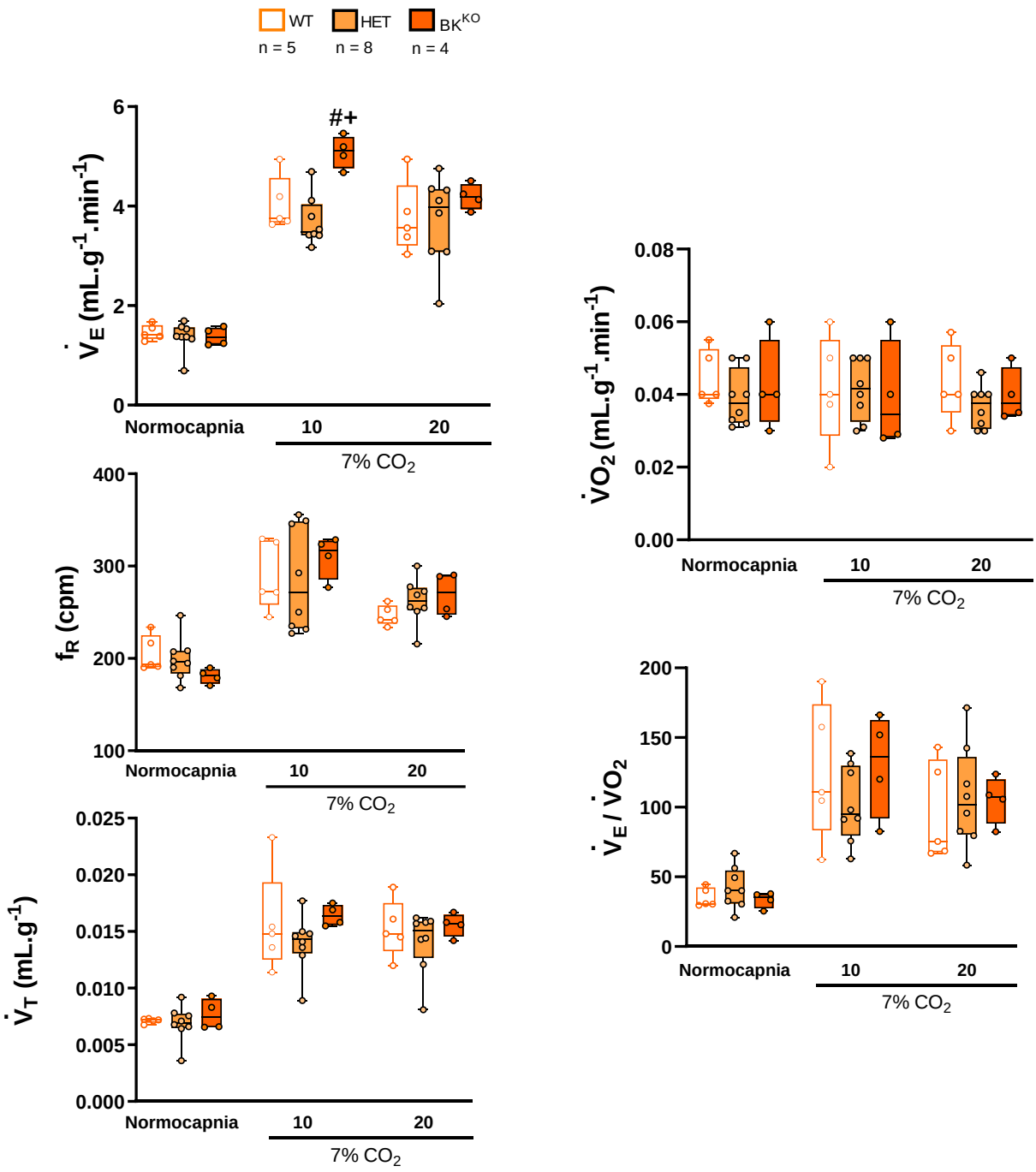

Figure 6. Ventilation ($\dot{V}_E$), tidal volume (V_T), respiratory rate (f_R), oxygen consumption ($\dot{V}O_2$) and air convection requirement ($\dot{V}_E / \dot{V}O_2$) of WT (n=5), HET (n=8) and BK^{KO} (n=4) P6-7 females under room air condition and under hypercapnic exposure (7% CO_2). Values are expressed as median with individual data points. # Indicates difference from WT group; + Indicates difference from HET group.

The results from experiments conducted on males aged P6-7 are presented in Figure 7. Under room air conditions, no difference was observed among groups. Additionally, exposure to hypercapnia resulted in a similar increase in all ventilatory and metabolic parameters across all three groups.

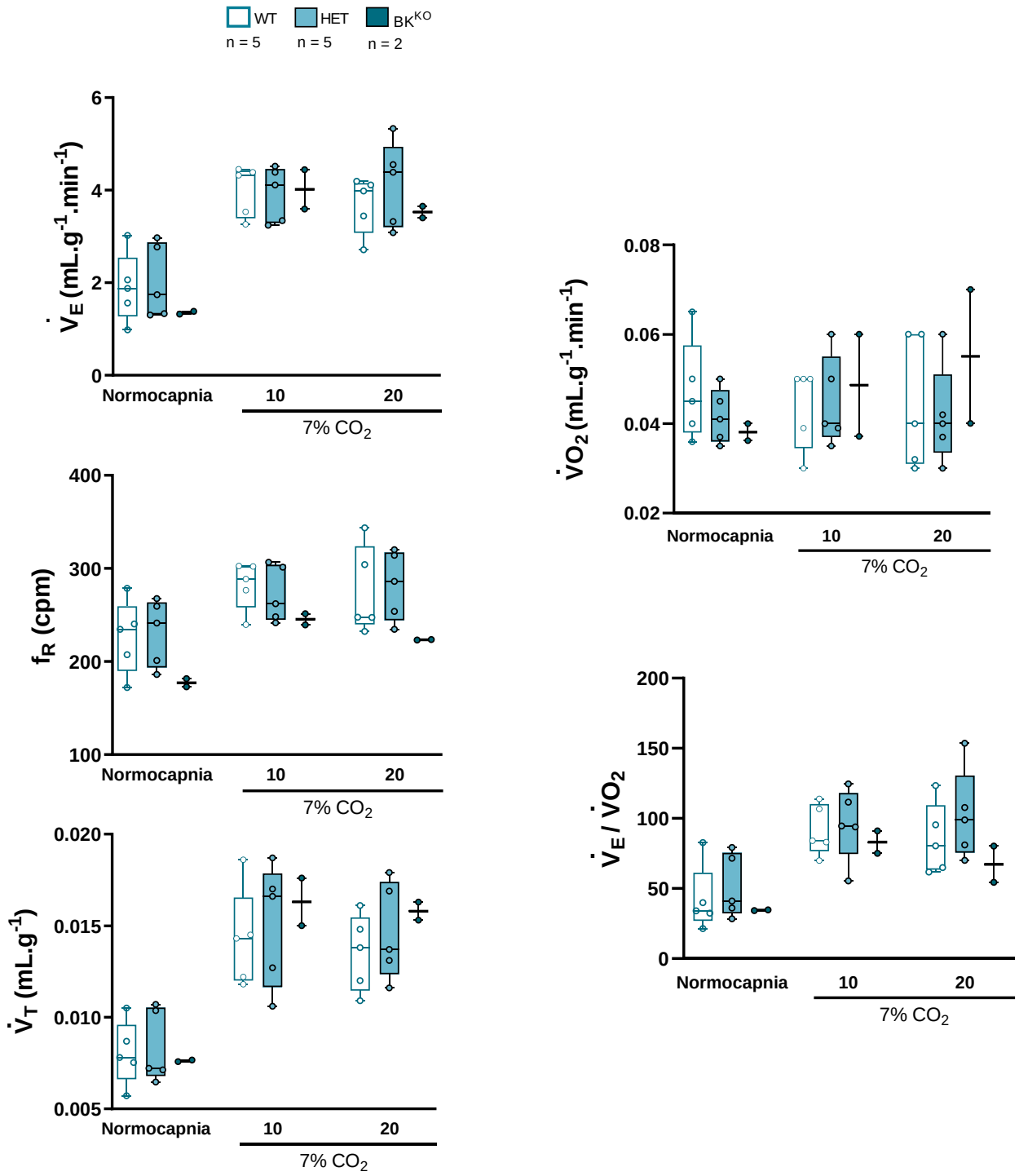

Figure 7. Ventilation ($\dot{V}_E$), tidal volume (V_T), respiratory rate (f_R), oxygen consumption ($\dot{V}O_2$) and air convection requirement ($\dot{V}_E / \dot{V}O_2$) of WT (n=5), HET (n=5) and BK^{KO} (n=2) P6-7 males under room air condition and under hypercapnic exposure (7% CO_2). Values are expressed as median with individual data points.

The results of ventilatory and metabolic measurements during exposure to room air and hypercapnia observed in the females at age P12-13 are shown in Figure 8. During normocapnia, there were no differences among the groups. During hypercapnic exposure, there was an increase in all ventilatory parameters and in the $\dot{V}_E / \dot{V}O_2$ of the three groups.

BK^{KO} females showed a greater ventilatory response to CO_2 compared to the HET group ($p=0.02$), and showed greater V_T compared to the WT group ($p=0.037$).

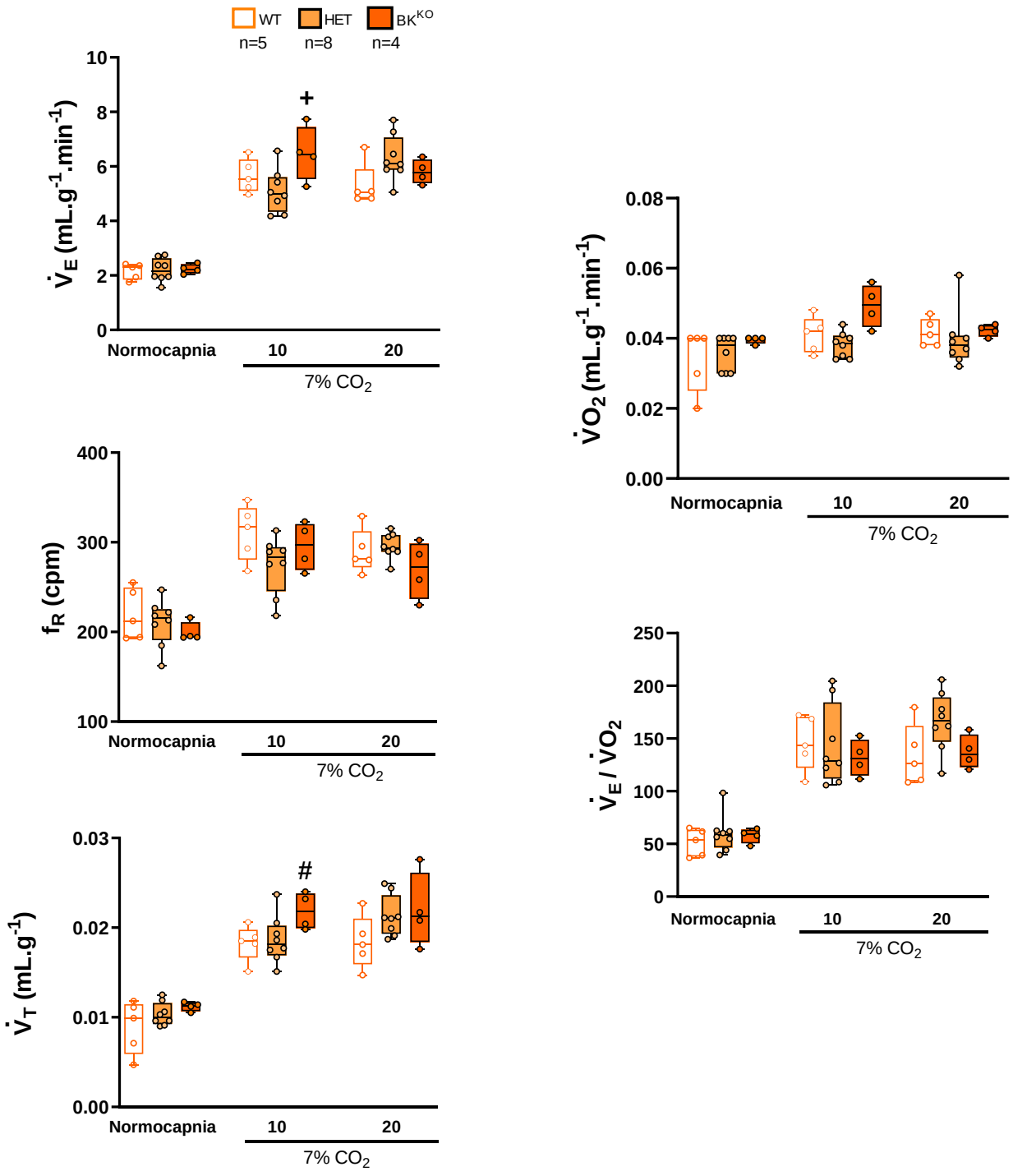

Figure 8. Ventilation ($\dot{V}_E$), tidal volume (V_T), respiratory rate (f_R), oxygen consumption ($\dot{V}O_2$) and air convection requirement ($\dot{V}_E$ / $\dot{V}O_2$) of WT (n=5), HET (n=8) and BK^{KO} (n=4) P12-13 females under room air condition and under hypercapnic exposure (7% CO_2). Values are expressed as median with individual data points. # Indicates difference from WT group; + Indicates difference from HET group.

Figure 9 shows the results of the variables measured for males at ages P12-13. During normocapnia, there was no difference among groups. Under hypercapnia, there was an increase in all the ventilatory parameters and $\dot{V}_E / \dot{V}O_2$ in the three groups compared to normocapnia. BK^{KO} males presented a higher $\dot{V}_E$ and V_T compared to WT mice at 10 min of CO_2 exposure ($p < 0.05$).

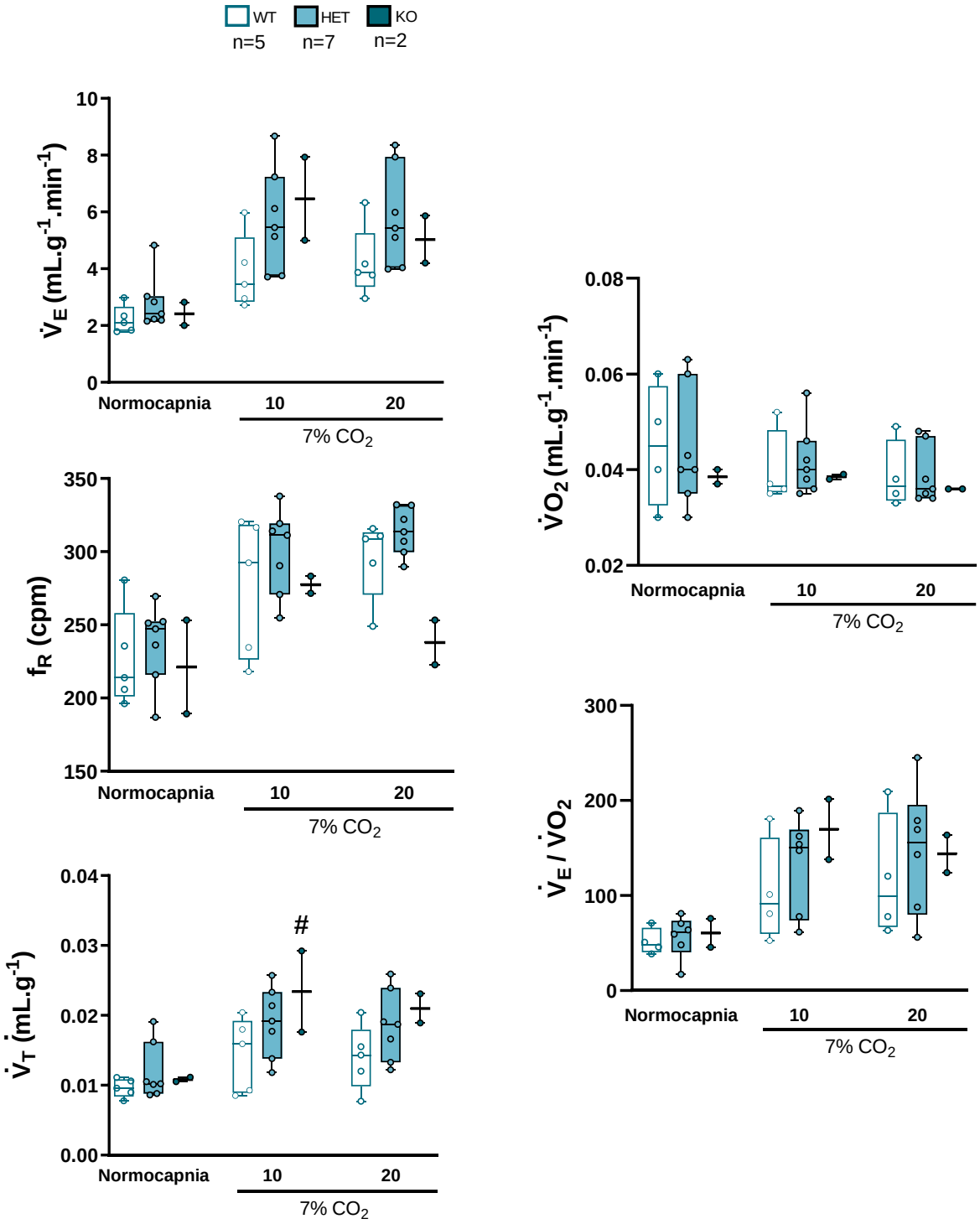

Figure 9. Ventilation ($\dot{V}_E$), tidal volume (V_T), respiratory rate (f_R), oxygen consumption ($\dot{V}O_2$) and air convection requirement ($\dot{V}_E / \dot{V}O_2$) of WT (n=5), HET (n=5) and BK^{KO} (n=1) P12-13 males under room air condition and under hypercapnic exposure (7% CO_2). Values are expressed as median with individual data points. # Indicates difference from WT group.

Evaluation of ventilation and metabolism during normocapnia and hypercapnia in juvenile (P30) and adult (P80) wildtype, heterozygous and knockout mice for BK channels

Figures 10 to 13 present the ventilatory and metabolic responses of juvenile and adult female and male mice under normocapnic and hypercapnic conditions, respectively.

Figure 10 illustrates the ventilatory and metabolic responses in juvenile females during these conditions. During normocapnia, no significant differences were observed in ventilatory and metabolic parameters between the groups. However, under hypercapnic exposure, all groups demonstrated an increase in ventilatory parameters and $\dot{V}_E / \dot{V}O_2$. Specifically, in juvenile BK^{KO} females, there was a greater increase in V_E ($p=0.027$) and V_T ($p=0.014$) compared to the WT group during hypercapnia (Figure 10).

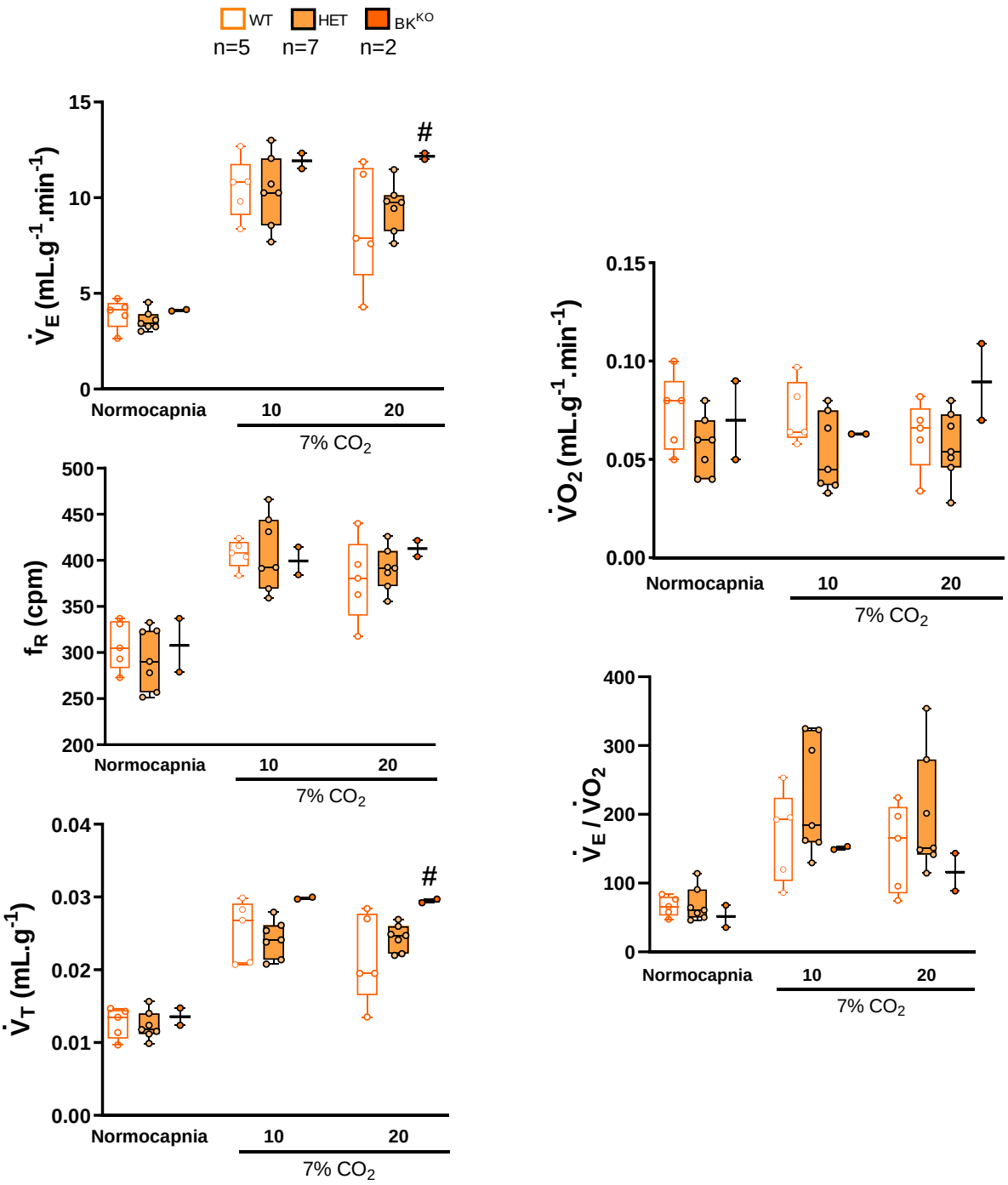

Figure 10. Ventilation ($\dot{V}_E$), tidal volume (V_T), respiratory rate (f_R), oxygen consumption ($\dot{V}O_2$) and air convection requirement ($\dot{V}_E / \dot{V}O_2$) of WT (n=5), HET (n=7) and BK^{KO} (n=2) juvenile females under room air condition and under hypercapnic exposure (7% CO_2). Values are expressed as median with individual data points. # Indicates difference from WT group.

Figures 11 show the ventilatory and metabolic responses of juvenile males during normocapnia and hypercapnia. During normocapnia, a lower f_R was observed in BK^{KO} males compared to HET mice ($p = 0.019$).

Male BK^{KO} animals showed an elevated metabolic rate at 10 and 20 min of CO_2 exposure compared to WT and HET mice [10 minutes: BK^{KO} vs WT ($p=0.002$); BK^{KO} vs. HET ($p=0.008$), 20 minutes: BK^{KO} vs WT ($p=0.008$), and BK^{KO} vs HET ($p=0.026$)]. Consequently, the air convection requirement was lower in the BK^{KO} group compared to the HET group (HET vs. BK^{KO}, $p=0.034$) (Figure 11).

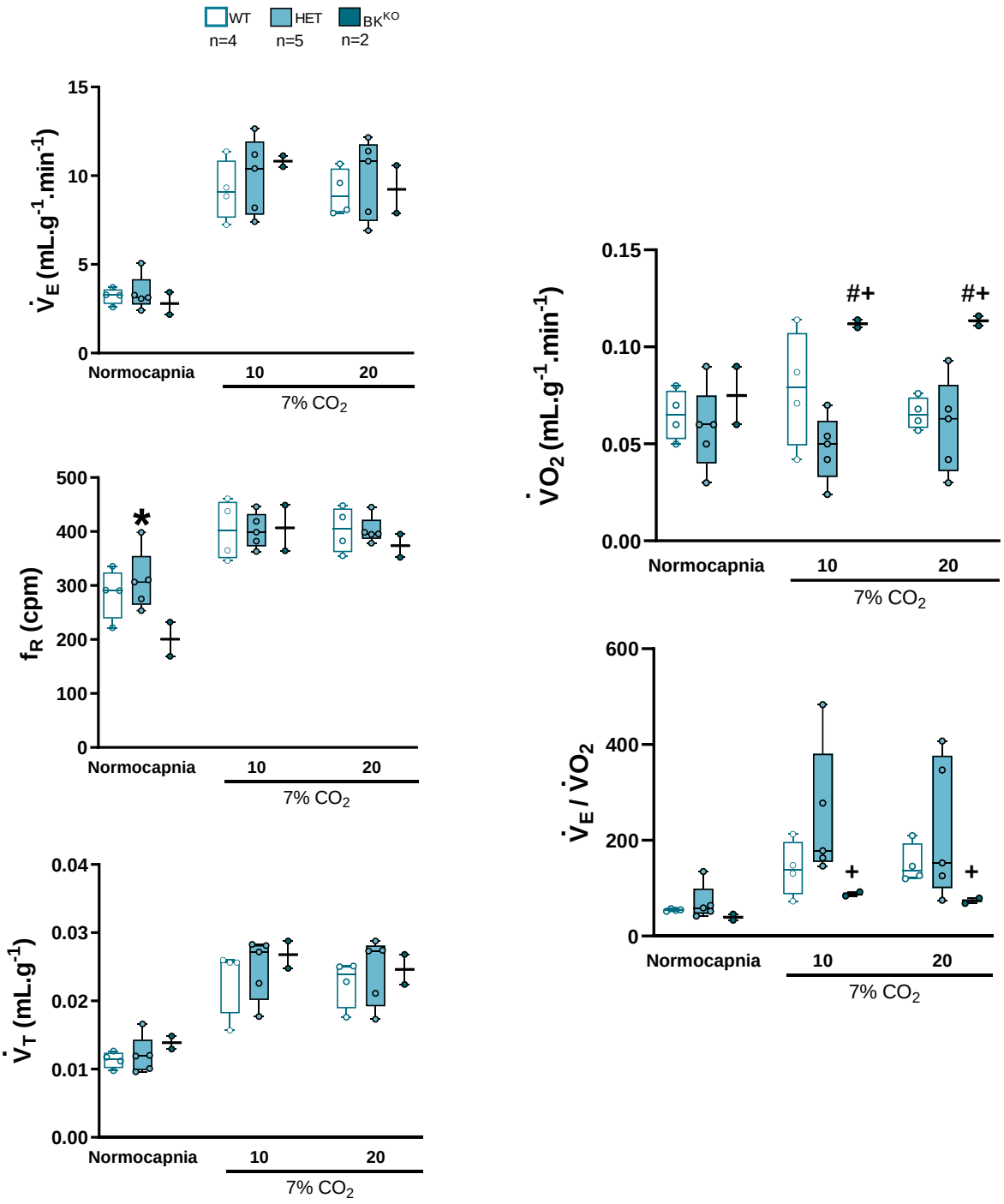

Figure 11. Ventilation ($\dot{V}_E$), tidal volume (V_T), respiratory rate (f_R), oxygen consumption ($\dot{V}O_2$) and air convection requirement ($\dot{V}_E / \dot{V}O_2$) of WT (n=4), HET (n=5) and BK^{KO} (n=2) juvenile males under room air condition and under hypercapnic exposure (7% CO_2). Values are expressed as median with individual data points. # Indicates difference from WT group; + Indicates difference from HET group; * Indicates difference from BK^{KO} group.

In adult animals, CO_2 exposure led to an increase in all ventilatory and metabolic responses across both sexes (Figures 12 and 13). Females showed no differences in ventilatory variables among groups during normocapnic exposure; however, under CO_2, the BK^{KO} group exhibited higher oxygen consumption compared to the WT ($p=0.014$) and HET groups ($p=0.017$) (Figure 12).

In contrast, male animals displayed no differences between groups under hypercapnic conditions (Figure 13).

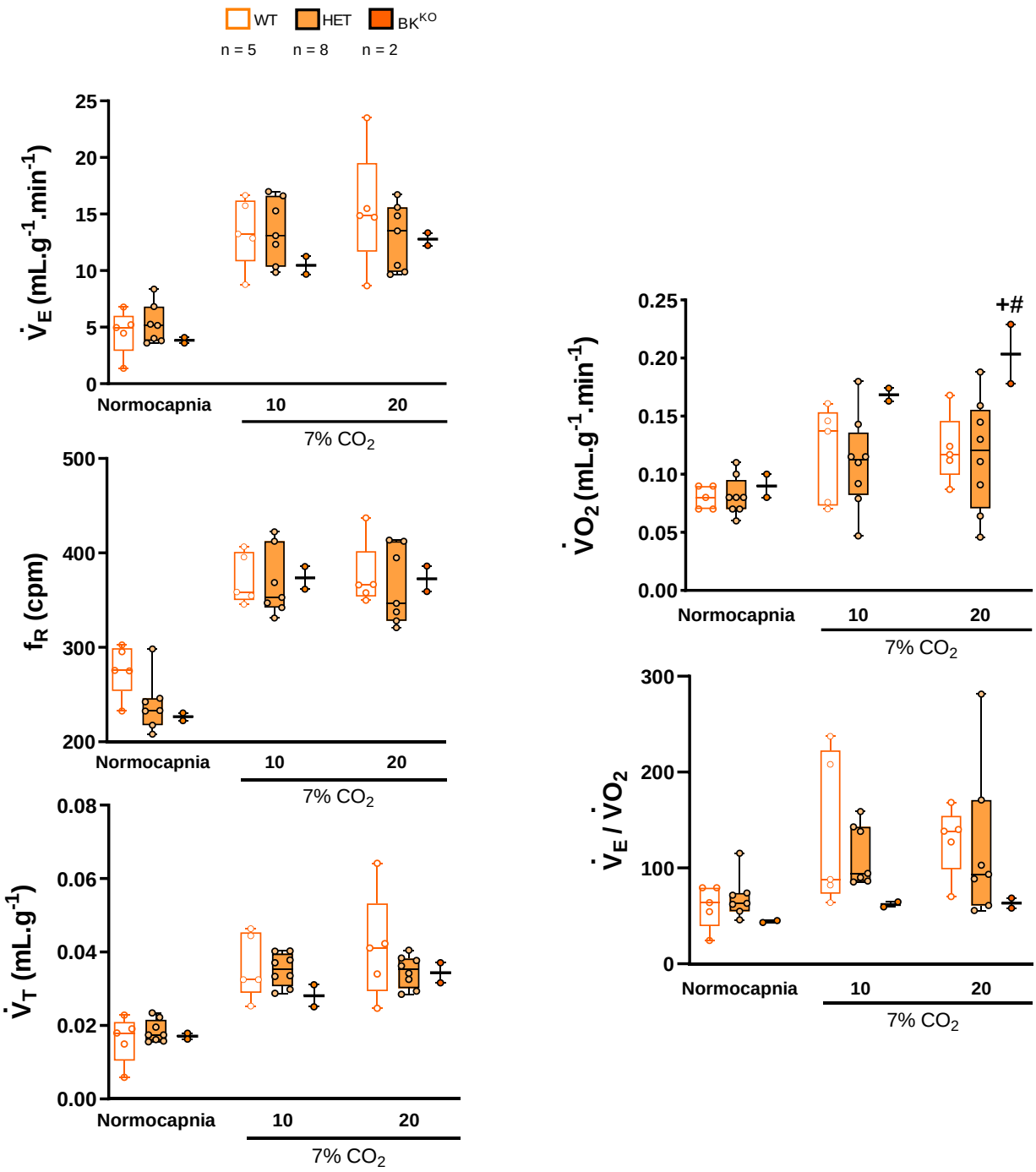

Figure 12. Ventilation ($\dot{V}_E$), tidal volume (V_T), respiratory rate (f_R), oxygen consumption ($\dot{V}O_2$) and air convection requirement ($\dot{V}_E / \dot{V}O_2$) of WT (n=5), HET (n=7) and BK^{KO} (n=2) adult females under room air condition and under hypercapnic exposure (7% CO_2). Values are expressed as median with individual data points. # Indicates difference from WT group; + Indicates difference from HET group.

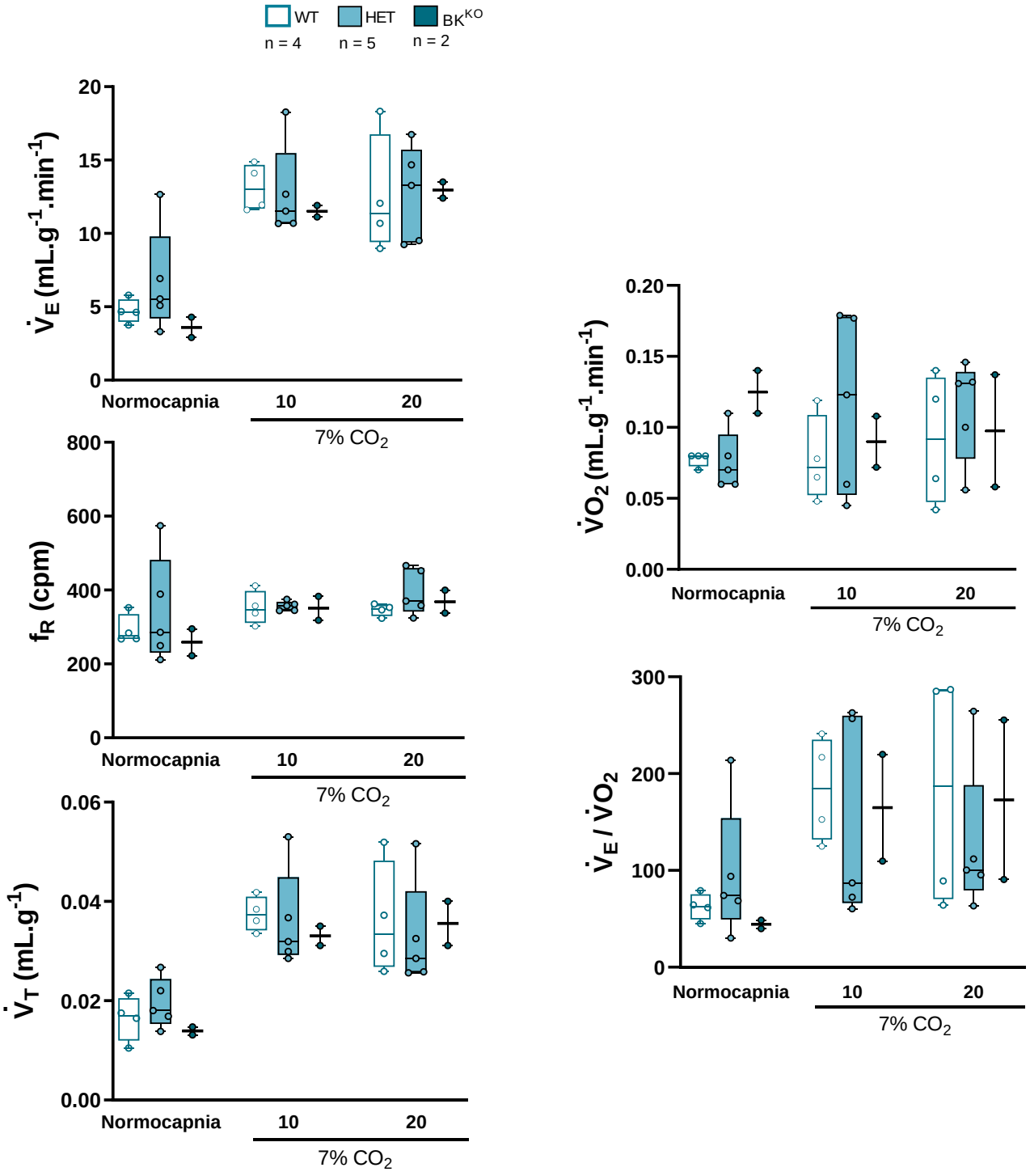

Figure 13. Ventilation ($\dot{V}_E$), tidal volume (V_T), respiratory rate (f_R), oxygen consumption ($\dot{V}O_2$) and air convection requirement ($\dot{V}_E / \dot{V}O_2$) of WT (n=4), HET (n=5) and BK^{KO} (n=2) adult males under room air condition and under hypercapnic exposure (7% CO_2). Values are expressed as median with individual data points.

Discussion

In the present study we used knockout mice for BK channels in order to evaluate to what extent the lack of BK channels affects respiratory and metabolic parameters during development in male and female mice. Our main findings indicate that the absence of BK channels did not alter ventilatory and metabolic parameters during normocapnic conditions. However, it intensified the hypercapnic ventilatory response, particularly in females at P6, P12, and the juvenile stage. In juvenile males and adult females, the most pronounced effect was observed in the metabolic rate. These results suggest that these channels act as a “brake”, limiting the CO_2 ventilatory response.

The use of BK channel knockout (BK^{KO}) mice has provided an efficient approach to studying the effects of the absence of these channels on respiratory control. This technique allows the exclusion of variables that could interfere with the specific analysis of the role of BK channels, making the results more accurate. However, the creation and maintenance of this animal model presents challenges, such as the lower viability of the knockout mice during the neonatal phase, which limits the number of individuals available for the experiments and may influence the sample representativeness of the results obtained. Previous studies have observed high mortality rates in knockout pups (Meridith et al., 2004; Halm et al., 2017) attributed to difficulties in sucking and swallowing during breastfeeding, which may cause the lower body mass of the BK^{KO} mice also observed in the present study.

Despite these limitations, the BK^{KO} model has proved useful not only in studying the ventilatory and metabolic response to CO_2/pH, but also in investigating the influence of BK channels on other physiological systems. Studies such as that by Sausbier et al. (2005) reveal that silencing the Slo1 gene, responsible for coding the subunit that forms BK channels, may have implications for several physiological systems besides respiration, which should be taken into account when analyzing the results. In addition, maintaining colonies of genetically modified mice requires specific laboratory infrastructure and advanced genetic manipulation techniques, which can represent a significant methodological barrier.

Although previous studies have reported ataxia in BK^{KO} mice (Sausbier et al., 2004), we did not observe any changes in chewing or righting reflexes in the present study. According to Knaus et al. (1996), despite the widespread expression of BK channels in the central nervous system, they appear to play a surprisingly modest role in normal brain function. This suggests that their broad neuronal distribution may have evolved primarily to support brain function under stress or adverse conditions—a view supported by evidence showing that BK channels enhance neuron survival in ischemic conditions. Therefore, our study corroborates with this view, since no major changes in motor reflex were found in the BK^{KO} mice.

During neonatal development in mice, BK channels exhibit a dynamic expression profile across various tissues, including the nervous system, where they play a crucial role in cell excitability and synaptic function (Contet et al., 2016). The literature review also highlights the critical role of BK channels in the development of chemosensitivity. Studies such as those by Putnam et al. (2001, 2004) and Davis (2006) provide a solid basis on changes in the ventilatory response to CO_2 during development. Such research points to a critical period in postnatal development when CO_2 sensitivity is particularly susceptible to variation, as also observed by Bavis and Mitchell (2008) and Wong-Riley and Liu (2020). Comparing these results with those obtained in this study allows us to contextualize the importance of BK channels in the maturation of this response and in the control of neuronal excitability throughout life.

Accordingly, our work sought to investigate the role of BK channels in the ventilatory response to hypercapnia, focusing on ontogenetic development and the sexual differences observed throughout this process. The data obtained from the research suggests that these channels act as an important regulatory mechanism of chemosensitivity, especially during the neonatal period, when ventilatory control is still maturing. In particular, an exacerbated response to hypercapnia was observed in female neonate mice knocked out for BK channels, indicating that, in the absence of this channel, there is a deregulation of the ventilatory response. This control mechanism, or “brake”, which seems to be mediated by BK channels, is critical to avoid neuronal hyperexcitability in the face

of CO_2 variations and consequent hypercapnic acidosis, which can result in the ventilatory response to hypercapnia.

Interestingly, a study by Wang and Kim (2017) using rat carotid body glomus cells reported that BK channels remain mostly closed at rest under normoxic conditions. During hypoxia-induced depolarization, BK channels activate to provide a hyperpolarizing force, which limits the extent of the hypoxic response. Therefore, this data suggests that BK channels are important to limit the cell hyperexcitability under stress conditions such as hypoxia, hypercapnia, ischemia or other situations. Similarly, the BK channels in locus coeruleus neurons are not active under normocapnic conditions and are activated by hypercapnia in a HCO_3^- - dependent fashion (Imber et al., 2014). In fact, BK channels in CO_2/pH chemosensitive neurons of locus coeruleus neurons play a limiting role (Braking pathway) in the neuronal chemosensitive response (Imber et al., 2014). Hypercapnic acidosis activates BK channels, causing K^+ efflux from the neuron, which hyperpolarizes the LC neurons and thus reduces the firing rate response to hypercapnic acidosis. According to the authors, the activation of BK channels during acidic conditions (caused by increased CO_2) suggests that they play a protective role by reducing excessive neuronal activity. This response may help prevent neuronal hyperexcitability and potential damage under prolonged high CO_2 exposure.

Another interesting finding of Imber et al. (2014) was that there is a significant change in the development of the intrinsic chemosensitivity of LC neurons. In neonatal rats less than 10 days postnatal (P10), a high percentage (70-80%) of LC neurons are chemosensitive, and the magnitude of the response, measured by the chemosensitivity index (CI; Wang and Richerson, 1999), was very high (around 235%), similar to some of the most chemosensitive neurons measured in vitro in the medullary raphe (Wang et al., 1998). Neither the percentage of neurons nor the chemosensitivity index was affected by the blockade of electrical synapses (carbenoxolone) in LC neurons from young neonates (<P10), indicating that these neuronal responses to hypercapnia are intrinsic (Hartzler et al., 2007; Nichols et al., 2008). In contrast, in LC neurons from older neonates (>P10), a high percentage of neurons were also activated

by hypercapnia, but the magnitude of the response was significantly lower (CI of 125%; a CI of 100% indicates a non-chemosensitive neuron).

In the current study, most of the effects of BK^{KO} in ventilation was observed from P6-7 until juvenile stage, mainly in females. This could be related to the different expression of BK channels during development. In fact, BK channel expression patterns shift throughout early development, suggesting that its role in regulating excitability may be developmentally dependent (MacDonald et al., 2006). Total BK mRNA levels increase several-fold from late embryonic development to the first postnatal week in rodents (MacDonald et al., 2006). Thus, it appears that in the initial stages of development (P0), this channel does not play a significant role in modulating the hypercapnic ventilatory response. However, future studies are needed to compare this expression in males and females in mice.

Regarding sex differences, previous study demonstrate that BK expression is significantly higher in hippocampi from female rats at P0, P2, P4, P6, and P8 (Hunsberger and Mynlieff, 2020). Interestingly, 17β-estradiol directly activates BK channels, reducing arterial contraction. Additionally, 17β-estradiol increases the expression of the BK channel β1 subunit, which further enhances channel activity through DNA demethylation (Valverde et al., 1999). Therefore, the greater effect in females observed in the present study might be due to the predominant role of this channel in this sex.

Finally, this research provides important contributions to understanding the specific mechanisms by which BK channels modulate the ventilatory response to CO_2/pH, especially at different ages and between the sexes. The identification of these mechanisms not only expands our knowledge of ventilatory regulation, but also suggests new possibilities for investigating the implications of BK channel dysfunction in respiratory and neurological conditions. The interaction between sex hormones and BK channels is particularly relevant for understanding how sex differences can influence the ventilatory response in pathological contexts. In summary, the results of this dissertation provide new evidence on the central role of BK channels in the control of ventilation throughout development, offering a

solid basis for future research on the regulation of chemosensitivity and its clinical implications.

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