PROGRAMA INTERINSTITUCIONAL DE PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS - UFSCar/UNESP

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PARTICIPAÇÃO DA RAFE BULBAR NA REGULAÇÃO DA TEMPERATURA CORPORAL EM AVE PRECOCE

Jaboticabal – SP

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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 Doutora em Ciências Fisiológicas.

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ROLE OF THE MEDULLARY RAPHE IN THE REGULATION OF BODY TEMPERATURE IN PRECOCIOUS BIRDS

Dissertation submitted to the Joint Graduate Program in Physiological Sciences, PIPGCF - UFSCar/UNESP as a requirement for the PhD degree in Physiological Sciences.

Supervisor: Dr. Kênia Cardoso Bícego

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Figure 5. Effect of intra-raphe microinjection of the $GABA_A$ receptor antagonist bicuculline on oxygen consumption ($\dot{\mathbf{V}}\mathbf{O_2}$), pulmonary ventilation ($\dot{\mathbf{V}}_\mathbf{E}$), tidal volume ($V_T$), breathing frequency (*f*) and respiratory equivalent ($\dot{\mathbf{V}}_\mathbf{E}/\dot{\mathbf{V}}\mathbf{O_2}$) of 1 week old chicks at 31ºC and 26ºC. Left, 31ºC; right, 26ºC. The arrow indicates the time of microinjection of 0.05 or 0.5 mmol^{-1} bicuculline or vehicle (saline) control into the medullary raphe (intra-raphe) or the nuclei surrounding the medullary raphe (peri-raphe). The number of animals is shown between parentheses. Data are means±s.e.m. *Significant difference (P<0.05) from saline control at the same time point. Open symbols, significant difference (P<0.05) over time from the pre-injection value in the same treatment.

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Supplementary Figure 1. Effect of intra-raphe microinjection of the NMDA receptor antagonist AP5 (A) and the $GABA_A$ receptor antagonist bicuculline (B) or vehicle on oxygen consumption ($\dot{\mathbf{V}}\mathbf{O_2}$), pulmonary ventilation ($\dot{\mathbf{V}}_\mathbf{E}$), tidal volume ($V_T$), breathing frequency (*f*) and respiratory equival*e*nt ($\dot{\mathbf{V}}_\mathbf{E}/\dot{\mathbf{V}}\mathbf{O_2}$) of one-week-old chicks at 36ºC. Arrow indicates the time of microinjection. Number of animals is shown between parentheses. Data are means ± s.e.m.

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conditions. Arrow indicates the time of microinjection. Number of animals is shown between parentheses. Intra-raphe: microinjection located in the medullary raphe. Data are means ± s.e.m *significant difference ($p < 0.05$) from vehicle at the same time. Open symbols mean significant difference ($p < 0.05$) over time from the pre-injection value in the same treatment.

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Figure 3. Effect of medullary raphe serotonergic neurons lesions (anti-SERT-SAP; 0.5 µM) on body temperature (Tb) of chicks at 26° C (A), 31°C (B) and 36°C (C). Data are means±s.e.m.

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LISTA DE ABREVIAÇÕES

5-HT	serotonina
5-HT-ir	neurônios imunorreativos para serotonina
A	amplitude da onda
ANT	translocase do nucleotídeo adenina
AP5	ácido DL-2-amino-5-fosfonopentanóico
APO/POA	área pre-óptica do hipotálamo anterior
avUCP	proteínas desacopladoras de prótons de aves
BAT	tecido adiposo marrom
BSA	soro albumina bovina
c-Fos-ir	neurônios imunorreativos para c-Fos
c-Fos/TrpOH-ir	neurônios imunorreativos para c-Fos e triptofano hidroxilase
CO_2	gás carbônico
CTz	corpus traprezoideum
CVC	vasoconstrição cutânea
DH	corno dorsal com neurônios sensoriais secundários
DMH	hipotálamo dorsomedial
DRG	gânglios da raiz dorsal com os neurônios sensoriais primários
ε	emissividade
f	frequência respiratória
FeO_2	fração de oxigênio expirado
FiO_2	fração de oxigênio inspirado
FRe	fluxo de ar
GABA	ácido γ-aminobutírico
GAD1+	neurônios positivos para GABA
Glu	glutamato
GRD	gânglio da raiz dorsal da medula espinal
IPC/HLI	índice de perda de calor
IML	coluna intermediolateral
LPBd	núcleo parabraquial dorsal
LPBel	núcleo parabraquial lateral
MnPO	núcleo pré óptico mediano

MPA	área pré-óptica medial
NMDA	N-metil D-Aspartato
O_2	oxigênio
P_B	pressão barométrica
PB	solução tampão fosfato
PBS	solução tampão fosfato salino
Pb_{H2O}	pressão de vapor de água do animal
P_{cal}	pressão de calibração
Pc_{H2O}	pressão de vapor de água da câmara
PFA	paraformoldeido
PG	prostaglandina
PGE_2	prostaglandina E_2
R	núcleo da rafe
Rgc	núcleo reticular gigantocelular
RPa	rafe pallidus
RPaM	núcleo reticular paramediano
RMg	rafe magnus
ROb	rafe obscurus
RQ	quociente respiratório
rRPa	rafe pallidus rostral
SERCA	ATPase-Ca^{+2} do retículo sarcoplasmático
SNC/CNS	sistema nervoso central
STPD	condições padrão de temperatura, pressão e ar seco
Ta	temperatura ambiente
Tc/Tb	temperatura corporal
T_{ch}	temperatura da câmara
TNZ	zona termoneutra
TrpOH	triptofano hidroxilase
TrpOH-ir	neurônios imunorreativos para triptofano hidroxilase
Ts	temperatura da superfície
V_{cal}	volume de calibração
$\dot{V}_E$	ventilação pulmonar
VeD	núcleo vestibular descendente
$\dot{V}O_2$	consumo de oxigênio

V_T	volume corrente

SÍNTESE

RESUMO

Inúmeras evidências em roedores indicam que regiões encefálicas caudais, como a rafe, estão envolvidas na ativação de efetores de produção (termogênese dependente e independente de tremor) e conservação (vasoconstrição periférica) de energia térmica. Por outro lado, nas aves, que tiveram uma trajetória evolutiva para aquisição da endotermia totalmente separada dos mamíferos, esse cenário de regulação neural da temperatura corporal não está esclarecido. Diante disso, o presente estudo teve como objetivo investigar a participação da rafe bulbar na ativação de termoefetores de frio (termogênese e vasoconstrição periférica) e de calor (perda de calor seca e evaporativa) em pintinhos na primeira semana de vida pós-eclosão. Para isso, foram mensurados temperatura corporal, consumo de oxigênio (índice termogênico), ventilação pulmonar (taquipnea térmica ao calor ou hiperpnea ao frio) e índice de perda seca de calor (a partir de temperatura da pele indicando vasoconstrição ou vasoldilatação) em animais com inibição e ativação da rafe e submetidos a frio (26^{o}C), calor (36^{o}C) ou ambiente neutro (31^{o}C). As inibições e ativações foram realizadas com microinjeção local de antagonistas de receptor GABA e de NMDA de glutamato. Os resultados indicaram a existência de influências excitatórias glutamatérgicas e gabaérgicas na rafe bulbar de pintinhos modulando a termogênese, e a estimulação glutamatérgica prevenindo a taquipnéia, sem terem qualquer papel nas respostas de defesa ao calor.

Sendo a rafe a principal região serotoninérgica do encéfalo, também foi verificada a participação de neurônios serotoninérgicos dessa região nas respostas termoefetoras de pintinhos expostos ao frio e ao calor. Entretanto a análise da ativação dos neurônios da rafe bulbar dos pintinhos mostrou que são ativados no frio (aumento de expressão de c-Fos) e estão envolvidos na termogênese, mas não parecem ser aqueles que sintetizam

serotonina, uma vez que uma baixa porcentagem de neurônios serotoninérgicos expressaram c-Fos e houve uma ausência de efeito da lesão específica de neurônio serotoninérgico na atividade dos termoefetores. Além de neurônios serotoninérgicos, foi demonstrado que a rafe bulbar de pintinhos também possui neurônios GABAérgicos, e a lesão desses neurônios afetou a Tc, o consumo de O_2 e influenciou o padrão ventilatório nas condições de frio e termoneutra. Dessa forma, os resultados indicam que, ao contrário dos mamíferos, a modulação das respostas termogênicas e ventilatórias ao frio pela rafe bulbar em pintos de galinha não envolve neurônios serotoninérgicos. Entre outros fenótipos de neurônios possivelmente envolvidos nesta modulação neural, evidenciamos um papel para os neurônios GABAérgicos da rafe. Tais resultados abrem uma nova perspectiva relacionada à regulação neural da temperatura corporal em vertebrados endotérmicos.

Palavras-chave: termorregulação, termogênese, índice de perda de calor, ofego, glutamato, GABA, serotonina

ABSTRACT

Numerous evidences in rodents indicate that caudal brain regions, such as the raphe, are involved in the activation of production effectors (tremor-dependent and tremor-independent thermogenesis) and conservation (peripheral vasoconstriction) of thermal energy. On the other hand, in birds, which had an evolutionary trajectory for acquiring endothermy totally separate from mammals, this scenario of neural regulation of body temperature is not clear. Therefore, the present study aimed to investigate the participation of medullary raphe in the activation of thermoeffectors of cold (thermogenesis and peripheral vasoconstriction) and heat (dry and evaporative heat loss) in chicks in the first week of post-hatch life. For this, body temperature, oxygen consumption (thermogenic index), pulmonary ventilation (thermal tachypnea to heat or hyperpnea to cold) and dry heat loss index (from skin temperature) were measured in animals with inhibition and activation of rafe and subjected to cold (26°C), heat (36°C) or neutral environment (31°C). Inhibitions and activations were performed with local microinjection of GABA and NMDA glutamate receptor antagonists. And the results indicated the existence of glutamatergic and GABAergic excitatory influences on the medullary raphe of chicks modulating thermogenesis, and glutamatergic stimulation preventing tachypnea, without having any role in the defense responses to heat.

Since the raphe is the main serotonergic region of the brain, the participation of serotonergic neurons in this region in the thermoeffector responses of chicks exposed to cold and heat was also verified. However, analysis of the activation of chick medullary raphe neurons showed that they are activated in the cold (increased expression of c-Fos) and are involved in thermogenesis, but do not appear to be those that synthesize serotonin, since a low percentage of neurons serotonergics expressed c-Fos and there was no effect

of serotonergic neuron-specific injury on thermoeffector activity. In addition to serotonergic neurons, the medullary raphe of chicks was also shown to have GABAergic neurons, and the lesion of these neurons affected Tc, O_2 consumption, and influenced the ventilatory pattern under cold and thermoneutral conditions. Thus, the results indicate that, unlike in mammals, the modulation of thermogenic and ventilatory responses to cold by medullary raphe in chicken chicks does not involve serotonergic neurons. Among other phenotypes of neurons possibly involved in this neural modulation, we evidence a role for GABAergic raphe neurons. Such results open a new perspective related to the neural regulation of body temperature in endothermic vertebrates.

Keywords: thermoregulation, thermogenesis, heat loss index, panting, glutamate, GABA, serotonin

Mecanismos termoefetores em aves

Um dos principais fatores ambientais que afeta os seres vivos é a temperatura. Assim, a manutenção da temperatura corporal (Tc) dentro de certos limites, por mecanismos termorreguladores, é extremamente importante, pelo fato de sua influência em variáveis fisiológicas e bioquímicas, como a função imunológica, contratilidade muscular, atividade neuronial, atividade endócrina, ventilação, frequência cardíaca, taxa metabólica (Randall D; Burggren W; French K, 1997). Com isso, é extremamente importante o desenvolvimento de estratégias autonômicas e comportamentais, para evitar grandes efeitos da temperatura nos seus processos fisiológicos, ou ainda apresentar tolerância fisiológica frente a variações térmicas.

Os animais endotérmicos (aves e mamíferos) dependem de fontes internas de energia térmica (do próprio metabolismo) para a manutenção da sua Tc dentro de uma faixa estreita de variação, que é característica de cada espécie, frente a variações da temperatura ambiente (Ta). O fato de manterem a Tc elevada e sem grandes variações está relacionado a um alto custo energético, que é mantido por uma elevada taxa metabólica, associada a uma alta demanda por alimento. Para regularem sua Tc, utilizam mecanismos autonômicos (involuntários) e comportamentais (voluntários), de perda e ganho de energia térmica, que pode resultar em alteração da taxa metabólica (Bicego et al., 2007; IUPS Thermal Commission, 2001)

Para a dissipação do calor corporal, diante de um ambiente quente, são ativados mecanismos de perda de calor, que são divididos em perda seca (sensível) e evaporativa (latente). Os mecanismos de perda seca de calor envolvem a vasodilatação periférica, com aumento do fluxo sanguíneo cutâneo, o que facilita a troca de calor com o ambiente, mas

que é insuficiente como mecanismo termolítico em condições muito quentes. Neste caso, ocorre a ativação de mecanismos de perda evaporativa de calor, que na maioria das aves ocorre por meio do ofego, ou taquipnea térmica, isto é, ventilação com o bico aberto e padrão ventilatório alterado para aumento da frequência respiratória e redução do volume corrente. Esse padrão ventilatório promove aumento da ventilação do espaço morto anatômico. Isto facilita a evaporação e o resfriamento das vias aéreas superiores e evita uma alcalose respiratória pela excessiva eliminação de CO_2, uma vez que não há alteração nas trocas gasosas de repouso, pois não há aumento do volume corrente (Menuam and Richards, 1975; Mortola and Maskrey, 2011). A taquipnea térmica pode induzir redução de até 2ºC na Tc, como já foi demonstrado em frangos, e representar um importante papel de proteção do sistema nervoso central (SNC), por manter a temperatura encefálica menor do que a do restante do corpo, o que é chamado de resfriamento encefálico seletivo (Arad et al., 1984).

Por outro lado, para as respostas de ganho de calor frente ao frio, as aves, diminuem o fluxo sanguíneo periférico (vasoconstrição cutânea), diminuindo assim, a perda de calor para o ambiente e conservando o calor produzido no interior do corpo. Quando esse mecanismo não é mais suficiente para manter a Tc, ocorre um aumento da energia térmica por meio da ativação da termogênese dependente e/ou independente de tremor, ambas no músculo esquelético. A termogênese independente de tremor é um mecanismo ainda não completamente entendido nas aves, podendo envolver a liberação de cálcio do retículo sarcoplasmático (Bicego et al., 2007; Bicudo et al., 2002) e/ou a atuação de proteínas desacopladoras de prótons mitocondriais como uma avUCP ou uma translocase de nucleotídeo adenina (ANT) (Raimbault et al., 2001; Vianna et al., 2001). Apesar das demonstrações de que a avUCP, expressa no músculo esquelético de frango e pato, ter sua expressão aumentada quando esses animais são aclimatados ao frio (Collin

et al., 2003; Raimbault et al., 2001), ainda não há evidencias diretas de que essa proteína seja importante para a produção de calor nas aves. Nesse sentido, a ANT parece ser a proteína envolvida diretamente na geração de calor mitocondrial em aves, pelo menos em frangos (Walter and Seebacher, 2009). Em relação aos mecanismos termogênicos dependentes de cálcio, sugere-se o possível envolvimento da proteína sarcolipina, desacopladora da função da ATPAse de cálcio do retículo sarcoplasmático (SERCA), inibindo a atividade de transporte de Ca^{+2} para o retículo mantendo alto gasto energético do transportador, e consequente termogênese (Rowland et al., 2015). Até o momento, somente há evidencias do papel da sarcolipina na termogênese muscular de mamíferos, mas há grande probabilidade de ter papel importante em aves também (Rowland et al., 2015).

Além dos mecanismos termorreguladores autonômicos, os endotérmicos também mantém a utilização de mecanismos comportamentais, sendo estes considerados mais eficazes e econômicos porque requerem demandas energéticas mais baixas e podem ser mantidos por mais tempo do que as respostas autonômicas (Briese and Cabanac, 1991). Estão relacionados à procura por um ambiente quente ou frio, ao contato com superfícies quentes ou frias, à utilização de posturas corporais encolhidas ou estiradas e ao comportamento de agrupar-se ou não (principalmente nos mais jovens), o que evita ou facilita a troca de calor do animal com o ambiente (revisado por Bicego *et al.*, 2007). Existem evidências que o reaquecimento passivo a partir do estado de torpor ou de hibernação, ao tomar banho de sol ou pela termorregulação social (agrupamento), parece ser comum em mamíferos e aves e essas estratégias comportamentais podem reduzir os custos de reaquecimento em torno de 15 a 85%, dependendo da espécie (Geiser et al., 2004). Tais mecanismos são mais antigos dentro da escala filogenética e estão presentes

em todos os grupos de vertebrados, apresentando um papel vital em condições térmicas mais severas (Bicego et al., 2007).

Ao longo do desenvolvimento ontogenético, a endotermia das aves precoces, como galinhas, frangos e patos, aparece na fase pré-eclosão do ovo, pois, no início, os embriões apresentam características de ectotérmicos (Seebacher et al., 2006; Szdzuy et al., 2008). Os pintinhos de galinha são quase completamente coberto por penugem e apresenta capacidade termorreguladora e locomotora logo após a eclosão do ovo (Dawson and Whittow, 2000; Mortola, 2009; Toro-Velasquez et al., 2014). Com isso, o estudo de sua fisiologia térmica pode ocorrer desde essa fase inicial da vida pós-eclosão, por ser uma fase crítica para o desenvolvimento do animal, já que a exposição a estressores térmicos e não-térmicos pode induzir alterações fisiológicas e/ou processo patológicos mais tarde na vida adulta (Soriano and Branco, 2010; Yahav and McMurtry, 2001).

Ao observar os mecanismos termoefetores recrutados em cada situação, como frio, calor, febre ou anapirexia (Bicego et al., 2007), percebe-se que tais respostas termorreguladoras são integradoras de vários sistemas sendo o mais diretamente afetado o sistema cardiorrespiratório, seja na aquisição de maior aporte de O_2 e eliminação de CO_2 em casos de frio, seja na distribuição de sangue entre o interior do corpo e a superfície corporal (vasodilatação/vasoconstrição periférica), ou ainda como mecanismo exclusivo para perda de calor, como o ofego, indicando uma interação de redes neurais envolvidas em todos esses efetores.

Regulação neural da temperatura corporal (Tc) em aves

A regulação da Tc, tanto por mecanismos autonômicos quanto comportamentais depende da integração pelo encéfalo dos sinais térmicos vindos do ambiente externo e do interior do corpo. Em mamíferos, a temperatura da superfície do corpo é detectada por

sensores térmicos cutâneos (Boulant, 1998a; Gentle, 1989; Necker, 1972; Necker, 1977; Necker and Reiner, 1980), os quais levam as informações sobre as alterações da Ta ao SNC, de modo que os efetores sejam rapidamente ativados e a Tc regulada (Bratincsák e Palkovits, 2004) e a temperatura interna é detectada por termorreceptores localizados nas vísceras e vasos e por neurônios termossensíveis centrais, como a área pré-optica do hipotálamo anterior (APO), tronco encefálico e medula espinal e possivelmente outras regiões (Boulant, 1998a; Boulant, 1998b; Boulant, 2000; Boulant, 2006; Boulant and Dean, 1986; Dimicco and Zaretsky, 2007; Hammel et al., 1976; Helfmann et al., 1981; Mercer and Simon, 1984; Morrison et al., 2008; Necker and Rautenberg, 1975; Schmidt, 1976a; Schmidt and Simon, 1982). Nas aves, também há evidências de receptores na pele e no sistema nervoso central, como na medula espinhal e no hipotálamo.

Os canais iônicos receptores de potencial transitório (TRPs: transiente receptor potential channels), respondem a diversos estímulos (Cui et al., 2011; Talavera et al., 2008; Voets and Nilius, 2003), incluindo mudanças térmicas, nesse caso são chamados de termo-TRPs. No entanto, evidências funcionais de um papel termorregulador do termo-TRP estão disponíveis apenas para alguns canais, incluindo TRPM8, TRPV3 e TRPV4 em roedores (Almeida et al., 2012; Peier et al., 2002; Scarpellini et al., 2019; Vizin et al., 2015; Wang and Siemens, 2015). Em aves não se sabe muito sobre a descrição dos TRPs. Saito and Shingai (2006) demonstraram a detecção de uma cópia de cada gene codificador de TRPV1, V2, V3, V4, M8 e A1 em frangos e também foi observada a presença de TRPV4 em sensores epiteliais em pombos (Cabo et al., 2013).

Em mamíferos e talvez em todos os vertebrados, a APO é definida como uma das principais regiões encefálicas envolvidas na regulação da Tc (Bicego et al., 2007). A APO é considerada em mamíferos uma região termosensível, pois detecta alterações térmicas locais, e termointegradora, uma vez que recebe informações sobre a temperatura

de várias regiões do organismo por meio de receptores periféricos (Boulant, 2006). Essa região contém neurônios sensíveis ao calor, os quais aumentam sua atividade com o aumento da temperatura, inibindo mecanismos de ganho de calor e ativando mecanismos de perda de calor, e neurônios insensíveis à variação de temperatura, que se conectam por meio de sinapses excitatórias e inibitórias com os neurônios efetores de perda e de produção de calor, respectivamente (Matsuda et al., 1992, Butler e Hodos, 1996, Boulant, 1998a, Boulant, 1998b, Boulant, 2000, Boulant, 2006, Bícego et al., 2007).

Em relação às vias de controle neural, o grupo dos pesquisadores Shaun Morrison & Kazuhiro Nakamura (Morrison, 2016; Morrison and Nakamura, 2011; Morrison and Nakamura, 2019; Morrison et al., 2008; Nakamura and Morrison, 2007; Nakamura and Morrison, 2010; Nakamura and Morrison, 2011) contribuíram de forma significativa para elucidar vias aferentes e eferentes de termorregulação em ratos. Esses autores sugeriram modelos que mostram que em ambientes quentes, os neurônios de segunda ordem da medula espinal, depois de receberem as informações dos termorreceptores periféricos, ativam o núcleo parabraquial dorsal por neurotransmissão glutamatérgica (Glu: glutamato) que, por sua vez, envia aferências também glutamatérgicas ao núcleo pré-óptico mediano (MnPO). Do MnPO, neurônios excitatórios (Glu) seguem em direção à área pré-óptica medial (MPA), ativando então esta região. A MPA, provavelmente por meio dos neurônios sensíveis ao calor, inibe, via neurotransmissão gabaérgica (GABA: ácido gamma-aminobutírico), regiões encefálicas caudais (hipotálamo dorso medial e núcleo rostral da rafe) responsáveis pela ativação dos efetores de produção e conservação de energia térmica. Se esses efetores são inibidos pela MPA, a produção e a conservação de energia térmica são reduzidas, regulando a Tc. No caso de ambiente frio, os receptores cutâneos transmitem a informação periférica aos neurônios espinhais que seguem em direção ao núcleo parabraquial lateral e, daí, ao MnPO. Entretanto, do MnPO, eferências

gabaérgicas são enviadas à MPA, inibindo esta região e, portanto, desinibindo as regiões caudais que ativam os efetores de produção e conservação de calor, controlando assim a Tc; (Morrison and Nakamura, 2011;Figura 1).

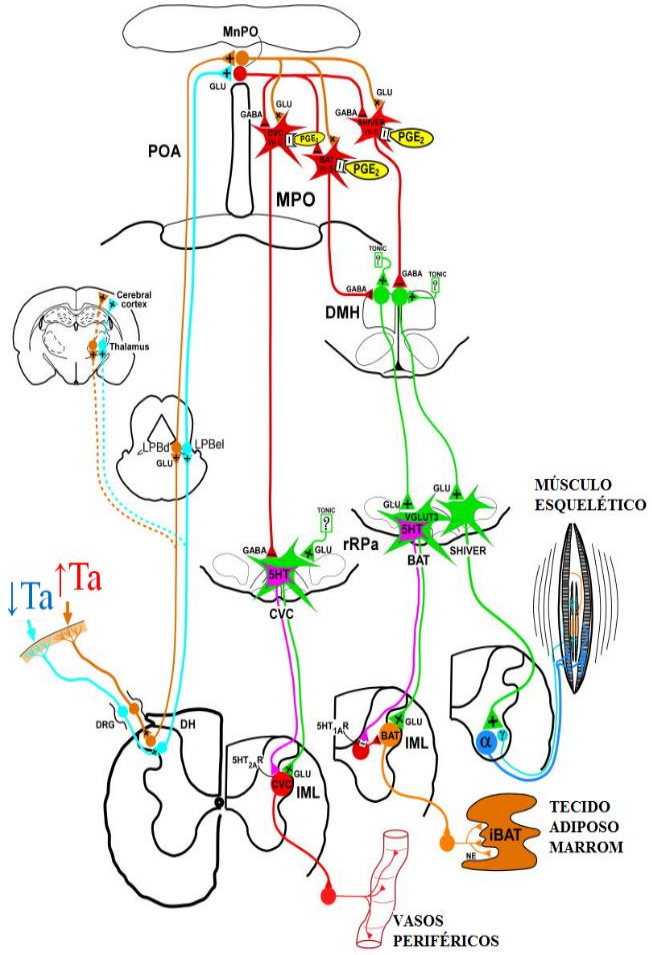


Figura 1. Esquema proposto por (Morrison and Nakamura, 2011) para a via termossensora de regulação da temperatura corporal. Em ambiente quente, o MnPO envia aferências excitatórias para a MPA, que inibe os neurônios eferentes de produção e conservação de calor, reduzindo então a produção e aumentando a perda de calor. Em ambiente frio, o MnPO inibe a MPA e desinibe os neurônios efetores de produção e conservação de calor, aumentando-as. GRD: gânglio da raiz dorsal da medula espinal; LPBd: núcleo parabraquial dorsal; LPBel: núcleo parabraquial lateral; MnPO: núcleo pré óptico mediano; MPA: área pré-óptica medial; Glu: glutamato; IML: coluna intermediolateral; CVC: vasoconstrição cutânea; BAT: tecido adiposo marrom; DRG: gânglios da raiz dorsal com os neurônios sensoriais primários; DH: corno dorsal com neurônios sensoriais secundários; PG: prostaglandina; rRPa: rafe pallidus rostral; 5-HT: serotonina; DMH; hipotálamo dorsomedial.

Nas aves, essa neuroregulação central da Tc está menos definida, e alguns estudos indicam uma termosensibilidade hipotalâmica semelhante à dos mamíferos, enquanto outros estudos mostram diferentes circuitos neurais para essa função (revisado

por Bicego *et al.*, 2007). A importância do hipotálamo anterior e da APO na regulação da Tc foi demonstrada em alguns trabalhos em pombos, aves domésticas e pardais que tiveram essas regiões lesionadas (Kanematsu et al., 1967; Lepkovsky et al., 1968; Mills and Heath, 1972; Necker and Gnuschke, 1989; Rogers, 1923). Nos pombos, as lesões anteriores à comissura anterior, que incluem a APO, afetam apenas a resposta à perda de calor (ofegos), enquanto as lesões posteriores à comissura anterior inibem a produção de calor (tremores) (Necker and Gnuschke, 1989). Este último é semelhante ao observado em mamíferos, em que o hipotálamo posterior está envolvido na regulação do tremor (Nagashima et al., 2000; Tanaka et al., 2001). Em relação à termossensibilidade hipotalâmica, estudos em pombos, pingüins, codornas, frangos e patos (Hammel et al., 1975; Rautenberg et al., 1972; Richards and Avery, 1978; Schmidt, 1976b; Simon et al., 1976; Simon Oppermann et al., 1978; Snapp et al., 1977) indicam que essa função é fraca e insignificante para a termorregulação desses animais, uma vez que o aquecimento da APO promove respostas termorreguladoras moderadas a fracas e o resfriamento da APO desencadeia alterações termoefetoras não relacionadas ao estímulo de frio, como uma redução na produção de calor metabólico. Em patos, por exemplo, as respostas de defesa ao frio (ativação de termogênese) e ao calor (ativação de respostas de perda de calor) são coerentemente estimuladas pelo resfriamento e aquecimento, respectivamente, de uma região no troco encefálico entre a ponte e o mesencéfalo (Martin et al., 1981). Entretanto, esse cenário em aves parece mais complexo, já que há estudos que indicam que pardais, emus e gansos apresentam termossensibilidade hipotalâmica semelhante à dos mamíferos (Bicego et al., 2007; Rautenberg, 1971). Além disso, a APO e o tronco encefálico rostral funcionam como sítios termossensíveis que alteram comportamentos relacionados à termorregulação em pombos (Schmidt, 1976a) .

Dessa forma, a regulação térmica neural das aves apresenta variação espécie-

específica, distinto do que é relatado em mamíferos, talvez pelo número reduzido de espécies diferentes de mamíferos utilizadas para comparação, ou por ser essa diversidade uma característica das aves mesmo. Outra possibilidade é que temos poucos estudos mais detalhados sobre essa regulação neural nas aves que permita dizer que são realmente diferentes ou não. Sendo necessária ainda muita investigação para se descrever as vias neurais envolvidas na regulação da Tc em aves.

Nos circuitos termorreguladores encefálicos, os principais neurotransmissores envolvidos são o glutamato (Glu; excitatório), o GABA (inibitório) e a serotonina (5-HT; principal neurotransmissor dos núcleos da rafe). O Glu e o GABA são amplamente distribuídos pelo encéfalo de mamíferos (Curtis and Johnston, 1974; Danbolt, 2001; Gaviraghi, 2000; Meister, 2007; Sivilotti and Nistri, 1991) e também de aves (Csillag et al., 1987; Granda and Crossland, 1989; Henley et al., 1989; Ottiger et al., 1995; Riters and Bingman, 1994; Stewart et al., 1988; Sun et al., 2005; Veenman and Reiner, 1994; Veenman et al., 1994). Vários estudos demonstraram que o GABA pode modular a Tc em diferentes espécies (Frosini et al., 2004; Frosini et al., 2006; Quéva et al., 2003; Sallagundala et al., 2007; Tsai et al., 2002; Yekimova and Pastukhov, 2002) e alguns autores sugerem que os mecanismos fundamentais de influência GABAérgica em neurônios sensíveis e insensíveis à temperatura na APO de aves foram conservados durante a evolução (Sallagundala et al., 2007). A serotonina é uma monoamina altamente conservada entre as espécies (Beliveau et al., 2017), desempenhando um papel importante como neurotransmissor em inúmeras funções corporais (Berger et al., 2009; Olivier, 2015; Sodhi and Sanders-Bush, 2004), incluindo a regulação da Tc (Hodges et al., 2008; Madden and Morrison, 2010; Morrison, 2004; Morrison and Nakamura, 2011; Nakamura and Morrison, 2007; Nakamura and Morrison, 2011).

Em vertebrados, os neurônios serotoninérgicos estão localizados no sistema

denominado rafe (núcleos da rafe), o qual possui projeções rostrais e caudais para várias regiões do encéfalo (Hay-Schmidt, 2000; Jacobs and Azmitia, 1992; Lauder, 1993). Em roedores, os núcleos da rafe são grupamentos de neurônios dispostos em uma coluna que se estende no eixo rostro-caudal, do mesencéfalo ao bulbo (Hay-Schmidt, 2000). A rafe bulbar por sua vez apresenta três subdivisões, sendo classificadas em núcleo obscuro da rafe (ROb), núcleo pálido da rafe (RPa) e o núcleo magno da rafe (RMg) que apresentam fenótipos de neurônios heterogêneos, todos tendo como principal neurotransmissor a 5-HT (Gao and Mason, 2001; Mason, 1997). A organização anatômica dos sistemas serotonérgicos encefálicos é notavelmente conservada entre os vertebrados (Parent, 1981), dessa forma a rafe bulbar em aves (galinhas, por exemplo) segue uma topografia equivalente à dos mamíferos, porém, sem a identificação de subáreas (Kuenzel and Masson, 1988; Okado et al., 1992). Os neurônios 5-HT no tronco encefálico de aves são divididos em vários grupos com base em características histológicas (localização, tamanho, forma e imunorreatividade; Yamada et al., 1984) ou reatividade química fluorescente para a detecção de monoaminas (Dubé and Parent, 1981; Ikeda and Gotoh, 1971).

Além de neurônios serotoninérgicos, nos mamíferos, a rafe também possui neurônios gabaérgicos, além de outros fenótipos, com coexpressão de neurotransmissores em alguns neurônios (Belin et al., 1983; Gras et al., 2002; Kachidian et al., 1991; Millhorn et al., 1987; Shikanai et al., 2012; Stamp and Semba, 1995). Neurônios serotoninérgicos da rafe bulbar sintetizam outros neurotransmissores além da serotonina, como GABA e glutamato e diferentes tipos de peptídeos, como a substância P (Blessing et al., 1986; Millhorn et al., 1987; Nakamura et al., 2004; Sasek et al., 1990; Stornetta et al., 1999; Stornetta et al., 2004). A atividade dos neurônios 5-HT está sob controle exercido por diferentes sistemas de neurotransmissores, incluindo a própria serotonina, noradrenalina,

dopamina, histamina, glutamato, glicina e vários neuropeptídeos (Jacobs and Azmitia, 1992) e o neurotransmissor inibitório GABA. O GABA desempenha um papel essencial na regulação dos neurônios 5-HT, atuando através de interneurônios gabaérgicos locais, bem como aferências GABAérgicas que se projetam para os núcleos da rafe, vindas de neurônios gabaérgicos localizados em regiões rostrais, incluindo a APO (Gervasoni et al., 2000).

Diante do que foi exposto, testamos a hipótese que a rafe bulbar modula a ativação de termoefetores de frio (termogênese e vasoconstrição periférica) e de calor (perdas de calor seca e evaporativa) em pintinhos na primeira semana de vida pós-eclosão. Assim, o presente trabalho está apresentado em dois capítulos cujos objetivos gerais foram:

Capítulo 1

Investigar o efeito das inibições glutamatérgica e gabaérgica na rafe bulbar sobre a termorregulação de pintinhos.

Capítulo 2

Investigar o papel de neurônios específicos, serotoninérgicos e gabaérgicos, da rafe bulbar na termorregulação de pintinhos.

Capítulo 1

No Capítulo 1, foi investigado o efeito das inibições de receptores ionotrópicos glutamatérgicos (NMDA) e gabaérgicos ($GABA_A$) da rafe bulbar, por meio de microinjeção dos antagonistas AP5 (0,5 e 5 mmol l^{-1}) e bicuculina (0,05 e 0,5 mmol l^{-1}), respectivamente, sobre a Tc, consumo de oxigênio, ventilação pulmonar e IPC em pintinhos de 1 semana de idade expostos a condições termoneutra (31ºC), fria (26ºC) e quente (36ºC). Para isso, dois dias antes dos experimentos, os animais tiveram um mini sensor de temperatura implantado na cavidade celomática, e no dia do experimento, após exposição à temperatura ambiente experimental durante 1.5 h, as medidas de Tc, consumo de oxigênio e ventilação ou Tc e IPC foram realizadas a cada 20 min por 2h, antes e após as microinjeções dos antagonistas ou veículo. Detalhes desses métodos são apresentados no Capítulo 1 adiante.

Capítulo 2

No Capítulo 2, foi analisada a ativação dos neurônios serotoninérgicos frente a estímulos térmicos através da imunoistoquímica dupla para c-Fos (índice de ativação neuronal) e 5-HT. Além disso, também foi investigado o efeito de lesões específicas de neurônios serotoninérgicos e gabaérgicos da rafe bulbar, utilizando as imunotoxinas, anti-SERT-SAP e anti-GAT1-SAP, respectivamente, sobre a Tc, consumo de oxigênio e a ventilação pulmonar em pintinhos de 1 semana de idade expostos a condições termoneutra (31ºC) e fria (26ºC). Nesse caso, a exposição ao calor foi investigada somente em alguns protocolos, devido aos resultados negativos obtidos nos experimentos do Capítulo 1. Para as lesões, sete dias antes dos experimentos, os animais receberam a microinjeção das imunotoxinas ou veículos e tiveram um mini sensor de temperatura

implantado na cavidade celomática. No dia do experimento, após exposição à temperatura ambiente experimental por 1h, as medidas de Tc, consumo de oxigênio e ventilação ou Tc e IPC foram realizadas a cada 20 min por 2h. Detalhes desses métodos são apresentados no Capítulo 2 adiante.

Capítulo 1

Demonstramos que os antagonismos dos receptores NMDA (AP5; 0,5; 5 mmol l^{-1}) e $GABA_A$ (bicuculina; 0,05; 0,5 mmol l^{-1}) da rafe bulbar reduziram a Tc e o consumo de oxigênio dos pintinhos a 31ºC e a 26°C, com resultado mais expressivo na condição de frio (resultados com as doses menores dos antagonistas). O AP5 aumentou a frequência respiratória dos animais durante a exposição ao frio. A 31ºC, o índice de perda de calor foi maior nos grupos bicuculina e AP5 (doses maiores) do que nos grupos salina no início da redução da Tc. Os tratamentos não afetaram nenhuma das variáveis testadas a 36°C.

Capítulo 2

Observou-se aumento da expressão de c-Fos em neurônios da rafe bulbar apenas nos animais expostos ao frio, em comparação àqueles em condições termoneutra e de calor, mas com somente cerca de 10% dos neurônios correspondendo aos serotoninérgicos. Além disso, o anti-SERT-SAP foi eficaz na destruição de cerca de 70% dos neurônios serotonérgicos da rafe bulbar, mas sem afetar nenhuma das variáveis avaliadas nas condições neutra, fria e quente. Já o anti-GAT1-SAP lesou 55% dos neurônios GABAergicos da rafe bulbar, promovendo um aumento da Tc, do consumo de O_2 e influenciando o padrão ventilatório nas condições de frio e termoneutra.

Os resultados do presente estudo indicam a existência de influências excitatórias glutamatérgicas e gabaérgicas na rafe bulbar de pintinhos modulando a termogênese, e a estimulação glutamatérgica prevenindo a taquipnéia, sem terem qualquer papel nas respostas de defesa ao calor. A inesperada influência estimuladora do GABA na rafe bulbar dos pintinhos pode ter relação com uma fase ainda imatura dos neurônios afetados pelo GABA, ou uma modulação de interneurônios inibitórios da própria rafe. De qualquer maneira, essa influência de excitação dupla na rafe bulbar pode fornecer um mecanismo neural protetor para dar suporte à termogênese durante o início da vida, quando o gasto de energia para crescimento e para manter a homeotermia é alto (Dawson and Whittow, 2000; Mortola, 2009; Toro-Velasquez et al., 2014). Esta nova demonstração de um papel termorregulador para a rafe bulbar em aves sugere uma regulação neuroquímica da Tc pelo tronco encefálico convergente em vertebrados endotérmicos.

Além disso, a análise da ativação dos neurônios da rafe bulbar dos pintinhos mostrou que são ativados no frio (aumento de expressão de c-Fos) e estão envolvidos na termogênese, mas não parecem ser aqueles que sintetizam serotonina, uma vez que houve uma baixa porcentagem de dupla marcação c-Fos/TrpOH-ir e ausência de efeito da lesão específica de neurônio serotoninérgico na atividade de termoefetores. Por outro lado, foi demonstrada a presença de neurônios gabaérgicos na rafe bulbar e esses neurônios parecem estar envolvidos na termogênese de pintinhos, pois a lesão de quase 60% desses neurônios promoveu um aumento da Tc, do consumo de O_2 e influenciou o padrão ventilatório nas condições de frio e termoneutra.

Dessa forma, diferente do que acontece em mamíferos, a modulação das respostas termogênicas e ventilatórias ao frio pela rafe bulbar em pintos de galinha não envolve

neurônios serotoninérgicos. Entre outros fenótipos de neurônios possivelmente envolvidos nesta modulação neural, evidenciamos um papel para as células GABA da rafe. Tais resultados abrem uma nova perspectiva relacionada à regulação neural da temperatura corporal em vertebrados endotérmicos. Tais diferenças em uma via neural termorreguladora podem estar bem inseridas no cenário de vias evolutivas distintas para endotermia em aves e mamíferos, como por exemplo os distintos efetores de termogênese sem tremores (apenas músculo vs BAT e músculo, respectivamente).

CAPÍTULO 1

A thermoregulatory role of the medullary raphe in birds - Journal of Experimental Biology (2021) 224, jeb234344. doi:10.1242/jeb.234344

(cópia do artigo publicado anexada no Apêndice dessa Tese)

ABSTRACT

The brainstem region medullary raphe modulates non-shivering and shivering thermogenesis and cutaneous vasomotion in rodents. Whether the same scenario occurs in the other endothermic group, i.e. birds, is still unknown. Therefore, we hypothesised that the medullary raphe modulates heat gain and loss thermoeffectors in birds. We investigated the effect of glutamatergic and GABAergic inhibitions in this specific region on body temperature (Tb), oxygen consumption (thermogenesis), ventilation (O_2 supply in cold, thermal tachypnea in heat) and heat loss index (cutaneous vasomotion) in 1 week old chicken exposed to neutral (31ºC), cold (26ºC) and hot (36ºC) conditions. Intra-medullary raphe antagonism of NMDA glutamate (AP5; 0.5, 5 mmol l^{-1}) and $GABA_A$ (bicuculline; 0.05, 0.5 mmol l^{-1}) receptors reduced Tb of chicks at 31ºC and 26ºC, mainly as a result of an O_2 consumption decrease. AP5 transiently increased breathing frequency during cold exposure. At 31ºC, heat loss index was higher in the bicuculline and AP5 groups (higher doses) than in the saline groups at the beginning of the Tb reduction. No treatment affected any variable tested at 36ºC. The results suggest that glutamatergic and GABAergic excitatory influences on the medullary raphe of chicks modulate thermogenesis, and glutamatergic stimulation prevents tachypnea, without having any role in warmth-defence responses. A double excitation influence on the medullary raphe may provide a protective neural mechanism for supporting thermogenesis during early life, when energy expenditure to support growth and homeothermy is high. This novel demonstration of a thermoregulatory role for the raphe in birds suggests a convergent brainstem neurochemical regulation of Tb in endotherms.

Keywords: Brainstem, GABA, Glutamate, Chick, Oxygen Consumption, Heat Loss Index, Thermal Tachypnea

Regulation of body temperature (Tb) by the activation/inhibition of thermoeffectors depends on the integration by the central nervous system (CNS) of thermal signals coming from the body surface, which are under the influence of the external environment, and from the inner body (Bicego et al., 2007; Romanovsky, 2014). In mammals and birds, body surface temperature is detected by skin thermal sensors (Boulant, 1998a; Gentle, 1989; Necker, 1972; Necker, 1977; Necker and Reiner, 1980), and core temperature by inner sensors and central thermosensitive neurons in the preoptic area of the anterior hypothalamus (POA), brainstem and spinal cord (Boulant, 1998a; Boulant, 1998b; Boulant, 2000; Boulant, 2006; Boulant and Dean, 1986; Dimicco and Zaretsky, 2007; Hammel et al., 1976; Helfmann et al., 1981; Mercer and Simon, 1984; Morrison et al., 2008; Necker and Rautenberg, 1975; Schmidt, 1976a; Schmidt and Simon, 1982). Following integration of all of the thermal information coming from the periphery and the body core in the hypothalamic regions (Bicego et al., 2007; Morrison, 2016), the activity of thermoeffectors is controlled by more caudal regions located in the brainstem, as is the case for the raphe nuclei in the medulla, as demonstrated in rodents (Morrison, 2016).

The raphe nuclei are a group of neurons, distributed throughout the midline of the midbrain to the medulla (Dimicco and Zaretsky, 2007; Morrison et al., 2008), which are involved in circuits that regulate life-sustaining thermoregulatory and respiratory networks (Morrison, 2016; Ray et al., 2011). In addition, the caudal raphe nuclei are known to be related to thermogenesis, cardiovascular and gastric functions in mammals through neuronal connections to the hypothalamus (Berthoud et al., 2005; Madden and Morrison, 2006; Morrison et al., 2008). The arrangement of the medullary raphe in birds

(e.g. chicken; Kuenzel and Masson, 1988) follows an equivalent topography in mammals (Paxinos and Watson, 2005), but without identification of subareas such as those described in rodents, i.e., the obscurus, pallidus and magnus nuclei of the raphe.

Specifically regarding thermoregulation in rodents, the medullary raphe's rostral nuclei receive excitatory (glutamatergic) projections from the dorsomedial hypothalamus (DMH) that modulate the activity of their pre-motor neurons, which project into the spinal cord, for control of non-shivering (brown adipose tissue) and shivering thermogenesis (Morrison, 2016; 1999; Morrison and Nakamura, 2011; Morrison et al., 2012). The medullary raphe also plays a role in skin vasomotion, receiving excitatory projections (glutamatergic) from the median preoptic nucleus (MnPO) and inhibitory (GABAergic) projections from the medial preoptic area (MPA; Morrison, 2016; 1999. Such neural pathways are stimulated by skin cooling (increased thermogenesis and skin vasoconstriction) and inhibited by skin warming (decreased thermogenesis and skin vasodilation), respectively.

In birds, a group that evolved endothermy through a completely separate pathway from mammals (Legendre and Davesne, 2020), much less is known about the neural circuitry for thermoregulation. Some studies suggest a hypothalamic thermosensitivity similar to that of mammals, while others show involvement of different neural circuits for this function (reviewed by Bícego et al., 2007). For example, chickens (Richards, 1970), sparrows (Mills and Heath, 1972), emus (Jessen et al., 1982), and geese (Helfmann et al., 1981) show similar hypothalamic thermosensitivity to mammals, while ducks have a primary thermosensitive region located outside of the hypothalamus, between the rostral brainstem and the midbrain (Martin et al., 1981). Similar to mammals, glutamatergic and GABAergic neurotransmissions are widely distributed throughout the brain in birds (Csillag et al., 1987; Granda and Crossland, 1989; Henley et al., 1989; Ottiger et al., 1995;

Riters and Bingman, 1994; Stewart et al., 1988; Sun et al., 2005; Veenman et al., 1994). There is also evidence for GABA modulation of Tb in birds (Sallagundala et al., 2007; Yakimova et al., 2005; Yekimova and Pastukhov, 2002), affecting the firing rate and thermal coefficient of hypothalamic neurons in juvenile chickens (Sallagundala et al., 2007). However, no data exist, however, about caudal brain regions that control thermoeffector activity in any species of bird, and we hypothesize that the medullary raphe plays an analogous thermoregulatory role to that which is demonstrated in mammals.

The main thermoeffectors in birds are shivering and non-shivering thermogenesis (Aulie, 1976; Aulie and Tøien, 1988; Bicego et al., 2007; Bicudo et al., 2002) and cutaneous vasoconstriction (Johansen and Bech, 1983; Richards, 1971) in cold conditions, and thermal tachypnea (Bícego and Mortola, 2017; Richards, 1971) (Bícego and Mortola, 2017; Richards, 1970) and cutaneous vasodilation (Richards, 1970; Richards, 1971) in warm conditions. In contrast to placental mammals, birds do not possess brown adipose tissue; thus, both shivering and non-shivering thermogenesis are based on skeletal muscle activity (Aulie and Tøien, 1988; Marjoniemi and Hohtola, 2000; Rowland et al., 2015). Precocious birds, including Galliformes, become endothermic at the end of incubation (Dzialowski et al., 2007; Nichelmann and Tzschentke, 2002; Price et al., 2018; Seebacher, 2009; Seebacher et al., 2006; Szdzuy et al., 2008). They hatch covered in plumage, possessing a well-developed nervous system and muscular and locomotor function, and can elicit thermogenic responses to cold (Khandoker et al., 2004; Mathiu et al., 1991; Mortola, 2009; Mortola and Maskrey, 2011; Toro-Velasquez et al., 2014) and thermal tachypnea when exposed to heat (Bícego and Mortola, 2017). As precocious birds, chicks are excellent models for studying thermal physiology at this early stage of life and, thus, were used in the present study.

In view of the considerations outlined above, we aimed to test the hypothesis that the medullary raphe plays a role in the activation of cold-defense thermoeffectors (thermogenesis and peripheral vasoconstriction) and the inhibition of warmth-defense thermoeffectors (thermal tachypnea and peripheral vasodilation) in chicks during the first week after hatching. To this end, we investigated the effects of antagonism of $GABA_A$ (by bicuculline) and glutamate NMDA (by AP5) receptors in the medullary raphe on O_2 consumption (index of thermogenesis), pulmonary ventilation, and heat loss index (HLI; index of cutaneous vasoconstriction/vasodilation) in 1 week old chicks under different thermal conditions.

Animals

Fertile *Gallus gallus* (Linnaeus, 1758) eggs, purchased from local commercial hatcheries (Globoaves and Pluma Agro Avicola, SP, Brazil), were incubated in a serial manner (37ºC, 65% relative humidity, turning every 2 h) to obtain only a few chicks of the same age per day for experiments. Experiments were performed on 1 week old chicks weighing 90-110 g. After hatching, chicks were reared in temperature-controlled chambers (Premium Ecológica, Belo Horizonte, MG, Brazil) at 31-32ºC, which is considered to be in thermoneutrality (thermoneutral zone, TNZ) for chicks at that age, as they show normal behaviors, such as eating and drinking with no huddling or panting, at these temperatures. Animals were under a light-dark cycle of 14 h:10 h (lights on at 06:00 h) and were fed a standard diet (initial feed for chicks) and water *ad libitum*. All experiments were randomly performed between 08:00 h am and 17:00 h pm to avoid any influence of the daily Tb cycle, and were conducted with the approval of the local Animal Care and Use Committee (CEUA) from FCAV/UNESP (protocol 013907/17).

Drugs

The N-methyl-D-aspartate (NMDA) receptor antagonist AP5 (DL-2-Amino-5-phosphonopentanoic acid; molecular weight: 197.1 g mol^{-1}), the $GABA_A$ receptor antagonist bicuculline (molecular weight: 367.35 g mol^{-1}) and the $GABA_A$ receptor agonist muscimol (molecular weight: 114.1 g mol^{-1}) were purchased from Sigma-Aldrich Brasil Ltda. (São Paulo, SP, Brazil). These drugs were dissolved in pyrogen-free saline.

Microinjections

The drugs were dissolved in 1% Evans Blue saline solution (vehicle) and microinjected into the medullary raphe of unanesthetized chicks, according to the method developed in our laboratory based on previous reports (Coleone et al., 2009; Davis et al., 1979). The animal's head was fixed to an acrylic stereotaxic apparatus, built to order (Bonther, Ribeirão Preto, SP, Brazil), and microinjection was performed using a dental injection needle (Mizzy, 200 μm outer diameter), connected by a PE-10 cannula to a Hamilton syringe (5 μL) and an automatic microinjector (model 310, Stoelting Co., Wood Dale, IL, USA). In animals at this early stage of post-hatching development, the skull has not yet fully ossified and hardened preventing the guide cannula from being attached to it. In addition, chicks grow rapidly, which could alter the position of the guide cannula after five days of post-operative recovery. After numerous tests, based on the stereotaxic atlas of chick brain (Kuenzel and Masson, 1988), the microinjection coordinates were determined to be between 1.5 and 2.5 mm posterior to the intersection of the longitudinal and transverse sutures at the midline, and 13 mm deep from the skull surface. As the skull is not visible during the injection procedure, the comb and ear were used as references for the midline and transverse suture, respectively (see Fig. 1A). The microinjection needle was inserted into the upper hole of the acrylic apparatus, puncturing the animal's soft skull and brain tissue at the time of injection. A volume of 50 nL was injected for 20 s, and the needle was withdrawn after another 20 s to prevent reflux. This method of injection, which lasts 40 s in total, is considered not to be stressful to the chicks, as previous demonstrations showed no change in feeding behavior, body temperature or plasma corticosterone levels (Coleone et al., 2009; Furuse et al., 1997; Saito et al., 2005).

Determination of body temperature (Tb)

Tb was obtained by implanting a mini temperature sensor (12.5 mm in length, 2.1 mm in diameter; biotag ATP12, Biomark HPR Plus Reader, Boise, ID, USA) inside the coelomic cavity. For this, each chick was anesthetized inside a chamber with 5% isoflurane in pure O_2 for ~2 min for induction, and 1% isoflurane in pure O_2 for maintenance, through a mask during surgery. A biotag was inserted into the coelomic cavity through the skin and abdominal muscle via an application needle, and the tiny hole was closed with surgical glue (Dermabond Topical Skin Adhesive; Johnson & Johnson, Sao Paulo, Brazil). At the end of the surgery, antibiotic (enrofloxacin, intramuscular; 10 mg kg^{-1}; Bayer SA, São Paulo, SP, Brazil) and analgesic anti-inflammatory (flunixin meglumine, intramuscular; 2.5 mg kg^{-1}; MSD Saúde Animal, São Paulo, SP, Brazil) agents were administered. The entire procedure lasted 10-15 min. Experiments were performed at least two days after sensor implantation. Individual Tb was recorded in real-time by telemetry with the appropriate reader for BioTherm sensors (Biomark HPR Plus Reader, Boise, ID, USA) and transferred to a computer (BioTherm software). The Tb was corrected using a linear regression equation obtained from the curves of the relationship between temperatures measured by each mini sensor and by a mercury thermometer (0.1°C range).

Oxygen consumption ($\dot{V}O_2$)

Oxygen consumption ($\dot{V}O_2$) was determined using an open-flow respirometry system. The chicks were placed individually inside a respirometry chamber (3 L), positioned inside of a climate-controlled chamber (FANEM, São Paulo, SP, Brazil). Room air was continuously drawn from the respirometer by a flowmeter (MSF; Sable Systems, Las Vegas, NV, USA). A subsample of that air (180 mL min^{-1}; SS4; Sable Systems, Las Vegas, NV, USA) was pulled through a water vapour pressure analyser

(RH300; Sable Systems, Las Vegas, NV, USA) to an O_2 analyser (PA-10; Sable Systems, Las Vegas, NV, USA) after air-drying (Drierite, with indicator, 8 mesh, Sigma-Aldrich Brazil Ltda., São Paulo, SP, Brazil). The values for water vapour pressure (WVP; kPa) and barometric pressure (P_B; kPa), were later used to correct the flow. Thus, $\dot{V}O_2$ was determined based on the flow rate and the difference in gas concentrations at the inlet (baseline) and outlet of the chamber. All of the analysers and flowmeters were connected to an analog-to-digital converter (PowerLab; ADInstruments, Sydney, NSW, Australia), and signals were recorded with the appropriate software (LabChart; ADInstruments, Sydney, NSW, Australia). The O_2 analyser was calibrated before each experiment using nitrogen (0%, oxygen) and dry ambient air (20.95% oxygen). As CO_2 was neither analysed nor scrubbed, $\dot{V}O_2$ was calculated using the following equation (Koteja, 1996a): $\dot{V}O_2 = [FRex (FIO_2 - FEO_2)] / [1 - FIO_2 (1 - RQ)]$, where FRex is the excurrent flow rate, FIO_2 is the incurrent fractional concentration of oxygen (baseline), FEO_2 is the excurrent fractional concentration of oxygen, and RQ is the respiratory quotient (considered to be 0.85). Data are shown in standard conditions of temperature, pressure and dry air (STPD).

Pulmonary ventilation ($\dot{V}_E$)

Pulmonary ventilation ($\dot{V}_E$) was measured using the barometric method (reviewed by Mortola and Frappell, 1998). This method is based on the principle that the volume of gas within a closed chamber with an animal inside expands during inspiration, because air is heated and humidified during its passage from the chamber to the airways. The opposite occurs during expiration. Thus, it is possible to detect the waves of ventilatory oscillation by means of a pressure transducer connected to the experimental chamber to determine the breath volume and respiratory rate of the animal. Based on the recordings, we determined: respiratory rate (*f*; peak pressure count), tidal volume (V_T), and their

product (V_T x f = $\dot{V}_E$). The V_T was determined from the following formula (Drorbaugh and Fenn, 1955): $V_T = A\ (V_{cal} / P_{cal})\ [Tb\ (P_B - P_{H2O,chamber})] / \{[Tb\ (P_B - P_{H2O,chamber})] - [T_{chamber}\ (P_B - P_{H2O,bird})]\}$, where A is the wave amplitude (pressure oscillations), V_{cal} is the calibration volume, P_{cal} is the calibration pressure, Tb is the body temperature, P_B is the barometric pressure, $P_{H2O,\ chamber}$ is the water vapour pressure inside the chamber, $T_{chamber}$ is the temperature inside the respirometry chamber, and $P_{H2O,bird}$ is the saturation vapour pressure at the bird's core temperature. $\dot{V}_E$ was periodically recorded for a maximum of 2 min each time the respirometer was sealed for baseline air analysis for $\dot{V}O_2$ calculation. The V_{cal} was determined during each experiment by injecting a known volume of air (3 mL) into the animal's chamber using a graduated syringe. This injection promoted a pressure difference in the chamber (P_{cal}), used to calculate the compliance of the chamber (V_{cal}/P_{cal}). A Thermistor Pod sensor (ADInstruments; recorded in LabChart) monitored the ambient temperature (Ta) within the respirometer.

During the heat exposure (36^{o}C) experiments, $T_{chamber}$ tends to approach Tb, a situation that reduces the sensitivity of the method. Thus, an adaptation of the barometric technique based on a previous study (Szdzuy and Mortola, 2007) was applied in order to create a significant difference between Tb and $T_{chamber}$. The animals were placed individually inside a chamber composed of two separate compartments connected through a polyethylene tube of short length (~20 cm) and large diameter (~4 cm). The animal´s compartment (3 L) was kept inside a climate chamber at the highest Ta (FANEM, São Paulo, SP, Brazil), while the other compartment (1 L) remained outside at room temperature (~25^{o}C) to achieve a desirable Tb-Ta gradient. The calculation of V_T was performed using the same formula above, with the difference being those weighted averages, relative to the volumes of the two compartments, were used for $T_{chamber}$ and $P_{H2O,\ chamber}$.

From the O_2 consumption and pulmonary ventilation data, the ventilatory equivalent, which is the ratio of $\dot{V}_E/\dot{V}O_2$, and the lung extraction efficiency of O_2, which is $(\dot{V}O_2/\dot{V}_E \cdot FiO_2) \cdot 100$ (where FIO_2 is the inspired fraction of O_2; Barros et al., 2001) were calculated. The first calculation refers to the air convection requirement, and the second refers to the percentage of O_2 extracted from the lungs during each ventilation.

Heat loss index (HLI)

The surface temperature (Ts) of the feet, which are considered to be thermal windows in birds, was measured through infrared thermographic images using a camera sensitive to infrared radiation (Flir E40, Portland, OR, USA), allowing for inferences of cutaneous vasodilation or vasoconstriction (Almeida et al., 2006; Cristina-Silva et al., 2017; Scott et al., 2008; Tattersall and Milsom, 2003). Skin temperature (emissivity 0.95), Tb and Ta were used to calculate the animal's HLI, according to the formula: HLI = (Ts - Ta) / (Tb - Ta) (Romanovsky et al., 2002). This index ranges from 0 to 1, with 0 indicating maximum vasoconstriction, and 1 indicating maximum vasodilation.

For the determination of HLI, a separate setup had to be prepared, because the infrared rays do not cross the acrylic walls of the respirometry chamber. Two chicks at a time, implanted with a biotag to measure Tb, were placed inside a homemade apparatus built with plastic walls and a black net floor, which allowed for infrared images to be taken of the feet from below (through the net floor). A wall made of the same net as the floor separated the two chicks, which allowed them to see but not touch each other. Infrared images of *black tape (emissivity 0.95), glued to the bottom of the net floor close to the chick´s feet, were used for* Ta determinations.

Experimental designs

All the protocols were performed on unanesthetized 1 week old chicks.

Protocol 1: Effect of NMDA and $GABA_A$ receptor activities in the medullary raphe on Tb, $\dot{V}O_2$ and $\dot{V}_E$ at 31, 26 and 36ºC

Chicks were placed individually inside a respirometer at 30-31ºC for habituation (~40 min), followed by $\dot{V}O_2$, $\dot{V}_E$ and Tb measurements. Following this period, the animal received microinjection of the glutamate NMDA receptor antagonist AP5 (0.5 or 5 mmol l^{-1}), the $GABA_A$ receptor antagonist bicuculline (0.05 or 0.5 mmol l^{-1}), the $GABA_A$ receptor agonist muscimol (1 mmol l^{-1}), or vehicle (saline; 50 nL) into the raphe, and was then returned to the respirometer. The chick was continuously monitored for O_2 consumption, ventilation and Tb for ~2 hours after injection. Calculation of $\dot{V}O_2$ and $\dot{V}_E$ was based on the last 4 min (before each baseline) of each subsequent 20 min-recording interval. Tb was recorded every 20 min. Based on the results obtained at 31ºC, the doses of the drugs that did not affect Tb were chosen for the protocols to be performed under acute thermal stimulation of cold (26ºC) and hot (36ºC) conditions. The drug doses and temperatures were based on previous studies in rats and chickens (Amaral-Silva et al., 2017; Coleone et al., 2009; Dantonio et al., 2016; Dimicco and Zaretsky, 2007; De Menezes et al., 2009; Nakamura and Morrison, 2010; Zaretsky et al., 2003) and on pilot experiments. We used two doses of the NMDA and $GABA_A$ antagonists at TNZ to allow us to choose the one that did not have any effect under this condition (i.e. below the dose that showed effects) and to test it in the cold and hot conditions. This is because it would be difficult to interpret results under thermal stresses using a dose that already affected Tb at the TNZ, because of the superimposed effect of the antagonist *per se*, reducing the specificity of the results obtained during thermal challenges. Animals were exposed to 25-26ºC or 35-36ºC for ~1,5 hours, and then placed inside the respirometer and maintained at the same respective temperature for measurements, as described above. The

protocol using the $GABA_A$ agonist muscimol was performed at 31ºC, only to confirm the opposite effect compared to the antagonist bicuculline (see Discussion). Different groups of animals were used for different treatments and Ta.

Protocol 2: Effect of NMDA and $GABA_A$ receptor activities in the medullary raphe on Tb and HLI at 31, 26 and 36ºC

After a 40-min habituation interval inside the apparatus used to determine HLI, chicks previously exposed for ~1.5h to one of the three temperatures had their Tb measured and infrared images of their feet taken. Then, they received a microinjection of AP5 (0.5 mmol l^{-1}), bicuculline (0.05 mmol l^{-1}) or vehicle (saline; 50 nL) into the medullary raphe. Tb was then measured and infrared images were taken every 20 min for the next 2 hours. Different groups of animals were used for different treatments and Ta.

Histology

Following completion of the experiments, animals were deeply anesthetized with 2,2,2-tribromoethanol (250 mg kg^{-1}; Sigma-Aldrich Brasil Ltda., São Paulo, SP, Brazil) intramuscularly, and perfused through the left ventricle of the heart with saline, and then with 10% formalin. The brain was kept immersed in 10% formalin solution for at least two days, and after fixation and paraffin embedding, 15-µm sections were cut from the region of interest (medullary raphe) on a microtome (Leica RM2255, Wetzlar, Hesse, Germany). The sections were stained using the Nissl method for confirmation of the microinjection sites. Animals with microinjections located in the medullary raphe were termed the ‘intra-raphe’ group, whereas those with having microinjections located in the nuclei surrounding the medullary raphe were considered to be of the ‘peri-raphe’ group. The rate of success of intra-raphe injections was 65%.

Statistical analyses of data

The data are presented as means ± s.e.m. The results obtained were tested using a one-way analysis of variance (ANOVA) (factor: Ta) to compare pre-injection values of body mass, Tb, *f*, $\dot{V}_E/\dot{V}O_2$, lung O_2 extraction efficiency and HLI in different thermal conditions. As $\dot{V}O_2$ and $\dot{V}_E$ change allometrically with body mass, an analysis of covariance (ANCOVA) was performed to check whether the effect of the Ta was influenced by the body mass of chicks in the different groups. Repeated measures two-way ANOVA (factors: time and treatment) was used to analyze the effect of injection over time in each thermal condition. Differences among means were assessed by Sidak post-hoc test, and were considered significant at $p < 0.05$.

Effect of different temperatures on physiological variables of intact 1 week old chicks

Table 1 shows the Tb, $\dot{V}O_2$, $\dot{V}_E$, V_T, *f*, $\dot{V}_E/\dot{V}O_2$, lung O_2 extraction efficiency and body mass (from the protocol 1), and Tb, HLI and body mass (from the protocol 2) of intact chicks, i.e., at the pre-injection time, without any pharmacological treatment. In the protocol 1, the Tb of the chicks exposed for 2hs to 26ºC did not differ from that of the group at 31ºC ($p = 0.0851$), whereas exposure to 36ºC resulted in higher Tb compared to that at 31ºC ($p = 0.0007$). Body mass was different among the 3 groups of animals ($p = 0.0003$). Based on the ANCOVA, it was observed that 2hs of exposure to different temperatures affected O_2 consumption and ventilation independent of the body mass of chicks. Specifically, $\dot{V}O_2$ increased in cold ($p = 0.0188$) and decreased in heat ($p = 0.0003$) compared to the group at 31ºC. Total ventilation increased in animals at 26ºC ($p < 0.0001$) as a result of the higher V_T ($p < 0.0001$) and unchanged *f* ($p = 0.9580$), compared to the group at 31ºC. In contrast, the higher ventilation at 36ºC ($p = 0.0011$), was due to a different respiratory pattern, characterized by a lower V_T ($p < 0.0001$) and a higher *f* ($p < 0.0001$), which is a typical tachypneic response. The respiratory equivalent increased in the group exposed to heat ($p < 0.0001$), but did not change in the cold ($p = 0.3400$), compared to the chicks at 31ºC. Exposure to heat ($p < 0.0001$), but not to cold ($p = 0.2238$), reduced the lung O_2 extraction efficiency of chicks. In the protocol 2, the body mass of chicks maintained at 31ºC was higher than that of animals used in the experiments of cold exposure ($p = 0.0251$), but did not differ from the group used for heat stress ($p = 0.1135$). Tb responses to the different Ta followed the same pattern as in the protocol 1, i.e., they did not differ between cold and neutral conditions ($p = 0.0630$), but were higher

in the heat (p = 0.0009). Cold exposure induced cutaneous vasoconstriction, as the HLI was significantly lower (p < 0.0001), whereas heat exposure did significantly change this variable (p = 0.1106), compared to the thermoneutral condition.

Table 1. Variables measured before any pharmacological treatment in 1 week old chicks exposed for 2h to different ambient temperatures in protocols 1 and 2.

	Ta= 26°C	**Ta= 31°C**	**Ta= 36°C**
Protocol 1:			
Tb (°C)	41.0±0.1^{b} (18)	41.3±0.1^{b} (46)	41.8±0.1^{a} (23)
$\dot{V}O_2$ (mL min^{-1} STPD)	4.7±0.4^{a} (18)	3.4±0.2^{b} (46)	2.5±0.1^{c} (23)
$\dot{V}_E$ (mL min^{-1})	190.7±10.3^{a} (18)	119.2±4.8^{b} (46)	173.8±10.4^{a} (23)
V_T (mL)	2.9±0.1^{a} (18)	1.9±0.1^{b} (46)	0.7±0.1^{c} (23)
***f* (breaths min^{-1})**	69.3±4.2^{b} (18)	61.4±2.3^{b} (46)	300.1±24.8^{a} (23)
$\dot{V}_E/\dot{V}O_2$	45.3±4.5^{b} (18)	38.6±2.2^{b} (46)	69.7±2.9^{a} (23)
Lung O_2 extraction efficiency (%)	12.1±1.1^{a} (18)	14.1±0.7^{a} (46)	7.6±0.4^{b} (23)
Body Mass (g)	119.0±2.8^{a} (18)	104.8±2.6^{b} (46)	119.3±3.1^{a} (23)
Protocol 2:			
Tb (°C)	40.7±0.1^{b} (26)	41.0±0.1^{b} (34)	41.6±0.1^{a} (25)
HLI	0.23±0.06^{b} (26)	0.66±0.04^{a} (34)	0.82±0.05^{a} (25)
Body Mass (g)	96.4±4.9^{b} (26)	112.2±4.3^{a} (34)	109.1±2.9^{ab} (25)

Tb, body temperature; $\dot{V}O_2$, oxygen consumption; $\dot{V}_E$, pulmonary ventilation; V_T, tidal volume; *f*, breathing frequency; $\dot{V}_E/\dot{V}O_2$, respiratory equivalent; HLI, heat loss index. The number of animals in each group is shown in parentheses. Different letters indicate significant differences among groups ($p< 0.05$). All values are means±s.e.m.

Micro-injection sites at the medullary raphe

Figure 1 shows head and brain coordinates for the actual sites of microinjection in the medullary raphe. The intra-raphe microinjections (Fig. 1C) were mainly located between 1.0 mm anterior and 1.6 mm posterior to the intersection of the longitudinal (midline under the comb) and transverse (between the ears) skull sutures (Fig. 1A). A representative photomicrograph of the microinjection site in the medullary raphe is shown in Fig. 1B.

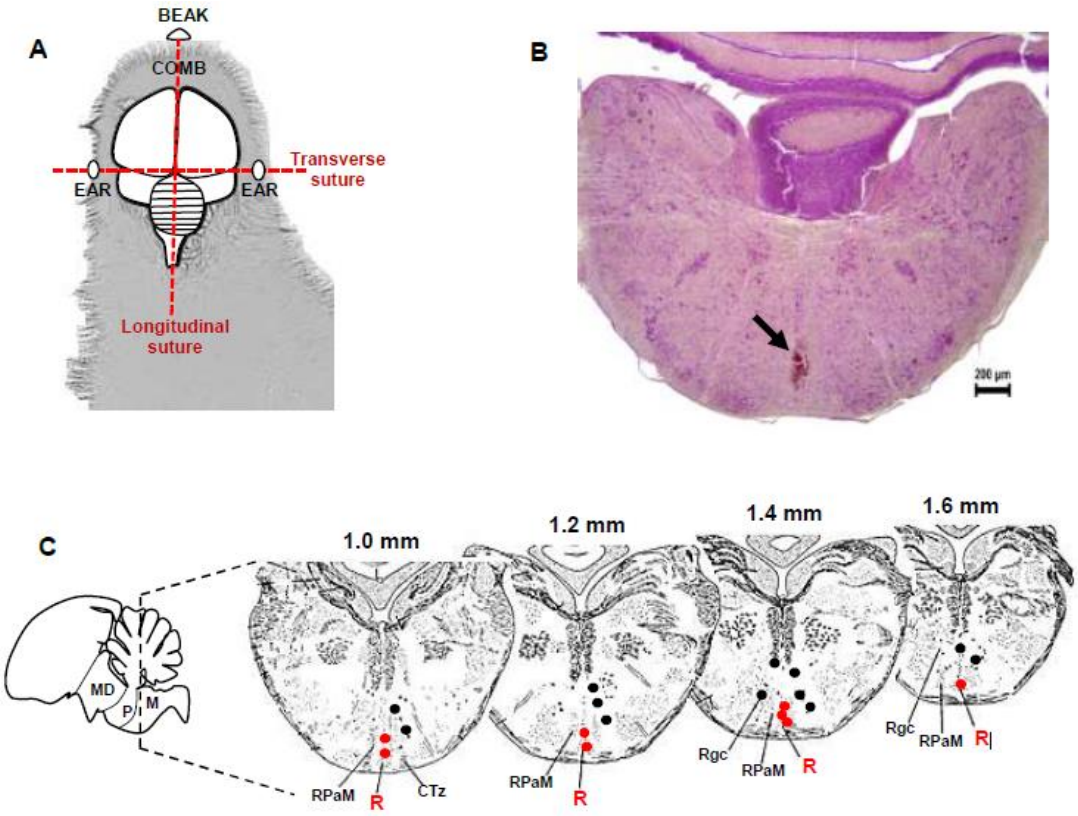


Figure 1. Head and brain coordinates for microinjections of chicks and the actual sites of injection into the medullary raphe. A) Schematic drawing showing the intersection between sutures (longitudinal and transverse) and the references used during the injection procedure, comb, ear and beak, as the skull is not visible. B) Photomicrograph of the brain of a 1 week old chick representative of the groups showing the typical microinjection site (black arrow). C) Schematic drawing of a lateral view of a chick brain showing the subdivisions of the brainstem: midbrain (MD), pons (P) and medulla (M). Coronal sections of representative diagrams show microinjection sites intra-raphe (red circles) and peri-raphe (black circles) at distances (in mm) caudal to the intersection between skull sutures (adapted from the atlas of Kuenzel and Masson, 1988). R=medullary raphe, CTz= corpus trapezoideum; RPaM=paramedian reticular nucleus; Rgc=gigantocellular reticular nucleus.

Effect of changes in the NMDA and $GABA_A$ receptor activities in the medullary raphe on Tb, $\dot{V}O_2$, and $\dot{V}_E$ of 1 week old chicks under different ambient temperatures

Figure 2 shows the effects of vehicle or AP5 (0.5 or 5 mmol l^{-1}) or saline control treatment on the Tb of chicks maintained at 31ºC (Fig. 2A), 26ºC (Fig. 2B) or 36ºC (Fig. 2C). Tb did not change after intra-raphe microinjection of saline or 0.5 mmol l^{-1} AP5 at 31ºC, but decreased with the highest dose (5 mmol l^{-1}) of AP5 from 60 to 100 min after

the microinjection (interaction between time and treatment: $p = 0.0141$, $F_{(3,31)} = 1.956$). In the cold, the chosen dose of 0.5 mmol l^{-1} AP5 caused a decrease in the Tb of animals exposed to cold 40-100 min after the microinjection (time effect: $p < 0.0001$, $F_{(2,15)} = 6.167$; treatment effect: $p = 0.0109$, $F_{(2,15)} = 6.521$). At 36ºC, however, Tb was not different between animals treated with saline or 0.5 mmol l^{-1} AP5 (treatment effect: $p = 0.9447$, $F_{(2,15)} = 0.05707$). Peri-raphe microinjections caused no effect on the Tb of chicks in any of the thermal conditions tested.

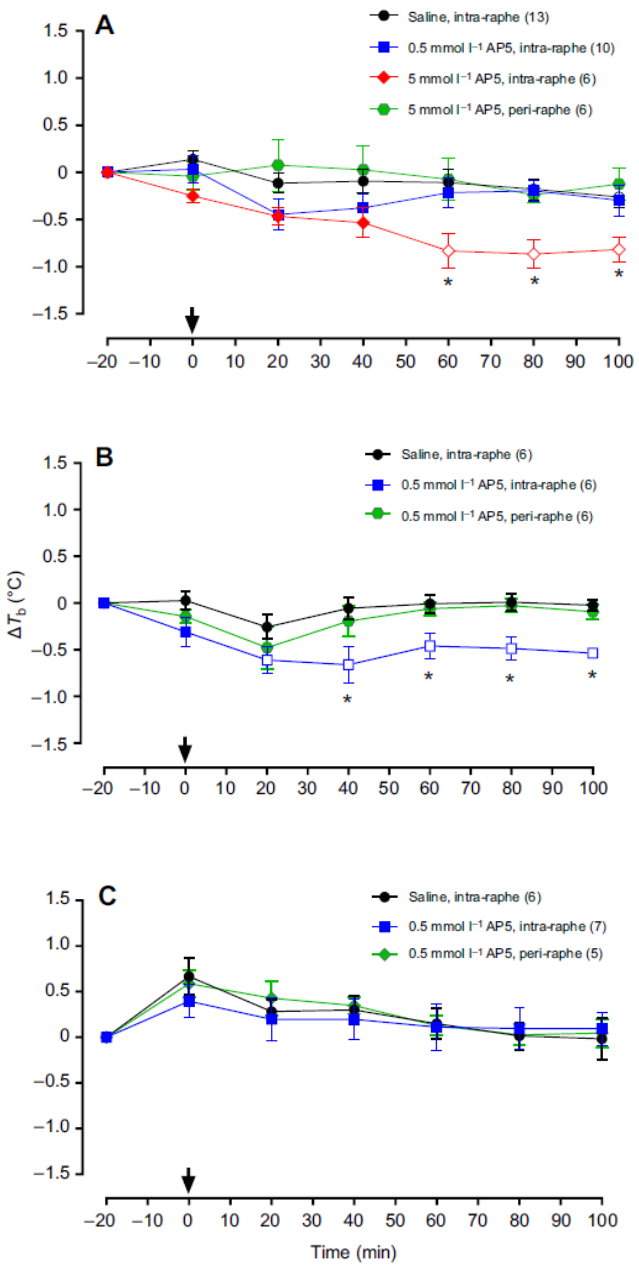


Figure 2. Effect of intra-raphe microinjection of the NMDA receptor antagonist AP5 on the body temperature (Tb) of 1 week old chicks at different ambient temperatures (Ta). Chicks were exposed to a Ta of (A) 31ºC, (B) 26ºC (B) and (C) 36ºC. The arrow indicates the time of microinjection of 0.5 or 5 mmol-1 AP5 or vehicle (saline) control into the medullary raphe (intra-raphe) or the nuclei surrounding the medullary raphe (peri-raphe). The number of animals is shown between parentheses. Data are means±s.e.m. *Significant difference (P<0.05) from saline control at the same time point. Open symbols, significant difference (P<0.05) over time from the pre-injection value in the same treatment.

Figure 3 shows the effects of vehicle, bicuculline (0.05 or 0.5 mmol l^{-1}) or muscimol (1 mmol l^{-1}) or saline control treatments on the Tb of chicks maintained in the different thermal conditions. The lower dose of bicuculline (0.05 mmol l^{-1}) did not affect

the Tb of animals at 31ºC, whereas the higher dose (0.5 mmol l^{-1}) decreased Tb 40-100 min after microinjection (interaction between time and treatment: $p = 0.0426$, $F_{(3,37)} = 1.681$) (Fig. 3A). The dose of bicuculline that did not affect Tb at 31ºC (0.05 mmol l^{-1}) was selected for treatment at 26ºC and 36ºC. Bicuculline decreased the Tb of chicks at 26ºC (time effect: $p = 0.0042$, $F_{(2,17)} = 3.419$; treatment effect: $p = 0.0061$, $F_{(2,17)} = 7.118$), but did not affect Tb at 36ºC (treatment effect: $p = 0.6189$, $F_{(2,18)} = 0.4928$). Because of to the unexpected effect of bicuculline on Tb (Fig. 3A), i.e., a reduction instead of an increase, the $GABA_A$ agonist muscimol was used to confirm the opposite effect compared to that of the $GABA_A$ antagonist. Intra-raphe microinjection of muscimol (1 mmol l^{-1}) in chicks at 31ºC increased Tb, compared with that of the saline and peri-raphe groups, at 40-100 min after the injection (interaction between time and treatment: $p = 0.0036$, $F_{(2,30)} = 2.545$; Fig. 3B).

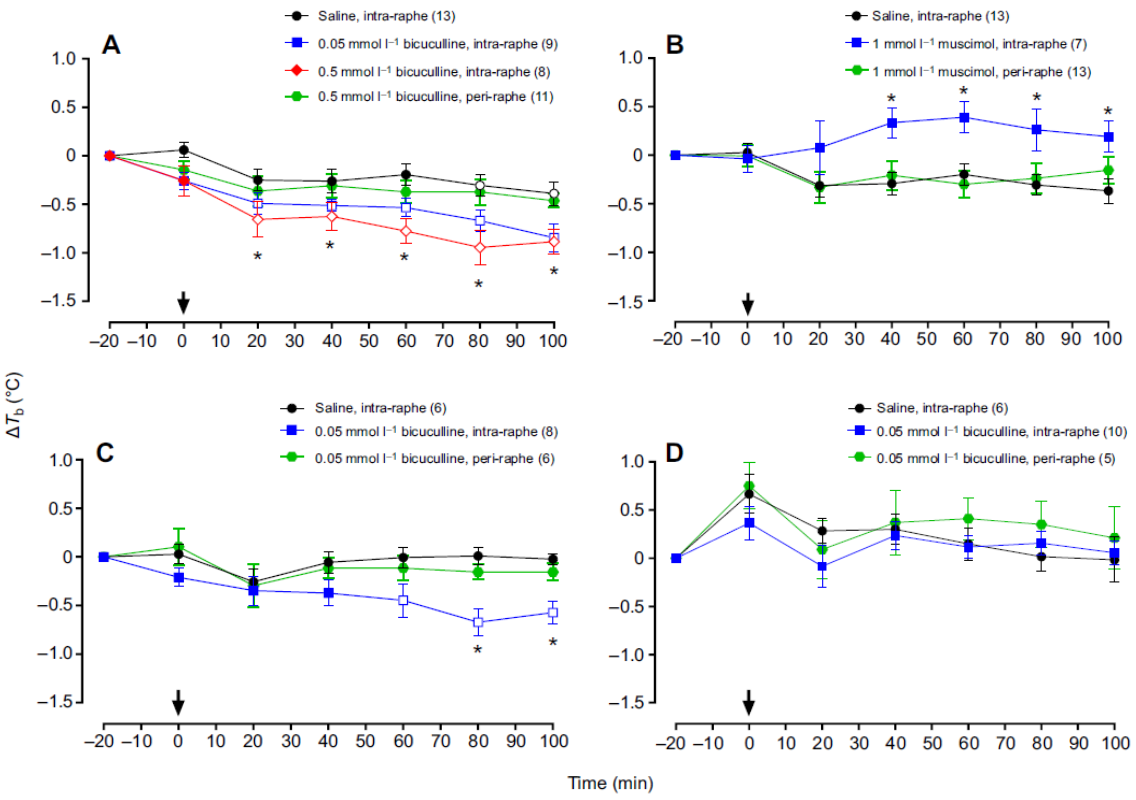


Figure 3. Effects of the intra-raphe microinjection of the $GABA_A$ receptor antagonist bicuculline and agonist muscimol on the Tb of 1 week old chicks at different Ta. Chicks were exposed to a Ta of (A) 31 ºC, (C) 26 ºC and (D) 36ºC for bicuculline treatment, and (B) 31 ºC for muscimol treatment. The arrow indicates the time of microinjection of 0.05 or 0.5 $mmol^{-1}$ bicuculline, 1 $mmol^{-1}$ muscimol or vehicle (saline) control into the medullary raphe (intra-raphe) or the nuclei surrounding the medullary raphe (peri-raphe). The number of animals is shown between parentheses. Data are means±s.e.m. *Significant difference ($P<0.05$) from saline control at the same time point. Open symbols, significant difference ($P<0.05$) over time from the pre-injection value in the same treatment.

Figure 4 shows the effects of the NMDA antagonist AP5 treatment on $\dot{V}O_2$, $\dot{V}_E$, V_T, f and $\dot{V}_E/\dot{V}O_2$ in chicks exposed to 31ºC and 26ºC. At 31ºC, intra-raphe microinjection of 5 mmol l^{-1}, but not 0.5 mmol l^{-1}, AP5 decreased $\dot{V}O_2$ 40-60 min after microinjection, compared to the saline control group (time effect: $p = 0.0003$, $F_{(3,31)} = 4.504$; treatment effect: $p = 0.0311$, $F_{(3,31)} = 3.363$). No effect of the treatments was observed for $\dot{V}_E$ (treatment effect $p = 0.9358$, $F_{(3,31)} = 0.1393$), V_T (treatment effect: $p = 0.2535$, $F_{(3,31)} = 1.428$), f (treatment effect: $p = 0.3836$, $F_{(3,31)} = 1.052$), or the respiratory equivalent (treatment effect $p = 0.2845$, $F_{(3,31)} = 1.323$). At 26ºC, oxygen consumption decreased after microinjection of 0.5 mmol l^{-1} AP5 (time effect: $p = 0.0007$, $F_{(2,15)} = 4.270$; treatment

effect: $p = 0.0050$, $F_{(2,15)} = 7.228$). No treatment effect was observed for $\dot{V}_E$ (time effect: $p = 0.0025$, $F_{(2,15)} = 3.636$; treatment effect: $p = 0.5531$, $F_{(2,15)} = 0.6121$), V_T (treatment effect: $p = 0.6366$, $F_{(2,15)} = 0.4631$) or the respiratory equivalent (time effect: $p = 0.0007$, $F_{(2,15)} = 4.221$; treatment effect: $p = 0.1267$, $F_{(2,15)} = 2.322$). In contrast, f increased significantly 20 and 40 min after AP5 microinjection at 26ºC (time effect: $p = 0.0011$, $F_{(2,15)} = 4.050$; treatment effect: $p = 0.0127$, $F_{(2,15)} = 5.702$). The peri-raphe injection groups were not affected by any treatment in any of the thermal conditions tested.

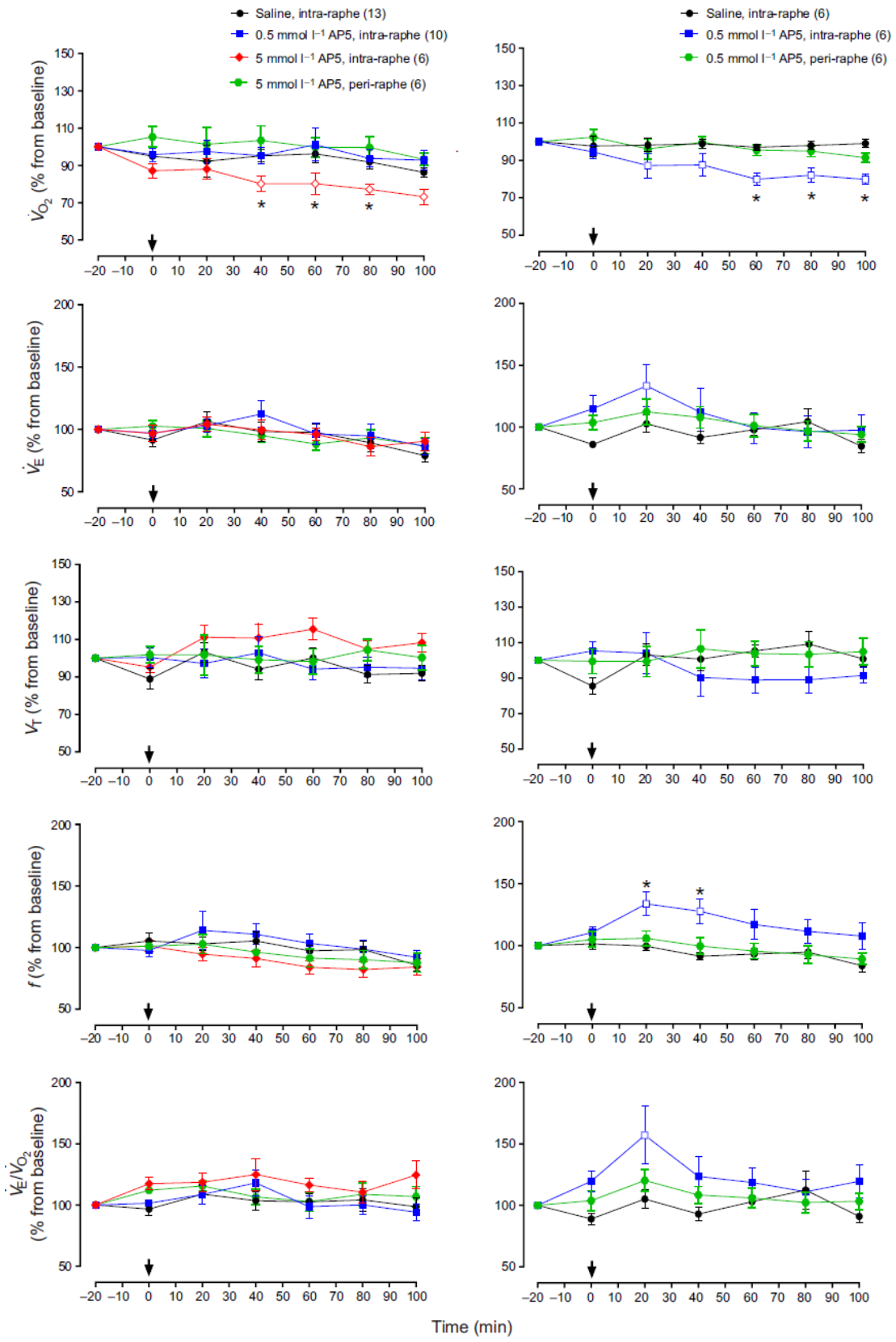


Figure 4. Effect of intra-raphe microinjection of the NMDA receptor antagonist AP5 on oxygen consumption ($\dot{V}O_2$), pulmonary ventilation ($\dot{V}_E$), tidal volume (V_T), breathing frequency (*f*) and respiratory equivalent ($\dot{V}_E/\dot{V}O_2$) of 1 week old chicks at 31ºC and 26ºC. Left, 31ºC; right, 26ºC. The arrow indicates the time of microinjection of 0.5 or 5 mmol^{-1} AP5 or vehicle (saline) control into the medullary raphe (intra-raphe) or the nuclei surrounding the medullary raphe (peri-raphe). The number of animals is shown between parentheses. Data are means±s.e.m. *Significant difference ($P<0.05$) from saline control at the same time point. Open symbols, significant difference ($P<0.05$) over time from the pre-injection value in the same treatment.

Intra-raphe bicuculline at the highest dose (0.5 mmol l^{-1}), but not 0.05 mmol l^{-1}, reduced oxygen consumption 60–100 min after microinjection in chicks at 31ºC (interaction between time and treatment: $p = 0.0420$, $F_{(3,37)} = 1.854$; Fig. 5). In contrast, bicuculline did not affect $\dot{V}_E$ (time effect: $p < 0.0001$, $F_{(3,37)} = 8.571$; treatment effect: $p = 0.2887$, $F_{(3,37)} = 1.301$), V_T (treatment effect: $p = 0.2828$, $F_{(3,37)} = 1.319$), *f* (treatment effect: $p = 0.7084$, $F_{(3,37)} = 0.7923$), or the respiratory equivalent (time effect: $p = 0.055$, $F_{(3,37)} = 3.149$; treatment effect: $p = 0.2085$, $F_{(3,37)} = 1.589$). At 26ºC, oxygen consumption decreased 80-100 min after intra-raphe microinjection of 0.05 mmol l^{-1} bicuculline (time effect: $p = 0.0422$, $F_{(2,17)} = 2.274$; treatment effect: $p = 0.0038$, $F_{(2,17)} = 7.895$) (Fig. 5). Similar to the results at 31ºC, no effect of the bicuculline treatment was observed on $\dot{V}_E$ (time effect: $p < 0.0001$, $F_{(2,17)} = 5.579$; treatment effect $p = 0.1684$, $F_{(2,17)} = 1.982$), V_T (treatment effect $p = 0.1666$, $F_{(2,17)} = 1.995$), *f* (treatment effect: $p = 0.7356$, $F_{(2,17)} = 0.3127$) or the respiratory equivalent (treatment effect: $p = 0.0902$, $F_{(2,17)} = 2.781$).

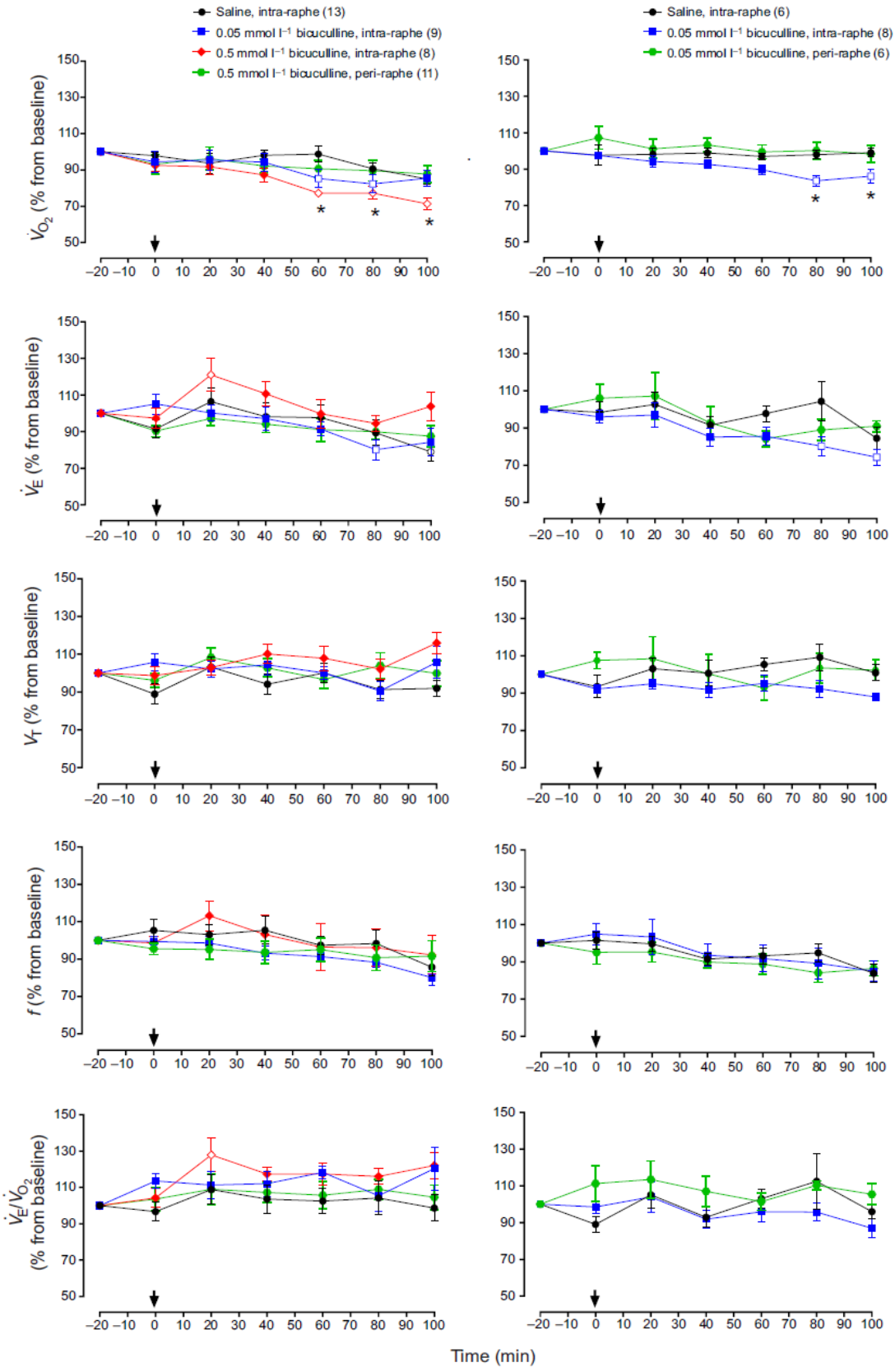


Figure 5. Effect of intra-raphe microinjection of the $GABA_A$ receptor antagonist bicuculline on oxygen consumption ($\dot{V}O_2$), pulmonary ventilation ($\dot{V}_E$), tidal volume (V_T), breathing frequency (*f*) and respiratory equivalent ($\dot{V}_E/\dot{V}O_2$) of 1 week old chicks at 31ºC and 26ºC. Left, 31ºC; right, 26ºC. The arrow indicates the time of microinjection of 0.05 or 0.5 $mmol^{-1}$ bicuculline or vehicle (saline) control into the medullary raphe (intra-raphe) or the nuclei surrounding the medullary raphe (peri-raphe). The number of animals is shown between parentheses. Data are means±s.e.m. *Significant difference ($P<0.05$) from saline control at the same time point. Open symbols, significant difference ($P<0.05$) over time from the pre-injection value in the same treatment.

AP5 and bicuculline treatment had not effect on Tb, $\dot{V}O_2$, $\dot{V}_E$, V_T, *f*, $\dot{V}_E/\dot{V}O_2$ (Supplementary Fig. 1) and HLI (Supplementary Fig. 2) tested at 36ºC.

Chicks maintained at 31ºC showed higher HLI 60-80 min after injection of 5 mmol l^{-1} of AP5 injection compared to saline treatment, because of a significant decrease in the vehicle group and a nonsignificant increase in the AP5 group at this time interval (Fig. 6A; time effect: $p = 0.0414$, $F_{(2,18)} = 2.168$; treatment effect: $p = 0.0239$, $F_{(2,18)} = 4.628$). These chicks showed a reduction in Tb from 80 to 120 min after microinjection of 5 mmol l^{-1} AP5 (interaction between time and treatment: $p < 0.0001$, $F_{(2,18)} = 5.963$; Fig. S3). At 26ºC, 0.5 mmol l^{-1} AP5 did not change the HLI of chicks (Fig. 6B; time effect: $p = 0.0019$, $F_{(1,15)} = 3.513$; treatment effect: $p = 0.0514$, $F_{(1,15)} = 4.432$), even with a reduction in Tb at 100–120 min (time effect: $p < 0.0001$, $F_{(1,15)} = 9.815$; treatment effect: $p = 0.0062$, $F_{(1,15)} = 9.929$; Fig. S3). In animals at 31°C, 0.5 mmol l^{-1} increased HLI at 60-80 min (interaction between time and treatment: $p = 0.036$, $F_{(2,19)} = 2.487$; Fig. 6C), while Tb decreased 80-120 min after injection (interaction between time and treatment: $p = 0.0055$, $F_{(2,19)} = 3.071$; Fig. S3). Treatment with 0.5 $mmol^{-1}$ bicuculline did not affect HLI at 26ºC (time effect: $p = 0.0022$, $F_{(1,16)} = 3.434$; treatment effect: $p = 0.0872$, $F_{(1,16)} = 3.295$; Fig. 6D), even with the reduction in Tb (interaction between time and treatment: $p = 0.0078$, $F_{(1,16)} = 2.897$; Supplementary Fig. 3). Thermographic images in Fig. 6 illustrate the colder feet (image ii) observed in chicks at 26ºC compared to chicks at 31ºC (image i). At 31ºC, warmer feet can be seen 80 min after intra-raphe injection of 5 $mmol^{-1}$ of AP5 (image iii; Fig. 6) and bicuculline (image iv; Fig. 6), compared to saline injection at 80 min.

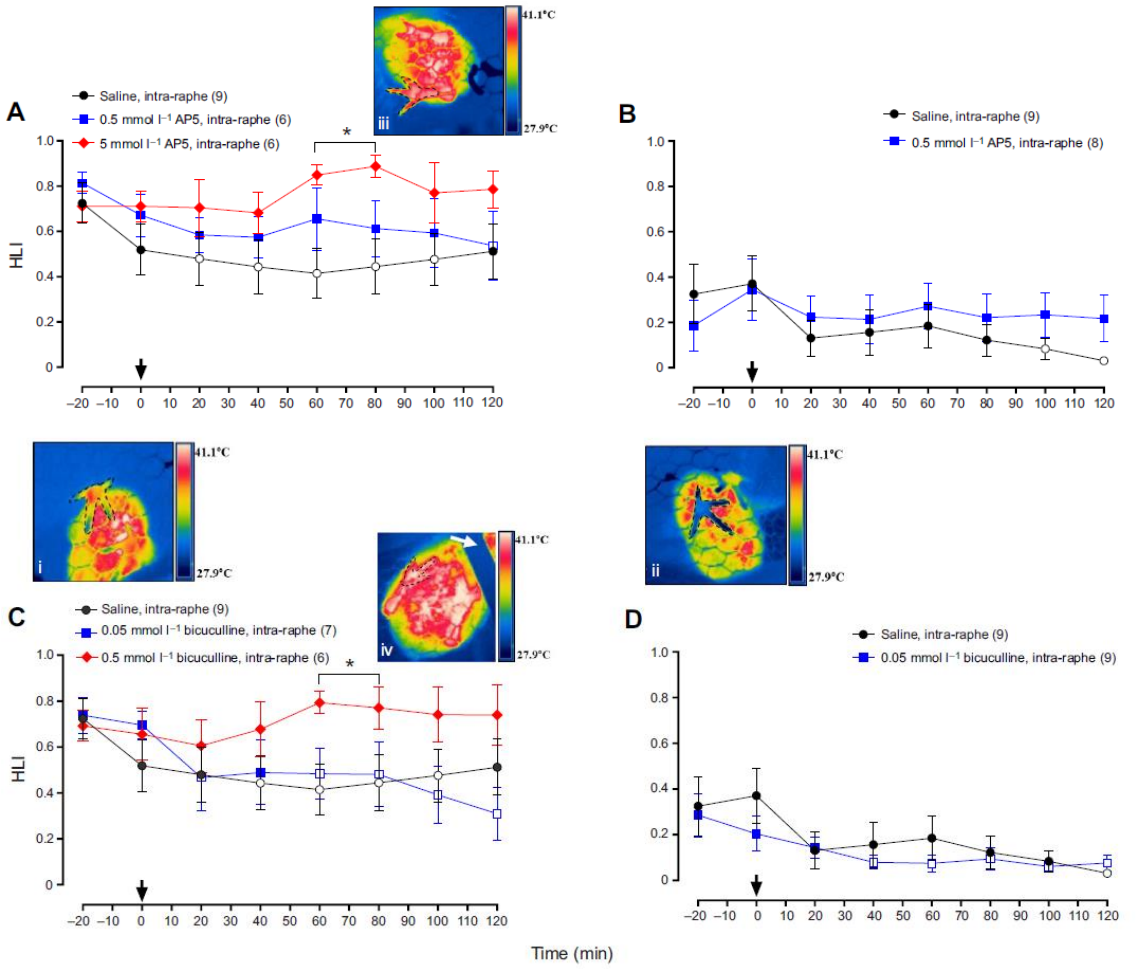


Figure 6. Effect of intra-raphe microinjection of the NMDA receptor antagonist AP5 or the $GABA_A$ receptor antagonist bicuculline on the heat loss index of 1 week old chicks at 31ºC and 26ºC. Left, 31ºC; right, 26ºC. The arrow indicates the time of microinjection of 0.5 or 5 $mmol^{-1}$ AP5 (A, B), 0.05 or 0.5 $mmol^{-1}$ bicuculline (C, D) or vehicle (saline) control into the medullary raphe (intra-raphe). The number of animals is shown between parentheses. Data are means±s.e.m. *Significant difference ($P<0.05$) from saline control at the same time point. Open symbols, significant difference ($P<0.05$) over time from the pre-injection value in the same treatment. Thermographic images of the feet of representative chicks exposed to 31ºC (i) and 26°C (ii) before microinjection, and at 31ºC 80 minutes after 5 mmol l^{-1} AP5 (iii) or 0.5 mmol l^{-1} bicuculline (iv) microinjection are also shown. The white arrow in iv indicates the black tape (emissivity= 0.95) on the floor for determination of Ta.

To our knowledge, the present study provides the first evidence of the involvement of the medullary raphe in thermoregulation of a bird species. We demonstrate in chicken chicks that the medullary raphe under excitatory glutamatergic modulation plays a role in thermogenesis stimulation and respiratory heat loss inhibition (Fig. 7A-C), while a GABAergic influence in this region is also involved in thermogenesis stimulation (Fig. 7A, D, E). In contrast, autonomic heat loss responses activated during heat exposure do not seem to be neurally modulated by the Raphe in these animals (no treatment effects at 36ºC).

In endotherms, acute decreases and increases in Ta activate physiological mechanisms involved in heat production/conservation and heat loss, respectively. A reduction in Ta stimulates cutaneous vasoconstriction, achieving its maximum at the lower critical temperature of the TNZ, when shivering and/or non-shivering thermogenesis is activated (Scholander et al., 1950; Bícego et al., 2007). At 26ºC, chicks showed pronounced skin vasoconstriction (heat conservation), higher O_2 consumption (thermogenesis activation) and higher ventilation as a result of increased V_T (gas exchange facilitated), leaving Tb unchanged (Table 1). In this case, the air convection requirement and lung O_2 extraction efficiency were not affected, indicating an adequate balance of air convection necessary to match the O_2 supply and demand in this condition. In contrast to cold, heat exposure increased the Tb of chicks (hyperthermia), combined with an increased in breathing frequency (*f*) and a reduced V_T, characterizing a thermal tachypnea, or panting (Bicego et al., 2007; Mortola and Maskrey, 2011). Thermal tachypnea is indeed the main evaporative heat loss response activated in Galliformes and Passeriformes (McKechnie et al., 2016), and becomes functional even before hatching in chickens (Bícego and Mortola, 2017). The change in the ventilatory pattern to rapid and

superficial ventilation is a strategy that facilitates heat loss without altering resting gas exchange owing to greater ventilation of the anatomical dead space (Mortola and Maskrey, 2011). Under conditions of intense thermal stress, hyperventilation can occur with increased V_T and gas exchange, resulting in respiratory alkalosis as a result of excessive CO_2 elimination (Mortola atnd Maskrey, 2011). In chicks in the present study, despite the increased air convection requirement indicating hyperventilation, the respiratory pattern retained with a high *f* and low V_T, which might have prevented alkalosis, especially considering the reduced O_2 lung extraction efficiency (Table 1), which may indicate a reduced CO_2 release as well. Depending on the species of adult birds and the severity of heat stress, metabolic rate may rise, remain constant or decrease (Salt, 1952; 1964; Arad and Marder, 1982; Mckechnie et al., 2016). Chicks maintained at 36ºC for 2 h had lower oxygen consumption than those at 31ºC, which may reflect metabolic reduction of some internal organs as a consequence of a shift in the blood perfusion from the inner body to the skin and/or may be related to the reduced lung O_2 extraction efficiency (Table 1). Future investigations of organ-by-organ metabolism and lung perfusion of chicks in the heat will enable testing of these hypotheses. Taken together, the results in intact chicks at different Ta (pre-microinjection values; Table 1) indicate that 1 week old broiler chicks are able to activate autonomic thermoeffectors in both cold (~5ºC below 31ºC) and hot (~5ºC above 31ºC) conditions, being more sensitive to the latter stimulus. Thus: i) 31ºC seems to be well inside the TNZ of these chicks; ii) 36ºC is clearly above TNZ, as evidenced by the activation of thermal tachypnea, a known evaporative heat loss response; and iii) 26ºC seems to be below TNZ, as evidenced by intense cutaneous vasoconstriction, higher thermogenesis and an unchanged air convection requirement.

The intra-raphe microinjections of AP5 and bicuculline in chicks promoted a reduction in Tb at 31ºC (higher doses) and at 26ºC (lower doses), together with a metabolic reduction. This indicates a stimulatory role for the raphe on thermogenesis in these animals, similar to that which is observed in rats (Morrison et al., 2012; Morrison et al., 2014). In these mammals, the raphe receives glutamatergic stimulatory afferents from more rostral regions, such as the dorsomedial hypothalamus (DMH), which, in turn, is inhibited by GABAergic neurons from the medial preoptic area (MPA) (Morrison et al., 2012; Morrison et al., 2014). During exposure to cold, there is inhibition of the MPA, which disinhibits the DMH, consequently stimulating neurons of the medullary raphe that activate both the sympathetic flow for non-shivering thermogenesis in the brown adipose tissue (Morrison, 1999) and cutaneous vasoconstriction in the tail pathways (Blessing and Nalivaiko, 2001; Tanaka et al., 2002), as well as shivering (Morrison and Nakamura, 2011; Morrison et al., 2012, 2014). A functional connection between the medullary raphe and skeletal muscle, the main site for shivering and non-shivering thermogenesis in birds (Bicudo et al., 2002; Rowland et al., 2015), still need to be demonstrated. Moreover, glutamatergic stimulation of the raphe in chicks also seems to be important for keeping *f* low during cold exposure to avoid increased dead space ventilation and, thus, heat loss through evaporation. Connections between the medullary raphe and the respiratory nuclei have been demonstrated in rats (Connelly et al., 1989; Ptak et al., 2009) and, based on our results, appear to be present in chickens as well.

The hypothermic effect of bicuculline, similar to that of AP5, in the medullary raphe of chicks was confirmed by the hyperthermic effect of the $GABA_A$ agonist muscimol. Metabolic reduction was also a consistent result for $GABA_A$ and NMDA antagonism. Thus, it appears that both glutamate and GABA activate raphe neurons to induce thermogenesis in chicks. These results are intriguing as they suggest an excitatory effect

of GABA, classically known to be the major inhibitory neurotransmitter in the brain of mammals and birds (Herlenius and Lagercrantz, 2004; Wu and Sun, 2015). There are at least two explanations for such results. First, there is a possibility that GABAergic neurotransmission in the raphe is at an immature phase of its development in the chicks. At least in rodents, GABA changes from an excitatory to an inhibitory action during postnatal development (Herlenius and Lagercrantz, 2004; Miles, 1999). During the initial stage of development, the intracellular concentration of Cl^- is high in many brain neurons of rats, which results in depolarization of the plasma membrane (excitation), caused by the opening of Cl^- channels on GABA receptors upon activation by GABA (Herlenius and Lagercrantz, 2004; Miles, 1999). During brain maturation, a membrane KCC2 transporter pumps Cl^- out to keep intracellular concentrations of this ion low, which causes the cell to hyperpolarize (inhibition) when GABA binds its receptor. Some studies indicate that this may be the case for birds too (Antrobus et al., 2012; Curry and Lu, 2016), which makes our results even more interesting because of the possibility of observing this functional difference of GABA in a region involved in thermoregulation in chicks. Second, there is evidence to suggest that the physiological functions modulated by the raphe in rodents may be coordinated, in part, by a specialized subset of serotoninergic neurons, distinguishable and, perhaps, functionally divisible by coexpression of various neurotransmitters, such as glutamate, GABA, thyrotropin releasing hormone, and substance P (Hennessy et al., 2017). Furthermore, the raphe also has GABAergic neurons, and these neurons are also involved in other aspects of homeostatic regulation, such as Tb, heart rate and blood pressure regulation (Cao and Morrison, 2003; Cao et al., 2006; Cerri et al., 2013; DiMicco et al., 2006; Zaretsky et al., 2003). According to Iceman et al. (2014), the GABAergic raphe neurons may overlap responses to multimodal stimuli, consistent with their diverse homeostatic roles. Thus, the antagonists of glutamatergic and

GABAergic receptors may be acting on a different subset of neurons present in the raphe, which participate in different functions. Independent of the mechanistic explanation, the existence of two excitatory influences in the raphe of chicks, i.e., glutamatergic and GABAergic, may indicate a protective neural mechanism for supporting thermogenesis during the initial phase of life, when passive heat loss to the environment is more accentuated and high energy is expended to maintain homeothermy and growth.

Regarding the exposure to heat, we did not observe any effect of treatments with glutamate and GABA receptor antagonists on any of the variables tested (Figs. 2C, 3D, S1 and S2). These results indicate that the medullary raphe plays no role in the neural regulation of Tb during a heat challenge in early life in precocious birds, which contradicts the reported role of this brain region in heat conservation inhibition in adult rodents subjected to heat (Morrison and Nakamura, 2011). However, it should be noted that there is no corresponding study in young rodents.

In conclusion, the present study demonstrates, for the first time, a specific thermoregulatory role of the medullary raphe for the activation of heat production and conservation in chicks. Glutamatergic and GABAergic influences on the raphe seem to be important for thermogenesis activation, while specific glutamate stimulation of the raphe is also relevant to inhibit a respiratory heat loss pathway (Fig. 7). Two doses of the respective antagonists were tested at temperatures within the TNZ, and the lower dose, which had no significant effect under these conditions, changed Tb and thermoeffectors during cold exposure. This means that at temperatures within the TNZ, raphe would be minimally affected by glutamate and GABA neurotransmissions, but in the cold, there would be increased activation of these neural pathways from skin thermoreceptors. The absence of any effect of the antagonists at 36^{o}C adds support to the specificity of the role of raphe in the neural modulation of thermoeffectors for heat production and

conservation, but not for heat loss; the neural control of the latter requires further investigation. Moreover, the double influence of glutamate and GABA neurotransmission in the raphe for activation of thermogenesis in chicks suggests a protective neural mechanism for maintaining a high metabolic rate at this age, when passive heat loss is facilitated, and energy is needed to maintain homeothermy and growth. Finally, the present results suggest a convergent neural regulation of thermogenesis in endotherms, as birds and mammals evolved endothermy through independent pathways (Legendre and Davesne, 2020; Polymeropoulos et al., 2018).

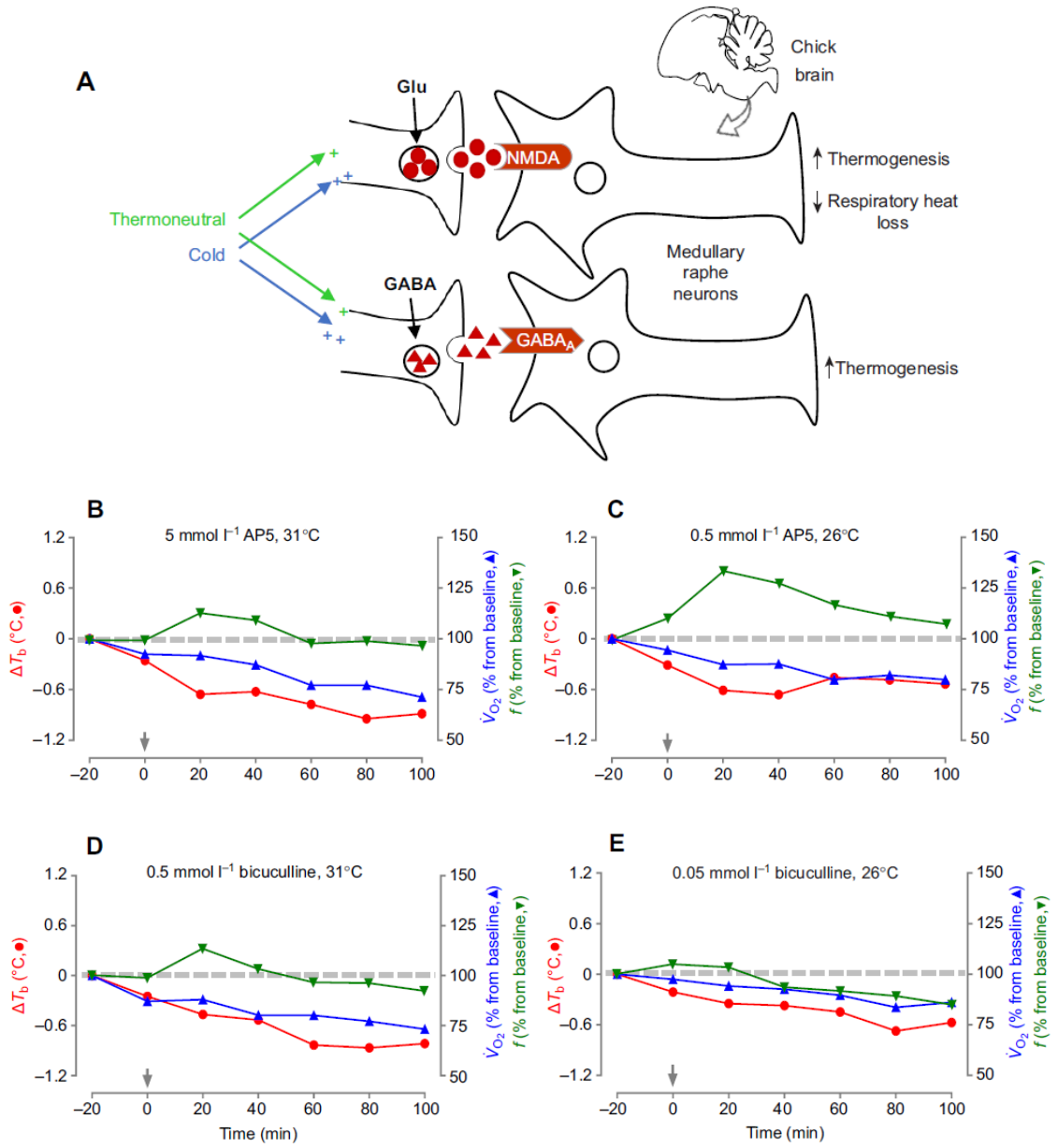
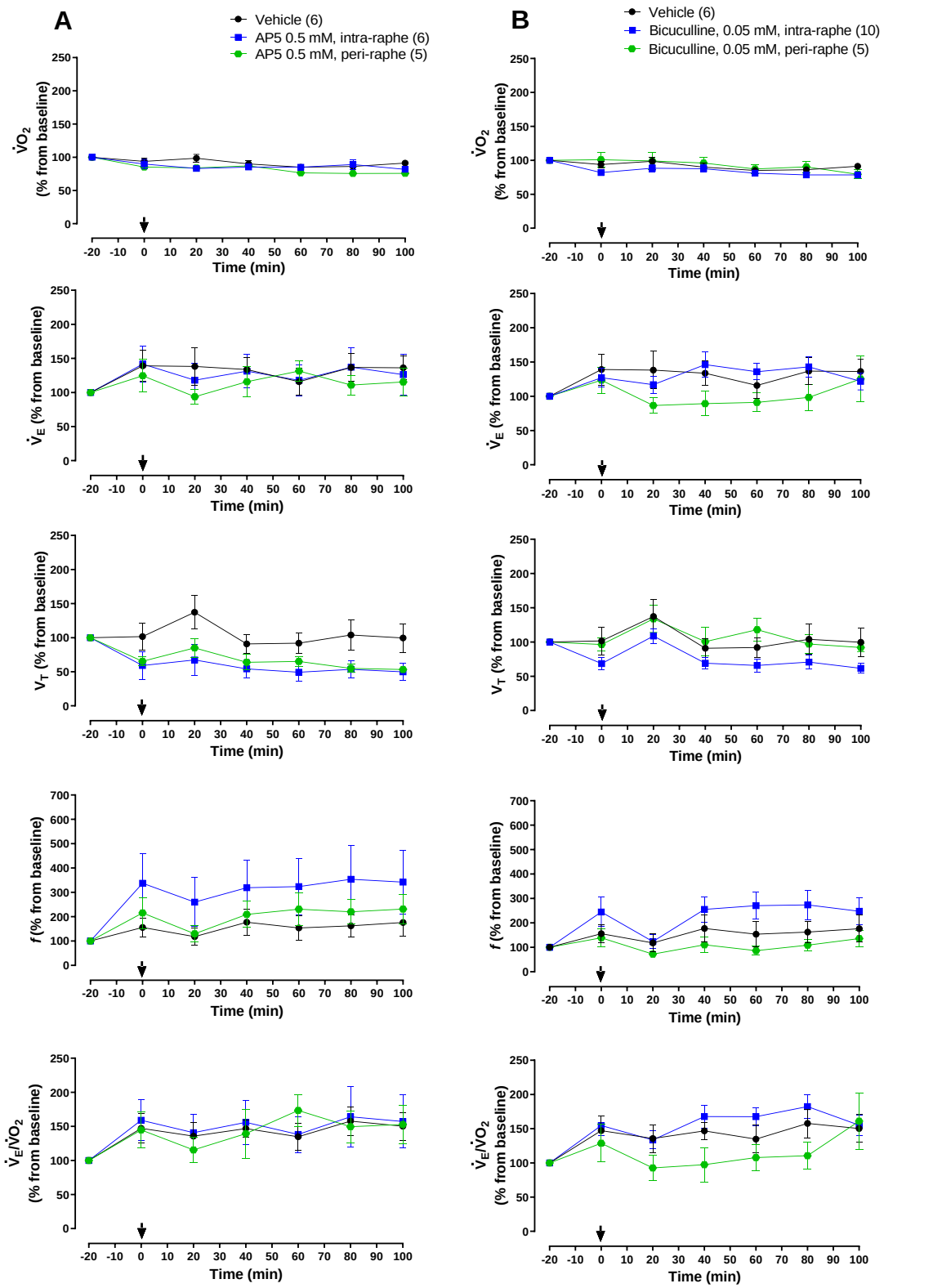
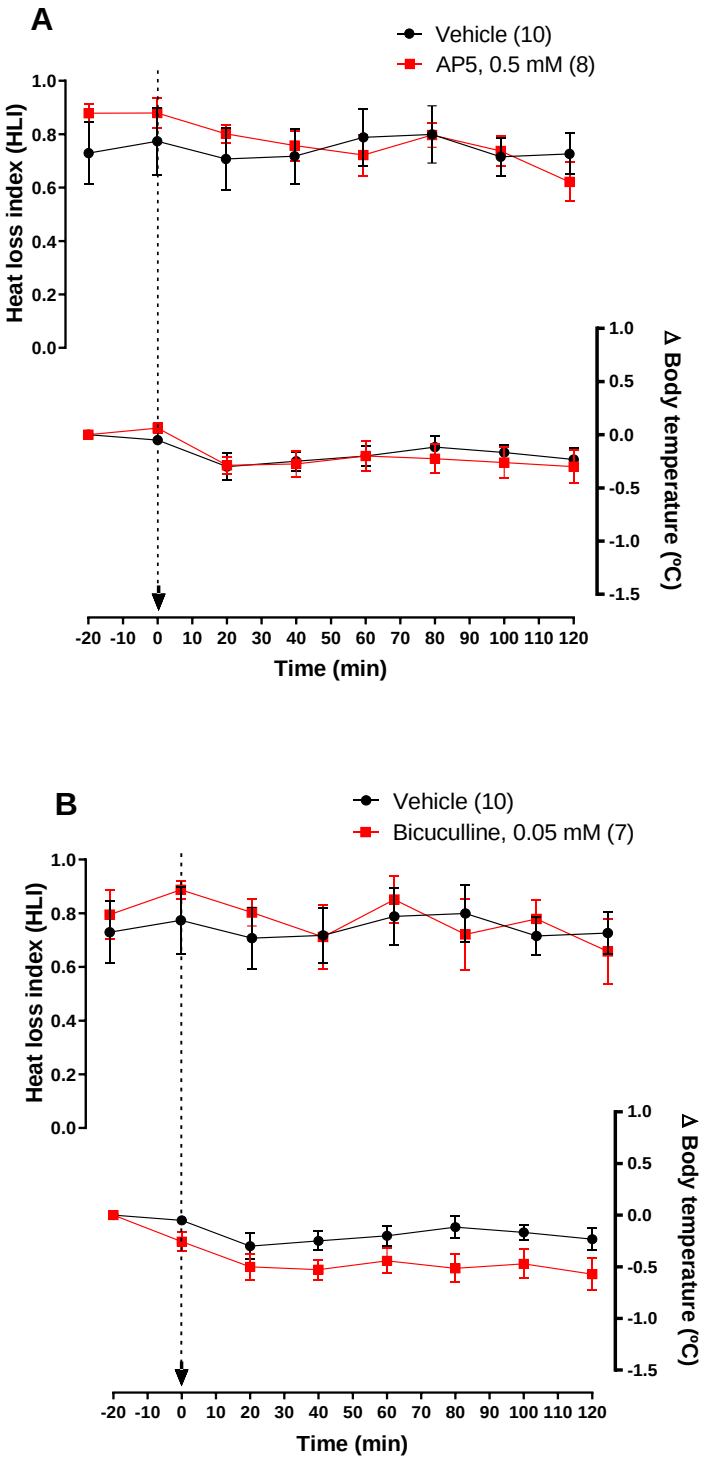


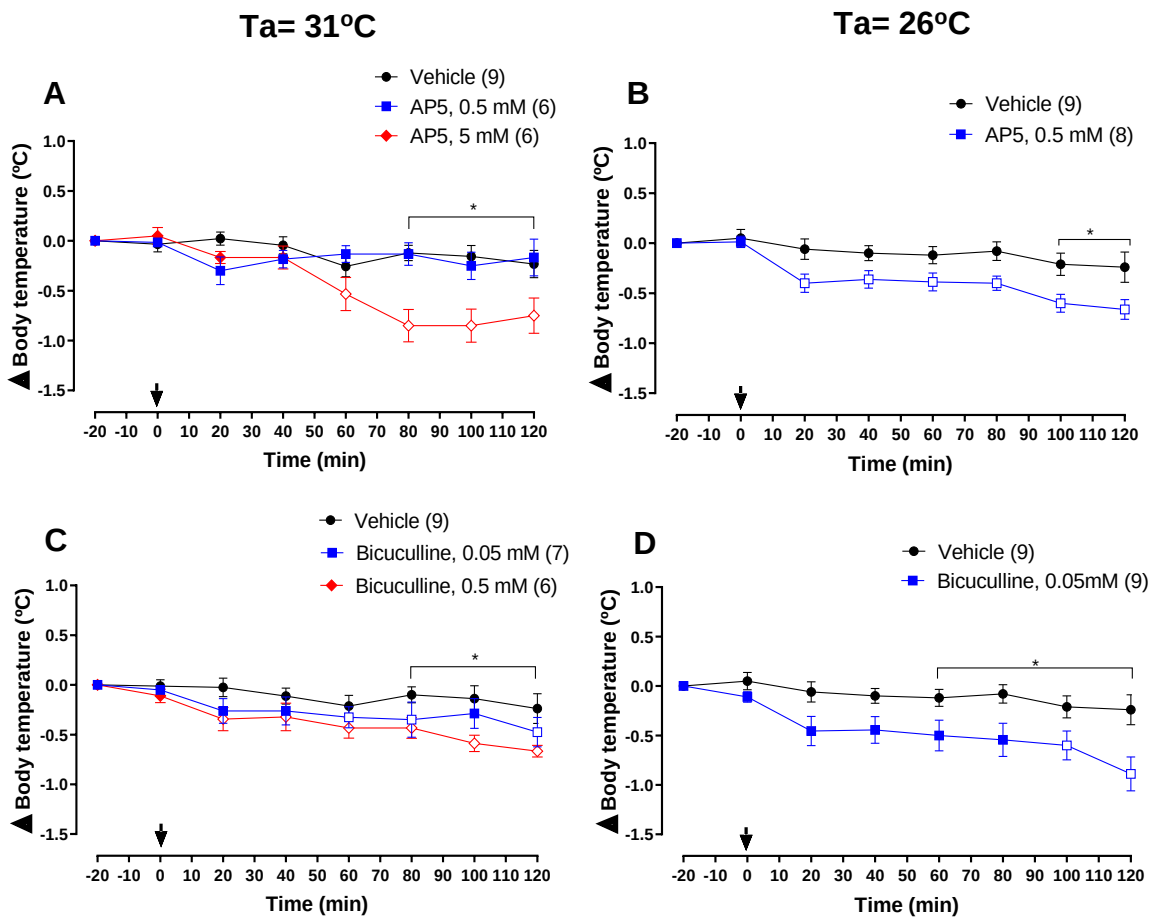
Figure 7. Thermoregulatory role of the medullary raphe in the activation of heat production and conservation in chicks. (A) Schematic drawing depicting glutamatergic and GABAergic influences on medullary raphe neurons modulating thermoeffectors involved in heat gain and heat loss in 1 week old chicks at thermoneutrality and in the cold. Glutamate (Glu) binds on NMDA receptors in raphe neurons, increasing thermogenesis and inhibits respiratory heat loss to keep Tb constant. The main results (B, C) that support this idea are those for the reduction of Tb by the NMDA antagonist AP5 (5 mmol l^{-1} at 31ºC and 0.5 mmol l^{-1} at 26ºC, C), firstly by an increase in f associated with a slight decrease in $\dot{V}O_2$ and then only by decreased $\dot{V}O_2$. These responses are more accentuated at 26ºC (C, at a lower dose) than at 31ºC (B). The influence of GABA on $GABA_A$ receptors in raphe neurons is more complex (see details in the text), but it seems to show a similar effect on thermogenesis (A, D, E) but minimum effect on f (D).



Supplementary Figure 1. Effect of intra-raphe microinjection of the NMDA receptor antagonist AP5 (A) and the $GABA_A$ receptor antagonist bicuculline (B) or vehicle on oxygen consumption ($\dot{\mathbf{V}}\mathbf{O_2}$), pulmonary ventilation ($\dot{\mathbf{V}}_\mathbf{E}$), tidal volume ($V_T$), breathing frequency (*f*) and respiratory equival*ent* ($\dot{\mathbf{V}}_\mathbf{E}/\dot{\mathbf{V}}\mathbf{O_2}$) of one-week-old chicks at 36ºC. Arrow indicates the time of microinjection. Number of animals is shown between parentheses. Data are means ± s.e.m.



Supplementary Figure 2. Effect of intra-raphe microinjection of the NMDA receptor antagonist AP5 (A) and the $GABA_A$ receptor antagonist bicuculline (B) or vehicle on the heat loss index of one-week-old chicks at 36ºC. The arrow indicates the time of microinjection. Number of animals is shown between parentheses. Data are shown as means ± s.e.m.



Supplementary Figure 3. Effect of intra-raphe microinjection of the NMDA receptor antagonist AP5 (A and B) and the $GABA_A$ receptor antagonist bicuculline (C and D) or vehicle on the body temperature of one-week-old chicks at 31ºC and 26ºC. These body temperature data are from the chicks used in protocol 2 to calculate heat loss index (see Fig. 6) under different treatments and conditions. Arrow indicates the time of microinjection. Number of animals is shown between parentheses. Intra-raphe: microinjection located in the medullary raphe. Data are means ± s.e.m. *significant difference ($p < 0.05$) from vehicle at the same time. Open symbols mean significant difference ($p < 0.05$) over time from the pre-injection value in the same treatment.

CAPÍTULO 2

GABAergic, but not serotonergic neurons of the medullary raphe inhibits cold-induced thermogenic response in precocious birds

ABSTRACT

In vertebrates, serotoninergic neurons are located almost exclusively in the raphe, being a neurotransmitter that plays an important role in numerous functions, such as the regulation of body temperature (Tb). In addition to serotonergic neurons, in mammals, raphe also has GABAergic neurons and other phenotypes. Recently we showed that medullary raphe plays a role in thermogenesis regulation in chicken chicks, but the nature of the neurons involved was not addressed. Thus, the aim of this study was to investigate the specific participation of serotoninergic and GABAergic neurons of the medullary raphe in the thermogenesis of one-week-old chicks. To this end, animals received intracerebroventricular microinjection (fourth ventricle) of anti-SERT-SAP or anti-GAT1-SAP, which promotes specific chemical damage to 5-HT neurons and GABAergic neurons respectively, or vehicles (IGG-SAP and PBS), 7-8 days before the experiments. Body temperature was measured in chicks implanted with a mini temperature sensor in the coelomatic cavity, and oxygen consumption (index of thermogenesis) was determined using open-flow respirometry under neutral (31ºC), cold (25ºC) and warm (36°C) conditions for 2 hs. Protocols were approved by the local Animal Care and Use Committee (CEUA of FCAV/Unesp, nº 013907/17). An increase in c-Fos expression in raphe neurons was observed only in animals exposed to cold, compared to those in thermoneutral and heat conditions, but with only about 10% of neurons corresponding to serotonergic ones. In addition, the lesion of anti-SERT-SAP was effective in destroying about 70% of the serotonergic neurons of the medullar raphe, but without affecting any of the variables evaluated in neutral, cold and warm conditions. The anti-GAT1-SAP, on the other hand, damaged 55% of the GABAergic neurons of the bulbar raphe promoting a reduction in Tc, O_2 consumption and ventilation, in cold and thermoneutral conditions.

Thus, the results indicate that, different like occurs in mammals, the modulation of thermogenic and ventilatory responses to cold by medullary raphe in chicken chicks does not involve serotonergic neurons. Among other phenotypes of neurons possibly involved in this neural modulation, we evidence a role for GABAergic raphe neurons. Such results open a new perspective related to the neural regulation of body temperature in endothermic vertebrates.

Keywords: Brainstem, c-Fos, Serotonin, GABA, Chick, Body Temperature, Oxygen Consumption, Thermal Tachypnea

Serotonin [5-hydroxytryptamine (5-HT)] is a monoamine neurotransmitter highly conserved across species (Beliveau et al., 2017; Marin et al., 2020), which plays an important role in numerous functions (Berger et al., 2009; Olivier, 2015; Sodhi and Sanders-Bush, 2004), including the regulation of body temperature (Tb) (Hodges et al., 2008; Madden and Morrison, 2010; Morrison, 2004; Morrison and Nakamura, 2011; Nakamura and Morrison, 2007; Nakamura and Morrison, 2011). In the vertebrate brain, serotonergic neurons are located almost exclusively in a system called raphe (raphe nuclei; Steinbusch, 1981), in an well conserved anatomical organization (Parent, 1981), which has rostral and caudal projections to all regions of the brain (Hay-Schmidt, 2000; Jacobs and Azmitia, 1992; Lauder, 1993).

The raphe nuclei are clusters of neurons arranged in a column that extends along the rostrocaudal axis, from the midbrain to the medulla (Hay-Schmidt, 2000). In mammals, the medullary raphe has three subdivisions, the obscurus (ROb), the pallidus (RPa) and the magnus (RMg) raphe nuclei and, although they present heterogeneous neuron phenotypes, the main neurotransmitter is 5-HT (Gao and Mason, 2001; Jacobs and Azmitia, 1992; Kiyasova and Gaspar, 2011; Lidov and Molliver, 1982; Mason, 1997). Regarding birds, the arrangement of the medullary raphe follows a topography equivalent to that of mammals, but the subareas and different neuron populations were not identified (Kuenzel and Masson, 1988; Okado et al., 1992). 5-HT neurons in the avian brainstem are divided into several groups based on histological features (location, size, and shape) and immunoreactivity (Yamada et al., 1984) or fluorescence chemical reactivity for the detection of monoamines (Dubé and Parent, 1981; Ikeda and Gotoh, 1971).

Serotonergic neurons in the medullary raphe synthesize others transmitters in addition to serotonin, such as γ-aminobutyric acid (GABA), glutamate and different types of peptides, including substance P (Blessing et al., 1986; Millhorn et al., 1987; Nakamura, 2004; Sasek et al., 1990; Stornetta et al., 1999; Stornetta et al., 2004). The activity of 5-HT neurons is under control exerted by different neurotransmitter systems, including serotonin itself, noradrenaline, dopamine, histamine, glutamate, GABA, glycine and several neuropeptides (Jacobs and Azmitia 1992). The raphe nuclei also have different types of neurons, such as GABAergic (Cao and Morrison, 2003; Cerri et al., 2013; DiMicco et al., 2006; Iceman et al., 2014; Zaretsky et al., 2003). The inhibitory neurotransmitter GABA plays an essential role in the regulation of 5-HT neurons, operating through local GABAergic interneurons as well as distal GABAergic afferences that project to the raphe nuclei (Gervasoni et al. 2000).

We recently reported the first evidence of a thermoregulatory role of medullary raphe in chicken chicks (Cristina-Silva et al., 2021). Our results suggest that glutamatergic and GABAergic influences on the medullary raphe of chicks stimulate thermogenesis, besides the glutamatergic stimulation prevents tachypnea, without having any role in warmth defense responses. In that study, the nature of the medullary raphe neurons involved in thermogenesis was not addressed, but based on the literature in mammals, we speculate that they are serotonergic and possibly GABAergic. The thermogenic effect of medullary raphe is well known in rodents. Specifically the rostral raphe pallidus (rRPa) is the primary site where descending signals affect sympathetic outflow to brown adipose tissue (BAT; Morrison, 2004a; 2004b; Tan & Knight, 2018), a main site for nonshivering thermogenesis in those animals. Serotonin may be the neurotransmitter in this case, because cats exposed to cold increase 5-HT neuron activity on medullary raphe (Martín-Cora et al., 2000), and knockout mice without central 5-HT

neurons, rapidly become hypothermic when exposed to cold (Hodges and Richerson, 2008).

GABA is classically known to be the major inhibitory neurotransmitter in the brain (Herlenius and Lagercrantz, 2004; Wu and Sun, 2015). Thus, it is intriguing the observation of similar responses between GABAergic and glutamatergic inhibitions on the raphe thermogenic effect in chicks (Cristina-Silva et al., 2021). This may suggest this region has different population of neurons, possibly expressing GABA, which would be inhibited by the GABAergic influence. In fact, mammalian raphe nuclei also has GABAergic neurons beside serotonergic and other phenotypes, with possible co-expression of neurotransmitters in some neurons (Belin et al., 1983; Gras et al., 2002; Kachidian et al., 1991; Millhorn et al., 1987; Shikanai et al., 2012; Stamp and Semba, 1995).

Based on what was presented above, we aim to test two hypotheses: i) there are populations of serotonergic and GABAergic neurons in the medullary raphe; and ii) these neurons are involved in the thermoregulatory function of the medullary raphe. To this end, we performed immunostochemistry for c-Fos (index for neuronal activation) and 5-HT and in situ hybridization for GABA (for identification of neurons). Then, we investigated the effect of specific lesions of serotonergic and GABAergic neurons in the medullary raphe on body temperature, oxygen consumption (index of thermogenesis) and pulmonary ventilation in one-week-old chicks exposed to different ambient temperatures.

Animals

The experiments were performed in one-week-old chicks of *Gallus gallus domesticus* (Cobb 500 lineage), weighing 110-130 g with no sex distinction. Fertile eggs weighing 60 ± 3 g or 1-day-old chicks weighting 50-60 g were acquired from a commercial local hatchery (Globoaves and Pluma Agro Avicola, SP, Brazil). Eggs were incubated in a serial manner, a few per day, in an automatic incubator (Premium Ecologica, Belo Horizonte, Brazil) with standard temperature (37.5°C) and relative humidity (60%), and rotation every 2 h. This serial incubation was done in order to obtain a few chicks of the same age per day for respirometry experiments. On the day 19 of incubation, eggs were transferred to a hatcher (Premium Ecologica, Belo Horizonte, Brazil) kept at 37.5°C and 60% humidity. After hatching, chicks were reared in temperature-controlled chambers (Premium Ecológica, Belo Horizonte, MG, Brazil) in a decreasing range of 32.5ºC-30.5°C, from day 0 to day 8. Animals were under a light-dark cycle of 14:10 h (lights on at 6:00 am) and with a standard diet (initial feed for chicks; Rostagno et al., 2005) and water *ad libitum*. All experiments were performed between 8:00 am and 5:00 pm and were conducted with approval of the local Animal Care and Use Committee (CEUA) from FCAV/UNESP, in agreement with the guidelines of the National Council of Control in Animal Experimentation (CONCEA-Brazil).

Drugs

The anti-SERT-SAP (IT-23; Advanced Target System, USA) was used to promote specific chemical damage to 5-HT neurons. Anti-SERT-SAP is an immunotoxin that uses the saporin toxin (SAP, ribosome-inactivating protein) conjugated to the antibody for the

5-HT reuptake transporter (anti-SERT), conferring specificity on serotonergic neurons. A concentration of 0.5 μM of anti-SERT-SAP was used for each chick (Da Silva et al., 2011; Da Silva et al., 2013; Dias et al., 2007; Nattie et al., 2004).

The anti-GAT1-SAP (IT-32; Advanced Target System, USA) was used for eliminating cells that express GABA-1 transporter in multiple species (GAT-1). GAT-1 is a sodium-coupled neurotransmitter transporter responsible for moving GABA across cell membranes and the conjugated with SAP eliminates GABAergic neurons, sparing other neurons. The concentration of anti-GAT1-SAP was 1.5 μM (Pang et al., 2011; Radley et al., 2009).

SAP conjugates are powerful and specific lesioning agents using ribosome-inactivating protein, saporin (from the seeds of the plant, *Saponaria officinalis*) and they are bound to a targeting agent (anything that is recognized on the cell surface and internalized). After the conjugate to be administered in the cells (*in vitro* or *in vivo*), the targeting agent seeks out and binds to its target on the cell surface, the conjugate is internalized, and saporin breaks away from the targeting agent and inactivates the ribosomes, which causes protein inhibition and, ultimately, cell death.

The control animals received IgG-SAP (IT-18; Advanced Target System, USA) or phosphate buffered saline (0.01M, pH 7.4). Mouse IgG-SAP is a chemical conjugate of pre-immune mouse IgG antibody and the ribosome-inactivating protein, saporin. This control molecule is the same molecular weight, consists of similar, comparable materials and is synthesized with the same protocols as the targeted conjugates. The difference is the cell-specific targeting agents are replaced with "blanks," antibodies or peptides that have no specificity, and no ability to target cells.

Surgical procedure and lesions

All surgical procedures were performed on 0-1 day-old animals (50-60 g), i.e., 7-8 days before the experiment, ensuring the time required for neuronal lesion (Nattie et al., 2004). First, chicks were anesthetized inside a chamber with 5% isoflurane in pure O_2 for ~2 min for induction and received intramuscular injections of antibiotic (enrofloxacin, intramuscular; 10 mg kg^{-1}; Bayer SA, São Paulo, SP, Brazil) and analgesic anti-inflammatory (flunixin meglumine, intramuscular; 2.5 mg kg^{-1}; MSD Saúde Animal, São Paulo, SP, Brazil) agents to prevent infection and post-surgical discomfort. Then, the animals were positioned and fixed in a stereotaxic apparatus (David Kopf Instruments, CA, USA) to perform the surgical procedure with 1% isoflurane in pure O_2 for maintenance in an open circuit connected to an agent-specific vaporizer (VetCase Brasmed, São Paulo, Brazil). The skin was cleaned with 2% chlorhexidine diglyconate, followed by a local anaesthetic and vasoconstrictor (lidocaine hydrochloride 2%; 0.1 mL; Biofarm Quimica e Farmacêutica Ltda, São Paulo, Brazil) injected under skin. After this, a rostro-caudal incision was made in the dorsal region of the head to expose the skullcap to perform anti-SERT-SAP, anti-GAT1-SAP or respective vehicles injections.

Anti-SERT-SAP was injected in the fourth ventricle (4V) and the location for microinjections was determined in pilot experiments, based on the stereotaxic atlas of chick´s brain (Kuenzel and Masson, 1988). The 4V´s coordinates were established by injecting 1 µL of 1% Evans blue solution, which marked the target region and the brainstem caudally. The starting point was the meeting point of the coronal and sagittal skull sutures, and from there, the turret, positioned at 90° (with the incisor bar +6.5 mm), followed 1.2 mm posterior and at this point, the skull was perforated by the tip of a 30-gauge (30G) needle and inserted 6 mm ventrally, reaching the 4V. A volume of 1 µL was slowly injected over a time of 5 min (rate of 0.2 µL min^{-1}) to allow drug diffusion. The needle remained in place for an additional 3 minutes to prevent backflow. Microinjections

were performed in the rostral portion of the fourth ventricle so that, due to the rostrocaudal cerebrospinal fluid flow, the toxin had contact with the serotonergic neurons distributed in the raphe along the brainstem and, thus, the lesion covered a large extension of the medullary raphe (Okado et al., 1992).

The anti-GAT1-SAP injections were made directly into the medullary raphe, to avoid an unspecific effect based on lesion of GABA neurons from other regions. The location of this region in 0-1-day-old chicks was also determined in pilot experiments, based on the stereotaxic atlas of chick´s brain (Kuenzel and Masson, 1988) by injecting 0.1 µL of 1% Evan's blue solution. The coordinates were defined with the turret positioned at 90° and the incisor bar at +6.5 mm, from the meeting point of the coronal and sagittal skull sutures. The tip of a 30G needle was positioned, followed -1.7 mm antero-posterior and -9.7 mm dorso-ventral, where a volume of 0.1 µL was slowly injected for 30 s (rate of 0.2 µL min^{-1}), and the needle was withdrawn after another 30 s to prevent reflux.

The injections were performed using a microinjector pump (model 310; Stoelting Co., Wood Dale, IL, USA), through a 30G needle connected to the Hamilton syringe (5 µL; Mizzy, 200 µm outer diameter; Sutter Instrument Co., Novato, CA, USA) with the aid of a polyethylene tube (PE-10). At the end of microinjection, the skin of the animal was sutured with surgical glue (DermabondTopical Skin Adhesive; Johnson & Johnson, Sao Paulo, Brazil). Control group animals underwent the same procedure, but received microinjection of IgG-SAP (0.5 or 1.5 µM) or PBS (0.01M, pH 7.4).

After being submitted to stereotaxic surgery, the animals remained anesthetized with 1% isoflurane in pure O_2 for maintenance, through a mask, for the implantation of a mini temperature biotag (12.5 mm length and 2.1 mm in diameter; BioTherm13, 134.2 kHz FDX-B; Biomark, Boise ID, USA) in the coelomic cavity. The insertion was made

through the skin and abdominal muscle by means of an application needle (pit tag implanter; Animalltag, São Carlos, Brazil). The tiny hole was closed with 2-octyl cyanoacrylate surgical glue (Dermabond Topical Skin Adhesive; Johnson & Johnson, Sao Paulo, Brazil). Animals were then observed until recovering from anesthesia, which took ~5 min, and the entire surgical procedure (stereotaxic surgery + sensor implantation) lasted 20 minutes. After completely recovering from anesthesia, chicks were reallocated to a clean temperature-controlled chamber (Premium Ecológica, Belo Horizonte, MG, Brazil).

Determination of body temperature (Tb)

Individual Tbs were recorded in real-time by telemetry with an appropriate reader for the mini temperature sensors (Biomark HPR Plus Reader, Boise, ID, USA) implanted in the coelomic cavity.At the end of the experiment, Tb data were downloaded using BioTerm software.

Oxygen Consumption ($\dot{V}O_2$)

Oxygen consumption ($\dot{V}O_2$) was determined using an open flow respirometry system. Chicks were placed individually in a respirometric chamber (3 L) in a temperature-controlled room or in a climate-controlled chamber (FANEM, São Paulo-Brazil). Air was continuously pulled from the respirometer or from the external ambient (baseline) by a flowmeter (MFS; Sable Systems, USA) at a rate of 1000 ml min^{-1}. A subsample of this air (180 mL min^{-1}; SS4; Sable Systems, Las Vegas, NV, USA) was pulled trought a water vapor pressure analyzer (RH300; Sable Systems, Las Vegas, NV, USA) and then through a drying column (Drierite, with indicator, 8 mesh, Sigma-Aldrich Brasil Ltda., São Paulo, SP, Brazil) to a calibrated O_2 analyzer (PA-10; Sable Systems,

Las Vegas, NV, USA). All system was connected to an analog-to-digital converter (PowerLab; ADInstruments, Sydney, NSW, Australia), and the signals were recorded in a computer with the appropriate software (LabChart; ADInstruments, Sydney, NSW, Australia). The values of water vapor pressure (WVP; kPa), in addition to barometric pressure (PB; kPa), were later used to correct flow. The gas analyzers were properly calibrated using nitrogen as 0% and dry ambient air as 20.95% oxygen. Thus, $\dot{V}O_2$ was determined based on the flow rate and the difference in concentration of oxygen fraction at the entrance and the exit of the chamber. Recording times were repeated each 20 min; gas from the respirometer was recorded for 18 min (only the final 2 min were analyzed) and from the baseline for 2 min. As CO_2 was neither analyzed nor scrubbed, oxygen consumption was calculated using the following equation (Koteja, 1996b): $\dot{V}O_2$ = [FRe (FiO_2 - FeO_2)] / [1 - FiO_2 (1 - RQ)], where FRe is the excurrent flow rate (outflow), FiO_2 is the incurrent fractional concentration of oxygen (baseline), FeO_2 is the excurrent fractional concentration of oxygen and RQ is the respiratory quotient (considered as 0.85). Values were presented in standard conditions of temperature, pressure and dry air (STPD).

Pulmonary ventilation ($\dot{V}_E$)

The pulmonary ventilation ($\dot{V}_E$) was measured using the barometric method (reviewed by Mortola and Frappell, 1998). In this method, the pressure signal is obtained from a pressure transducer (ADInstruments, Sydney, NSW, Australia), converted by an analogue-digital converter (Powerlab; ADInstruments, Sydney, NSW, Australia) and recorded using LabChart (ADInstruments, Sydney, NSW, Australia). Thus, it was possible to detect the waves of ventilatory oscillation to determine the tidal volume (V_T) and respiratory rate (*f*) of the animal, and their product was used to calculate ventilation:

(V_T x f) = $\dot{V}_E$. The breathing signal was periodically recorded for 2 min during the baseline, when the chamber was closed and based on the recording and volume calibration was performed during each experiment by injecting a known volume of air into the animal's chamber (3 mL) using a graduated syringe. The ambient temperature (Ta) within the respirometer was monitored by a Thermistor Pod sensor (ADInstruments, Sydney, NSW, Australia). The V_T was determined from the following formula (Drorbaugh and Fenn, 1955): $V_T = A\ (V_{cal} / P_{cal})\ [Tb\ (P_B - Pc_{H2O})] / \{[Tb\ (P_B - Pc_{H2O})] - [T_{ch}\ (P_B - Pb_{H2O})]\}$, where A is the wave amplitude, V_{cal} is the calibration volume, P_{cal} is the calibration pressure, Tb is the body temperature, P_B is the barometric pressure, Pc_{H2O} is the water vapor pressure inside the chamber, T_{ch} is the temperature inside the respirometry chamber, and Pb_{H2O} is the water vapor pressure of the air inside the animal´s body.

During the heat exposure (36^{o}C) experiments, an adaptation of the barometric technique was applied (Szdzuy and Mortola, 2007) in order to create a significant difference between Tb and $T_{ch.}$ Thus, animals were placed individually inside a chamber comprised of two separate compartments connected through a polyethylene tube of short length (~20 cm) and large diameter (~4 cm). The animal´s compartment (3 L) was kept inside a climate chamber at the highest Ta (FANEM, São Paulo, SP, Brazil), while the other compartment (1 L) remained outside at room temperature (~25^{o}C), at a lower Ta. The calculation of V_T was performed using the same formula above, with the difference being those weighted averages, relative to the volumes of the two compartments, were used for T_{ch} and Pc_{H2O} (Cristina-Silva et al., 2021).

Besides the above mentioned measurements, two other calculations were done. The ratio of $\dot{V}_E/\dot{V}O_2$, which refers to the air convection requirement, and the lung extraction efficiency of O_2, i.e. the percentage of O_2 extracted from the lungs during each

ventilation, which is $(\dot{V}O_2/\dot{V}_E \cdot FiO_2) \cdot 100$ (Barros et al., 2001; Cristina-Silva et al., 2021; FiO_2 = inspired fraction of O_2).

Heat loss index (HLI)

The HLI, was calculated based on the feet skin temperature (Ts), Tb and Ta, according to the formula: HLI = (Ts - Ta) / (Tb - Ta) (Romanovsky et al., 2002). The feet Ts (emissivity 0.95) was measured using an infrared sensitive camera (Flir E40, Portland, OR, USA). Ta was determined by infrared images of a black tape (emissivity 0.95) glued to the bottom of the net floor close to the chick´s feet. Tb was obtained by a mini temperature sensor (Biomark HPR Plus Reader, USA) implanted in the animal. This index ranges from 0 (maximum vasoconstriction) to 1 (maximum vasodilation). This method allow inferences of cutaneous vasodilation or vasoconstriction in different species of mammals and birds (Almeida et al., 2006; Amaral-Silva et al., 2021; Cristina-Silva et al., 2017; Cristina-Silva et al., 2021; Scott et al., 2008; Tattersall and Milsom, 2003).

Brain Collection and Preparation for immunohistochemistry and RNA scope

Immediately after the end of the experiments, animals were deeply anesthetized and perfused through the left ventricle with 0.01M phosphate-buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde (PFA) in 0.2 M phosphate-buffered (PB), being injected with 100 mL/ 100g body mass of each solution at a rate of 12 mL min^{-1}. The brains were removed from the skull and postfixed with 4% PFA in 0.2 M PB, pH 7.4, at 4°C for about 12 hours and then immersed in 30% sucrose solution dissolved in 0.01 M PBS, pH 7.4, at 4°C for at least 48 hours. Then, brains were rapidly frozen in isopentane in dry-ice, and stored at -20 °C and fixed in Tissue Tek OCT (Sakura Finetek, Torrance, CA, USA). Serial coronal sections (35 μm) of the brainstem were made using a cryostat

microtome (CM1860 – Ag Protect; Leica, Wetzlar, Germany). Three series of 35 µm coronal sections of the rostrocaudal extension of the raphe were obtained and stored in cryoprotective solution at -20 ºC.

Immunohistochemistry

Double-immunostaining (immunoperoxidase) for c-Fos protein (neuron activation) and TrpOH (serotonergic neuron) in the medullary raphe – assessment of serotonergic neurons activated by thermal stimuli

Neuronal activation can be quantified based on an indirect marker, the expression of the c-Fos protein (product of c-fos proto-oncogene), which is detected in the nuclei of the cells by immunohistochemistry (Bullitt, 1990; de Carvalho et al., 2016; Fujiwara et al., 1987; Hunt et al., 1987; Mölders et al., 1987; Perrin-Terrin et al., 2016).

After washing in PBS to remove the cryoprotectant solution, the tissues were incubated in an antigen recovery solution (Citrate Buffer, pH 6.0; ScyTek Laboratories, Logan, Utah, USA) at 70-75ºC (in a water bath inside microtubes) for 30 min. After that, the sections remained for 20 min at room temperature and were washed three times in PBS, then the tissues were immersed in 1% hydrogen peroxide solution in PBS for 10 minutes and were washed three times in PBS. Tissue sections were then incubated in a blocking solution of 1% BSA in PBS containing 0.2% Triton for 30 min, followed by overnight incubation with the primary anti-c-Fos antibody (1:1000; IgG de rabbit; sc-253, Santa Cruz Biotechnology) diluted in PBST solution (PBS 0.01M, 0.1% BSA and 0.2% Triton X-100). After incubation in the primary antiserum, sections were washed four times in PBS and incubated for two hours with biotinylated secondary antibody, anti-rabbit IgG (1:400, BA-1000, Vector Laboratories, Burlingame, California, USA) in PBST solution. The sections were then washed three times in PBS and incubated for 1.5 hours

with the avidin-biotin-peroxidase complex (1:1000; PK-6100; ABC Kit Peroxidase Elite Vectastain, Vector Laboratories, Burlingame, California, USA) diluted in PBS. c-Fos immunoreactivity was revealed after immersing the sections in a solution containing 0.02% chromogen 3,3′-diaminobenzidine tetrahydrochloride, 0.0015% hydrogen peroxide, and 0.05% nickel ammonium sulfate in PBS for 10 min.

After immunohistochemical assays for c-Fos protein, sections were washed four times in PBS, followed by immunohistochemical labeling for detection of tryptophan hydroxylase (TrpOH), a key enzyme in the synthesis pathway of 5-HT (Höglund et al., 2019), as described below. Initially, tissues were incubated overnight with anti-TrpOH primary antibody (T0678; mouse IgG, 1:1000, Sigma-Aldrich Brasil Ltda; São Paulo, SP, Brazil) diluted in PBST solution. After primary antibody incubation, sections were washed four times in PBS and incubated for two hours with biotinylated secondary antibody, anti-mouse IgG (1:500, BA-9200, Vector Laboratories, Burlingame, California, USA). Sections were then washed four times in PBS and incubated for 1.5 h hours in avidin-biotin-peroxidase complex (1:1000; PK-6100; ABC Kit Peroxidase Elite Vectastain, Vector Laboratories, Burlingame, California, USA) diluted in PBS. The set of sections for TrpOH immunoreactivity was revealed after immersing the sections in a solution containing 0.02% chromogen, 3,3′-diaminobenzidine tetrahydrochloride and 0.0015% hydrogen peroxide in PBS for 10 min. Sections were then washed four times in PBS to stop the reaction.

Coronal sections were mounted on gelatin-coated glass slides, then went through a process of dehydration in a graded alcohol series, cleared in xylol, and coverslipped using Fluoromount Aqueous Mounting Medium (F4680; Sigma-Aldrich Brazil Ltda; São Paulo, SP, Brazil).

Immunofluorescence for 5-HT - assessment of anti-SERT-SAP chemical lesion effectiveness and placement

To confirm the correct site and effectiveness of the chemical lesions, 5-HT immunoreactivity was performed using the free-floating method. The sections were washed with PBS to remove the cryoprotectant solution and incubated in an antigen recovery solution (Citrate Buffer, pH 6.0; ScyTek Laboratories, Logan, Utah, USA) at 70-75ºC (in a water bath inside microtubes) for 30 min and remained for 20 min at room temperature. After washes in PBS (3 x 5min), they were pre-incubated with 1% bovine serum albumin (BSA) in PBS containing 0.3% Triton for 1 h to prevent non-specific binding. The sections were incubated for 48 hs at 4°C with rabbit anti-5-HT primary antibody (S5545, Sigma-Aldrich, St. Louis, MO, USA) at a dilution of 1:1000 in PBS containing 0.3% Triton and 1% BSA (solution used in the dilution of all primary and secondary antibodies). The next day after washing with PBS (3x5min), the sections were incubated with the goat anti-rabbit secondary antibody (DyLight 594, Thermo Fisher Scientific Inc., Waltham, MA, USA) at a dilution of 1:250 for 3h in the dark. After washing in PBS, sections were mounted on gelatin-coated slides, dried and coverslipped using ProLong™ Gold for preserving fluorescence longer.

In situ hybridization

RNAscope for GABA in the medullary raphe – assessment of GABAergic neurons and of anti-GAT1-SAP chemical lesion effectiveness and placement

Serial sections (20 µm) were mounted on charged glass slides, dried for 1 hr at room temperature and stored at −80°C. On the day of the RNAScope assay, the slides were thawed and pretreated as follows to improve adhesion of thicker tissue sections as described in Biancardi *et al.*, (2020). The slices were placed in 4% PFA for 1 hr at 4°C,

rinsed two times in PBS 0.01 M and placed in an oven for 15 min at 60°C. Slides were then gradually dehydrated in ethanol and heated again for 15 min at 60°C. The pretreatment then concluded with the H_2O_2 incubation for 10 min at room temperature and the remaining steps as described in RNAScope Multiplex Fluorescent Assay kit version 2 (Wang et al., 2012).

Following pretreatment, slides were air-dried for 30 min and incubated in protease III for 30 min at 40°C. Slides were then rinsed in distilled water and incubated in RNAscope oligonucleotide probe *Gallus gallus* glutamate decarboxylase 1 (Gg-GAD1-C1; #320269, ACDBio, Newark, CA) for 2 hr at 40°C. The slides were washed 2 x 2 min in wash buffer at room temperature and three signals amplification systems were used to detect multiple target RNAs at 40°C. Then, the sections were incubated with HRP-blocker, washed 2 x 2 min in wash buffer at room temperature and incubated with Opal 570 (1:1000 in TSA Buffer) for 30 min at 40°C. After washes (2 x 2 min) in wash buffer at room temperature, the slides were coverslipped with antifade mounting medium with DAPI (H-1500; Vectashield HardSet, Vector Laboratories, Burlingame, California, USA).

Analysis and Quantification of immunohistochemistry and in situ hybridization

Immunostaining of c-Fos and TrpOH was captured through a camera (Leica DMC 2900) attached to a microscope (Leica DM1000 LED). c-Fos-immunoreactive (c-Fos-ir) neurons could be identified by the presence of black pigmentation restricted to the neuronal nucleus, whereas TrpOH immunoreactive neurons (TrpOH-ir) were identified by the presence of brown-orange pigmentation restricted to the neuronal cytoplasm. Neurons with labeling were photographed using an image analysis system (Las V45; Leica).

Cells that showed 5-HT immunoreactivity (5-HT-ir) or GAD1 positive (GAD1+) were analyzed by optical microscopy (AXIO IMAGER Z2; Carl Zeiss do Brasil Ltda., São Paulo, SP, Brazil), and neurons with labeling were photographed using an image analysis system (AxioVision).

The slides were analyzed under an optical microscope without prior knowledge of the experimental groups (blind test) and immunoreactive neurons were count using a computerized image analysis system (NIH System, Image J developed by the US National Institutes of Health and made available via the internet: http://www.rsb.info.nih.gov/nih-image). The results were expressed as mean number of positive cells per slice. The rostro-caudal sections containing the medullary raphe were analyzed and the identification of the medullary raphe was made according to the atlas for chicks (Kuenzel & Masson, 1988), with length along the rostrocaudal ranging from +0.2 mm anteroposterior to -3.0 mm posterior.

Experimental protocols

All the protocols were performed on unanesthetized one-week-old chicks.

Protocol 1: Effects of thermoneutral, cold and hot conditions on the activity of the medullary raphe serotonergic neurons

To reduce the nonspecific c-Fos expression, animals were kept in temperature-controlled chambers (Premium Ecológica, Belo Horizonte, MG, Brazil), where the experiments took place. On the day of the experiment, one group of animals remained at neutral condition (30-31°C) all the time. The other two groups of animals were exposed to cold (25-26°C) and hot (35-36°C) conditions for 2 hours (Cristina-Silva et al., 2021), followed by 30-60 min of recovery at thermoneutral condition prior to sample collection. This extra time in thermoneutrality was necessary, because in response to a specific

stimulus an increase in mRNA levels for c-fos is observed after 5 to 20 min (Greenberg and Ziff, 1984), and the consequent c-FOS protein synthesis can be detected by immunohistochemistry at 20 to 90 min post stimulation (Bullitt, 1990; Curran and Morgan, 1987; Perrin-Terrin et al., 2016).

Protocol 2: Effect of the medullary raphe serotonergic neurons lesion (anti-SERT-SAP) on Tb, $\dot{V}O_2$, $\dot{V}_E$ and HLI in thermoneutral, cold and hot conditions

Seven days after surgical intervention, chicks were exposed to 25–26, 30-31°C or 35–36°C for 1 h. Then, they were individually placed inside the respirometer for habituation (20-30 min), at the same respective temperatures, for recording of Tb, $\dot{V}O_2$ and $\dot{V}_E$ every 20 min for approximately 2 hours. Different groups of animals were used for different treatments and ambient temperatures.

Separate experiments were performed to determine the IPC in different conditions. Thus, seven days after surgical intervention, other groups of animals were exposed to 25–26, 30-31°C or 35–36°C for 1 h. Then two animals at a time were placed inside the apparatus used to determine HLI for habituation (20-30 min), at the same respective temperature, and had their Tb measured and infrared images of their feet taken every 20 min for the next 2 h (Cristina-Silva et al., 2021). Different groups of animals were used for different treatments and Ta.

Protocol 3: Demonstration of the presence of GABAergic neurons in the medullary raphe and the effect of their lesion (anti-GAT1-SAP) on Tb, $\dot{V}O_2$ and $\dot{V}_E$ in neutral and cold conditions

Seven days after surgical intervention, chicks were exposed to 25–26 or 30-31°C for 1 h. Then, they were individually placed inside the respirometer for habituation (20-

30 min), at the same respective temperatures, and for recording of Tb, $\dot{V}O_2$ and $\dot{V}_E$ every 20 min for approximately 2 hours. Different groups of animals were used for different treatments and ambient temperatures.

Data analysis

Data are presented as mean ± s.e.m. The results of c-FOS-ir, TrpOH-ir, c-FOS-ir/TrpOH-ir, 5-HT-ir or GAD1+ neurons were compared using one-way analysis of variance (ANOVA) (factor: Ta). The results to compare PBS 0.01M and IgG-SAP 0.5 µM or PBS 0.01M and IgG-SAP 1.5 µM also were tested using a one-way ANOVA (factor: Ta) to compare values of body mass, Tb, $\dot{V}O_2$, $\dot{V}_E$, V_T, *f*, $\dot{V}_E/\dot{V}O_2$, lung O_2 extraction efficiency and HLI. As $\dot{V}O_2$ and $\dot{V}_E$ change allometrically with body mass, an ANCOVA was performed to check whether the effect of Ta was influenced by the body mass of chicks in the different groups. We performed a Student's unpaired t-test to analyze the effect of the medullary raphe serotoninergic or GABAergic neurons lesion on Tb, $\dot{V}O_2$, $\dot{V}_E$, V_T, *f*, $\dot{V}_E/\dot{V}O_2$, lung O_2 extraction efficiency in each thermal condition. Differences between means were considered significant for $p < 0.05$

Effects of neutral, cold and hot exposures on the activation of the medullary raphe serotonergic neurons

Fig. 1A shows the number of c-Fos immunoreactive neurons (c-Fos-ir) per section counted in medullary raphe at different ambient temperatures. The group submitted to cold (26^{o}C) showed increased c-Fos immunostaining ($p = 0.0002$, $F_{(2, 17)} = 14.96$) when compared to thermoneutral condition (31^{o}C). The number of c-Fos-ir cells did not differ between neutral and heat conditions ($p = 0.7671$). Fig. 1A also shows that the number of double-immunostained neurons (c-Fos-ir/TrpOH-ir) per section in the medullary raphe did not differ among the 3 groups ($p = 0.1015$, $F_{(2, 17)} = 2.625$). About 9.4% of the total c-Fos-ir neurons correspond to serotonergic neurons at 26°C, 15.4% at 31°C and 7.8% at 36°C. The number of TrpOH immunoreactive neurons (TrpOH-ir) per section did not differ between neutral, cold and heat conditions in medullary raphe ($p = 0.9552$; Fig. 1B). In the representative photomicrographs of medullary raphe serotonergic neurons and double-labeled to c-Fos and TprOH it can be observed a higher number of c-Fos-ir neurons of the cold group (Fig. 1C and 1F) when compared with neutral (Fig. 1D, 1G) and heat (Fig. 1E, 1H) groups.

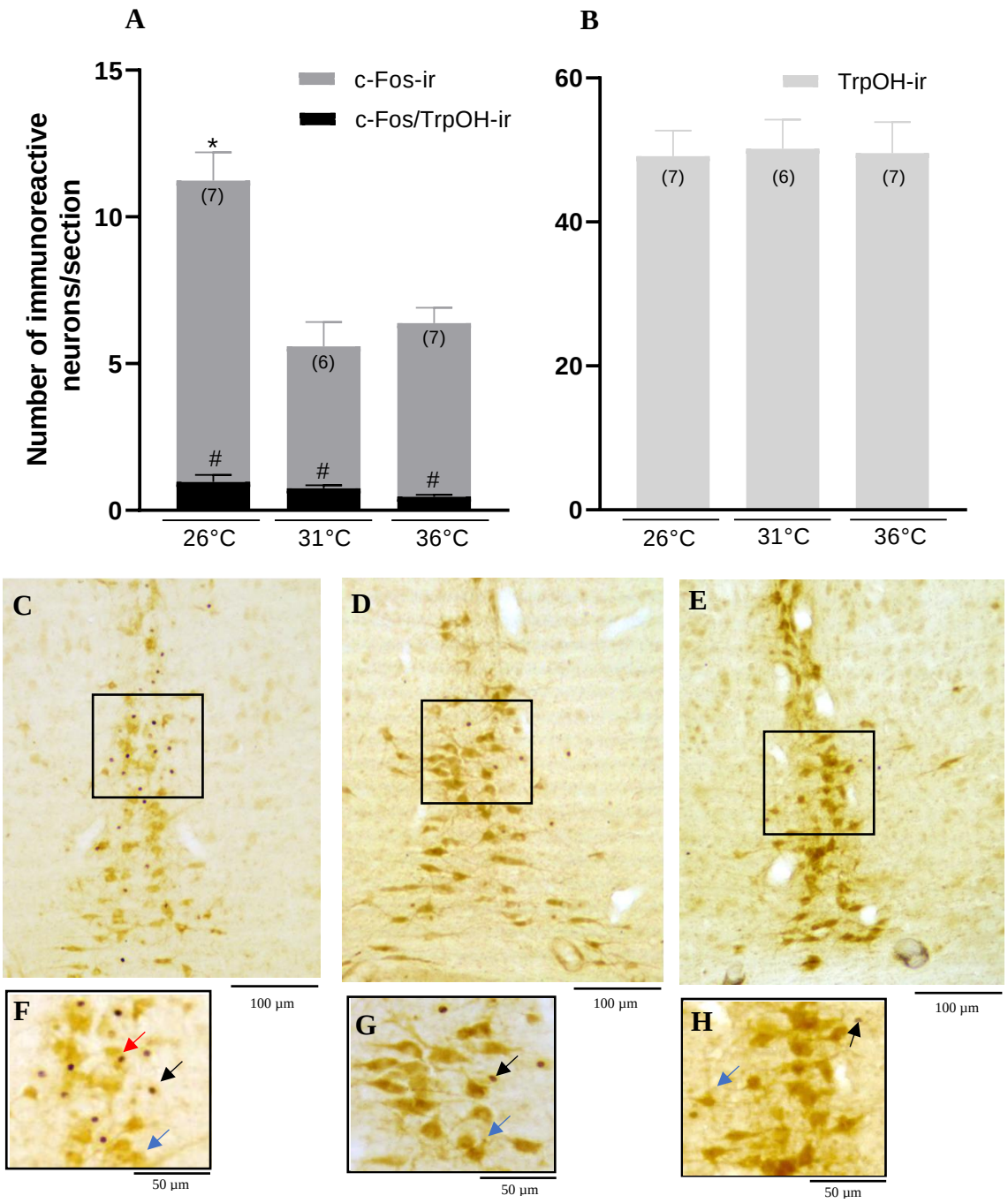


Figure 1. A-B) Number (mean ± S.E.M.) of c-Fos (c-Fos-ir), c-Fos/TprOH-immunoreactive neurons (c-Fos/TrpOH-ir) and TprOH-immunoreactive neurons (TrpOH-ir) in the medullary raphe in different conditions: cold (26°C), neutral (31°C) and heat (36°C). Number of animals is shown between parentheses. *Significant difference ($p < 0.05$) among c-Fos-ir groups. *Significant difference ($p < 0.05$) between c-Fos-ir and c-Fos/TrpOH-ir groups. **C-H)** Photomicrographs of medullary raphe (coronal sections) double-labeled to c-Fos and tryptophan hydroxylase (TprOH) of representative animals exposed at 26°C (C, F), 31°C (D, G) and 36°C (E, H). Black boxes indicate regions shown at higher magnification in the bottom. Black arrow indicate example of c-Fos-immunoreactive neurons (c-Fos-ir). Blue arrow indicate example of TrpOH-immunoreactive neurons (TrpOH-ir). Red arrow indicate example of c-Fos + TrpOH-ir double-immunostained neurons.

Effect of the medullary raphe serotonergic neurons lesion (anti-SERT-SAP) on Tb, $\dot{V}O_2$, $\dot{V}_E$ and HLI in thermoneutral, cold and hot conditions

Treatment with anti-SERT-SAP immunotoxin was effective for inducing chemical lesion of medullary raphe 5-HT neurons. Animals treated with anti-SERT-SAP showed a reduction in the number of 5-HT neurons on medullary raphe ($p < 0.0001$; $F_{(3,35)} = 109.0$), of approximately 70%, when compared to the control group treated with IgG-SAP at 31°C, 26°C and 36°C (Fig. 2A). Figs. 2B-E shows representative photomicrographs in a coronal view of 5-HT-ir cells from serotonergic nuclei in the brainstem of one-week-old chicks after IgG-SAP (Fig. 2B and 2D) or anti-SERT-SAP (Fig. 2C and 2E) injection in the fourth ventricle, demonstrating the effectiveness of the chemical lesion. Successful damage to serotonergic neurons was mainly revealed by the disappearance of 5-HT-ir cells.

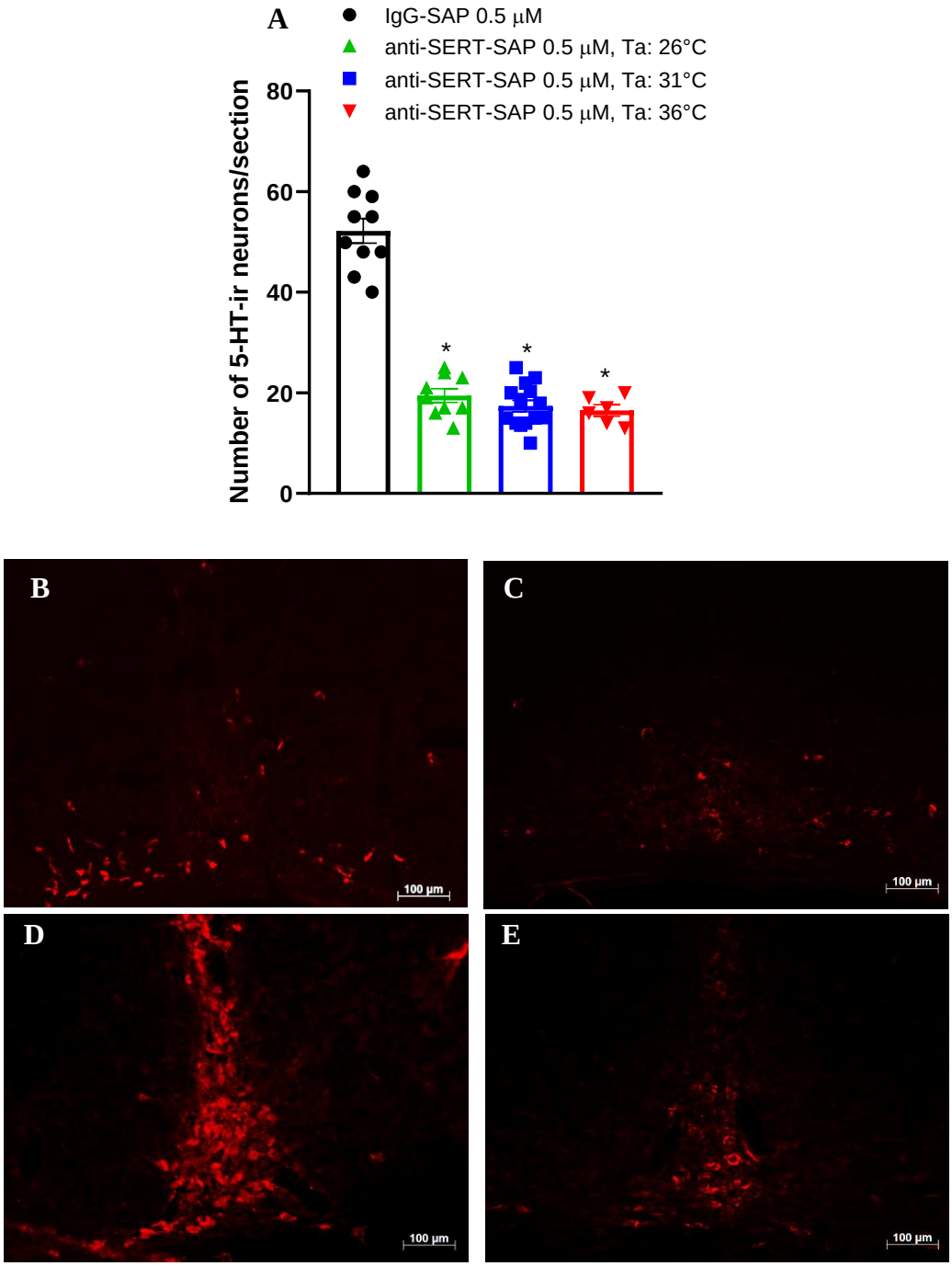


Figure 2. A) Effectiveness of the lesion of serotonergic neurons in the medullary raphe of chicks at 31°C, 26°C and 36°C. The number of 5-HT-imunorreactive neurons (5-HT-ir) was reduced in the medullary raphe of these animals, demonstrating the effectiveness of the lesion. All values are expressed as the mean ± s.e.m. ∗Significant difference ($p < 0.05$) between lesioned groups (anti-SERT-SAP in the 4th ventricle) and the control one (IgG-SAP in the 4th ventricle). B-E) Representative immunohistochemical photomicrographs (coronal sections) of medullary raphe serotonergic neurons (5HT-ir) of one-week-old chicks under a 10× objective. The cell bodies of the serotonergic neurons were identified in red, with a fluorescent marker, in control (IgG-SAP, B and D) and lesioned (anti-SERT-SAP, C and E) animals. The number of serotonin-positive neurons was drastically reduced in lesioned ones.

Figure 3 shows the Tb results of chicks that suffered or not the lesion of the serotonergic neurons of the medullary raphe at 26, 31 and 36°C. There was no difference in Tb values between animals treated with IgG-SAP and animals that suffered serotonergic neuron lesion in any of the ambient temperatures (26°C: p= 0.2535; 31°C: p= 0.2232; 36°C: p= 0.8531). The number of 5-HT-ir cells from medullary raphe the animals that received PBS 0.01 M or IgG-SAP 0.5 µM also did not show differences (p= 0.5985).

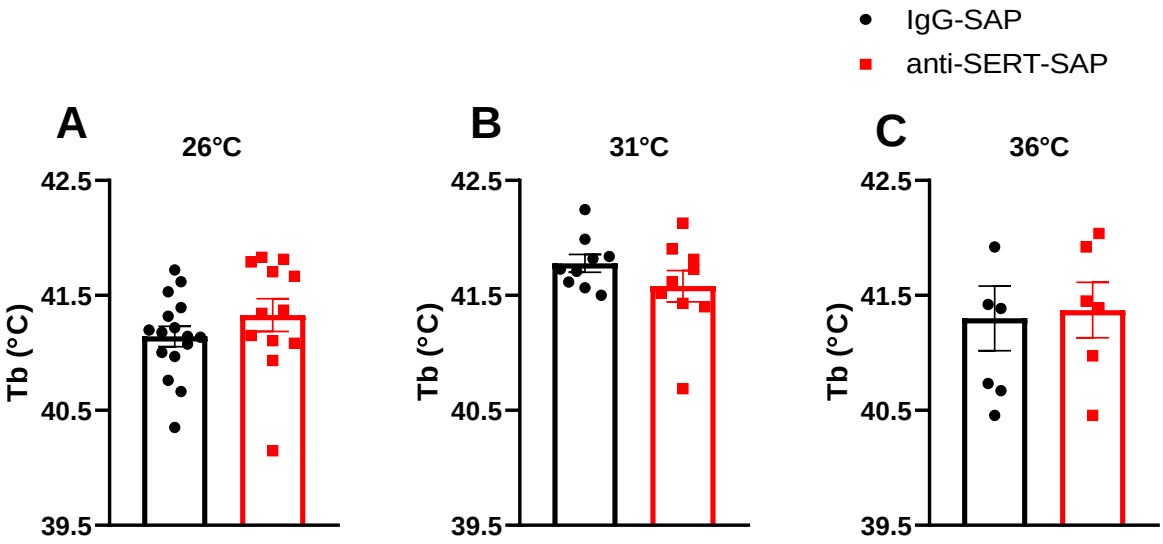


Figure 3. Effect of medullary raphe serotonergic neurons lesions (anti-SERT-SAP; 0.5 µM) on body temperature (Tb) of chicks at 26° C (A), 31°C (B) and 36°C (C). Data are means±s.e.m.

Figure 4A shows that there was no difference in $\dot{V}O_2$ between animals that suffered or not the serotonergic neurons lesion at 31°C (p=0.9785). In figures 4B, C and D it is observed that there was no difference in $\dot{V}_E$ (p=0.3687), in V_T (p=0.3764), and in *f* (p=0.1876) in the groups that suffered the chemical lesion of serotonergic neurons in relation to the vehicle group. In Figure 4E and F, we observe that there was a difference in the respiratory equivalent in relation to the treatments (p=0.1547), but not in the oxygen extraction (p=0.6823; Fig. 4F).

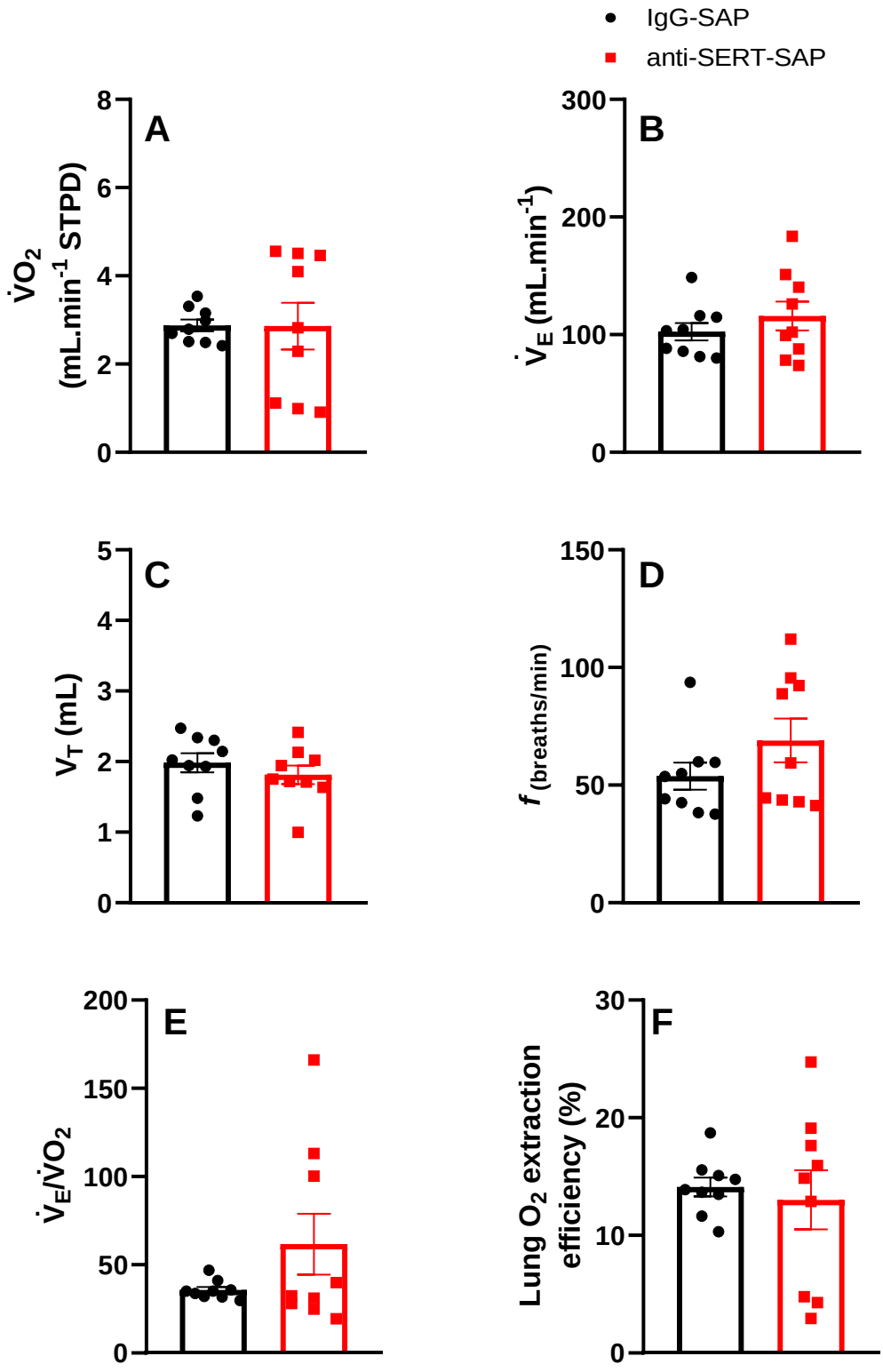


Figure 4. Effect of medullary raphe serotonergic neurons lesions (anti-SERT-SAP; 0.5 µM) on oxygen consumption ($\dot{V}O_2$, A), pulmonary ventilation ($\dot{V}_E$, B), tidal volume (V_T, C), breathing frequency (*f*, D), respiratory equivalent ($\dot{V}_E/\dot{V}O_2$, E) and lung O_2 extraction efficiency (F) from chicks at 31°C. Data are means±s.e.m.

Figure 5A shows that there was no difference in $\dot{V}O_2$ between animals that suffered or not the serotonergic neurons lesion at 26°C (p=0.8145). In figures 5B, C and D, it is observed that there was no difference in $\dot{V}_E$ (p=0.4568), in V_T (no treatment effect p=0.0760), not in f (p=0.4469) in the groups that suffered the lesion of serotonergic neurons in relation to the vehicle group. In Figure 5E and F, we observe that there was no difference in the respiratory equivalent in relation to the treatments (p=0.6422), not in oxygen extraction (p=0.6061; Figure 5F).

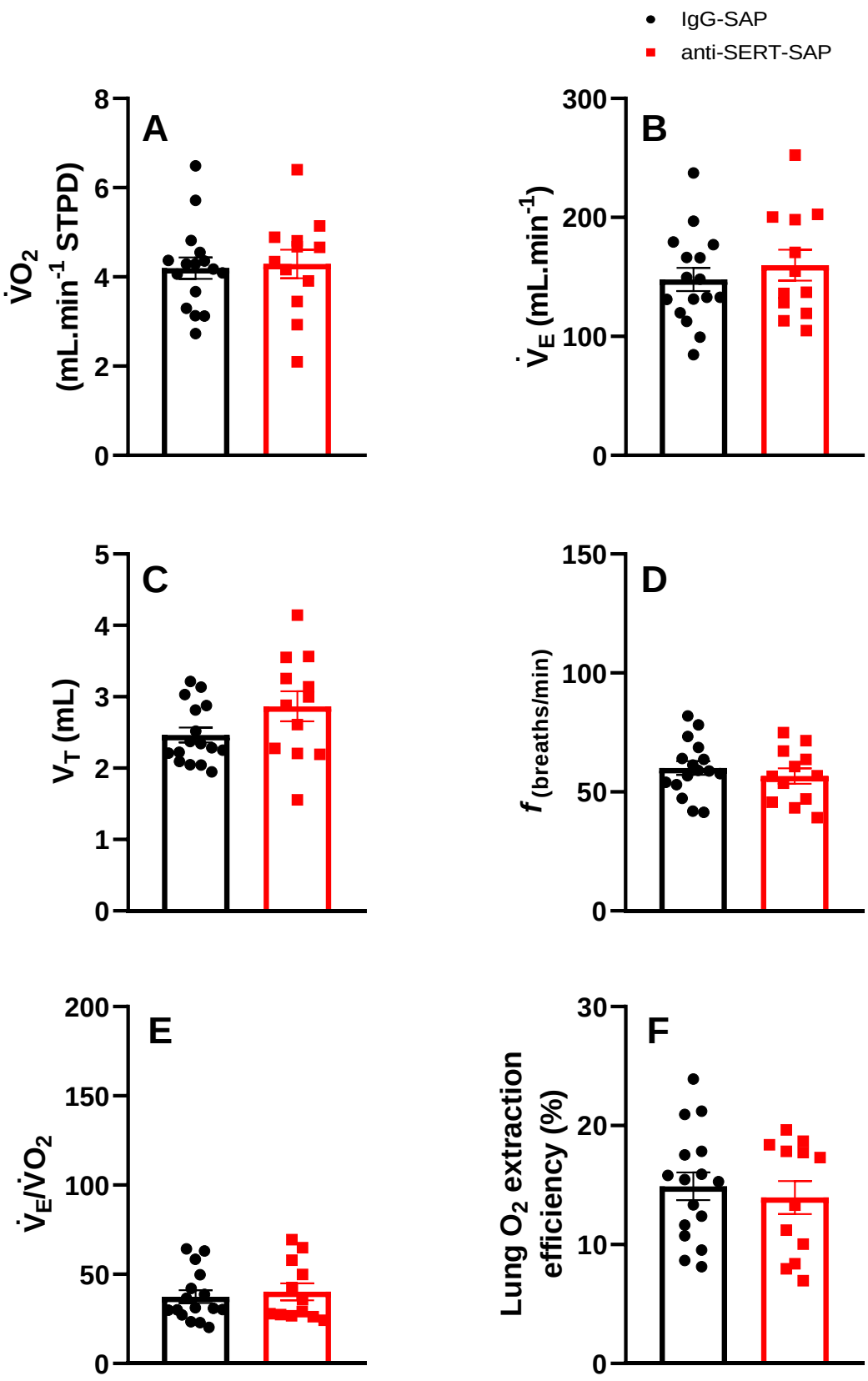


Figure 5. Effect of medullary raphe serotonergic neurons lesions (anti-SERT-SAP; 0.5 µM) on oxygen consumption ($\dot{V}O_2$, A), pulmonary ventilation ($\dot{V}_E$, B), tidal volume (V_T, C), breathing frequency (*f*, D), respiratory equivalent ($\dot{V}_E/\dot{V}O_2$, E) and lung O_2 extraction efficiency (F) from chicks at 26°C. Data are means±s.e.m.

Figure 6A shows that there was no difference in $\dot{V}O_2$ between animals that suffered or not serotonergic neurons lesion at 36°C (p=0.3394), and animals that suffered neuron injury showed an increase in oxygen consumption). In figures 6B, C and D, it was observed that there was no difference in $\dot{V}_E$ (p=0.9088), in V_T (p=0.6447), not in *f* (p=0.7439) in the groups that suffered the injury of serotonergic neurons in relation to the vehicle group. In Figure 6E and F, we observe that there was no difference in the respiratory equivalent in relation to treatments (p=0.0569), nor in oxygen extraction (p=0.2160; Fig.6F).

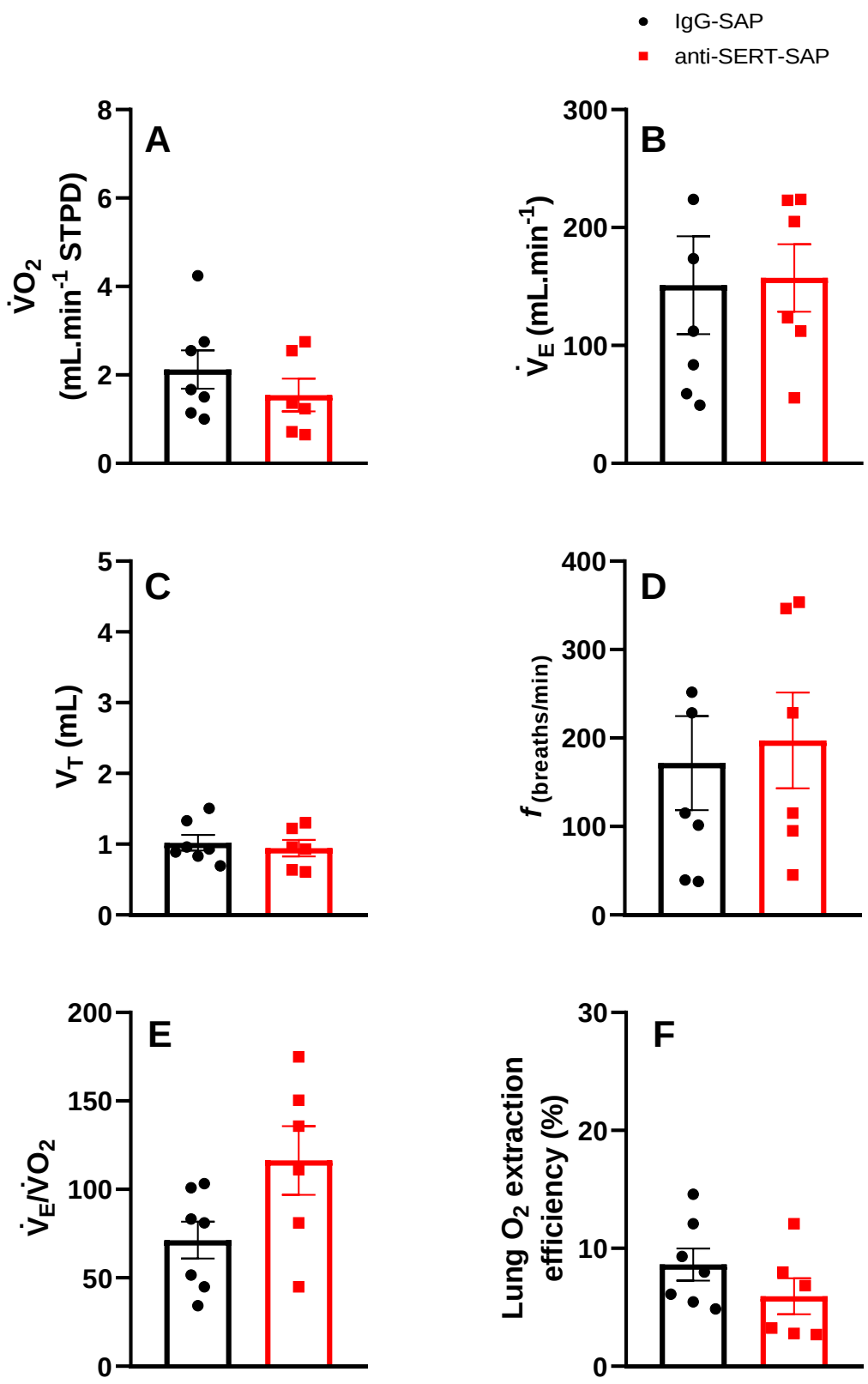


Figure 6. Effect of medullary raphe serotonergic neurons lesions (anti-SERT-SAP; 0.5 µM) on oxygen consumption ($\dot{V}O_2$, A), pulmonary ventilation ($\dot{V}_E$, B), tidal volume (V_T, C), breathing frequency (*f*, D), respiratory equivalent ($\dot{V}_E/\dot{V}O_2$, E) and lung O_2 extraction efficiency (F) from chicks at 36°C. Data are means±s.e.m.

Figure 7 shows the Tb results of chicks that suffered or not the lesion of the serotonergic neurons of the medullary raphe at 26°C (Fig. 7A), 31°C (Fig. 7B) and 36°C (Fig. 7C). There was no difference in Tb between animals treated with IgG-SAP and animals with serotonergic neurons lesioned in any of the ambient temperatures (26°C: p= 0.0573; 31°C: p= 0.5141; 36°C: p= 0.3446). In figures 11D, E and F, it is observed that there was no difference in HLI at 26°C (p=0.9581), 31°C (no treatment effect p=0.4261), and 36°C (p=0.0629) in the groups that suffered the lesion of serotonergic neurons in relation to the vehicle group.

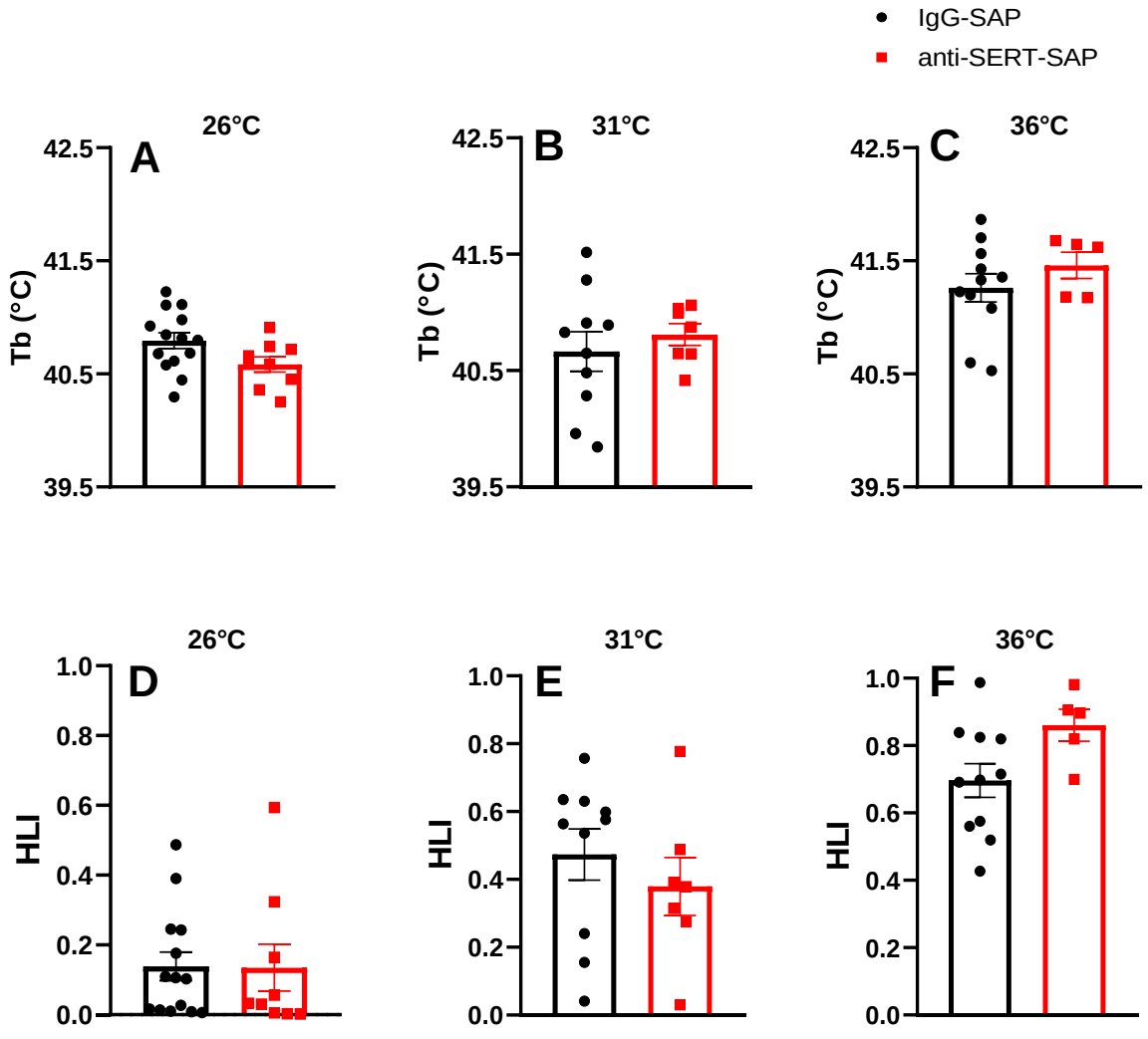


Figure 7. Effect of medullary raphe serotonergic neurons lesions (anti-SERT-SAP; 0.5 µM) on body temperature (Tb; A, B and C) and heat loss (HLI; D, E and F) from chicks at 26, 31 and 36°C. Data are means±s.e.m.

Supplementary Table 1 shows the comparison of the effects of vehicles used (PBS 0.01 M and IgG-SAP 0.5 µM) on Tb, $\dot{V}O_2$, $\dot{V}_E$, V_T, *f*, $\dot{V}_E/\dot{V}O_2$, lung O_2 extraction efficiency, HLI and body mass at different conditions (26°C, 31°C and 36°C). No one of the vehicles affected any of the variables tested. Also was observed the effect of Ta in the chicks that received the vehicle IgG-SAP: the Tb of the chicks that were exposed to 26ºC did not differ from that of the group at 31°C ($p = 0.0851$), but the animals showed higher $\dot{V}O_2$ ($p = 0.0046$) from those of the group at 31ºC, whereas exposure to 36ºC did not change Tb ($p = 0.1938$) and $\dot{V}O_2$ ($p = 0.9297$) compared to those at 31ºC. Total ventilation ($p = 0.5474$), V_T ($p = 0.1247$), *f* ($p =1.0000$), respiratory equivalent ($p = 0.9998$) and lung O_2 extraction efficiency ($p=0.9967$) in animals at 26ºC were not different from the group at 31ºC. In contrast, at 36°C chicks showed higher ventilation ($p = 0.0194$), due to a different respiratory pattern characterized by a lower V_T ($p = 0.0017$) and a higher *f* ($p = 0.0441$), which is a typical tachypneic response. The respiratory equivalent increased in the group exposed to heat ($p = 0.0010$) and reduced the lung O_2 extraction efficiency of chicks ($p = 0.0465$) compared to the chicks at 31ºC. Based on the ANCOVA, it was observed that ~2 hs of exposure to different temperatures affected O_2 consumption and ventilation independent on the body mass of chicks. In the group of chicks for HLI measurements, the body mass of chicks maintained at 31ºC also did not differ from that of animals at 26°C ($p = 0.9839$) and 36°C ($p = 0.9462$). Tb responses to the different Ta did not differ between cold and neutral conditions ($p = 0.9627$), but were higher in the heat ($p = 0.0102$). Cold exposure induced cutaneous vasoconstriction, as the HLI was significantly lower ($p < 0.0047$), whereas heat exposure did significantly change this variable ($p = 0.1734$), compared to the thermoneutral condition.

Demonstration of the presence of GABAergic neurons in the medullary raphe and the effect of their lesion (anti-GAT1-SAP) on Tb, $\dot{V}O_2$ and $\dot{V}_E$ in thermoneutral and cold conditions

The microinjection of anti-GAT-SAP immunotoxin intra-medullary raphe was effective for inducing chemical lesion of medullary raphe GABA neurons in the chicks. Fig. 8A shows that the treatment with anti-GAT1-SAP caused a reduction of approximately 55% in the number of GABAergic neurons (GAD1+; $p < 0.0001$; $F_{(2,25)} = 88.75$), when compared to the control group treated with IgG-SAP. The intra-raphe microinjections were mainly located between 1.0 mm anterior and 1.6 mm posterior to the intersection between the longitudinal and transverse skull sutures. The Fig 8B shows a schematic drawing of coronal section of a chick brain showing the raphe nuclei at 1.2 mm caudal (adapted from the atlas of Kuenzel and Masson, 1988). Representative photomicrographs in a coronal view of GAD1+ neurons in the medullary raphe after IgG-SAP or anti-GAT1-SAP injections are shown in Fig. 8C and 8D. Successful damage to GABAergic neurons was mainly revealed by the disappearance of GAD1-ir cells.

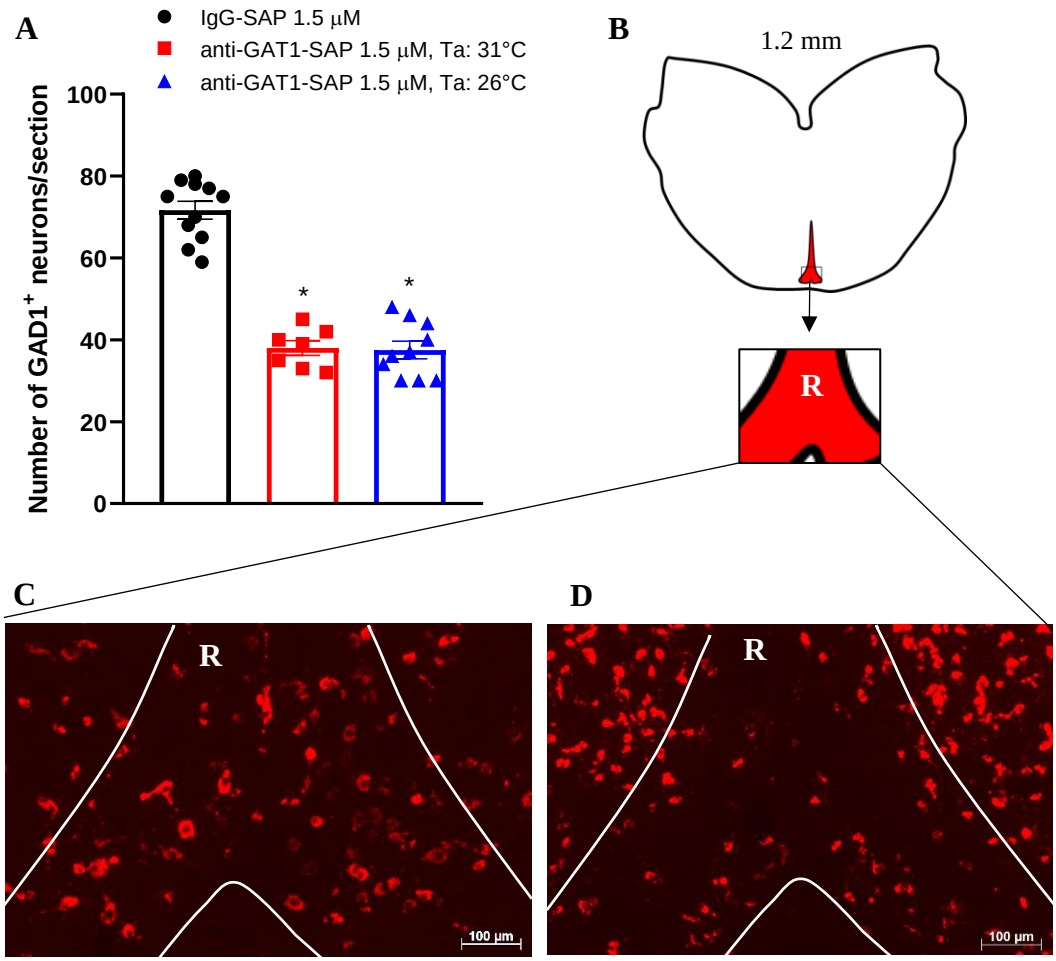


Figure 8. A) GABAergic neurons in the medullary raphe and effectiveness of their lesion in one week-old-chicks. The number of GAD1 positive neurons (GAD1+) was reduced in the medullary raphe of these animals, demonstrating the effectiveness of the lesion. All values are expressed as the mean ± s.e.m. *Significant difference ($p < 0.05$) in the number of GAD1+ cells between lesioned groups (anti-GAT1-SAP) and control (IgG-SAP). **B)** Schematic drawing of coronal section of a chick brain showing the raphe nuclei at 1.2 mm caudal to the intersection between skull sutures (adapted from the atlas of Kuenzel and Masson, 1988). **C-D)** Representative in situ hybridization (RNA scope) photomicrographs (coronal sections) of medullary raphe GABAergic neurons under a 20× objective. The cell bodies of the GABAergic neurons were identified in red, with a fluorescent marker, in control (**C**) and lesioned (**D**) animals. The number of GAD1+ neurons was drastically reduced in lesioned ones. Scale bar = 100 μm. R= raphe nuclei.

Figure 9 shows the effect of medullary raphe GABAergic neurons lesions on Tb of chicks at 31°C or 26°C. In both conditions, the lesioned animals presented a significant increase in Tb compared to the control (IgG-SAP) group ($p= 0.0006$ and $p= 0.0046$, respectively).

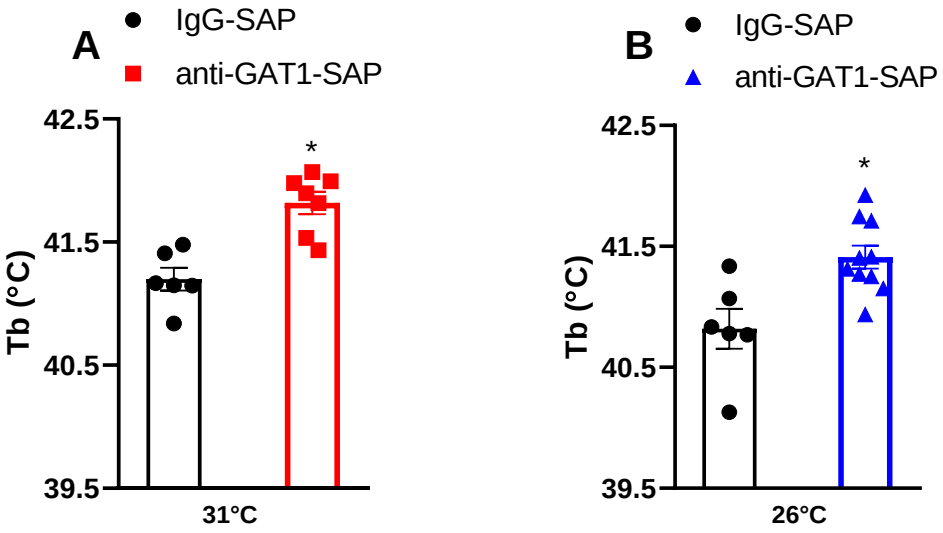


Figure 9. Effect of medullary raphe GABAergic neurons lesions (anti-GAT-SAP; 1.5 µM) on body temperature (Tb) of one-week-old chicks at 31°C (A) and 26°C (B). Data are means±s.e.m. *Significant difference (P<0.05) from control (IgG-SAP) at the same time point.

Figure 10 shows the effects of medullary raphe GABAergic neurons lesions on $\dot{V}O_2$, $\dot{V}_E$, V_T, *f*, $\dot{V}_E/\dot{V}O_2$ and lung O_2 extraction efficiency in chicks at 31°C. The lesioned animals showed an increase on $\dot{V}O_2$ and $\dot{V}_E$ compared to the control group (treatment effect: p = 0.0083 and p = 0.0468, respectively). No effect of the treatments was observed for V_T (treatment effect: p = 0.1745), respiratory equivalent (treatment effect p = 0.0953) or lung O_2 extraction efficiency (treatment effect p = 0.1136) but *f* increased significantly (treatment effect: p = 0.0103).

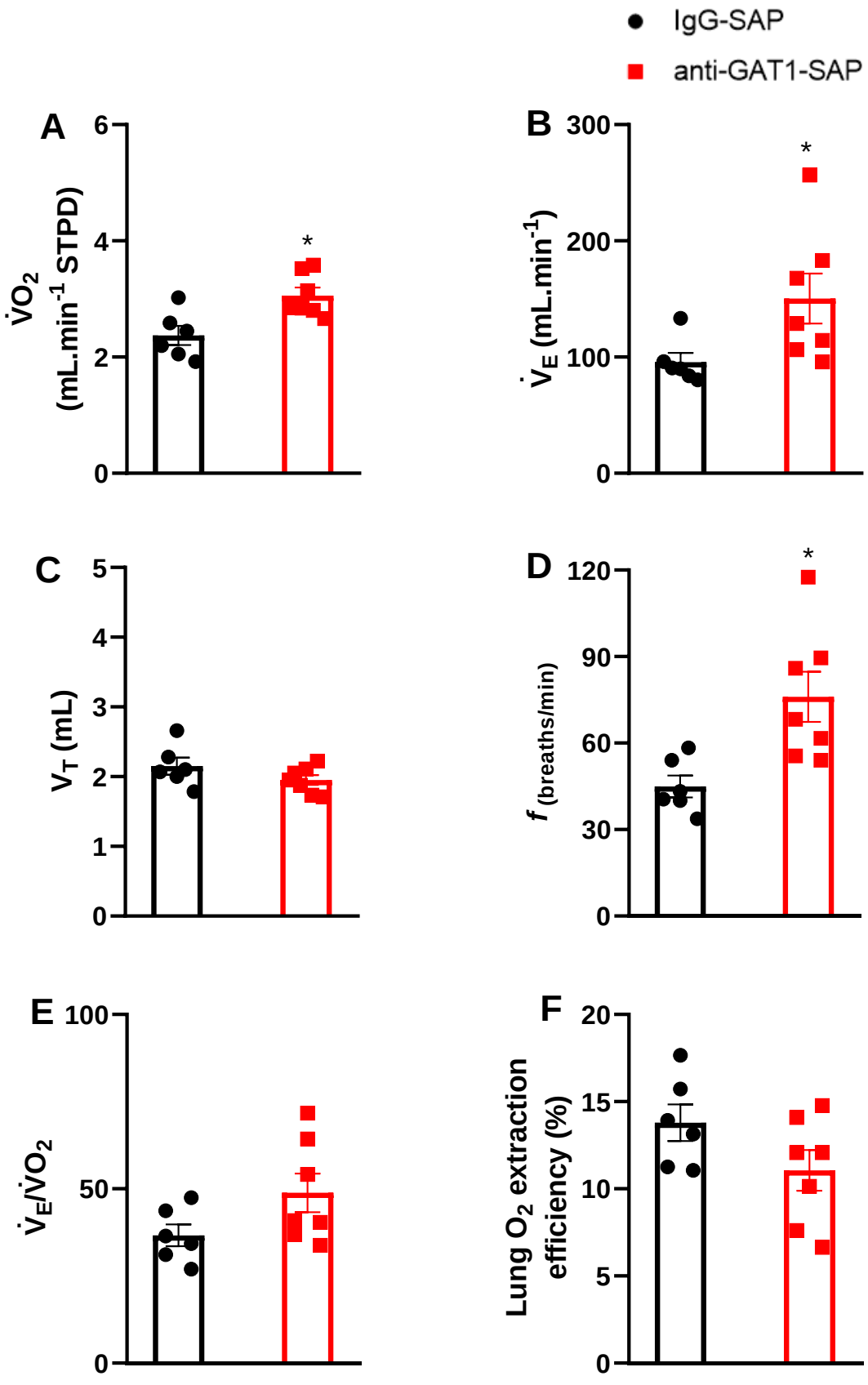


Figure 10. Effect of medullary raphe GABAergic neurons lesions (anti-GAT-SAP; 1.5 µM) on oxygen consumption ($\dot{V}O_2$, A), pulmonary ventilation ($\dot{V}_E$, B), tidal volume (V_T, C), breathing frequency (*f*, D), respiratory equivalent ($\dot{V}_E/\dot{V}O_2$, E) and lung O_2 extraction efficiency (F) from chicks at 31°C.

Figure 11 shows the effects of medullary raphe GABAergic neurons lesions (anti-GAT-SAP; 1.5 µM) on $\dot{V}O_2$, $\dot{V}_E$, V_T, *f* and $\dot{V}_E/\dot{V}O_2$ in chicks at 26°C. In this condition, $\dot{V}O_2$ and $\dot{V}_E$ increased after medullary raphe GABAergic neurons lesions (treatment effect: p = 0.0319 and p= 0.0133, respectively). No treatment effect was observed for *f* (treatment effect: p = 0.2689), the respiratory equivalent (treatment effect: p = 0.0636) or lung O_2 extraction efficiency (treatment effect: p = 0.0905). In contrast, V_T increased significantly (treatment effect: p = 0.0400).

Supplementary Table 2 shows the comparison of the effects of vehicle used (IgG-SAP 1.5 µM) with PBS 0.01 M on Tb, $\dot{V}O_2$, $\dot{V}_E$, V_T, *f*, $\dot{V}_E/\dot{V}O_2$, lung O_2 extraction efficiency, HLI and body mass at different conditions (31°C and 26°C). Both vehicles did not affect any of those variables tested on each condition.

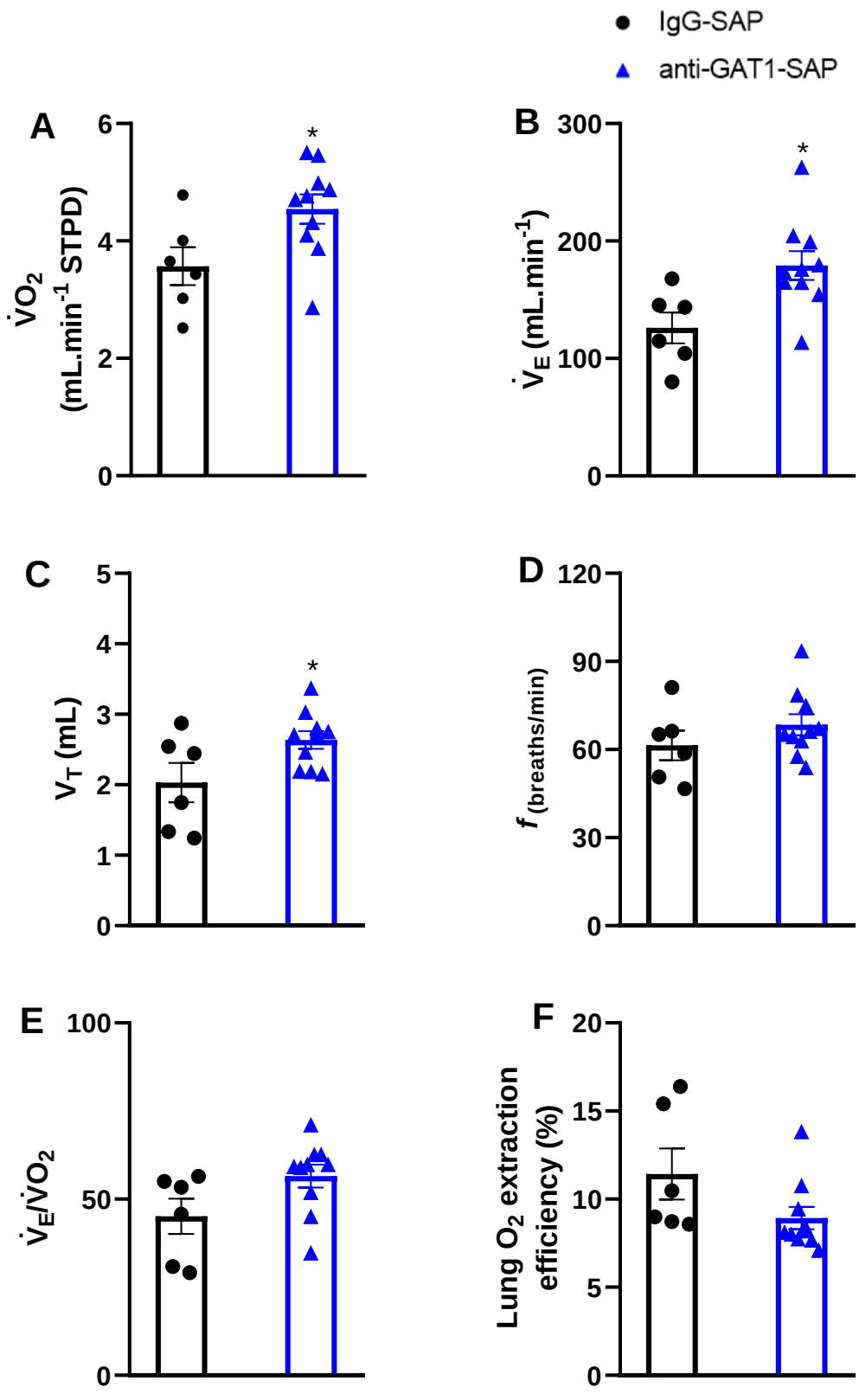


Figure 11. Effect of medullary raphe GABAergic neurons lesions (anti-GAT-SAP; 1.5 µM) on oxygen consumption ($\dot{V}O_2$, A), pulmonary ventilation ($\dot{V}_E$, B), tidal volume (V_T, C), breathing frequency (f, D), respiratory equivalent ($\dot{V}_E/\dot{V}O_2$, E) and lung O_2 extraction efficiency (F) in one-week-old chicks at 26°C.

Surprising results from our study indicate that the modulation of thermogenic and ventilatory responses to cold by the medullary raphe in chicken chicks does not involve its serotonergic neurons. This was evident because of the higher number of c-Fos-positive neurons in the medullary raphe in the cold, but very few of them were serotonergic. Besides that, selective lesion of the 5-HT cells did not affect Tb and activity of thermoeffectors. In contrast, GABA neurons demonstrated to be present in the medullary raphe seem to be relevant for thermoregulation in chicks, as their lesion increased Tb and O_2 consumption and influenced the ventilatory pattern in cold and in thermoneutral conditions.

Our study is the first one to show a higher expression of c-Fos in the medullary raphe of a species of bird, induced by a cold stimulus, similar to what is demonstrated in rodents (Bonaz and Taché, 1994; Bratincsák and Palkovits, 2004; Cano et al., 2003; Morrison et al., 1999). In contrast to cold, hot conditions seem not to activate neurons in the medullary Raphe in chicken chicks. This reinforces the idea of a thermorregulatory role of the medullary raphe in chicks mainly in the cold, but not in the heat (Cristina-Silva et al., 2021). In our previous report (Cristina-Silva et al., 2021), chicks exposed to heat showed no effect of the intra medullary raphe injections of the antagonists of glutamate NMDA and $GABA_A$ receptors on body temperature, oxygen consumption, ventilation and heat loss index. In contrast, in adult rats, both cold (4^oC) and heat (37^oC) exposures for 2 hours increase c-Fos expression in the raphe pallidus (Bratincsák & Palkovits, 2004), the main raphe region demonstrated to be involved in thermoeffectors modulation in those animals (Morrison and Nakamura, 2011). Cats exposed to cold also increase the activity of 5-HT neuron on medullary raphe (Martín-Cora et al., 2000)**.** Taken together, our results

and literature data suggest differences between birds and mammals in the thermal activation of the medullary raphe.

The serotonergic neurons of chicks´ brain were found in the midline of the brainstem, where the raphe is located, in agreement with Okado *et al.* (1992). This distribution is also equivalent in mammals (Azmitia, 1999; Gao and Mason, 2001; Jacobs and Azmitia, 1992; Kiyasova and Gaspar, 2011; Lidov and Molliver, 1982; Mason, 1997). We observed that anti-SERT-SAP was effective in deleting about 70% of the serotonergic neurons in the chick´s medullary raphe. This indicates that the sodium dependent serotonin transporter (SERT) in chickens (Larsen et al., 2004) is similar to that in rodents (Abe et al., 2016; Blakely et al., 1991; Hoffman et al., 1991; Kroeze et al., 2016; Mayser et al., 1991; Miceli et al., 2017; Qian et al., 1997; Ramamoorthy et al., 1993; van der Doelen et al., 2017; Zoratto et al., 2017) and shows immunotoxin specificity (Da Silva et al., 2011; Da Silva et al., 2013; Dias et al., 2007; Nattie et al., 2004). Even after this significant reduction in the number of 5-HT-ir neurons, no effect on heat loss (at 36^{o}C) and heat gain (at 26^{o}C) thermoeffector activities were observed. In fact, from the total of c-Fos positive neurons, about 10% corresponded to serotonergic ones at 26°C, 15% at 31°C and only 8% at 36°C, indicating a small activation of these neurons during changes in ambient temperature. This absence of a thermoregulatory role by 5-HT in chicks is another contrast with data in mammals. In rats, 5-HT seems to be a key neurotransmitter involved in the modulation by the medullary raphe of shivering (Nakamura and Morrison, 2011) and non-shivering (Nakamura and Morrison, 2007) thermogenesis. Moreover, knockout mice, with complete absence of central 5-HT neurons, rapidly become hypothermic when exposed to an ambient temperature of 4°C (Hodges et al., 2008)**.** This failure of thermoregulation was caused by impaired shivering and nonshivering thermogenesis, whereas thermosensory function and heat conservation

response were normal (Hodges et al., 2008). Besides of this evidence, the thermoregulatory role of medullary raphe in mammals seems to be stimulus-dependent as it does not influence responses to hypoxia (Dias et al., 2007; Gargaglioni et al., 2003). Regarding birds, a definitive role of brain serotonin in thermoregulation is still a pending question. In pigeons, intrahypothalamic injection of 5-HT inhibits panting (Pyörnilä et al., 1978), which could indicate a possible neural connection between hypothalamus and raphe. Such hypotheses, however, is not supported by our present results. Moreover, the injection of 5-HT might induce a pharmacological rather than a physiological thermoregulatory effect.

Our present study (Figs 1, 3C and 6) together with our previous report (Cristina-Silva et al., 2021) confirm the absence of a modulation by the medullary raphe on warmth-defense responses in chicken chicks. It is still unknown if this scenario changes in older chickens. If so, it will probably be related to a network rearrange between medullary raphe and other connected regions rather than to changes in the population of serotonergic neurons, as the distribution of 5-HT positive cells in the brainstem is established before hatch in chickens (Okado *et al.*, 1992).

As serotonergic neurons are not involved in the thermogenic effect of the medullary raphe in chicks, what kind of neurons do that? GABAergic cells, possibly among others, are likely to be involved in this function. Firstly, we provided evidence of the presence of GABAergic neurons, in addition to the serotonergic ones, in this region, as it occurs in mammals (Cao and Morrison, 2003; Cerri et al., 2013; DiMicco et al., 2006; Iceman et al., 2014; Zaretsky et al., 2003). Secondly, the lesion of about 60% of the GABA cells was capable of affecting the Tb, $\dot{V}O_2$ and $\dot{V}_E$ of chicks at the two temperatures tested, 31°C and 26°C. Tb increased in both thermal conditions in lesioned animals, which resulted from a higher metabolic rate (oxygen consumption). Higher Tb

is also observed after intra medullary raphe injection of the $GABA_A$ agonist muscimol, while the $GABA_A$ antagonist bicuculine reduces both Tb and O_2 consumption (Cristina-Silva et al., 2021). Thus, at least GABAergic, but not serotonergic, neurons are involved in the modulation of metabolic rate by the medullary raphe in chicken chicks, mainly in the cold (present study; Cristina-Silva et al., 2021). These results, however, do not exclude a possible involvement of other neuron phenotypes in the thermogenesis modulation by raphe. This is because the antagonism of NMDA glutamatergic receptors in the medullary raphe of chicks decreases Tb and thermogenesis (Cristina-Silva et al., 2021), suggesting the participation of other than serotonergic and GABAergic cells in such responses.

The GABA lesion affected differently the ventilatory pattern in chicks, causing a tachypneic response in thermoneutrality (higher frequency without change in tidal volume) and a higher ventilation based on tidal volume increase (no change in frequency) in the cold. Some studies in mammals demonstrated that medullary raphe responds differently in relation to breathing activity, depending on the neuronal population and specific region affected. A subset of GABAergic cells within the medullary raphe is thought to be involved in processes such as CO_2 chemosensation, temperature, pain, stress, and cardiovascular regulation (Cao and Morrison, 2003; Cao et al., 2006; Iceman et al., 2014; Zaretsky et al., 2003). Regarding chicks in thermoneutrality, GABA neurons in medullary raphe may tonically inhibit breathing frequency, as evidenced by both specific lesions (Fig. 10) and glutamate antagonism with AP5 (Cristina-Silva et al., 2021). Alternatively, tachypnea might be a secondary effect, as a response against the thermogenesis and hyperthermia induced by GABA lesion.

In conclusion, our results indicate that, in contrast to mammals, the modulation of thermogenic and ventilatory responses to cold by the medullary raphe in chicken chicks

does not involve serotonergic neurons. Among other neuron phenotypes possibly involved in this neural modulation, we provide evident of a role for raphe GABA cells. Such results open a new perspective related to the neural regulation of body temperature in endothermic vertebrates. Such differences in a thermoregulatory neural pathway might be well inserted in the scenario of distinct evolutionary pathways for endothermy in birds and mammals, as for example the distinct nonshivering thermogenesis effectors (only muscle *vs* BAT and muscle, respectively).

Supplementary Table 1. Comparison between the effects of the vehicles PBS (0.01M) and IgG-SAP (0.5 µM) on the variables measured in protocols 1 and 2 at different conditions (26°C, 31°C and 36°C) in one-week-old chicks.

	26°C		**31°C**		**36°C**	
	PBS 0.01 M	**IgG-SAP 0.5 µM**	**PBS 0.01 M**	**IgG-SAP 0.5 µM**	**PBS 0.01 M**	**IgG-SAP 0.5 µM**
	(n=10)	*(n=16)*	*(n=12)*	*(n=9)*	*(n=9)*	*(n=7)*
Tb (°C)*	41.3±0.1^{b}	41.1±0.1^{b}	41.4±0.1^{ab}	41.4±0.1^{ab}	41.6±0.1^{a}	41.6±0.3^{a}
$\dot{V}O_2$ (mL min^{-1} STPD)	3.9±0.3^{a}	4.2±0.2^{a}	2.6±0.1^{b}	2.9±0.1^{b}	1.7±0.3^{b}	2.1±0.4^{b}
$\dot{V}_E$ (mL min^{-1})	141.6±13.4^{b}	147.9±9.6^{b}	96.9±5.3^{b}	102.6±7.3^{b}	156.43±23.9^{a}	151.1±41.6^{a}
V_T (mL)	2.4±0.2^{a}	2.5±0.1^{a}	2.0±0.1^{a}	1.9±0.1^{a}	0.8±0.1^{b}	1.0±0.1^{b}
***f* (breaths min^{-1})**	59.5±2.4^{b}	60.1±2.9^{b}	49.1±3.3^{b}	53.9±5.7^{b}	240.7±55.5^{a}	171.8±53.2^{a}
$\dot{V}_E/\dot{V}O_2$	39.0±5.2^{b}	37.4±3.6^{b}	39.3±3.5^{b}	35.7±1.7^{b}	93.7±7.0^{a}	71.3±10.5^{a}
Lung O_2 extraction efficiency (%)	14.1±1.4^{a}	14.9±1.2^{a}	13.5±1.2^{a}	14.1±0.8^{a}	5.6±0.5^{b}	8.6±1.3^{b}
Body Mass (g)	121.5±7.8	131.5±7.8	112.9±5.2	119.4±9.1	94.8±5.9	92.0±11.8
	(n=5)	*(n=14)*	*(n=7)*	*(n=10)*	*(n=7)*	*(n=11)*
Tb (°C)*	40.4±0.08^{b}	40.8±0.07^{b}	40.9±0.1 ^{b}	40.7±0.1^{b}	41.2±0.2^{a}	41.3±0.1^{a}
HLI	0.08±0.03^{b}	0.14±0.04^{b}	0.54±0.15^{a}	0.47±0.07^{a}	0.73±0.04^{a}	0.70±0.05^{a}
Body Mass (g)	99.3±7.7	108.4±5.0	90.5±6.4	113.6±7.9	111.7±5.8	106.3 ±4.7

*Tb, body temperature. There are two different values for Tb because the experiments with respirometry and for HLI determination were done in different groups of animas; $\dot{V}O_2$, oxygen consumption; $\dot{V}_E$, pulmonary ventilation; V_T, tidal volume; *f*, breathing frequency; $\dot{V}_E/\dot{V}O_2$, respiratory equivalent; HLI, heat loss index. The number of animals in each group is shown in parentheses. Different letters indicate significant differences among groups ($p< 0.05$). All values are mean ± s.e.m.

Supplementary Table 2. Comparison between the effects of the vehicles PBS (0.01M) and IgG-SAP (1.5 µM) on the variables measured in protocol 3 at different conditions (31°C and 26°C) in one-week-old chicks.

	26°C		**31°C**	
	PBS 0.01 M	**IgG-SAP 1.5 µM**	**PBS 0.01 M**	**IgG-SAP 1.5 µM**
	(n=10)	*(n=6)*	*(n=12)*	*(n=6)*
Tb (°C)*	41.3±0.1^{ab}	40.8±0.2^{b}	41.5±0.1^{a}	41.2±0.1^{ab}
$\dot{V}O_2$ (mL min^{-1} STPD)	3.9±0.3^{a}	3.6±0.3^{a}	2.6±0.1^{b}	2.6±0.2^{b}
$\dot{V}_E$ (mL min^{-1})	141.6±13.4^{a}	126.0±13.1^{a}	96.9±5.3^{b}	95.7±7.8^{b}
V_T (mL)	2.4±0.2	2.0±0.3	2.0±0.1	2.1±0.1
***f* (breaths min^{-1})**	59.5±2.4	61.4±5.1	49.1±3.3	44.9±3.8
$\dot{V}_E/\dot{V}O_2$	39.0±5.2	45.1±5.0	39.3±3.5	36.3±3.2
Lung O_2 extraction efficiency (%)	14.1±1.4	11.4±1.4	13.5±1.2	12.1±1.9
Body Mass (g)	121.5±7.8	106.8±14.9	112.9±5.2	130.1±6.7

Tb, body temperature; $\dot{V}O_2$, oxygen consumption; $\dot{V}_E$, pulmonary ventilation; V_T, tidal volume; *f*, breathing frequency; $\dot{V}_E/\dot{V}O_2$, respiratory equivalent; HLI, heat loss index. The number of animals in each group is shown in parentheses. Different letters indicate significant differences among groups ($p< 0.05$). All values are mean ± s.e.m.

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