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RESEARCH ARTICLE

A thermoregulatory role for the medullary raphe in birds

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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 hypothesized that the medullary raphe modulates heat gain and loss thermoeffectors in birds. We investigated the effect of glutamatergic and GABAergic inhibition in this specific region on body temperature (T_b), 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 (31°C), cold (26°C) and hot (36°C) 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 T_b of chicks at 31 and 26°C, mainly as a result of an O_2 consumption decrease. AP5 transiently increased breathing frequency during cold exposure. At 31°C, heat loss index was higher in the bicuculline and AP5 groups (higher doses) than in the saline groups at the beginning of the T_b reduction. No treatment affected any variable tested at 36°C. 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-defense 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 T_b in endotherms.

KEY WORDS: Brainstem, GABA, Glutamate, Chick, Oxygen consumption, Heat loss index, Thermal tachypnea

INTRODUCTION

Regulation of body temperature (T_b) 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, 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,b, 2000, 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, 1976; 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 Bicego 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 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 T_b in birds (Sallagundala

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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 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 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, 1970) and cutaneous vasodilation (Richards, 1970, 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.

MATERIALS AND METHODS

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 (37°C, 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 31–32°C, 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 and 17:00 h to avoid any influence of the daily T_b 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 5 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, T_b or plasma corticosterone levels (Coleone et al., 2009; Furuse et al., 1997; Saito et al., 2005).

Determination of T_b

T_b 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 2 days after sensor implantation. Individual T_b was recorded in real time by telemetry with the appropriate reader for BioTherm sensors (Biomark HPR Plus Reader) and transferred to a computer (BioTherm software). T_b 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).

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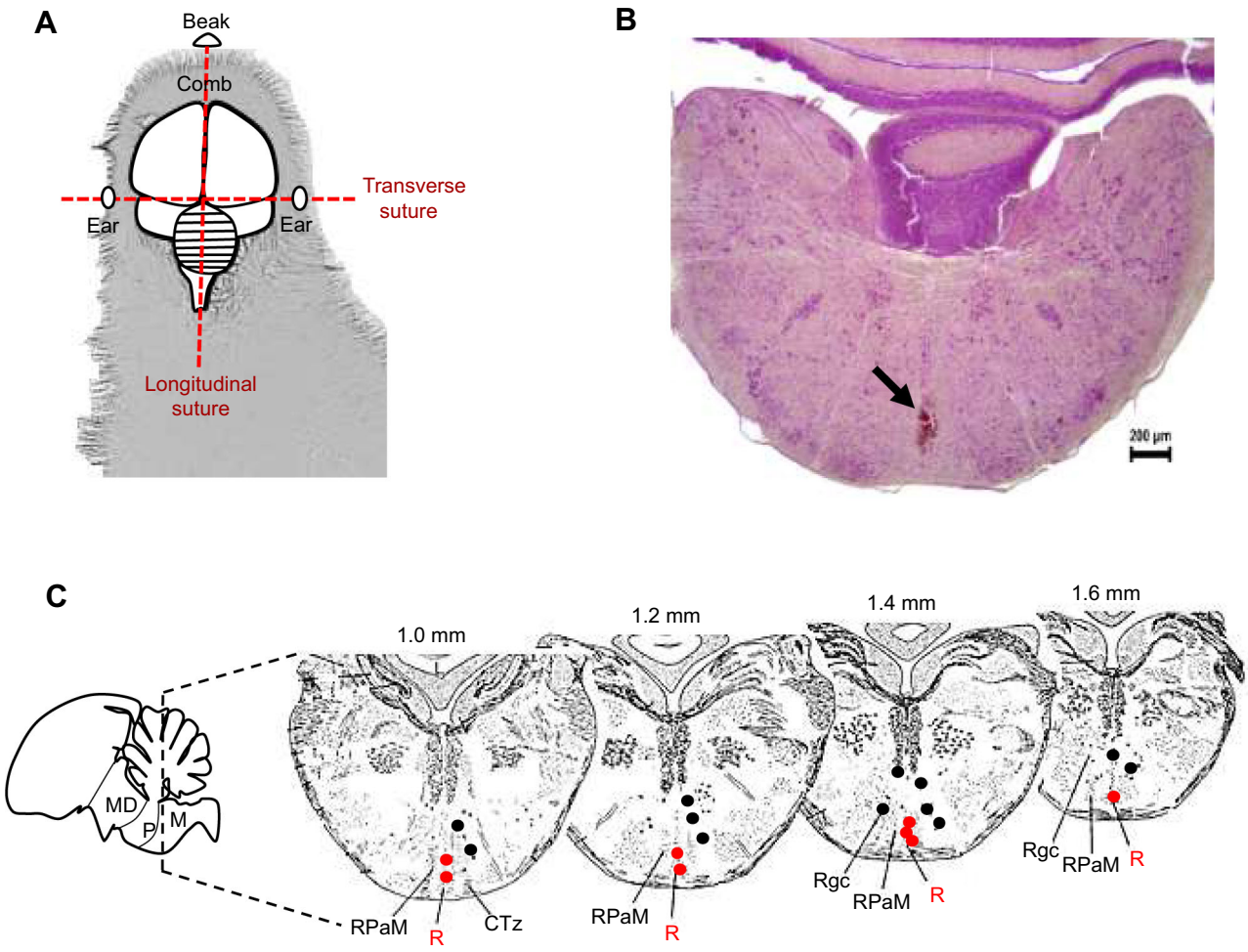

Fig. 1. Head and brain coordinates for microinjection of chicks and the actual sites of injection into the medullary raphe. (A) Schematic drawing showing the intersection between sutures (longitudinal and transverse) and the references used during the injection procedure (comb, ear and beak), as the 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 intra-raphe (red circles) and peri-raphe (black circles) microinjection sites at distances 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.

Oxygen consumption

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 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) was pulled through a water vapor pressure analyzer (RH300, Sable Systems) to an O_2 analyzer (PA-10, Sable Systems) after air drying (Drierite, with indicator, 8 mesh, Sigma-Aldrich Brazil Ltda). The values for water vapor 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 concentration at the inlet (baseline) and outlet of the chamber. All of the analyzers 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). The O_2 analyzer was calibrated before each experiment using nitrogen (0% oxygen) and dry ambient air (20.95% oxygen). As CO_2 was neither analyzed nor scrubbed, $\dot{V}_{O_2}$ was calculated using the following equation (Koteja, 1996):

$$\dot{V}_{O_2} = [FR_{ex}(F_{IO_2} - F_{EO_2})]/[1 - F_{IO_2}(1 - RQ)], \quad (1)$$

where FR_{ex} is the excurrent flow rate, F_{IO_2} is the incurrent fractional concentration of oxygen (baseline), F_{EO_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

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 ($V_T \times f = \dot{V}_E$). V_T was determined from the following formula (Drorbaugh and Fenn, 1955): $V_T = A(V_{cal}/P_{cal})[T_b(P_B - P_{H_2O,chamber})]/\{[T_b(P_B - P_{H_2O,chamber})] - [T_{chamber}(P_B - P_{H_2O,bird})]\}$, where A is the wave amplitude (pressure oscillations), V_{cal} is the calibration volume, P_{cal} is the calibration pressure, P_B is the barometric pressure, $P_{H_2O,chamber}$ is the water vapor pressure inside the chamber, $T_{chamber}$ is the temperature inside the respirometry chamber, and $P_{H_2O,bird}$ is the saturation vapor 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. 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 (T_a) within the respirometer.

During the heat exposure (36°C) experiments, $T_{chamber}$ tends to approach T_b, 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 T_b 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 liters) was kept inside a climate chamber at the highest T_a (FANEM), while the other compartment (1 liter) remained outside at room temperature (~25°C) to achieve a desirable T_b–T_a gradient. The calculation of V_T was performed using the formula above, with the difference being that weighted averages, relative to the volume of the two compartments, were used for $T_{chamber}$ and $P_{H_2O,chamber}$.

From the O_2 consumption and pulmonary ventilation data, the ventilatory equivalent, which is $\dot{V}_E/\dot{V}_{O_2}$, and the lung extraction efficiency of O_2, which is $[\dot{V}_{O_2}/(\dot{V}_E \cdot F_{I_{O_2}})] \times 100$ (where $F_{I_{O_2}}$ 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.

HLI

The surface temperature (T_s) 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). T_s (emissivity 0.95), T_b and T_a were used to calculate the animal's HLI, according to the formula: $HLI = (T_s - T_a)/(T_b - T_a)$ (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 T_b, 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 T_a determination.

Experimental design

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

Protocol 1: effect of NMDA and $GABA_A$ receptor activity in the medullary raphe on T_b, $\dot{V}_{O_2}$ and $\dot{V}_E$ at 31, 26 and 36°C

Chicks were placed individually inside a respirometer at 30–31°C for habituation (~40 min), followed by $\dot{V}_{O_2}$, $\dot{V}_E$ and T_b measurements. Following this period, the animal received a 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 T_b for ~2 h 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. T_b was recorded every 20 min. Based on the results obtained at 31°C, doses of the drugs that did not affect T_b were chosen for the protocols performed under acute thermal stimulation of cold (26°C) and hot (36°C) conditions. 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 stress using a dose that already affected T_b 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 25–26 or 35–36°C for ~1.5 h, 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 31°C, only to confirm the opposite effect to the antagonist bicuculline (see Discussion). Different groups of animals were used for different treatments and T_a.

Protocol 2: effect of NMDA and $GABA_A$ receptor activity in the medullary raphe on T_b and HLI at 31, 26 and 36°C

After a 40 min habituation interval inside the apparatus used to determine HLI, chicks previously exposed for ~1.5 h to one of the three temperatures had their T_b 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. T_b was then measured and infrared images were taken every 20 min for the next 2 h. Different groups of animals were used for different treatments and T_a.

Histology

Following completion of the experiments, animals were deeply anesthetized with 2,2,2-tribromoethanol (250 mg kg^{-1}; Sigma-Aldrich Brasil Ltda) 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 2 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 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 ANOVA (factor: T_a) to compare pre-injection values of body mass, T_b, 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 ANCOVA was performed to check whether the effect of T_a 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 Šidák *post hoc* test, and were considered significant at $P<0.05$.

RESULTS

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

Table 1 shows the T_b, $\dot{V}_{O_2}$, $\dot{V}_E$, V_T, f, $\dot{V}_E/\dot{V}_{O_2}$, lung O_2 extraction efficiency and body mass (from protocol 1), and T_b, HLI and body mass (from protocol 2) of intact chicks, i.e. at the pre-injection time, without any pharmacological treatment. In protocol 1, the T_b of chicks exposed for 2 h to 26°C did not differ from that of the group at 31°C ($P=0.0851$), whereas exposure to 36°C resulted in higher T_b compared with that at 31°C ($P=0.0007$). Body mass was different among the three groups of animals ($P=0.0003$). Based on the ANCOVA, it was observed that 2 h 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 with the group at 31°C. Total ventilation increased in animals at 26°C ($P<0.0001$) as a result of the higher V_T ($P<0.0001$) and unchanged f ($P=0.9580$), compared with the group at 31°C. In contrast, the higher ventilation at 36°C ($P=0.0011$) 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 with the chicks at 31°C. Exposure to heat ($P<0.0001$), but not to cold ($P=0.2238$), reduced the lung O_2 extraction efficiency of chicks. In protocol 2, the body mass of chicks maintained at 31°C was higher than that of animals used exposed to cold ($P=0.0251$), but did not differ from the group exposed to heat stress ($P=0.1135$). T_b responses to the different T_a followed the same pattern as in 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 not significantly change this variable ($P=0.1106$), compared with the thermoneutral condition.

Micro-injection sites at the medullary raphe

Fig. 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.

Effect of changes in NMDA and $GABA_A$ receptor activity in the medullary raphe on T_b, $\dot{V}_{O_2}$ and $\dot{V}_E$ of 1 week old chicks under different T_b

Fig. 2 shows the effects of AP5 (0.5 or 5 mmol l^{-1}) or saline control treatment on the T_b of chicks maintained at 31°C (Fig. 2A), 26°C (Fig. 2B) or 36°C (Fig. 2C). T_b did not change after intra-raphe microinjection of saline or 0.5 mmol l^{-1} AP5 at 31°C, but decreased with the highest dose (5 mmol l^{-1}) of AP5 from 60 to 100 min after microinjection (interaction between time and treatment: $P=0.0141$, $F_{3,31}=1.956$). In the cold, 0.5 mmol l^{-1} AP5 caused a decrease in T_b 40–100 min after microinjection (time effect: $P<0.0001$, $F_{2,15}=6.167$; treatment effect: $P=0.0109$, $F_{2,15}=6.521$). At 36°C, however, T_b 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 T_b of chicks in any of the thermal conditions tested.

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

	T_a=26°C	T_a=31°C	T_a=36°C
Protocol 1			
T_b (°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			
T_b (°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[a,b] (25)

T_b, 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.

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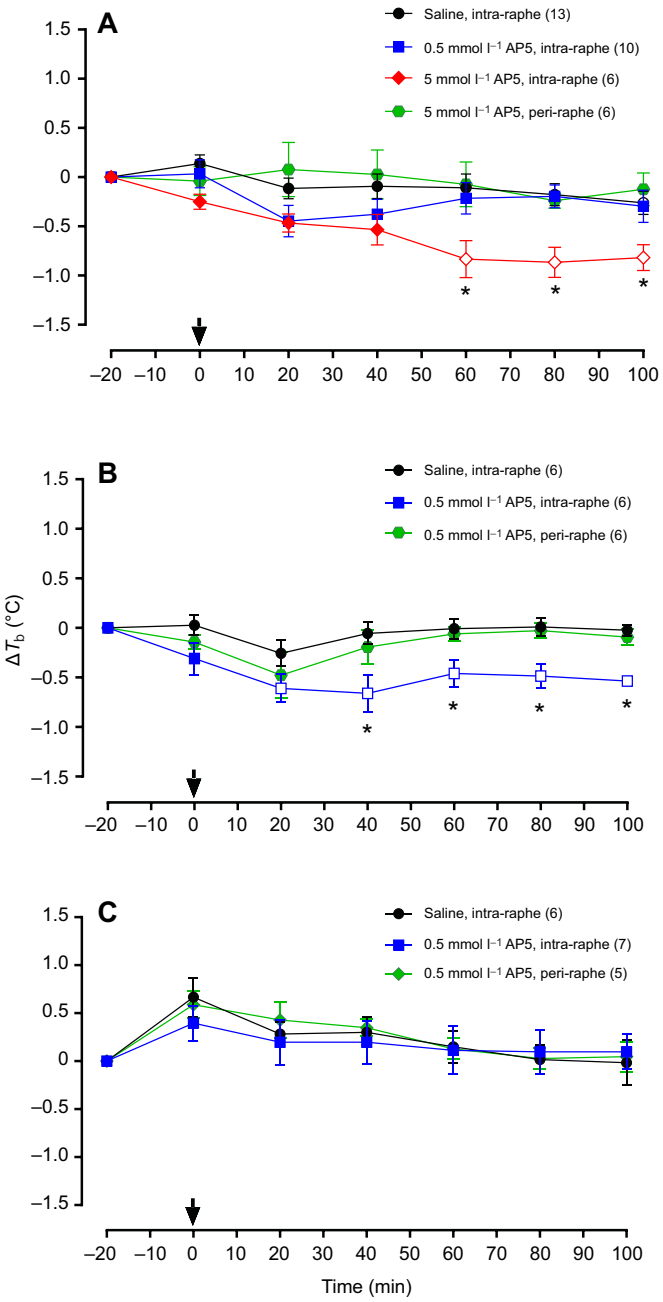

Fig. 2. Effect of intra-raphe microinjection of the NMDA receptor antagonist AP5 on the body temperature (T_b) of 1 week old chicks at different ambient temperatures (T_a). Chicks were exposed to a T_a of (A) 31°C, (B) 26°C and (C) 36°C. The arrow indicates the time of microinjection of 0.5 or 5 mmol l^{-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 in 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.

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

Fig. 4 shows the effects of the NMDA antagonist AP5 on $\dot{V}_{O_2}$, $\dot{V}_E$, V_T, f and $\dot{V}_E/\dot{V}_{O_2}$ in chicks exposed to 31°C and 26°C. At 31°C, 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 with 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 26°C, 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 20–40 min after AP5 microinjection at 26°C (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.

Intra-raphe bicuculline at 0.5 mmol l^{-1}, but not 0.05 mmol l^{-1}, reduced oxygen consumption 60–100 min after microinjection in chicks at 31°C (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 26°C, 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 31°C, no effect of 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).

AP5 and bicuculline treatment had no effect on T_b, $\dot{V}_{O_2}$, $\dot{V}_E$, V_T, f, $\dot{V}_E/\dot{V}_{O_2}$ (Fig. S1) and HLI (Fig. S2) at 36°C.

Chicks maintained at 31°C showed higher HLI 60–80 min after injection of 5 mmol l^{-1} AP5 compared with saline treatment, because of a significant decrease in the saline group and a non-significant increase in the AP5 group at this time interval (time effect: P=0.0414, $F_{2,18}$=2.168; treatment effect: P=0.0239, $F_{2,18}$=4.628; Fig. 6A). These chicks showed a reduction in T_b 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 26°C, 0.5 mmol l^{-1} AP5 did not change the HLI of

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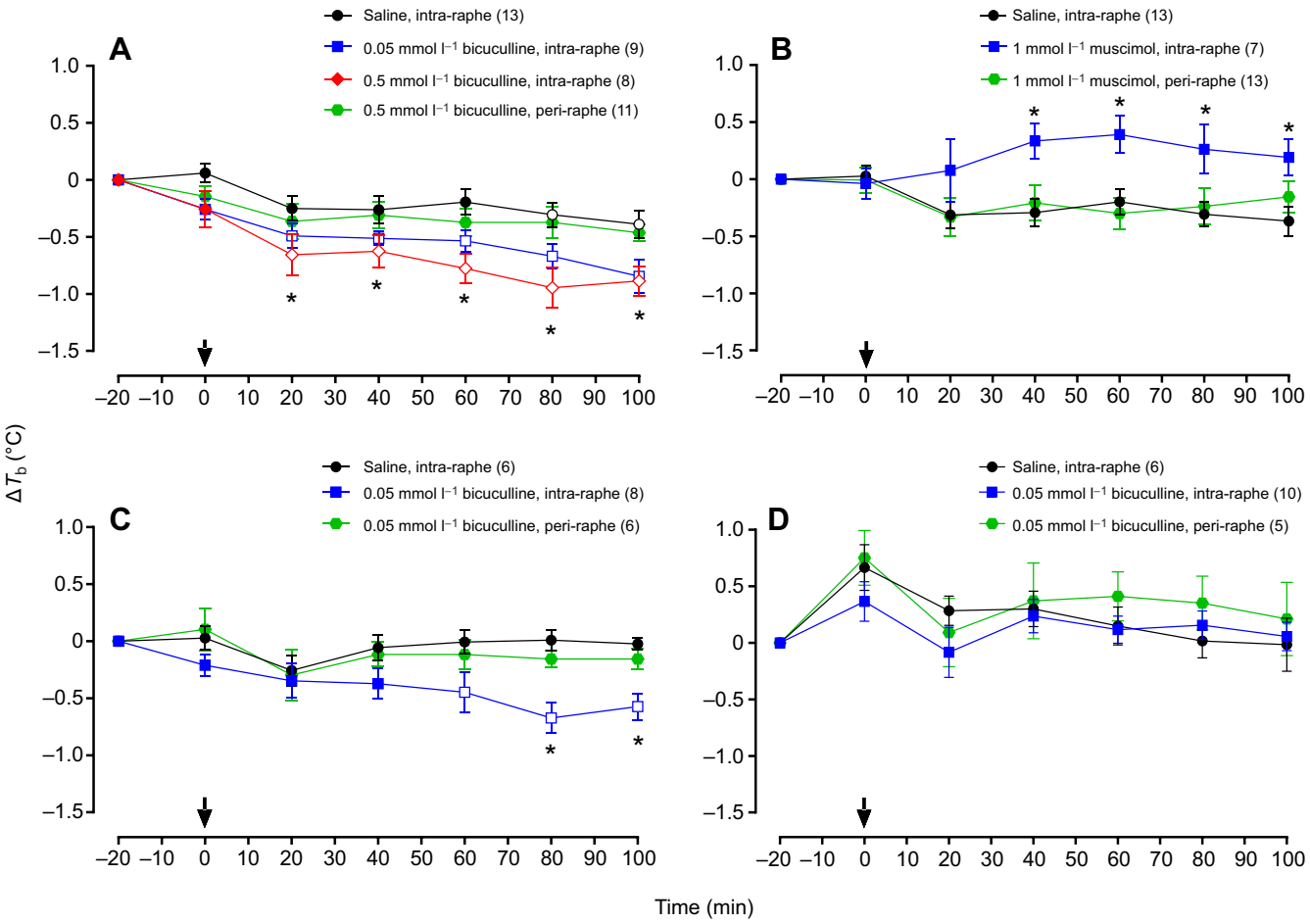

Fig. 3. Effect of intra-raphe microinjection of the $GABA_A$ receptor antagonist bicuculline and agonist muscimol on the T_b of 1 week old chicks at different T_a. Chicks were exposed to a T_a of (A) 31°C, (C) 26°C and (D) 36°C for bicuculline treatment, and (B) 31°C for muscimol treatment. The arrow indicates the time of microinjection of 0.05 or 0.5 mmol l^{-1} bicuculline, 1 mmol l^{-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 in 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.

chicks (time effect: P=0.0019, $F_{1,15}$=3.513; treatment effect: P=0.0514, $F_{1,15}$=4.432; Fig. 6B), even with a reduction in T_b 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} bicuculline increased HLI at 60–80 min (interaction between time and treatment: P=0.036, $F_{2,19}$=2.487; Fig. 6C), while T_b decreased 80–120 min after injection (interaction between time and treatment: P=0.0055, $F_{2,19}$=3.071; Fig. S3). Treatment with 0.05 mmol l^{-1} bicuculline did not affect HLI at 26°C (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 T_b (interaction between time and treatment: P=0.0078, $F_{1,16}$=2.897; Fig. S3). Thermographic images in Fig. 6 illustrate the colder feet (image ii) observed in chicks at 26°C compared with chicks at 31°C (image i). At 31°C, warmer feet can be seen 80 min after intra-raphe injection of 5 mmol l^{-1} AP5 (image iii; Fig. 6) and bicuculline (image iv; Fig. 6), compared with saline injection at 80 min.

DISCUSSION

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 36°C).

In endotherms, acute decreases and increases in T_a activate physiological mechanisms involved in heat production/conservation and heat loss, respectively. A reduction in T_a 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; Bicego et al., 2007). At 26°C, 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 T_b 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 T_b of chicks (hyperthermia), combined with an increase in breathing frequency (f) and a reduced V_T, characterizing a thermal tachypnea, or panting (Bicego et al., 2007; Mortola and

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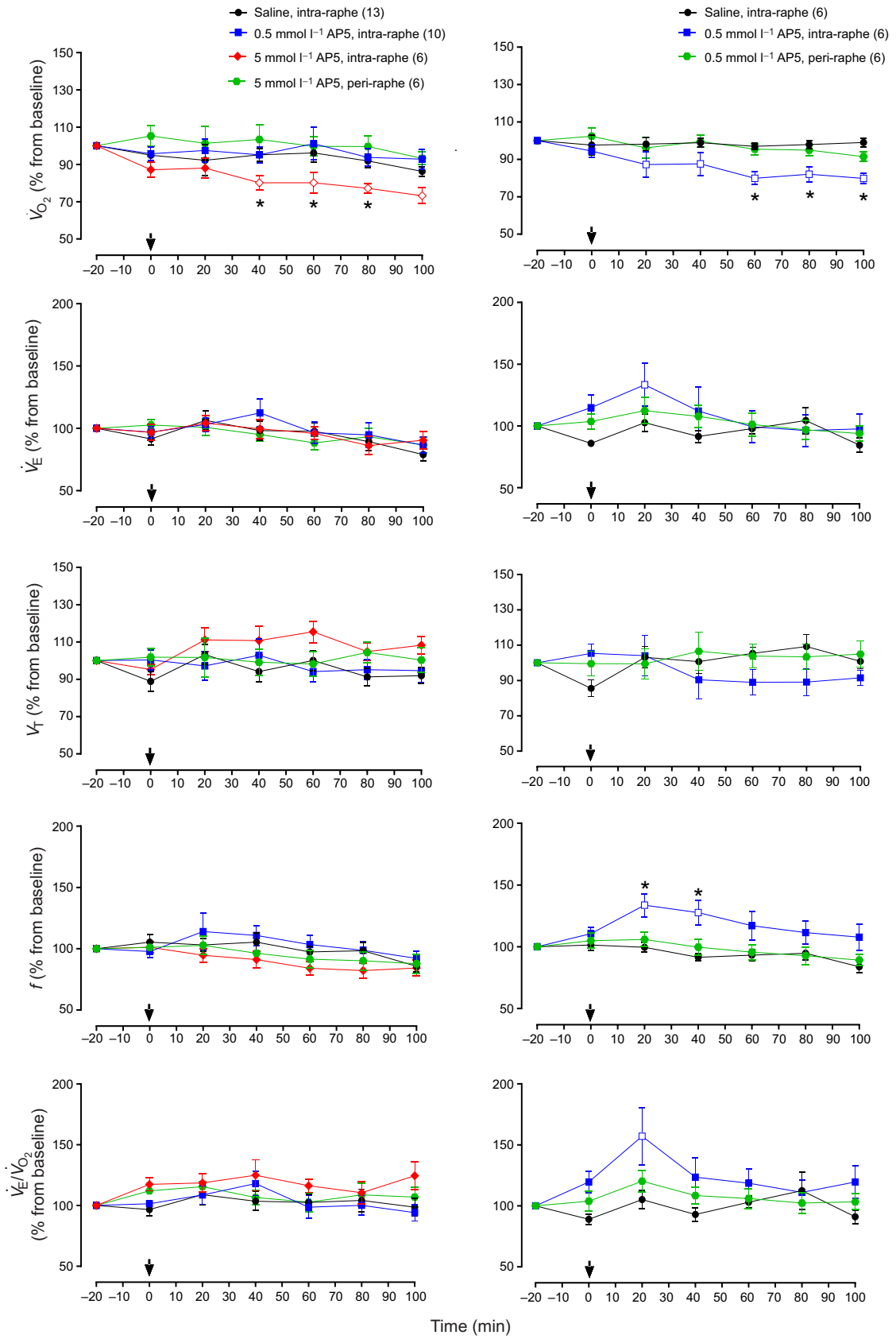

Fig. 4. Effect of intra-raphe microinjection of the NMDA receptor antagonist AP5 on oxygen consumption ($\dot{V}_{O_2}$), pulmonary ventilation ($\dot{V}_E$), tidal volume (V_T), breathing frequency (f) and respiratory equivalent ($\dot{V}_E/\dot{V}_{O_2}$) of 1 week old chicks at 31 and 26°C. Left, 31°C; right, 26°C. The arrow indicates the time of microinjection of 0.5 or 5 mmol l^{-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 in 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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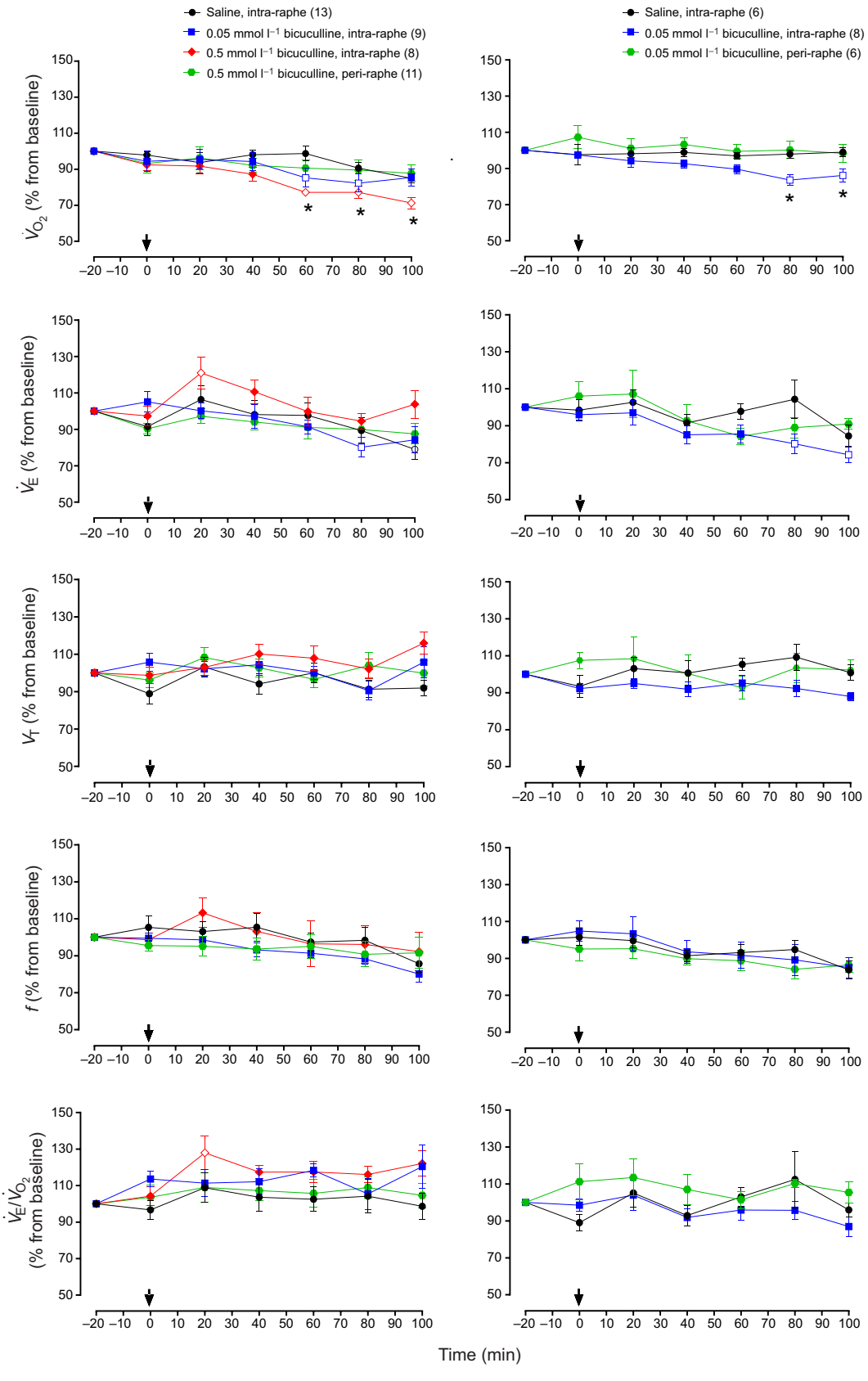

Fig. 5. Effect of intra-raphe microinjection of the $GABA_A$ receptor antagonist bicuculline on $\dot{V}_{O_2}$, $\dot{V}_E$, V_T, f and $\dot{V}_E/\dot{V}_{O_2}$ of 1 week old chicks at 31 and 26°C. Left, 31°C; right, 26°C. The arrow indicates the time of microinjection of 0.05 or 0.5 mmol l^{-1} bicuculline 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 in 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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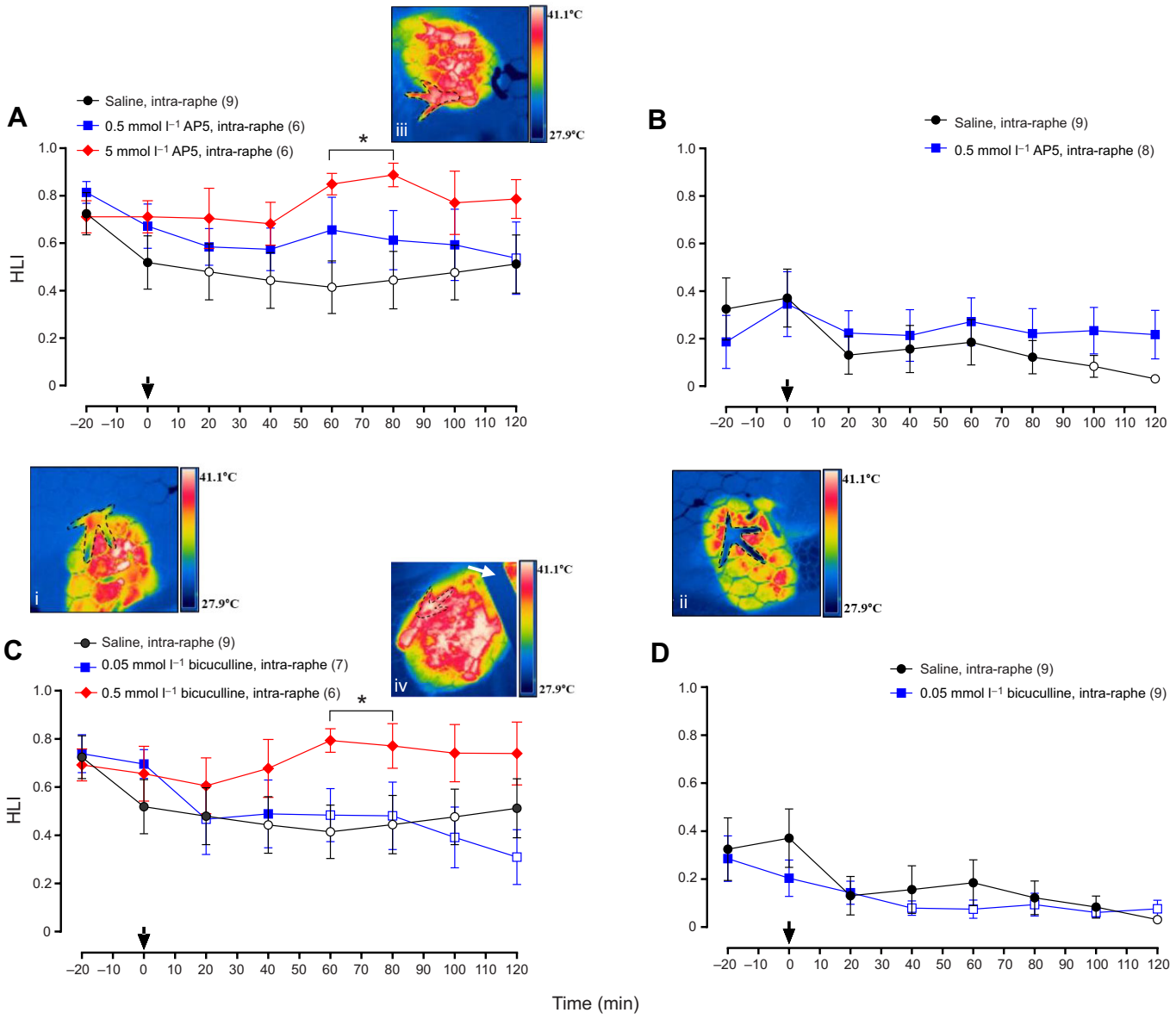

Fig. 6. Effect of intra-raphe microinjection of the NMDA receptor antagonist AP5 or the $GABA_A$ receptor antagonist bicuculline on the heat loss index (HLI) of 1 week old chicks at 31 and 26°C. Left, 31°C; right, 26°C. The arrow indicates the time of microinjection of 0.5 or 5 mmol l^{-1} AP5 (A,B), 0.05 or 0.5 mmol l^{-1} bicuculline (C,D) or vehicle (saline) control into the medullary raphe (intra-raphe). The number of animals is shown in 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 31°C (i) and 26°C (ii) before microinjection, and at 31°C 80 min after 5 mmol l^{-1} AP5 (iii) or 0.5 mmol l^{-1} bicuculine (iv) microinjection are also shown. The white arrow in iv indicates the black tape (emissivity 0.95) on the floor for determination of T_a.

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 and Maskrey, 2011). In chicks in the present study, despite the increased air convection requirement indicating hyperventilation, the respiratory pattern retained 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 bird 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 36°C for 2 h had lower oxygen consumption than those at 31°C, 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

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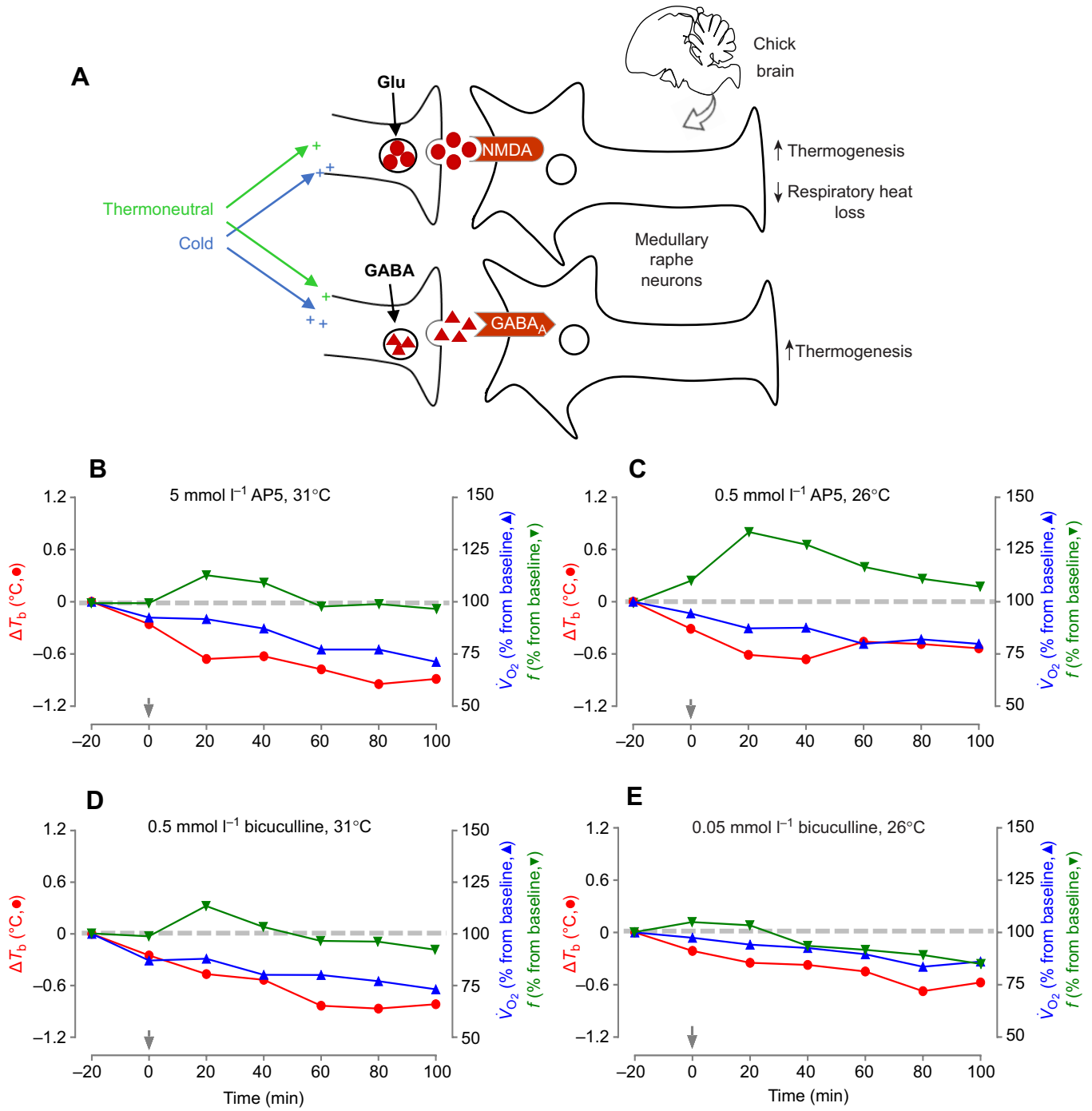

Fig. 7. Thermoregulatory role of the medullary raphe in the activation of heat production and conservation in chicks. (A) Schematic drawing depicting glutamatergic and GABAergic influences on medullary raphe neurons modulating thermoeffectors involved in heat gain and heat loss in 1 week old chicks at thermoneutrality and in the cold. Glutamate (Glu) binds to NMDA receptors in raphe neurons, increasing thermogenesis and inhibiting respiratory heat loss to keep T_b constant. The main results (B,C) that support this idea are those for the reduction of T_b by the NMDA antagonist AP5 (5 mmol l^{-1} at 31°C, B; and 0.5 mmol l^{-1} at 26°C, 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 26°C (C, at a lower dose) than at 31°C (B). The influence of GABA on GABA$_A$ receptors in raphe neurons is more complex (see Discussion), but it seems to have a similar effect on thermogenesis (A,D,E) but minimum effect on f (D).

T_a (pre-microinjection values; Table 1) indicate that 1 week old broiler chicks are able to activate autonomic thermoeffectors in both cold (~5°C below 31°C) and hot (~5°C above 31°C) conditions, being more sensitive to the latter stimulus. Thus: (i) 31°C seems to be well inside the TNZ of these chicks; (ii) 36°C is clearly above TNZ, as evidenced by the activation of thermal tachypnea, a known evaporative heat loss response; and (iii) 26°C 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 T_b at 31°C (higher doses) and at 26°C (lower doses), together with a metabolic reduction. This indicates a

stimulatory role for the raphe on thermogenesis in these animals, similar to that observed in rats (Morrison et al., 2012, 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, 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 needs 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 co-expression 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 T_b, 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 treatment with glutamate and GABA receptor antagonists on any of the variables tested (Figs 2C and 3D; Figs S1 and S2). These results indicate that the medullary raphe plays no role in the neural regulation of T_b 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 for inhibition of the 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 T_b 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°C adds support to the specificity of the role of the 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).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: C.C.-S., K.C.B.; Formal analysis: C.C.-S., L.H.G., K.C.B.; Investigation: C.C.-S., K.C.B.; Writing - original draft: C.C.-S., L.H.G., K.C.B.

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Supplementary information

Supplementary information available online at
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