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An adenosine A_{2A} agonist injected in the nucleus of the solitary tract prolongs the laryngeal chemoreflex by a GABAergic mechanism in decerebrate piglets

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Abstract

Hyperthermic prolongation of the laryngeal chemoreflex (LCR) in decerebrate piglets is prevented or reversed by gamma-aminobutyric acid A (GABA_A) receptor antagonists and adenosine A_{2A} (Ad-A_{2A}) receptor antagonists administered in the nucleus of the solitary tract (NTS). Therefore, we tested the hypothesis that enhanced GABA_A activity and administration of the Ad-A_{2A} agonist, CGS-21680, would prolong the LCR under normothermic conditions. We studied 46 decerebrate piglets ranging from 3 to 8 post-natal days of age. Focal injection into the NTS of 100 nl of 0.5 M nipecotic acid, a GABA reuptake inhibitor, significantly ($P < 0.05$) prolonged the LCR under normothermic conditions in 10 of 11 animals tested. Injecting 100 nl of 5–12.5 microM CGS-21680 unilaterally or bilaterally into the NTS also prolonged the LCR under normothermic conditions ($n=15$), but the effect was smaller than the effect of unilateral injection of nipecotic acid. Systemic administration of the GABA_A receptor antagonist, bicuculline, prevented the CGS-21680-dependent prolongation of the LCR in normothermic animals ($n = 11$). We conclude that thermal prolongation of the LCR depends on a thermally sensitive process or set of neurons in the NTS, which, when activated by elevated brain temperature, enhance adenosinergic and GABAergic function in the region of the NTS. These results emphasize the importance of a thermally sensitive integrative site in the dorsal medulla that, along with sites in the ventral medulla, determine the response to laryngeal chemoreflex stimulation.

Keywords

SIDS; laryngeal chemoreflex; hyperthermia; adenosine; nucleus of the solitary tract

Introduction

Neonatal animals are peculiarly susceptible to respiratory inhibition by reflex mechanisms. The diving reflex, the Hering-Breuer inflation reflex and the laryngeal chemoreflex (LCR) are all present in neonatal animals and particularly potent. Among these reflexes, the LCR is probably the most frequently elicited in the normal course of events, and many investigators have suggested that the LCR contributes to the pathogenesis of Sudden Infant Death Syndrome (SIDS) (Thach 2001, 2005; Leiter & Böhm 2007). Elevated body temperature increases the strength of the LCR, but the thermal enhancement of the LCR diminishes as animals mature (Curran *et al.* 2005; Xia *et al.* 2008b). Hyperthermia reduces the stimulation threshold for laryngeal adduction by electrical stimulation of the superior laryngeal nerve (SLN) in puppies, but this effect is absent in adult dogs (Haraguchi *et al.* 1983). In lightly

anesthetized rat pups, the LCR is enhanced by elevating body temperature by 1.5–2.0°C from postnatal day 3 (P3) to P25, but the magnitude of thermal prolongation of the LCR wanes during the first 21 days of life, even though the LCR (unmodified by body temperature) persists beyond 21 days of age in rats (Xia *et al.* 2008b). On the other hand, focally warming the area in and around the nucleus of the solitary tract (NTS) ~2.0°C reversibly prolonged the LCR, even though body temperature was held constant at ~38°C (Xia *et al.* 2006). Therefore, thermal prolongation of the LCR seems to originate in the central nervous system (CNS) from an elevated temperature in the region of the NTS.

The duration of laryngeal apnea induced by electrical stimulation of the superior laryngeal nerve in normothermic decerebrate neonatal piglets was shortened by intracisternal or intravenous administration of bicuculline, a GABA_A receptor antagonist (Abu-Shaweesh *et al.* 2001). Unilateral microdialysis of gabazine (also a GABA_A receptor antagonist) in the dorsal medulla near the NTS reversed the thermal prolongation of the LCR in decerebrate piglets (Xia *et al.* 2007), but unlike the effect of intracisternal GABA_A antagonists, focal gabazine treatment in the region of the NTS did not change the duration of the LCR under normothermic conditions.

Adenosine may also modify the LCR and modify the effect of hyperthermia on the LCR. Activation of presynaptic Ad-A_{2A} receptors seems to enhance GABA release (Phillis 1998; Ochi *et al.* 2000; Hong *et al.* 2005). Therefore, the effect of adenosine antagonists on the LCR has been attributed to blockade of adenosine A_{2A} (Ad-A_{2A}) receptors on GABAergic neurons in the medulla (Martin *et al.* 2004; Wilson *et al.* 2004; Mayer *et al.* 2006). Consistent with such an hypothesis, Ad-A_{2A} agonists injected into the cistern of decerebrate piglets enhanced apnea elicited by SLN stimulation, and this Ad-A_{2A} effect was blocked by GABA_A antagonists (Abu-Shaweesh 2007). The site of Ad-A_{2A} action within the brainstem was not further explored in this study. Blocking Ad-A_{2A} receptors focally in the dorsal medulla near the NTS reversed the thermal prolongation of the LCR in decerebrate piglets (Xia *et al.* 2008a), but, once again, unlike the effect of cisternally administered adenosinergic agents, focal administration of Ad-A_{2A} and GABA_A antagonists in the NTS did not alter the LCR under normothermic conditions. Thus, thermal prolongation of the LCR seems to depend on GABAergic and adenosinergic mechanisms specifically in the region of the NTS. If blocking Ad-A_{2A} receptors prevents thermal prolongation of the LCR by interfering with GABAergic mechanisms, we reasoned that 1) activation of GABA receptors in the dorsal medulla should prolong the LCR even when body temperature is not elevated; 2) activation of Ad-A_{2A} receptors should prolong the LCR in the absence of any elevation of body temperature, but 3) Ad-A_{2A} receptor-dependent normothermic prolongation of the LCR within the NTS should be prevented by blocking GABA_A receptors if Ad-A_{2A} receptors achieve their effects by enhancing GABA release. We tested these hypotheses in a series of studies in normothermic decerebrate piglets using focal injections in the NTS of nipecotic acid, which blocks reuptake of GABA from the extracellular space and enhances activation of GABA receptors, and injection of a selective Ad-A_{2A} agonist in the presence or absence of systemically administered bicuculline to block GABA_A receptors.

Methods

Experiments were performed on 46 piglets, 24 of whom were male, ranging in age from 3 to 8 days (5.3 ± 1.2 days; mean $\pm$ SEM) with an average weight of 2.3 ± 0.5 kg. The Institutional Animal Care and Use Committee of Dartmouth College approved all surgery and experimental protocols.

Surgical preparation

Animals were anesthetized with 2% halothane (2-Bromo-2-chloro-1,1,1-trifluoroethane; Halocarbon Laboratories, NJ) in O₂. A rectal probe was inserted, and body temperature was maintained between 38 and 39°C using a heating pad. Femoral arterial and venous catheters were inserted to measure blood pressure and administer drugs, respectively. Each animal was tracheotomized and artificially ventilated (Harvard Apparatus Dual Phase Respirator, South Natick, MA) to maintain the end-tidal CO₂ concentration at approximately 5%. After exposing the carotid sinus regions bilaterally, the internal and external carotid arteries were ligated to facilitate decerebration. The vagus nerves were sectioned bilaterally to prevent entrainment of the phrenic rhythm to the mechanical ventilator (Petrillo *et al.* 1983; Graves *et al.* 1986). The animal was placed prone with the head in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). The skull was opened; the animal was decerebrated at the level of the superior colliculi; and all brain tissue rostral to the section was removed by suction. Following decerebration, halothane anesthesia was discontinued. Each animal was paralyzed using pancuronium bromide (1 mg/kg, iv; Elkins-Sinn Inc., Cherry Hill, NJ), and supplemental doses of pancuronium were given as required, usually at a rate of 0.5 mg/kg/hr. A phrenic nerve was exposed and sectioned, and the central cut end was placed on a bipolar recording electrode to monitor respiratory output. Phrenic activity was amplified (Gould Universal Amplifier, Cleveland, OH), and the moving time average (“integrated activity”) was calculated electronically (100 ms time constant; CWE, Ardmore, PA). Integrated phrenic nerve activity, body temperature, end-tidal CO₂ and blood pressure were recorded on a computer (PowerLab, ADI, Australia) for later analysis.

A 26 gauge needle attached to a 0.5 µl syringe (SGE Analytical Sciences, Austin, TX) was inserted into the dorsal medulla perpendicular to the dorsal surface using the obex as a visual reference point (Niblock *et al.* 2005); there is no stereotaxic atlas for neonatal piglets. The needle for microinjections was placed in the medulla approximately 5–10 minutes before any tests of the LCR were performed. Respiratory activity was stable at the time of each injection. All drugs injected into the medulla were combined with 0.5 micron diameter fluorescent microbeads (Fluoresbrite® YG Microspheres, Polysciences, Inc., Warrington, PA). The beads were added to the drugs dissolved in the solvent vehicle (5 µl stock solution of microbeads, which contained 3.64×10^{11} particles/ml, were added to 100 µl vehicle) and distributed within the solvent by shaking before being aspirated into the injection syringe.

We placed a pharyngeal catheter (PE-90) through a nostril and positioned the tip just above the larynx. The catheter was filled with water, and 0.1 ml of water was injected into the larynx using a computer controlled syringe pump each time that we elicited the LCR. Water remained in the catheter between tests, and as a consequence, the temperature of the water injected was near body temperature, which varied in different conditions. However, laryngeal water receptors do not respond to the temperature of the stimulus (Xia *et al.* 2005). The larynx was suctioned periodically as needed. At least 5 minutes elapsed between tests of the LCR, and the LCR was not tested unless phrenic respiratory activity was stable.

Neuroanatomy

At the conclusion of each experiment, each piglet was killed with an injection of 500 mg/kg pentobarbital sodium followed by 5–10 ml of saturated potassium chloride administered I.V. The brainstem was removed from the animal, placed in cryo-embedding medium (Tissue-Tek O.C.T. 458, Sakura Finetek, Torrance, CA) and frozen in isopentane at –70°C. Brainstems were sectioned (50 µm) in a cryostat at –18°C, mounted on gelatinized glass slides, fixed for 15 min in 4% paraformaldehyde in phosphate buffered saline (pH 7.0) and stained with cresyl violet (Luna 1992; Bandroft & Cook 1994). The location of the microinjection was identified using the location of fluorescent microbeads. The distribution

of fluorescent microbeads was examined under fluorescent light using a TRITC filter (excitation 540 ± 25 nm; dichroic mirror 565 nm) on a Nikon Eclipse E800 microscope (Nikon Instruments Inc., Melville, NY). We recorded the location of the highest concentration of beads as the center of the injection, and we noted the rostro-caudal extent of spread of the beads.

Experimental protocols

In previous studies, the few animals that failed to demonstrate hyperthermic prolongation of the LCR also failed to respond to drugs injected or dialyzed into the NTS. Therefore, studies began with a control period during which the body temperature was held at approximately 38°C, and the LCR was elicited three times. Next, the body temperature was elevated approximately 2.5°C by warming with a heating pad, and the LCR was stimulated three more times. After this series of tests, each animal was cooled by swabbing it with isopropyl alcohol. It usually took approximately 30 minutes to reduce body temperature to the control level, after which the LCR was tested three times in a second control, normothermic period. In the fourth condition, the drug to be tested was injected into the medulla, and testing of the LCR began no sooner than 10 minutes after the injection. The onset of the drug effects seemed delayed in some animals, so we assessed the LCR at 5 min intervals 4–6 times. There were some deviations to this basic pattern in specific experiments as described below.

Nipecotic acid blocks reuptake of GABA by GABA transporter proteins and may, at higher concentrations, directly activate GABA_A receptors (Barrett-Jolley 2001). We injected nipecotic acid (100 nl of 0.5 M nipecotic acid in normal saline; Sigma-Aldrich, St. Louis, MO) unilaterally into the caudal NTS to test the hypothesis that activation of GABA receptors in the absence of elevated body temperature would prolong the LCR. There were four test conditions in this study: an initial normothermic control period, a hyperthermic test period to prove that elevated body temperature prolonged the LCR in each animal, a second normothermic control period after each animal was cooled back to its original body temperature and a final test period in which nipecotic acid was injected into the NTS to assess the effect of increasing GABA activation on the LCR when the animal was normothermic.

As a control for the saline containing injections and for the passage to time, we conducted an identical series of tests: normothermic control, hyperthermia, second normothermic control and test period after a focal injection of saline in the NTS without administering any drug.

In the third experiment, we used CGS-21680, a selective Ad-A_{2A} agonist, to activate adenosine receptors. The protocol was identical to the one used for nipecotic acid except that CGS-21680 (Tocris Bioscience, Ellisville, MO) was injected into each animal in the fourth condition. CGS-21680 is relatively insoluble in water, and a 4 mM stock solution of CGS-21680 was made in dimethylsulfoxide (DMSO). The final concentration of CGS-21680 used ranged between 5 µM and 12.5 µM as we tried to find the optimal dose. All the injections had a 100 nl volume. Most of the injections were made unilaterally, but as the dose was escalated, we also made bilateral injections of 8 µM CGS-21680 (100 nl of fluid injected on both sides of the dorsal brainstem). We have previously demonstrated that similar injections of DMSO alone in the NTS do not modify the thermal prolongation of the LCR (Xia *et al.* 2008a), and control studies with DMSO were not repeated in this set of experiments to avoid needless use of experimental animals.

In the final set of experiments, we made measurements of the LCR in a normothermic control period and a hyperthermic period. Each piglet's body temperature was reduced to the control value by cooling. At this point, a systemic dose of bicuculline methiodide (Tocris), a GABA_A receptor antagonist, was given (0.2 mg/Kg I.V.) in five animals and the LCR was

tested 3 times. Subsequently, CGS-21680 (8 μ M in 100 nl), a selective Ad-A_{2A} agonist, was injected bilaterally in the NTS in these animals. We used bilateral injections of CGS-21680 to maximize the likelihood of a response to Ad-A_{2A} receptor activation. The order of the third and fourth test conditions was reversed in a second group of 6 animals so that CGS-21680 (12.5–20.0 μ M in 100 nl) was administered bilaterally in the NTS; the LCR was tested three times; an intravenous dose of bicuculline was then given; and the LCR was tested three final times.

Data analysis and statistics

We defined the duration of the LCR as the period of respiratory instability (defined as variability of phrenic amplitude and/or respiratory timing) from the beginning of the breath during which the water stimulus was delivered to the onset of at least five regular breaths. These five breaths did not need to have the same frequency or amplitude as the control breaths; we simply required that they be regular (van der Velde *et al.* 2003; Curran *et al.* 2005; Xia *et al.* 2006). By measuring the LCR duration (rather than phrenic amplitude, for example), we kept the definition of the LCR simple and applied it consistently to all animals. We also measured the longest apnea duration of each reflex trial, which is less subject to interpretation than the restoration of regular breathing. Apnea was defined as the cessation of phrenic activity greater than the duration of the two breaths preceding the breath during which the stimulus was delivered. However, apnea did not occur in all tests of the LCR. Measuring both the LCR duration and apnea duration, when present, provided a more complete analysis of the LCR. Stimulation of the LCR may induce bradycardia as well as apnea. However, we did not analyze the heart rate responses because the animals were vagotomized.

Each of the four studies was analyzed separately using a one-way repeated measures analysis of variance (ANOVA, SYSTAT 9.0, SPSS, Inc, Chicago, IL). The duration of apnea and the duration of the LCR were not normally distributed, and the variances were inhomogeneous among treatment conditions (Curran *et al.* 2005). Therefore, statistical analyses were performed on log transformed data for these two variables. When the ANOVA indicated that significant differences existed among the treatments, specific pre-planned comparisons were made using orthogonal contrasts and P-values adjusted by the Bonferroni method. We first compared the two normothermic conditions (the first and third treatments). If these conditions were not significantly different, then we made orthogonal contrasts between the means of the normothermic control conditions and each of the treatment conditions (hyperthermia or the post-injection condition). If the first and third normothermic periods differed significantly, the hyperthermic and post-injection data were compared to the normothermic period immediately preceding each of these test conditions. Data are presented as the mean $\pm$ the standard error of the mean.

Results

Effect of hyperthermia and nipecotic acid on the LCR

An example of the responses of integrated phrenic nerve activity during each of the experimental treatments in one piglet is shown in Fig. 1. The injection site in this piglet was caudal to the obex (the circled solid symbol in Fig. 2). In the control condition, body temperature was 38.6°C, and introducing 0.1 ml of water into the larynx (arrow) caused apnea (solid line), and a slightly longer but still brief period of respiratory instability (hatched bar). After elevating the animal's body temperature to 40.9°C, 0.1 ml water injected into the larynx disrupted respiratory activity and regular respiratory activity was not resumed for approximately 18 sec, much longer than the disruption when the animal was normothermic. The animal was cooled to its control temperature, and the LCR was tested

again. Just as in the initial normothermic control period, the apnea and respiratory disruption were short lived. After injection of nipecotic acid into the NTS, the LCR was tested a final time while the animal was still normothermic. Despite the normal body temperature, the LCR was markedly prolonged.

The average values of the LCR duration, the longest apnea duration, respiratory frequency and body temperature during each of these four experimental conditions (normothermic control, hyperthermia, normothermic control and normothermic injection of nipecotic acid in the NTS) are shown in Fig. 2. The sites of injection of nipecotic acid are shown in schematic cross-sections of the piglet brainstem in the same figure. When the temperature was increased from a control value of $38.6 \pm 0.02^{\circ}\text{C}$ to $41.1 \pm 0.1^{\circ}\text{C}$, the apnea duration increased by approximately 50% (Fig. 2; $P < 0.05$), and the LCR duration increased approximately threefold, also a significant change ($P < 0.05$). The respiratory frequency rose slightly, but this was not significant. These changes in apnea and LCR duration were reversed when the body temperature was cooled to $38.5 \pm 0.1^{\circ}\text{C}$ so that apnea and LCR duration and respiratory frequency were not significantly different from the initial normothermic control period. After focal injection of nipecotic acid, body temperature remained stable on average, $38.6 \pm 0.1^{\circ}\text{C}$, and respiratory frequency did not change from the preceding three conditions. However, the apnea and LCR durations were increased more than two-fold ($P < 0.05$ for both variables). Thus, focally blocking GABA reuptake and enhancing activation of GABA_A receptors in the NTS was associated with significant enhancement of the LCR even though body temperature was not elevated.

We started using smaller injections of drugs and added fluorescent microbeads to define the location of the injection. We had some trouble using this method initially, and we were unable to define the site of injection in three animals (we used too few beads in each injection initially). Moreover, the microinjection needle was flexible, and the fluorescent beads were spread over, to us, a surprisingly long rostro-caudal distance. The maximum spread of beads, which occurred in three animals, extended over multiple cross-sections of the brain as far apart as 1900 μm . Thus, the injections in the nipecotic acid study were not as focally restricted as we had hoped, but all of them were still in the region of the NTS.

We analyzed the data from each experiment as an ‘intention to treat’ study; meaning that we analyzed all the results from all the animals as if each animal responded similarly to the treatments. This was not actually the case. One animal failed to show any thermal prolongation of the LCR when its body temperature was elevated, and as we have seen before, this animal did not respond to the nipecotic acid injection. Thus, thermal prolongation of the LCR must be present to investigate the normothermic neuropharmacology of this circuit within the NTS. The nipecotic acid injection in this animal was made near the central canal just caudal to the obex (circled open symbol in the -0.56 cross-section, Fig. 2). In addition, the animal with the most caudal injection of nipecotic acid had a thermal response (the average apnea and LCR durations in this animal were increased by 190% and 143%, respectively, during hyperthermia compared to the average normothermic responses), but there was no response to nipecotic acid injection (apnea and LCR durations were 82% and 76% of the average initial control values). All of the other nipecotic acid injections were associated with prolonged apnea and LCR durations during normothermia in animals that also demonstrated hyperthermic prolongation of the LCR. However, one of these ‘effective’ injections was made into the loose tissue overlying the dorsal surface of the medulla rostral to the obex. The drug injection, as reflected by the distribution of fluorescent microbeads, did not really penetrate the brain tissue ($+3.28$ cross-section, Fig. 2), and the drug effect may have reflected fairly superficial dispersal of the nipecotic acid.

Effect of hyperthermia and saline injection on the LCR

We conducted a series of control studies in 6 piglets to confirm that focal saline injections into the NTS do not modify the LCR. The results of these studies and the sites of focal injections are shown in Fig. 3. The pattern and magnitude of response to hyperthermia was identical to the nipecotic injected animals: when body temperature was raised significantly by approximately 2°C, apnea duration and LCR duration were increased significantly ($P < 0.05$ for both variables), and respiratory frequency did not change. The hyperthermic effect on the LCR was also reversed by restoring a normothermic body temperature, just as in the previous experiment. Focal injection of a 100 nl of saline did not prolong the duration of apnea or the LCR, change the respiratory frequency significantly or change the body temperature. Thus, the effects of nipecotic acid injection into the NTS originate from the nipecotic acid, not the saline vehicle. The sites of saline injection overlapped the sites of nipecotic acid injection, and we were able to locate all the sites of injection.

Effect of hyperthermia and CGS-21680 injection on the LCR

Identically designed studies were conducted to test the effect of the Ad-A_{2A} agonist, CGS-21680, on the LCR, and the average responses of 15 animals are shown in Fig. 4. The prolongation of apnea and LCR durations after CGS-21680 treatment was more variable than either the response to hyperthermia in the same animals or the response to nipecotic acid in the initial experiment that we did. Therefore, we varied the concentration of CGS-21680 used during the study (1 animal received 5 µM CGS-21680, 9 animals received a 10 µM dose, one animal received a 12.5 µM dose, and 4 animals received two 10 µM injections, one on either side of the midline – all to try to optimize the response to the drug). The escalating dose had no clear effect on the response, and the data from all of these animals were pooled in the following analysis. All the animals in this experiment demonstrated significant prolongation of apnea duration and the LCR when they were made hyperthermic (Fig. 4; $P < 0.05$ for both apnea and LCR duration). There was also a significant increase in the respiratory frequency when body temperature was elevated in each animal ($P < 0.05$). Apnea and LCR durations, the frequency and body temperature all returned to control levels after the animal was cooled to the normothermic range. Focal injection of CGS-21680 increased the respiratory frequency significantly ($P < 0.05$) despite the absence of any change in body temperature. The duration of the LCR was increased significantly ($P < 0.05$) after treatment with CGS-21680, although the magnitude of the increase was much less than after nipecotic acid or in the presence of hyperthermia. Apnea duration increased slightly, but this was not statistically significant.

When respiratory drive is increased, the duration of the LCR tends to diminish (Lawson 1982; van der Velde *et al.* 2003). Therefore, the increase in respiratory frequency after CGS-21680, to the extent it represented an increase in respiratory drive, might have shortened the LCR (thereby explaining the relatively modest effects of the CGS-21680 treatment). However, there was no correlation between the increase in respiratory frequency and the apnea or LCR durations among the animals tested, and we cannot, therefore, attribute the small effect of CGS-21680 on the LCR to any change in respiratory drive.

We have done control studies of DMSO injections in the past using higher concentrations of DMSO than were used in this study, and there was no effect of DMSO on the LCR (Xia *et al.* 2008a). Nonetheless, we repeated these control studies in three animals using bilateral injections of 0.4% DMSO in 100 nl into the NTS. The injections were in the same sites identified in the CGS-21680 studies, but there was no effect of DMSO on respiratory frequency or apnea and LCR duration (data not shown). We have never seen any effect of DMSO on the LCR at any dose we studied in any of the control studies ($n = 12$) that we have done in the context of studying thermal prolongation of the LCR in neonatal piglets.

The locations of the injections in the CGS-21680 treated animals are shown in Fig. 4. In all the animals in the CGS-21680 treatment group, hyperthermia prolonged either the apnea or LCR duration in individual animals by a minimum of 40% compared to the control value in each animal (and the average increase in apnea or LCR duration during hyperthermia among all animals was ~300%). The same was not true of the apnea and LCR durations after treatment with CGS-21680. In four of fifteen animals, the apnea or LCR duration decreased or was no more than 10% greater than the preceding normothermic response (the site of injection(s) in these animals are shown with open circles). In the remaining 'responder' animals, bilateral injections (solid circles yoked together in Fig. 4) and unilateral injections seemed equally effective in prolonging the LCR under normothermic conditions. Superficial injections in the most caudal area of the NTS were ineffective in the study of CGS-21680. Beyond that, however, effective and ineffective injections were intermixed anatomically and spread over a relatively long rostro-caudal distance centered on the obex. Effective injection sites frequently involved the NTS, but injections deep to the NTS and generally in the dorsal half of the brainstem also prolonged the LCR.

Effect of hyperthermia, systemic bicuculline and CGS-21680 on the LCR

In this set of studies, we elicited the LCR under normothermic and hyperthermic conditions. In six animals, we restored normothermia and administered bicuculline (0.2 mg/Kg I.V.), a GABA_A receptor antagonist, which reversed the thermal prolongation of the LCR in a previous study (Böhm *et al.* 2007), and then injected CGS-21680 focally in the region of the NTS. All six animals received bilateral injections of 8 μ M CGS-21680. In five additional animals, we restored normothermia and injected CGS-21680 bilaterally in the NTS first (12.5–20.0 μ M), tested the LCR and then gave bicuculline systemically. If Ad-A_{2A} agonists work through a GABAergic mechanism, then treatment with bicuculline should 'occlude' the effect of administering the Ad-A_{2A} agonist. The average responses for the two sequences of study are shown in Fig. 5. In both sets of studies, hyperthermic treatment significantly prolonged apnea and LCR durations ($P < 0.02$ for both variables) as in the previous studies. Apnea and LCR durations, respiratory frequency and body temperature were not significantly different after systemic administration of bicuculline from control normothermic values (bicuculline did not alter the normothermic response regardless of the order of focal injection of CGS-21680). On the other hand, the response to CGS-21680 injection differed depending on the order of treatment with respect to bicuculline (as we expected). When CGS-21680 was given under normothermic conditions before bicuculline, apnea and LCR durations and respiratory frequency were increased, but these effects were reversed after bicuculline treatment. When CGS-21680 was given after bicuculline treatment, the drug had no effect on apnea or LCR duration, but respiratory frequency was still increased even though each animal was normothermic. When these results are combined with similar studies shown in Fig 4, it is apparent that CGS-21680 treatment during normothermia significantly increased apnea and LCR duration and the respiratory frequency compared to normothermic control conditions ($P < 0.05$ for all three comparisons; $n = 20$). Thus, bicuculline occluded the effect of CGS-21680 treatment regardless of the order of treatment.

The locations of the injections of CGS-21680 in the bicuculline treated animals are shown in Fig. 5. All the injections were in or near the NTS, and the locations of the CGS-21680 injections before and after bicuculline treatment were not different from the locations of injections of CGS-21680 in the preceding experiment (see Fig. 4).

Discussion

The main findings in this study are that hyperthermia prolongs the LCR in decerebrate piglets, and thermal prolongation of the LCR can be recapitulated under normothermic

conditions by blocking reuptake of GABA focally within the NTS. Furthermore, focal injection of the Ad-A_{2A} agonist, CGS-21680 in the region of the NTS partially recapitulates the prolongation of the LCR under normothermic conditions. The effect of activating the Ad-A_{2A} receptors in or near the NTS seems to depend on activation of GABA_A receptors since bicuculline, a GABA_A receptor antagonist, blocked CGS-21680-dependent normothermic prolongation of the LCR. These findings are consistent with the hypothesis that hyperthermia increases adenosine levels in the NTS, and adenosine binds to Ad-A_{2A} receptors that amplify GABAergic mechanisms and prolong the LCR (Abu-Shaweesh *et al.* 2001; Abu-Shaweesh 2007; Xia *et al.* 2008a). These results highlight the existence of a thermally sensitive adenosinergic and GABAergic circuit in the dorsal medulla that is separate from but contributes to the ventral medullary circuit mediating respiratory inhibition associated with the LCR (Remmers *et al.* 1986; Czyzyk-Krzeska & Lawson 1991).

Neuronal circuitry of the LCR

The LCR is elicited when water receptors in the laryngeal mucosa are activated. Afferent information from these receptors is carried to the central nervous system by the SLN and has its primary termination on neurons in the caudal NTS (Patrickson *et al.* 1991; Hayakawa *et al.* 2001). From the NTS the information ramifies throughout the brainstem, and the reflex respiratory inhibition originating from sensory activation in the larynx ultimately inhibits inspiratory and expiratory neurons and stimulates post-inspiratory neurons in the region of the nucleus ambiguus in the ventral medulla (Remmers *et al.* 1986; Czyzyk-Krzeska & Lawson 1991). Apnea after stimulation of the larynx results, therefore, from prolonged expiratory time; the post-inspiratory neurons remain persistently depolarized and prevent the normal sequential activation of expiratory neurons that would lead to the next breath (Remmers *et al.* 1986). The exact pathways between the NTS and the effector neurons in the ventral respiratory group that actually change the respiratory pattern have not been established. Some of the necessary elements are clear, however, even if their pattern of connectivity is not.

Focal heating of the NTS prolongs the LCR (Xia *et al.* 2006), and therefore, there must be a thermal sensor of some sort within the NTS. There are temperature sensitive neurons in and around the NTS in the rabbit (Inoue & Murakami 1976), but we have not established that similar temperature sensitive neurons exist in piglets or that such neurons play a role in thermal prolongation of the LCR. Even if such neurons exist in piglets, the relationship of thermally sensitive neurons to the primary sensory neurons has not been established. One might imagine thermally sensitive neurons (perhaps containing thermally sensitive transient receptor potential (TRP) channels) that release ATP, which is then converted to adenosine, or that release adenosine through nucleoside transporters in the region of GABAergic interneurons in the NTS -and GABAergic neurons are plentiful in the NTS (Bailey *et al.* 2008; Okada *et al.* 2008). These GABAergic interneurons may then mediate laryngeal chemoreflex inhibition of respiration by acting on more ventral structures. However, it seems equally plausible that hyperthermia might enhance metabolic activity within the NTS, enhance adenosine production from ATP, and in this way activate Ad-A_{2A} receptors and augment the activity of GABAergic neurons that ultimately interact with neurons in the ventral respiratory group – one need not posit a necessary role for thermally sensitive neurons; a thermally sensitive metabolic process would suffice.

It also seems clear that the Ad-A_{2A} receptor activation is upstream of GABA_A receptors that may provide the final output from the dorsal brainstem to the effector neurons in the ventral respiratory group. The Ad-A_{2A} receptors may be presynaptic on the GABAergic neurons, but our data allow us to state only that they are upstream from GABA_A receptors in the reflex circuitry and that participation of Ad-A_{2A} receptor activation in thermal prolongation

of the LCR has an essential dependence on a GABAergic step in the circuitry. Based on these findings, it is our hypothesis that stimulation of the LCR augments GABAergic neurotransmission within the NTS, and activation of Ad-A_{2A} receptors during or by hyperthermia enhances GABA release within the NTS. It seems likely that this adenosine-dependent process is thermally sensitive (the GABAergic mechanism(s) may or may not be thermally sensitive) since blocking Ad-A_{2A} receptors in the NTS completely prevented the thermal prolongation of the LCR. These adenosinergic and GABAergic effects are probably downstream from the primary relay neurons receiving laryngeal afferent information, but upstream from bulbospinal neurons in the ventral respiratory group. However, the neuroanatomical details of the circuit among the interneurons within the NTS responsible for thermal prolongation of the LCR remain unknown.

GABA seems to have a dominant role mediating the LCR. Focal administration of gabazine in the NTS (Xia *et al.* 2007) completely blocked the hyperthermic prolongation of the LCR. Moreover, nipecotic acid injected in the NTS prolonged the LCR even under normothermic conditions, and the effect was potent – unilateral nipecotic acid injections achieved the same magnitude increase in the LCR duration as heating the entire animal. Focal Ad-A_{2A} receptor activation in the NTS prolonged the apnea duration and the LCR during normothermia, especially at the higher doses used, but the magnitude of the response, even after bilateral injection of CGS-21680 into the NTS, was smaller than the response to whole animal warming or to focal nipecotic acid treatment within the same area of the NTS. The increases in apnea and LCR durations after CGS-21680 treatment were 68% and 72%, respectively, of the hyperthermic responses in the same animals (n = 20). In addition, the response to Ad-A_{2A} receptor activation was not the exact inverse of blocking Ad-A_{2A} receptors – blocking Ad-A_{2A} receptors unilaterally within small regions of the NTS completely reversed the thermal prolongation of the LCR (Xia *et al.* 2008a) whereas Ad-A_{2A} receptor activation under normothermic conditions only partially mimicked the response to hyperthermia. It is difficult to compare nipecotic acid to CGS-21680 since they work by different mechanisms, and the extent of GABA enhancement probably does not equal the extent of Ad-A_{2A} enhancement in our studies. Even within the manipulations of Ad-A_{2A} activation that we have used in the present and previous studies, the binding affinity of the Ad-A_{2A} agonist is less than the binding affinity of the Ad-A_{2A} antagonist that we used previously (the K_D of SCH-58261, the antagonist, is ~0.7–2.3 μM, and the K_D of CGS-21680 ranges from 15–60 μM); the volumes of distribution of the injections may have differed; and the sites of injection were not identical. Notwithstanding these limitations, it still seems likely that activation of Ad-A_{2A} receptors amplifies the release of GABA within the NTS to augment the prolongation of the LCR during hyperthermia, but the primary and more potent effector neurotransmitter remains GABA.

Our results differ from studies of SLN stimulation in the presence of more widely distributed CGS-21680 and bicuculline in anesthetized piglets (Abu-Shaweesh 2007) in that focal administration of these agents within the region of the NTS does not alter the normothermic features of the LCR, whereas intracisternal administration of bicuculline reduced the intensity of SLN-induced apnea even during normothermic conditions. It may be that the dorsal circuit in the NTS mediates thermal prolongation of the LCR, and a ventral medullary circuit, which is also GABAergic, controls the LCR duration during normothermia. Consistent with such an hypothesis, a GABA_A receptor antagonist focally injected into the dorsal medulla inhibit only the thermal prolongation of the LCR (Xia *et al.* 2007), but focal activation of GABA_A receptors in the rostral ventral medulla prolonged the LCR even under normothermic conditions (van der Velde *et al.* 2003). On the other hand, systemic administration of bicuculline did not significantly reduce the duration of the LCR in decerebrate piglets (Böhm *et al.* 2007) as intracisternal administered of bicuculline had (Abu-Shaweesh *et al.* 2001; Wilson *et al.* 2004; Abu-Shaweesh 2007). These last studies

used similar doses of bicuculline, but systemic administration of these doses of bicuculline probably resulted in lower CNS levels of the drug than were present after intracisternal administration. We suspect that the divergent levels of CNS bicuculline are responsible for the greater reduction in apnea duration during SLN stimulation in normothermic piglets compared to the lack of any effect of I.V. bicuculline on the manifestations of the LCR in normothermic animals as previously shown (Böhm *et al.* 2007) and as shown in the current study. In all of these studies, relatively low doses of GABA_A receptor antagonists were selected to avoid altering the baseline cardiorespiratory values.

The manifestations of the LCR change as animals mature. Thermal prolongation of the LCR and respiratory disruption are prominent in newborns, but the thermal sensitivity is lost as the neonatal period ends. The respiratory disruption of the LCR persists after thermal sensitivity is lost, but at some point in infancy, the respiratory disruption disappears as well, and the LCR consists of coughing and swallowing, which are the primary manifestations of the LCR in the adults (Thach 2001). What changes in the neural circuitry of the LCR are responsible for these age-dependent changes are not known, but neonatal animals behave as if the maturation process occurs first in the dorsal medulla and then later in the ventral medulla. Loss of sensitivity to adenosine may be a key element in this process. For example, systemic or intracisternal administration of CGS-21680 causes respiratory depression and apnea when given in neonatal rats at age P14 (Mayer *et al.* 2006). As neonatal rats mature, this respiratory depression diminishes. No respiratory disruption is evident when adult rats are given CGS-21680. Loss of adenosinergic inputs may contribute more widely to maturation of a variety of respiratory responses. The biphasic response to hypoxia, which also depends on adenosine in part (Runold *et al.* 1986), and the thermal sensitivity of the LCR and respiratory disruption associated with the LCR are transformed into more adult patterns of responses over similar periods of neonatal development.

Respiratory effects of hyperthermia and CGS-21680

Hyperthermia often increases the respiratory frequency in decerebrate piglets, but this effect has been inconsistent among animals within each study and among studies (Curran *et al.* 2005; Böhm *et al.* 2007; Xia *et al.* 2008a). Injection of CGS-21680 consistently increased the respiratory frequency, and this effect was present even after GABA_A receptors were blocked. Therefore, the effect of Ad-A_{2A} receptor activation within the NTS on respiratory frequency does not seem to depend on GABA. Once again, the effects of adenosine receptor agonists and antagonists were not symmetrical opposites of each other. Focal injection of the Ad-A_{2A} antagonist, SCH-58261, did not reduce the respiratory frequency under hyperthermic or normothermic conditions. As noted above, the SCH-58261 injections should have been more potent than CGS-21680 injections (more of the Ad-A_{2A} antagonist was given and it has a higher affinity and selectivity for the Ad-A_{2A} receptor). However, the sites of injection were not identical, and added to the differences among injection sites, it is also the case that the level of decerebration varies slightly among animals and this may have a significant effect on the respiratory frequency (St. John 1979).

Limitations of the methods

As we have noted before (Curran *et al.* 2005; Xia *et al.* 2006; Xia *et al.* 2008a), the nature of the decerebrate preparation is a major limitation of our studies. Moreover, thermal stress caused by increased room temperatures or heavily insulated bed clothes has been identified as a risk factor for SIDS (Williams *et al.* 1996; Kleeman *et al.* 1998; Guntheroth & Spiers 2001; Blair *et al.* 2008), but this does not mean necessarily that infants who died of SIDS had an elevated body temperature. In this respect, our studies of decerebrate piglets may not accurately mimic the thermal risk factors for SIDS. Finally, we do not know the exact site of the injections that we made. We tried to make microinjections to isolate the neuronal

circuitry of the LCR within the NTS. There were two problems. First, it is difficult to restrict the injections to small areas of the brainstem, and the distribution of the injections, at least as reflected by the fluorescent microbeads, was larger than we anticipated. We injected low concentrations of the drugs to try to isolate the site of action, but this meant that there was also a smaller physiological effect to observe. For example, we escalated the dose of CGS-21680 from 5 to 20 μ M and gave injections bilaterally. This had the advantage of increasing the biological effect of the treatment, but when we increased the drug concentration, we also reduced the focality of the effect and reduced the spatial resolution of our injections. In the final analysis, we confirmed that the hyperthermic modulation of the LCR seems to originate in the NTS, but we did not obtain any information about involvement of specific sub-nuclei of the NTS.

Thermal stresses, adenosine, the LCR and SIDS

Thermal stress is a risk factor for SIDS (Williams *et al.* 1996; Kleeman *et al.* 1998; Guntheroth & Spiers 2001; Blair *et al.* 2008), and the LCR, which is commonly elicited during sleep in neonates, may begin the process that leads to SIDS (Downing & Lee 1975; Page *et al.* 1996; Thach 1997, 2005; Leiter & Böhm 2007). Therefore, we hypothesize that thermal stress, by enhancing the LCR through GABAergic and adenosinergic mechanisms, may increase the likelihood of prolonged apneas that result ultimately in sudden death in infants in whom the LCR and the thermal effects on the LCR are particularly strong. For these reasons, adenosinergic mechanisms may contribute to the pathogenesis of SIDS, and by the same token, adenosine receptor antagonists may have potential as a preventive therapy in neonates at risk for SIDS (Hunt *et al.* 1983). There has, however, never been a trial of adenosine receptor antagonist therapy to prevent SIDS because, first, it has been difficult to identify infants at high risk for SIDS and second, the pleiotropic effects of adenosine receptor antagonists have made investigators reluctant to use such drugs in neonates. Enthusiasm for a prophylactic therapy for SIDS would be increased by evidence that adenosine receptor antagonists can be used safely in neonates and evidence that such drugs intervene with real specificity in the pathogenesis of SIDS. It turns out that brief use of caffeine in neonates was remarkably safe (and subsequent neurological development was actually improved following caffeine therapy) (Schmidt *et al.* 2006a; Schmidt *et al.* 2006b), and our data suggest that adenosinergic mechanisms in the dorsal medulla may adversely prolong the LCR and contribute to the maintenance of apneic events leading to sudden death.

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Figure 1.

Integrated phrenic activity is shown during four tests of the LCR in a female piglet (post-natal age 6 days). The control response to laryngeal injection of 0.1 ml of water at a normal body temperature is shown in the top panel. Elevating the body temperature (second panel) prolonged the duration of the LCR. The hyperthermic prolongation of the apnea duration and LCR duration was reversible when body temperature was returned toward the initial control value (third panel). After injection of nipecotic acid, apnea and LCR durations were prolonged even though body temperature remained at the normothermic value. The arrow pointing down indicates the time when 0.1 ml was injected into the larynx in each test; the solid horizontal line indicates the duration of apnea; and the thick dashed line indicates the duration of the LCR. 'BT' indicates body temperature. The location of the injection in this animal is shown in the schematic anatomical drawings in Fig. 2 as a circled solid circle on the most caudal cross-section of the piglet brainstem.

Figure 2.

Body temperature (BT), respiratory frequency, the duration of the LCR and the duration of apnea have been plotted as functions of experimental conditions ('Ctr1,' control normothermia condition; 'Hyp,' hyperthermic condition; 'Ctr2,' second normothermic control period; and 'Nip,' after injection of nipecotic acid. '*' indicates $P < 0.05$ compared to mean of the two control conditions, and 'NS' indicates that no significant difference existed between the treatments indicated. Schematic cross-sections starting caudal to the obex of the neonatal piglet medulla are shown on the right side of the figure. Nipecotic injections in the NTS that altered the LCR are shown with filled circles, and open circles show the locations of nipecotic injections that did not alter the LCR. The solid symbol with the larger circle around it marks the site of the injection in the animal from which the data in Fig. 1 were taken. Anatomical abbreviations: 7, facial nucleus; RO, raphé obscurus; RP, raphé pallidus; RM, raphé magnus; DMX, dorsal vagal motor nucleus; ION, inferior olivary nucleus; HG, hypoglossal motor nucleus; NTS, nucleus tractus solitarius; NA, nucleus ambiguus.

Figure 3.

The symbol conventions are the same as in Fig. 2 except that the final treatment consisted of saline injection (Sal) alone.

Figure 4.

The symbol conventions are the same as in Fig. 2 except that the final treatment consisted of CGS-21680 injection (A2A). Bilateral injections are indicated by the yoked pairs of injection sites.

Figure 5.

The symbol conventions are the same as in Fig. 2, but the protocol was more complicated. Average responses of normothermic animals treated with bicuculline and then CGS-21680 are shown on the left, and average responses of animals treated first with CGS-21680 and then bicuculline are shown on the right. Statistical comparisons are described in the text. In the anatomical cross-sections at the bottom of the figure, the filled circles represent the animals treated with bicuculline then CGS-21680 and the filled squares represent the animals treated with CGS-21680 first and then bicuculline.