Published in final edited form as:

Respir Physiol Neurobiol. 2013 March 1; 186(1): 73–80. doi:10.1016/j.resp.2013.01.002.

Influence of age, body temperature, GABA_A receptor inhibition and caffeine on the Hering-Breuer inflation reflex in unanesthetized rat pups

Ashley V. Arnal, Julie L. Gore, Alison Rudkin, Donald Bartlett Jr., and J.C. Leiter

Department of Physiology & Neurobiology, Dartmouth Medical School, Lebanon, NH 03756, USA

Abstract

We measured the duration of apnea induced by sustained end-inspiratory lung inflation (the Hering Breuer Reflex; HBR) in unanesthetized infant rat pups aged 4 days (P4) to P20 at body temperatures of 32°C and 36°C. The expiratory prolongation elicited by the HBR lasted longer in the younger pups and lasted longer at the higher body temperature. Blockade of adenosine receptors by caffeine following injection into the cisterna magna (ICM) significantly blunted the thermal prolongation of the HBR. Blockade of gamma-amino-butyric acid A (GABA_A) receptors by pre-treatment with ICM bicuculline had no effect on the HBR duration at either body temperature. To test the hypothesis that developmental maturation of GABAergic inhibition of breathing was modifying the response to bicuculline, we pretreated rat pups with systemically administered bumetanide to lower the intracellular chloride concentration, and repeated the bicuculline studies. Bicuculline still did not alter the HBR at either temperature after bumetanide treatment. We administered PSB-36, a selective adenosine A1 receptor antagonist, and this drug treatment did not modify the HBR. We conclude that caffeine blunts the thermal prolongation of the HBR, probably by blocking adenosine A2a receptors. The thermally-sensitive adenosinergic prolongation of the HBR in these intact animals does not seem to depend on GABA_A receptors

Keywords

Lung inflation; reflex apnea; body temperature; rat pups; bicuculline; bumetanide; caffeine

1. INTRODUCTION

Elevated body temperature seems to inhibit respiratory drive in fetal and neonatal animals. Warming at the time of birth, especially if warm water is applied to the face, suppresses breathing in fetal sheep (Dawes, 1968), and bathing fetal sheep in a warm liquid promotes sleep and apnea and reduces the respiratory response to carbon dioxide (Moss et al., 1983). Elevated environmental temperatures, without elevated body temperature, increase the incidence of apneas in pre-term and term infants (Bader et al., 1998; Perlstein et al., 1970). Moreover, reflex apneas are prolonged in infant mammals by mild increases in body temperature. The laryngeal chemoreflex (LCR) and respiratory inhibition associated with

© 2013 Elsevier B.V. All rights reserved.

Corresponding author: J.C. Leiter, Department of Physiology and Neurobiology, Dartmouth Medical School, One Medical Center Drive, Lebanon, NH 03756, Tel: 603-650-7723, Fax: 603-650-6130, james.c.leiter@dartmouth.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

superior laryngeal nerve stimulation are both prolonged in neonatal rats, piglets and dogs when body temperature is elevated by approximately 2 °C (Curran et al., 2005; Haraguchi et al., 1983; Xia et al., 2008b). In addition, the expiration promoting Hering Breuer Reflex (HBR) induced by lung inflation in neonatal rats lasted longer at high (36°C) than at low (32°C) ambient temperature (Merazzi and Mortola, 1999a; b). In these studies of the HBR, the environmental temperatures were accompanied by closely related differences in body temperature, and the metabolic rate diminished as body temperature rose. Therefore, the duration of the HBR was prolonged as the metabolic rate declined, and Merazzi and Mortola speculated that the duration of apneas was inversely related to the metabolic rate. On the other hand, Merazzi and Mortola found that the respiratory drive during asphyxial stimulation increased as body temperature increased, which should have shortened the HBR. Therefore, the prolongation of apnea at higher temperatures was associated with a reduced metabolic rate, but apnea prolongation could not be attributed to a slower rise in respiratory drive due to the reduction in metabolism.

The duration of apnea associated with the laryngeal chemoreflex (LCR) is increased when body temperature is elevated ~2 °C in decerebrate, neonatal piglets and neonatal rats (Curran et al., 2005; Xia et al., 2008b), and this effect originates centrally within the NTS (Xia et al., 2006). It is our hypothesis that thermal activation of pre-synaptic TRPV1 receptors, which are present on vagal afferents terminating in the NTS (Doyle et al., 2002; Jin et al., 2004), enhances glutamate release in the NTS and mediates, in part, the thermal enhancement of the LCR (Xia et al., 2011). Moreover, the thermal prolongation of the LCR is associated with activation of adenosine type A2a receptors in the region of the NTS (Duy et al., 2010), which can act pre-synaptically to enhance GABA release (Hong et al., 2005; Ochi et al., 2000; Phillis, 1998), and so adenosine and GABA seemed to participate in the thermal prolongation of the LCR as well. Thus, at least two thermally sensitive synaptic mechanisms seem to exist in the region of the NTS that significantly alter the duration of respiratory inhibition associated with the LCR. Given the similarities between thermal prolongation of apnea in the LCR and the thermal prolongation of apnea in the HBR in neonatal animals, we tested the hypothesis that thermal prolongation of the HBR in neonatal animals would be modified by the same neurotransmitters, adenosine and GABA (Xia et al., 2008a; Xia et al., 2007), that mediate, in part, thermal prolongation of the LCR in piglets.

2. METHODS

We measured Hering-Breuer responses to lung inflation in 142 unsedated, neonatal, Sprague-Dawley rat pups ranging in age between postnatal day 4 (P4) and P20. All procedures were approved by the Institutional Animal Care and Use Committee of Dartmouth College.

2.1 Eliciting the HBR

Our method of testing the Hering Breuer reflex was patterned after that of Matsuoka and Mortola (1995). The animals were studied in a water-jacketed glass plethysmograph from which the head protruded through an air-tight latex collar into an anterior chamber. We incorporated a small air leak into the plethysmograph to allow plethysmographic pressure to equilibrate with atmospheric pressure during control conditions (Matsuoka and Mortola, 1995). The anterior chamber was ventilated with room air at a constant flow rate of 100 ml/min. Flow changes through the anterior chamber were recorded by means of a small pneumotachograph made in the lab, which was connected to a differential pressure transducer (Validyne model P-45-28-271, Northridge, CA) to determine the breathing pattern and to measure the duration of apneas. Lung inflations were performed by rapid, sustained exposure of the posterior chamber to a negative 5 cm H₂O pressure source. We tried to apply the negative chamber pressure during inspiration, and the following expiratory

period was, in general, prolonged. Once the animal resumed breathing, the extrathoracic suction pressure was removed, allowing the lungs to return to their resting volume. Each pup was tested in random order at two body temperatures: 32° and 36°C. A small thermistor was placed in the rectum of each animal, and body temperature was servo-controlled (Physiotemp Instruments, Inc., model TCAT-2AC controller, Clifton, NJ) by regulating the temperature of the water circulating in the wall of the plethysmographic chamber (see Fig. 1.; (Merazzi and Mortola, 1999b). These temperatures were chosen based on observations of the range of body temperatures measured in week-old, huddling rat pups kept with their mothers at an ambient temperature of 25°C (Schmidt et al., 1986) and based on previous studies of the thermal sensitivity of the HBR (Merazzi and Mortola, 1999a; b). Five inflations, separated by at least two minutes, were performed at each temperature.

2.3 Drug studies

We studied the effects of blocking adenosine receptors, blocking GABA_A receptors and blocking GABA_A receptors after pre-treatment with bumetanide on the duration of the Hering-Breuer reflex at 32°C and 36°C body temperatures. The rat pups were pretreated with either caffeine, a non-selective inhibitor of adenosine receptors; PSB-36, an adenosine A1 receptor antagonist; bicuculline, an inhibitor of GABA_A receptors; or with 0.9% saline or DMSO in saline, whichever was the vehicle for each particular drug treatment. Each of these drugs was administered into the cisterna magna (ICM). Pre-treatment with bumetanide was administered intraperitoneally (IP). For the intracisternal treatments, each animal was briefly anesthetized with 2% isoflurane (Abbot Laboratories, North Chicago, IL), the skin over the base of the skull was incised, and 4 µl of 2.5 mM, 10 mM or 20 mM mM bicuculline methiodide (Sigma-Aldrich, St. Louis, MO), 10 mM PSB-36 (Tocris Bioscience, Bristol, UK) or 10 mM caffeine (Sigma-Aldrich, St. Louis, MO) was injected into the cisterna magna through a 30 g. needle. The effect of each drug or combination of drugs was compared separately to its own set of controls in animals injected with 4 µl of the vehicle alone. After the ICM injection, the skin was closed with a suture, and a small amount of lidocaine gel was applied to the wound. After recovery from the anesthetic (30–40 min), the animal was placed in the plethysmograph and studied as described above.

For the combined study of the effect of bumetanide on the response to bicuculline, each pup received 0.25 mgs/kg bumetanide (Sigma-Aldrich, St. Louis, MO) in saline by IP injection approximately 30 minutes before the animal was anesthetized and given intracisternal bicuculline or the saline control as described above. Bumetanide blocks the Na⁺-K⁺-2Cl⁻ co-transporter and reduces the intracellular chloride ion concentration. This change in chloride transport and the reversal potential of chloride is thought to make activation of GABA_A receptors, which may have a depolarizing and excitatory effect in immature neurons, more hyperpolarizing and inhibitory, which is the typical response to GABA_A receptor activation in mature neurons (Dzhala et al., 2005).

2.4 Analysis and statistics

The response to each inflation was expressed as an “inhibitory ratio” (IR; (Matsuoka and Mortola, 1995): the ratio of the duration of the expiratory pause induced by the inflation to the average expiratory time of the three breaths immediately preceding the inflation. The caffeine, PSB-36, bicuculline and bumetanide/bicuculline studies were performed at different times and have been analyzed separately. Rats are an altricial species, and in previous studies, we noticed differences in the duration of the LCR between the early (P4–P10) and late prenatal period (P11–P20), and other investigators have observed that the duration of apnea elicited by the HBR declines as animals mature (Cross et al., 1960; Matsuoka and Mortola, 1995; Rabbette and Stocks, 1998). Therefore, the mean IR values for the 5-trial sets were subjected to analysis of variance with temperature as a within-subject,

repeated factor and age and drug treatment were between-subjects factors. When the ANOVA indicated that significant differences existed between treatments, specific pre-planned comparisons were made using P-values adjusted by the Bonferroni method. $P < 0.05$ was taken as the criterion for statistical significance. Data are reported as the mean $\pm$ the standard error of the mean (SEM).

3. RESULTS

The rat pups tolerated the procedures well. They appeared to recover fully from the anesthesia and surgery associated with the ICM injection. After a brief period of restlessness in the plethysmograph, each animal settled down and permitted the study to be conducted without difficulty.

Representative lung inflations from a P6 control pup at 32°C and 36°C are shown in Fig. 1 along with the applied chamber pressures. At the lower body temperature, the inflation maneuver increased expiratory time from 3.6 sec to 24.0 sec (Fig. 1, A), which corresponded to an IR of 6.6. When the animal was warmed to 36°C, the same inflation maneuver resulted in an 52.6 sec apnea and an IR of 11.0 (Fig. 1, B). Thus, greater body temperature was associated with longer apnea duration in this example.

3.1 Effect of caffeine on thermal prolongation of the HBR

The average responses of all the pups in the caffeine study are shown in Fig. 2. The test animals received 4 μ l injections of 10 mM caffeine into the cisterna magna approximately 30 minutes before the HBR was tested. The relative prolongation of apnea during sustained maintenance of inflation is shown for the P4–P10 pups in Fig. 2, A as a function of treatment with caffeine or saline control and as a function of body temperature. The analogous data are shown for the older animals (P11–P20) in Fig. 2, B. First, rectal temperature did not vary among the different treatment groups at each temperature tested; the thermal control of body temperature was equally effective in all treatment groups. There was a significant main effect of temperature prolonging the HBR regardless of animal age ($P = 0.001$). The apneas resulting from lung inflation at 36°C body temperature was prolonged compared to apneas elicited at 32°C, and this was true for both age groups. The effect of caffeine on apnea duration, however, differed as a function of temperature. Caffeine had no significant effect on apnea duration at 32°C body temperature, but significantly shortened the apnea duration at 36°C (a significant temperature by drug treatment interaction apparent across both age groups; $P = 0.031$). In addition, apnea durations were significantly shorter across all treatment conditions in the older group of animals than in the younger group (a main effect of age; $P = 0.004$). Thus, the pattern of responses to caffeine treatment was similar in both age groups though the apnea durations were consistently shorter in the older animals.

3.2 Effect of bicuculline on thermal prolongation of the HBR

Based on our studies of thermal prolongation of the LCR in decerebrate piglets, we believed that adenosine enhances GABA release during reflex apnea at higher body temperatures (Duy et al., 2010). To test the hypothesis that a GABAergic mechanism in the lower brainstem also prolongs the HBR at higher body temperatures, we assessed the effect of intracisternal administration of bicuculline, a GABA_A receptor antagonist, on the duration of the HBR at 32°C and 36°C in the two age groups of pups defined above. The average responses of the neonatal rats in this study are shown in Fig. 3. We studied three doses of bicuculline, but there were no dose effects, and data from all of the bicuculline-treated animals have been pooled into a single bicuculline treatment group in Fig. 3. The results for the P4–P10 pups are shown in Fig. 3, A, and the results from older P11–P20 pups are shown

in Fig. 3, B. As before, elevating the body temperature prolonged the HBR in control and drug-treated animals (a main effect of temperature $P < 0.001$), and apnea was significantly shorter at both body temperatures in older compared to younger animals (main effect of age group; $P = 0.001$). Bicuculline, however, had no significant effect on the duration of the apnea (we had expected bicuculline to shorten the duration of reflex apnea at high temperatures, indicative of a decrease in GABAergic inhibition). The absence of any effect of 2.5 mM bicuculline was such a surprise to us that we repeated the study and increased the dose of bicuculline to 10 mM, and we still saw no effect of bicuculline on the duration of the HBR. In an additional four animals, we tried 20 mM, but there was still no effect of bicuculline on the duration of the HBR. Despite the large number of animals and elevated doses of bicuculline, there was no interaction between bicuculline and temperature (in contrast to the response to caffeine; see Fig. 2). There was a trend toward shortened apnea durations at both temperatures after bicuculline treatment, but this trend was not significant ($P = 0.21$.) Thus, we found no evidence of GABAergic modulation of the duration of the HBR.

3.3 Effect of bumetanide on thermal prolongation of the HBR

The intracellular chloride concentration can be high in neurons in neonatal animals since immature neurons express $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter 1 (NKCC1), which transports chloride into neurons and elevates the GABA reversal potential (Dzhala et al., 2005; Yamada et al., 2004). Activation of GABA_A receptors in this setting is depolarizing and may be excitatory. The absence of a GABAergic effect on apnea duration was not what we expected, but bicuculline might not have altered thermal prolongation of the HBR if GABA_A were not having an inhibitory effect in immature neonatal rats. The intracellular chloride concentration achieves its adult state after the expression of $\text{K}^+\text{-Cl}^-$ cotransporter 2 (KCC2) develops, and the response to GABA_A receptor activation becomes inhibitory (Ben-Ari, 2002). The chloride gradient can be made more like the adult pattern by treatment with bumetanide, which blocks chloride entry into neurons so that GABA_A receptor activation favors inward movement of chloride even in neonatal animals (Dzhala et al., 2005; Yamada et al., 2004). Therefore, we administered bumetanide to decrease the intracellular chloride concentration in neurons and expose any inhibitory action of GABA to test the hypothesis that bicuculline would prolong the HBR once bumetanide enhanced the inhibitory effect of GABA. We conducted this study only in younger animals, and the average responses of the treatment and control groups are shown in Fig. 4. The combination of bumetanide and bicuculline did not change the duration of the HBR or the thermal prolongation of the HBR. The responses in the bumetanide plus bicuculline treated animals mimicked those of the animals treated with bicuculline alone, as in the prior experiments. The HBR tended to be longer at the higher body temperature, but this change did not reach statistical significance in this group of animals.

3.4 Effect of PSB-36 on the duration of the HBR

The absence of any apparent role for GABA_A receptors in the thermal prolongation of the HBR prompted us to re-evaluate the caffeine response. We had assumed that caffeine acted through A2a receptors as it does in the thermal prolongation of the LCR (Duy et al., 2010), but adenosine A1 receptors are also present in the brain. Therefore, we decided to determine whether selectively blocking adenosine A1 receptors might recapitulate the effects of caffeine on thermal prolongation of the HBR. The average responses of a group of neonatal rat pups treated with ICM injection of vehicle alone (DMSO in saline) compared to the responses of a group of animals that received 10 mM ICM PSB-36, a selective adenosine A1 receptor antagonist, are shown in Fig. 5 at two body temperatures. As in previous studies, elevated body temperature significantly prolonged the HBR in both age groups (a main effect of body temperature; $P < 0.001$). In addition, older animals tended to have shorter

HBR durations at both body temperatures (a main effect of age group; $P=0.061$). The age effect was less striking than in the other drug studies because the inhibitory ratio was relatively short in the younger group of animals compared to the other groups studied (see Figures 2 and 3). This may be related to the distribution of ages within each age group: the younger group in this study had a disproportionate number of animals at the upper end of the age range (P9 and P10) compared to the other series of drug studies. Most importantly however, PSB-36 had no effect on HBR duration, and there were no interactions between drug treatment and age or body temperature.

4. DISCUSSION

We tested the hypothesis that blocking adenosine and GABA_A receptors in the brainstem would reduce the thermal prolongation of the HBR, and we expected the effect of these drugs to be greater in younger animals. As others have found, the HBR was prolonged in neonatal rat pups when body temperature was elevated, and the HBR was more potent - the expiratory prolongation lasted longer - in younger animals. Blocking adenosine receptors with caffeine reduced the thermal prolongation of the HBR, and this effect was apparent in both age groups we studied. The caffeine effect seems to operate through adenosine A2a receptors since treatment with a selective adenosine A1 receptor antagonist did not modify the thermal prolongation of the HBR or any other aspect of the reflex as we studied it. Contrary to our expectations, blocking GABA_A receptors did not blunt the thermal prolongation of the HBR - the GABA_A antagonist, bicuculline, had little effect on any aspect of the HBR. Moreover, systemic pretreatment with bumetanide did not change the response to bicuculline; we found no evidence that GABA_A receptor activation might have an excitatory effect in these neonatal animals. We conclude that activation of adenosine A2a receptors when body temperature is elevated prolongs the HBR, but this effect of adenosine did not depend on modulation of GABAergic mechanisms in our experiments.

4.1 Neural circuitry of the HBR

The neural circuitry of the HBR within the brainstem has been studied intensively although there are still some unresolved issues. The HBR is mediated by lung volume-related information derived from slowly adapting stretch receptors (SARs) in the lung. The lung volume-related information is transmitted centrally in vagal afferent fibers that innervate second order neurons in the NTS called pump neurons. Pump neurons are defined by the absence of intrinsic respiratory rhythmicity and the tight coupling of activity of the neurons to changes in lung volume (Ezure and Tanaka, 2004). The majority of pump neurons identified have been GABAergic, but a small number of these neurons may co-release glycine (Ezure and Tanaka, 2004). However, the HBR reflex is thought to be mediated by excitatory pump neurons that project to expiratory neurons in the ventral respiratory group with a decrementing pattern of firing (E-dec neurons) (Hayashi et al., 1996). Excitation of the E-dec neurons inhibits a subset of inspiratory neurons and prolongs the expiratory phase of the respiratory rhythm. The existence of excitatory pump neurons has been inferred from the short latency, disynaptic pathway between vagal stimulation and depolarization of the E-dec neurons (Hayashi et al., 1996), though the anatomical location of these excitatory second order neurons has not, so far as we know, been identified in the NTS. Given that there are few synapses in the reflex arc of the HBR, most of the control of the duration of the response to lung inflation is invested in the activity of these putative excitatory pump neurons. The afferent, vagal sensory nerves terminating in the NTS are glutamatergic, and there is a good deal of pre-synaptic regulation of glutamate release from these afferents (Bailey et al., 2008; Doyle et al., 2002; Fernandes et al., 2011; Peters et al., 2010). In addition, the activity of pump neurons is regulated by respiration-phasic and tonic inhibitory inputs. Early during inspiration, there is a glycinergic input (Miyazaki et al., 1999). There is also a tonic GABAergic input that is thought to suppress pump neuron activity by as much

as 40% under resting conditions (Miyazaki et al., 1999) and may set the gain of the pump neurons (Zuperku and McCrimmon, 2002). Finally, there are many GABAergic interneurons and GABAergic collateral inputs from second order neurons within the NTS that feed back onto pump neurons (Bailey et al., 2008).

There are multiple potential thermally sensitive processes within the complex neural circuitry of the HBR. There is a theoretical possibility that the effect of temperature originates at the level of the pulmonary stretch receptors. Schoener and Frankel (1972) found that some of the SARs that they studied in adult rats increased their firing rates slightly with temperature at constant tidal volumes. The effect was small, however, and most of the receptors were thermally insensitive near normal body temperature. Hence, the thermal modulation of SAR activity seems unlikely as a source for the thermal prolongation of the HBR. Moreover, laryngeal afferents from water receptors in the larynx, which mediate the LCR, have no apparent thermal sensitivity in the range in question (Xia et al., 2005), but the LCR is also prolonged when body temperature or just the NTS temperature is elevated (Curran et al., 2005; Xia et al., 2006). Therefore, thermally-sensitive, central, neural mechanisms seem to be more likely sources of the thermal prolongation of both the HBR and the LCR.

TRPV1 channels are well represented in the NTS, and thermal excitation of pre-synaptic TRPV1 receptors on vagal afferents could sensitize the HBR (Mezey et al., 2000; Patterson et al., 2003; Sun et al., 2009). These TRPV1 channels enhance glutamate release on to second order neurons in the NTS over a temperature range that encompasses 32–36° C (Peters et al., 2010; Shoudai et al., 2010). However, TRPV1 channels are expressed on unmyelinated C-fibers (Doyle et al., 2002), and the slowly-adapting stretch receptors that mediate the HBR are myelinated. Therefore, thermal activation of TRPV1 channels would have to sensitize stretch receptor afferent activity by some indirect modulation of pump neuron activity.

The thermal prolongation of the HBR that was blocked by caffeine is probably mediated by adenosine A2a receptors. Adenosine is an endogenous neuromodulator that exerts inhibitory effects on respiration in immature and, to a lesser extent, adult animals. For example, caffeine and other methylxanthines are frequently used to treat apnea in premature infants (Abu-Shaweesh and Martin, 2008). These adenosine receptor antagonists behave as if they were respiratory stimulants because activation of pre-synaptic adenosine A2a receptors enhances release of GABA (Hong et al., 2005; Ochi et al., 2000; Phillis, 1998), and the stimulating effect of caffeine on respiration has been attributed to blocking the pre-synaptic adenosinergic enhancement of GABA release (Mayer et al., 2006; Zaidi et al., 2006). Adenosine also binds to adenosine A1 receptors in the central nervous system (CNS), which can be presynaptic, but on glutamatergic neurons where their activation decreases glutamate release and reduces excitation at glutamatergic synapses (Arrigoni et al., 2005). Though adenosine A1 receptors are present in the brainstem, and caffeine blocks both receptor types approximately equally, the respiratory effects of caffeine have been attributed to blocking adenosine A2a receptors (Abu-Shaweesh and Martin, 2008). We examined the possibility that A1 receptors were mediating the thermal prolongation of the HBR. The thermal prolongation was, however, unchanged by treatment with a specific adenosine A1 antagonist. We conclude, therefore, that caffeine also modifies the duration of the HBR by acting on adenosine A2a receptors,

Caffeine treatment reduced the duration of the HBR at 36°C, which is consistent with the idea that caffeine is a respiratory stimulant by virtue of blocking the actions of adenosine. It seems, however, that adenosine enhanced the HBR only at elevated body temperatures. It seems possible that the elevated temperature in the NTS increased metabolism so that there

was a mismatch between metabolic demand and the supply of oxygen; a circumstance in which neurons may depolarize and release ATP, which is converted by extracellular ectonucleotidases into adenosine (Dale and Frenguelli, 2009; Latini and Pedata, 2001; Sperlagh and Vizi, 2011). The thermal stress that we applied, however, seems too modest to have led to such severe metabolic disruption that neurons could not maintain normal ATP levels and normal transmembrane ion concentration differences. It seems more likely that elevated temperature within the NTS may increase neuronal activity, which may increase ATP release and result in greater accumulation of extracellular adenosine. Alternatively, adenosine may be released directly from neurons or astrocytes by some other, as yet undefined, thermal process. For example, elevating the temperature of hippocampal brain slices increased extracellular adenosine levels without significantly changing ATP levels (Masino et al., 2001). The thermally sensitive process responsible for the increased adenosine levels in the extracellular space in this study was not identified, but some thermally sensitive process that increased intracellular adenosine levels and increased release of adenosine into the extracellular space through nucleoside transport processes seems more plausible than metabolic stress, ATP depletion, cellular depolarization and subsequent breakdown of extracellular ATP to adenosine since changes in ATP levels were not well correlated with the observed changes in adenosine levels.

Caffeine-mediated blunting of the thermal prolongation of the HBR cannot be attributed to modulation of GABA release in our experiments. We found no evidence that bicuculline modified the HBR either at the low or the high body temperature. The failure of bicuculline to affect the HBR might result if GABAergic activation were not inhibitory, but excitatory or depolarizing as described in immature neurons in some preparations (Dzhala et al., 2005; Yamada et al., 2004). Bumetanide treatment of neonatal rat pups did not, however, change the thermal prolongation of the HBR, nor did it alter the response to bicuculline. Even though there are changes in the expression of chloride transporters in the NTS over the age range that we studied (Liu and Wong-Riley, 2012), there appear to be no functional consequences of these changing levels of expression of the transporters for the HBR since the reflex was functional and consistently responsive to lung inflation and temperature. Moreover, the entire concept of GABAergic depolarization on the basis of changes in the chloride reversal potential has recently been called into question (Bregestovski and Bernard, 2012).

4.2 Limitations of the study

Adenosine A2a receptors are G-protein-coupled receptors coupled to G_s , and our results are consistent with the hypothesis that activation of post-synaptic adenosine A2a receptors on excitatory pump neurons directly increases pump neuron activity and thereby prolongs the duration of the HBR. The idea that adenosine acts post-synaptically on pump neurons is difficult for us to accept. First, these results are not consistent with our previous studies of the LCR in which bicuculline treatment occluded the effects of adenosine A2a receptor antagonists (Duy et al., 2010). Moreover, there is abundant evidence that adenosine A2a receptors are post-synaptic on GABAergic neurons in other studies and other parts of the brain (Abu-Shaweesh, 2007; Cunha, 2001; Hong et al., 2005; Mayer et al., 2006; Zaidi et al., 2006). Second, the absence of any effect of bicuculline is suspect since there are so many GABA_A receptors within the NTS and so many opportunities within the circuitry of the HBR for GABA to modulate the HBR (Ezure and Tanaka, 2004; Fernandes et al., 2011; Kubin et al., 2006; Miyazaki et al., 1999; Wasserman et al., 2002).

Before accepting the idea that GABA_A receptors do not modulate the HBR in neonatal rats and that adenosine A2a receptors are post-synaptic on excitatory pump neurons (for which there is no evidence one way or the other), we want to recognize a variety of limitations in our study that may have masked an effect of bicuculline on the HBR. Foremost among them,

we gave bicuculline by intracisternal injection, which does not limit bicuculline to any specific location, but bathes the entire pons and medulla with the drug. We did this because it allowed us to study the animals without anesthesia. The main disadvantage of ICM treatment is that bicuculline may have effects outside the NTS. Moreover, the ubiquity of GABA_A and GABA_B receptors and the multiple interconnections among neurons means that blocking one set of GABA receptors may cancel the effect of activation of another set. To address this issue, we are currently repeating the bicuculline studies using microinjections in the NTS, but these studies must be done in anesthetized animals. The limitations associated with ICM injection apply to the caffeine experiments as well, and we need to confirm with microinjections that the caffeine effects that we observed originate in the NTS and reflect antagonism of adenosine A2a receptors.

In addition to the lack of anatomical specificity, the effects of bicuculline treatment are not restricted to GABA_A receptors. Bicuculline also blocks small-conductance, calcium-activated potassium (SK) channels (Khawaled et al., 1999; Stocker et al., 1999). The SK channels are present in the NTS, and depending on the pre- or post-synaptic location of the SK channels and the neurotransmitter released by neurons expressing the SK channels, inhibition of these currents may excite or inhibit various reflex processes within the NTS (Butcher et al., 1999). Gabazine, a GABA_A receptor antagonist that does not inhibit SK channels and only inhibits phasic GABA_A currents (Gao and Smith, 2010; Semyanov et al., 2004), blocked the thermal prolongation of the LCR in neonatal piglets when administered directly within the NTS (Xia et al., 2007), and we need to re-examine the role of GABA in the thermal prolongation of the HBR using drugs, such as gabazine, which have greater receptor specificity than bicuculline.

We based our initial hypothesis that the neurochemical control of the HBR ought to resemble the LCR on piglet data (Duy et al., 2010; Xia et al., 2011). It may be that there are significant differences between piglets and neonatal rats. It could be that glycine is more important in rodents and GABA has a less important role in modulation of the HBR (and we are currently investigating this possibility). Moreover, piglets are precocious when born and rats are altricial. This may be particularly important since the development of synaptic networks is almost certainly delayed in newborn rat pups compared to neonatal piglets. However, the development of inhibitory synaptic mechanisms usually proceeds from GABAergic mechanisms in immature animals to glycinergic mechanisms in more mature animals (Singer and Berger, 2000), which ought to mean that GABA plays a dominant role in neonatal rats – something we could not confirm in our studies.

Last, our hypotheses were based on the apparently similar thermal sensitivity of the HBR and the LCR, the similar patterns of respiratory inhibition, and the idea that reflexes with similar thermal responses and respiratory effects might be convergent on the same second order neurons in the NTS. However, convergence at the level of second order neurons in the NTS is an unusual event (Bailey et al., 2008). If there is a convergent effect on apnea duration, it seems more likely in light of the current study that this convergence occurs at the level of the ventral respiratory group and E-dec or pre-inspiratory neurons, and the neurochemical control of the LCR and HBR within the NTS may actually be quite different.

4.3 Maturation of the Hering-Breuer reflex

The duration of the HBR in the control animals decreased as the age of the pups increased, which is consistent with reports of apneic behavior in human infants (Cross et al., 1960; Rabette et al., 1994). Furthermore, Smejkal et al. (1985), studying urethane-anesthetized rat pups, found that the apneic response to lung inflation lasted longer in P1 than in P8 animals. There is a similar age-related decline in the inhibitory magnitude of the LCR in humans (Thach, 2001) and rats (Xia et al., 2008b; Xia et al., 2010). A common feature in the

neuropharmacology of these reflexes is the inhibitory action of adenosine, and so we suspect that an age-related decline in the expression or activity of A_{2a} receptors or changes in the synthesis and metabolism of adenosine may underlie this age-dependent diminution in potency of the HBR and LCR.

4.3 Summary

We confirmed that the expiratory prolongation elicited by lung inflation is greater in younger neonatal pups and greater at higher body temperature. Caffeine blocked the thermal prolongation of the HBR, and it is probable that this action was mediated by blocking adenosine A_{2a} receptors. GABA_A receptors do not seem to play any role in the thermal prolongation of the HBR, but there are significant limitations inherent to the intracisternal administration of drugs that we used in these studies. Our results are consistent with the hypothesis that thermally sensitive chemical processes within the brainstem mediate the thermal prolongation of the HBR.

Acknowledgments

This work was supported by grants 36379 and 42707 from the NICHD. Ashley Arnal was a Presidential Scholar at Dartmouth College.

References

- Abu-Shaweesh JM. Activation of central adenosine A_{2A} receptors enhances superior laryngeal nerve stimulation-induced apnea in piglets via a GABAergic pathway. *J Appl Physiol.* 2007; 103:1205–1211. [PubMed: 17656623]
- Abu-Shaweesh JM, Martin RJ. Neonatal apnea: What's new? *Pediatr. Pulmonol.* 2008; 43:937–944.
- Arrigoni E, Crocker AJ, Saper CB, Greene RW, Scammell TE. Deletion of presynaptic adenosine A₁ receptors impairs the recovery of synaptic transmission after hypoxia. *Neurosci.* 2005; 132:575–580.
- Bader D, Tirosh E, Hodgins H, Abend M, Cohen A. Effect of increased environmental temperature on breathing patterns in preterm and term infants. *J Perinatol.* 1998; 18:5–8. [PubMed: 9527936]
- Bailey TW, Appleyard SM, Jin YH, Andresen MC. Organization and properties of GABAergic neurons in the solitary tract nucleus (NTS). *J Neurophysiol.* 2008; 99:1712–1722. [PubMed: 18272881]
- Ben-Ari Y. Excitatory actions of GABA during development: The nature of the nurture. *Nat Rev.* 2002; 3:728–739.
- Bregestovski P, Bernard C. Excitatory GABA: How a correct observation may turn out to be an experimental artifact. *Front Pharmacol.* 2012; 3:65. [PubMed: 22529813]
- Butcher WJ, Kasparov S, Paton FJ. Differential effects of apamin on neuronal excitability in the nucleus tractus solitarius of rats studied in vitro. *J Auton Nerv Syst.* 1999; 77:90–97.
- Cross KW, Klaus M, Tooley WH, Weisser K. The response of the new-born baby to inflation of the lungs. *J Physiol.* 1960; 151:551–565. [PubMed: 13813016]
- Cunha RA. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. *Neurochem Int.* 2001; 38:107–125. [PubMed: 11137880]
- Curran AK, Xia L, Leiter JC, Bartlett D Jr. Elevated body temperature enhances the laryngeal chemoreflex in decerebrate piglets. *J Appl Physiol.* 2005; 98:780–786. [PubMed: 15542573]
- Dale N, Frenguelli BG. Release of adenosine and ATP during ischemia and epilepsy. *Curr Neuropharmacol.* 2009; 7:160–179. [PubMed: 20190959]
- Dawes, GS. Foetal and Neonatal Physiology: A Comparative Study of the Changes at Birth. Year Book Medical Publishers, Inc; Chicago, IL: 1968.

- Doyle MW, Bailey TW, Jin YH, Andresen MC. Vanilloid receptors presynaptically modulate cranial visceral afferent synaptic transmission in nucleus tractus solitarius. *J Neurosci.* 2002; 22:8222–8229. [PubMed: 12223576]
- Duy MP, Xia L, Bartlett D Jr, Leiter JC. An adenosine A_{2A} agonist injected in the NTS prolongs the LCR by a GABAergic mechanism in decerebrate piglets. *Exp Physiol.* 2010; 95:774–787. [PubMed: 20418346]
- Dzhala VI, Talos DM, Sdrulla DA, Brumback AC, Mathews GC, Benke TA, Delpire E, Jensen FE, Staley KJ. NHCC1 transporter facilitates seizures in the developing brain. *Nature Med.* 2005; 11:1205–1213. [PubMed: 16227993]
- Ezure K, Tanaka I. GABA, in some cases together with glycine, is used as the inhibitory transmitter by pump cells in the Hering-Breuer reflex pathway of the rat. *Neurosci.* 2004; 127:409–417.
- Fernandes LG, Jin YH, Andresen MC. Heterosynaptic crosstalk: GABA-glutamate metabotropic receptors interactively control glutamate release in solitary tract nucleus. *Neurosci.* 2011; 174:1–9.
- Gao H, Smith BN. Tonic GABAA receptor-mediated inhibition in the rat dorsal motor nucleus of the vagus. *J Neurophysiol.* 2010; 103:904–914. [PubMed: 20018836]
- Haraguchi S, Fung RQ, Sasaki R. Effect of hyperthermia on the laryngeal closure reflex. Implications in the sudden infant death syndrome. *Ann Otol Rhinol Laryngol.* 1983; 92:24–28. [PubMed: 6824275]
- Hayashi F, Coles SK, McCrimmon DR. Respiratory neurons mediating the Breuer-Hering reflex prolongation of expiration in rat. *J Neurophysiol.* 1996; 16:6526–6536.
- Hong ZY, Huang ZL, Qu WM, Eguchi N, Urade Y, Hayaishi O. An adenosine A_{2A} receptor agonist induces sleep by increasing GABA release in the tuberomammillary nucleus to inhibit histaminergic systems in rats. *J Neurochem.* 2005; 92:1542–1549. [PubMed: 15748171]
- Jin YH, Bailey TW, Li B, Schild JH, Andresen MC. Puringergic and vanilloid receptor activation releases glutamate from separate cranial afferent terminals in nucleus tractus solitarius. *J Neurosci.* 2004; 24:4709–4717. [PubMed: 15152030]
- Khawaled R, Bruening-Wright A, Adelman JP, Maylie J. Bicuculline block of small-conductance calcium-activated potassium channels. *Pflugers Arch.* 1999; 438:314–321. [PubMed: 10398861]
- Kubin L, Alheid GF, Zuperku EJ, McCrimmon DR. Central pathways of pulmonary and lower airway vagal afferents. *J Appl Physiol.* 2006; 101:618–627. [PubMed: 16645192]
- Latini S, Pedata F. Adenosine in the central nervous system: release mechanisms and extracellular concentrations. *J Neurochem.* 2001; 79:463–484. [PubMed: 11701750]
- Liu Q, Wong-Riley MT. Postnatal development of Na(+)-K(+)-2Cl(−) co-transporter 1 and K(+)-Cl(−) co-transporter 2 immunoreactivity in multiple brain stem respiratory nuclei of the rat. *Neurosci.* 2012; 210:1–20.
- Masino SA, Latini S, Bordoni F, Pedata F, Dunwiddie TV. Changes in hippocampal adenosine efflux, ATP levels, and synaptic transmission induced by increased temperature. *Synapse.* 2001; 41:58–64. [PubMed: 11354014]
- Matsuoka T, Mortola JP. Effects of hypoxia and hypercapnia on the Hering-Breuer reflex of the conscious newborn rat. *J Appl Physiol.* 1995; 78:5–11. [PubMed: 7713842]
- Mayer CA, Haxhiu MA, Martin RJ, Wilson CG. Adenosine A_{2A} receptors mediate GABAergic inhibition of respiration in rats. *J Appl Physiol.* 2006; 100:91–97. [PubMed: 16141383]
- Merazzi D, Mortola JP. Effects of changes in ambient temperature on the Hering-Breuer reflex of the conscious newborn rat. *Ped Res.* 1999a; 45:370–376.
- Merazzi D, Mortola JP. Hering-Breuer reflex in conscious newborn rats: effects of changes in ambient temperature during hypoxia. *J Appl Physiol.* 1999b; 87:1656–1661. [PubMed: 10562605]
- Mezey A, Toth ZE, Cortright DN, Arzubi MK, Krause JE, Elde R, Guo A, Blumberg PM, Szallasi A. Distribution of mRNA for vanilloid receptor subtype 1 (VR1), and VR1-like immunoreactivity, in the central nervous system of the rat and human. *Proc Natl Acad Sci.* 2000; 97:3655–3660. [PubMed: 10725386]
- Miyazaki M, Tanaka I, Ezure K. Excitatory and inhibitory synaptic inputs shape the discharge pattern of pump neurons of the nucleus tractus solitarii in the rat. *Exp Brain Res.* 1999; 129:191–200. [PubMed: 10591893]

- Moss IR, Mautone AJ, Scarpelli EM. Effect of temperature on regulation of breathing and sleep/wake state in fetal lambs. *J Appl Physiol*. 1983; 54:536–543. [PubMed: 6403494]
- Ochi M, Koga K, Kurokawa M, Kase H, Nakamura JI, Kuwana Y. Systemic administration of adenosine A_{2A} receptor antagonist reverses increased GABA release in the globus pallidus of unilateral 6-hydroxydopamine-lesioned rats: A microdialysis study. *Neurosci*. 2000; 100:53–62.
- Patterson LM, Zheng H, Ward SM, Berthoud HR. Vanilloid receptor (VR1) expression in vagal afferent neurons innervating the gastrointestinal tract. *Cell Tissue Res*. 2003; 311:277–287. [PubMed: 12658436]
- Perlstein PH, Edwards NK, Sutherland JM. Apnea in premature infants and incubator-air-temperature changes. *N Engl J Med*. 1970; 282:461–466. [PubMed: 5411868]
- Peters JH, McDougall SJ, Fawley JA, Smith SM, Andresen MC. Primary afferent activation of thermosensitive TRPV1 triggers asynchronous glutamate release at central neurons. *Neuron*. 2010; 65:657–669. [PubMed: 20223201]
- Phillis JW. Inhibitory action of CGS 21680 on cerebral cortical neurons is antagonized by bicuculline and picrotoxin - is GABA involved? *Brain Res*. 1998; 807:193–198. [PubMed: 9757034]
- Rabbette PS, Stocks J. Influence of volume dependency and timing of airway occlusions on the Hering-Breuer reflex in infants. *J Appl Physiol*. 1998; 85:2033–2039. [PubMed: 9843523]
- Schmidt I, Barone A, Carlisle HJ. Diurnal cycle of core temperature in huddling, week-old rat pups. *Physiol Behavior*. 1986; 37:105–109.
- Semyanov A, Walker MC, Kullmann DM, Silver RA. Tonically active GABA A receptors: modulating gain and maintaining the tone. *Trends Neurosci*. 2004; 27:262–269. [PubMed: 15111008]
- Shoudai K, Peters JH, McDougall SJ, Fawley JA, Andresen MC. Thermally active TRPV1 tonically drives central spontaneous glutamate release. *J Neurosci*. 2010; 30:14470–14475. [PubMed: 20980604]
- Singer JH, Berger AJ. Development of inhibitory synaptic transmission to motoneurons. *Brain Res Bull*. 2000; 53:553–560. [PubMed: 11165791]
- Sperlagh B, Vizi ES. The role of extracellular adenosine in chemical neurotransmission in the hippocampus and Basal Ganglia: pharmacological and clinical aspects. *Curr Top Med Chem*. 2011; 11:1034–1046. [PubMed: 21401497]
- Stocker M, Krause M, Pedarzani P. An apamin-sensitive Ca²⁺-activated K⁺ current in hippocampal pyramidal neurons. *Proc Natl Acad Sci*. 1999; 96:4662–4667. [PubMed: 10200319]
- Sun H, Li DP, Chen SR, Hittelman WN, Pan HL. Sensing of blood pressure increase by transient receptor potential vanilloid 1 receptors on baroreceptors. *J Pharmacol Exp Ther*. 2009; 331:851–859. [PubMed: 19726694]
- Thach BT. Maturation and transformation of reflexes that protect the laryngeal airway from liquid aspiration from fetal to adult life. *Am J Med*. 2001; 111:69S–77S. [PubMed: 11749929]
- Wasserman AM, Ferreira M Jr, Sahibzada N, Hernandez YM, Gillis RA. GABA-mediated neurotransmission in the ventrolateral NTS plays a role in respiratory regulation in the rat. *Am J Physiol*. 2002; 283:R1423–R1441.
- Xia L, Bartlett D Jr, Leiter JC. An adenosine A_{2A} antagonist injected in the NTS reverses thermal prolongation of the LCR in decerebrate piglets. *Respir Physiol Neurobiol*. 2008a; 164:358–365. [PubMed: 18775519]
- Xia L, Bartlett D Jr, Leiter JC. TRPV1 channels in the nucleus of the solitary tract mediate thermal prolongation of the LCR in decerebrate piglets. *Respir Physiol Neurobiol*. 2011; 176:21–31. [PubMed: 21276877]
- Xia L, Damon T, Niblock MM, Bartlett D Jr, Leiter JC. Unilateral microdialysis of gabazine in the dorsal medulla reverses thermal prolongation of the laryngeal chemoreflex in decerebrate piglets. *J Appl Physiol*. 2007; 103:1864–1872. [PubMed: 17823299]
- Xia L, Damon TA, Leiter JC, Bartlett D Jr. Focal warming of the nucleus of the solitary tract prolongs the laryngeal chemoreflex in decerebrate piglets. *J Appl Physiol*. 2006; 102:54–62. [PubMed: 16959905]
- Xia L, Leiter JC, Bartlett D Jr. Laryngeal water receptors are insensitive to body temperature in neonatal piglets. *Respir Physiol Neurobiol*. 2005; 150:82–86. [PubMed: 15993656]

- Xia L, Leiter JC, Bartlett D Jr. Laryngeal apnea in rat pups: effects of age and body temperature. *J Appl Physiol.* 2008b; 104:269–274. [PubMed: 17962578]
- Xia L, Leiter JC, Bartlett D Jr. Gestational nicotine exposure exaggerates hyperthermic enhancement of laryngeal chemoreflex in rat pups. *Respir Physiol Neurobiol.* 2010; 171:17–21. [PubMed: 20097312]
- Yamada J, Okabe A, Toyoda H, Kilb W, Luhmann HJ, Fukuda A. Cl⁻ uptake promoting depolarizing GABA actions in immature rat neocortical neurones is mediated by NKCC1. *J Physiol.* 2004; 557:829–841. [PubMed: 15090604]
- Zaidi SIA, Jafri A, Martin RJ, Haxhui MA. Adenosine A_{2A} receptors are expressed by GABAergic neurons of medulla oblongata in developing rat. *Brain Res.* 2006; 1071:42–53. [PubMed: 16413509]
- Zuperku EJ, McCrimmon DR. Gain modulation of respiratory neurons. *Respir Physiol Neurobiol.* 2002; 131:121–133. [PubMed: 12107000]

Highlights

Caffeine administered into the fourth ventricle blunted thermal prolongation of the Hering-Breuer Reflex

Caffeine probably achieved this effect by blocking adenosine A2a receptors.

Blocking GABAa receptors by administration of bicuculine into the fourth ventricle had no effect on the thermal prolongation of the Hering-Breuer Reflex.

Figure 1.

Examples of tracings of airflow into and out of the head-out plethysmograph and of plethysmographic pressure demonstrating apnea following elicitation of the inspiratory HBR. Note that an increased body temperature was associated with greater expiratory prolongation when the HBR was elicited. The flow rate during the application of negative chamber pressure was slightly greater than zero due to the small air leak incorporated into the plethysmograph. However, the applied pressure was consistent throughout the test period. The dashed lines superimposed on the airflow tracings indicate the beginning and end of each apnea. Inspiration is up in these tracings. Flow was measured in arbitrary units ('arb units') since we used the flow tracing only to determine respiratory timing (and not minute ventilation), and inspiration ('Insp') was up and expiration ('Exp') was down.

Figure 2.

The average inhibitory ratio in P4–P10 (A) and the P11–P20 age groups (B) at warm and cool body temperatures after caffeine treatment (gray) or vehicle treatment (black). The ‘**’ indicates a main effect of age (vertical bracket) or body temperature (horizontal bracket). The ‘*’ indicates that apnea duration was significantly shorter after caffeine treatment at 36°C body temperature compared to the vehicle control treatment (a significant temperature by drug treatment interaction).

Figure 3.

The average inhibitory ratio in P4–P10 (A) and the P11–P20 age groups (B) at warm and cool body temperatures after bicuculline treatment (gray) or vehicle treatment (black). The ‘**’ indicates a main effect of age (vertical bracket) or body temperature (horizontal bracket). All three bicuculline treatment groups (2.5, 10 and 20 mM/kg) have been combined since there were no differences among these doses.

Figure 4.

The average inhibitory ratio in P4–P10 rat pups at warm and cool body temperatures after 2.5 mM/kg ICM bicuculline treatment (gray) or vehicle treatment (black). The pups that received bicuculline were pretreated 30 minutes before the ICM injections with 0.25 mg/Kg bumetanide IP. There was no effect of the bumetanide/bicuculline treatment on the duration of the HBR at either temperature.

Figure 5.

The average inhibitory ratio in P4–P10 (A) and the P11–P20 age groups (B) at warm and cool body temperatures after ICM treatment with a specific adenosine A1 antagonist, PSB-36 (gray) or vehicle treatment (black). The ‘***’ indicates a main effect of body temperature (horizontal bracket). There was no significant main effect of drug, and there were no significant drug interaction terms.