Unilateral microdialysis of gabazine in the dorsal medulla reverses thermal prolongation of the laryngeal chemoreflex in decerebrate piglets

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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 103: 1864–1872, 2007. First published September 6, 2007; doi:10.1152/japplphysiol.00524.2007.—The laryngeal chemoreflex (LCR) is elicited by water in the larynx and leads to apnea and respiratory disruption in immature animals. The LCR is exaggerated by the elevation of brain temperature within or near the nucleus of the solitary tract (NTS) in decerebrate piglets. Thermal prolongation of reflex apnea elicited by superior laryngeal nerve stimulation is reduced by systemic administration of GABA<sub>A</sub> receptor antagonists. Therefore, we tested the hypothesis that microdialysis within or near the NTS of gabazine, a GABA<sub>A</sub> receptor antagonist, would reverse thermal prolongation of the LCR. We examined this hypothesis in 21 decerebrate piglets (age 3–13 days). We elicited the LCR by injecting 0.1 ml of water into the larynx before and after each piglet’s body temperature was elevated by ∼2.5°C and before and after 2–5 mM gabazine was dialyzed unilaterally and focally in the medulla. Elevated body temperature failed to prolong the LCR in one piglet, which was excluded from analysis. Elevated body temperature prolonged the LCR in all the remaining animals, and dialysis of gabazine into the region near the NTS (n = 10) reversed the thermal prolongation of the LCR even though body temperature remained elevated. Dialysis of gabazine in other medullary sites (n = 10) did not reverse thermal prolongation of the LCR. Gabazine had no consistent effect on baseline respiratory activity during hyperthermia. These findings are consistent with the hypothesis that hyperthermia activates GABAergic mechanisms in or near the NTS that are necessary for the thermal prolongation of the LCR.

sudden infant death syndrome; laryngeal chemoreflex; hyperthermia; gamma-aminobutyric acid; gabazine; microdialysis; nucleus of the solitary tract

ACCORDING TO THE TRIPLE RISK MODEL of sudden infant death syndrome (SIDS) (11), infants who die of SIDS have some underlying vulnerability, probably related to reduced numbers or affinity of receptors for serotonin (21, 38, 39), glutamate (37), and/or acetylcholine (20), and they are exposed to some exogenous stressor(s) during a critical period of development (the peak incidence of SIDS is between 2 and 6 mo of age). Many putative stressors have been identified from epidemiological studies of SIDS infants, and we recently examined two interacting stressors, the laryngeal chemoreflex (LCR) and hyperthermia. The LCR is elicited when fluids with a low chloride content or low pH stimulate receptors in the laryngeal mucosa with afferents in the superior laryngeal nerve (SLN) (4, 9, 24). The reflex is a complex behavioral response that consists of apnea and respiratory inhibition, redistribution of blood flow to vital organs (12), as well as airway clearance mechanisms such as coughing and swallowing (45, 47). The respiratory inhibition associated with the LCR is prominent in newborns, including normal infants (45). Many investigators have suggested that the LCR may begin a process that starts with apnea and leads to SIDS in a small number of infants who possess some critical vulnerability (9, 11, 25, 36, 46).

Environmental hyperthermia and thermal stress are also well recognized risk factors for SIDS (13, 41, 43). Many of the features of the LCR can be elicited by electrical stimulation of the SLN (8), and the threshold of such electrical stimulation necessary to elicit the laryngeal adductor reflex was reduced as body temperature was elevated in puppies (14). The thermal reduction in threshold waned as animal age increased from the newborn period to adulthood, and there was little or no effect of temperature on the threshold of the laryngeal adductor reflex in adult dogs. More recently, we found that elevating the body temperature of decerebrate neonatal piglets markedly prolonged the apnea and respiratory disruption associated with the LCR (7). This effect of hyperthermia is centrally mediated since there was no temperature-dependence of laryngeal water receptor activity measured from single fibers in the SLN over the body temperature range from 38.3 to 40.6°C (49). Moreover, elevating the temperature in or adjacent to the nucleus of the solitary tract (NTS), while body temperature was held constant at the normal level, was sufficient to prolong the LCR (50).

Reflex apnea following electrical stimulation of the SLN seems to depend on GABAergic mechanisms within the medulla since systemic or intracisternal administration of bicuculline, a GABA<sub>A</sub> receptor antagonist, shortened apnea duration after SLN stimulation in piglets (1). Systemic administration of GABA<sub>A</sub> receptor antagonists also blocked the thermal prolongation of reflex apnea after SLN stimulation (5). We have not determined where in the central nervous system the GABAergic processes mediating thermal prolongation of the LCR reside. Unilateral heating of the region of the NTS, without elevating brain temperature in other regions of the medulla, prolonged the LCR, suggesting that thermally sensitive GABAergic processes within the NTS might be sufficient to mediate the thermal prolongation (50). To test this hypoth-
esis, we used unilateral focal dialysis of gabazine, a GABA<sub>A</sub> receptor antagonist, to block GABAergic activity in discrete regions of the medulla. It was our expectation that blocking GABA<sub>A</sub> receptors in the NTS would abrogate the thermal prolongation of the LCR but that blocking GABA<sub>A</sub> receptors elsewhere in the medulla would not.

**METHODS**

Experiments were performed on 21 piglets ranging in age from 3 to 13 days (7.7 ± 0.7 days; mean ± SE) with an average weight of 3.0 ± 0.2 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) in O<sub>2</sub>. A rectal probe was inserted, and body temperature was maintained between 37 and 38°C using a heating pad. Femoral arterial and venous catheters were inserted to measure blood pressure and administer drugs, respectively. Each animal was tracheostomized and artificially ventilated (Harvard Apparatus Dual Phase Respirator, South Natick, MA) to maintain the end-tidal CO<sub>2</sub> concentration at ~5%. The carotid sinus regions were exposed bilaterally, and 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. The animal was placed prone, and the head was positioned in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). The skull was opened, and the animal was decerebrated at the level of the superior colliculi. All brain tissue rostral to the section was removed by suction. Following decerebration, halothane anesthesia was discontinued, and each animal was paralyzed using pancuronium bromide (1 mg/kg iv; Elkins-Sinn, Cherry Hill, NJ). Supplemental doses of pancuronium were given as required, usually at a rate of 0.5 mg·kg<sup>−1</sup>·h<sup>−1</sup>. 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 CO<sub>2</sub>, and blood pressure were recorded on a computer (PowerLab, ADI) for later analysis.

A dialysis probe was placed through a guide tube in the midline on the left side of the medulla from the dorsal surface of the medulla, which was exposed as part of the preparation for decerebration. The probe was positioned by using visual landmarks on the dorsal surface (the obex primarily), the lateral distance from the midline and an estimate of the depth of the target within the medulla based on previous studies in piglets (35). Throughout each experiment, the medullary site was dialyzed at a rate of 8.5 μl/min with an artificial cerebrospinal fluid (aCSF) solution equilibrated with 5% CO<sub>2</sub>/pH 7.4 (this dialysis flow rate caused no volume transduction of fluid across the dialysis membrane). The aCSF had the following composition (in mM): 152 sodium, 3.0 potassium, 2.1 magnesium, 2.2 calcium, 131 chloride, 26 bicarbonate, and 5 dextrose. At least 60 min elapsed after placement and perfusion of the dialysis probe before any tests of the LCR were performed.

To stimulate the LCR, 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 very near body temperature. The larynx was suctioned periodically as needed. At least 10 min elapsed between tests of the LCR, and the LCR was not tested unless phrenic respiratory activity was stable.

**Experimental protocols.** Studies began with a control period during which the body temperature was held at 37–38°C, and the LCR was elicited three times during dialysis with aCSF. Next, the body temperature was elevated ~2.5°C by warming the heating pad. Once the body temperature reached a stable elevated temperature, the LCR was stimulated three more times. At this point in the protocol, the dialysis was switched from aCSF alone to aCSF containing gabazine. We varied the concentration of gabazine between 2 and 5 mM but saw no differences in responses among these doses. As a result, the majority of animals (13/21 animals) received 2.5 mM gabazine in the dialysate. Dialysis with gabazine was given for 30 min, and testing of the LCR began no sooner than 10 min after the dialysate containing gabazine began perfusing the medulla. After 30 min of gabazine dialysis, the dialysate was changed back to aCSF for the remainder of the experiment. The animal’s temperature remained elevated during and after the dialysis with gabazine until three tests of the LCR were completed. Subsequently, the animal was cooled by swabbing it with isopropyl alcohol until the body temperature was reduced to the control value, and the LCR was stimulated a final three times in this follow-up normothermic period. Thus the majority of hyperthermic and normothermic tests of the LCR after gabazine dialysis were performed while the animal was paralyzed using pancuronium bromide.

**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 intravenously. Microinjections of 20–50 μl of 1% potassium permanganate were made into the medulla through a broken microdialysis probe passed through the microdialysis guide tube to mark the location of the tip of the probe and site of dialysis. The permanganate formed an insoluble tar, which was not removed during subsequent tissue processing (44). The brain stem was removed from the animal, placed in cryo-embedding medium (Tissue-Tek OCT 458, Sakura Finetek, Torrance, CA), and frozen in isopentane at ~70°C. Brain stems were sectioned (50 μm) in a cryostat at ~18°C, and sections were mounted on gelatinized glass slides, fixed over night in 10% formalin in phosphate-buffered saline (pH 7.0), and stained with cresyl violet (2, 27). We expressed the location of each probe using three dimensions in millimeters: a mediolateral dimension (midline = 0), a dorsoventral dimension (dorsal surface of the medulla = 0), and a rostrocaudal dimension (obex = 0; rostral, positive; caudal, negative).

**Data analysis and statistics.** We defined the duration of respiratory disruption by 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 (7, 47, 50). The respiratory disruption measured in this way included both periods of unstable respiratory activity and apneas. To avoid bias in the measurement of the LCR, we kept the definition simple and applied it consistently across all animals. In addition, we measured apnea duration, which is less subject to interpretation. Apnea was defined as a cessation of breathing greater than the duration of the two breaths preceding the breath during which the stimulus was delivered. Apnea did not occur in all tests of the LCR; therefore, we varied both the LCR and apnea durations provided a more complete analysis of the response. 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.

We analyzed these experiments using a three-way repeated-measures ANOVA (SYSTAT 9.0, SPSS, Chicago, IL). The average response from each animal in each set of test conditions was used in this analysis. The location of the dialysis probe (the NTS region or not near the NTS region) was a between-subjects factor, and treatment with gabazine (control vs. drug) and temperature (control vs. hyper-
GABAZINE BLOCKS THERMAL PROLONGATION OF THE LCR

Results

Examples of the responses of integrated phrenic nerve activity taken from two piglets during each of the experimental treatments are shown in Fig. 1. The left panel shows results from a piglet with the dialysis probe in the NTS just rostral to the obex, and the right panel shows data from a piglet with the dialysis probe ventral to the NTS at the level of the facial nucleus (see Fig. 2). In the control condition in the piglet with the dialysis probe in the NTS, body temperature was 38.6°C, and introducing 0.1 ml of water into the larynx (arrow) caused brief apnea, but regular respiratory activity was quickly restored. After elevating the animal’s body temperature to 41°C, 0.1 ml of water injected into the larynx elicited a much longer apnea (~9 s long) before regular respiratory activity resumed. After gabazine dialysis (2.0 mM), the LCR was tested, and the apnea duration was once again relatively short, despite the fact that the rectal temperature remained elevated at 40.9°C. When body temperature was reduced to the control level (38.0°C), the response to stimulation of the LCR after gabazine dialysis was similar to the initial control condition at the control body temperature before hyperthermia and also similar to the apnea duration and respiratory disruption during hyperthermia after gabazine dialysis, but still much shorter than the LCR response during hyperthermia and dialysis of aCSF alone. In the piglet represented by the data in the right set of panels, note that hyperthermia prolonged the LCR (just as in the left set of panels), but gabazine treatment did not reverse the thermal prolongation of the LCR. Even after restoration of normothermia, the preceding treatment with gabazine outside the NTS seemed to prolong the LCR compared with the initial normothermic condition.

We compared the average responses of animals with the dialysis probe in the region of the NTS to all the other animals. To make the test of the hypothesis that the GABAergic prolongation of the LCR originates in or near the NTS more robust, we used a liberal definition of the area of the NTS. This was done for two reasons. First, we do not know exactly how far the dialysate diffuses from the tip of the dialysis probe, and the region of diffusion can be large and irregular (6, 32). Moreover, dialysate may diffuse preferentially along the dialysis probe and probe track. Therefore, dialysis probes in the region of the NTS or probe tips that were ventral to the NTS, but passed through the NTS, may still have affected neurons within the NTS. Second, by defining the NTS as the NTS and surrounding tissue, we enhance the stringency of our analysis; we increased the likelihood of including sites that were not involved in the thermal prolongation of the LCR, and we reduce the likelihood of any retrospective selection bias by the investigators based on the response to treatment. Using a liberal definition of the region of the NTS, we found that 11 animals had probes in the region of the NTS (circles; Fig. 2) and that 10 animals had probes outside the NTS (squares). Results from one animal with the probe in the NTS were rejected from analysis because this animal demonstrated no prolongation of the LCR during hyperthermia alone (marked by x in Fig. 2) and, therefore, had no thermal prolongation of
the LCR against which to compare the effect of gabazine. In the remaining 20 piglets, elevating body temperature \( \pm 2.5°C \) consistently prolonged the LCR regardless of the location of the dialysis probe, and the location of the probe had no effect on the magnitude of the thermal prolongation of the LCR in the absence of gabazine.

The average responses of body temperature, the duration of apnea, and the duration of respiratory disturbance after eliciting the LCR are shown in Fig. 3 for animals with probes in the region of the NTS (left) and all other locations (right). There was a significant three-way interaction for both apnea duration \( (P = 0.025) \) and the duration of respiratory disruption \( (P = 0.002) \), indicating that the pattern of responses to gabazine treatment and changing body temperature differed significantly in the two anatomical groups. To identify the specific differences, we made two sets of comparisons: we compared each treatment combination (body temperature and dialysate) to the control condition (normothermia and aCSF dialysis), and we compared each treatment combination sequentially to detect changes in the response as the combined treatments evolved.

Apnea duration was significantly prolonged by elevating body temperature an average of \( 2.8 \pm 0.1°C \) in the piglets with the probes in the region of the NTS \( (P < 0.05) \). After gabazine was added to the dialysate, apnea duration fell significantly compared with hyperthermia alone \( (P < 0.05) \) in those animals with the dialysis probe in the region of the NTS, and the apnea duration during hyperthermia and gabazine dialysis was not different from the initial control condition, even though body temperature remained elevated. In the final condition (after treatment with gabazine dialysis but during normothermia), apnea duration was not different from the first control response and also not different from the preceding hyperthermic response during gabazine dialysis. An exactly similar pattern was seen in terms of the duration of LCR: the LCR was significantly prolonged during hyperthermia and gabazine dialysis compared with the initial control condition \( (P < 0.05) \), but the LCR was not different from the normothermic control value during gabazine dialysis and hyperthermia: gabazine dialysis in the region of the NTS completely blocked the thermal prolongation of the LCR. In the final treatment condition, the LCR duration during normothermia after gabazine dialysis was not different from the initial control condition and not different from the preceding hyperthermic condition during gabazine dialysis.

The pattern of responses was quite different in the animals in which the dialysis probes were not in the region of the NTS.
Apnea duration was significantly prolonged by elevating body temperature an average of 2.8 ± 0.1°C in the piglets with the probes outside the NTS ($P < 0.05$). After gabazine was added to the dialysate, apnea duration actually increased significantly compared with hyperthermia and aCSF dialysis ($P < 0.05$), and the apnea duration during hyperthermia and gabazine dialysis was also significantly longer than the initial duration in the initial control condition ($P < 0.05$). In the final condition after treatment with gabazine dialysis during normothermia, apnea duration was significantly less than during hyperthermia and gabazine dialysis ($P < 0.05$) but also significantly greater than the initial normothermic control condition ($P < 0.05$).

Thus the apnea length did not return to the initial control level in the non-NTS dialysis group as it did in the NTS group. A similar pattern occurred for the duration of respiratory disruption after stimulating the LCR: the LCR was significantly prolonged during hyperthermia and aCSF dialysis ($P < 0.05$) and remained significantly elevated during hyperthermia after gabazine dialysis compared with the initial control value ($P < 0.05$). The LCR duration during hyperthermia after gabazine treatment was slightly prolonged compared with hyperthermia and aCSF dialysis, but this difference was not statistically significant ($P = 0.35$). Finally, the LCR duration fell when the animals were made normothermic after gabazine dialysis, and the LCR duration was significantly less than during hyperthermia after gabazine dialysis ($P < 0.05$) but also significantly longer than during the initial normothermic control aCSF dialysis condition ($P < 0.05$).

Despite differences in the response of apnea and the LCR duration, gabazine had no effect on the body temperature. The only significant outcome of the ANOVA on body temperature was a main effect of hyperthermia (there was no interaction between the site of dialysis groups and any other factor in the ANOVA, which indicates that the pattern and magnitude of temperature change was similar and well controlled among all the experimental animals). Thus body temperature rose significantly during hyperthermia in all the animals ($P < 0.05$) and fell significantly when the animals were cooled in the final condition ($P < 0.05$).

Respiratory responses to gabazine and hyperthermia. The duration of the LCR is modified by the level of respiratory drive. Interventions that increase the drive to breathe often shorten the duration of the LCR; for example, increased levels of inspired CO$_2$ significantly shorten the duration of reflex apnea after SLN stimulation (23). Therefore, we determined whether respiratory activity was altered by hyperthermia and/or gabazine treatment to determine whether the effect of gabazine was really specific to the LCR or a manifestation of a more general effect of hyperthermia or gabazine on respiratory activity. We analyzed peak integrated phrenic nerve activity, respiratory frequency, and minute phrenic activity, as shown in Table 1. The end-tidal CO$_2$ level, which we controlled by adjusting the ventilator volume and frequency, is also shown to confirm that the respiratory stimulus was stable. There were some changes in respiratory activity, but with the exception of respiratory frequency, these did not vary as a consistent function of the hyperthermic or gabazine treatments. Moreover, there were no differences in these responses between the anatomical groupings based on the placement of the dialysis probes in striking contrast to the anatomically divergent responses of the LCR and apnea durations during hyperthermia and gabazine dialysis. Peak integrated phrenic nerve activity fell in all animals after the initial normothermic condition ($P < 0.05$). Respiratory frequency increased significantly in all animals during hyperthermia ($P < 0.05$), and frequency remained elevated after gabazine dialysis in all animals regardless of the site of dialysis. Respiratory frequency fell to the initial control level once body temperature was cooled to the initial level in all animals. Minute phrenic nerve activity was significantly reduced in all animals in the final condition (normothermia after gabazine dialysis), but the small reductions in peak integrated phrenic nerve activity and respiratory frequency that brought about this change in minute activity were not statistically significant.

Anatomical assessment of GABAergic-mediation of thermal prolongation of the LCR. An alternative way to assess the association of a given area of the medulla with a particular response to treatment is to define responsiveness quantitatively and then determine statistically if the magnitude of the response is significantly associated with any particular location. We defined gabazine responsiveness during hyperthermia as the ratio of the LCR duration during hyperthermia and dialysis.
with gabazine to the LCR duration during hyperthermia and wasCSF dialysis (the results were identical when apnea duration was used to define responsiveness). The three anatomical coordinates for all animals were regressed on gabazine responsiveness during hyperthermia in a stepwise fashion, adding each anatomical direction in all possible orders. More caudal and dorsal locations were significantly associated with reductions in the LCR during hyperthermia after gabazine dialysis (the average reduction in LCR duration during hyperthermia was ~65%), whereas more rostral and ventral locations were significantly associated with a slight prolongation of the LCR during hyperthermia after gabazine dialysis (the LCR was on average ~6% longer during hyperthermia after gabazine dialysis). However, no combination of these individual terms and interaction between these terms improved the fit of the model beyond the correlation that was available from each of these terms alone. This indicates that those dialysis locations that were caudal and dorsal were associated with significant shortening of the LCR duration after gabazine dialysis during hyperthermia, and rostral and ventral areas were associated with enhancement of the thermal effect on the LCR duration. However, there was insufficient independent variation among the dorsal and caudal dimensions to assess the separate contribution of these dimensions to the response to gabazine dialysis during hyperthermia. The lateral dimension relative to the midline was not correlated with gabazine responsiveness.

**DISCUSSION**

The main finding of this study is that gabazine dialysis in the dorsal medulla in or adjacent to the NTS reversed thermal prolongation of the LCR in decerebrate piglets even though body temperature remained elevated, and other aspects of respiratory activity that we measured were not consistently altered by gabazine dialysis during hyperthermia. In addition, dialysis of gabazine in regions of the medulla other than the NTS did not shorten the duration of apnea or the duration of the LCR during hyperthermia. Thus a thermally sensitive GABAergic process within or near the NTS seems to be necessary to prolong the LCR during hyperthermia in decerebrate neonatal piglets.

**The circuitry of the LCR and thermal prolongation of the LCR.** The afferent limb of the LCR originates from nerve endings in the larynx that are sensitive to water and other liquids. Afferent sensory information from the larynx is conducted to the central nervous system in the SLN, and the first synapse within the central nervous system occurs in the NTS (15, 40). Secondary interneurons process and distribute sensory information from the larynx throughout the brain stem. Electrical stimulation of the SLN or stimulation of the laryngeal mucosa with water inhibited inspiratory neurons and inspiratory neurons in the ventral respiratory group and stimulated postinspiratory neurons (8, 42). Stimulation of the SLN seemed to prolong expiratory time by preventing inhibition of postinspiratory neurons in the ventral respiratory group; these neurons were depolarized in a state labeled “postinspiratory apneusis” (42), and the transition between the depolarized “apneustic” state in postinspiratory neurons early in expiration and their hyperpolarized state during late expiration seemed to require the activity of chloride-dependent inhibitory postsynaptic potentials (8, 42). Reflex prolongation of the postsynaptic period after SLN stimulation was associated with the suppression or absence of inhibitory postsynaptic potentials that usually hyperpolarized postinspiratory neurons in the ventral respiratory group, terminated the effect of SLN stimulation and prepared the way for the final phase of expiration and the next inspiration. Thus ventral medullary sites seem to mediate the effects of SLN stimulation on the LCR during normothermic conditions.

The effects of hyperthermia, on the other hand, may be mediated by direct effects of hyperthermia on neurons within the dorsal medulla or by specific temperature-sensitive neurons. Any direct effects of hyperthermia on primary or secondary sensory neurons processing information from the larynx must reside within the NTS, since isolated heating of the region of the NTS was sufficient to elicit the entire phenomenon of thermal prolongation of the LCR. Thermal prolongation does not seem to originate within the ventral respiratory group despite the evidence that the efferent control of phrenic activity seems to require some processing within this group of neurons (8, 42). There are thermally sensitive neurons (both hot and cold sensitive cells) within the dorsal medulla in the region of the NTS in rabbits (18), and a plausible alternative is that the thermal effects on the LCR that we observed may be mediated by these temperature-sensitive neurons, which in turn change and probably enhance the excitability of those neurons within the NTS that integrate laryngeal afferent information so that the inhibitory potency of this information is enhanced and the LCR is prolonged. Whatever the neuronal source of the thermal information, the inhibitory circuitry responsible for the

**Table 1. Peak integrated phrenic nerve activity, respiratory frequency, minute activity, and the end-tidal CO₂ as a function of treatment condition**

<table>
<thead>
<tr>
<th>Dialysate Located</th>
<th>aCSF Gabazine Treatment</th>
<th>aCSF Gabazine Treatment</th>
<th>aCSF Gabazine Treatment</th>
<th>aCSF Gabazine Treatment</th>
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<tbody>
<tr>
<td>In or Near the NTS (n = 10)</td>
<td>Hyperthermia</td>
<td>Control</td>
<td>Hyperthermia</td>
<td>Control</td>
</tr>
<tr>
<td>Integrated phrenic activity, arbitrary units</td>
<td>3.4±0.4</td>
<td>2.6±0.3†</td>
<td>2.9±0.5</td>
<td>2.8±0.4</td>
</tr>
<tr>
<td>Respiratory frequency, breaths/min</td>
<td>36.7±2.3</td>
<td>46.0±5.2*</td>
<td>49.4±5.2*</td>
<td>39.0±4.8</td>
</tr>
<tr>
<td>Minute activity, arbitrary units/min</td>
<td>125.1±20.3</td>
<td>116.3±21.0</td>
<td>139.7±24.9</td>
<td>105.3±17.5</td>
</tr>
<tr>
<td>end-tidal CO₂, %</td>
<td>5.3±0.1</td>
<td>5.2±0.1</td>
<td>5.3±0.1</td>
<td>5.3±0.1</td>
</tr>
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</table>

Values are means ± SE. There were no interactions between any of these variables and the dialysis probe location. Therefore, all the statistical marks indicate main effects; even though the statistical marks are present on both left and right panels of the table for ease of viewing, they represent the results of a pooled analysis of all 20 animals. NTS, nucleus of the solitary tract; aCSF, artificial cerebrospinal fluid. *Significant main effect of hyperthermia on respiratory frequency both with and without gabazine dialysis (P < 0.05). †Significant reduction in integrated phrenic activity between the control and initial hyperthermic conditions (P < 0.05). ‡Significant reduction in minute phrenic activity in the final condition compared to the initial control condition (P < 0.05).
thermal prolongation of the LCR seems to be completely integrated within the NTS or in the volume of tissue just surrounding the NTS.

Focal dialysis of gabazine in the ventral regions of the medulla significantly accentuated the thermal prolongation of apnea and slightly prolonged the duration of the LCR. Moreover, when these animals were made normothermic after gabazine dialysis (and we believe that the effect of gabazine persisted in this condition), the LCR and apnea durations were both significantly prolonged. It seemed that there was a gabazine effect in the ventral medulla that was present whether the animal was hyperthermic or not. This finding is not consistent with previous studies in which cisternally perfused or systemically administered GABA_A receptor antagonists shortened the duration of reflex apnea after SLN stimulation (1, 5). That divergent responses should originate from studies using focal dialysis as opposed to treatments that affect the entire brain stem should not be too surprising; the volumes of tissue affected by cisternal perfusion or systemic administration and focal dialysis are probably orders of magnitude different. More importantly, the diversity of effects of GABA_A receptor antagonists suggests that there are at least two separate neuronal processes involved in controlling the LCR. The thermal prolongation of the LCR seems to depend on GABA_A receptors in the dorsal medulla in or near the NTS, and GABA_A receptors in this area participate in the thermal prolongation of the LCR, but possibly not other aspects of the LCR (since the normothermic apnea and LCR durations were not different from the initial normothermic control condition). By contrast, normothermic control of the LCR seems to involve ventral GABAergic processes (8, 42, 47). Moreover, gabazine dialysis outside the NTS actually seemed to prolong the LCR in both normothermic and hyperthermic conditions. The supposition that at least two separate sites control the hyperthermic and normothermic aspects of the LCR also receives some support from recent work in rat pups in which we found that the maturational decline in the potency of thermal prolongation of the LCR differed from the maturational pattern of apnea duration elicited during stimulation of the LCR (Xia L, Leiter JC, Bartlett DJr, unpublished observations). Even within the ventral medulla, there may be multiple sites of GABAergic action since the responses to focal dialysis in the ventral medulla differ significantly from the responses to cisternal and systemic administration of GABA_A receptor antagonists. The thermal effect on the LCR was reduced in 1 of 10 animals with dialysis probes outside the region of the NTS. However, the LCR duration during hypercapnia was shortened by ~29% in this animal after gabazine dialysis. In contrast, the average reduction in the LCR duration after gabazine dialysis in animals with dialysis probes in or near the NTS was ~65%. Thus the gabazine “response” of this single animal with a ventrally located dialysis probe probably represents simple stochastic variation in the duration of the LCR, but it may point to the existence of GABAergic sites distant from the NTS that also modulate the thermal prolongation of the LCR.

The site of action of gabazine on the thermal prolongation of the LCR seemed to be in or adjacent to the NTS, but the dialysates diffuse over surprisingly large and unpredictable distances (6, 32). Therefore, we are unable to define a truly discrete site of action of gabazine in the dorsal medulla: our power to discriminate among sites is poor, and the response may actually originate from a fairly diffuse volume of tissue in the dorsal medulla. We can only say that the GABAergic processes necessary for thermal prolongation of the LCR are in the caudal and dorsal medulla near the NTS. This less restrictive definition of the site of action of gabazine is consistent with the regression analysis of dialysis probe tip coordinates as well. We are better able to say that the rostral and ventral areas of the medulla do not contain the GABAergic processes that mediate the thermal prolongation of the LCR since the LCR was, if anything, prolonged by gabazine dialysis in areas outside the NTS compared with hyperthermia during aCSF dialysis in these sites (Fig. 2, bottom). Finally, the fact that unilateral microdialysis disrupted a process that is presumably represented bilaterally in the medulla indicates that the reflex response to heating and water in the larynx is dependent on neural activity that is to a considerable extent integrated by both sides of the medulla. Unilateral disruption of the thermal prolongation of the LCR implies that GABAergic activity may be present anatomically on both sides of the medulla, but these GABAergic neurons function as a single interdependent circuit susceptible to disruption at any single anatomical point within the circuit.

Enhancing or blunting respiratory drive by elevating the inspired CO_2 or lesioning or inhibiting central chemosensory regions may shorten or lengthen the LCR (23, 26, 34, 47). Hyperthermia did increase the respiratory frequency, but the LCR was prolonged despite the increase in respiratory frequency and the increase in respiratory drive this may reflect. Furthermore, gabazine dialysis did not reduce the respiratory frequency during hyperthermia but did reduce the duration of the LCR when dialyzed into the NTS and surrounding tissue. Thus the thermal prolongation of the LCR and the shortening of the LCR after blocking GABA_A receptors in the region of the NTS cannot be attributed to nonspecific changes in respiratory drive.

We do not know how GABAergic mechanisms within or adjacent to the NTS actually modulate the characteristics of the LCR, but we imagine that increasing the temperature of neurons in this region may increase, through a GABAergic mechanism, the activity of interneurons within or adjacent to the NTS that increase the duration and strength of the LCR by altering the activity patterns of inspiratory, postsynaptic, and expiratory neurons within the ventral respiratory group of neurons. This proposed circuit is quite similar to the putative organization of the Hering-Breuer reflex, in whichafferent pulmonary stretch receptor information is also integrated within the NTS. The central processing of the Hering-Breuer reflex relies on “pump cells,” second-order relay neurons within the NTS that are largely GABAergic and to a lesser extent glycinegic (10). These inhibitory pump cells project to a variety of neurons within the ventral respiratory group that control the duration and depth of each breath (22). It is interesting to note that the inspiratory inhibitory Hering-Breuer reflex was also prolonged in rodents when body temperature was elevated (30, 31). In addition, some laryngeal afferents project directly to pump cells. It seems possible, therefore, that pump cells also receive excitatory thermal inputs. It will be interesting to determine whether the LCR and the inspiratory Hering-Breuer reflex, both of which are inhibitory and prolong expiration, share a common pathway to control the neural activity of the ventral respiratory group neurons.
**Limitations of the methods.** As we have noted before (7, 50), the nature of the decerebrate preparation is a major limitation of our studies. Thermoregulatory processes originating above the brain stem are not present in decerebrate animals, and effective thermoregulation might limit the effects of brain heating and reduce the thermal prolongation of the LCR that we observed. Moreover, inputs from the midbrain and cortex provide an excitatory drive that may blunt the prolongation of the LCR that we observed. The usual airway clearance mechanisms that remove the water from the larynx and airway, such as swallowing and coughing, are limited or absent in the decerebrate, paralyzed animal, and the animals were tracheostomized. Thus the stimulus of the LCR may remain in the larynx longer than in intact animals. The animals in our study were mechanically ventilated, which prevented the development of hypoxia and hypercapnia during the LCR, and both of these stimuli may shorten the duration of the LCR in intact animals (23, 47, 48). Nonetheless, studies in decerebrate animals do reveal the direction of change in the LCR, and the central mechanisms within the medulla responsible for the thermal prolongation of the LCR are still accurately represented. Finally, there are other manifestations of the LCR that we did not examine. For example, we examined only phrenic nerve activity; it is difficult to examine hypoglossal activity or other aspects of upper airway control in decerebrate tracheostomized neonatal piglets since they have little apparent hypoglossal activity. We do not know whether hyperthermia may alter the cardiovascular aspects of the LCR or other aspects of upper airway muscle activation during the LCR since we studied only phrenic nerve activity.

**Thermal stresses and the LCR: implications for SIDS.** We have discussed the implications of the thermal prolongation of the LCR in the pathogenesis of SIDS previously (7, 50). The current study emphasizes once again that interactions among stressors such as the LCR and hyperthermia may cause significant respiratory inhibition. It is entirely plausible, therefore, that the LCR may initiate apneas that start the chain of events leading to SIDS, as many previous investigators have suggested (24, 25, 29, 36, 46). Many previous studies have focused on the importance of excitatory respiratory reflexes (e.g., ventilatory responses to hypoxia and hypercapnia) in the pathogenesis of SIDS (16, 17), and the results of these previous studies suggest that reduced excitatory reflex response may predispose infants to SIDS. The current study emphasizes that increased potency of inhibitory reflexes, whether enhanced as a result of inborn differences in reflex potency or as a result of environmental factors, may also increase the likelihood of SIDS.

Our studies of the LCR and hyperthermia also emphasize the importance of interactions among risk factors for SIDS. These interactions are consistent with the triple risk model of SIDS and indicate that combinations of rare events may create a lethal “perfect storm” from individual factors that are probably not life-threatening when each of them occurs alone. Implicit in this perfect storm analogy is the idea that the ultimate causes and processes of SIDS will be identified only from studies in which multiple facets of neonatal physiology and development are studied simultaneously.

The current study also points out the need for continued studies of human infants who died of SIDS. There is no evidence that GABAergic neurotransmission is abnormal in babies who die of SIDS; there have been no studies, for example, of GABAergic receptors analogous to the studies of serotonin receptors in the brains of human infants who died of SIDS (39). On the other hand, maternal smoking, which is a significant risk factor for SIDS (3, 33), may increase the expression of GABA receptors or increase the potency of GABAergic activity. For example, prenatal nicotine exposure in rats enhances inhibitory neurotransmission in respiratory-related areas of the medulla (28). It would be interesting to determine in future studies of human infants who died of SIDS whether inhibitory neurotransmitter mechanisms are also abnormal in these babies and whether neurotransmitter receptor abnormalities exist in or adjacent to the NTS.

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**REFERENCES**
