Arousal from sleep in response to intermittent hypoxia in rat pups is modulated by medullary raphe GABAergic mechanisms

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Darnall RA, Schneider RW, Tobia CM, Zemel BM. Arousal from sleep in response to intermittent hypoxia in rat pups is modulated by medullary raphe GABAergic mechanisms. Am J Physiol Regul Integr Comp Physiol 302: R551–R560, 2012. First published December 7, 2011; doi:10.1152/ajpregu.00506.2011.—Arousal is an important defense against hypoxia during sleep. Rat pups exhibit progressive arousal impairment (habituation) with multiple hypoxia exposures. The mechanisms are unknown. The medullary raphe (MR) is involved in autonomic functions, including sleep, and receives abundant GABAergic inputs. We hypothesized that inhibiting MR neurons with muscimol, a GABA_A receptor agonist, or preventing GABA reuptake with nipecotic acid, would impair arousal and enhance arousal habituation and that blocking GABA_A receptors with bicuculline would enhance arousal and attenuate habituation. Postnatal day 15 (P15) to P25 rat pups were briefly anesthetized, and microinjections with aCSF, muscimol, bicuculline, or nipecotic acid were made into the MR. After a ~30-min recovery, pups were exposed to four 3-min episodes of hypoxia separated by 6 min of normoxia. The time to arousal from the onset of hypoxia (latency) was determined for each trial. Latency progressively increased across trials (habituation) in all groups. The overall latency was greater after muscimol and nipecotic acid compared with aCSF, bicuculline, or noninjected controls. Arousal habituation was reduced after bicuculline compared with aCSF, muscimol, nipecotic acid, or noninjected controls. Increases in latency were mirrored by decreases in chamber [O_2] and oxyhemoglobin saturation. Heart rate increased during hypoxia and was greatest in muscimol-injected pups. Our results indicate that the MR plays an important, not previously described, role in arousal and arousal habituation during hypoxia and that these phenomena are modulated by GABAergic mechanisms. Arousal habituation may contribute to sudden infant death syndrome, which is associated with MR serotoninergic and GABAergic receptor dysfunction.

γ-aminobutyric acid; sudden infant death syndrome; GABA_A receptor; habituation

IN HUMAN INFANTS, AROUSAL is an important protective mechanism against hypoxia during sleep. Spontaneous arousals and those in response to exogenous stimuli are affected by many factors: sleep position (3, 29, 37), sleep efficiency (30), prenatal exposure to cigarette smoking (28, 38), and stage of development (39). It has been hypothesized that arousal impairment may play an important role in the etiology of sudden infant death syndrome (SIDS) (32, 40, 43, 46). Moreover, many SIDS infants have repeated episodes of apnea and hypoxia in the days or weeks prior to death (44, 56). We propose that repeated exposure to hypoxia during sleep leads to progressive lengthening of the time to arousal in response to hypoxia that might contribute to sudden death.

We and others have shown in newborn and infant rodents (17, 18, 21), lambs (25, 41, 42), and piglets (71) that repeated brief exposures to mild hypoxia result in a progressive lengthening of the time to arousal with each successive hypoxia exposure. A similar phenomenon has been described in human infants in response to repeated tactile (55) and auditory (47) stimuli. The phenomenon of progressive lengthening of the time to arousal with repeated exposures to hypoxia has been referred to as “habituation” by some investigators and refers to the waning over time of a physiological response to repetitive stimuli. The concept of “habituation” to a hypoxic stimulus has been discussed in detail previously (17).

We have previously hypothesized that arousal “habituation” could be explained by hypoxia-induced biochemical processes with relatively long time constants, resulting in arousal inhibition, such that with brief alternating periods of hypoxia and normoxia, there is lingering inhibition between exposures resulting in a progressive cumulative inhibitory process (17). Brain levels of GABA, adenosine, glycine, and opiates all increase during hypoxia and are potential candidate inhibitory neuromodulators. There is little or no information linking these substances with arousal from sleep in response to hypoxia. One study in newborn lambs demonstrated that blockade of opioid receptors did not reverse the progressive lengthening of the time to arousal in response to repeated hypoxia exposures (48). Adenosine is released from cells into the extracellular space during hypoxia when oxygen needs no longer match oxygen supply (16) and activation of both GABA_A and serotonin 1A (5-HT_1A) receptors in this region disrupts sleep and promotes wakefulness (11, 13, 15, 34).

Our major objective was to determine whether the time to arousal in response to hypoxia and/or its progressive lengthening (habituation) in response to repeated exposure to hypoxia are mediated or modulated by medullary raphe GABAergic mechanisms. We focused on the medullary raphe because this region has been implicated in the pathogenesis of SIDS (46, 60). To determine whether the medullary raphe contributes to arousal during hypoxia in P15 and P25 rat pups, we locally inhibited medullary raphe neuronal activity with muscimol, a GABA_A receptor agonist and then exposed them to four brief periods of hypoxia separated by normoxia. To determine the role of endogenous GABA in arousal, we locally blocked GABA_A receptors in the medullary raphe with bicuculline and blocked reuptake of GABA with nipecotic acid before arousal testing. We hypothesized that in P15 and P25 rat pups, activation of medullary raphe GABA_A receptors with muscimol or blocking GABA transporters with nipecotic acid would delay arousal and increase arousal habituation and that blocking GABA_A receptors with bicuculline would enhance arousal and reduce arousal habituation.

MATERIALS AND METHODS

Subjects. All procedures were approved by the Animal Care and Use Committee of Dartmouth College. Litters bred in-house to

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AROUSAL DURING HYPOXIA IS MODULATED BY RAPHE GABAERGIC MECHANISMS

Sprague-Dawley rats (Harlan Laboratories) were housed in standard cages in the Dartmouth College animal facility with standard light-dark cycling (7 AM–7 PM). The day of birth was logged as postnatal day 0 (P0). Rat pups were studied at two developmental time points, P15 ± 2 or P25 ± 2. Cages were brought to the laboratory for experiments between 9 AM and 5 PM.

Surgery. Arousal to hypoxia was tested after a surgical microinjection procedure. Pups were anesthetized with isoflurane (in O2) (induction 3%; maintenance 2%) and placed in a neonatal rat adapter (World Precision Instruments) on a stereotaxic frame (Kopf). Body temperature (Tb) was maintained with a heating pad and lamp. The skull was leveled between bregma and lambda, lidocaine (2% jelly) was applied to the scalp, a midline incision was made, and a 2-mm diameter hole was drilled in the skull over the injection site. Agent or vehicle was administered in a single injection in each subject. Microinjections targeted the midline medullary raphe at the rostrocaudal level of the facial nucleus. The target area encompassed portions of the raphe pallidus, obscurus and magnus (see Location of injections).

Stereotaxic coordinates relative to lambda were (rostrocaudal, mediolateral, doroventral) P15 (-2.3, 0.0, -10.0) and P25 (-2.4, 0.0, -10.3). The atlas of Paxinos and Watson (2005) was used as a guide for comparison of young rat brain stem to adult. Fused silica tubing (TSP05192, 185 μm OD, 51 μm ID; Polymicro Technologies) positioned by a stereotaxic micromanipulator and connected to a Picospritzer (General Valve) delivered injectate incrementally over several minutes. The fused silica tubing selected was the smallest that was rigid enough to remain straight when entering brain tissue with minimal clogging. Volume was visually measured by miniscus travel along a millimeter ruler. Pups received one 50-nl injection of either the GABA_A receptor antagonist bicuculline methiodide (1 mM; Sigma), GABA_A receptor agonist muscimol hydrobromide (2 mM; Sigma), or artificial cerebrospinal fluid (aCSF) (in mM: 150 Na, 3 K, 1.4 Ca, 0.8 Mg, 1 P, 155 Cl, at pH 7.4) with fluorescent latex microspheres (Polysciences) added for locating injection sites in brain tissue. The scalp was sutured, and care was taken to maintain Tb, while the animal recovered from anesthesia. Posture, locomotor activity, response to touch, and control of Tb recovered after ~30 min, at which point the animal was readied, and the arousal experiment began.

Arousal test. While recovering from surgery, pups were fitted with a vest that held small flat-surface ECG electrodes to record heart rate. A typical experiment is illustrated in Fig. 1, showing the changes in chamber [O2], HbO2Sat, HR, movement (MOVE), and f50. As shown, HR and f50 increase prior to arousal, which is associated with an abrupt change in body movement.

Because all of our studies were performed in a thermoneutral environment, we elected to rely solely on stereotypical behavior to identify arousal to wakefulness. Using these criteria, the observer recorded arousal from sleep after the onset of hypoxia (time to arousal or arousal latency). A typical experiment is illustrated in Fig. 1. After an acclimation period (~10 min) in the chamber, pups were exposed to four 3-min trials of hypoxia. Each trial was initiated during QS. During each trial chamber [O2] decreased to 10% over a period of 50 s and then remained constant for an additional 130 s. This was followed by a ~6-min period of normoxia. There was some variation in the recovery time to allow the pup to achieve QS before the onset of the next hypoxia trial. Thus the total cycle time (hypoxia + recovery) for each trial was ~9 min, and the total time for several experiments was ~40 min. In a separate group of P15 pups, room air from a compressed gas source was substituted for 10% oxygen to provide a measure of the spontaneous arousal rate and to confirm that there were no other nonhypoxia-related stimuli (sounds, abrupt changes in pressure, etc.) that might have affected arousal.

In many animal species, arousal to mild hypoxia is depressed during AS compared with QS (25, 41). In contrast, in human infants, arousal to mild hypoxia is depressed during QS (59). Although all of the pups in these experiments were in a state of quiet immobility at the onset of hypoxia, ~50% of the P15 pups developed myoclonic twitching before arousal, indicating a transition into AS (17, 65); we were, therefore, not able to separate out arousals by state. After arousal, during the remainder of hypoxic trial, P15 pups were often active for short periods of time and then slept; P25 pups were more active, frequently reentering sleep late in the trial.

Location of injections. At the conclusion of experiments, animals were euthanized, and the brains were removed and stored at ~80°C. Frozen brain stems were sectioned (50 μm) on a cryostat, and alternate sections were either stained with cresyl violet or left unstained to locate injected microspheres under a fluorescent microscope (Nikon). The section of brain stem with the greatest fluorescence signal from injected beads established the location of the injection. The same section was used to measure distances to the ventral surface and to midline of the injection center. A graphical record was made for each injection on a brain stem diagram (Adobe Illustrator). The rostrocaudal dimension of the bead spread was variable but was
estimated from the number of slices encompassing the beads; the extent of mediolateral diffusion or spread for each 50-nl injection was not calculated. The obex, the caudal pole of the facial nucleus, and the facial nerve served as landmarks for the determination of injection sites. Images were viewed with Neurolucida software (Microbrightfield). Our goal was to evaluate the effects of microinjections in the medullary raphe that included portions of the raphe pallidus, obscurus, and magnus. Therefore, injections located from 0.5 mm caudal of the obex to the caudal pole of the facial nucleus, within 0.75 mm of midline, and between the ventral surface to within 0.5 mm of the 4th ventricle; or from the caudal pole of the facial nucleus to 0.2 mm rostral of the facial nerve, within 1.2 mm of the midline, and from the ventral surface to 1.8 mm from the 4th ventricle were considered successful (see Fig. 2). The target area reflects the medial-lateral and dorsoventral dimensions of raphe obscurus and magnus. The results of experiments were pooled from all injections that met the above criteria.

Data reduction and analysis. A total of 140 pups received microinjections and were studied as described above. For this report, data were analyzed from 85 experiments that met the microinjection criteria indicated above (see Table 1). The arousal latency, the chamber [O2], and HbO2Sat measured at the time of arousal were initially compared using ANOVA for repeated measures (Systat v12) with “trial” (1–4) set as a repeated measure, and “group” (aCSF, MUS, BIC, NIP), “age” (P15 and P25), and “sex” set as grouping factors. Initial analyses showed no main effect of age or sex, and no interaction with each other, group, or trial; thus, final analyses were done on age and sex, data combined, with group as the only grouping factor. Evaluation of the interaction between group and trial provided information about any differences in the change of latency, chamber [O2], and HbO2Sat over the four hypoxia trials. We also compared the change in the latencies over the four hypoxia trials by calculating a linear slope (change in latency/trial). An additional group of animals not exposed to anesthesia or injections was evaluated to assure that the anesthesia or injection of aCSF did not affect arousal characteristics.

To analyze HR and fR, ECG and movement signals were filtered and peak detection algorithms (ADInstruments) were used to determine instantaneous rates. The resulting rate data were smoothed as necessary. HR and fR were analyzed at two points: 1) a baseline period consisting of the average of the data over the 5 s prior to the onset of hypoxia for each trial, and 2) the peak value achieved during each 3-min hypoxia trial. The latter was determined by visual inspection of the raw data, as the peak did not always occur near the end of the trial and the movement artifact after arousal necessitated choosing periods of “clean” data. In addition, to ascertain the continuous changes in HR, fR, and chamber [O2] up until the time of arousal, we analyzed data every 5 s for the first 40 s of each hypoxia trial.

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**Fig. 1.** An example of an experiment after a medullary microinjection of artificial cerebrospinal fluid (aCSF) illustrating the period from the onset of hypoxia to the time of arousal. Note that there is an increase in both heart rate and respiratory rate (fR) prior to arousal, with the suggestion that respiratory rate starts to increase prior to heart rate. The onset of hypoxia and the time of arousal are indicated by vertical dashed lines. Arousal is heralded by an abrupt increase in body movement. MOVE, movement, CHO2, chamber [O2].

**Fig. 2.** A and B: schematic diagram showing the locations of microinjections. Boxes have been drawn to indicate the boundaries to determine the accuracy of the microinjections. Dimensions for the postnatal day 25 (P25) animals were scaled so that both postnatal day 15 (P15) and P25 data could be displayed on the same diagram. C: unstained section showing an example of the determination of lesion foci with fluorescent microbeads. The arrow points to the center of the injection. The facial nuclei (nVII) have been identified for reference.
RESULTS

Information about the pups and baseline data obtained before the onset of the first hypoxia trial for the two age groups are shown in Table 1. Age and weight did not differ significantly across groups. Females weighed less than males at P25 but not at P15 \((P > 0.016)\). Mean baseline body temperature, averaged across trials, for P15 and P25 pups combined, was higher in the BIC pups \((36.38 \pm 0.14^\circ C; P = 0.002)\), MUS \((36.49 \pm 0.19^\circ C; P = 0.030)\), and NIP \((35.87 \pm 0.14^\circ C; P < 0.001)\) pups. Mean baseline HR, averaged across the four trials of hypoxia, was higher in the P25 pups \([468.8 \pm 4.9\) beats per minute \((bpm)\) for P25 vs. 445.3 \(\pm 5.6\) bpm for P15; \(P = 0.002)\] but did not vary among groups. There was no main effect of age on metabolic rate, measured either as oxygen consumption \(\left(V_\text{O}_2\right)\) or carbon dioxide production \(\left(V_\text{CO}_2\right)\). **Table 1. Demographic and baseline data obtained before the first hypoxia trial**

<table>
<thead>
<tr>
<th>Variable</th>
<th>aCSF</th>
<th>Muscimol</th>
<th>Bicuculline</th>
<th>Nipicotic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P15</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number</td>
<td>18</td>
<td>16</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>Number analyzed</td>
<td>13</td>
<td>7</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>Male/female</td>
<td>7/6</td>
<td>2/5</td>
<td>4/4</td>
<td>8/3</td>
</tr>
<tr>
<td>Age, days</td>
<td>14.9 (\pm 0.4)</td>
<td>16.0 (\pm 0.4)</td>
<td>14.9 (\pm 0.4)</td>
<td>16.2 (\pm 0.2)</td>
</tr>
<tr>
<td>Weight, g</td>
<td>36.9 (\pm 3.1)</td>
<td>36.9 (\pm 2.3)</td>
<td>34.2 (\pm 1.7)</td>
<td>36.9 (\pm 1.6)</td>
</tr>
<tr>
<td>Temperature, (^\circ C)</td>
<td>35.9 (\pm 0.1)</td>
<td>36.0 (\pm 0.3)</td>
<td>36.7 (\pm 0.3)*</td>
<td>35.2 (\pm 0.2)</td>
</tr>
<tr>
<td>% HbO2 Sat</td>
<td>98.3 (\pm 0.4)</td>
<td>97.9 (\pm 1.0)</td>
<td>98.1 (\pm 0.9)</td>
<td>98.1 (\pm 0.8)</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>455 (\pm 11)</td>
<td>432 (\pm 17)</td>
<td>445 (\pm 15)</td>
<td>449 (\pm 12)</td>
</tr>
<tr>
<td>(f_s), breaths/min</td>
<td>136 (\pm 8)</td>
<td>111 (\pm 13)</td>
<td>93 (\pm 15)</td>
<td>137 (\pm 11)</td>
</tr>
<tr>
<td>(V_\text{O}_2), ml(\text{min}^{-1})kg(^{-1})</td>
<td>38.7 (\pm 3.3)</td>
<td>24.0 (\pm 1.7)</td>
<td>26.4 (\pm 2.2)</td>
<td>33.8 (\pm 2.4)</td>
</tr>
<tr>
<td>(V_\text{CO}_2), ml(\text{min}^{-1})kg(^{-1})</td>
<td>27.1 (\pm 0.1)</td>
<td>22.4 (\pm 1.8)</td>
<td>24.9 (\pm 1.0)</td>
<td>26.7 (\pm 1.6)</td>
</tr>
<tr>
<td>RQ</td>
<td>0.75 (\pm 0.05)</td>
<td>0.93 (\pm 0.02)</td>
<td>0.97 (\pm 0.07)</td>
<td>0.80 (\pm 0.02)</td>
</tr>
</tbody>
</table>

\(\text{aCSF, MUS, or NIP, P15, and P25 combined (} P < 0.002, \text{ with no main effect of age.) \text{For heart rate, P25 > P15 (} P = 0.002, \text{ with no difference among groups.) \text{For} \ V_\text{CO}_2, \ aCSF > MUS or NIP, P15, and P25 combined (} P < 0.05, \text{ with no main effect of age.)} \text{For HbO2 Sat, MUS or NIP, P15 and P25 combined (} P = 0.05), with no main effect of age.}\)

In a separate group of 18 P15 pups, spontaneous arousal rates were determined in experiments in which air was substituted for 10% oxygen after microinjections with aCSF, muscimol, or bicuculline \((aCSF, n = 6; MUS, n = 6; BIC, n = 6)\). We did not test nipeptic acid in these experiments. During these “air control” experiments, chamber \(O_2\), HbO2Sat, and baseline HR did not change across trials and did not differ among groups. The arousal latency during air breathing (spontaneous arousal rate) was higher after microinjection...
with muscimol than after microinjection with aCSF (P = 0.022) or bicuculline (P = 0.001). Moreover, the arousal latencies during air breathing were significantly greater than during hypoxia for pups microinjected with muscimol (P < 0.001) and aCSF (P = 0.010) but not bicuculline. Figure 4 compares the mean latency, averaged across trials, for the hypoxia and air control experiments in P15 pups microinjected with aCSF, bicuculline, and muscimol.

**Chamber oxygen concentration and oxyhemoglobin saturation.** The decreases across hypoxia trials in chamber [O2] and HbO2Sat, measured at the time of arousal, mirrored the increases in arousal latency and are illustrated in Fig. 5. The mean chamber [O2], averaged across the four trials of hypoxia, varied by group (P = 0.005). Mean chamber [O2] was lowest in the MUS group compared with both the aCSF (P = 0.007) and BIC (P = 0.001) groups and highest in the BIC group compared with MUS (P = 0.001) and NIP (P = 0.022) groups. Similarly, the mean HbO2Sat varied by group (P < 0.001). The mean HbO2Sat values were lowest in the MUS group compared with the NIP (P = 0.009), aCSF (P < 0.001), and BIC (P < 0.001) groups. The mean HbO2Sat values in the NIP pups were also lower than in the BIC pups (P = 0.009). The HbO2Sat values in the BIC group were also higher than the MUS and NIP groups but not significantly different from those in the aCSF group. The rates of decrease in chamber [O2] and HbO2Sat, measured at the time of arousal, across the four trials of hypoxia, measured by calculating a linear slope (%/trial), were not different among the groups. It is important to note that the data for HbO2Sat were incomplete in the aCSF (12/26 pups) and the NIP (15/27 pups) groups.

**Heart rate.** Baseline HR, measured before the onset of hypoxia, increased over the four trials of hypoxia in the MUS pups but not in the other groups (P < 0.001). After the onset of hypoxia, HR increased before arousal in all groups (P < 0.001), and the rate of increase, measured as a linear slope (bpm/20 s), averaged across all four trials was greatest in the MUS pups (P = 0.023). In addition, the slope increased over the four trials of hypoxia (P = 0.027), but there were no effects of group or age. The increase of HR from baseline to peak was also evaluated. Similar to baseline HRs, peak heart rates were greater in the P25 pups (P < 0.001), but there was no effect of group. There was also no effect of group or age on the change in HR from baseline to peak.

**Respiratory rate.** Baseline fR, measured before each hypoxia exposure, increased across the four trials of hypoxia (P = 0.008), but there was no effect of group or age. Baseline fR ranged from 117 breaths/min (b/m) before trial 1 to 124 b/m before trial 4. As expected, fR increased during each hypoxia exposure. The increase started within ~5 s after the onset of hypoxia, and in many cases, preceded the increase in HR. Changes in HR and fR in relationship to the onset of hypoxia and the time of arousal in a single experiment are shown in Fig. 1. The increase in fR over the first 40 s of hypoxia (before arousal) did not differ among groups and averaged 29 b/m. Respiratory rate continued to increase after arousal and peaked at various times during the 3-min hypoxia trial. The increase from baseline to peak fR was not different across trials or among groups and averaged 38 b/m.

**Body temperature.** Our study chamber was designed to provide a near-thermoneutral environment. Thus, in a steady state, the pup would not be required to produce extra heat in an attempt to maintain Tb. It is unlikely, however, given the nature of our experiments, that a complete steady state was achieved. Although there was no main effect of group on the mean Tb, averaged across the four trials of hypoxia, there was an effect of group on Tb after placement in the chamber, before the first hypoxia trial (P < 0.001). More specifically, BIC pups had higher Tb than aCSF (P = 0.009), MUS (P < 0.001), or NIP pups (P < 0.001). The Tb of NIP pups was lower than aCSF pups (P = 0.003), and the Tb of MUS pups trended lower than aCSF pups (P = 0.072). Tb increased across the four hypoxia trials in all groups, the greatest increase occurring in the MUS group and the lowest in the aCSF group. Figure 6 shows the initial and final Tb in the four groups across the four trials of hypoxia.

**DISCUSSION**

The most important finding in this study was that arousal from sleep in response to intermittent hypoxia is modulated by medullary raphe GABAergic mechanisms. Arousal latency was prolonged by activating GABA<sub>A</sub> receptors in the medullary raphe with muscimol or blocking the reuptake of GABA with nipeptic acid. However, muscimol and nipeptic acid did not alter the degree of “habituation” or the progressive lengthening of latency over successive trials of hypoxia. In contrast, blocking GABA<sub>A</sub> receptors with bicuculline resulted in a reduction in the degree of habituation compared with the other groups.

Our results indicate that the medullary raphe plays an important role in arousal in response to hypoxia. Moreover, P15 pups microinjected with muscimol had longer latencies between spontaneous arousals, and after bicuculline injections, latencies in response to hypoxia were not shorter than those between spontaneous arousals. Taken together, this suggests a role of the medullary raphe in spontaneous arousals, as well as those in response to hypoxia. Caution must be taken, however, in the interpretation of the spontaneous arousal results. This was a small group of P15 pups, tested to make sure that there were no stimuli, other than hypoxia, that could affect arousal and not specifically designed to determine the role of the medullary raphe in spontaneous arousals.

The role of this region of the brain stem in sleep and arousal has not been completely elucidated and has generally not been included in discussions of ascending arousal pathways. However, we previously showed in newborn piglets that activating GABA<sub>A</sub> receptors with muscimol, diazylized locally into medullary regions, including the midline raphe nuclei, as well as the more lateral paragigantocellularis lateralis (PGCL), results in a disruption of sleep architecture and promotes wakefulness (14). Similarly, activating 5-HT<sub>1A</sub> receptors in both the midline raphe and PGCL promotes wakefulness and decreases REM.
sleep (11, 34). Others have described similar shifts to wakefulness after lidocaine injection into the raphe magnus and rostral ventromedial medulla, a region implicated in antinociception (7).

Role of GABA in arousal and arousal habituation. Acute hypoxemia is associated with increases in the extracellular concentration (measured with microdialysis) of both excitatory and inhibitory neurotransmitters and/or neuromodulators in the brain stem, including glutamate, GABA, taurine, adenosine, and serotonin in both anesthetized and conscious animals (33, 36, 63, 66). Most of these studies have focused on respiration-related areas, including the nucleus tractus solitarii (NTS) and ventrolateral medulla. To our knowledge, there have been no studies specifically focused on the medullary raphe.

In the rodent, there is a broad distribution of the GABA-synthesizing enzyme, glutamate decarboxylase-containing neurons throughout the medullary raphe with the highest density near the ventral surface and extending laterally (35, 45, 51). Thus, the source of GABA in the medullary raphe most likely is from GABA-producing interneurons. However, there may also be substantial projections of remote GABAergic neurons into this region. Finally, some neurons of other phenotypes, including 5-HT neurons, express multiple neurotransmitters, including GABA. It is also clear that GABA-producing neurons are present very early in gestation (50), with a distribution pattern at P15–P25 very similar to that of the adult. GABA_{A} receptors are expressed by neurons of many phenotypes. Muscimol would, therefore, be expected to inhibit the activity of almost all neurons expressing GABA_{A} receptors and would not

Fig. 5. A: chamber [O_{2}] at the time of arousal over four trials of hypoxia after medullary raphe microinjection of aCSF, muscimol, bicuculline, and nipeptic acid. Mean chamber [O_{2}] at arousal was lower after microinjection of muscimol than after microinjections of aCSF and bicuculline and was higher after microinjection of bicuculline than after microinjections with muscimol or nipeptic acid. By trial 4, pups microinjected with muscimol aroused at lower chamber [O_{2}] compared with those microinjected with aCSF (P = 0.011) or bicuculline (P = 0.001) (*). In contrast, pups microinjected with bicuculline aroused at higher chamber [O_{2}] than those injected with muscimol (P = 0.001) or nipeptic acid (P = 0.019) (+). B: HbO_{2}Sat at the time of arousal over four trials of hypoxia after medullary raphe microinjection of aCSF, muscimol, bicuculline, and nipeptic acid. Similar to chamber [O_{2}], mean HbO_{2}Sat (averaged over all four trials) at the time of arousal was lowest after microinjections of muscimol compared with those microinjected with aCSF, bicuculline, or nipeptic acid. Pups microinjected with bicuculline aroused at higher mean HbO_{2}Sats compared with those microinjected with nipeptic acid or muscimol. By trial 4, pups injected with muscimol aroused at significantly lower HbO_{2}Sats compared with those injected with aCSF (P = 0.001) or bicuculline (P = 0.002) (*), but HbO_{2}Sats at arousal were not different than in pups microinjected with nipeptic acid. Similarly, pups injected with nipeptic acid aroused at lower HbO_{2}Sats than those injected with aCSF. In contrast, pups injected with bicuculline aroused at significantly higher HbO_{2}Sats than pups injected with either muscimol (P = 0.002) or nipeptic acid (P = 0.014) (+). All values are expressed as the means ± SE.

Fig. 6. Body temperatures before trial 1 and trial 4 of hypoxia. Initial body temperatures were greater in pups microinjected with bicuculline compared with the other groups (*), and temperatures were lowest after microinjections of muscimol (+) and nipeptic acid. By the last trial, there were no differences in body temperature between the groups. Values are expressed as the means ± SE.
help determine the source of GABA. Thus, our results after muscimol microinjection could be interpreted as a generalized inhibition of medullary raphe neuronal activity.

Nicotic acid, with a high affinity for the GABA transporters, GAT1 and GAT3, would be expected to block the reuptake of GABA into both neurons (GAT1) and glia (GAT3) (31, 49) and may also provide insight as to changing levels of endogenous GABA. The results of our experiments with nicotic acid suggest that hypoxia results in an increase in ambient GABA and when reuptake is inhibited, there is an enhanced progressive increase providing a source of increasing tonic inhibition. The use of more specific antagonists to GABA transporters may have provided more specific information about the location of GABA reuptake but would not have distinguished between GABA released by local GABA-producing interneurons, remote GABA neurons projecting to the raphe or neurons of other phenotypes that coproduce GABA.

In contrast, blocking GABA_A receptors with bicuculline did not shorten arousal latency compared with controls but resulted in an elimination of habituation. There was no successive increase in arousal latency across multiple trials of hypoxia. These data indicate that activation of GABA_A receptors are necessary for arousal habituation.

Stimulating GABA receptors with muscimol microinjection could be interpreted as a generalized increase in arousal latency across multiple trials of hypoxia. These data indicate that activation of GABA_A receptors are necessary for arousal habituation.

Neurophysiological and anatomical origin of arousal in response to hypoxia. It is generally thought that arousal from sleep, either spontaneously occurring, or in response to an external stimulus, is mediated by 1) ascending cholinergic, serotonergic, noradrenergic, and histaminergic pathways necessary for “cortical” arousal and 2) descending pathways that modulate breathing, spinal motor activity, and sympathetic activity that controls body temperature, heart rate and blood pressure, components of “subcortical” arousals. Thach and colleagues (54, 55, 68) described an “arousal sequence” in human infants in response to hypercapnia and tactile stimuli that consists of a spinal withdrawal, followed by an augmented breath, a startle (all subcortical events), and finally a change in the EEG and full awakening. We have described this same sequence in the newborn piglet (12). Moreover, in both the piglet and human infant, heart rate and blood pressure changes (subcortical) precede EEG changes (cortical) (2). Our observations have confirmed that a similar sequence also occurs in newborn and infant rodents (17). These observations, taken together, suggest that most arousals, either spontaneous or in response to a stimulus, originate in the brain stem. However, there is at least one report that hippocampal theta rhythms appear before heart rate, and blood pressure changes in the conscious rabbit in response to carotid chemoreceptor stimulation with phenylbiguanide (72). Thus, it remains unclear whether the entire arousal process, specifically in response to hypoxia, follows this caudal-rostral progression.

Arousal in response to hypoxia is impaired after denervation of the carotid body (10, 24, 26). Sensory information from the carotid bodies is transmitted largely to the medial and the commissural subnuclei of the nucleus tractus solitarii (com NTS) with some afferents terminating in the ventrolateral medulla (27). Secondary afferent neurons project widely to other areas in the brain stem, targeting groups of neurons controlling respiration, heart rate, blood pressure, body temperature, and the upper airway, including the medullary raphe. Although neuronal pathways responsible for increasing ventilation, heart rate, and blood pressure and inhibiting brown fat thermogenesis in response to carotid body stimulation have been partially explained (1, 8, 22, 52, 53, 67), little is known about the specific pathways responsible for arousal in response to hypoxia.

Our results suggest that the medullary raphe either lies in an arousal pathway or modulates other ascending arousal pathways activated by hypoxia. There are several lines of evidence suggesting that exposure to intermittent hypoxia activates medullary raphe neurons. Long-term facilitation associated with exposure to acute intermittent hypoxia requires the activation of medullary 5-HT neurons that project to the spinal cord (4, 5). Stimulation of the carotid sinus nerves induces c-Fos-like protein in regions of the medullary raphe (23). Multiarray extracellular recordings further suggest that midline raphe neurons are critical components of a larger raphe-ponto-medullary network of neurons with respiratory related activity and respond to peripheral chemoreceptor stimulation in concert with the entire network (58). Although there is clear evidence of direct projections of medullary raphe 5-HT neurons to the NTS (69), the existence of direct projections from the com NTS to the medullary raphe is less clear. The com NTS does project to the ventrolateral medulla, retrotrapezoid nucleus, the hypothalamus, amygdala, and the midbrain periaqueductal gray, all of which, in turn, project to the medullary raphe. In addition, a recent paper by Takakura et al. (67) that focused on the retrotrapezoid nucleus suggests that anterograde labeling from the com NTS is present medial to the facial nucleus (para-pyramidal region) extending into the raphe. Neurons in this region were not further characterized in this report, but labeling could indicate an oligo- or mono-synaptic pathway from the com NTS to the medullary raphe in the rostral-caudal dimension of the facial nucleus (67). Some of these pathways are likely components of the raphe-ponto-medullary network referred to above. Further, the effects of hypoxia on sympathetic outflow to brown fat might involve inhibition of presym pathetic neurons in the medullary raphe by GABAergic projections from the com NTS (53, 58). These data all suggest that neurons in the medullary raphe are activated during peripheral chemoreceptor stimulation. Our experiments, however, did not differentiate between specific raphe nuclei that undoubtedly have different embryonic origins, functions, and projections. Although activation of neurons during hypoxia most likely occurs in all raphe nuclei, and activation itself may be critical in the arousal process, further investigation is necessary to determine whether arousal functions are located in specific raphe regions, analogous to the role of the raphe pallidus in thermoregulation (57).

How information might be transmitted from the caudal medullary raphe to more rostral groups of neurons involved in cortical arousal is not known. Neurons in the nearby nucleus

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paragigantocellularis lateralis project to the locus coeruleus, a region important for maintaining vigilance (70). Our previous experiments in piglets, in which muscimol was dialyzed into the PGCL found that muscimol promoted wakefulness, whereas small microinjections into the medullary raphe in the current experiments prolonged arousal. The reason for the discrepancy is not known, but it may involve varying functions of the two regions studied, or species differences. We speculated previously that decreasing neuronal activity in the PGCL might interrupt normal sensory integration or dampening that might result in heightened responses to sensory input resulting in agitation and sleep disruption (15).

Effects of anesthesia and surgery on arousal. We were initially concerned that the anesthesia and surgery, by itself, would affect arousal latency, and care was taken to assure that motor activity, heart rate, and body temperature were normal before starting each experiment. Moreover, the pattern of arousal latency across the four trials of hypoxia in pups microinjected with aCSF was almost identical to the arousal response in pups that had not experienced anesthesia, surgery, or microinjection, indicating that there was little observable effect of the anesthesia or surgery itself on arousal.

Pharmacological issues. The diameter of a sphere that would contain 50 nl would be ~0.67 mm, which was very close to the 0.63 mm that we measured from the rostral-caudal spread of fluorescent beads in our injections. It is likely that our measurements of spread underestimated the actual extent of the effects of the pharmacological agents that we used. We cannot be sure that some of the injected agents did not extend laterally into more lateral reticular areas, perhaps including the medial edge of the lateral column of 5-HT neurons contained in the PGCL. In addition, the pharmacodynamics of the various agents that we used have not been completely elucidated. Thus, there might have been waning effects of some agents over the course of the arousal experiments after microinjection. There is considerable experience with muscimol. For example, bilateral injections of 1.75 mM in 30 nl into the ventral respiratory group in which strong inspiratory-related multiunit activity could be recorded eliminates phrenic nerve electrical activity for up to 2 h (67). Less is known about the duration of bicuculline and nipecotic acid under our experimental conditions. The elevated body temperature and the inability to measure HbO2 Sat from the tail in pups (increased vasoconstriction) with raphe injections of bicuculline indicate that the effects for this agent lasted for the duration of our experiments.

Is the role of medullary raphe specific to arousal in response to hypoxia? Whether the role of the medullary raphe is unique to arousal in response to hypoxia, or whether this region is important for arousal in response to other sensory stimuli or spontaneous arousal is unknown. Our air control experiments in a small group of P15 pups suggest that the medullary raphe is involved in spontaneous arousal. Further experiments will need to be performed to confirm these preliminary findings. In human infants, habituation has been demonstrated after exposure to repeated tactile (55) and auditory (47) stimulation. However, progressive impairment of arousal could not be demonstrated in the one study in which human infants were exposed to repeated exposure to 15% oxygen (61). Moreover, there is some evidence in humans that the type of stimulus does not affect the pattern of arousal, suggesting there are common components of the responsible neural pathways (62).

Perspectives and Significance

In summary, to the best of our knowledge, this is the first demonstration that the medullary raphe plays a role in arousal and arousal habituation in response to hypoxia and perhaps also in the frequency of spontaneous arousals. In addition, we have shown that inhibition of neuronal activity during hypoxia by activating GABA_A receptors or increasing levels of ambient GABA, prolongs arousal and that specifically blocking GABA_A receptors prevents arousal habituation. These findings have important implications for SIDS research and may shed some light on causation. A large subset of infants (up to 70%) who die of SIDS have decreases in 5-HT_1A and GABA_A receptor binding and decreases in tissue 5-HT and TPH2, the synthesizing enzyme for 5-HT, in the medullary raphe and other medullary nuclei containing 5-HT neurons (20, 46, 60). Taken together, these findings suggest decreased 5-HT neuronal activity and involvement of GABAergic mechanisms. We postulate that these defects may decrease the capacity to increase neuronal activity required for a timely arousal. Arousal from sleep is the first line of defense when faced with stressors, such as hypoxia during sleep, and an arousal deficit has long been suggested as an hypothesis for the etiology of SIDS (32, 40, 43).

The results of these and our previous experiments also suggest that repeated exposure to brief periods of hypoxia can cause a progressive arousal impairment to subsequent exposures to hypoxia. Meny, Kelly, and colleagues (44, 56) described repeated apnea and bradycardia events in monitor recordings of infants who subsequently died. In addition, our own review of the cardiorespiratory recordings of infants who died while attached to their home monitors confirms that many of these infants had clusters of apnea and bradycardia, and presumed hypoxia, in the weeks and months preceding death (R. A. Darnall, unpublished results). We hypothesize that repeated episodes of hypoxia will increase the risk for impaired arousal in response to a subsequent hypoxia event. Thus, the combination of a brain stem abnormality and exposure to repeated episodes of hypoxia resulting in blunting of arousal may be particularly dangerous and significantly increase the probability of sudden death.

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No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: R.A.D. and R.W.S. were responsible for conception and design of the research; R.A.D., R.W.S., C.M.T., and B.M.Z. analyzed the data; R.A.D. and R.W.S. interpreted the results of experiments; R.A.D. and R.W.S. prepared the figures; R.A.D. and R.W.S. drafted the manuscript; R.A.D. and R.W.S. edited and revised the manuscript; R.A.D., R.W.S., C.M.T., and B.M.Z. approved the final version of the manuscript; R.W.S., C.M.T., and B.M.Z. performed the experiments.
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