Nasal Trigeminal Inputs Release the A5 Inhibition Received by the Respiratory Rhythm Generator of the Mouse Neonate

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INTRODUCTION

In mammals, respiration, which is crucial to sustain life by allowing tissue oxygenation, is spontaneous since birth. Most of our knowledge on the respiratory rhythmogenesis has been obtained from in vitro neonatal rodent preparations in which the respiratory rhythm generator (RRG), located in a small region of the rostral ventrolateral medulla, generates rhythmic motor outputs such as the phrenic bursts controlling the diaphragm (Rekling and Feldman 1998; Smith et al. 1991; Suzue 1984; for reviews Ballanyi et al. 1999; Hilaire and Duron 1999). However, it has recently been shown that in the rostral ventrolateral medulla of the rat the RRG contains two distinct oscillator circuits, the pre-Bötzinger complex and the para-facial respiratory group (Onimaru and Homma 2003) whose coupled activity is necessary to produce normal eupneic breathing (Janczewski et al. 2002; Mellen et al. 2003). Even though the peripheral deafferentation and the low bath temperature used in vitro are likely contributing to the low respiratory frequency produced by the isolated RRG (Smith et al. 1990), its activity is still modulated by endogenous substances released from central structures present in the preparation, such as noradrenaline from the pontine noradrenergic A5 nuclei (for reviews, Ballanyi et al. 1999; Hilaire and Duron 1999). Indeed, ponto-medullary preparations from neonatal rats display spontaneous rhythmic phrenic bursts although the RRG is inhibited by the A5 neurons terminals which activate the medullary α2 adrenoceptors (Errchidi et al. 1990, 1991; Hilaire et al. 1989). However, in ponto-medullary preparations from neonatal mice, no rhythmic phrenic bursts occur although they are induced by the elimination of the A5 neurons, and so far, only an activation of the RRG through medullary α1 adrenoceptors has been shown (Hilaire et al. 1997; Viemari and Hilaire 2002; Viemari et al. 2003). Therefore questions arise: how the A5 inhibition received by the neonatal mouse RRG is mediated and how it is possible to prevent it.

The RRG of both adults and neonates is regulated by numerous peripheral inputs such as those arising from the carotid bodies, the lungs, the ribcage, and the upper airways, which provide respiratory adjustments to meet both internal and environmental variations (Hlastalva and Berger 1996). Of interest in this study are the trigeminal afferents from the nasal cavity that are known to project to the A5 nuclei (Caous et al. 2001; Panneton 1990; Panneton et al. 2000). Furthermore, the trigeminal afferent neurons derive from Mash1-positive neural precursors and depend on the Rnx homeobox for proper formation (Qian et al. 2002). The mutant mice for Rnx that have an altered maturation of their trigeminal afferents as well as no A5 neurons present lethal respiratory deficits at birth (Shirasawa et al. 2000). Therefore the trigeminal afferents and the A5 nuclei may have a significant functional role in the respiratory rhythmogenesis, at least in mouse neonates.

Using electrophysiological, pharmacological, and histological approaches, we show that as in the rat, the RRG in the mouse is inhibited by the A5 neurons through medullary α2 adrenoceptors in such a way that no respiratory activity is displayed in isolated ponto-medullary preparations and that the trigeminal afferent inputs can modulate the strength of the A5 inhibition received by the RRG.

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METHODS

Animals

The experimental procedures were carried out in keeping with the European guidelines for care and use of laboratory animals (Council Directive 86/6009/EEC). The experiments were performed from postnatal day 0 to 5 (P0–P5) on neonatal mice belonging to the OF1 mouse strain (Iffa-Credo breeding center, Saint Germain sur l’Arbresle, France).

In vivo experimental protocols

Whole-body plethysmography (Burnet et al. 2001; Viemari et al. 2003) was used to measure the breathing parameters of awake mouse neonates. The animal chamber (25 ml), equipped with a temperature sensor (Checktemp1, Hanna Instruments, Lingoldshein, France) was connected to a reference chamber of identical volume. Both chambers were immersed in a thermostated water bath (27°C). The pressure difference between the two chambers, generated by the inward-outward respiratory flow, was measured with a differential pressure transducer connected to a sine-wave carrier demodulator (Validyne CD15, Northbridge, CA). The signals were amplified, filtered (0.1–5 Hz), fed to an A/D converter (sampling frequency: 1 kHz), and stored on a PC disk via Spike 2 interface and software (Cambridge Electronic Design, Cambridge, UK). The calibrations were performed by injecting 10 μl of air into the animal chamber. At the end of the recording session, the mouth temperature was measured by a miniature thermistor probe (YSI 555) connected to its thermistor thermometer 8402-20 (Cole-Palmer Instruments, Vernon Hills, IL). We assumed that the body temperature was equal to the mouth temperature and that the breathing mixture in the lungs was fully saturated with water vapor at body temperature. For each neonate (P3–P5), the breathing parameters (respiratory frequency, RF; tidal volume, VT) were measured during 3 min, first under control condition and second under test condition; sometimes, a third measure was performed to check recovery from test condition. In each condition, the minute ventilation was calculated for each animal. Four test conditions were examined: a nasal occlusion was performed by placing a small piece of hypoallergenic adhesive plaster (Urgo, Chenôve, France) gently touching the lower ribs. The frequency of the respiratory movements of awake neonates (P1–P2) was monitored under control condition (3 min) and under administration of a nasal continuous positive airway pressure (20 mmHg of H2O) delivered through a nasal mask during 2 min. After the neonates (n = 8) were set in lateral decubitus on a piece of adhesive plaster to minimize their leg movements, the respiratory movements were recorded by the deformations of a strain gauge (Beam, Marseille, France) gently touching the lower ribs.

In vitro experimental protocols

The mouse neonates (P0–P3) were ether-anesthetized and decerebrated; the brain stems and the cervical spinal cords were dissected out and placed ventral sides up in a 2-ml chamber superfused with artificial cerebrospinal fluid (ACSF) at 27 ± 0.25°C (mean ± SD), renewed at a rate of 2 ml/min (Bou-Flores et al. 2000). The ACSF [containing (in mM) 129 NaCl, 3.35 KCl, 1.26 CaCl2, 1.15 MgCl2, 21 NaHCO3, 0.58 NaH2PO4, and 30 glucose (Sigma-Chimie, St Quentin, France)] was oxygenated and equilibrated (pH ∼ 7.4 at 27°C) by bubbling carbogène (95% O2–5% CO2). In the pharmacological experiments, this was replaced by another ACSF in which bioreactive substances (Sigma-Chimie) were dissolved: noradrenaline at 25 μM (NA-ACSF) or α2 adrenoreceptor antagonists, either piperoxane at 50 μM (PIP-ACSF) or yohimbine at 50 μM (YO-ACSF). In some of the experiments, a patch-clamp microelectrode (1-μm diameter tip) was lowered within the ventral pons into the A5 nucleus where a solution of either ACSF or NA (1 mM) was pressure-ejected. The ejected volume was estimated ~20 nl for a pressure pulse lasting 2 s.

The signals were amplified, filtered (0.1–3 kHz), and the phrenic activity was measured by a home-made leaky integrator (time constant: 50 ms). All the signals were printed on a Gould TA 2000 (Gould Electronique, Longjumeau, France).

Two types of preparations were used either with intact brain stems (ponto-medullary preparations) or after pons elimination (medullary preparations). However, in some pharmacological experiments, a physical barrier done with a thin plastic sheet was set at the ponto-medullary junction of the intact brain stem to apply different types of ACSF onto the pons and the medulla. The rhythmic phrenic bursts produced in vitro by the isolated respiratory network were recorded from the fourth cervical ventral root (C4) with a suction electrode and in some experiments a simultaneous recording of either the trigeminal (Vth) or the facial (VIIth) cranial nerves was made with a second suction electrode. Tungsten microelectrodes (5–10 MΩ, Frederick Haer, Brunswick, ME) were used to record the extracellular unitary activity of medullary neurons. The signals were amplified (104) and band-pass filtered (0.1–3 kHz for nerve activity; 0.3–6 kHz for unitary activity), and the phrenic nerve activity was then ampliﬁed through a home-made leaky integrator (time constant: 50 ms). All the signals were printed on a Gould TA 2000 (Gould Electronique, Longjumeau, France).

Trans-neuronal tracing experiments

We used the trans-neuronal infection of the rabies virus to localize the neurons belonging to the respiratory network (Aistic et al. 1993; Burnet et al. 2001; Gaytan et al. 2002; Ugolini 1995). To do so, an incision (<5 mm) was made above and parallel to the 9th and 10th ribs of cold-anesthetized P1 neonates to visualize the diaphragm through the thin intercostal muscles. A solution of the Challenge Virus Standard fixed strain of rabies virus (2 μl; 2.5 × 10^7 PFU/μl) was slowly injected through a needle inserted into the right part of the diaphragm. After the injections, the neonates were warmed until recovery and the phrenic nerve activity was then integrated through a home-made maintained in depression. After 2 days of survival, they were anesthetized and killed; the brain stems and the spinal cords were dissected out and placed in 4% paraformaldehyde in PBS, pH 7.4.
7.4. The nervous tissues were serially sectioned (70 μm thickness) in the coronal plane by means of a vibraslicer (Campden Instruments, Longborough, UK). The virus was detected by immuno-histochemistry: the mouse monoclonal antibody 31G10 (Raux et al. 1997) specific to the phosphoprotein, a constituent of the rabies nucleocapsid, diluted 1:5,000, was used as primary antibody and a goat antimouse IgG rhodamine-coupled (Jackson ImmunoResearch, West Grove, PA) diluted 1:200 was used as secondary antibody. The mouse monoclonal antibody 31G10 (Raux et al. 1997) was used to detect the phosphoprotein, a constituent of the rabies nucleocapsid, diluted 1:200 was used as secondary antibody. The infected neurons were visualized under an Olympus BX50 microscope equipped with fluorescence (Olympus Optical, Hamburg, Germany), and photographed.

**Statistical analysis**

The data were analyzed with SigmaStat software (SPSS ASC GmbH, Erkrath, Germany). First the assumption of normality and homoscedasticity of the samples were verified (Zar 1984). Then the statistical significance of the effects of either drug application or nerve stimulation was assessed by Dunnett’s tests following a one-way ANOVA; in other cases, Student’s paired or unpaired t-test were used to assess the statistical significance. The values were expressed as means ± SE, and statistical significance was taken at $p \leq 0.05$.

**RESULTS**

**In vivo nasal afferent inputs maintain a high respiratory frequency**

**Nasal occlusion.** Visual observations of awake neonates ($n = 8$; P3–P5) revealed that they breathe through their noses: their nostrils showed tinyc rhythmic movements at a high frequency while their mouths were tightly close with sealed lips. A nasal occlusion induced a switch from nasal to oral breathing with a wide opening of the mouth and an apparent decrease of the respiratory frequency. Plethysmography measurements, before (Fig. 1A1) and after 3 min of nasal occlusion (Fig. 1A2), showed that the nasal occlusion significantly decreased the mean respiratory frequency by $45 \pm 5\%$ of the mean control value ($212 \pm 10$ respiratory cycles/min, c/min; Fig. 1A1, histogram) and significantly increased the mean tidal volume by $70 \pm 12\%$ of the mean control value ($12 \pm 2$ μl); the mean minute ventilation was slightly but not significantly decreased (by $5 \pm 8\%$ of the mean control value, $2.42 \pm 0.02$ ml/min). When the nasal occlusion was stopped, the breathing parameters resume their mean control values (not shown).

**Nasal applications.** Visual observations of awake neonates ($n = 5$; P3–P5) when small drops of lidocaine were applied onto the nostrils showed rhythmic bubbles within the fluid due to respiratory nasal airflow as the fluid was inhaled within ~10 s and a slight opening of the mouth. Comparing breathing parameters measured by plethysmography before (Fig. 1B1) and after 3 min of nasal applications of lidocaine (Fig. 1B2) revealed a significant decrease of the mean respiratory frequency by $34 \pm 4\%$ of the mean control value (185 ± 14 c/min; Fig. 1B, histogram) and a significant increase of the mean tidal volume by $48 \pm 15\%$ of the mean control value ($11 \pm 5$ μl); the mean minute ventilation was slightly but not significantly decreased (by $10 \pm 7\%$ of the mean control value, 2.04 ± 0.09 ml/min). The lidocaine-induced modifications of the breathing parameters were long-lasting; the mean control values were restored in ~2 h (3 neonates tested).

When similar nasal applications were made with physiological saline ($n = 2$) or when lidocaine was applied on the snout ($n = 3$), no significant variations of the breathing parameters were observed (not shown).

**Continuous positive airway pressure.** The respiratory movements of awake neonates ($n = 9$; P1–P2) were recorded as they breathed air through a nasal mask. In control condition (Fig. 1C1 and histogram), the mean respiratory frequency ($132 \pm 5$ c/min) of these neonates was significantly lower than the one measured in P3–P5 neonates as previously reported (Viemari et al. 2003). The administration of a nasal continuous positive airway pressure (20 mm of H2O, Fig. 1C2) during 3 min increased the mean respiratory frequency by $29 \pm 6\%$ of the mean control value (Fig. 1C1 and histogram) while the amplitude of the respiratory movements decreased. The mean control frequency resumed within 2 min of the withdrawal of the airway pressure. However, if the pressure was increased to 60 mm of H2O or further up, the respiratory frequency was drastically depressed ($n = 3$; not shown).

Therefore these in vivo experiments show that nasal afferent inputs play a significant role in sustaining a high respiratory frequency in neonatal mice.

In vitro electrical stimulation of the trigeminal nerve activates the RRG in quiescent ponto-medullary preparations

In vitro, ponto-medullary preparations from P0 to P3 neonatal mice (Fig. 2C) are quiescent and the lack of active nasal afferent inputs may well be accounted for the lack of respiratory activity. In these isolated preparations ($n = 23$), we tested the hypothesis that nasal afferent inputs help the RRG to sustain a high respiratory frequency by recording the phrenic burst occurrences while stimulating the ipsilateral trigeminal nerve (Vth cranial nerve; Fig. 2C) through which the nasal afferent fibers enter the brain stem.

When the central end of the cut left Vth nerve was stimulated every 10–20 min by single trains (30- or 40-s duration, 2–5 Hz) of negative electric shocks (0.7-ms duration, 10–100 μA), rhythmic phrenic bursts were triggered within and after the trains of stimuli (Figs. 2, 5, and 6). For a given ponto-medullary preparation, increasing the intensity of the electric shocks within similar trains (40-s duration, 3 Hz) increased the number of phrenic bursts within the trains and therefore the respiratory frequency (Fig. 2A). In addition, increasing the shock intensity above threshold values that induce phrenic bursts within the train most often triggered rhythmic phrenic bursts persisting $\geq 2$–3 min after the train had ceased (21 of 23 preparations) before the preparations became quiescent. Furthermore, when repetitive trains of stimuli (40-s, 5 Hz; 0.7 ms, 30 μA) were delivered to the Vth nerve in inactive ponto-medullary preparations (4–5 trains in 20–30 min; Fig. 2B1), on-going phrenic bursts were induced at a low frequency (3.2 ± 1.1 c/min; Fig. 2B2) at least up to the termination of the experiments 45–60 min later in 10 of the 14 tested preparations. We have also checked that trains of stimuli applied to the Vth, which triggered rhythmic phrenic bursts, never induced any activity in either of the silent contralateral Vth ($n = 5$) or VIIth ($n = 4$) nerves.

In our experiments, no phrenic burst was triggered at a shock intensity $< 15 \mu$A even for a long train duration ($\geq 60$-s duration). In addition, variations in the number of phrenic burst occurrences, within and after the trains, were seen between preparations for similar trains of stimuli. In this work, the

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relationship between the number of the phrenic burst occurrences and either the train duration and frequency or the shock intensity has not been closely studied as it may well depend on the position of the stimulating extracellular microelectrode on the nerve. In consequence, we have systematically chosen train and shock characteristics able to trigger phrenic bursts within and after the trains.

Attempts were made to activate the silent medullary RRG of

\[ \text{FIG. 1. Sensory afferent inputs from the nasal cavity are required for sustaining a high respiratory frequency in neonatal mice. Breathing of awake mouse neonates is shown as plethysmographic recordings (P3–5 old in A and B) or as rib cage movements (P1–2 old in C) in control (1) and test (2) conditions and the mean respiratory frequency (RF ± SE in cycles/minute: c/min) is displayed in the histograms (C, control; ■, test). A: when a nasal occlusion is performed (n = 8), the mean respiratory frequency decreases and the tidal volume increases (compare the amplitude of the plethysmographic traces in 1 and 2). B: when the nasal afferents are anesthetized by a nasal application of lidocaine (n = 5), the mean respiratory frequency decreases and the tidal volume increases. C: when a continuous positive airway pressure (CPAP, 20 mm of H}_2 \text{O) is applied by a nasal mask (n = 9), the mean respiratory frequency increases while the respiratory movement amplitude decreases. * , statistically significant difference at } P < 0.05. \]
inactive ponto-medullary preparations by direct stimulation within the RVLM in a region known to reset the phrenic rhythm (Viemari et al. 2003) (gray area in Fig. 2C). Single trains (30- to 60-s duration, 2 Hz) of negative electric shocks (30–100 μA, 0.7-ms duration) induced rhythmic phrenic bursts at a frequency ~5 c/min only during the occurrences of the stimulation in all the experiments (n = 5; not shown). In addition, repetitive trains of stimuli (≤5 in 10–20 min) failed to trigger phrenic bursts after the stimulation stopped.

Therefore taken together, these in vitro experiments show that the activation of trigeminal afferent fibers is able to release the inhibition received by the RRG in quiescent ponto-medullary preparations, whereas direct stimulation of the RRG is not able to do so.

In vitro the pontine noradrenergic A5 nuclei inhibit the RRG via the activation of medullary α2 adrenoceptors

It has been shown that the A5 nuclei inhibit the RRG activity in vitro (Errchidi et al. 1990, 1991; Viemari and Hilaire 2002; Viemari et al. 2003). Indeed, in our experiments when the pons was eliminated by section (see Fig. 2C, ↔), on-going rhythmic phrenic bursts were displayed in medullary preparations, and unilateral electrolytic lesion of an A5 nucleus in ponto-medullary preparations induced on-going rhythmic phrenic bursts whose frequency could be doubled by the electrolytic lesion of the contralateral A5 nucleus as shown in Fig. 3, A and B, respectively. Rhythmic phrenic bursts with a mean burst frequency of 6.7 ± 0.5 c/min (n = 15) were immediately induced by elimination of the pons (Fig. 3A, 1 and 2); unilateral electrolytic lesions of an A5 nucleus induced phrenic bursts at a frequency ranging 3–5 c/min (Fig. 3B1; n = 10), whereas bilateral lesions doubled the frequency 7.8 ± 0.7 c/min (Fig. 3B2; n = 7). The mean phrenic burst frequency after ponto-medullary sections and after bilateral lesions of the A5 nuclei was not significantly different.

In addition, noradrenaline (NA) pressure-ejected within an A5 nucleus so as to selectively inhibit the activity of the A5 neurons through the activation of their α2 adrenergic autoreceptors (Andrade and Aghajanian 1982; Huangfu and Guyenet 1997a) is able to trigger a bout of rhythmic phrenic bursts (Viemari et al. 2003) (Fig. 3D). In our preparations, NA ejections within an A5 nucleus induced rhythmic phrenic bursts in all the tested ponto-medullary preparations (n = 7) and for pressure pulses of 2-s duration, the phrenic burst frequency ranged from 2 to 3 c/min for several minutes, then decreased until the preparations became silent (Fig. 3D1); similar effects were seen if similar 2-s pressure pulses were reapplied 5–10 min later. However, the effects of NA seemed dose-dependent since the phrenic burst frequency and the time for the preparations to become silent increased when the duration of the pressure pulses increased (Fig. 3, 2 s in D1, 4 s in D2, and 6 s in D3). Furthermore, in three of the seven preparations when several ejections of NA within an A5 nucleus were repeated up to four to five times within 20–30 min, they induced phrenic bursts—at a frequency ranging from 5 to 7 c/min—which persisted at least up to the termination of the experiments 45–60 min later (not shown), whereas when ACSF was repeatedly pressure-ejected within an A5 nucleus in quiescent ponto-medullary preparations (n = 2), no phrenic burst activity was elicited (not shown). When the α2 adrenoceptor antagonist yohimbine (YOH-ACSF, 50 μM) was applied onto inactive ponto-medullary preparations (Fig. 3E1, n = 5) for 20 min, it induced rhythmic phrenic bursts at a low frequency (2–3 c/min) within 10–15 min after the beginning of the superfusion (Fig. 3E2), and washing the preparations with ACSF led to the disappearance of the phrenic bursts within the same delay. Therefore the activity of the A5 nuclei in isolated ponto-medullary preparations of P0–P3 neonatal mice is involved in the lack of RRG activity.

It has already been demonstrated that in active medullary preparations of mouse neonates, the superfusion of NA-ACSF (25 μM, 5 min) enhances the occurrences of rhythmic phrenic bursts through the activation of medullary α1 adrenoceptors (Viemari et al. 2003). Indeed, in our experiments when the pons was eliminated by section (see Fig. 2C, ↔), on-going rhythmic phrenic bursts were displayed in medullary preparations, and unilateral electrolytic lesion of an A5 nucleus in ponto-medullary preparations induced on-going rhythmic phrenic bursts whose frequency could be doubled by the electrolytic lesion of the contralateral A5 nucleus as shown in Fig. 3, A and B, respectively. Rhythmic phrenic bursts with a mean burst frequency of 6.7 ± 0.5 c/min (n = 15) were immediately induced by elimination of the pons (Fig. 3A, 1 and 2); unilateral electrolytic lesions of an A5 nucleus induced phrenic bursts at a frequency ranging 3–5 c/min (Fig. 3B1; n = 10), whereas bilateral lesions doubled the frequency 7.8 ± 0.7 c/min (Fig. 3B2; n = 7). The mean phrenic burst frequency after ponto-medullary sections and after bilateral lesions of the A5 nuclei was not significantly different.
and Hilaire 2002) (Fig. 4B). Here we show that the medullary α2 adrenoceptors are also involved in the regulation of the RRG activity. In active medullary preparations, we compared the effects of NA applications (25 μM; 5 min) when the preparations were superfused either by ACSF (n = 8) or by the α2 adrenoceptor antagonist piperoxane (50 μM; PIP-ACSF; n = 5). NA applications either alone (NA-ACSF; Fig. 4B) or with piperoxane (PIP-ACSF + NA; Fig. 4, A and B) significantly increased the phrenic burst frequency. However, the blockage of the medullary α2 adrenoceptors by piperoxane potentiated a phrenic burst frequency increase: during the fifth minute of NA applications, the phrenic burst frequency reached 171 ± 11% of the mean control value when ACSF was applied alone and 234 ± 21% of the mean control value when PIP-ACSF was applied in control condition (Fig. 4B). Therefore NA application alone activates the RRG via a facilitation through medullary α1 (Viemari and Hilaire 2002) and/or a release of inhibition through medullary α2 adrenoceptors, this last being the only one in rats (Errchidi et al. 1990, 1991). The blockade of the medullary α2 adrenoceptors releases the inhibition received by the RRG and thus increases the phrenic burst frequency.

To test whether the medullary α2 adrenoceptors that release the inhibition of the RRG involved medullary release of NA by A5 neuron terminals, a physical barrier was placed over the brain stem at the ponto-medullary junction in inactive ponto-medullary preparations (n = 15). Surprisingly, setting it induced rhythmic phrenic bursts at a low frequency (1–3 c/min) in 12 of the 15 preparations. When the medulla was superfused with α2 adrenoceptor antagonist piperoxane (50 μM; 5 min) while the pons was with ACSF, the three inactive preparations displayed rhythmic phrenic bursts at a low frequency (2–4 c/min), and the phrenic burst frequency of the 12 active ones significantly increased during the last 3 min of piperoxane applications (163 ± 12% of the previous mean frequency; Fig. 4C). In addition, when rhythmic phrenic bursts were induced by the electrolytic lesions of both the A5 nuclei in previously inactive ponto-medullary preparations (n = 4), the applications of PIP-ACSF (5 min) onto the medulla and of ACSF onto the pons did not significantly alter the mean phrenic burst frequency (Fig. 4D). Therefore in ponto-medullary preparations, medullary terminals from the pontine A5 neurons release NA, which silences the RRG through the activation of medullary α2 adrenoceptors.

It is worth noting that NA applications, when the medullary α2 adrenoceptors were blocked, induced a specific respiratory activity (4 of 5 preparations; Fig. 4A) in which phrenic bursts fired in rhythmical groups of three- to five (at a frequency between 20 and 30 c/min inside the groups) separated by long-lasting expiratory pauses (~10–15 s) similar to that obtained with NA applications alone (5 of 8 preparations) as it has been shown by Viemari et al. (2003). Therefore our experiments confirmed that this firing pattern results from the activation of medullary α2 adrenoceptors by NA.

However, as, in vitro, the activity of the A5 nuclei is able to silence the RRG, whereas the Vth afferent inputs activate it, the question which arises is whether the Vth afferent pathways to the RRG include connections through the A5 nuclei that can release the A5 inhibition received by the RRG.

**A5 nuclei are involved in the respiratory response to trigeminal nerve stimulation**

In inactive ponto-medullary preparations, the electrical stimulation of the Vth nerve elicits rhythmic phrenic bursts. To know whether the A5 nuclei were implicated in this respiratory response, we performed an unilateral electrolytic lesion of the left A5 nucleus so as to trigger rhythmic phrenic bursts (2.7 ± 0.4

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*Images and diagrams not included in the text.*
FIG. 4. The medullary $\alpha_2$ adrenoceptors mediate the A5 inhibition received by the respiratory rhythm generator (RRG). A: the respiratory activity in medullary preparations is shown as the integrated inspiratory activity of recordings from the C1 phrenic nerve. When the preparation is perfused with piperoxane, an $\alpha_2$ adrenoceptor antagonist, dissolved in ACSF (PIP-ACSF, 50 $\mu$M; $\lambda$–3) the addition of NA (25 $\mu$M, 5 min; 2) to the perfusion medium produces an increase in the phrenic burst frequency and rhythmical groups of phrenic bursts. B: the histograms show the mean variations ($\pm$ SE) of the phrenic burst frequency (PBf) as % of the control values (control medium is either ACSF or PIP-ACSF, white bars) for the 5 min of NA applications. When NA is applied in ACSF (NA-ACSF), the mean phrenic burst frequency (gray bar) significantly increases to 174% of the mean control value ($n$ = 8). When NA is applied in PIP-ACSF, the mean phrenic burst frequency (black bar) significantly increases to 234% ± 21% of control value ($n$ = 5). Therefore the blockage of the medullary $\alpha_2$ adrenoceptors unmasks their inhibitory effects on the RRG (compare gray and black bars). C and D: after setting a barrier at the ponto-medullary junction, PIP-ACSF was perfused onto the medulla for 5 min, whereas ACSF was onto the pons. The histogram shows the mean phrenic burst frequency (PBf ± SE) every minutes as % of the control values (100% is the average frequency of the phrenic bursts over the 5 min prior to the PIP applications) when the A5 nuclei are intact (C, $n$ = 12) or when bilateral lesions of the A5 nuclei are performed (D, $n$ = 4). PIP (black bars) significantly increases the phrenic burst frequency during the last 3 min of its application and during the 1st minute of washing the medulla with ACSF only when the A5 nuclei are intact. *, statistically significant difference at $P < 0.05$.

c/min) in previously inactive ponto-medullary preparations ($n$ = 5). Single trains of stimuli (30-s, 4 Hz; 30 $\mu$A, 0.7 ms) applied to the right Vth nerve (ipsilateral side to the phrenic nerve recordings; Fig. 5A1) significantly increased the phrenic burst frequency during the stimulation (5.8 ± 0.8 c/min; ~214% of the mean control value). After the cessation of the stimulation, the phrenic burst frequency remained facilitated for 2–3 min with a mean phrenic burst frequency significantly increased during the first minute after the stimulation (4.7 ± 0.5 c/min; ~174% of the mean control value). In every preparation, an electrolytic lesion of the contralateral A5 nucleus was performed to eliminate the remaining A5 inhibition received by the RRG; consequently, the phrenic burst frequency rose to 7.2 ± 1.1 c/min. When single trains of stimuli (35-s, 4 Hz; 30 $\mu$A, 0.7 ms) were then applied to the right Vth nerve (Fig. 5A2), they significantly increased the phrenic burst frequency during the stimulation (10.9 ± 1.2 c/min; ~151% of the mean control value) although the phrenic burst frequency returned to the mean control value within the first minute after the stimulation (6.9 ± 1.1 c/min; ~95% of the mean control value).

To test that the stimulated Vth afferent fibers were not damaged by the electrolytic lesion of the A5 nucleus ipsilateral to the Vth nerve stimulation, we pressure-ejected NA into this intact nucleus (Fig. 5B2) in two active ponto-medullary preparations obtained by the lesion of the contralateral A5 nucleus (Fig. 5B1). As previously, the poststimulus response to the Vth nerve stimulation (40-s, 4 Hz; 30 $\mu$A, 0.7 ms) was suppressed although it reappeared ~20 min later. In addition, when inactive ponto-medullary preparations (Fig. 5C1; $n$ = 4) were superfused by the $\alpha_2$ adrenoceptor antagonist yohimbine (YOH-ACSF, 50 $\mu$M), the poststimulus response to the Vth nerve stimulation (40-s, 2 Hz; 30 $\mu$A, 0.7 ms) was also suppressed (Fig. 5C2).

These experiments suggest that the A5 nuclei are implicated in some parts of the respiratory response to trigeminal nerve stimulation, that is in maintaining an increased phrenic burst frequency after the offsets of the Vth nerve stimulation.

MLRt is also involved in the respiratory response to trigeminal nerve stimulation

ELECTRICAL ACTIVATION OF THE MLRT MIMICS VTH NERVE STIMULATION. In inactive ponto-medullary preparations ($n$ = 10), single trains (30-s, 5 Hz) of negative electric shocks (40 $\mu$A, 0.7 ms) applied either within the MLRt (Fig. 6A1) or to the
Vth nerve (Fig. 6A2)—ipsilateral to the phrenic nerve recordings—induced rhythmic phrenic bursts during the stimulation that persisted for 2–3 min after the cessation of the stimulation. In addition, repetitive trains of stimuli (4–5 trains in 20–30 min), applied within the MLRt in six of seven tested preparations, induced phrenic bursts at a low frequency (4.1 ± 0.9 c/min) at least up to the termination of the experiments 45–60 min later (not shown). In three of the preparations, single trains of stimuli applied to the Vth nerve when the ipsilateral MLRt was destroyed by electric coagulation (Fig. 6A3) did not induce phrenic nerve activity, suggesting that MLRt is implicated in an afferent pathway between trigeminal afferent fibers and the RRG.

MLRt contains neurons belonging to the respiratory network. The *trans*-neuronal infection property of the rabies virus was used to localize the neurons belonging to the respiratory network of mouse neonates (*n* = 8) with the diaphragm as port of entry. After 2 days of infection (Fig. 6C), bilaterally well-defined groups of infected neurons were present within the medulla. In addition to the RVLM neurons, a well-infected group of neurons was seen in the MLRt located under the trigeminal nucleus and clearly separated from the respiratory group of neurons of the RVLM although some dendritic projections from both RVLM and MLRt neurons overlapped.

Extracellular unitary recordings from 35 neurons within the
MLRt were performed in medullary preparations \((n = 7)\). Among these, 15 fired rhythmic phasic bursts of action potentials timed with the phrenic bursts (Fig. 6B1) and 20 fired tonically at a frequency between 1 and 5 Hz, some of them presenting a slight increase in frequency during the occurrence of the phrenic bursts (Fig. 6B2). In addition, in another set of medullary preparations, applying single trains (30-s, 5 Hz) of negative electric shocks (40 µA, 0.7 ms) ipsilateral to the phrenic nerve recording to either the MLRt (1) or to the Vth nerve (2) produces a similar respiratory activity (i.e., rhythmic phrenic bursts occurring during and after the offset of the stimulation), whereas similar Vth nerve stimulation when the ipsilateral MLRt is electrolytically lesionned (3) does not induce any phrenic nerve activity. B: extracellular unitary recordings (bottom traces) of neurons in the MLRt show neurons that display phase discharge patterns locked in phase with the inspiratory phrenic bursts (1) and neurons that display tonic discharge patterns with a slight increase in firing frequency locked in phase with the inspiratory phrenic bursts (2). C: in the histological section of the medulla, the rabies virus infected neurons in the MLRt and the RVLM (d, dorsal; l, lateral) show that they belonged to the respiratory network.

**DISCUSSION**

In this study on mouse neonates, our results show the following. 1) In vivo the RRG is spontaneously active, which is necessary to sustain life, and its output activity can be modified by the stimulation of nasal afferents that produces a sustained respiratory frequency. 2) In vitro ponto-medullary brain stem-spinal cord preparations are inactive due to a powerful inhibition of the RRG arising from the pontine A5 nuclei. 3) This inhibition, mediated by medullary \(\alpha_2\) adrenergic receptors, is released either by the inactivation or the lesion of the A5 neurons or by the stimulation—ipsilateral to the phrenic nerve recording—of either the trigeminal nerve or the medullary lateral reticular area. In addition 4), we show that the medullary lateral reticular area contains respiratory network-connected neurons, some of them displaying inspiratory-modulated firing patterns, whose activity is not necessary for the RRG to elicit rhythmic phrenic bursts when the A5 inhibition is suppressed.

**Nasal afferent inputs participate in the regulation of the respiratory frequency in mouse neonates**

Except for its motor branch, the Vth cranial nerve (trigeminal nerve) conveys sensory information from the nose, the mouth, and the oro-facial regions through different types of sensory afferents whose activation induces autonomic responses. Of interest in this study are the sensory afferents originating from the nose apart from the olfactory ones. Nasal nociceptors, when stimulated, induce cardiorespiratory responses (Dutschmann and Herbert 1996, 1998; Dutschmann and Paton 2002; McCulloch and Panneton 1997; McCulloch et al. 1999a,b; Panneton 1990), which include apnea and bradycardia. In the rat, these trigeminal afferents project to brain stem nuclei involved in cardiovascular and respiratory responses (Caous et al. 2001; Dutschmann and Herbert 1996, 1998; Panneton 1991; Panneton et al. 2000). The stimulation of nonnociceptive nasal afferents also induce respiratory responses. Airflow-sensitive receptors, which are active during inspiration and silent during nasal occlusion, elicit variations of the respiratory frequency (Sant’Ambrogio et al. 1995; Sekizawa et al. 1996; Tsubone 1989). They may be implicated in the sneeze reflex (Sant’Ambrogio et al. 1995), although this reflex is mainly induced by the activation of the nasal mechano-sensitive afferents (Sekizawa and Tsubone 1996;
Wallos et al. 1991), which modify the nasal resistance during nose breathing and both the respiratory pattern and the nose airflow during sneezing (Sekizawa et al. 1998). Nasal-pressure-sensitive afferents respond to either negative or positive pressure applied to the upper airway (Tsubone 1990). When delivered by a facial mask, negative airway pressure increases the inspiratory effort (Meessen et al. 1994) and the respiratory frequency by decreasing the expiratory time (Hirsch and Bishop 1981), whereas positive airway pressure decreases the respiratory frequency and the tidal volume (Durand et al. 1983). The activation of these afferents by positive pressure helps to maintain the upper airway patency (Mathew et al. 1982; Tsubone 1990) and can be used to treat sleep obstructive apnea (Massa et al. 2002; Stepnowky and Moore 2003). Nasal occlusion (which suppresses airflow afferent activity) leads through an increase of successive inspiratory efforts (which may stimulate other kinds of nasal afferents such as pressure- or mechano-sensitive ones) to oral breathing (de Almeida et al. 1994; Harding 1986; Harding et al. 1991; Mathew et al. 1982) with a decreased respiratory frequency (de Almeida et al. 1994; Mathew et al. 1982).

Our observations show that the mouse neonates that are normally nose breathers switch to oral breathing during nasal occlusion with a reduced respiratory frequency which is half that of infants (de Almeida et al. 1994), and with an increased tidal volume. Local anesthesia of nasal afferents, which blocks their activation, reduces the respiratory frequency, whereas saline application into the nasal cavity or snout anesthesia has no effect. Therefore when nasal afferent inputs are reduced (occlusion) or suppressed (anesthesia), the respiratory activity is depressed, whereas a low continuous positive airway pressure, which stimulates the nasal nonnociceptive afferents, produces the opposite effect namely an increase of the respiratory frequency and a reduced amplitude of the rib cage movements. However, in these intact neonates, a high continuous positive airway pressure induces a drastic reduction of the respiratory activity that may arise from pulmonary stretch-receptor reflex to protect the lungs from inflation (Burnet and Hilaire 1999).

Therefore in vivo the activity of nasal trigeminal afferents, probably arising from the nonnociceptive ones which are normally stimulated in nonthreatening conditions, seems to help the respiratory rhythm generator to sustain a high-frequency breathing activity.

**Trigeminal inputs facilitate the RRG of mouse neonate in vitro**

In vitro, when the Vth nerve is stimulated by single trains of electric shocks in quiescent ponto-medullary preparations, the RRG is transiently activated as shown by a sequence of rhythmic phrenic bursts, whereas when repetitive trains are delivered over a short-time period, the RRG displays a long-lasting activation shown by on-going rhythmic phrenic bursts.

First, the induced phrenic bursts may have nonrespiratory functions as the diaphragm is implicated in other behaviors such as suckling, swallowing, and vomiting (Grelot et al. 1992; Larson et al. 1994; Montague and Hilaire 1991; Ono et al. 1998). In brain stem preparations of neonatal rats, motor activities can be recorded from the Vth, the VIIth, and the XIIth cranial nerves, which correspond either to specific oral-motor activities or respiratory activity (Katakura et al. 1995; Koizumi et al. 1999, 2002; Tanaka et al. 1999). As, under our experimental conditions, nerve activity in the Vth or VIIth cranial nerves was never recorded spontaneously or in response to the contralateral Vth nerve stimulation, this suggests that our Vth nerve stimulation very probably activated neuronal pathways that impinge on the respiratory network.

Second, as the Vth nerve afferents that we stimulated in our experimental conditions only produced an increase in the respiratory frequency in either quiescent or active preparations, we may suggest that either the stimulating electrode position and/or the strength of the electric socks have recruited nonnociceptive trigeminal afferents, although the implication of other Vth afferents cannot be excluded.

Third, the long-lasting rhythmic phrenic bursts in response to repetitive Vth nerve stimulation may have arisen from the activation of nervous structures that have a modulatory effect on the RRG as Vth afferents project to spinal trigeminal nuclei whose neurons relay information to nuclei which are involved in autonomic responses (Allen et al. 1996; Caous et al. 2001; Dawid-Milner et al. 2001; Dutschman and Herbert 1996, 1998; Esser et al. 1998; McCulloch and Panneton 1997; Panneton 1991; Panneton et al. 2000) such as the brain stem noradrenergic nuclei (e.g., A5 nuclei), the Kölliker-Fuse, and the parabrachial nuclei.

**Noradrenergic A5 nuclei exert a potent inhibition of the RRG activity through medullary α2 adrenoceptors**

In rats, the A5 neurons are spontaneously active in vitro (Huangfu and Guyenet 1997b) although their activities can be inhibited by the activation of their somatic α2 autoreceptors (Andrade and Aghajanian 1982; Huangfu and Guyenet 1997a). In vitro, the A5 neurons exert an inhibitory modulation on the neonatal rat RRG through medullary α2 adrenoceptors (Err-chidi et al. 1990, 1991; Hilaire et al. 1989), but this inhibition is not strong enough to prevent respiratory rhythogenesis in ponto-medullary preparations. On the contrary in mice, the A5 neurons exert a very potent inhibition on the neonatal mouse RRG as no respiratory rhythmogenesis occurs in ponto-medullary preparations. On the other hand, the suppression of the A5 nuclei (either by electrolytic lesions or by pons elimination) also releases the A5 inhibition and allows the on-going respiratory rhythmogenesis. We do not yet know the mechanism by which the A5 inhibition is released for long periods. However, it is known that both exogenously applied and synaptically released NA induce long-term facilitation at cerebellar synapses (Mitoma and Koshishi 1999) and that the A5 neurons participate in long-lasting respiratory responses such as those following acute hypoxia (Coles and Dick 1996) and bilaterally carotid sinus nerve transection (Roux et al. 2000). Therefore we suggest that the Vth afferents may be involved in a pathway that induces a long-lasting modulation of the A5 neurons; this produces a long-term facilitation of the RRG activity.
In medullary preparations, direct applications of NA onto the preparations produce a phrenic burst frequency decrease in rats through the activation of RRG medullary \( \alpha_1 \) adrenoceptors (Errchidi et al. 1990, 1991), whereas they produce a phrenic burst frequency increase in mice through the activation of RRG medullary \( \alpha_2 \) adrenoceptors (Viemari and Hilaire 2002; Viemari et al. 2003). In mice, however, we show here that the phrenic burst frequency increase, in response to NA application, is further enhanced after the blockage of the medullary \( \alpha_2 \) adrenoceptors, suggesting the implication of both facilitatory \( \alpha_1 \) and inhibitory \( \alpha_2 \) adrenoceptors in the regulation of mouse neonatal breathing. In addition, we report that in mouse ponto-medullary preparations, the application of an \( \alpha_2 \) adrenoceptor antagonist onto the medulla increases the phrenic burst frequency solely when the A5 nuclei are intact. These results show that the A5 neurons release endogenous NA that inhibits the RRG through the activation of medullary \( \alpha_2 \) adrenoceptors in mice as well as in rats.

Since we demonstrated that the A5 neurons exert an inhibitory modulation on the in vitro RRG of neonatal rats (Errchidi et al. 1990, 1991; Hilaire et al. 1989), it has also been shown for the fetal mouse RRGs in vitro (Viemari et al. 2003) and for the adult rat RRG in vivo. Indeed in adult rats, the A5 neurons, which are connected to the respiratory network (Dobbins and Feldman 1994), fire with respiratory-modulated discharge patterns, mostly postinspiratory ones (Guyenet et al. 1993), and their activation lowers the respiratory frequency (Dawid-Milner et al. 2001; Jodkowski et al. 1997). Furthermore, the A5 neurons are implicated in the respiratory responses triggered by hypoxia (Coles and Dick 1996; Dick and Coles 2000; Guyenet et al. 1993; Roux et al. 2000) and hypercapnia (Haxhiu et al. 1996) and in the cardiorespiratory responses induced by the stimulation of nociceptive trigeminal afferents (Allen and Pronych 1997; Dutschmann and Paton 2002; Esser et al. 1998; McCulloch and Panetton 1997).

In vitro trigeminal afferent inputs modulate the A5 inhibition received by the RRG

In ponto-medullary preparations of mouse neonates, the pontine A5 nuclei seem responsible for the inactivity of the RRG as their elimination or inactivation releases the RRG activity (see RESULTS) (Viemari and Hilaire 2002; Viemari et al. 2003). However, trigeminal afferent inputs are able to activate the RRG so that the respiratory activity is increased in vivo or elicited in vitro and therefore may interact with the A5 neuronal pathway. Indeed when the A5 nuclei are intact, the stimulation of the Vth nerve is able to trigger a sequence of rhythmic phrenic bursts in silent ponto-medullary preparations both during and after the stimulation. In addition, when the A5 nucleus, ipsilateral to the nerve stimulation, is intact while the contralateral A5 nucleus is lesioned, the Vth nerve stimulation induces an increased phrenic burst response both during and after the stimulation, whereas when the A5 nuclei are lesioned, the increased phrenic burst in response to the Vth nerve stimulation occurs only during the stimulation. This suggests that the trigeminal afferent inputs are able to activate the RRG through at least two neuronal pathways, one that produces a short-term activation of the RRG whether the A5 nuclei are intact or not and another one that produces a long-lasting release of the A5 inhibition received by the RRG. The latter may arise from the second-order sensory neurons within the trigeminal sensory nuclei that project to the A5 nucleus where the neurons would be silenced or to the RRG where the A5 inhibition would be released at the level of the medulla. These hypotheses may be supported by anatomical data showing projections of sensory neurons within the trigeminal sensory nuclei to both the A5 nuclei and the medullary reticular formation (Caous et al. 2001; Panetton 1990; Panetton et al. 2000).

MLRt may be involved in the respiratory response to the Vth nerve simulation

Within the MLRt, we show a new group of neurons, anatomically connected to the respiratory network, which are probably different from the para-facial respiratory neurons, recently described in neonatal rats (Onimaru and Homma 2003). First, MLRt neurons are located more laterally and caudally within the medulla; second, they display inspiratory-modulated firing patterns and third, their elimination does not suppress the respiratory rhythm in medullary preparations. Yet these neurons may be involved in an afferent pathway between the Vth afferent fibers and the RRG as the MLRt stimulation induces a respiratory response during and after the stimulation in a similar way as the Vth nerve stimulation does and as the MLRt lesion suppresses the respiratory response to Vth nerve stimulation. On the one hand, one cannot exclude that the MLRt lesion has damaged Vth afferent fibers and that the MLRt stimulation has not activated dendritic extensions of RVLM neurons, local interneurons, and/or Vth afferent fibers. On the other hand, the implication of MLRt neurons in this pathway may be supported by anatomical data showing projections from Vth afferents to the medullary reticular formation (Caous et al. 2001; Panetton 1990; Panetton et al. 2000). In addition, the mechanical activation of some of the MLRt neurons when a physical barrier is set over the brain stem at the ponto-medullary junction may explain why previously silent ponto-medullary preparations display rhythmic phrenic bursts.

In conclusion, trigeminal afferents, MLRts and A5 neurons seem linked together to define the activity level of the RRG. In vitro the total absence of Vth nerve afferent inputs may facilitate the A5 neuron activity, leading to the silencing of the RRG. The results lead us to the hypothesis that the activation of the RRG by the trigeminal afferents in these neonatal mouse preparations involves at least two pathways: one that activates the RRG and may act through the MLRt and one that releases the A5 inhibition received by the RRG. However, only intracellular recordings from the different neurons will help to clarify their connections and their implications in the regulation of the RRG activity.

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