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Activation of alpha-2 noradrenergic receptors is critical for the generation of fictive eupnea and fictive gasping inspiratory activities in mammals *in vitro*

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Abstract

Biogenic amines are not just 'modulators', they are often essential for the execution of behaviors. Here, we explored the role of biogenic amines acting on the pre-Bötzinger complex (pre-BötC), an area located in the ventrolateral medulla which is critical for the generation of different forms of breathing. Isolated in transverse slices from mice, this region continues to spontaneously generate rhythmic activities that resemble normal (eupneic) inspiratory activity in normoxia and gasping in hypoxia. We refer to these as 'fictive eupneic' and 'fictive gasping' activity. When exposed to hypoxia, the pre-BötC transitions from a network state relying on calcium-activated nonspecific cation currents (I_{CAN}) and persistent sodium currents (I_{Nap}) to one that primarily depends on the I_{Nap} current. Here we show that in inspiratory neurons I_{Nap} -dependent bursting, blocked by riluzole, but not I_{CAN} -dependent bursting, required endogenously released norepinephrine acting on alpha2-noradrenergic receptors ($\alpha 2$ -NR). At the network level, fictive eupneic activity persisted while fictive gasping ceased following the blockade of $\alpha 2$ -NR. Blockade of $\alpha 2$ -NR eliminated fictive gasping even in slice preparations as well as in inspiratory island preparations. Blockade of fictive gasping by $\alpha 2$ -NR antagonists was prevented by activation of 5-hydroxytryptamine type 2A receptors (5-HT2A). Our data suggest that gasping depends on the converging aminergic activation of 5-HT2AR and $\alpha 2$ -NR acting on riluzole-sensitive mechanisms that have been shown to be crucial for gasping.

Keywords

A1C1 neurons; gasping; norepinephrine; pacemaker; respiration

Introduction

Neuromodulators are critical for maintaining network activity and synchrony, and for determining the configuration of neuronal networks (Nadim *et al.*, 2008; Doi & Ramirez, 2008; Grashow *et al.*, 2009; Carrillo-Reid *et al.*, 2009; Viemari & Tryba, 2009). The mammalian respiratory network located within the pre-Bötzinger complex (pre-BötC) is essential for breathing (Feldman & Del Negro, 2006; Tan *et al.*, 2008), and it is ideal for studying the mechanisms underlying neuromodulation (Doi & Ramirez, 2008). When

isolated in transverse slices or inspiratory island preparations, the pre-BötC continues to generate inspiratory rhythmic activity (Johnson *et al.*, 2001; Smith *et al.*, 1991).

Quantitative characterizations of the population activity generated in transverse slice preparations reveal that these activities resemble three distinct forms of inspiratory activities: fictive eupneic, fictive gasping and fictive sigh activity (Lieske *et al.*, 2000; Ruangkittisakul *et al.*, 2008). During the transition from normoxia into hypoxia, this portion of the respiratory network is reconfigured into a rhythm that has many of the characteristics of gasping (Lieske *et al.*, 2000). During this reconfiguration, mechanisms of synaptic inhibition are significantly reduced and this is thought to contribute to (i) the loss of expiratory activity (Ramirez *et al.*, 1997), (ii) the reconfiguration of post-inspiratory activity into inspiratory activity (Ramirez *et al.*, 1997), (iii) the change in inspiratory activity from a bell-shaped to a decrementing activity pattern (Lieske *et al.*, 2000) and (iv) the release of bursting properties in a subpopulation of pacemaker neurons (Tryba *et al.*, 2003). The only pacemaker neurons that remain active throughout hypoxia are those in which bursting depends on the persistent sodium current (I_{NaP}); these so-called cadmium-insensitive (CI) pacemaker neurons are also sensitive to riluzole (Peña *et al.*, 2004). Interestingly, gasping, but not fictive eupnea is sensitive to riluzole, suggesting that these CI pacemaker neurons become the sole driver of gasping (Peña *et al.*, 2004). Consistent with this hypothesis is the observation that gasping in the arterially-perfused brainstem and *in vivo* preparations is also abolished by riluzole (Paton *et al.*, 2006; Peña & Aguileta, 2007; Peña, 2009). Based on these combined cellular and systems level findings we proposed that blockade of either the calcium-activated nonspecific cation current (I_{CAN}) or I_{NaP} alone is not sufficient to block inspiratory rhythm generation at the network level (Peña *et al.*, 2004). Our discovery that bursting in CI pacemaker neurons depends on endogenous activation of 5-HT_{2A}R, and that blockade of 5-HT_{2A}R is sufficient to abolish fictive gasping, was consistent with this hypothesis (Peña *et al.*, 2004; Tryba *et al.*, 2006). However, our findings were not reproduced in the perfused model (Toppin *et al.*, 2007). This discrepancy may be explained by the hypothesis that gasping depends on more than one neuromodulator and that blocking only one modulatory mechanism is not sufficient to eliminate this activity. Here, we demonstrate that bursting in CI pacemaker neurons and fictive gasping are also dependent on α 2- noradrenergic receptor (α 2-NR) activation. We propose that noradrenergic and serotonergic inputs converge on riluzole-sensitive mechanisms that are critical for the generation of gasping.

Materials and methods

Medullary transverse slice preparation and inspiratory island preparation

Brainstem transverse slice preparation from CD1 mice [home-bred; postnatal day (P)6–12] were prepared using a technique previously described in detail (Ramirez *et al.*, 1996). The most important steps are summarized here. All surgical and experimental procedures conformed to guidelines from the French Ministry for Agriculture and Fisheries and the Institutional Animal Care and Use Committee (IACUC) at the Seattle Children's Research Institute. The mice were quickly decapitated. The isolated brainstem was then placed in ice-cold artificial cerebrospinal fluid (aCSF) bubbled with carbogen (95% O₂ and 5% CO₂). The aCSF contained (in mM): NaCl, 128; KCl, 3; CaCl₂, 1.5; MgCl₂, 1; NaHCO₃, 24; NaH₂PO₄, 0.5; and D-glucose, 30; pH 7.4. The brainstem, glued rostral end up onto an agar block, was mounted in a vibratome (Leica Microsystems, Waukegan, IL, USA) and serially sliced until the rostral boundary of the preBötC (Smith *et al.*, 1991) was identified by anatomical landmarks such as disappearance of the facial nucleus and appearance of the inferior olive, the nucleus ambiguus and the hypoglossal nucleus. A single 650- μ m-thick slice was then taken. Because the slices also contained regions caudal to the pre-BötC we refer to the area encompassed in the slice as the ventral respiratory group (VRG). Slices

were transferred into a recording chamber, continuously superfused with oxygenated aCSF and maintained at a temperature of 30 ± 0.5 °C. To initiate and maintain fictive inspiratory rhythmic activity the potassium concentration of the perfusate was raised from 3 to 8 mM over 30 min. Only one slice was obtained per animal.

The VRG island preparation (Johnson *et al.*, 2001) was made by taking a rhythmically active slice preparation (650 μ m) and micro-dissecting with a sharp razor blade to give a wedge-shaped piece of tissue that contained the pre-BötC and the surrounding VRG.

Hypoxic conditions

Fictive gasping was induced by an hypoxic stimulus in the following manner: the incoming bath aCSF, which was saturated with carbogen during control conditions, was made hypoxic by saturating it with 95% N₂ and 5% CO₂ (Lieske *et al.*, 2000; Tryba *et al.*, 2006; Hill *et al.*, 2011). In this study we refer to this stimulus as ‘application of hypoxic aCSF’.

After the slice was stabilized for 30 min under baseline conditions we exposed the slice to the hypoxic aCSF for 10 min. After 10 min of hypoxic aCSF, the inspiratory network activity was allowed to recover for 30 min to baseline conditions in aCSF equilibrated with 95% O₂ and 5% CO₂. This recovery period is based on a protocol used in previous studies (Blitz & Ramirez, 2002; Tryba *et al.*, 2006). Using this protocol, fictive gasping activity generated in two consecutive 10-min applications of hypoxia were not significantly different when separated by a 30-min recovery in oxygenated aCSF (Tryba *et al.*, 2006). In the present study, we performed the following protocol: after recovery from the first hypoxic exposure we applied yohimbine for 10 min and then exposed the slice to the second hypoxic exposure in the continued presence of yohimbine.

Fictive gasping has been characterized by a short rise time and short burst duration compared to fictive eupnea (Lieske *et al.*, 2000). The transition from eupnea into gasping can be gradual, both *in vitro* (Lieske *et al.*, 2000) and *in vivo* (Wang *et al.*, 1993). During this transition, bursts with varying rise time and burst duration are typical, a characteristic which is referred to as ‘pre-gasping’ *in vivo* (Wang *et al.*, 1993). In the present study we evaluated the effect of yohimbine on gasping and not on the transitory phase that characterizes pre-gasping. For this reason we evaluated the effect of yohimbine on the gasping activity measured during the last 2 min of hypoxic aCSF exposure at a time when the rise-time as well as burst duration were significantly different from fictive eupnea.

Drugs and solutions

A cocktail of antagonists for NMDA receptors [CPP-(RS), 10 μ M; Tocris Cookson, Ellisville, MO, USA], non-NMDA receptors (CNQX, 20 μ M; Tocris Cookson), glycine receptors (strychnine, 1 μ M; SIGMA-RBI, St Louis, MO, USA) and GABA_A receptors (bicuculline free base, 20 μ M; SIGMA-RBI) was used to block fast synaptic transmission (Peña *et al.*, 2004). Note that the bicuculline free base used in the present study has a very different pharmacology than the commonly used bicuculline salts (e.g. bicuculline methiodide), and the free base does not block apamin-sensitive calcium-activated potassium currents (Debarbieux *et al.*, 1998; Seutin & Johnson, 1999). This cocktail does not block neuromodulator-evoked influences on bursting of the neurons. All drugs were initially solubilized in dimethylsulfoxide (DMSO; SIGMA-RBI). In some experiments yohimbine hydrochloride (SIGMA-RBI) rauwolscine, RX 821002 and / or DOI [(±)-2,5-dimethoxy-4-iodophenyl-2-aminopropane hydrochloride] was added to aCSF. Each drug was applied only once in a given experiment.

Electrophysiology

Extracellular recordings—In the transverse slice preparation, population activity recordings were obtained with suction electrodes positioned on the surface of the slice in the area of the nucleus ambiguus, i.e. dorsal to the pre-BötC. The signals were amplified 2000 times, filtered (low-pass 1.5 KHz, high-pass 250 Hz), rectified and integrated using an electronic filter (time constant of 30–50 ms). Integrated population activity from the VRG was always in phase with integrated inspiratory activity of the hypoglossal motor nucleus (Telgkamp & Ramirez, 1999). Therefore, it was used as a marker for inspiratory population activity (Fig. 1A). In the present study we refer to the activity generated within the transverse slice preparation and the island preparation as ‘inspiratory’ to reflect the fact that the population activity generated within the pre-BötC in slices is in phase with inspiratory activity. However, calling the activity ‘inspiratory’ does not mean that all neurons within the pre-BötC are inspiratory. Both *in vivo* and *in vitro* studies have demonstrated that the pre-BötC also contains other types of respiratory neurons including neurons rhythmically active in phase with expiration and post-inspiration (Ramirez & Lieske, 2003; Ramirez *et al.*, 1997; Schwarzacher *et al.*, 1995).

Intracellular recordings—We recorded inspiratory pre-BötC neurons (one neuron per slice) using the blind patch-clamp recording method. The slice preparation was placed rostral side up and recordings were made from neurons that were no deeper than 300 μm from the surface. Inspiratory neurons were first identified in the cell-attached mode, which revealed their discharge pattern in phase with population activity. Experiments were then performed in whole-cell configuration with the neuron recorded in current-clamp (Holding current, 0 nA). We have previously demonstrated that the whole-cell configuration does not alter the firing pattern of the recorded neuron (Peña *et al.*, 2004). In the present study we characterized the effect of yohimbine on pacemaker neurons. As we published previously (e.g. Tryba *et al.*, 2008, 2003; Thoby-Brisson & Ramirez, 2001), neurons are considered pacemakers if they continue to exhibit voltage-dependent intrinsic bursting properties following blockade of ionotropic glutamate, GABAA and glycinergic receptors. To be considered a pacemaker these neurons must meet several criteria. Although we have already provided these criteria in detail elsewhere (Tryba *et al.*, 2008, 2003; Thoby-Brisson & Ramirez, 2001), we briefly describe the key attributes for identifying pacemakers. Pacemaker neurons often exhibit ectopic bursting when embedded in the inspiratory network, as exemplified in Ramirez *et al.* (2004). After isolating a neuron from chemical synaptic input by bath-applying CNQX, CPP, strychnine and bicuculline, a neuron is considered a potential pacemaker if it: (i) continues to burst in the absence of VRG population bursts; and (ii) exhibits voltage-dependent bursting properties. Specifically, a brief depolarizing current injection needs to evoke a burst or a hyperpolarizing current needs to terminate an ongoing burst. It is expected that either of these reset the ongoing pacemaker bursting rhythm. Depolarizing current injection increases, and injected hyperpolarizing current decreases, the bursting frequency. For examples of voltage dependency in inspiratory pacemaker neurons, the reader is referred to figures and experiments already published in Tryba *et al.* (2008, 2003, 2006); Viemari & Ramirez (2006); Thoby-Brisson & Ramirez (2001). After synaptic isolation with CNQX, CPP, bicuculline and strychnine, we routinely use cadmium (Cd^{2+}), a broad-spectrum calcium channel blocker, to discriminate between different types of pacemaker neurons. Two basic types of inspiratory bursting pacemaker neurons can be discriminated based on their sensitivity to cadmium (Thoby-Brisson & Ramirez, 2001; Peña *et al.*, 2004): Neurons in which bursting is abolished by 200 μM Cd^{2+} are referred to as cadmium-sensitive (CS) pacemaker neurons (Fig. 2); these neurons have been shown to be typically flufenamic acid (FFA)-sensitive (Peña *et al.*, 2004). Neurons that continue to burst in 200 μM Cd^{2+} are referred to as CI pacemaker neurons (Fig. 3; also Peña *et al.*, 2004). These pacemaker neurons are riluzole-sensitive (Peña *et al.*,

2004). We use 200 μM Cd^{2+} because this concentration ensures the blockade of all voltage-activated calcium currents in VRG neurons, as previously demonstrated (Elsen & Ramirez, 1998). We also assume that following bath application of 200 μM Cd^{2+} all calcium-dependent chemical synaptic transmission should be fully blocked. Although it would be an interesting issue to investigate whether CI and CS pacemaker neurons are present in different rostrocaudal locations, this very specific question is beyond the scope of the present study. Here and in all our prior studies, we aimed at producing slices consistently within the same rostrocaudal plane. During the course of this and all of our prior studies over the past decade, we consistently recorded CI and CS pacemakers. However, the exact rostrocaudal boundaries of the pre-BötC have not been determined in our study for mice. However, despite this uncertainty, it seems plausible to assume that at least some of the recorded pacemaker neurons were located within the preBötC.

The patch electrodes were pulled from filamented borosilicate glass tubes (G150F-4; Warner Instruments, Hamden, CT, USA) and filled with a solution containing (in mM) K-gluconic acid, 140; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 1; EGTA, 10; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2; Na_2ATP , 4; and HEPES, 10. The composition of this intracellular solution and the lack of adverse effects on pacemaker activity have already been addressed (Peña *et al.*, 2004; Del Negro *et al.*, 2005).

Data analysis

All recordings were stored on a personal computer using AxoTape (Version 2.0; Axon Instruments, Union City, CA, USA) and analyzed offline using customized analysis software written with IGOR Pro (Wavemetrics, Lake Oswego, OR, USA). Bursts were automatically detected by the IGOR program as described in detail in our previous study (Viemari & Ramirez, 2006). This computerized method was only reliable when used in conjunction with qualitatively good recordings that were characterized by a good signal-to-noise ratio. In the case of intracellular recordings this automated program could detect bursts, but the amplitude and burst duration values were not useful because of the presence of action potentials. For this reason we used cursor values to assess changes in the membrane potential (V_m) of automatically detected, intracellularly recorded bursts. Unless noted otherwise, measured parameter values are reported in the text as mean $\pm$ SEM. Statistical significance is considered at the 95% confidence level, and Friedman, Kruskal–Wallis, Mann–Whitney rank-sum and Wilcoxon tests were used for analysis as appropriate.

The mean rise time and duration of the VRG population bursts obtained in each preparation were assessed for the two last minutes of a 10-min hypoxic exposure. The VRG population burst rise time was measured between 20 and 80% of the burst onset, as described in more detail in Lieske *et al.* (2000). Burst duration was measured as the width at half-maximum amplitude. To analyze the irregularity of the VRG population, we calculated an irregularity score (IS) by applying a formula for consecutive cycle length values: $S_n = 100 \times \text{ABS} - (P_n - P_{n-1}) / P_{n-1}$, where S_n is the score of nth cycle, P_n is its period, P_{n-1} is the period of preceding burst and ABS is the absolute value. To estimate the cycle duration variability we also calculated the coefficient of variability of cycle duration, defined as the ratio between the SD and the mean cycle duration as measured during 30–60 successive cycles (Viemari *et al.*, 2004).

Results

Fictive eupneic activity required activation of $\alpha 2$ -NR

The transverse medullary slice preparation contains medullary noradrenergic groups (Fig. 1A; also Zanella *et al.*, 2006) which continue to exert an endogenous modulatory effect on the inspiratory rhythm. Medullary slice preparations also generate fictive sighs (Fig. 1B), but

the effect of yohimbine on fictive sighs was not investigated in the present study. The $\alpha 2$ -NR antagonist yohimbine (20 μ M) blocked the endogenous activation of $\alpha 2$ -NR and significantly depressed the mean inspiratory rhythm frequency, by $58 \pm 6\%$ (from 0.22 ± 0.03 to 0.1 ± 0.02 Hz; Mann–Whitney test, $P = 0.008$, $n = 9$; Fig. 1, B and C3). To assess the effects of this blockade on the regularity of the rhythm we calculated the coefficient of variability (mean / SD: Cvd; Viemari *et al.*, 2004) and the IS (Telgkamp *et al.*, 2002). Yohimbine (20 μ M, Fig. 1, C1 and C2) increased the Cvd by $126 \pm 32\%$ and IS of the cycle period by $166 \pm 32\%$ (0.26 ± 0.04 vs. 0.58 ± 0.09 for Cvd, Mann–Whitney test, $P = 0.017$; and 28 ± 4 vs. 66 ± 7 for IS, Mann–Whitney test, $P = 0.008$, $n = 9$; Fig. 1, C1 and C2). To determine whether the action of yohimbine was on $\alpha 2$ -NR, we used other $\alpha 2$ -NR antagonists rauwolscine and RX 821002. The effects of yohimbine were mimicked by rauwolscine (20 μ M) and RX 821002 (20 μ M; Fig. 1C). Rauwolscine ($n = 4$) decreased significantly the rate of inspiratory rhythm, by $56 \pm 3\%$ (from 0.18 ± 0.01 to 0.08 ± 0.01 Hz, $P = 0.010$). The Cvd and IS of the cycle period increased respectively by 120 ± 11 and $75 \pm 4\%$ (0.25 ± 0.01 vs. 0.55 ± 0.02 for Cvd, Mann–Whitney test, $P = 0.024$; and 32 ± 1 vs. 56 ± 2 for IS, Mann–Whitney test, $P = 0.029$; Fig. 1C). Application of RX 821002 (Fig. 1C; $n = 3$) had the same effect than that caused by yohimbine and rauwolscine, i.e. it decreased the rate of inspiratory rhythm by $59 \pm 5\%$ (Mann–Whitney test, $P = 0.024$) and increased significantly the Cvd and IS, by $167 \pm 30\%$ (Mann–Whitney test, $P = 0.029$) and $122 \pm 20\%$ (Mann–Whitney test, $P = 0.046$), respectively.

Blockade of $\alpha 2$ -NR affected CI but not CS bursting properties

Here we recorded from 14 pacemaker neurons that continued to generate bursting activity in a cocktail of antagonists for NMDA receptors, non-NMDA receptors, glycine receptors and GABAA receptors. This cocktail blocks synaptic transmission involving ionotropic glutamate receptors and receptor-coupled anion channels. Following application of the cocktail we bath-applied yohimbine (20 μ M). In all six recorded CS pacemaker neurons yohimbine did not affect burst duration (1.12 ± 0.22 vs. 1.54 ± 0.37 s, Wilcoxon test, $P = 0.42$), burst amplitude (17.62 ± 6.10 vs. 20.00 ± 6.45 mV, Wilcoxon test, $P = 0.42$), burst area (9.15 ± 4.16 vs. 12.13 ± 7.04 a.u., Wilcoxon test, $P = 0.84$) or burst frequency (0.47 ± 0.07 vs. 0.45 ± 0.11 Hz, Wilcoxon test, $P = 1$). Yohimbine completely abolished burst generation in seven of eight CI pacemaker neurons after 10 min but in one neuron yohimbine completely abolished burst generation after 15 min. (Fig. 3D and E; $n = 8$, Friedman test, Dunn's post-test). Yohimbine did not affect the membrane potential of either CS (-64.2 ± 3.2 vs. -64.03 ± 3.6 mV) or CI pacemaker neurons (-58.8 ± 5.4 vs. -59.2 ± 4.7 mV). Our data indicate that bursting in CI pacemaker neurons depends on $\alpha 2$ -NR activation (shown here) as well as 5-HT_{2A}R activation as previously demonstrated by Tryba *et al.* (2006). We tested the hypothesis that gasping activity also depends on $\alpha 2$ -NR activity.

Fictive gasping required activation of $\alpha 2$ -NR

In hypoxia the inspiratory network reconfigures into a network state that we refer to as fictive gasping (Lieske *et al.*, 2000). This pattern is characterized by a change in shape of the integrated VRG population burst. We calculated both the rise time and the VRG burst duration during the last 2 min of the 10-min hypoxic exposure. Compared to control, the rise time and the VRG burst duration were significantly shorter in hypoxic conditions (0.41 ± 0.02 vs. 0.31 ± 0.02 s, Mann–Whitney test, $P = 0.029$ for duration and 0.2 ± 0.02 vs. 0.12 ± 0.03 s, Mann–Whitney test, $P = 0.029$). Here, we evaluated the effects of blocking $\alpha 2$ -NR on the VRG population in hypoxia. The inspiratory rhythm was recorded in seven slices during 10-min exposures to a hypoxic aCSF. All seven preparations exhibited fictive gasping under these conditions (Fig. 4A). After 10 min of hypoxia the inspiratory network activity was allowed to recover (30 min) to baseline conditions in aCSF equilibrated with carbogen. After allowing the inspiratory network to recover, we applied yohimbine (20 μ M)

and exposed the slice to a second application of the hypoxic aCSF. Following blockade of $\alpha 2$ -NR the frequency of the VRG burst population decreased dramatically and ceased during the last 2 min of the second hypoxic exposure (Fig. 4C). Fictive gasping that we characterized during this time window was abolished (Fig 4B; Kruskal–Wallis test, Dunn's post-test, $P < 0.05$). Despite the continuous application of yohimbine, inspiratory VRG burst population returned during the period of recovery in all preparations.

To further investigate the role of neuromodulators we repeated the same set of experiments after exposure to DOI 20 μ M (a 5-HT_{2A}R agonist; $n = 5$, Fig. 5A and B) before application of yohimbine. 5-HT_{2A}R antagonists have also been shown to abolish gasping (Tryba *et al.*, 2006). Application of DOI excited the inspiratory network (Peña & Ramirez, 2002). Subsequent application of yohimbine did not significantly depress the rate of the VRG burst population (Fig. 5C; Friedman test) and fictive gasping was not abolished during the last 2 min of hypoxic exposure, which differs from the situation in which we antagonized $\alpha 2$ -NR alone.

Fictive gasping was generated in inspiratory islands

It is already known that in the inspiratory island, a preparation that contains the pre-BötC (Fig. 6A; also Johnson *et al.*, 2001), generates inspiratory activity *in vitro*, but whether this network was sufficient to elaborate gasping was not investigated. In a set of experiments we exposed inspiratory islands to hypoxia. In all experiments fictive gasping was induced (Fig. 6B, $n = 5$) during hypoxia. Fictive gasping generated in the inspiratory island has characteristics similar to those generated in slice preparations. Specifically, the rise time and the burst duration of fictive gasping were significantly shorter than those of fictive eupnea (0.40 ± 0.03 vs. 0.28 ± 0.06 s, Mann–Whitney test, $P = 0.01$ for duration; and 0.18 ± 0.03 vs. 0.12 ± 0.02 s, Mann–Whitney test, $P = 0.024$; Fig. 6C). However, the values of the rise time and the burst duration of fictive eupnea or fictive gasping in the inspiratory island were not significantly different from those obtained in slices.

Yohimbine (20 μ M) application abolished fictive gasping (Fig. 6D, $n = 5$) similar to that observed in the slice preparation. These experiments suggest that A1C1 neurons (catecholaminergic nuclei A1C1 in the ventral part of medulla near the VRG) preserved in the inspiratory islands are sufficient to sustain gasping *in vitro*.

Discussion

Activation of $\alpha 2$ -NR sustained fictive gasping

Here we showed that a subpopulation of inspiratory pacemaker neurons, the CI pacemaker neurons, is endogenously activated by $\alpha 2$ -NRs. This has important implications for the hypoxic response of the respiratory network. During hypoxia CI pacemaker neurons remain active while CS pacemaker neurons and many non-pacemakers are inhibited, rendering the remaining network sensitive to the blockade of CI pacemaker neurons (Peña *et al.*, 2004). Consistent with the hypothesis that gasping is dependent on CI pacemaker activity, fictive gasping is also eliminated following blockade of endogenous 5-HT_{2A}R activation (Tryba *et al.*, 2006). However, a subsequent study suggested that blockade of 5-HT receptor activation alone produced no significant effect on gasping in the arterially-perfused brainstem preparation (Toppin *et al.*, 2007; St-John & Leiter, 2008). In Pet-1 knockout mice, in which serotonin levels are reduced, gasping was also still generated (St-John *et al.*, 2009). However, in this study it was not clear whether the respiratory network was fully reconfigured. The *in vivo* preparation was exposed to just 40 s of ischemia and, while the inspiratory burst had a shorter rise time, the duration of time of inspiration, one of the key criteria for gasping, was not different from eupnea (St-John *et al.*, 2009). In another study, it

was demonstrated in the Pet-1 knockout mouse that the onset of gasping and the restoration of eupnea are delayed once hypoxic apnea develops (Erickson & Sposato, 2009). Thus, while it is unclear to what extent gasping depends on serotonergic activation, none of these studies addressed whether gasping may also involve noradrenergic drive. Our findings indicate that noradrenaline is probably an additional neuromodulator important for gasping, as fictive gasping was eliminated following blockade of endogenous $\alpha 2$ -NR activation. We demonstrated that $\alpha 2$ -NR activation modulates riluzole-sensitive bursting mechanisms in a similar way to the activation of 5-HT_{2A}R (Peña & Ramirez, 2002). We also demonstrated that $\alpha 2$ -NR activation does not affect I_{CAN} -dependent bursting in CS pacemaker neurons as has previously been shown for 5-HT_{2A}R (Pena & Ramirez, 2002; Tryba *et al.*, 2006). The noradrenergic modulatory effect on inspiratory network activity appears to be in close proximity to the pre-BötC as yohimbine also blocked gasping-like activity in inspiratory islands. However, even in this preparation we cannot exclude the possibility that presynaptic terminals from other brain regions were still intact and were releasing neuromodulators. However, what we can conclude is that this island preparation will probably include A1C1 catecholaminergic neurons (Zanella *et al.*, 2006) because this region probably overlaps with the preBötC.

Activation of $\alpha 2$ -NRs was required to generate regular fictive eupneic activity

Endogenous 5-HT_{2A}R and $\alpha 2$ -NR activation are also involved in the generation of normal inspiratory activity. Under baseline conditions, significant depression and increased irregularity of the inspiratory rhythm occurs following blockade of 5-HT_{2A}R (Peña & Ramirez, 2002) and, as shown here, also following blockade of $\alpha 2$ -NR. In both cases, the irregularity could potentially be attributed to the specific loss of riluzole-sensitive mechanisms under normoxic conditions (Peña & Ramirez, 2002). It is important to note that the blockade of the aminergic receptors abolished specifically bursting and not the generation of action potentials. Taken together our studies (Peña & Ramirez, 2002; Viemari & Ramirez, 2006) support the hypothesis that noradrenergic and serotonergic endogenous drive are necessary to sustain the riluzole-sensitive bursting mechanism which is important for the control of inspiratory frequency and regularity, and also for gasping. In control conditions, however, inspiratory rhythm generation did not entirely rely on this riluzole-sensitive mechanism. This is not surprising given that other rhythm-generating and modulatory mechanisms also contribute to inspiratory rhythm generation. These other mechanisms include I_{CAN} (Peña *et al.*, 2004), various excitatory synaptic mechanisms (Peña *et al.*, 2004; Lieske & Ramirez, 2006a, b; Pace *et al.*, 2007; Del Negro & Hayes, 2008; Rubin *et al.*, 2009) and inhibitory mechanisms (Ramirez *et al.*, 1997; Shao & Feldman, 1997; Busselberg *et al.*, 2001) as well as various additional modulatory mechanisms (Doi & Ramirez, 2008, 2010). We did not investigate the intracellular mechanisms that underlie this modulatory effect. However, it is well established that $\alpha 2$ -NRs produce an intracellular inhibition of adenylate cyclase via G protein αi . $\alpha 2$ -NR receptors can also be linked to increased phosphoinositide turnover via G protein $\beta \gamma$ stimulation of Phospholipase C and subsequent activation of protein kinase C (PKC) (Boehm *et al.*, 1996; Gesek, 1996; Dorn *et al.*, 1997; Talaia *et al.*, 2006; Doi & Ramirez, 2008). In the inspiratory network, blockade of endogenous activation of 5-HT_{2A}R reduced fast and persistent sodium current (Peña & Ramirez, 2002). Peña & Ramirez (2002) also showed that activation of PKC masked the effect of the 5-HT_{2A}R blockade. Here, we demonstrated that activating 5-HT_{2A}R with DOI, presumably acting via PKC, prevented the abolition of gasping in the presence of yohimbine.

Common pathways may mediate the effects of 5-HT_{2A}R and $\alpha 2$ -NR

Thus, our study suggests that 5-HT_{2A}R and $\alpha 2$ -NR activation may possibly share the same PKC pathway. The effects on riluzole-sensitive mechanisms, CI pacemaker neurons and the

effects on gasping at the network level that depend on the same mechanisms also suggest that these two neuromodulators could potentially act on the persistent sodium current via the PKC pathway. However, the exact intracellular mechanisms that govern the observed convergence on the same type of bursting mechanism will require further investigation. At the systems level, the present study is consistent with a prior *in vivo* study in which we demonstrated that the effects on breathing caused by NE and 5-HT were not additive (Doi & Ramirez, 2010). Together, these findings support the perspective that these two neuromodulators act on common downstream targets.

Data presented here suggest that the noradrenergic release which is important for inspiratory rhythm generation and gasping arises from A1C1. This is interesting as neurons in this region are particularly active during hypoxia (Teppema *et al.*, 1997). Although an important role for medullary noradrenergic neurons in cardiorespiratory regulation has been well established (Champagnat *et al.*, 1979; Guyenet, 1991; Haxhiu *et al.*, 2001), it is much less clear whether these neurons also play a crucial role in inspiratory rhythmogenesis. A large percentage of tyrosine hydroxylase (TH)-expressing neurons within the A1 noradrenergic group are activated during hypercapnia and hypoxia (Teppema *et al.*, 1997) whereas the rostral end of C1 shows a reduced level of TH-Fos colocalisation. The A1 and A2 noradrenergic cell groups are also activated after paradoxical sleep (PS) or rapid eye movement (REM) sleep deprivation, indicating that those noradrenergic neurons might also contribute to PS inhibition (Leger *et al.*, 2009). During REM sleep there is a significant decrease in both noradrenergic and serotonergic neuronal activity (Luppi *et al.*, 2006), which could potentially increase the probability of a resuscitation failure. While these considerations cannot prove any link between aminergic disturbances and a failure to resuscitate, it is conceivable that an imbalance in these important modulators could have detrimental effects that are worsened in a state-dependent manner. Doi & Ramirez (2010) investigated state-dependent neuromodulation but state dependency is difficult to replicate in *in vitro* slice preparations and was therefore not investigated in the present study. Nevertheless, our data support an important role for medullary noradrenergic inputs that converge partly with serotonergic inputs. However, it must be emphasized that medullary noradrenergic input is not the only noradrenergic drive important for breathing, and neurons from the pontine noradrenergic nuclei are probably as important (Viemari, 2008; Doi & Ramirez, 2008, 2010).

Potential clinical implications

The finding that the inspiratory network depends critically on converging endogenous aminergic drive during hypoxia but not during normoxic conditions has potentially interesting clinical implications. Gasping is a vital mechanism for survival in response to several life-threatening conditions (Fewell, 2005; Thach, 2008), and gasping is a critical component in the arousal response that leads to spontaneous recovery from hypoxic apnea (Adolph, 1969; Jacobi & Thach, 1989). A disturbance in the generation of gasping and the arousal response has been implicated in Sudden Infant Death Syndrome (SIDS; Poets *et al.*, 1999; Sridhar *et al.*, 2003). Moreover, SIDS has also been associated with disturbances in serotonergic and noradrenergic systems (Obonai *et al.*, 1998; Ozawa *et al.*, 2003; Weese-Mayer *et al.*, 2004; Viemari, 2008; Gaultier & Gallego, 2008; de Pontual *et al.*, 2003; Kinney, 2005; Weese-Mayer *et al.*, 2008; Duncan *et al.*, 2010). Although the direct link between a disturbance in aminergic modulation and SIDS remains unclear the convergence of serotonin and norepinephrine onto the same neurons that are critical for gasping suggests that a disturbance in noradrenergic mechanisms could have equally detrimental consequences as a disturbance in serotonin. At the same time, this convergence may constitute an important safety mechanism protecting the network against the disturbance of a single modulator. The dependency on more than one factor is indeed one of the core

hypotheses of the triple risk model according to Filiano & Kinney (1994). Thus, a disturbance in one neuromodulator may increase the risk for SIDS but other risk factors (e.g. Allen *et al.*, 2011; Rubens *et al.*, 2008) must be present to ultimately cause SIDS.

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Abbreviations

5-HT2AR	5-hydroxytryptamine type 2A receptors
A1C1	catecholaminergic nuclei A1C1
aCSF	artificial cerebrospinal fluid
CI	cadmium-insensitive
CS	cadmium-sensitive
Cvd	coefficient of variability (mean / SD)
FFA	flufenamic acid
I_{CAN}	calcium-activated nonspecific cation current
I_{Nap}	persistent sodium current
IS	irregularity score
P	postnatal day
PKC	protein kinase C
pre- BötC	pre-Bötzinger complex
VRG	ventral respiratory group
α2-NR	alpha2-noradrenergic receptor

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Fig. 1.

Blockade of α 2NRs decreased the frequency of fictive eupneic activity and increased the irregularity. (A) Sites of integrated VRG ($\int$ VRG) and whole-cell patch-clamp recordings (membrane potential; V_m) are illustrated in the schematic of the brainstem slice preparation including the catecholaminergic nuclei A1C1 (in the ventral part of medulla near the VRG) and A2C2 (in the dorsal medulla). (B) Fictive eupnea and fictive sigh inspiratory activities in (B1) control conditions and (B2) after application of yohimbine (20 μ M). (C) Histograms summarize the differences between fictive eupneic activity in control and after application of yohimbine. We compared (C1) the Cvd and (C2) the IS to evaluate the irregularity, and (C3) the frequency; ** $P < 0.01$, * $P < 0.05$. Results are expressed as mean $\pm$ SEM. VRG, ventral respiratory group; NA, nucleus ambiguus; IO, inferior olive; Sp5, spinal trigeminal nucleus.

Fig. 2.

Yohimbine did not affect the bursting properties of CS inspiratory pacemaker neurons. (A) Recording of an inspiratory pacemaker neuron that produced bursts when embedded in the network. (B) This neuron continued to burst in the presence of the cocktail. (C) Application of yohimbine (20 μ M) did not change the bursting properties of the CS pacemaker neuron. (D) Application of 200 μ M cadmium abolished burst properties of this particular type of pacemaker neuron. Loss of bursting properties was confirmed by current injection. (E) The histogram represents the time course of the burst frequency of synaptically isolated CS pacemaker neurons during a 10-min bath application of yohimbine. Each bin represents the burst frequency for a time window of 1 min. The percentage of control frequency was calculated based on the mean frequency 1 min before bath application of yohimbine (grey bin). Results are expressed as mean $\pm$ SEM.

Fig. 3.

Yohimbine abolished the burst properties of CI inspiratory pacemaker neurons. (A) Recording of an inspiratory pacemaker neuron in the network. (B) This neuron continued to burst after blockade of the fast synaptic transmission by the cocktail and (C) after application of cadmium. (D) Yohimbine (20 μ M) abolished the burst properties of the CI pacemaker neuron. (E) The histogram represents the time course of the burst frequency of synaptically isolated CI pacemaker neurons ($n = 8$) during a 10-min bath application of yohimbine. Each bin represents the burst frequency for a time window of 1 min. The percentage of control frequency was calculated based on the mean frequency 1 min before bath application of yohimbine (grey bin); *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, Friedman test. Results are expressed as mean $\pm$ SEM.

Fig. 4.

Blocking α 2-NRs eliminated gasping in the transverse slice preparation *in vitro*. (A) Gasping was evoked after application of hypoxic aCSF. (B) The blockade of α 2-NRs prevented generation of gasping in the transverse slice preparation. (C) Histogram showing fictive inspiratory activity during application of hypoxic aCSF (control, open bins). After application of yohimbine the VRG burst frequency decreased and ceased during the last 2 min of application of the hypoxic aCSF (filled bins, $n = 6$). The asterisks indicate significant differences between control burst frequency and following yohimbine application. The onset of the 10-min application of the hypoxic aCSF was at the origin. VRG, ventral respiratory group; NA, nucleus ambiguus; IO, inferior olive; Sp5, spinal trigeminal nucleus. $*P < 0.05$, Kruskal-Wallis test, Dunn's post-test. Results are expressed as mean $\pm$ SEM.

Fig. 5.

Activation of 5-HT_{2A} receptors prevented the abolition of gasping *in vitro*. (A) Gasping was evoked after application of hypoxic aCSF. (B) Application of DOI (20 μ M) prevented the effect of blocking α ₂-NRs in the transverse slice preparation. (C) Histogram showing fictive inspiratory activity during application of hypoxic aCSF (control, open bins). After application of DOI and yohimbine the VRG burst frequency was maintained during the last minutes of application of hypoxic aCSF (filled bins, $n = 5$). The onset of the 10-min application of hypoxic aCSF was at the origin.

Fig. 6. Blocking $\alpha 2$ -NRs eliminated gasping in the inspiratory island preparation *in vitro*. (A) Schematic of the inspiratory island that generated rhythmic inspiratory activity. (B) Gasping was evoked after application of hypoxic aCSF. (C1) Characteristics of gasping in the inspiratory island. VRG burst during application of hypoxic aCSF in black and in control in grey. (C2 and 3) Histograms show fictive eupnea (open bars) and fictive gasps (solid bars). C2 shows the shorter rise time of fictive gasps and (C3) the shorter burst duration. (D) Blockade of alpha-2 noradrenergic receptors prevents generation of gasping in the inspiratory island preparation. $*P < 0.05$, Mann–Whitney test. Results are expressed as mean $\pm$ SEM.