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Blockade of dorsolateral pontine 5HT1A receptors destabilizes the respiratory rhythm in C57BL6/J wild-type mice

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ABSTRACT

The neurotransmitter serotonin (5HT) acting via 5HT1a receptors (5HT1aR) is a potent determinant of respiratory rhythm variability. Here, we address the 5HT1aR-dependent control of respiratory rhythm variability in C57BL6/J mice. Using the *in situ* perfused preparation, we compared the effects of systemic versus focal blockade of 5HT1aRs. Blocking 5HT1aRs in the Kölliker-Fuse nucleus (KFn) increased the occurrence of spontaneous apneas and accounted for the systemic effects of 5HT1aR antagonists. Further, 5HT1aRs of the KFn stabilized the respiratory rhythm's response to arterial chemoreflex perturbations; reducing the recovering time, *e.g.*, the latency to return to the baseline pattern. Together, these results suggest that the KFn regulates both intrinsic and sensory determinants of respiratory rhythm variability.

1. Introduction

At the Oxford Conference on Breathing and Modelling, we presented recent modeling and experimental work suggesting that the Kölliker-Fuse nuclei (KFn), a component of the ponto-medullary respiratory pattern generator (Dutschmann and Dick, 2012), regulates the gain of both intrinsic and extrinsic sources of respiratory rhythm variability. Intrinsic sources are channel- and synaptic-noise within rhythm generating nuclei whereas the extrinsic sources are afferent feedbacks arising from sensory sources such as pulmonary stretch receptors (Dhingra et al., In review). In addition, variability of respiration is linked to the postnatal maturation of the KFn (Dutschmann et al., 2008) and disrupting the maturation processes of the KFn can enhance respiratory variability and cause pathologic breathing irregularities observed in neurodevelopmental disorders such as Rett syndrome (Stettner et al., 2007; Dhingra et al., 2013). Recent evidence suggests that increased breathing variability is associated with reduced GABAergic innervation of the KFn (Abdala et al., 2016).

The underlying synaptic mechanisms that generate intrinsic and extrinsic variability of the respiratory rhythm depend on the serotoninergic 1a receptor (5HT1aR), which modulates the respiratory control network. The 5HT1aR is an inhibitory receptor that decreases respiratory neuron excitability *via* $G_{i/o}$-dependent mechanisms (see Richter et al., 2003). Systemic administration of a 5HT1aR agonist reduced the occurrence spontaneous central apneas, and therefore intrinsic respiratory rhythm variability, in C57BL6/J and Mecp2-/y mice (Yamauchi et al., 2008). Moreover, Pet-1 and Lmx1b knock-out mice which lack serotonergic neurons show increased respiratory rhythm variability in early postnatal life (Erickson et al., 2007). Further, 5HT-dependent respiratory rhythm variability may depend selectively on 5HT1aR-dependent neurotransmission because mice lacking the 5HT1aR also have increased in breathing variability at baseline despite an increase in serotonin release (Barrett et al., 2012).

Here, we test whether systemic or focal application of the selective 5HT1aR antagonist WAY100635 increases the variability of the baseline respiratory rhythm, and delays the recovery of the baseline pattern after an arterial chemoreflex-mediated perturbation of the respiratory rhythm.

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2. Methods

Experimental protocols were approved by the Case Western Reserve University Institutional Animal Care and Use Committee, and were performed with strict adherence to all American Association of Accreditation of Laboratory Animal Care and National Institutes of Health guidelines.

2.1. In situ *perfused brainstem preparation*

Experiments were performed in female, adult (3 mo. postnatal) wild-type mice maintained on a background (C57BL6/J x 129 Sv x Balb/c, $n = 8$) to minimize genotype-dependent effects. The *in situ* perfused preparation was prepared as described previously (Dhingra et al., 2013). Briefly, mice were deeply anesthetized with isofluorane (1.5–3%, Piramal Healthcare, Andra Pradesh, IN). Once the animal failed to respond to a noxious paw pinch, the animal was transected below the diaphragm and submerged in ice-cold ACSF. The skull was quickly removed for precollicular decerebration, and cerebellectomy. The lungs were removed, and the right phrenic, hypoglossal and vagal nerves were dissected for recording efferent activity. The descending aorta was also isolated for cannulation. The preparation was then transferred to the recording chamber. The descending aorta was cannulated, and the preparation was immediately perfused with an artificial cerebrospinal solution (aCSF), which had a pH 7.4 (in mM: 125 NaCl, 3 KCl, 1.25 KH_2PO_4, 2.5$CaCl_2$, 1.25 $MgSO_4$, 25 $NaHCO_3$, 10 D-Glucose and 1.25% (w/v) Ficoll) maintained at 31° C *via* a recirculating heat exchanger and bubbled with a gas mixture containing 94% O_2 and 6% CO_2. Within minutes of aCSF perfusion, the preparation showed respiratory movements. The activity of the phrenic, vagal and hypoglossal nerves were recorded using on bipolar suction electrodes. Nerve activity was amplified (20000x, Grass P511, West Warwick, RI, USA), filtered (0.003–3 kHz), digitized (10 kHz, Power1401, CED, Cambridge, UK) and stored on a computer using Spike2 software (CED, Cambridge, UK). Finally, the preparation was paralyzed *via* a bolus of vecuronium bromide (1 mg/250 mL perfusate) to minimize motion artifacts. Typical flow rate and perfusion pressure were 18–21 mL/min and 40–60 mmHg, respectively.

2.2. Experimental protocol

Once a stable respiratory rhythm was established, baseline respiratory output was recorded for 5 min. The chemoreflex was evoked *via* a bolus of NaCN (0.1 mL, 0.03% (w/v)). In the first protocol ($n = 3$), we applied the 5HT1aR antagonist WAY100635 systemically in the perfusate (10 μM, Sigma–Aldrich, St. Louis, MO, USA). Respiratory output was again recorded for 5 min to assess respiratory effects of 5HT1aR blockade. The chemoreflex was evoked 10 min after WAY100635 administration to assess whether 5HT1aR blockade modulated the respiratory chemosensitivity.

In the second protocol ($n = 5$), we microinjected WAY100635 into the dorsolateral (dl) pons using triple-barreled microinjection pipettes. Triple barreled pipettes were pulled using a vertical puller (Narishige International USA, Amityville, NY, USA) from standard glass capillaries (1.2/0.68 mm ID/OD, World Precision Instruments, Sarasota, FL, USA). Drugs were delivered *via* a pressure microinjection system (Veradyne). Injected volumes were calibrated using a reticule to monitor the movement of the meniscus within the pipette. Respiratory related areas in the dl pons were identified by mapping with glutamate microinjections (50 nL, 10 mM). Sites where glutamate microinjection evoked a transient slowing of the respiratory pattern were selected for subsequent microinjection of WAY100635 (100 nL, 10 mM, Sigma–Aldrich) and Alexa 488-conjugated microspheres (Invitrogen, Grand Island, NY, USA). The same procedure was repeated on the contralateral side to achieve bilateral administration of the drug in the dl pons. Effective sites were typically found at the following coordinates: AP: 0.2–0.5 mm caudal to the inferior colliculus, ML: midline ± 1.6–2.2 mm, Depth: 1.5–2 mm below surface.

2.3. Histology

At the end of the experiment, the brainstem was removed, and postfixed overnight in 4% PFA/PBS. The tissue was transferred into a 20% sucrose/PBS solution for cryoprotection. The fixed tissue was then sectioned on a freezing microtome (50 μm, SM2010R, Leica Biosystems, Buffalo Grove, IN, USA). Pontine sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and imaged (Eclipse 80i, Nikon Instruments, Melville, NY, USA; Retiga 200R, QImaging, Surrey, BC, CAN) for *post-hoc* identification of the injection sites.

2.4. Data analysis

Phrenic nerve activity (PNA) was used as an index of fictive respiratory output. From 5 min epochs recorded before and after administration of WAY100635, we measured the mean duration of inspiration (T_I) and expiration (T_E), and plotted the instantaneous respiratory period (T_{TOT}) to identify central apneas, and quantify their frequency. A central apnea was defined as a respiratory period more than twice the mean respiratory period in the epoch. The apnea index reflects the frequency of these events. The apnea index was measured from epochs that did not include spontaneous swallows, which were characterized simultaneous brief bursts in hypoglossal and vagal nerve activities. From chemoreflex responses, we measured the peak and minimum respiratory frequencies during the trial. Additionally, we measured the latency for recovery from chemosensory perturbation as the time from the onset of the chemoreflex to the first cycle with a duration comparable to the respective baseline duration.

All data are presented as means ± SEM. Statistical comparisons were made with a one-way ANOVA with Bonferroni correction for multiple comparisons.

3. Results

To assess the role of brainstem- versus dl pontine-5HT1aRs on intrinsic respiratory variability, we analyzed the respiratory pattern and variability before and after systemic or focal blockade of 5HT1aRs with WAY100635 *in situ* (Fig. 1).

Representative traces of PNA are shown before and after systemic administration of WAY100635 in Fig. 1A and B. Systemic blockade of 5HT1aRs did not evoke any change in the respiratory period (TTOT) or the durations of inspiration or expiration (Fig. 1L). However, systemic administration reduced the amplitude of PNA (baseline, 0.44 ± 0.08 AU versus after systemic WAY100635, 0.27 ± 0.06 AU, $p < 0.05$, Fig. 1M). Moreover, systemic administration of WAY100635 tended to increase respiratory rhythm variability. Representative plots of the respiratory period before and after systemic 5HT1aR blockade is shown in Fig. 1C and D. Enhanced respiratory variability was observed as an increase in the frequency of spontaneous central apneas (baseline, 0.35 ± 0.35 apneas/min versus after systemic WAY100635, 1.15 ± 0.86 apneas/min, Fig. 1N). A representative central apneic event is presented in Fig. 1E. As described by Abdala et al. (2010), central apneas consisted of prolonged bursts of hypoglossal and vagal nerve activity, and was also associated with low-amplitude activity in PNA. The pattern of respiratory motor outputs during central apneas after systemic administration of WAY100635 was qualitatively the same as that during baseline.