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RESEARCH ARTICLE | *Nervous System Pathophysiology*

Phrenic long-term depression evoked by intermittent hypercapnia is modulated by serotonergic and adrenergic receptors in raphe nuclei

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Stipica Safic I, Pecotic R, Pavlinac Dodig I, Dogas Z, Valic Z, Valic M. Phrenic long-term depression evoked by intermittent hypercapnia is modulated by serotonergic and adrenergic receptors in raphe nuclei. *J Neurophysiol* 120: 321–329, 2018. First published April 4, 2018; doi:10.1152/jn.00776.2017.—Intermittent hypercapnia evokes prolonged depression of phrenic nerve activity (phrenic long-term depression, pLTD). This study was undertaken to investigate the role of 5-HT and α2-adrenergic receptors in the initiation of pLTD. Adult male urethane-anesthetized, vagotomized, paralyzed, and mechanically ventilated Sprague-Dawley rats were exposed to a protocol of acute intermittent hypercapnia (AIHc; 5 episodes of 15% CO_2 in air, each episode lasting 3 min). The experimental group received microinjection of the selective 5-HT_{1A} receptor agonist 8-hydroxy-2-(dipropylamino)tetralin hydrobromide (8-OH-DPAT), the broad-spectrum 5-HT antagonist methysergide, or the α2-adrenergic antagonist yohimbine, whereas the control group received microinjection of 0.9% saline into the caudal raphe region. Peak phrenic nerve activity (pPNA) and burst frequency (*f*) were analyzed during baseline (T0), during 5 hypercapnic episodes (THc1–THc5), and at 15, 30, and 60 min after the end of the last hypercapnic episode. In the control group, pPNA decreased 60 min after the end of the last hypercapnic episode compared with baseline values, i.e., pLTD developed ($P = 0.023$). In the 8-OH-DPAT group, pPNA significantly decreased at T15, T30, and T60 compared with baseline values, i.e., pLTD developed ($P = 0.01$). In the methysergide and yohimbine groups, AIHc did not evoke significant changes of the pPNA at T15, T30, and T60 compared with baseline values. In conclusion, activation of 5-HT_{1A} receptors accentuated induction of pLTD, whereas blockade of α2-adrenergic receptors prevented development of pLTD following AIHc in anesthetized rats. These results suggest that chemical modulation of 5-HT and α2-adrenergic receptors in raphe nuclei affects hypercapnia-induced pLTD, offering important insights in understanding the mechanisms involved in development of respiratory plasticity.

NEW & NOTEWORTHY Hypercapnia is a concomitant feature of many breathing disorders, including obstructive sleep apnea. In this study, acute intermittent hypercapnia evoked development of phrenic long-term depression (pLTD) 60 min after the last hypercapnic episode that was preserved if the selective 5-HT_{1A} receptor agonist 8-hydroxy-2-(dipropylamino)tetralin hydrobromide was microinjected in the caudal raphe region before the hypercapnic stimulus. This study highlights that both 5-HT and adrenergic receptor activation is needed for induction of pLTD in urethane-anesthetized rats following intermittent hypercapnia exposure.

central nervous system; phrenic nerve; rats; respiratory plasticity

INTRODUCTION

Respiratory plasticity is an intrinsic feature of the respiratory control network characterized by long-lasting expressions that are based on prior experience (Mitchell and Johnson 2003). Lately, much attention has focused on the exposure to intermittent hypoxia and its effects on breathing stability, particularly in sleep apnea disorder (Mateika and Narwani 2009; O'Halloran 2016). It has been shown that the benefit or detriment of intermittent hypoxia depends on the dose of hypoxia and timing of intermittent exposures, where more severe episodes are deleterious and more moderate episodes are beneficial (Mateika and Narwani 2009). However, stimulus of intermittent hypercapnia is an often overlooked consequence of recurrent apneas that triggers a form of respiratory plasticity known as long-term depression (LTD), which is manifested as a progressive decrease in respiratory motor activity that lasts hours after the end of the hypercapnic stimulus (Baker et al. 2001; Stipica et al. 2016; Valic et al. 2016). There is evidence that the serotonergic system is involved in modulation of respiratory plasticity at the spinal (Baker-Herman and Mitchell 2002) and supraspinal level involving the caudal raphe region (Pecotic et al. 2009; Valic et al. 2010). Moreover, modulation of serotonergic receptors located on the raphe neurons can elicit various respiratory effects, possibly as a result of activation of either autosynaptic or postsynaptic receptors (Nucci et al. 2008; Pavlinac et al. 2011). It has been shown that the 5-HT system also influences brain noradrenergic neurons via dense 5-HT projections to the noradrenergic neurons of the locus coeruleus (LC) (Arai et al. 1997; Vertes and Kocsis 1994). Previous studies provided evidence for an inhibitory role of 5-HT receptors on the function of LC noradrenergic neurons, possibly by the mechanism of a tonic inhibition (Aston-Jones et al. 1991; Bobker and Williams 1989), whereas α2-adrenoceptor antagonists have been shown to increase LC neuron firing activity (Engberg 1992; Sanghera and German 1983; Sanghera et al. 1990).

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Intermittent hypercapnic exposure activates the noradrenergic system, which sends inhibitory projections to raphe serotonergic neurons, respiratory neurons, and the phrenic motor nucleus (Kinkead et al. 2001). In our previous study (Valic et al. 2016), systemic administration of the broad-spectrum serotonergic antagonist methysergide before exposure to acute intermittent hypercapnia (AIHc) enhanced phrenic long-term depression (pLTD), indicating the involvement of 5-HT receptors in pLTD (Valic et al. 2016). On the contrary, in a protocol in which rats were pretreated with systemic administration of the α2-adrenergic antagonist yohimbine, development of pLTD was attenuated (Bach and Mitchell 1998).

Taking into consideration that lesioning of 5-HT neurons modulates the firing activity of noradrenergic firing neurons (Haddjeri et al. 1997), we hypothesized that chemical modulation of serotonergic and adrenergic receptors at the supraspinal level would evoke opposite effects on LTD of phrenic nerve activity following AIHc in anesthetized rats. Therefore, this study was undertaken to investigate the role of 5-HT_{1A} and α2-adrenergic receptors in the initiation of pLTD, exploring whether the microinjection of 8-hydroxy-2-(dipropylamino)tetralin hydrobromide (8-OH-DPAT), methysergide, or yohimbine into the caudal raphe nuclei, before the onset of an AIHc protocol, would modulate pLTD in anesthetized rats.

MATERIALS AND METHODS

Ethical standards. The protocol for this study was approved by the Ethical Committee for Biomedical Research of the University of Split School of Medicine (Split, Croatia). All experiments were performed in accordance with National Research Council's guide for the care and use of laboratory animals.

General procedures. Experiments were carried out on 35 adult male Sprague-Dawley (Harlan, Udine, Italy) rats weighing 280–330 g (animal facility of the University of Split School of Medicine). Anesthesia was achieved by intraperitoneal injection of a 20% solution of urethane in 0.9% saline (1.2 g/kg; supplemental dose 0.2 g/kg). The adequacy of anesthesia was assessed by the absence of corneal and withdrawal reflex and by changes in the blood pressure after noxious paw pinch.

Bilateral cannulation of the femoral artery and vein was performed for blood pressure monitoring, blood sampling, and intravenous drug injection. Blood samples of 0.2 ml of arterial blood were taken at regular intervals for determination of blood gases and pH (RapidLab 348; Bayer Diagnostics, Sudbury, UK). Arterial partial pressure of carbon dioxide (Pa_{CO_2}) and pH were maintained within physiological limits by adjustment of ventilation or infusion of bicarbonates, if needed. The physiological "limit" for Pa_{CO_2} is 35–45 mmHg, and was maintained in our study in that range (40.2–45.2 mmHg), and that for pH is 7.2–7.3. However, in our study animals were exposed to hyperoxia in between hypercapnic stimuli, and arterial partial pressure of oxygen (Pa_{O_2}) was in the range 190–285 mmHg. All animals received continuous infusion of the saline (0.6 $ml \cdot h^{-1} \cdot kg^{-1}$) to

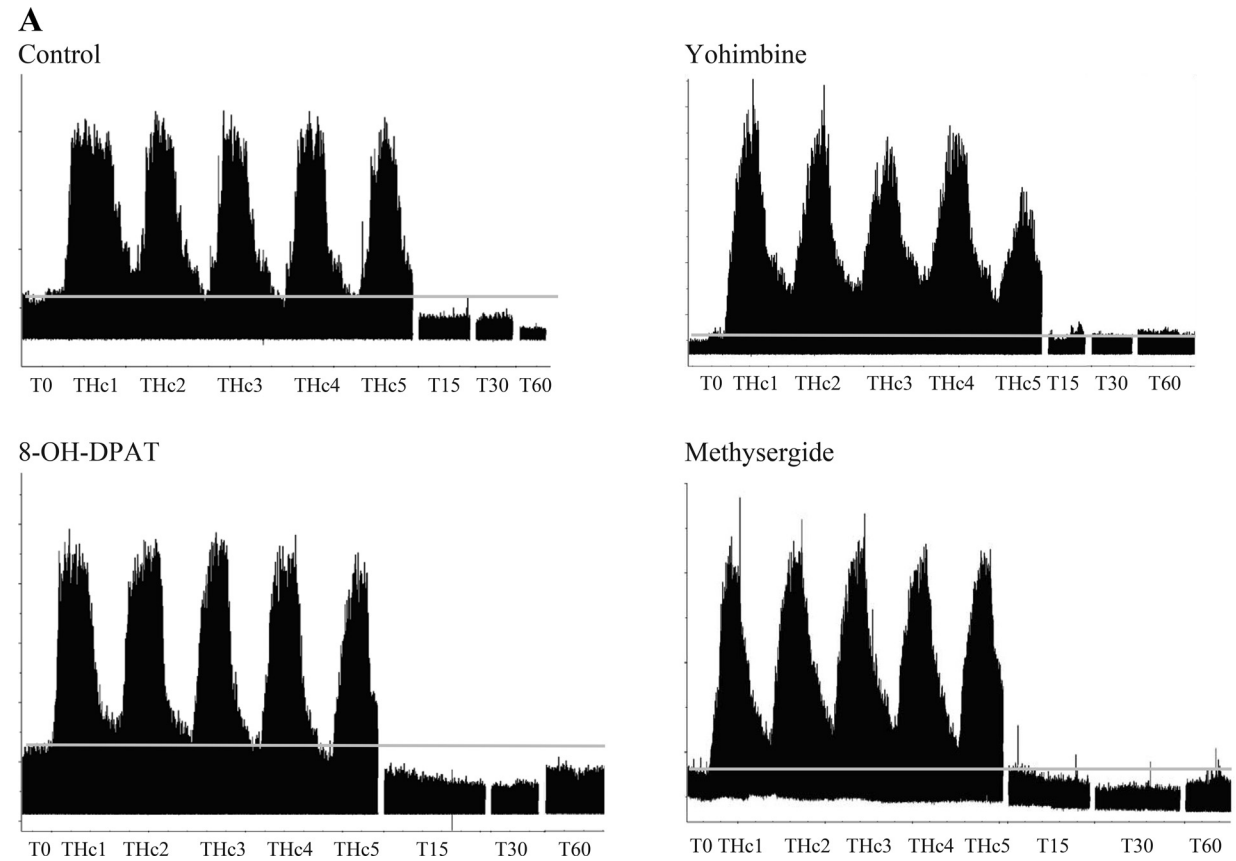

Fig. 1. Responses of phrenic nerve activity following acute intermittent hypercapnic stimulus in 4 experimental animals. *A*: compressed neurogram of the phrenic nerve showing phrenic long-term depression (pLTD) as a function of time. *B*: integrated phrenic nerve activity (IPNA; in μV) and raw phrenic nerve activity (RPNA; in μV). In the control group and the group that received microinjection of 8-hydroxy-2-(dipropylamino)tetralin hydrobromide (8-OH-DPAT) into the caudal raphe region before exposure to acute intermittent hypercapnia, pLTD was evoked at 60 min after the last hypercapnic episode (T60). In the groups that received microinjection of yohimbine or methysergide, pLTD was prevented. T0, baseline; THc1–THc5, 5 hypercapnic exposures; T15 and T30, 15 and 30 min after the end of the last hypercapnic episode, respectively.

improve the stability and vitality of the preparations throughout the experimental protocol. All animals were vagotomized bilaterally to avoid pulmonary stretch receptors input and to prevent spontaneous breathing attempts. Rectal temperature was monitored by digital thermometer and maintained between 37 and 38.5°C.

Cannulation of the trachea was performed through the midline ventral neck incision. The animals were artificially ventilated (volume-controlled ventilation) through a tracheostomy using respirator for small animals (SAR 830-P; CWE, Ardmore, PA). The initial ventilatory parameters were respiratory rate of 50 breaths/min, inspiratory pressure of 12 cmH_20, inspiratory flow range of 250–350 ml/min, and positive end-expiratory pressure of 2–3 cmH_2O. Parameters were adapted according to blood gases and capnography throughout the experiment. Concentration of CO_2 was continuously monitored with a Gemini respiratory gas analyzer (CWE) and was adjusted by changing the inspiratory time. The animals were ventilated with a 50:50 nitrogen-oxygen mixture throughout the experiment. The animals were paralyzed with the neuromuscular blocking agent rocuronium bromide (Esmeron, Organon; 50 mg in 5 ml, 1.33 mg/kg).

After cannulation of the blood vessels, tracheostomy, and vagotomy, the rats were placed in a prone position in a stereotaxic instrument (Lab Standard; Stoelting, Wood Dale, IL). The right phrenic nerve was dissected at the level of the C5 nerve rootlet using a dorsal approach, mounted on bipolar silver wire electrodes, and covered with silicone gel to prevent it from drying. The obtained electrophysiological nerve signal was then amplified (Super Z, System 1000 Modular Instrumentation; CWE), filtered (bandpass 300 Hz–10

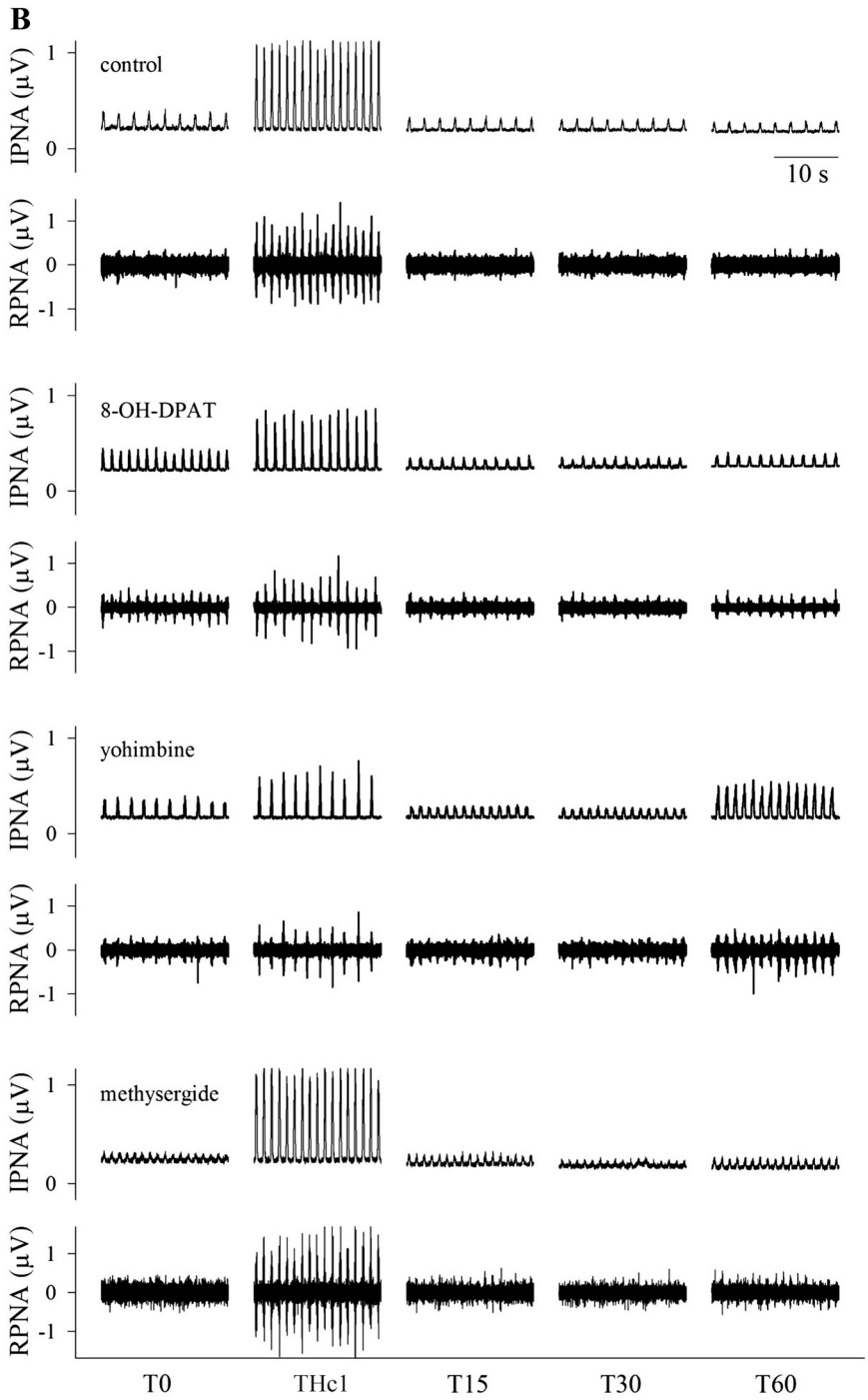

Fig. 1.—*Continued*

kHz), and rectified. After completion of the surgical procedures, a minimum of 30 min were allowed to pass in order for stabilization at a fraction of inspired oxygen (FiO_2) of 0.5.

Functional identification of raphe nuclei and microinjection technique. First, an occipital craniotomy was performed and the dorsal surface of the brain stem exposed. The dura was opened, and the cerebellum was carefully elevated and pushed rostrally under microscopic observation. This approach provided clear access to the obex, which was used as a reference point for measurements (Paxinos 2005). The rostrocaudal, mediolateral, and dorsoventral coordinates of the micropipette tip were determined with respect to the obex. Via a dorsal approach, a multibarrel glass micropipette with an external tip diameter of 30–50 μm was inserted in the medulla oblongata for stimulation of the caudal portion of the raphe nuclei.

Selective glutamate receptor agonist D,L-homocysteic acid (DLH; 20 ± 5 nl, 10 mm; Sigma-Aldrich, St Louis, MO) was microinjected. Adjustments of the micropipette position were made until a site was located at which a DLH-microinjection evoked an increase in peak phrenic nerve activity (pPNA) with no significant change in the respiratory frequency (Valic et al. 2010). Effective injection sites were found at the following coordinates: 0.2–0.3 mm rostral from the obex, in the midline, and 2.5–2.7 mm deep from the dorsal surface of the brain stem. Effects of DLH microinjections were transient, highly reproducible among animals, and related to the microinjection sites. A relatively small amount of solution (20 ± 5 nl) was used to affect only neurons adjacent to the injection site. Drug bolus was flushed with microinjection of 0.9% saline from another barrel. Following an observed increase in phrenic nerve activity, full recovery of phrenic nerve activity was allowed before further chemical modulation of 5-HT receptors of the caudal raphe portion.

A four-barrel micropipette was inserted at coordinates targeting raphe nuclei. The ejected solutions contained the vehicle (0.9% saline), DLH, diluted India ink solution for labeling purposes, and one of three examined solutions: the selective 5-HT_{1A} receptor agonist 8-OH-DPAT (1 mM, 20 ± 5 nl; Sigma-Aldrich), the broad-spectrum 5-HT receptor antagonist methysergide (4 mM, 20 ± 5 nl; Sigma-Aldrich), or the α2-adrenergic receptor antagonist yohimbine (2 mM, 20 ± 5 nl; Sigma-Aldrich), dissolved in 0.9% saline. For pressure injections, polyethylene tubing was sealed over the pipette and connected to a plastic syringe. With the use of positive pressure, a drug volume of 20 ± 5 nl was ejected. Ejected drug volumes were measured using the fluid meniscus movement in the pipette barrel on a microscope equipped with a fine eyepiece reticule.

Experimental design. Four groups (n = 35) of animals underwent the protocol, consisting of five acute hypercapnic exposures (AIHc). The 8-OH-DPAT group (n = 10) received 5-HT_{1A} receptor agonist 8-OH-DPAT, the methysergide group (n = 9) received nonselective 5-HT receptor antagonist methysergide, and the yohimbine group (n = 9) received yohimbine into the raphe nucleus. The control group (n = 7) received an equivalent amount of 0.9% of saline into the same site of the caudal raphe region, previously mapped with DLH. In the experimental groups (8-OH-DPAT, methysergide, and yohimbine), following identification of raphe nuclei with microinjection of the synaptic excitant DLH, the examined drug was injected at the same site. Two minutes after microinjection of the drug, the control blood sample was taken (0.2 ml drawn into a heparinized glass capillary tube; unused blood was returned to the animal). The AIHc protocol was then performed: five 3-min episodes of hypercapnia [fraction of inspired carbon dioxide ($FiCO_2$) = 0.15] separated by 3 min of hyperoxic recovery (at FiO_2 = 0.5). No blood samples were taken during hypercapnia to minimize the overall blood volume taken from the animals. Arterial blood samples were taken at the regular intervals of 15, 30, 45, and 60 min after the end of the last (fifth) hypercapnic episode.

To confirm that microinjection of the 8-OH-DPAT, methysergide, or yohimbine in the caudal raphe region did not have a long-lasting, nonspecific effect on phrenic nerve activity, the viability of experimental preparation was tested in rats microinjected with 8-OH-DPAT, methysergide, or yohimbine and then not exposed to the hypercapnic protocol (n = 6). There were no changes in the pPNA during a period of up to 90 min (pPNA decreased by 22.53% in 8-OH-DPAT-microinjected rats, by 9.18% in methysergide-microinjected rats, and by 0.51% in yohimbine-microinjected rats).

Histology. At the end of the experiment, diluted India ink (20 ± 5 nl) was injected to mark the injection sites. Each animal was then perfused transcardially under deep anesthesia with Zamboni's fixative (4% formalin and 15% saturated picric acid in 0.1 M phosphate buffer). The brain stem was removed and stored in 4% formaldehyde in 0.1 M phosphate buffer at 4°C until 50-μm coronal sections were cut with a vibratome (Vibratome Series 1000, Pelco 101; Vibratome, St Louis, MO). Native sections were examined microscopically. The locations of injection sites were defined on the basis of the distances from marked sites to known anatomical structures (Paxinos 2005).

Statistical methods. The moving-time average of the phrenic nerve activity was obtained using the MA-1000 Moving Averager (System 1000 Modular Instrumentation; CWE) with a 50-ms time constant. Processed signal (phrenic neurogram, PNG) was recorded using a PowerLab system (ADInstruments, Castle Hill, Australia) and analyzed using PowerLab software, Chart for Windows version 5.4.2, and Scope for Windows version 3.8 (ADInstruments).

Analysis of recorded phrenic nerve activity and blood pressure data was performed by sampling averaged nerve activity and blood pressure over 20-s periods at 10 predetermined time points. The time points for data analysis were as follows: before the first hypercapnia (T0), during five hypercapnic exposures (THc1–THc5), and at 15

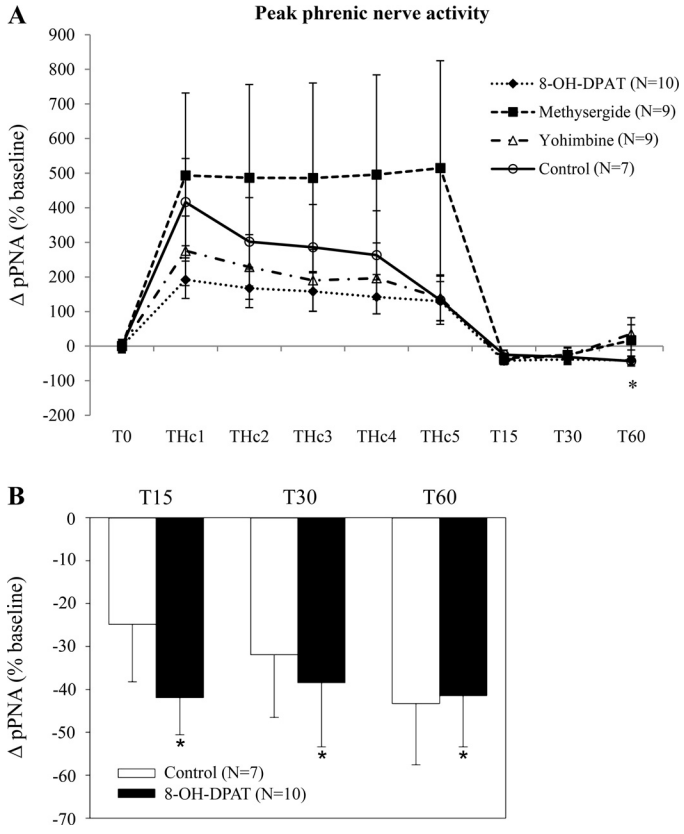

Fig. 2. Changes in peak phrenic nerve activity (ΔpPNA) in control and experimental groups. *A*: time course of changes in pPNA in the control and the 8-hydroxy-2-(dipropylamino)tetralin hydrobromide (8-OH-DPAT)-treated group reveals development of pLTD at 60 min after the last hypercapnic episode (T60; P = 0.0131), whereas in the methysergide- and yohimbine-treated groups, pLTD did not develop. *B*: bar graph shows changes of pPNA in the control and 8-OH-DPAT-treated groups at T15, T30, and T60. T0, baseline; THc1–THc5, 5 hypercapnic exposures.

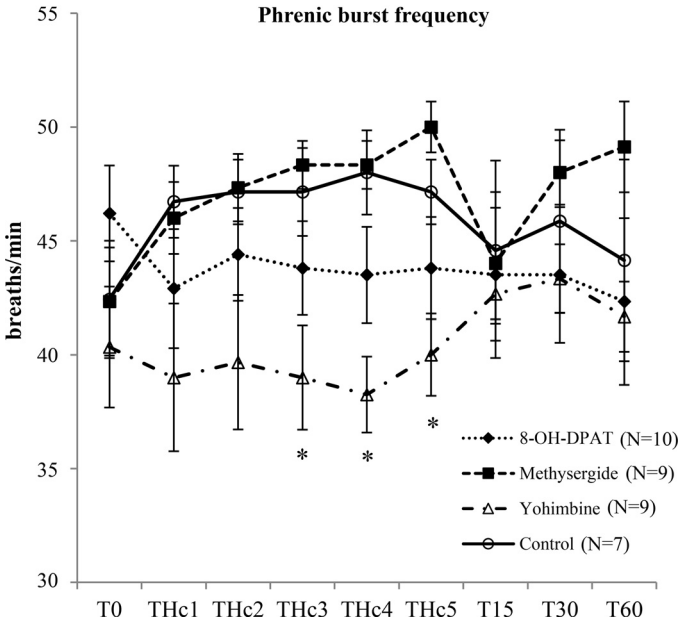

Fig. 3. Changes in phrenic nerve burst frequency (in breaths/min) in control and experimental groups. There were no significant changes in respiratory frequency at any time point during the experimental protocol within each group. *$P < 0.05$, significantly different from control group. 8-OH-DPAT, 8-hydroxy-2-(dipropylamino)tetralin hydrobromide. T0, baseline; THc1–THc5, 5 hypercapnic exposures; T15, T30, T60, 15, 30, and 60 min after the end of the last hypercapnic episode.

(T15), 30 (T30), and 60 min (T60) after the end of the last hypercapnic episode. The amplitude of integrated phrenic nerve activity was normalized as a percentage of the change from baseline activity in each animal.

In addition, phrenic motor output was analyzed in terms of phrenic burst frequency (*f*), inspiratory duration (Ti), expiratory duration (Te), and respiratory cycle duration (Ttot).

The data were analyzed using MedCalc (Ostend, Belgium) two-way repeated-measures analysis of variance (ANOVA; within and between groups) followed by Bonferroni post hoc correction. Two-way repeated-measures ANOVA and post hoc analyses were used to provide information for all pairwise comparisons of interest, both within and between groups at the same time points during the protocol. Data are means ± SE. A P value of 0.05 was considered statistically significant.

RESULTS

Control group. In the control group, AIHc evoked significant depression of the pPNA 60 min after the end of the last hypercapnic episode (pPNA decreased by 43.31 ± 14.28%, $F = 9.20$, df = 6, $P = 0.0230$, ANOVA; Figs. 1 and 2) compared with baseline values, i.e., pLTD was induced. There were no significant pPNA changes at 15 and 30 min after the end of the last hypercapnic episode (pPNA decreased by 24.86 ± 13.36% at 15 min, $F = 3.47$, df = 6, $P = 0.112$, and by 31.91 ± 14.66% at 30 min, $F = 4.74$, df = 6, $P = 0.0725$, ANOVA; Figs. 1 and 2). There were no significant changes in respiratory frequency at T15 (44.57 ± 3.95 breaths/min), T30 (45.86 ± 4.02 breaths/min), and T60 (44.14 ± 4.43 breaths/min) after the last hypercapnic episode compared with baseline frequency values (42.43 ± 2.57 breaths/min, $F = 0.4$, df = 6, $P = 1.000$, ANOVA; Fig. 3).

8-OH-DPAT group. In the group that received microinjection of 8-OH-DPAT into the caudal raphe region before the AIHc protocol, pPNA significantly decreased at T15 (41.95 ± 8.62%, $F = 23.71$; df = 9, $P = 0.0009$, ANOVA), T30 (38.45 ± 14.96%, $F = 6.61$, df = 9, $P = 0.0301$, ANOVA), and T60 (41.47 ± 11.9%, $F = 12.14$, df = 6, $P = 0.0131$, ANOVA; Figs. 1 and 2) compared with baseline values, i.e., pLTD was developed. No significant changes in respiratory frequency at T15 (43.50 ± 3.64 breaths/min), T30 (43.50 ± 2.97 breaths/min), and T60 (42.33 ± 3.66 breaths/min) after the last hypercapnic episode compared with baseline frequency values (46.20 ± 2.11 breaths/min) were observed in the 8-OH-DPAT group ($F = 2.75$, df = 7, $P = 1.000$, ANOVA; Fig. 3).

Methysergide group. In the group that received microinjection of the broad-spectrum 5-HT receptor antagonist methysergide, the AIHc protocol did not evoke significant changes in pPNA at T15 (decreased by 35.12 ± 14.92%), T30 (decreased by 25.64 ± 19.92%), and T60 (increased by 16.55 ± 45.17% compared with baseline values ($F = 1.62$, df = 6, $P = 1.000$, ANOVA; Figs. 1 and 2). There were no significant changes in

Table 1. *Relative changes in Ti, Te, and Ttot in control and experimental groups at different time points of protocol*

Group	T0	T15	T30	T60	THc1	THc2	THc3	THc4	THc5
Control									
Ti	0.550 ± 0.055	0.579 ± 0.041	0.612 ± 0.057	0.569 ± 0.038	0.380 ± 0.027	0.405 ± 0.047	0.423 ± 0.048	0.435 ± 0.050	0.429 ± 0.052
Te	0.793 ± 0.103	0.851 ± 0.176	0.758 ± 0.158	0.942 ± 0.174	0.930 ± 0.047	0.840 ± 0.068	0.813 ± 0.056	0.801 ± 0.059	0.798 ± 0.057
Ttot	1.351 ± 0.098	1.440 ± 0.168	1.374 ± 0.131	1.515 ± 0.179	1.307 ± 0.049	1.249 ± 0.045	1.243 ± 0.049	1.236 ± 0.055	1.229 ± 0.044
8-OH-DPAT									
Ti	0.506 ± 0.025	0.537 ± 0.019	0.559 ± 0.027	0.571 ± 0.054	0.425 ± 0.022	0.395 ± 0.018	0.369 ± 0.019	0.384 ± 0.019	0.376 ± 0.023
Te	0.847 ± 0.072	0.905 ± 0.103	0.875 ± 0.098	1.113 ± 0.173	0.910 ± 0.067	0.990 ± 0.064	1.018 ± 0.071	1.001 ± 0.075	1.002 ± 0.078
Ttot	1.365 ± 0.065	1.419 ± 0.092	1.420 ± 0.102	1.666 ± 0.214	1.361 ± 0.096	1.434 ± 0.117	1.421 ± 0.091	1.411 ± 0.079	1.404 ± 0.082
Methysergide									
Ti	0.560 ± 0.034	0.512 ± 0.049	0.456 ± 0.046	0.456 ± 0.036	0.330 ± 0.022	0.319 ± 0.023	0.310 ± 0.034	0.304 ± 0.030	0.312 ± 0.042
Te	0.778 ± 0.078	0.882 ± 0.068	0.849 ± 0.066	0.817 ± 0.071	0.941 ± 0.043	0.962 ± 0.033	0.920 ± 0.025	0.923 ± 0.025	0.919 ± 0.064
Ttot	1.345 ± 0.090	1.369 ± 0.075	1.293 ± 0.039	1.266 ± 0.069	1.250 ± 0.042	1.260 ± 0.040	1.219 ± 0.029	1.218 ± 0.026	1.221 ± 0.028
Yohimbine									
Ti	0.526 ± 0.031	0.552 ± 0.036	0.570 ± 0.033	0.584 ± 0.042	0.378 ± 0.019	0.389 ± 0.021	0.385 ± 0.023	0.402 ± 0.023	0.392 ± 0.020
Te	1.013 ± 0.101	0.804 ± 0.032	0.799 ± 0.061	0.832 ± 0.069	1.227 ± 0.119	1.200 ± 0.117	1.116 ± 0.077	1.080 ± 0.075	1.073 ± 0.066
Ttot	1.541 ± 0.098	1.360 ± 0.052	1.376 ± 0.065	1.419 ± 0.070	1.606 ± 0.125	1.591 ± 0.124	1.500 ± 0.083	1.483 ± 0.080	1.466 ± 0.067

Values are means ± SE (in seconds) of inspiratory time (Ti), expiratory time (Te), and total respiratory time (Ttot) in control group ($n = 7$) and in experimental groups microinjected with 8-hydroxy-2-(dipropylamino)tetralin hydrobromide (8-OH-DPAT; $n = 10$), methysergide ($n = 9$), and yohimbine ($n = 9$). T0, baseline value before the first hypercapnia; T15, T30, and T60, 15, 30, and 60 min after the last hypercapnic episode, respectively; THc1–THc5: first to fifth hypercapnic episodes.

Table 2. *MAP in control and experimental groups at different time points of protocol*

Group	MAP, mmHg								
	T0	T15	T30	T60	THc1	THc2	THc3	THc4	THc5
Control	129.8 ± 8.3	123.0 ± 8.4	124.0 ± 7.3	113.4 ± 10.2	128.4 ± 13.8	122.7 ± 9.4	117.1 ± 10.3	116.2 ± 9.1	106.4 ± 11.2
8-OH-DPAT	124.0 ± 6.9	123.4 ± 10.9	123.7 ± 13.7	118.7 ± 11.4	117.5 ± 10.2	117.2 ± 13.6	114.8 ± 14.9	110.8 ± 15.7	113.0 ± 14.3
Methysergide	117.1 ± 6.7	95.9 ± 9.0	105.1 ± 8.7	93.3 ± 7.9	113.8 ± 4.9	114.1 ± 6.3	113.9 ± 5.7	111.2 ± 7.7	110.8 ± 8.1
Yohimbine	129.3 ± 5.3	132.7 ± 3.9	128.8 ± 4.9	131.0 ± 6.3	119.4 ± 5.4	124.8 ± 7.0	127.2 ± 4.6	124.9 ± 6.2	124.4 ± 6.1

Values are means ± SE of mean arterial blood pressure (MAP) in control group ($n = 7$) and in experimental groups microinjected with 8-hydroxy-2-(dipropylamino)tetralin hydrobromide (8-OH-DPAT; $n = 10$), methysergide ($n = 9$), and yohimbine ($n = 9$). T0, baseline value before the first hypercapnia; T15, T30, and T60, 15, 30, and 60 min after the last hypercapnic episode, respectively; THc1–THc5: first to fifth hypercapnic episodes.

respiratory frequency at T15 (44.00 ± 2.45 breaths/min), T30 (48.00 ± 1.41 breaths/min), and T60 (49.13 ± 1.99 breaths/min) after the last hypercapnic episode compared with baseline frequency values (42.33 ± 2.37 breaths/min; $F = 0.79$, df = 2, $P = 1.000$, ANOVA; Fig. 3).

Yohimbine group. In the group that received microinjection of the selective α2-adrenergic antagonist yohimbine into the caudal raphe region, AIHc evoked a transient decrease in pPNA at T15 (decreased by 39.2 ± 14.6%; $F = 7.21$, df = 8, $P = 0.0277$, ANOVA), but there were no significant changes in pPNA at T30 (decreased by 26.85 ± 23.68%) and T60 (increased by 35.47 ± 46.7%; $F = 1.69$, df = 7, $P = 1.000$, ANOVA; Figs. 1 and 2) compared with baseline values, i.e., development of the pLTD was prevented. There were no significant changes in respiratory frequency at T15 (42.67 ± 1.30 breaths/min), T30 (43.33 ± 1.51 breaths/min), and T60 (41.67 ± 1.54 breaths/min) after the last hypercapnic episode compared with baseline frequency values (40.33 ± 2.65 breaths/min; $F = 0.47$, df = 3, $P = 1.000$, ANOVA; Fig. 3).

General parameters. Changes in pPNA during intermittent hypercapnic episodes (THc1–THc5) are shown in Figs. 1 and 2. The frequency response to intermittent hypercapnia (THc1–THc5) elicited nonsignificant changes in respiratory frequency during exposures to hypercapnic stimulus ($F = 1.43$, df = 6, $P = 1.000$, ANOVA; Figs. 1*B* and 3). With regard to respiratory cycle parameters, there were no significant changes in Ti, Te, and Ttot at 15, 30, and 60 min after the last hypercapnic stimulus compared with baseline values in any group of animals, as shown in Table 1. Mean arterial pressure (MAP) tended to decrease during the hypercapnic exposures and then returned to baseline levels, and it was successfully maintained relative to baseline in all experimental groups of animals until the end of the experiments, as shown in Table 2. There were no significant changes in Pa_{O_2} and Pa_{CO_2} values at any time point of the experiment in any experimental group, as shown in Table 3. The value of pH was maintained between 7.2 and 7.3 in all groups throughout the experiment. Microinjection sites into the caudal raphe region were verified histologically, as shown in Fig. 4.

DISCUSSION

This study demonstrated that exposure to acute intermittent hypercapnia evoked development of pLTD 60 min after the last hypercapnic episode. Furthermore, pLTD following episodic hypercapnia was preserved if the selective 5-HT_{1A} receptor agonist 8-OH-DPAT was microinjected in the caudal raphe region before episodic hypercapnic exposure. In addition, raphe administration of the nonselective 5-HT receptor antagonist methysergide caused nonsignificant changes in pPNA 60 min after the hypercapnic protocol. In the group that received microinjection of the selective α2-adrenergic antagonist yohimbine into the caudal raphe region, development of the pLTD was prevented. Thus we concluded that both 5-HT and adrenergic receptor activation is needed for induction of pLTD in urethane-anesthetized rats following intermittent hypercapnia exposure.

Chemical modulation of respiratory plasticity by 5-HT and α2-adrenergic receptors. It is well known that hypercapnia activates brain stem noradrenergic neurons (Bach and Mitchell 1998; Elam et al. 1981; Guyenet et al. 1993; Haxhiu et al. 1996). Norepinephrine released from these neurons can inhibit respiratory output by activating α2-adrenergic receptors (Errchidi et al. 1990, 1991; Hedrick et al. 1995, Hedrick et al. 1998; Kinkead et al. 2001), making it reasonable to propose that noradrenergic modulation might be involved in the underlying mechanisms of pLTD following acute hypercapnic exposure (Bach and Mitchell 1998; Errchidi et al. 1990).

Distinct forms of respiratory plasticity can be evoked under specific respiratory challenges including episodes of intermit-

Table 3. *Partial pressures of CO_2 and O_2 and pH in arterial blood in control and experimental groups at different time points of protocol*

Group	T0			T15			T30			T60		
	Pa_{CO_2}	Pa_{O_2}	pH	Pa_{CO_2}	Pa_{O_2}	pH	Pa_{CO_2}	Pa_{O_2}	pH	Pa_{CO_2}	Pa_{O_2}	pH
Control	45.2 ± 2.3	235.5 ± 16.0	7.3 ± 0.1	46.4 ± 2.7	212.3 ± 34.2	7.2 ± 0.02	46.5 ± 2.7	224.6 ± 17.1	7.3 ± 0.02	41.9 ± 2.3	194.7 ± 20.3	7.2 ± 0.03
8-OH-DPAT	43.3 ± 1.2	278.3 ± 7.7	7.3 ± 0.02	45.6 ± 2.2	272.3 ± 11.5	7.2 ± 0.02	41.7 ± 1.4	285.6 ± 9.2	7.2 ± 0.03	42.8 ± 2.1	262.8 ± 11.8*	7.2 ± 0.02
Methysergide	42.4 ± 2.0	281.9 ± 8.4	7.3 ± 0.02	45.0 ± 1.5	275.3 ± 6.9	7.3 ± 0.02	42.4 ± 1.4	268.5 ± 8.3	7.3 ± 0.02	41.1 ± 1.8	278.5 ± 20.3*	7.3 ± 0.03
Yohimbine	43.0 ± 1.4	235.5 ± 20.6	7.3 ± 0.01	45.7 ± 2.5	236.5 ± 20.1	7.2 ± 0.01	42.2 ± 1.1	236.1 ± 20.6	7.2 ± 0.02	40.2 ± 2.1	221.3 ± 27.1	7.2 ± 0.03

Values are means ± SE of partial pressure of carbon dioxide (Pa_{CO_2}), partial pressure of oxygen (Pa_{O_2}), and pH in control group ($n = 7$) and in experimental groups microinjected with 8-hydroxy-2-(dipropylamino)tetralin hydrobromide (8-OH-DPAT; $n = 10$), methysergide ($n = 9$), and yohimbine ($n = 9$). T0, baseline value before the first hypercapnia; T15, T30, and T60, 15, 30, and 60 min after the last hypercapnic episode, respectively; *$P < 0.05$, significantly different from control.

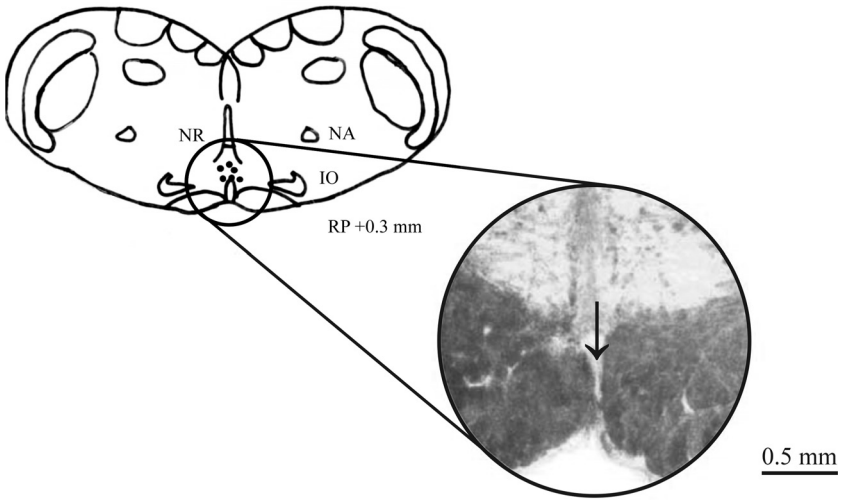

Fig. 4. Photomicrograph and schematic of transverse section through the caudal raphe region, illustrating the injection site (arrow) at which microinjection was performed. NR, nuclei raphe; NA, nucleus ambiguus; IO, inferior olive; RP +0.3 mm, raphe pallidus 0.3 mm rostral to obex.

tent hypoxia (Baker-Herman and Mitchell 2008) or intermittent hypercapnia (Bach and Mitchell 1998). Both forms of respiratory plasticity might oppose one another in a "push-pull" manner (Kinkead et al. 2001).

Previous research has shown that in anesthetized animals, intermittent hypercapnia-induced pLTD depends on chemical modulation of both 5-HT (Valic et al. 2016) and α2-adrenergic receptors (Bach and Mitchell 1998). Systemic administration of the broad-spectrum 5-HT receptor antagonist methysergide raised questions regarding specific type and precise location of the relevant 5-HT receptors at the spinal and/or supraspinal level (Valic et al. 2016). In our study, the selective 5-HT_{1A} agonist 8-OH-DPAT accentuated hypercapnia-induced pLTD, and the broad-spectrum 5-HT antagonist methysergide abolished pLTD. Methysergide has been shown to have an antagonistic effect on 5-HT type 1, 2, 4, 5, 6, and 7 receptors, as well as an agonistic effect on 5-HT_{1A} receptors, making it is reasonable to believe that another 5-HT receptor contributes to the development of pLTD. In our study, an agonist of 5-HT_{1A} receptors accentuated pLTD, suggesting that their activation in the caudal raphe region is needed for the development of pLTD. Similarly, systemic modulation of α2-adrenergic receptors has been shown to prevent development of pLTD acting through many sites at the supraspinal level, including α2-adrenergic receptors located on serotonergic raphe neurons to inhibit serotonin release (Haddjeri et al. 1996). Therefore, in this study, we investigated the effects of chemical modulation of α2-adrenergic receptors located in the caudal raphe region on the development of hypercapnia-induced pLTD.

Contribution of anatomical interconnections to pLTD development. Anatomical evidence has been provided by immunohistochemical studies indicating that both the caudal ventrolateral pons (A5) region and the LC (A6) send projections to relevant respiratory centers (Bach and Mitchell 1998; Dobbins and Feldman 1994), including raphe nuclei (Anderson et al. 1977; Baraban and Aghajanian 1981; Hermann et al. 1997; Jones and Yang 1985; Loizou 1969; Luppi et al. 1995). It was previously explained that a possible mechanism of long-term depression could depend on hypercapnia-induced release of norepinephrine acting on α2-adrenergic receptors located on raphe serotonergic neurons to inhibit raphe excitability and the release of serotonin (Bach and Mitchell 1998). Thus serotonin-dependent phrenic long-term facilitation (pLTF) might be diminished, and pLTD could be evoked (Bach and Mitchell 1998). Our working model concerning the mechanisms of distinct forms of respiratory plasticity is shown in Fig. 5. It is known that acute intermittent hypoxia activates raphe medullary neurons that promote the development of pLTF via the increased release of serotonin. On the contrary, acute intermittent hypercapnia activates brain stem noradrenergic neurons in LC (A6) and caudal ventrolateral pons (A5), triggering norepinephrine release. Hypercapnia-induced release of norepinephrine acting on α2 receptors leads to pLTD development, either directly, via projections to phrenic motoneurons, or indirectly, via α2 receptors located on raphe serotonergic neurons that inhibit release of serotonin and pLTF (Dobbins and Feldman 1994).

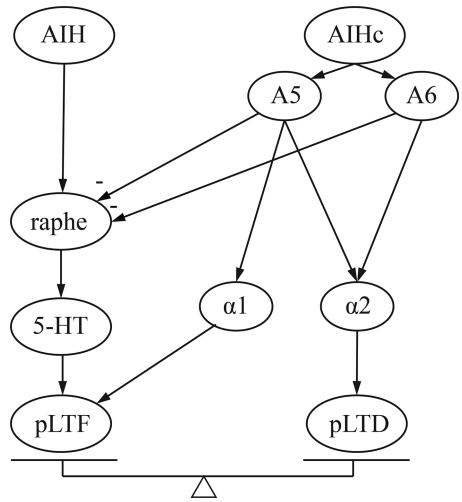

Fig. 5. Working model concerning the mechanisms of distinct forms of respiratory plasticity. Acute intermittent hypoxia (AIH) activates raphe medullary neurons that, by the increased release of serotonin, promote the development of phrenic long-term facilitation (pLTF). Acute intermittent hypercapnia (AIHc) activates brain stem noradrenergic neurons in the locus coeruleus (A6) and caudal ventrolateral pons (A5), triggering norepinephrine release. Hypercapnia-induced release of norepinephrine acting on α2 receptors leads to development of phrenic long-term depression (pLTD), either directly, via projections to phrenic motoneurons, or indirectly, via α2 receptors located on raphe serotonergic neurons that inhibit release of serotonin and development of pLTF.

Data from our previous study indicate that systemic injection of a selective 5-HT_{1A} receptor antagonist (Valic et al. 2016) produces effects similarly to the central administration of 5-HT_{1A} agonist into the medullary raphe region (Taylor et al. 2005). In the present study, pLTD was evoked if the selective serotonin receptor agonist 8-OH-DPAT was microinjected into the caudal raphe region before hypercapnic exposure. It is possible that activation of 5-HT_{1A} receptors in the caudal raphe region inhibited the activity of raphe neurons via modulation of 5-HT_{1A} autoreceptors and thus led to decreased release of serotonin (Baker-Herman and Mitchell 2002). Decreased release of serotonin might prevent its facilitatory effect responsible for pLTF development, and thus pLTD could be evoked. On the contrary, due to its antagonistic or agonistic effects on different serotonin receptors (Valic et al. 2016), methysergide might lead to increased release of serotonin, and thus enhance serotonin dependent pLTF, which might cause pLTD to be further diminished.

On the other hand, activation of α2-adrenergic receptors is needed for pLTD development following episodic hypercapnia (Bach and Mitchell 1998). Microinjection of yohimbine (antagonist of α2-adrenergic receptors) into the caudal raphe region might enable raphe excitation during hypercapnia, leading to increased release of serotonin, enhancement of serotonin-dependent pLTF, and attenuation of pLTD as seen in this study.

Experimental specificity and viability. In our study, MAP was maintained throughout the whole experiment and did not affect respiratory motor output. According to the results reported in this article, the time control preparations provided stability to our experimental design that allowed us to draw the conclusions we have offered. However, there was a slight difference in the preparation protocol compared with our previous studies. In this study, the protocol was conducted in background hyperoxia (50% O_2) to eliminate additional hypoxic stimulus between challenging episodes (Bach and Mitchell 1998; Stipica et al. 2016). Additionally, the raphe region was mapped with injection of the selective glutamate receptor agonist DLH, which evoked an increase in pPNA with no significant change in the respiratory frequency (Valic et al. 2010). Although effects of DLH microinjections were transient, allowing further chemical modulation of the affected neurons in the raphe region, they might have contributed to the enhanced pLTD magnitude seen in this study.

Clinical significance and perspectives. The biological and clinical significance of intermittent hypercapnia might be recognized in sleep-related breathing disorders. Bearing in mind that respiratory plasticity in humans depends on increased levels of carbon dioxide, intermittent hypercapnia could be a viable means of restoring respiratory functions in respiratory control (Gerst et al. 2011; Harris et al. 2006; Lee et al. 2009; Mateika and Syed 2013; Wadhwa et al. 2008; Yokhana et al. 2012).

The results of this study indicate that exposure to acute intermittent hypercapnia evoked development of pLTD that was preserved if the selective 5-HT_{1A} receptor agonist 8-OH-DPAT was microinjected in the caudal raphe region. On the other hand, microinjections of the selective α2-adrenergic antagonist yohimbine or the nonselective 5HT antagonist methysergide into the caudal raphe region prevented development of the pLTD. Thus we conclude that chemical modulation of 5-HT and α2-adrenergic receptors in raphe nuclei affects hypercapnia-induced pLTD in anesthetized animals.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

M.V. conceived and designed research; I.S.S., I.P.D., and M.V. performed experiments; I.S.S., I.P.D., and M.V. analyzed data; I.S.S., R.P., I.P.D., Z.D., and M.V. interpreted results of experiments; I.P.D., Z.V., and M.V. prepared figures; I.S.S., I.P.D., and M.V. drafted manuscript; I.S.S., R.P., I.P.D., Z.D., Z.V., and M.V. edited and revised manuscript; I.S.S., R.P., I.P.D., Z.D., Z.V., and M.V. approved final version of manuscript.

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