Blockade of α2-adrenergic receptors in the caudal raphe region enhances the renal sympathetic nerve activity response to acute intermittent hypercapnia in rats

Katarina Madirazza[1], Renata Pecotic[1,†], Ivana Pavlinac Dodig[1], Maja Valic[1] and Zoran Dogas[1]

[1]Department of Neuroscience, University of Split School of Medicine, Soltanska 2, 21000 Split, Croatia

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[†]Corresponding author:

Assoc Prof Renata Pecotic, MD, PhD

Department of Neuroscience, University of Split School of Medicine, Soltanska 2, 21000 Split, Croatia

E-mail: renata.pecotic@mefst.hr

Phone: +38521557951

SUMMARY

The study investigated the role of α2-adrenergic receptors of the caudal raphe region in the sympathetic and cardiovascular responses to the acute intermittent hypercapnia (AIHc). Urethane-anesthetized, vagotomized, mechanically ventilated Sprague-Dawley rats (n=38) were exposed to the AIHc protocol (5x3 min, 15% CO_2+50% O_2) in hyperoxic background (50% O_2). α2-adrenergic receptor antagonist–yohimbine was applied intravenously (1 mg/kg, n=9) or microinjected into the caudal raphe region (2 mM, n=12) prior to exposure to AIHc. Control groups of animals received saline intravenously (n=7) or into the caudal raphe region (n=10) prior to exposure to AIHc. Renal sympathetic nerve activity (RSNA), mean arterial pressure (MAP) and heart rate (HR) were monitored before exposure to the AIHc protocol (T0), during five hypercapnic episodes (THc1-5) and at 15 minutes following the end of the last hypercapnic episode (T15). Following intravenous administration of yohimbine, RSNA was significantly greater during THc1-5 and at T15 than in the control group ($P<0.05$). When yohimbine was microinjected into the caudal raphe region, AIHc elicited greater increases in RSNA during THc1-5 when compared to the controls (THc1:138.0±4.0% vs. 123.7±4.8%, P=0.032; THc2:137.1±5.0% vs. 124.1±4.5%, P=0.071; THc3:143.1±6.4% vs. 122.0±4.8%, P=0.020; THc4:146.1±6.2% vs. 120.7±5.7%, P=0.007 and THc5:143.2±7.7% vs. 119.2±7.2%, P=0.038). During THc1-5, significant decreases in HR from T0 were observed in all groups, while changes in MAP were observed in the group that received yohimbine intravenously. These findings suggest that blockade of the α2-adrenegic receptors in the caudal raphe region might have an important role in sympathetic responses to AIHc.

Key words: acute intermittent hypercapnia, sympathetic nervous activity, α2-adrenergic receptors, raphe nuclei, yohimbine

INTRODUCTION

Hypercapnia, which is a hallmark of many respiratory disorders, has been shown to have a substantial effect on various organ systems [1]. It is known to stimulate both peripheral and central chemoreceptors [2, 3], consequently driving hyperventilation, raised blood pressure and elevated sympathetic nerve activity [1, 4]. Hypercapnia-induced ventilatory response has been well described previously, such that the elevation of the arterial partial pressure of carbon dioxide ($PaCO_2$) leads to a strong activation of the areas controlling the respiratory activity, and the response seems to be largely dependent on the medullary raphe neurons [5-7]. Moreover, when administered intermittently, hypercapnia is documented to cause a long-term depression of breathing modulated by both, serotonergic and adrenergic receptors [8-12].

Although a large pool of evidence shows single episode or sustained hypercapnia potently stimulate the sympathetic nervous system [1, 3, 13-15], the effects of intermittent hypercapnia on sympathetic activity remain unclear. Recently, our laboratory demonstrated that the acute intermittent hypercapnia (AIHc) causes substantial activation of the sympathetic nervous system measured through the renal sympathetic nerve activity (RSNA), which is dependent on the background oxygen level [16]. Evaluating different protocols, we found that severe acute intermittent hyperoxic hypercapnia (15% CO_2+50% O_2) modestly activates the RSNA, but the magnitude of the response is significantly lower than in a normoxic background, suggesting the involvement of a central mechanism.

Central neural pathways controlling the cardiorespiratory outflow are complex and to this date not fully worked out [17]. Undoubtedly, central chemoreception evokes increases in sympathetic nervous activity by direct action on cardiovascular centers or mediated by the central respiratory network [17, 18]. Thus far, different regions have been proposed to act as central CO_2 chemoreceptors [14, 15, 18-20], among which locus coeruleus (LC) and raphe nuclei have been reported to be extremely responsive to elevations in $PaCO_2$ [6, 19-22].

Projections of the noradrenergic groups participate in increases in breathing and sympathetic nerve activity caused by hypercapnia or reduced pH in noradrenergic brainstem regions [20, 21]. Dense projections from LC to the caudal raphe region exist, where they can induce, either, excitatory effects via α1-adrenergic receptors or inhibitory effects via α2-adrenergic receptors [23]. Nuclei of the caudal raphe region (nucl. raphe magnus, obscurus and pallidus) send extensive descending projections to the spinal cord, innervating the dorsal and ventral horns and the intermediolateral cell column (IML) [24]. The caudal raphe region, comprised of mainly serotonergic neurons, has been implicated in many neurophysiological functions including the modulation of pain, respiration, motor activity and various autonomic functions [24-26]. It has been proposed that the caudal raphe nuclei are involved in sympathetic control by means of direct projections to the IML, innervating the preganglionic sympathetic neurons [23, 26-28], but also to other regions controlling the sympathetic activity, such as the rostral ventrolateral medulla (RVLM) [29].

In light of this, the aim of the present study was to determine the role of α2-adrenergic receptors of the caudal raphe region in the sympathetic and cardiovascular responses to the AIHc. The effects of blockade of α2-adrenergic receptors in the caudal raphe region on renal sympathetic nerve, arterial pressure and heart rate responses to acute intermittent hypercapnia were examined.

MATERIALS AND METHODS

All experimental procedures were designed in accordance with the European Guidelines on Laboratory Animal Care and were approved by the Ethical Committee for Biomedical Research of the University of Split School of Medicine (Split, Croatia) and the National Ethics Committee of the Veterinary Directorate, Ministry of Agriculture, Republic of Croatia. The animals used in this study were bred and maintained in controlled environment rooms (22-24°C; 55-70% relative humidity;12h-light:12h-dark cycle) with access to food and water *ad libitum* at the University of Split School of Medicine Animal Facility.

General procedures

Male Sprague-Dawley rats (n=38; body weight 280-350 g) were anesthetized by an intraperitoneal injection of urethane (20% urethane in 0.9% saline, dose 1.2 g/kg). The adequacy of anesthesia was determined by the hind paw withdrawal and corneal reflex loss. Additional doses of urethane (0.2 g/kg i.v.) were given, if required, in order to maintain a deep anesthesia before exposing the animals to the experimental protocol. Upon achieving the adequate level of anesthesia, femoral arteries and veins were catheterized for arterial blood pressure monitoring, blood gas sampling and drug/fluid administration, respectively. The trachea was cannulated and vagus nerves cut bilaterally at cervical level (C3-5) to facilitate the mechanical ventilation. The animals were ventilated using a 50:50 oxygen-nitrogen mixture throughout the experiment by means of a small animal ventilator (SAR 830-P, CWE, Ardmore, PA, USA). Physiological levels of pH and $PaCO_2$ were maintained by adjusting the frequency and/or inspiratory time parameters on the ventilator in accordance with the blood gas analysis results (RAPIDPoint 500; Siemens Healthcare Limited, Surrey, UK). Since the animals were maintained on a hyperoxic mixture, the arterial partial pressure of oxygen (PaO_2) was in the range of 250-360 mmHg. To ensure the overall stability of the preparation, the fluids were

supplemented continuously (0.6 $ml \cdot h^{-1} \cdot kg^{-1}$) and the animals' body temperature was maintained by a heating pad (FST, Heidelberg, Germany) throughout the experiment. The animals were then positioned in a stereotaxic frame (Lab Standard, Stoelting, Wood Dale, IL, USA) in a prone position and the left renal nerve was exposed using a retroperitoneal approach. The nerve was placed on a bipolar silver wire electrode and the surgical field was covered with silicone gel to secure the electrode, provide electrical noise isolation and prevent desiccation.

Recording parameters

The signal from the recording electrode was amplified (SuperZ, System 1000, CWE Inc., Ardmore, USA), filtered (300 Hz–10 kHz, bandpass filter), full wave rectified and integrated (MA-1000 PowerLab Moving Averager module for System 1000; 50 ms time constant). Chart 5.4.2. for Windows software (ADInstruments, Bella Vista, Australia) was used to obtain the RSNA electrograms, arterial blood pressure tracings (Memscap blood pressure transducer, Skoppum, Norway) and record the signals simultaneously in high resolution at a sampling rate of 20 kHz.

Raphe nuclei identification and microinjection technique

A partial occipital craniotomy was performed and the dura reflected to expose the dorsal surface of the brain stem and the obex. Relative to the obex, the initial coordinates for identification of the caudal raphe region were: +0.2 mm anterioposterior, 0.0 mm mediolateral and -2.5 mm dorsoventral and were determined based on our previous study [11] relying on the rat brain atlas of Paxinos and Watson [30]. A four-barrel glass micropipette (external tip diameter: 30-50 μm) was filled with the vehicle (0.9% saline), selective glutamate agonist D,L-homocysteic acid (DLH; 10 mM, Sigma-Aldrich, St Louis, MO, USA), selective α2-adrenergic receptor antagonist yohimbine (2 mM, Sigma-Aldrich) and diluted India ink solution. The tip of the micropipette was lowered into the position targeting the caudal raphe region according to the

initial coordinates. Following the placement of the micropipette, DLH (10 mM in 0.9% saline; 20±5 nl, Sigma-Aldrich) was microinjected and RSNA and blood pressure changes monitored. If no response was observed, the coordinates were finely adjusted until an increase in RSNA and a pressor response of >20 mmHg was evoked [29]. After the transient effects of DLH dissipated, the micropipette was flushed with the vehicle to ensure no glutamate agonist remained in the pipette. Microinjections were performed by a pressure ejection system using a large plastic syringe connected to the barrels by polyethylene tubing. The application of positive pressure allowed for the solution ejection until the final volume reached 20±5 nl. The volume ejected was controlled by monitoring the fluid meniscus under a monocular microscope with a finely graduated eyepiece.

Experimental design

The animals were allowed a 30-min stabilization period before the onset of recordings and the exposure to the experimental protocol. Then, the animals were subjected to the AIHc protocol, which consisted of 5 exposures to a hypercapnic gas mixture interspersed by 3 min recovery periods. The hypercapnic gas mixture applied was 15% CO_2+50% O_2 in N_2 and the duration of each episode was 3 min. During the recovery periods the animals were ventilated with a hyperoxic mixture of 50% O_2 in N_2. Two sets of experiments were performed: first, involving the systemic blockade of the α2-adrenergic receptors and, second, involving the central blockade of the α2-adrenergic receptors with their respective control groups. In the first set, the yohimbine group (n=9) received a bolus injection of yohimbine (1 mg/kg, 0.6 ml, Sigma-Aldrich) intravenously before exposure to the AIHc protocol. In order to comply with the ethical standards for animal experimentation, one set of data from our previous study was used as a control group (n=7) [16]. In this group, the animals were given the same volume of 0.9% saline in a bolus before exposure to the AIHc protocol under identical conditions as applied in this study. In the second set of experiments, the yohimbine group (n=12) received a

microinjection of α2-adrenergic receptor antagonist yohimbine (2 mM in 0.9% saline, 20±5 nl, Sigma-Aldrich) into the same caudal raphe site previously mapped using DLH and then exposed to the experimental protocol. In the control group (n=10), the same volume of 0.9% saline was microinjected into the caudal raphe region before the animals were subjected to the AIHc protocol. Before commencing the experimental protocol, an arterial blood sample (0.2 ml) was taken and arterial blood gas values measured. Another control arterial blood sample was taken 15 min following the end of the last hypercapnic episode. No blood samples were taken during the hypercapnic exposures to minimize the overall circulating volume loss and its possible effects on blood pressure and sympathetic activity. The experimental design is shown in Figure 1.

Histological processing

After the completion of each experiment, diluted India ink (20±5 nl) was deposited at the microinjection site and the animal was perfused transcardially with saline, followed by Zamboni's fixative (4% formaldehyde and 15% picric acid in 0.1 M PBS (phosphate buffered saline)). The brainstem was then removed and stored in Zamboni's fixative for 24 h at 4ºC. Following fixation, the brainstem was washed with 0.1 M PBS and cut in 50 µm coronal sections using a vibrating microtome (Vibratome Series 1000, Pelco 101; Vibratome, St Louis, MO, USA). The injection sites were verified using a conventional microscope and determined in respect to a reference section from the atlas of Paxinos and Watson [30].

Data analysis

Recorded variables were measured at 7 predetermined time points: immediately before the onset of the first hypercapnic episode (T0), during each of five hypercapnic episodes (THc1-THc5) and at 15 min following the end of the last hypercapnic episode (T15) using LabChart 8.1.13. for Windows software (ADInstruments). At each experimental time point, 20-second

intervals were used to analyze RSNA, MAP and HR. The integrated signal was used to quantify the RSNA by measuring the area under the curve (i.e., calculating the integral from the minimum) in arbitrary units. To allow for comparison within and across groups, RSNA values were reported as a percentage of the baseline activity for each experiment. MAP was derived from the blood pressure signal and expressed in mmHg. HR was calculated from the arterial blood pressure waveform using average cycling rate function and expressed in beats per minute. All data analyses were conducted in MedCalc statistical package, version 19.1.2 (MedCalc Software, Mariakerke, Belgium). The normality of the data distribution was verified using Shapiro-Wilk's test of normality for all studied variables. Two-way repeated measures ANOVA with a post-hoc Bonferroni correction was used for multiple comparisons within groups for all experimental variables. One-way ANOVA with Student-Newman-Keuls test was used for pairwise comparisons of corresponding experimental data points between groups. Data are reported as mean±standard error of the mean (SEM). Statistical significance was set at $P<0.05$.

RESULTS

Systemic blockade of α2-adrenergic receptors

Intravenous application of yohimbine evoked significant increases in RSNA during all five hypercapnic episodes when compared to the baseline (THc1: 169.2±10.0%, P=0.009; THc2: 178.9±11.0%, P=0.008; THc3: 180.7±13.2%, P=0.023; THc4: 179.0±12.7%, P=0.019; THc5: 173.2±11.5%, P=0.018; F=30.81, df=6, P<0.001, ANOVA, Figure 2), whereas in the control group a significant increase was observed only during the first hypercapnic episode (THc1: 126.0±5.2%, P=0.036; F=5.82, df=5, P=0.014, ANOVA, Figure 2). A significant interaction between hypercapnic episodes and treatment was found (F=10.84, df=6, P=0.001, ANOVA). Pair-wise comparisons revealed that RSNA activation was greater during all hypercapnic episodes (THc1-5) and at T15 in the yohimbine group than in the control group (Figure 2). At baseline, MAP was significantly lower in the yohimbine group in comparison to the control group (54.9±1.9 vs. 94.3±7.2 mmHg, F=35.57, df=1, P<0.001, ANOVA), but no significant differences in HR were observed. During the hypercapnic episodes, significant decreases in HR were observed in both studied groups. In the control group, AIHc did not evoke significant changes in MAP, while in the yohimbine group MAP was significantly higher at THc4, THc5 and T15 when compared to the baseline (71.1±3.7 mmHg, P=0.018; 73.4±3.3 mmHg, P=0.004; 67.3±3.3 mmHg, P=0.028 vs. 54.9±1.9 mmHg, respectively; F=11.31, df=6, P<0.001, ANOVA, Table 1).

Blockade of α2-adrenergic receptors in the caudal raphe region

Control group. A significant increase in RSNA was observed during the first three hypercapnic episodes when compared to the baseline (F=5.54, df=6, P=0.014, ANOVA; Figure 2). There were no significant changes in RSNA at 15 min following the end of the last hypercapnic episode when compared to the baseline (T15: 103.1±9.8% baseline, F=5.54, df=6, P=1.000,

ANOVA; Figure 2). No significant changes in MAP were observed during or following the AIHc protocol (F=1.37, df=6, P=0.280, ANOVA; Table 1). HR was significantly lower when compared to the baseline during all five hypercapnic episodes (F=143.85, df=6, P<0.001, ANOVA; Table 1). There were no significant changes in pH and $PaCO_2$ at 15 min following the last hypercapnic episode, while PaO_2 was significantly higher at T15 when compared to the baseline (299.5±5.3 mmHg vs. 286.6±3.9 mmHg, F=18.83, df=1, P=0.002, ANOVA; Table 2).

Yohimbine group. A significant increase in RSNA was observed during all five hypercapnic episodes when compared to the baseline. At T15, RSNA was significantly lower when compared to all five hypercapnic episodes, but not compared to the baseline (F=27.40, df=6, P<0.001, ANOVA; Figure 2). MAP was lower at T15 when compared to last three hypercapnic episodes, but not compared to the baseline (F=7.26, df=6, P=0.001, ANOVA; Table 1). During THc1-5, HR was significantly lower when compared to the baseline and T15 (F=78.57, df=6, P<0.001, ANOVA; Table 1). No differences in HR were observed between baseline and T15 (344.5±8.2 vs. 339.4±8.3 beats/min, F=78.57, df=6, P=1.000, ANOVA; Table 1). There were no significant changes in pH, $PaCO_2$ or PaO_2 at T15 when compared to the baseline (Table 2).

Control vs. yohimbine group. A significant interaction between hypercapnic episodes and treatment was found (F=4.44, df=6, P=0.012, ANOVA). AIHc elicited a greater increase in RSNA during all five hypercapnic episodes in the group that received a yohimbine microinjection into the caudal raphe region when compared to the control group (THc1: 138.0±4.0% vs. 123.7±4.8%, F=5.32, df=1, P=0.032; THc2: 137.1±5.0% vs. 124.1±4.5%, F=3.63, df=1, P=0.071; THc3: 143.1±6.4% vs. 122.0±4.8%, F=6.39, df=1, P=0.020; THc4: 146.1±6.2% vs. 120.7±5.7%, F=8.85, df=1, P=0.007 and THc5: 143.2±7.7% vs. 119.2±7.2% baseline, F=4.97, df=1, P=0.038, ANOVA, Figure 2). At T15 no differences in RSNA were observed between yohimbine and control groups (T15: 103.1±9.8% vs. 94.6±7.3% baseline, F=0.49, df=1, P=0.490, ANOVA, Figure 2).

Histological verification of the microinjection sites. Microinjection sites into the caudal raphe region were verified histologically, as shown in Figure 3.

DISCUSSION

This study demonstrated that the blockade of α2-adrenergic receptors by yohimbine enhanced the renal sympathetic nerve response to the acute intermittent hypercapnia in urethane-anesthetized rats. Moreover, when yohimbine was microinjected into the caudal raphe region, more pronounced increases in RSNA were evoked than in the control group, indicating that the caudal raphe region might have an important role in the regulation of sympathetic outflow during exposure to the AIHc.

The present finding that systemic blockade of α2-adrenergic receptors profoundly affects the RSNA response to AIHc is not unexpected as α2-adrenergic receptors are known to generally mediate a sympathoinhibitory role and are involved in blood pressure homeostasis [31, 32]. Following intravenous administration, yohimbine (α2-adrenergic receptor antagonist) acts via receptors located throughout the peripheral vasculature and enters the brain rapidly where it binds to α2-adrenergic receptors, widely distributed at multiple sites involved in sympathetic control [31, 33, 34]. Therefore, blocking the α2-adrenergic receptors systemically does not allow for drawing clear conclusions about the mechanism and the precise site of action of yohimbine.

Hypercapnia is known to potently activate distinct brainstem neuronal groups and might evoke different responses as a consequence of acute or chronic hypercapnic exposures [10, 11, 15, 35]. The conventional setting for subjecting animals to AIHc consists of cyclically changing the CO_2 fraction in different background gas mixtures and as such, the model yielded dose-dependent responses related to hypercapnia severity [8-12]. Previous studies have shown that the acute exposure to a severe hypercapnic stimulus (15% CO_2) lasting 30 min to 24 h results in changes of noradrenaline, dopamine and serotonin concentrations at various CNS regions [36, 37]. Many previous studies provided evidence that hypercapnia, by means of peripheral and central chemoreception, leads to increases in respiratory and sympathetic activity [1, 3, 14,

15]. The retrotrapezoid nucleus (RTN) is widely recognized as the principal site of the central CO_2 chemoreception [19], essential in respiratory response to hypercapnia [15]. However, the increase in sympathetic activity produced by hypercapnia is partially dependent on the activity of the RTN neurons [15], along with various medullary regions with chemoreception properties, including the raphe neurons [27, 38-40]. Thus, we aimed to investigate the role of the caudal raphe region in the sympathetic response to severe AIHc.

The caudal raphe region has been recognized as a source of input to numerous medullary and spinal sites, where it can exert an influence on many different functions, including cardiovascular control and autonomic activity [6, 39]. It has been established that the caudal raphe nuclei send extensive direct projections to the sympathetic preganglionic neurons of the IML cell column [24, 26, 39]. Moreover, the raphe nuclei may indirectly contribute to the overall sympathetic output by means of projections to other brainstem regions involved in sympathetic control such as the RVLM [29] and RTN [15, 41]. Additionally, the caudal raphe region has reciprocal connections with the RVLM [24, 29] and the LC [21, 23], regions that play a central role in the regulation of the autonomic activity at rest and in stressful conditions. Altogether, these anatomical projections suggest that the regulation of the arterial pressure, heart rate and sympathetic activity may be mediated by the caudal raphe region.

Previous studies proposed that hypercapnia-induced release of noradrenaline acting on α2-adrenergic receptors of the caudal raphe region leads to its inhibition and lowers the release of serotonin henceforth affecting the descending pathways [9, 11, 42-44]. Thus, the increased effect of AIHc on RSNA following α2-adrenergic receptor blockade in the caudal raphe region in our study might be a consequence of disinhibition of the caudal raphe region by the LC noradrenergic neurons. Based on the findings of this study, we might speculate that disinhibition of the caudal raphe region probably led to the increased direct input to the IML, but also via other regions that modulate the sympathetic activity such as the RVLM and RTN

[29, 41]. This speculation is supported by the results of similar studies that found that disinhibition of the raphe pallidus leads to increases in RSNA and splanchnic SNA [38, 45].

One might speculate whether anesthesia might affect the sympathetic nerve discharge. However, urethane anesthesia has been shown to have minimal effects on cardiorespiratory and sympathetic activity and is commonly used in experiments requiring the preservation of the reflex response loops as the results closely resemble those observed in conscious animals [15, 45, 46]. Another consideration is the possible recruitment of the serotonergic signaling in relatively robust exposures to severe hypercapnic stimuli (15% CO_2), which may activate the wake promoting pathways [5]. Considering this experimental design is used as a model of obstructive sleep apnea (OSA), we find this stimulus to be appropriate to evoke similar effects to those seen during the airway obstruction episodes in OSA, which, among other consequences, lead to hypercapnia and arousal. Yohimbine has been reported to have different affinities toward α2-adrenergic receptor subtypes and is also known to bind to α1-adrenergic receptors [47]. Since both α1- and α2-adrenergic receptors are present in the caudal raphe region [34], it is possible that the sympathoexcitatory effect observed in this study may be due to stimulation of the α1-adrenergic receptors. Moreover, yohimbine is known to evoke responses via other receptors, including serotonin 5-HT_{1A} receptors, at which it can act as a partial agonist [48, 49]. Since microinjections of yohimbine were performed into the caudal raphe region, it may be possible that some yohimbine bound to the 5-HT_{1A} receptors and influenced the observed effects. However, at concentration applied and considering the 80-fold higher affinity for α2-adrenergic than for $5HT_{1A}$ receptors in the rat [49], it is unlikely that yohimbine produced notable effects via $5HT_{1A}$ receptors in this study. Finally, the primary stimulus for the central chemoreceptor response to changes in $PaCO_2$ has not been identified up to now and it has been argued whether it is solely related to the changes in CO_2 or it combines several other physiological parameters such as extracellular/intracellular pH, bicarbonate concentration or a

combination of these stimuli [19, 50]. In this study, we used a paradigm centered around changing the inspiratory CO_2 fraction, which is likely to have produced hypercapnic acidosis. However, it is difficult to define the primary chemoreceptor stimulus eliciting the observed changes in the renal sympathetic nerve response using this type of *in vivo* experiment.

The results of the current study indicate that the chemical blockade of the α2-adrenergic receptors in the caudal raphe region enhanced the renal sympathetic nerve response to the acute intermittent hypercapnia in anesthetized rats. Thus, we conclude that the neurons in the caudal raphe region make a significant contribution to the renal sympathoexcitatory response evoked by severe acute intermittent hypercapnia.

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FIGURE LEGEND

Figure 1. Schematic representation of the surgical preparation of the experimental animals **(A)** and the experimental design with the protocols applied in four experimental groups **(B)**. A group of animals received α2-adrenergic receptor antagonist-yohimbine intravenously ($YOHIMBINE_{i.v.}$) and a control group received saline intravenously ($CONTROL_{i.v.}$) prior to exposure to the acute intermittent hypercapnia (AIHc) protocol. Subsequently, a group of animals received a microinjection of yohimbine into the caudal raphe region ($YOHIMBINE_{raphe}$) whereas a control group received a saline microinjection into the caudal raphe region ($CONTROL_{raphe}$) prior to exposure to the AIHc protocol. RSNA: renal sympathetic nerve activity; BP: blood pressure; ABS: arterial blood status; FiO_2: fraction of inspired oxygen; T_i: inspiratory time; Freq: ventilator frequency; T0: baseline conditions immediately preceding the first hypercapnic episode; THc1-5: five hypercapnic episodes; T15: 15 min following the end of the last hypercapnic episode.

Figure 2. (A) Time-course of changes in the renal sympathetic nerve activity (RSNA) during exposure to the acute intermittent hypercapnia (AIHc) protocol. RSNA response to AIHc was greater during all five hypercapnic episodes in a group that received yohimbine intravenously ($YOHIMBINE_{i.v.}$, n=8, dark blue, ♦) than in a control group that received saline intravenously ($CONTROL_{i.v.}$, n=7, orange, ●). Moreover, when yohimbine was microinjected into the caudal raphe region ($YOHIMBINE_{raphe}$, n=12, blue, ■), the RSNA response to AIHc was greater during all five hypercapnic episodes than in a control group that received saline into the same region ($CONTROL_{raphe}$, n=10, red, ▲) (*significantly different from corresponding baseline value; †significantly different from corresponding T15; $^{\#}$significantly different from respective control group; ANOVA, $P<0.05$). **(B)** Tracings of representative experiments in four

485 experimental groups, from top to bottom: an experiment involving the systemic
486 blockade of the α2-adrenergic receptors ($YOHIMBINE_{i.v.}$) and a respective control
487 experiment ($CONTROL_{i.v.}$) followed by an experiment with the blockade of the α2-
488 adrenergic receptors in the caudal raphe region ($YOHIMBINE_{raphe}$) and a respective
489 control experiment ($CONTROL_{raphe}$) each showing arterial blood pressure (BP; mmHg,
490 red), integrated renal sympathetic nerve activity (IRSNA; arbitrary units, a.u., blue) and
491 raw renal sympathetic nerve activity (RSNA; arbitrary units, a.u., green) at seven
492 experimental time points. Scale bar represents 20 seconds. T0: baseline conditions
493 immediately preceding the first hypercapnic episode; THc1-5: five hypercapnic
494 episodes; T15: 15 min following the end of the last hypercapnic episode.

495 **Figure 3.** Photomicrograph of a coronal section of the brainstem showing the microinjection
496 site in the caudal raphe region marked by diluted India ink dye (blue) and indicated by
497 the arrow. Microinjection coordinates were: +0.2 mm anterioposterior, 0.0 mm
498 mediolateral and -2.5 mm dorsoventral relative to the obex. Scale bar represents 500
499 µm.

Table 1. Mean arterial blood pressure (MAP, mmHg) and heart rate (HR, beats/min) in experimental groups at all experimental time points.

Experimental groups		T0	THc1	THc2	THc3	THc4	THc5	T15
Systemic α2-adrenergic receptor modulation								
Control (n=7)	MAP	94.3±7.2	95.7±4.3	100.1±5.32	99.9±5.7	98.9±5.5	98.7±5.4	99.0±7.0
	HR	371.4±8.0	337.0±7.7*,†	340.1±7.3*,†	338.4±7.5*,†	338.0±7.3*,†	336.9±7.3*,†	377.0±8.5
Yohimbine (n=9)	MAP	54.9±1.9$^{\#}$	64.5±4.0	67.6±4.3	71.9±5.3	71.1±3.7*	73.4±3.3*	67.3±3.3*
	HR	375.1±6.0	348.1±5.7*,†	348.1±5.9	348.2±5.8*,†	344.0±6.3*,†	345.4±5.1*,†	376.2±4.3
Central α2-adrenergic receptor modulation								
Control (n=10)	MAP	89.4±4.5	86.5±3.5	89.1±3.4	91.0±3.8	90.7±3.8	90.7±4.4	86.0±4.3
	HR	331.3±7.96	283.5±8.06*,†	281.6±6.53*,†	281.0±8.22*,†	279.6±7.56*,†	277.5±6.93*,†	327.2±6.96
Yohimbine (n=12)	MAP	82.0±3.4	82.1±3.6	85.0±3.9	88.3±3.8†	87.9±3.5†	88.1±3.7†	75.8±3.5
	HR	344.5±8.18	304.1±9.38*,†	306.4±9.66*,†	306.1±8.34*,†	304.6±8.71*,†	302.1±8.63*,†	339.4±8.33

Data are presented as mean±SEM. T0: baseline value immediately before the first hypercapnia; THc1: first hypercapnia; THc2: second hypercapnia; THc3: third hypercapnia; THc4: fourth hypercapnia; THc5: fifth hypercapnia; T15: 15 minutes following the end of the last hypercapnic episode. (* significantly different from respective baseline, † significantly different from respective T15, $^{\#}$ significantly different from respective control group; $P<0.05$)

Table 2. Arterial blood gas analysis results in four experimental groups showing pH values and partial pressures of carbon dioxide ($PaCO_2$, mmHg) and oxygen (PaO_2, mmHg) at two experimental time points.

Experimental groups	**T0**			**T15**		
	pH	**$PaCO_2$**	**PaO_2**	**pH**	**$PaCO_2$**	**PaO_2**
Systemic α2-adrenergic receptor modulation						
Control (n=7)	7.300±0.005	46.2±1.3	281.5±8.5	7.279±0.012	49.3±2.3	292.5±7.3
Yohimbine (n=9)	7.314±0.021	44.0±2.4	316.6±8.7	7.255±0.023	47.4±3.1	319.2±7.3
Central α2-adrenergic receptor modulation						
Control (n=10)	7.271±0.015	42.8±1.5	286.6±3.9	7.262±0.018	44.8±2.8	299.5±5.3*
Yohimbine (n=12)	7.288±0.016	44.3±1.5	312.3±8.8	7.293±0.020	43.7±2.8	313.1±12.9

Data are presented as mean±SEM. T0: baseline value immediately before the first hypercapnic episode; T15: 15 minutes following the end of the last hypercapnic episode. (* significantly different from respective baseline, $P<0.05$)

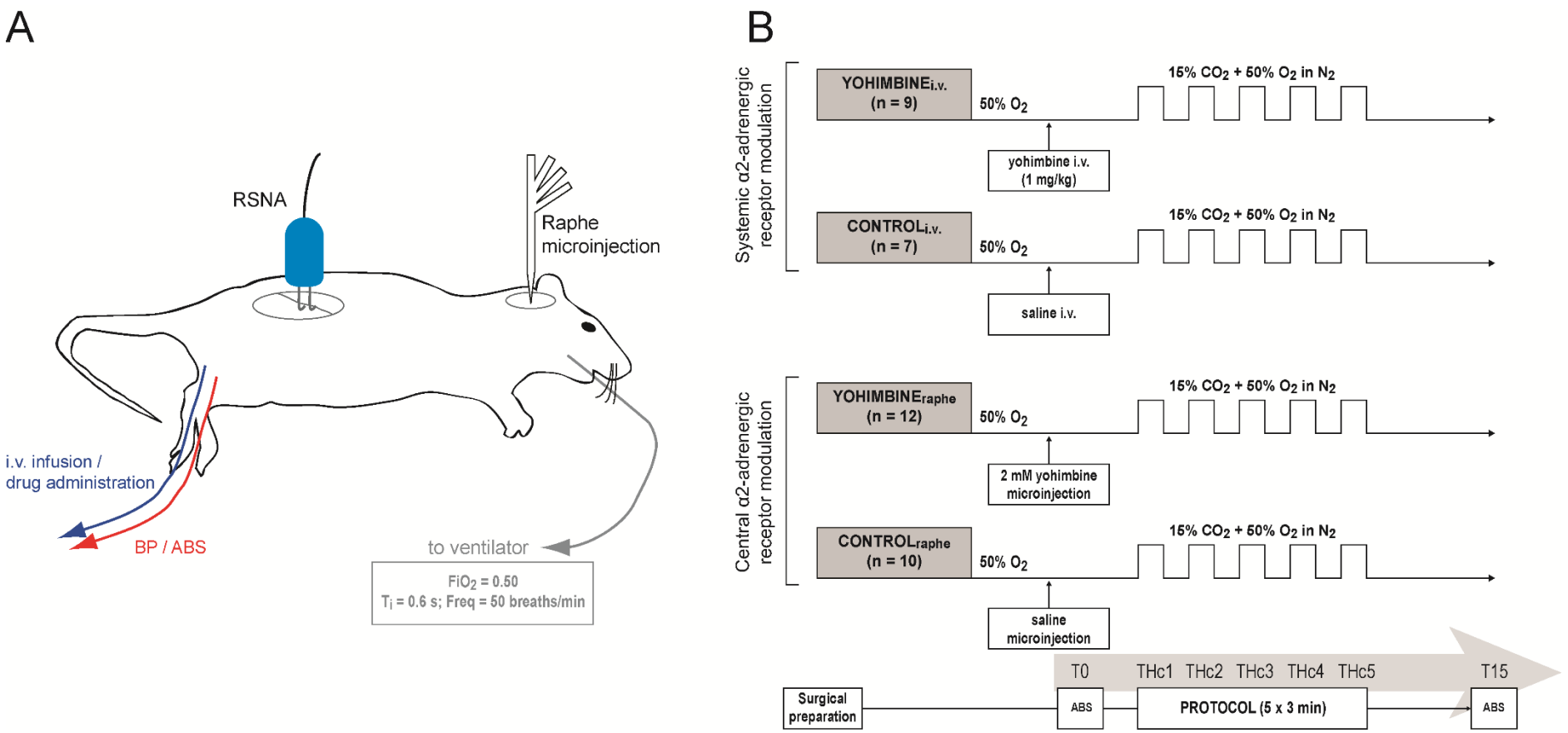
A
RSNA
Raphe
microinjection
i.v. infusion /
drug administration
BP / ABS
to ventilator
FiO2 = 0.50
Ti = 0.6 s; Freq = 50 breaths/min
B
Systemic α2-adrenergic receptor modulation
YOHIMBINEi.v.
(n = 9)
50% O2
15% CO2 + 50% O2 in N2
yohimbine i.v.
(1 mg/kg)
CONTROLi.v.
(n = 7)
50% O2
15% CO2 + 50% O2 in N2
saline i.v.
Central α2-adrenergic receptor modulation
YOHIMBINEraphe
(n = 12)
50% O2
15% CO2 + 50% O2 in N2
2 mM yohimbine
microinjection
CONTROLraphe
(n = 10)
50% O2
15% CO2 + 50% O2 in N2
saline
microinjection
T0
THc1 THc2 THc3 THc4 THc5
T15
Surgical
preparation
ABS
PROTOCOL (5 x 3 min)
ABS

Figure 1.

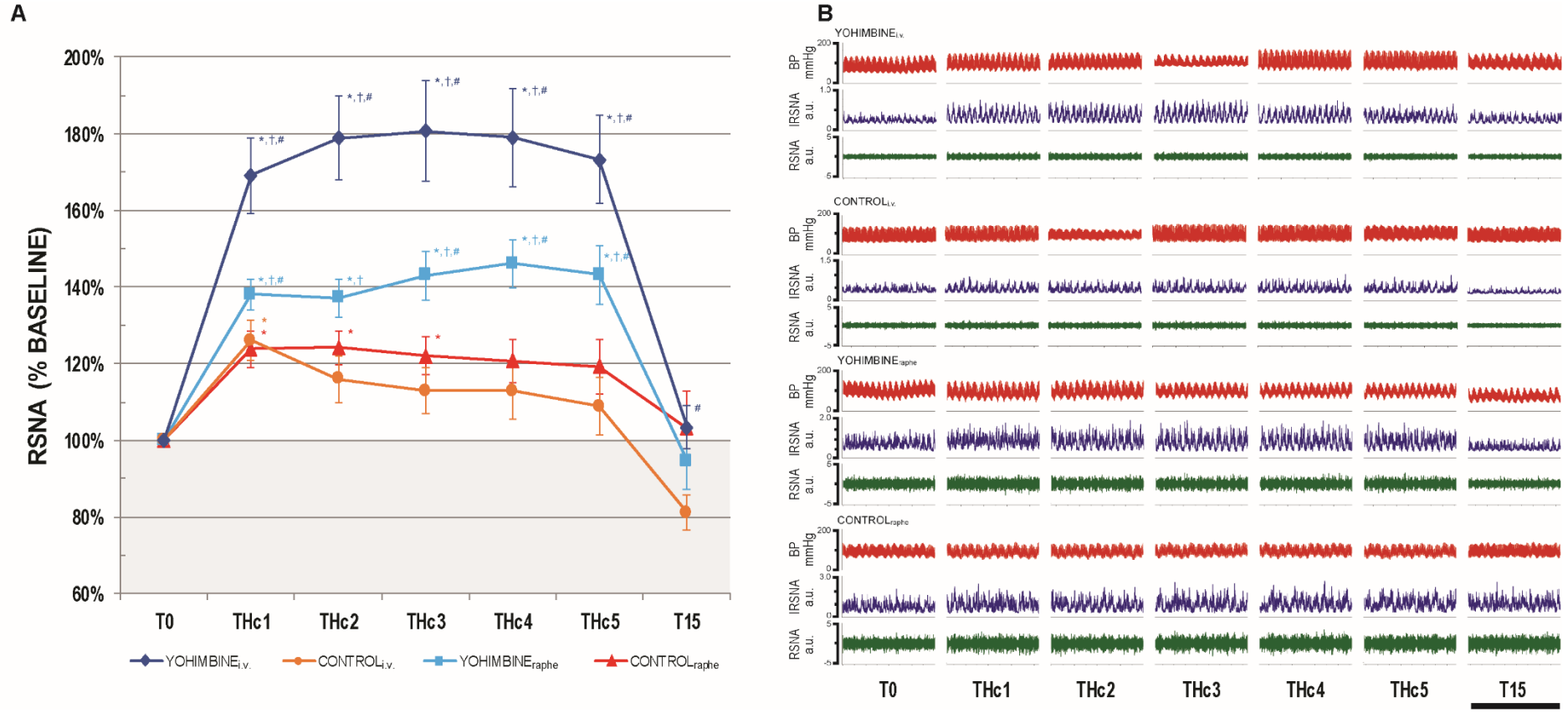
A
RSNA (% BASELINE)
200%
180%
160%
140%
120%
100%
80%
60%
T0
THc1
THc2
THc3
THc4
THc5
T15
YOHIMBINE i.v.
CONTROL i.v.
YOHIMBINE raphe
CONTROL raphe
B
YOHIMBINE i.v.
CONTROL i.v.
YOHIMBINE raphe
CONTROL raphe
BP mmHg
IRSNA a.u.
RSNA a.u.
T0
THc1
THc2
THc3
THc4
THc5
T15

Figure 2.

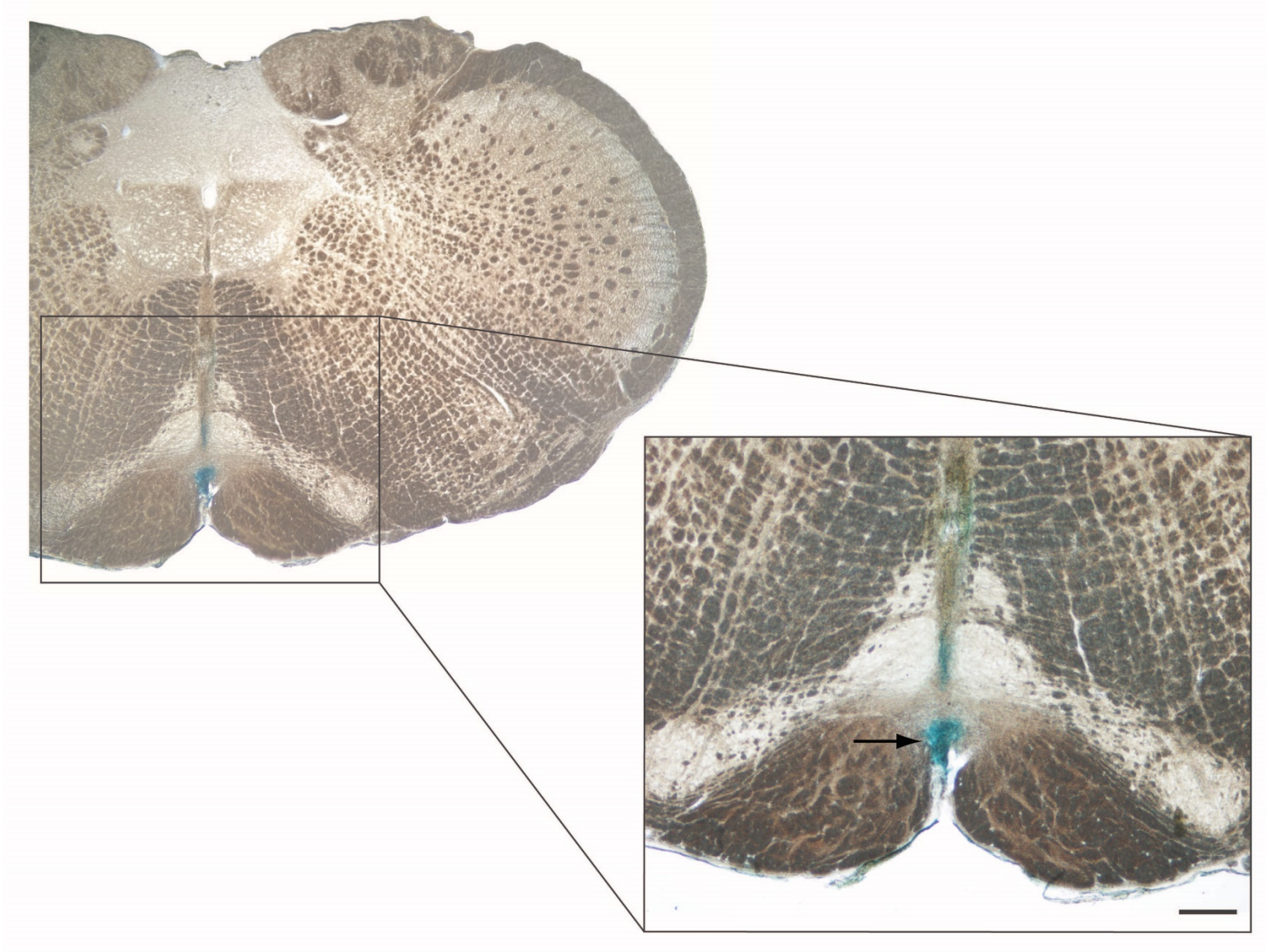

Figure 3.