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GABA mechanisms of the nucleus of the solitary tract regulates the cardiovascular and sympathetic effects of moxonidine

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ABSTRACT

The antihypertensive drugs moxonidine and clonidine are α_2-adrenoceptor and imidazoline (I_1) agonists. Previous results from our laboratory have shown that moxonidine can act in the commissural nucleus of the solitary tract (commNTS). In addition, some studies have shown that GABA or glutamate receptor blockade in the RVLM blunted the hypotension produced by these antihypertensive agents in spontaneously hypertensive rats. Therefore, in the present study we verify whether the cardiovascular and sympathetic effects produced by moxonidine in the commNTS are dependent on GABAergic or glutamatergic mechanisms. Mean arterial pressure (MAP) and splanchnic sympathetic nerve activity (sSNA) were recorded in urethane-anesthetized, and artificially-ventilated male Wistar rats (250–350 g). Injection of the GABAA antagonist bicuculline (25 pmol/50 nL) into the commNTS reduced the hypotension as well as the sympathoinhibition elicited by moxonidine. Prior injection of the glutamate receptor antagonist kynurenic acid (2.5 nmol/50 nL) into the commNTS was not effective in reducing the hypotension and sympathoinhibition elicited by moxonidine. Therefore, we conclude that the hypotensive and sympathoinhibitory effects elicited by microinjection of moxonidine into the commNTS are dependent on GABA receptors, but not ionotropic glutamate receptors.

1. Introduction

It is well established that the nucleus of the solitary tract (NTS) is considered the site of the first synapse of the visceral sensory inputs to the brainstem (Dampney, 1994; Guyenet, 2006). One of the neurotransmitter released by these afferents in the NTS is the L-glutamate (Talman et al., 1980; Dampney, 1994). Anatomical and immunohistochemical studies have shown that, besides the baroreflex pathway, the NTS sends monosynaptic inputs to the rostral ventrolateral medulla/C1 region (RVLM/C1) (Hancock, 1988; Morilak et al., 1989; Otake et al., 1992) and these projections may convey peripheral chemoreceptor signals (Colombari et al., 1996; Koshiya and Guyenet, 1996). The existence of pressor mechanisms in the NTS is supported by the increase in arterial pressure produced by L-glutamate injections into the NTS in conscious rats (Machado and Bonagamba, 1992; Colombari et al., 1994).

The NTS and the RVLM/C1 are considered the main brainstem regions of centrally acting antihypertensive drugs (Ernsberger et al., 1997; Guyenet, 1997; Ernsberger and Haxhiu, 1997; Totola et al., 2013). Preliminary results from our laboratory depicted that moxonidine act on α_2-adrenoceptors (α_2-AR) in the commissural aspect of the NTS (commNTS) to produce hypotension and sympathoinhibition in anesthetized and conscious rats (Totola et al., 2013).

Besides the α_2-adrenergic mechanisms related to the action of antihypertensive drugs such as moxonidine and clonidine, several studies have indicated that several neurotransmitters such as GABA (γ-aminobutyric acid) and glutamate are involved in the sympathoinhibition and hypotension elicited by moxonidine or clonidine (Tingley and Arnerić, 1990; Milhaud et al., 2000). Considering the existing evidence, our hypothesis is that GABAergic and glutamatergic mechanism might be responsible to the antihypertensive effects elicited by moxonidine into commNTS. However, due to the importance of GABA as well as glutamate receptors into the NTS for cardiovascular regulation, it might be interesting to investigate whether GABAergic and glutamatergic mechanisms are also important to mediate the antihypertensive effects of moxonidine into the commNTS.

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2. Materials and methods

2.1. Animals

Experiments were performed in 37 adult male Wistar rats weighing 280–320 g. All experimental protocols were in accordance with the Ethical Principles in Animal Research of the Brazilian College of Animal Experimentation (CONCEA) and were approved by the Ethics Committee for Animal Research of the Institute of Biomedical Sciences of the University of Sao Paulo (CEUA: Authorization No. 85/2010).

2.2. Surgery and anesthesia

Rats were deeply anesthetized with halothane (5% in 100% oxygen inspired air) for general surgical procedures, such as: a) tracheostomy for artificial ventilation; b) femoral artery and vein catheterization for arterial pressure measurement and administration of fluids and drugs, respectively; c) intraparenchymal injection by removal of the occipital bone and retracting the underlying dura-mater membrane for insertion of a pipette into the medulla oblongata via a dorsal transcerebellar approach (Takakura and Moreira, 2011); d) splanchnic sympathetic nerve isolation for subsequent nerve activity monitoring. The level of anesthesia was checked by a flexor reflex to the animal's paw pinching.

Splanchnic sympathetic nerve activity (sSNA) was recorded as previously described (Takakura and Moreira, 2011). Briefly, the right splanchnic nerve was isolated via a retroperitoneal approach, and the segment distal to the suprarenal ganglion was placed on a pair of Teflon-coated silver wires that had been bared at the tip (250 μm bare diameter; A-M Systems, www.amsystems.com). The nerves and wires were embedded in adhesive material (Kwik-Cast Sealant, WPI, USP, Sarasota, FL, USA), and the wound was closed around the exiting recording wires.

Upon completion of the surgical procedures, halothane was replaced by urethane (1.2 g/kg of body weight) slowly administered intravenously (i.v.). All rats were artificially ventilated with 100% oxygen throughout the experiment. The rectal temperature was maintained at 37 °C and the end tidal-CO_2were monitored throughout the experiment with a capnometer (CWE, Inc., Ardmore, PA, USA) that was calibrated twice per experiment against a calibrated CO_2/N_2mix. The adequacy of the anesthesia was monitored during a 20-min stabilization period by testing for the absence of withdrawal response and the lack of arterial pressure change to firm toe pinch. After these criteria were satisfied, the muscle relaxant pancuronium was administered at the initial dose of 1 mg/kg i.v. and the adequacy of anesthesia was thereafter gauged solely by the lack of increase in arterial pressure to firm toe pinch. Approximately hourly supplements of one-third of the initial dose of urethane were needed to satisfy these criteria during the course of the recording period (80 min).

2.3. In vivo recordings of physiological variables

Mean arterial pressure (MAP) and the discharge of the splanchnic nerve (sSNA) and the tracheal CO_2 were recorded as previously described (Taxini et al., 2011). Before starting the experiments, the ventilation was adjusted to have the end-expiratory CO_2 at 3–4% at steady-state (60–80 cycles/s; tidal volume 1–1.2 ml/100 g). All analog data (end-expiratory CO_2, MAP and sSNA) were stored on a computer via a micro 1401 digitizer (Cambridge Electronic Design, Cambridge, UK) and were processed off-line using version 6 of the Spike 2 software (Cambridge Electronic Design) as described previously (Takakura and Moreira, 2011; Taxini et al., 2011). The integrated splanchnic nerve activity (iSNA) was obtained after the rectification and smoothing ($\sigma =$ 2 s) of the original signal, which was acquired with a 30–3000 Hz band pass. The iSNA was normalized for each animal by assigning the value of 100 to the resting SNA and the value of 0 to the minimum value recorded after the administration of the ganglionic blockade (hexamethonium: 30 mg/kg, i.v.).

2.4. Intraparenchymal injections

All drugs were purchased from Sigma-Aldrich (Sigma Chemicals Co.) unless otherwise stated. Bicuculline methiodide (Bic: 25 pmol/50 nL), kynurenic acid (kyn: 2.5 nmol/50 nL) and moxonidine hydrochloride (moxo: 5 nmol/50 nL in sterile saline pH 7.4) were pressure injected (Picospritzer III, Parker Hannifin Corp, USA) (50 nL in 5 s) through single-barrel glass pipettes (20 μm tip diameter). Injections into the commNTS were made 400 μm caudal to the *calamus scriptorius*, in the midline and 0.3 to 0.5 mm below the dorsal surface of the brainstem. A mix of propylene glycol/water 2:1 was used as vehicle for bicuculline and moxonidine because these drugs are not soluble in saline. The solution of moxonidine, bicuculline and kynurenic acid contained a 5% dilution of fluorescent latex microbeads (Lumafluor, New City, NY, USA) for later histological identification of the injection sites (Takakura and Moreira, 2011; Takakura et al., 2011).

2.5. Histology

At the end of the experiment, the rats were deeply anesthetized with urethane and perfused through the heart with PBS (pH 7.4) followed by paraformaldehyde (4% in 0.1 M phosphate buffer, pH 7.4). The brains were removed and stored in fixative for 24 h at 4 °C. The medulla was cut in 40 μm thick coronal sections with a vibrating microtome (Vibratome 1000S Plus, USA). Sections were stored at −20 °C in a cryoprotectant solution (Schreihofer and Guyenet, 1997). The injections sites were confirmed with an Axioskop 2 microscope (Zeiss, Oberkochen, Germany). The section alignment between the brains was done relative to a reference section. To align the sections around NTS level, the mid-area postrema level was identified in each brain and assigned the level 13.8 mm (Bregma −13.8 mm) according to the atlas of Paxinos and Watson (1998). The coordinates of sections rostral and caudal of this reference section were calculated with respect to the reference section, using the number of intervening sections and the section thickness.

2.6. Experimental protocol

2.6.1. Effects of the combination of bicuculline and moxonidine injected into the commNTS on arterial pressure and sympathetic outflow

All experiments were performed in rats anesthetized with urethane (1.2 g/kg, i.v.). Recordings began 10 min after the connection of the arterial line to the pressure transducer. MAP and sSNA were continuously recorded for 80 min and were analyzed every 10 min. Control (baseline) values were recorded for 10 min and were analyzed immediately before the bicuculline (25 pmol/50 nL) or vehicle injection (first treatment). These values were used as a reference to calculate the changes produced by the treatments. After 10 min, moxonidine (5 nmol/50 nL) or vehicle was injected into the commNTS and the MAP and sSNA responses were evaluated for the next hour.

2.6.2. Effects of the combination of kynurenic acid and moxonidine injected into the commNTS on arterial pressure and sympathetic outflow

All experiments were performed in rats anesthetized with urethane (1.2 g/kg, i.v.). Recordings began 10 min after the connection of the arterial line to the pressure transducer. MAP and sSNA were continuously recorded for 80 min and were analyzed every 10 min. Control (baseline) values were recorded for 10 min and were analyzed immediately before the kynurenic acid (2.5 nmol/50 nL) or vehicle injection (first treatment). These values were used as a reference to calculate the changes produced by the treatments. After 10 min, moxonidine (5 nmol/50 nL) or vehicle was injected into the commNTS and the MAP and sSNA responses were evaluated for the next hour.

2.7. Statistics

Statistical analysis was done with Sigma Stat version 3.0 (Jandel Corporation, Point Richmond, CA). Data are reported as means ± SEM. Two-way parametric ANOVA followed by the Newman-Keuls multiple comparisons test were used as appropriate. Significance was set at $p < 0.05$.

3. Results

3.1. Histological analysis

Injections into the commNTS were located about 400 μm caudal to the *calamus scriptorius* as illustrated in Figs. 1A and 1B. A single injection was administered in or near the midline as represented in Figs. 1A-B, 2D and 3D. Based on the area of the distribution of the beads, the injectate spread bilaterally (approximately 500 μm from the injection center) and a little less in the rostrocaudal direction (approximately 300 μm from the injection center). The effective spread of the drugs was not determined and could have been larger than that of the microbeads due to the difference in molecular mass.

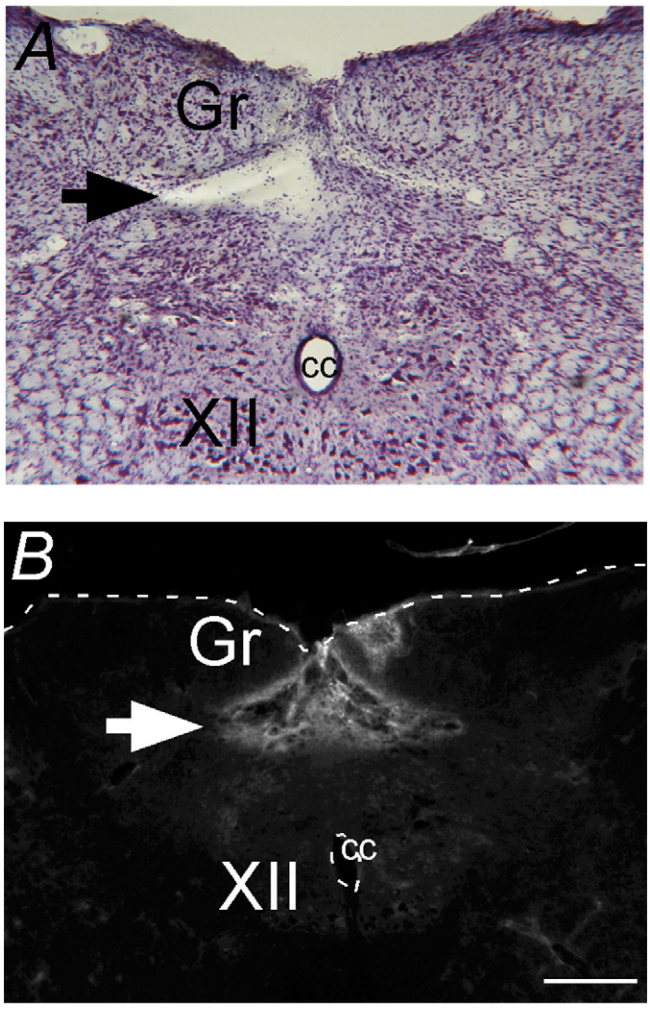

Fig. 1. Photomicrographs of typical midline injection into the commNTS in anesthetized rat (arrow). A) Nissl staining and B) fluorescent microbeads. Scale bar in B = 1 mm apply to all panels. Abbreviations: cc: central canal; Gr: gracile nucleus; XII: hypoglossal nucleus.

3.2. Effects of moxonidine injection into the commNTS in rats previously treated with bicuculline

The first set of experiments was designed to test the involvement of GABAergic mechanisms within the commNTS in the hypotension and sympathoinhibition elicited by moxonidine in rats. Bicuculline (25 pmol/50 nL) injected into the commNTS reduced moxonidine-induced (5 nmol/50 nL) hypotension (104 ± 2, vs. moxonidine: 89 ± 7 mm Hg) [$F(3, 41) = 57.13$, $p = 0.034$] and sympathoinhibition ($\Delta = -13 \pm 3\%$ vs. moxonidine: $\Delta = -24 \pm 2\%$ of inhibition) [$F(3, 41) = 62.34$, $p = 0.028$] (Fig. 2A–C). Bicuculline injected into the commNTS produced no change in the resting MAP (119 ± 6 vs vehicle: 117 ± 5 mm Hg, $p = 0.42$) and resting sSNA (2 ± 5, vs vehicle: 9 ± 7%, $p = 0.55$) (Fig. 2A–C).

3.3. Effects of moxonidine injection into the commNTS in rats previously treated with antagonist of glutamatergic ionotropic receptors

The next experiments attempted to evaluate the glutamatergic participation on cardiovascular and sympathetic effects elicited by moxonidine within the commNTS.

Pre-treatment with the broad spectrum ionotropic antagonist kynurenic acid (2.5 nmol/50 nL) into commNTS was not effective to attenuate the hypotension ($\Delta = -24 \pm 3$, vs. moxonidine: $\Delta = -25 \pm 9$ mm Hg) [$F(1, 28) = 0{,}18$, $p = 0.642$] and sympathoinhibition ($\Delta = -22 \pm 5\%$ vs. moxonidine: $\Delta = -28 \pm 9\%$ of inhibition) [$F(1, 28) = 0057$, $p = 0.62$] elicited by moxonidine (5 nmol/50 nL) into commNTS. The injection of kynurenic acid into commNTS produced no change in baseline MAP ($\Delta = 2 \pm 4$, vs. vehicle: $\Delta = 7 \pm 4$ mm Hg) $F(1, 28) = 0{,}0012$, $p = 0.078$], and SNA ($\Delta = 8 \pm 5$, vs. vehicle: $\Delta = 7 \pm 2\%$) [$F(1, 28) = 0{,}03$, $p = 0.4$] (Fig. 3A–C).

The lack of effect produced by a high dose of kyn prompted us to verify that identical injections of kyn could block the baroreflex-induced sympathoinhibition in response to intravenous (iv) injection of phenylephrine (5 μg/kg). For example, injection of kyn in the commNTS in 4 urethane anesthetized rats blocked the sympathoinhibitory effect ($3 \pm 3\%$, vs. saline: $-81 \pm 9\%$ of inhibition) ($p < 0.001$) elicited by iv injection of phenylephrine (data not shown).

Figs. 2D and 3D represents the injections that were located outside the commNTS region and these injections often reached the hypoglossal motor nucleus (7of 11) or the gracile nucleus (4 of 11). Injections of moxonidine (5 nmol/50 nL) outside the commNTS ($n = 11$) produced no significant changes in the resting MAP (106 ± 4, vs. vehicle: 109 ± 6 mm Hg) [$F(1, 24) = 0.18$, $p = 0.43$] and sSNA ($\Delta = -1 \pm 4\%$ vs. vehicle: $\Delta = 2 \pm 3\%$) [$F(1, 24) = 0.46$, $p = 0.07$] in anesthetized rats.

4. Discussion

The present study showed at least two important findings. First, the hypotensive and sympathoinhibitory effects elicited by moxonidine into the commNTS were significantly attenuated by the pre-treatment with bicuculline, a $GABA_A$ receptor antagonist. Second, previous injection of kynurenic acid was not effective to attenuate the decrease in MAP and sSNA produced by moxonidine. Based on the above evidence, it might be suggested that the cardiovascular and sympathetic effects elicited by moxonidine is dependent on GABAergic, but not glutamatergic mechanisms at the level of the commNTS.

4.1. Brainstem areas and the α_2 and imidazoline compounds

The NTS acts as the gateway to central nervous system for sensory information arising from internal organs. Neuroanatomical studies have shown that NTS integrates different types of sensory afferents and connects with other pontomedullary areas. Among many neurotransmitters and neuromodulators present at the level of the NTS,

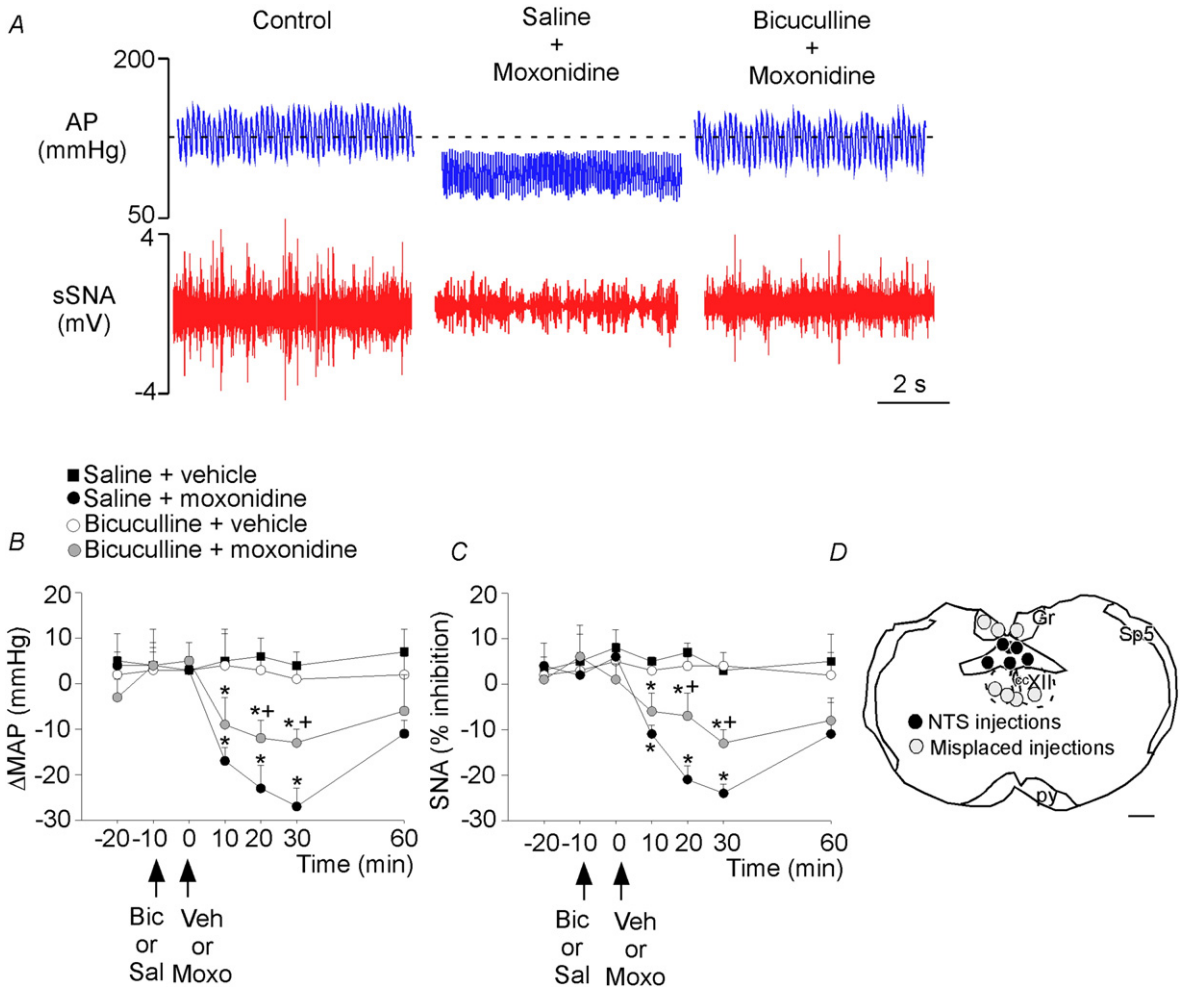

Fig. 2. Cardiovascular and sympathetic responses produced by combining moxonidine with bicuculline into the commNTS in urethane-anesthetized rats. (A) Recordings showing the changes in arterial pressure (AP) and splanchnic sympathetic nerve activity (sSNA) from rats treated with injections of saline + vehicle, saline + moxonidine (5 nmol/50 nL), and bicuculline (25 pmol/50 nL) + moxonidine into commNTS. B) and C) changes in mean arterial pressure (MAP) and sSNA, respectively, produced by injection of bicuculline or saline combined with moxonidine or vehicle injected into the commNTS in urethane-anesthetized rats. D) The center of injections into the commNTS in different rats tested is depicted in a single section (bregma = −14.3 mm, according to Paxinos and Watson, 1998). Scale = 1 mm. Abbreviations: cc, central canal; Gr, gracile nucleus; py, pyramide; Sp5, spinal trigeminal tract; XII, hypoglossal nucleus. Results in B–C are reported as means ± SEM. *Different from vehicle ($p < 0.05$), +Different from saline + moxonidine 2-way ANOVA with the Newman–Keuls post hoc test; N = 5–6/group of rats.

adrenergic mechanisms are particularly important in the regulation of arterial pressure (Carrettiero et al., 2012). The NTS contains a large number of catecholaminergic neurons and different subclasses of α-adrenergic receptors, such as α_1-and α_2-ARs (Duale et al., 2007; Carrettiero et al., 2012). Previous study from our laboratory showed that moxonidine injected in the commNTS produced a reduction in arterial pressure and sympathetic outflow and the pre-treatment with yohimbine or RX821002, specific α_2-AR antagonists, abolished the hypotension and the sympathoinhibition by moxonidine, suggesting the involvement of central α_2-AR in these responses at the level of the commNTS (Totola et al., 2013). The activation of α_2-AR may attenuate the activity of second-order commNTS neurons leading to an inhibition of the presympathetic RVLM/C1 neurons.

Centrally antihypertensive drugs have been used as a pharmacological treatment to reduce blood pressure (Edwards et al., 2012). Many brainstem, spinal cord and forebrain regions have been suggested to be crucial where clonidine or moxonidine-like drugs influence the autonomic nervous system (Isaac, 1980; Head et al., 1998; Totola et al., 2013). The NTS and RVLM/C1 regions are the main sites of action of these drugs (Lipski et al., 1976; Bousquet et al., 1981; Dominiak, 1994; Guyenet, 1997; Ernsberger et al., 1997; Hayar and Guyenet, 2000; Totola et al., 2013). The latter is considered to be the main region that sends sympathetic efferent projections to spinal cord and has been shown to contain imidazoline (I_1R) receptors as well as α_2-AR (Kino et al., 2005). Previous studies have proposed that α_2-AR and I_1R agonists act in separate brain regions to produce the antihypertensive effects, i.e. the I_1R in the RVLM/C1 and α_2-AR in the NTS, however, we can not ruled out an effect of moxonidine on I_1R at the level of the NTS (Head et al., 1997; Guyenet, 1997; Ernsberger et al., 1997; Carrettiero et al., 2012).

5. GABA mechanisms: cardiovascular and sympathetic effects of moxonidine

GABAergic mechanism appears to be involved in mediating the effects of central antihypertensive drugs (Jastrzebski et al., 1995; Zhang and Abdel-Rahman, 2002; Wang et al., 2007a, 2007b). In addition, GABAergic neurotransmission are proposed to play an important role in the hypotension and sympathoinhibition mechanisms of clonidine (Goźlińska and Czyzewska-Szafran, 1999). It was demonstrated in cats that microinjections of clonidine were more effective when injected in caudal regions of the ventrolateral medulla, i.e. in a region that contains the bulk of GABA neurons involved in the inhibition of presympathetic neurons of the RVLM/C1 region (Gatti et al., 1988; Cravo et al., 1991; Schreihofer and Guyenet, 2003). The results obtained by Marmo et al. (1987) indicate that $GABA_A$ receptor antagonist bicuculline was able to decrease the antihypertensive effects of clonidine in Wistar Kyoto

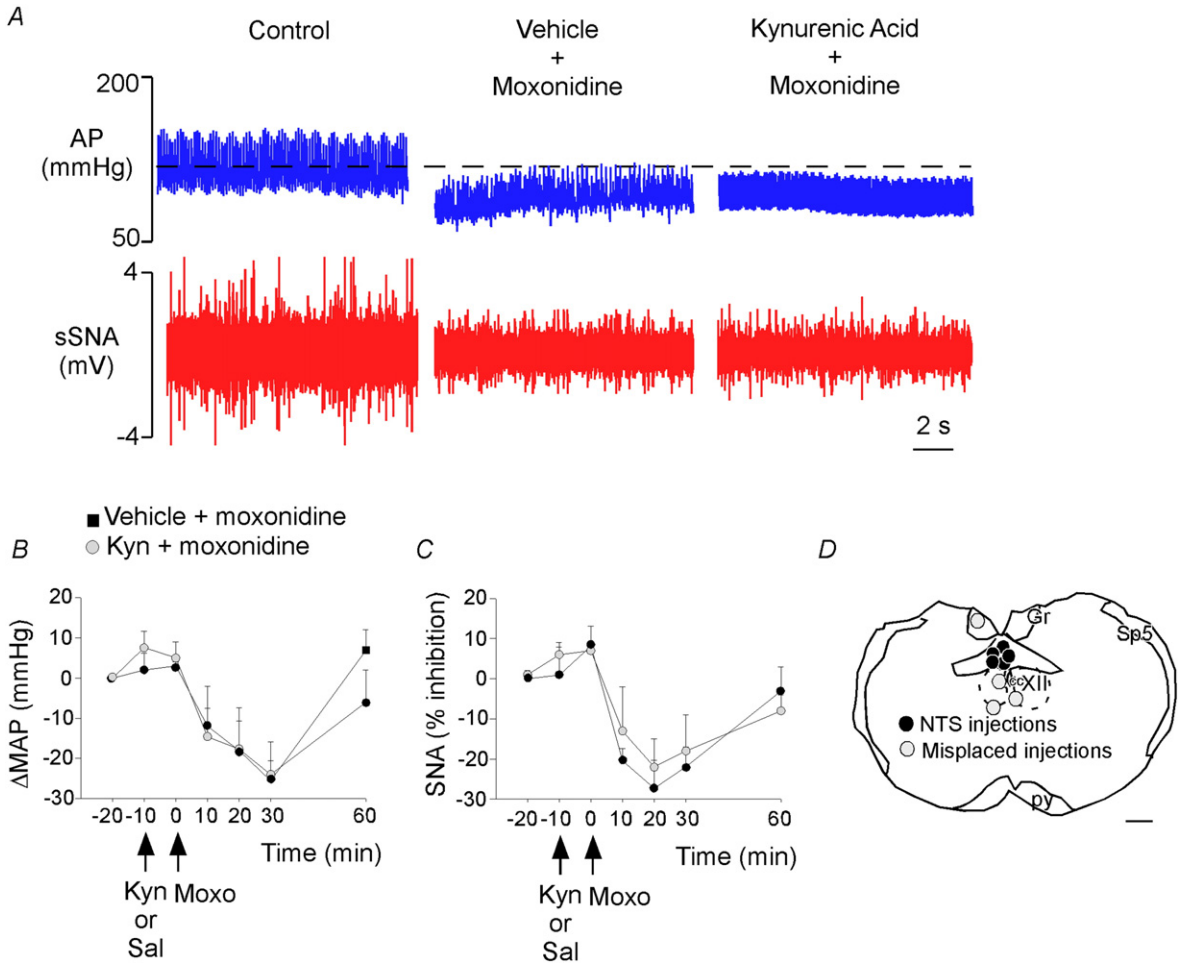

Fig. 3. Cardiovascular and sympathetic responses produced by combining moxonidine with kynurenic acid into the commNTS in urethane-anesthetized rats. (A) Recordings showing the changes in arterial pressure (AP) and splanchnic sympathetic nerve activity (sSNA) from rats treated with injections of vehicle + saline, vehicle + moxonidine (5 nmol/50 nL) or kynurenic acid (2.5 nmol/50 nL) + moxonidine into commNTS. B) and C) Changes in mean arterial pressure (MAP) and sSNA, respectively, produced by injection of kynurenic acid or vehicle combined with moxonidine (5 nmol/50 nl) injected into the commNTS in urethane-anesthetized rats. D) The center of injections into the commNTS in different rats tested is depicted in a single section (bregma = −14.3 mm, according to Paxinos and Watson, 1998). Scale = 1 mm. Abbreviations: cc, central canal; Gr, gracile nucleus; py, pyramide; Sp5, spinal trigeminal tract; XII, hypoglossal nucleus. Results in B–C are reported as means ± SEM. N = 5–6/group of rats.

and SHR rats. Similarly, in the present study, we observed that a GABAergic receptors within the commNTS could mediate the hypotensive and sympathoinhibition effects elicited by moxonidine. Nonetheless, the mechanisms responsible for the antihypertensive effects are still unknown, but it is plausible that the antihypertensive action of moxonidine relies on the $GABA_A$ receptor stimulation (Goźlińska and Czyzewska-Szafran, 1999), which directly or indirectly yields reduction in MAP and SNA.

Clonidine, an α_2-AR and I_1R agonist, evoked the release of inhibitory neurotransmitters such as GABA to hyperpolarize the RVLM/C1 neurons (Sun and Reis, 1995). In the present study, we suggest that moxonidine could act on postsynaptic α_2-AR, which is coupled to an inhibitory G protein (Gi), leading to an inhibition of a cAMP-dependent protein kinase A (PKA) compromising the proper function of the cellular machinery. In addition, moxonidine could also act on presynaptic α_2-AR, recruiting GABAergic transmission. We believe that, at the level of the commNTS, the activation of the mechanisms proposed above would be involved to trigger the reduction of commNTS activity, leading to a decrease in the downstream presympathetic neurons in the RVLM/C1 region. This notion could be certainly extended to many other brain regions that contribute to the sympathetic outflow and have a high density of GABAergic receptors such as the paraventricular nucleus of the hypothalamus and dorsolateral pons. Therefore, more studies are necessary to improve the understanding of the central mechanisms involved on moxonidine-induced cardiovascular and sympathetic responses.

6. Glutamatergic mechanisms: cardiovascular and sympathetic effects of moxonidine

Besides GABAergic mechanisms, others have also suggested the role of glutamate receptors on cardiovascular effects of clonidine and moxonidine (Lin et al., 2004). Previous studies already demonstrated that antagonism of N-methyl-D-aspartate receptor (NMDA) into RVLM/C1 region was able to blunt the hypotension and sympathoinhibition induced by rilmenidine in normotensive and hypertensive rats (Zhang and Abdel-Rahman, 2002; Wang et al., 2003, 2004). Because the NTS is an important brainstem region for the antihypertensive effects elicited by moxonidine (Totola et al., 2013) and it is well established the role of glutamatergic neurotransmission into NTS in cardiovascular regulation, we also investigated the role of glutamate receptors in the NTS on the cardiovascular and sympathetic responses elicited by moxonidine. The literature showed that the glutamatergic neurotransmission is crucial for the antihypertensive effects elicited by central antihypertensive drugs (Wang et al., 2003, 2004), however, at the level of the

commNTS, the pre-treatment of kynurenic acid was not effective to blunt the hypotension and the sympatholytic effects produced by moxonidine.

The molecular mechanisms in which moxonidine produce hypotension and sympathoinhibition are still poorly understood, but some possibilities would be plausible besides the one described above. Moxonidine acting on presynaptic α_2-AR, located on primary afferent solitary tract will cause the activation of the Gi-coupled protein to reduce intracellular cAMP and decrease the release of neurotransmitters in the synaptic cleft. One of the neurotransmitters could be the glutamate. Metabotropic glutamate receptors are often located presynaptically on glutamatergic and GABAergic terminals (Thompson et al., 2004). These metabotropic glutamate receptors provide a negative feedback, thus decreasing the release of neurotransmitters (Chen et al., 2002). This could be the mechanism by which moxonidine produce a reduction in blood pressure and sympathetic activity (present results; Wang et al., 2007a, b). Taken together, these findings strongly suggest that the cardiovascular and sympathetic inhibition evoked by moxonidine will depend on which brainstem area will be activated to produce hypotension and sympathoinhibition. Future studies will be necessary to better understand the role of moxonidine and the possible interaction between glutamatergic and GABAergic mechanisms.

In summary, we confirm the role of commNTS as an important site for action of moxonidine and we also showed that the hypotension and reduction in sympathetic activity elicited by moxonidine depends on the integrity of GABA, but not ionotropic glutamate mechanisms in the commNTS region.

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Author contribution

TBA, LTT, ACT, EC, and TSM designed research; TBA, LTT, and TSM performed research; TBA, LTT and TSM analyzed data; TBA, LTT and TSM wrote the paper.

Conflict of interest

None declared.

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