GABAergic mechanism in the rostral ventrolateral medulla contributes to the hypotension of moxonidine

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Aims

The depressor action of the centrally antihypertensive drug moxonidine has been attributed to activation of I1-imidazoline receptor in the rostral ventrolateral medulla (RVLM). The objective of this study was to determine the role of the γ-aminobutyric acid (GABA) mechanisms in the RVLM in mediating the effect of moxonidine in anaesthetized normotensive rats.

Methods and results

The relationship between the effects of microinjection or picoinjection of moxonidine and the functional state of GABA receptors at the level of the RVLM or pre-sympathetic neuron was determined. Microdialysis was performed to detect the effect of moxonidine on the release of GABA in the RVLM. Western blot analysis was carried out to test the effect of chronic intracerebroventricular injection of moxonidine on the protein expression of GABA receptors in the RVLM. Pre-treatment with the GABAA or GABAB receptor antagonist bicuculline (5 pmol) or CGP35348 (200 pmol), respectively, microinjected into the RVLM significantly attenuated the decrease in blood pressure and renal sympathetic nerve activity induced by moxonidine. In 22 moxonidine-sensitive pre-sympathetic neurons in the RVLM, picoinjection of bicuculline (100 fmol/5 nL) significantly attenuated the neuronal inhibition evoked by moxonidine (100 pmol/5 nL). The release of GABA in the RVLM was increased after intravenous moxonidine (50 µg/kg). Central infusion of moxonidine upregulated the protein expression of both GABAA and GABAB receptors in the RVLM.

Conclusion

The current data demonstrate that GABAergic mechanisms in the RVLM are responsible for the hypotension and sympathoinhibition of moxonidine.

Keywords

Centrally antihypertensive drug • Renal sympathetic nerve activity • Microdialysis • Pre-sympathetic neuron • Western blot analysis

1. Introduction

The centrally antihypertensive drug moxonidine, a selective agonist for I1-imidazoline receptors (I1R), has been demonstrated to lower blood pressure (BP) and sympathetic outflow primarily by an action within the rostral ventrolateral medulla (RVLM).1-3 It is well known that the RVLM plays a key role in mediating tonic and reflex control of the cardiovascular system. The activity of the pre-sympathetic neuron in the RVLM, which is barosensitive and directly projects to the spinal cord, contributes to the control of resting BP and sympathetic outflow.3 It has been documented that the RVLM I1R and/or α2-adrenergic receptor (α2AR) mediates the mechanism responsible for sympathoinhibition of centrally antihypertensive drugs.1,2,5-7 Interestingly, the neurotransmission of amino acids, such as glutamate and γ-aminobutyric acid (GABA), has been demonstrated to be involved in mediating the effects of centrally antihypertensive drugs.9-11
It has been widely demonstrated that the GABA receptors in the RVLM play an important role in mediating control of resting BP and sympathetic outflow. In the RVLM, endogenous GABA activates GABA receptors to produce a fall in BP. GABA receptors contain ionotropic A and C subtypes and metabotropic B subtypes. In general, the inhibitory GABAergic influence in the RVLM has been demonstrated to be mediated by GABA_A receptors. The injection of the GABA_A receptor agonist muscimol into the RVLM reduces BP, whereas its antagonist bicuculline elevates BP. However, the GABA_B receptor in the RVLM is also suggested to mediate cardiovascular regulation. Previous studies have provided interesting evidence to suggest a possible relationship between GABAergic neurotransmission and the effects of centrally antihypertensive drugs. For example, the depressor action of systemic injection of the centrally antihypertensive drug clonidine, a mixed agonist of I1R and α2AR, is significantly reduced by pre-treatment with systemic bicuculline. In an in vitro study, clonidine enhances the spontaneous release of GABA from synaptosomes prepared from the RVLM in hypertensive rats. In recent studies from our and other groups, a possible mechanism was proposed by which the selective I1R agonist moxonidine and rilmenidine stimulate the release of glutamate, which in turn triggers the release of GABA in the RVLM. For example, the depressor action of systemic injection of the centrally antihypertensive drug clonidine, a mixed agonist of I1R and α2AR, is significantly reduced by pre-treatment with systemic bicuculline. In an in vitro study, clonidine enhances the spontaneous release of GABA from synaptosomes prepared from the RVLM in hypertensive rats. In recent studies from our and other groups, a possible mechanism was proposed by which the selective I1R agonist moxonidine and rilmenidine stimulate the release of glutamate, which in turn triggers the release of GABA in the RVLM. Therefore, we determined the effects of blockade of GABA receptors in the RVLM on the cardiovascular response to moxonidine. We further determined the effects of moxonidine on the release of GABA in the RVLM by microdialysis. We also detected the protein expression of GABA receptors in the RVLM after the intracerebroventricular (ICV) injection of moxonidine for 1 week.

2. Methods

2.1 Animals

Male Sprague–Dawley rats (300 and 360 g) were supplied by Sino-British SIPPR/BK Laboratory Animal Ltd (Shanghai, China) in these experiments. All studies conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Institutional Care and Use Committee of the Second Military Medical University. The acute experiments, including the general surgical procedures, recording of renal sympathetic nerve activity (RSNA), RVLM microinjection, extracellular recording, and western blot, were carried out as previously described.

2.2 General surgical procedures

Briefly, rats were anaesthetized with urethane (800 mg/kg i.p.) and α-chloralose (40 mg/kg i.p.), and the trachea was cannulated to facilitate mechanical respiration. The right common carotid artery was catheterized for supplemental anaesthesia, which was given based on the BP response to nociceptive stimulus with paw pinch. Body temperature was kept at 37°C by a temperature controller.

2.3 Recording of RSNA

The RSNA was recorded as previously described. The renal sympathetic nerves were exposed and placed on a pair of recording electrodes. The distal terminal of the renal nerve was cut to avoid afferent activity. The RSNA was amplified and recorded. Baseline RSNA was taken as 100% from the absolute value after the noise level was subtracted.

2.4 RVLM microinjection

The rat was placed in a stereotaxic frame, and the dorsal surface of the medulla was surgically exposed. Microinjections were made from a four-barrel micropipette. The coordinates for RVLM were based on an atlas of the rat. The RVLM injection (100 nL) was made over a period of 5–10 s, and the RVLM was chemically identified by a pressor response to I-glutamate (1 nmol) microinjection. Based on previous studies, bicuculline and CGP35348 were chosen to selectively block GABA receptor A and B subtypes, respectively. As shown in the Supplemental data, the low dose of antagonists used in this study was tested for their efficiency for blocking GABA receptors in the RVLM. The injection interval between administration of antagonists and moxonidine was about 10 min. At the end of each experiment, the injection sites marked by 2% pontamine sky blue solution were confirmed to be located within the RVLM area, which are similar to those described previously.

2.5 Extracellular recording and pico-injection

The extracellular recording and pico-injection were carried out as previously described. A laminectomy (T1–3) was performed and a stimulating electrode was placed in the dorsolateral funiculus of spinal segment T2 to allow for antidromic stimulation. One barrel of a five-barrel micropipette (20–30 μm diameter) containing a carbon filament (7 μm diameter) was connected to a high impedance amplifier for extracellular recording. The neuronal discharge was amplified and discriminated for a standard pulse. This pulse output was proportional to the number of spikes per second. These signals were displayed online on the computer and recorded on the Powerlab system. As described in the Supplementary data, the pre-sympathetic unit was electrophysiologically identified by both barosensitivity and spinal cord projection. The remaining barrels of the micropipette were used for pico-injection of solutions. The volume (5 nL) of each pico-injection was measured by movement of the fluid meniscus along a reticule in each barrel. These parameters for pico-injection were based on previous studies. An excitatory response to an initial injection of I-glutamate (50 pmol/5 nL) was used to confirm that the recording was from the cell body and not the axon. The changes in discharge of the neuron in response to moxonidine were compared before and 3 min after pico-injection of bicuculline or vehicle. The interval between repeated pico-injections of moxonidine in a same neuron was at least 30 min. The recording site was marked at the end of the experiment by dye injection (5 nL), which are similar to those described previously.

2.6 ICV infusion and western blot analysis

The rats were anaesthetized with a combination of ketamine–diazepam and placed in a stereotaxic frame. A sterile brain cannula connected to an osmotic mini-pump (0.5 μL/h, model 1007D, Alzet) containing moxonidine was inserted into the right lateral cerebral ventricle according to rat atlas and fixed to the skull with dental cement. One week after ICV infusion of moxonidine (20 nmol/day) or aCSF, the rats were euthanized by overdose of anaesthetic and the brain stem was sectioned at 100 μm thickness. The RVLM tissue was punched according to the rat brain atlas. The protein concentration was measured and loaded onto a 7.5% SDS–PAGE gel and then transferred to a polyvinylidene fluoride membrane. The membrane was probed with primary antibody (GABA_A_Rx2, Santa Cruz, sc-7350; GABA_AR1, ABCOM, ab75239; 1:500) and secondary antibody. The protein bands were visually detected and analysed. The levels of target proteins were normalized to β-actin, which served as a loading control.
2.7 Brain microdialysis and high-performance liquid chromatography (HPLC)

The procedures for GABA-level measurement in the RVLM by microdialysis and high-performance liquid chromatography (HPLC) were described previously.25,26 The femoral artery and vein were cannulated for BP measurement and intravenous injection, respectively. After the RVLM was chemically identified by a pressor response to L-glutamate (1 nmol), a microdialysis probe (MAB6.14.2, Stockholm, Sweden) was unilaterally inserted into this region. Brain microdialysis was performed by perfusing the probe with aCSF at a rate of 2 μL/min with a microdialysis pump (Bioanalytical System, MD 1020, USA). A total volume of each dialysate sample (10 min) was 20 μL, which were obtained at least for 60 min for animal rest after the surgical operation. Three dialysates were collected for 10 min periods before injection and 10 and 20 min after intravenous administration of moxonidine (50 μg/kg in 0.2 mL) or saline (0.2 mL). The GABA in the sample was separated and measured by HPLC (LC-10 AT, Shimadzu, Japan) with a fluorescence detector (RF-10AXL, Shimadzu, Japan).

2.8 Data analysis

All values are expressed as mean ± SE. Because of the large variability of baseline activity of RSNA and neuronal discharge rate, percent change was used for comparison before and after treatments. Student’s t-test (paired or unpaired) was used for comparing the baseline data and the difference between pre- and post-injections. Statistical comparisons between different groups were made by the repeated measure one-way ANOVA followed by Newman–Keuls post hoc test. These analyses were performed by software (SigmaStat 3.5). Differences were considered to be significant at $P < 0.05$.

3. Results

3.1 Effect of blockade of GABA receptors in the RVLM on the moxonidine-induced cardiovascular inhibition

The basal MAP and HR in several groups are shown in Table 1. Figure 1A shows original tracings of BP, HR, and RSNA responses to unilateral moxonidine microinjected into the RVLM 10 min after pre-treatment with aCSF or GABA receptor antagonists. Similar to our previous work, unilateral injection of 5 nmol of moxonidine into the RVLM produced a significant fall in BP ($-25.6 ± 4.1$ mmHg), HR ($-56.5 ± 8.4$ b.p.m.), and RSNA ($-53.7 ± 6.5$%) following treatment with vehicle. However, the moxonidine-induced decrease in BP, HR, and RSNA was significantly blunted by pre-treatment with the GABAA receptor antagonist bicuculline (5 pmol) or the GABAB receptor antagonist CGP35348 (200 pmol) into the RVLM compared with aCSF pre-treatment (Figure 1B). We found that unilateral...
injection of bicuculline or CGP35348 did not significantly elevate basal BP, HR, and RSNA.

We further investigated the effects of bilaterally injected bicuculline (5 pmol) into the RVLM on the effect of systemic moxonidine administration (50 μg/kg) in nine rats (Table 1). As indicated in Figure 2, the magnitudes of reductions in BP and RSNA evoked by intravenous injection of moxonidine were significantly (P < 0.05) lower in bicuculline pre-treatment group than those in aCSF pre-treatment group. However, the HR response to intravenous moxonidine was not altered by bicuculline compared with aCSF treatment.

### 3.2 Effects of moxonidine on the bicuculline-induced pressor action in the RVLM

A total of 23 rats (basal BP and HR shown in Table 2) were used to determine the BP response to blockade of GABA<sub>A</sub> receptor in the RVLM after moxonidine treatment. As indicated in Figure 3, unilateral injection of 50 pmol of bicuculline into the RVLM produced a significant increase in BP (8.1 ± 1.0 mmHg). The peak change in BP was usually reached within 10 min after bicuculline injection. It was found that the peak increase in BP induced by bicuculline was significantly (P < 0.05) enhanced (18.7 ± 3.4 mmHg compared with bicuculline-pre-injection level) 10 min after injection of moxonidine (5 nmol) into the RVLM. However, in the presence of efaroxan (5 nmol, a mixed antagonist of I<sub>1</sub>R and α<sub>2</sub>AR) in the RVLM, moxonidine did not decrease baseline BP, but also did not further enhance the pressor action (10.5 ± 1.2 mmHg) induced by bicuculline injected subsequently. In contrast, pre-treatment with the selective α<sub>2</sub>R antagonist yohimbine (200 pmol) in the RVLM did not significantly modify the moxonidine-induced decrease in baseline BP and enhancement in the pressor action (16.2 ± 2.5 mmHg) evoked by bicuculline.

### 3.3 Effect of blockade of GABA<sub>A</sub> receptor on the moxonidine-induced inhibition of pre-sympathetic neurons in the RVLM

A total of 28 RVLM pre-sympathetic neurons were spontaneously active (9.6 ± 1.5 spikes/s) and recorded from 19 rats. Of 28 RVLM pre-sympathetic neurons tested for their response to picoinjection of moxonidine (100 pmol/5 nL), 22 were significantly inhibited by an average of 42.9%, while 6 did not respond. In general, the peak change in discharge of units was reached within 1 min after moxonidine picoinjection, and gradually returned to control levels within 5 min. These 22 moxonidine-sensitive neurons (10.4 ± 1.5 spikes/s) were further studied for detecting the interaction between moxonidine and bicuculline. Figure 4A shows the original tracings of the discharge of a RVLM unit in response to picoinjection of moxonidine before and 3 min after bicuculline (100 fmol). In 15 units (mean of 9.5 ± 1.4 spikes/s), the decrease in discharge frequency evoked by

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**Figure 2** The original tracings (A) and time course (B) of changes in BP, HR, and RSNA in response to intravenous injection of moxonidine (Mox, 50 μg/kg) after pre-treatment with aCSF or bicuculline (Bic, 5 pmol) injected into the RVLM bilaterally. n = 5 for each group. *P < 0.05 vs. aCSF + Mox at the corresponding time points.

**Table 2** Baseline values of MAP and HR in experimental groups for determining the effect of moxonidine on the pressor action evoked by bicuculline

<table>
<thead>
<tr>
<th></th>
<th>MAP (mmHg)</th>
<th>HR (b.p.m.)</th>
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<tbody>
<tr>
<td>aCSF + Bic</td>
<td>4</td>
<td>103 ± 4</td>
</tr>
<tr>
<td>Mox + aCSF</td>
<td>4</td>
<td>98 ± 3</td>
</tr>
<tr>
<td>Mox + Bic</td>
<td>5</td>
<td>99 ± 5</td>
</tr>
<tr>
<td>Efa + Mox + Bic</td>
<td>5</td>
<td>94 ± 4</td>
</tr>
<tr>
<td>Yoh + Mox + Bic</td>
<td>5</td>
<td>101 ± 5</td>
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Data are expressed as mean ± SE. n, the number of the rats in each group. Bic, bicuculline; Mox, moxonidine; Efa, efaroxan; Yoh, Yohimbine.
moxonidine picoinjection was significantly blunted after pre-
treatment with bicuculline (average: \(244.2\%\) to \(220.5\%\), \(P<0.05\)) (Figure 4B). Notably, picoinjection of bicuculline produced a transient (less than 30 s) increase in discharge of 9 units (average: \(36.8\%\) + \(4.8\%\)). In remaining 7 units (average: \(11.3\%\) + \(1.5\) spikes/s), in contrast, there is no significant difference of neuronal inhibition in response to moxonidine before and after aCSF picoinjection (average: \(241.6\%\) to \(239.1\%\), \(P>0.05\)).

3.4 Effect of moxonidine on the release of GABA in the RVLM

A total of 12 rats (basal BP and HR: \(96\%\) + \(4\) mmHg and \(406\%\) + \(12\) b.p.m., respectively) were studied to determine the release of GABA in the RVLM in response to intravenous injection of moxonidine. As indicated in Figure 5, the release of GABA in the RVLM was significantly increased by an average of \(67\%\) and \(50\%\) at the first 10 min and second 10 min period after moxonidine administration compared with the pre-injection level. In contrast, there is no significant change in the concentration of GABA after normal saline administration compared with the pre-injection level.

3.5 Effect of chronic ICV infusion of moxonidine on the protein expression of GABA receptors in the RVLM

A total of 10 rats were euthanized for western blot analysis after chronic ICV infusion of moxonidine or aCSF. Before the rats were euthanized, baseline BP and HR were measured under the anaesthesia. Both baseline BP (\(105\%\) + \(4\) mmHg) and HR (\(418\%\) + \(13\) vs. \(340\%\) + \(12\) b.p.m.) were significantly (\(P<0.05\)) reduced after chronic infusion of moxonidine compared with aCSF infusion. As indicated in Figure 6, protein expressions of both GABA_A and GABA_B receptor subunits (GABA_A-Rx2 and GABA_B-R1, respectively) in the RVLM were significantly upregulated following chronic ICV infusion of moxonidine. Based on the above evidence, it is suggested that the GABAergic mechanism in the RVLM contributes to the effects of the centrally antihypertensive drug moxonidine.

4. Discussion

There are several important findings obtained from the present study. First, the hypotension and sympathoinhibition evoked by moxonidine were significantly attenuated by pre-treatment with GABA_A or GABA_B receptor antagonists in the RVLM. Second, the pressor action evoked by GABA_A receptor blockade in the RVLM was significantly enhanced by moxonidine, which was prevented by the I\(_1\)R antagonist efaroxan. Third, systemic injection of moxonidine increased the concentration of GABA level in the RVLM. Finally, the protein expression of both GABA_A and GABA_B receptors in the RVLM was upregulated following chronic ICV infusion of moxonidine. Based on the above evidence, it is suggested that the GABAergic mechanism in the RVLM contributes to the effects of the centrally antihypertensive drug moxonidine.

In the present study, GABA receptor antagonists were first used for determining the role of GABA receptors in mediating the effect of moxonidine at the level of the RVLM. It should be noted that the dose (5 pmol) of the GABA_A receptor antagonist bicuculline was lower in the present study than a regular dose (100–200 pmol) used in previous studies, in which a potent and long-term increase in BP and RSNA is produced by bicuculline injected into the RVLM.\(^{12,15}\) In the present work, therefore, a low dose of bicuculline (5 pmol) was chosen to avoid an elevation in baseline BP, HR, and RSNA, because the similar levels of basal BP, HR, and RSNA was very important to compare the difference of moxonidine effect in the presence and absence of bicuculline in the RVLM. Such a low dose of bicuculline (5–10 pmol) was reported to effectively block the GABA_A receptor in the RVLM based on a previous study.\(^{21}\) In order to confirm the efficiency of 5 pmol of bicuculline against GABA_A receptor, we found that microinjection of 5 pmol of bicuculline into the RVLM significantly attenuated the baroreflex sensitivity control of RSNA, but also partially prevented the pressor action evoked by the GABA_A receptor agonist muscimol (see Supplementary data). On the other hand, 200 pmol of CGP35348, which is lower compared with a previous study (400 pmol),\(^{16}\) injected into...
the RVLM did not alter basal BP. However, we confirmed that 200 pmol of CGP35348 almost abolished the decrease in BP evoked by microinjection of the GABAB receptor agonist baclofen into the RVLM. Taken together, we suggest that the doses of bicuculline and CGP-35348 effectively antagonize, at least to some degree, GABAA and GABAB receptors in the RVLM, respectively.

The present data clearly showed that the decrease in BP, HR, and RSNA evoked by injection of moxonidine into the RVLM was significantly blunted by pre-treatment with the GABA or GABAB receptor antagonist bicuculline (Bic, 100 fmol) in the RVLM, respectively. The degree of attenuation of the effects of moxonidine was similar with bicuculline compared with CGP35348, suggesting a similar importance of GABA and GABAB receptors in the RVLM.

The present data clearly showed that the decrease in BP, HR, and RSNA evoked by injection of moxonidine into the RVLM was significantly blunted by pre-treatment with the GABA or GABAB receptor antagonist bicuculline or CGP-35348 in the RVLM, respectively. The degree of attenuation of the effects of moxonidine was similar with bicuculline compared with CGP35348, suggesting a similar importance of GABA and GABAB receptors in mediating the effects of moxonidine. Moreover, we further determined the role of GABA receptor in mediating the effects of moxonidine. We found that the hypotension and sympathoinhibition evoked by intravenous moxonidine were blunted by pre-treatment with bilaterally

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**Figure 4** Effect of GABA<sub>A</sub> receptor blockade on inhibition of RVLM pre-sympathetic neurons induced by moxonidine picoinjection. (A) Representative tracings showing the effects of picoinjection of moxonidine (Mox, 100 pmol) on spontaneous discharge of a RVLM pre-sympathetic neuron before (b) and after (d) picoinjection of bicuculline (Bic, 100 fmol, c). The neuron was first tested by an excitatory response to picoinjection of L-glutamate (L-Glu, 5 pmol, a). (B) The percent changes in discharge of RVLM pre-sympathetic neurons evoked by picoinjection of moxonidine (Mox, 100 pmol) following pre-treatment with aCSF (5 nL, n = 7) and bicuculline (Bic, 100 fmol, n = 15). *P < 0.05 vs. pre-level.

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**Figure 5** The release of GABA in the RVLM in response to intravenous injection of normal saline (n = 5) and moxonidine (Mox, 50 μg/kg, n = 8). *P < 0.05 vs. pre-injection group.
injection of bicuculline into the RVLM. This result indicates the importance of RVLM in processing the interaction between GABAergic mechanism and the effect of systemic moxonidine. Moreover, this interaction was further detected at the level of RVLM pre-sympathetic neuron, because BP and sympathetic outflow are dependent on activity of RVLM pre-sympathetic neuron. It was found that the moxonidine-induced decrease in ongoing activity of pre-sympathetic neuron was significantly blunted by pre-treatment with bicuculline picoinjection.

It should be noted that either bicuculline or CGP35348 did not completely abolish the effect of moxonidine. This phenomenon may be explained by three possibilities. First, although we did not study the effect of co-injection of both antagonists on the moxonidine-induced action, it is logical to speculate that pre-treatment with bicuculline and CGP 35348 together may produce a stronger impact on attenuating the cardiovascular response to moxonidine than pre-treatment with only one antagonist. Second, the low doses of both antagonists used in this work may not be enough to completely block their corresponding receptors. In fact, there is a clear limitation that the low doses of antagonists for blocking GABA receptors were performed in this work. As a result, there may be enough functional (unblocked) GABA receptors to mediate the effect of moxonidine. A possibility is that a greater counteraction against the effect of moxonidine would be produced by high dose of antagonists. Finally, in addition to GABAergic mechanism, the other mechanisms (e.g. glutamatergic, nitric oxide (NO), and noradrenergic) are also suggested to be involved in mediating the effect of centrally antihypertensive drugs.4,10,11,27 – 29

In the present study, we further determined the effect of moxonidine on the release of GABA and the protein expression of GABA receptors in the RVLM. The release of GABA in the RVLM was significantly increased after intravenously injected moxonidine, suggesting that moxonidine is capable of directly stimulating the release of GABA in the RVLM. The specific location of the action of systemic moxonidine is widely suggested to be located within the RVLM.1,2,19,30 However, there is no direct evidence confirming whether the presence of I1R or α2AR is in the GABAergic inter-neurons in the RVLM. Previous evidence from immunohistochemical studies has shown that I1R or α2AR has been suggested to be located in axon terminals of the rat brain.35,37 The electrophysiological studies suggest that centrally antihypertensive drugs (e.g. moxonidine and clonidine) pre-synaptically inhibit activity of the RVLM pre-sympathetic neurons.35,34 Collectively, these data from electrophysiological and microdialysis studies suggest the functional existence of pre-synaptic I1R or α2AR that is coupled with GABA release. In addition, we further tested the GABAa receptor subunit Rα2 and the GABAb receptor subunit R1 for observing the expression of GABA receptors, because these two subunits are expressed in the RVLM and are involved in cardiovascular regulation according to the previous studies.35 – 37 We found that BP was stably decreased by approximately 20 mmHg at day 7 after continuous infusion (ICV) of moxonidine with a micro-osmotic pump. It was found that the GABA receptors including A and B subunits in the RVLM were upregulated by moxonidine. Based on the evidence from microdialysis and western blot, we suggest that the release of GABA and GABA receptor expression are enhanced following moxonidine application.

There is a continuing and yet unresolved debate for the contribution of both I1R and α2AR to effects of centrally antihypertensive drugs.38,39 Based on previous evidence, moxonidine has been demonstrated to be a relatively selective agonist for I1R.1,2,3 Moreover, it is likely that I1R, but not α2AR in the RVLM, plays a crucial role in mediating the effect of centrally antihypertensive drugs.1,3 In this work, we found that efaroxan (a mixed antagonist for I1R and α2AR) but not yohimbine (a selective antagonist for α2AR) abolished the moxonidine-induced decrease in baseline BP, but also prevented the moxonidine-induced enhancement in the pressor action evoked by bicuculline (50 pmol) in the RVLM. Therefore, it is strongly suggested that only I1R accounts for mediating of the interaction between GABAergic neurotransmission and moxonidine. Another important question is how activation of I1R by moxonidine stimulates the release of GABA in the RVLM. Calcium influx may be a possible mechanism responsible for the release of GABA by moxonidine. It is well known that calcium influx is necessary to trigger the release of neurotransmitters such as GABA.40,41 It has been reported that I1R activation is capable of producing an increase in intracellular calcium concentration.12 Another possibility is a NO mechanism. Blockade of NO generation in the brain effectively antagonizes the effect of centrally antihypertensive drugs such as clonidine and moxonidine.19,27,28 Importantly, it has been demonstrated that NO signaling is an important trigger for the release of GABA.23,43 As indicated in the Supplementary data, this possibility was initially confirmed. We found that the moxonidine-induced enhancement in the pressor action evoked by GABA receptor blockade was completely abolished by pre-treatment with the NO synthase inhibitor L-NAME in the RVLM. In addition, it is not clear whether the release of GABA triggered by moxonidine is coupled with glutamate release in the present study. Although the release of glutamate in the RVLM was not detected following moxonidine administration in this work, blockade of glutamatergic mechanism antagonizes the hypotension of centrally hypertensive drugs.8 – 10 The spontaneous release of glutamate

Figure 6 Protein expression of GABA receptor subunits in punched RVLM samples measured by western blot in rats with chronic ICV infusion of aCSF and moxonidine (Mox). (A) Examples of visualized electrophoresis bands of target and β-actin protein. (B) Mean data of band densities of ratio of GABA receptor subunits to β-actin. n = 5 for each group. *P < 0.05 vs. aCSF.
in the RVLM is also enhanced by the centrally antihypertensive drug clonidine. Moreover, stimulation of glutamate receptors triggers GABA release and facilitates the GABAergic synaptic activity, whereas antagonism of glutamate receptor reduces GABA outflow from the septum. In accordance with these data, we do not exclude the possibility that the release of GABA is secondary to the glutamate release triggered by moxonidine.

In summary, the current data demonstrate that the hypotension and sympathoinhibition by moxonidine are dependent on the functional state of GABA receptors in the RVLM. Moreover, moxonidine stimulates the release of GABA and upregulates the GABA receptors in the RVLM. Therefore, it concludes that the enhanced GABAergic neurotransmission in the RVLM contributes to the hypotension and sympathoinhibition by moxonidine.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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**Conflict of interest:** none declared.

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