Non-NMDA receptors within caudal ventrolateral medulla are involved in transmission of baroreflex of rats[1]

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ABSTRACT

AIM: To investigate the role of non *N*-methyl-*D*-aspartate acid (non-NMDA) receptors within the caudal ventrolateral medulla (CVLM) in mediating the baroreflex. **METHODS:** In urethane-anesthetized, paralyzed, and artificially ventilated rats, the effects of 6-cyano-7-nitroquinoxaline-2,3-dinoe (CNQX, a selective non-NMDA receptors antagonist) locally injected into the CVLM on the depressor responses evoked by aortic nerve stimulation and the barosensitivity of the rostral ventrolateral medulla (RVLM) barosensitive neurons were observed. **RESULTS:** Bilateral microinjection of CNQX (200 pmol in 100 nL for each side) into the CVLM significantly ($P<0.01$) increased the blood pressure (BP) and heart rate, and markedly ($P<0.01$) attenuated the depressor response to the aortic nerve stimulation. CNQX (200 pmol in 100 nL) unilaterally injected into the CVLM significantly ($P<0.01$) increased the firing rate of the ispilateral RVLM barosensitive neurons and reduced the inhibitory responses of neurons evoked by stimulation of aortic nerve and elevation of BP, and partially inhibited the neuronal cardiac cycle-related rhythm. **CONCLUSION:** The CVLM played an important role in maintaining the tonic excitatory cardiovascular activities and transmitting the baroreceptor information via activation of non-NMDA receptors.

INTRODUCTION

Baroreflex is one of the principal mechanisms by which the central nervous system regulates peripheral cardiovascular activity[1,2]. At least three regions in medulla oblongata, the nucleus tractus solitarius (NTS), the caudal ventrolateral medulla (CVLM), and the rostral ventrolateral medulla (RVLM), have been found involved in the neurotransmission of baroreflex. The NTS receive primary afferent input from peripheral baroreceptors and project to the barosensitive neurons in the CVLM[2,3]. The CVLM neurons are tonically active, and exert a sympathoinhibitory effect by inhibiting the tonically active RVLM barosensitive neurons (presympathetic neurons), which play a critical role in the tonic and reflex control of peripheral cardiovascular activity[4,5].

Excitatory amino acid receptors within the CVLM are involved in regulation of cardiovascular activities, and classified into *N*-methyl-*D*-aspartate acid (NMDA) and non-NMDA subtypes[6-9]. Previous studies reported that, within the CVLM, the baroreflex is exclusively mediated by the NMDA subclass of excitatory amino acid receptors because locally injected NMDA receptor

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antagonists within the CVLM abolished the baroreflex[7,8]. Interestingly, electrophysiological information showed that the local blockade of the CVLM non-NMDA receptors attenuated the inhibitory response of the RVLM barosensitive neurons to the aortic nerve stimulation[9]. However, the roles of the CVLM non-NMDA receptors involved in the regulation of the cardiovascular activity have not been extensively studied, and litter is known about the role of the CVLM non-NMDA receptors in mediating the transmission of baroreflex.

Here, the present study was designed to reevaluate the role of non-NMDA receptors within the CVLM in the neurotransmission of baroreflex. Our aim was to explore the excitatory amino acid receptor mechanism of baroreflex in the central nervous system.

MATERIALS AND METHODS

General procedures Male Sprague-Dawly rats (weighing 270-350 g, Animal Center, Shanghai Institute of Family Planning, Grade II, Certificate No 152) were used in this study. After induction of anesthesia with sodium pentobarbitone (60 mg/kg, ip), catheters were placed into the left femoral artery and the left femoral vein for BP measurement and drug administration. Blood pressure (BP), electcardiogram (ECG), and heart rate (HR) were sequentially measured with analytic software (MPA2000, China) by a computer. The trachea was cannulated, and the rats were paralyzed with gallamine triethiodide (4 mg/kg, iv per 30 min) and artificially respirated with oxygen-enriched room air, maintaining end-tidal CO_2 at 4-5 %. Urethane was injected intravenously to maintain surgical anesthesia (1.1 g/kg). Anesthetics were supplemented when necessary. The anesthetized rats were fixed on a stereotaxic frame (Narishige, Japan). The left aortic nerve was exposed and isolated near its junction with the superior laryngeal nerve. A part of the occipital bone and the cerebellum were removed to expose the dorsal surface of the medulla. All exposed tissues were covered with warm mineral oil. Body temperature was maintained at around 37 ºC with a heating pad and an infrared heating lamp.

Microinjections Under the guidance of a sterotoxic apparatus, multi-barrel micropipette (tip diameter 20-30 μm) was inserted into the CVLM (0-0.3 mm rostral to the obex, 1.6-1.9 mm lateral to the midline, and 2.6-3.0 μm below the dorsal surface of the medulla). The three-barrel pipette was filled with *L*-glutamate (*L*-glu, Sigma) 50 mmol/L, 6-cyano-7- nitroquinoxaline-2,3-dinoe (CNQX, Sigma) 2 mmol/L, and 2 % Pontamine sky blue solution. CNQX was first solubilized with dimethyl sulfoxide, finally dissolved in 0.5 mol/L phosphate-buffered physiological saline (PBS), and the pH was adjusted close to 7.4. The dose used for the drugs was based on previous studies[6,9]. All drugs were administered into the CVLM in a volume of 100 nL by the microinjector (Narishige, Japan). The injections were made over a period of 5 s with a syringe. Accurate placement of the pipette and functional identification of the CVLM depressor area was accomplished by microinjection of *L*-glu (5 nmol in 100 nL). Microinjections of *L*-glu were used to identify sites of synaptic contact in the CVLM that participate in central cardiovascular regulation because this amino acid excites receptors only on neurons without affecting fibers of passage[10]. Finally, 50 nL of 2 % Pontamine sky blue dye was injected to mark the site for subsequent histological identification.

Electrophysiology Extracellular single-unit recording from neurons in the RVLM was made using a glass single-barrel electrode (4-10 MΩ, impedance). The glass microelectrode was filled with 2 % Pontamine sky blue dissolved in sodium acetate 0.5 mol/L. The RVLM was approached at the following parameters: 2.4-2.8 mm rostral to the obex, 1.7-2.0 mm lateral to the midline and 2.9-3.5 mm below the dorsal surface of the medulla. The microelectrode was inserted into the RVLM by a micromanipulator (Narishige, Japan). Signals were preamplified (100-3000 Hz bandpass) first by a microelectrode amplifier (MEZ8201, Nihon Kohden, Japan) and then by a biophysical amplifier, and subsequently fed into a time-amplitude window discriminator (TAWD-94, China) and simultaneously monitored on an oscilloscope (National, Japan). Corresponding to each spike, the window discriminator generated a digitized pulse for further computerized analysis. Digitized unit activity along with the BP and ECG were fed to another computer. Data were analyzed by software (Unit Spikes Analysis System, China) for averaging ECG and BP signals together with neural activity. The RVLM barosensitive neurons were identified by the following criteria[7,11]: (1) inhibition by short train stimulation of aortic nerve (3 pulse, 0.2 ms pulse width, 5 ms pulse interval, 50-250 μA, 1 Hz); (2) abrupt inhibition of spontaneous firing by a bolus injection of phenylephrine (10 mg/kg) which elevated BP by about 30 mmHg (1 mmHg=0.133 kPa); and (3) the presence of cardiac rhythmicity as demonstrated by R wave (of ECG) trig-

gered average. The sites for microinjection of drugs into the CVLM were ipsilateral to the RVLM barosensitive neurons tested. In addition, at the end of experiments, recording sites were also marked by iontophoresis of Pontamine sky blue dye (15 μA for 10 min, negative currents).

Baroreflex activation Baroreflex was activated by electrical stimulation of the left aortic nerve, which contains only baroreceptor afferent fibers in rats[12,13]. The aortic nerve was stimulated electrically for 30 s at supramaximal voltage (5 V) and pulse duration (2 ms)[8,13], while the frequency of nerve stimulation was adjusted for each rat to establish parameters that would elicit reproducible depressor responses of about -30 mmHg. Antagonist CNQX then was injected bilaterally into the CVLM. Ten and twenty minutes after drug microinjection, and the aortic nerve was again stimulated at the previously established parameters.

Histological procedure By the end of the experiment, the animal was transcardially perfused with 0.9 % NaCl and 10 % formalin. The brain stem was removed, stored overnight in 10 % phosphate-buffered formalin, and then transferred to fixative containing 30 % sucrose. Frozen brain tissue was sectioned in the coronal plane (50 μm) and stained with neutral red. The sites for both recording within the RVLM and injection within the CVLM were reconstructed from the dyespots according to the atlases[14]. Histological analysis showed that the centers of the microinjection sites in the CVLM (Fig 1 left panel) were localized just dorsal to the lateral reticular nucleus and ventrolateral to nucleus ambiguous. This area of the medulla has been shown to contain the cell bodies of sympathoinhibitory interneurons[6]. The right panel of Fig 1 shows a composite of the locations of the barosensitive neurons recording.

Statistical analysis BP is expressed as mean arterial pressure (MAP). Data are presented as means±SD. Difference was considered significant at $P<0.05$ level using the Student's *t*-test. Perstimulus time histograms (bin width 2 ms), ECG-triggered histograms (bin width 2 ms) and neuronal discharges time histograms during BP elevation (bin width 1 s) of single-unit activity were used to identify barosensitive neurons within the RVLM and to assess changes in the sensitivity of neurons to baroreceptor activation. The changes of barosensitivity were calculated as follows: expected count=average count in baseline or in excitatory periods (ECG-triggered histograms)/bin width×numbers of bin in inhibitory period; magnitude of changes in barosensitivity=[(expected count–actual count in inhibitory period)/expected count]×100. The magnitude of the barosensitivity of the RVLM barosensitive neurons in the control period was taken as a barosensitivity of 100 %. The histograms were constructed again after microinjection of the antagonist (CNQX). Analysis of histograms in the postantagonist period was done on the same bins used to analyze histograms in the preantagonist period. Falls in barore-

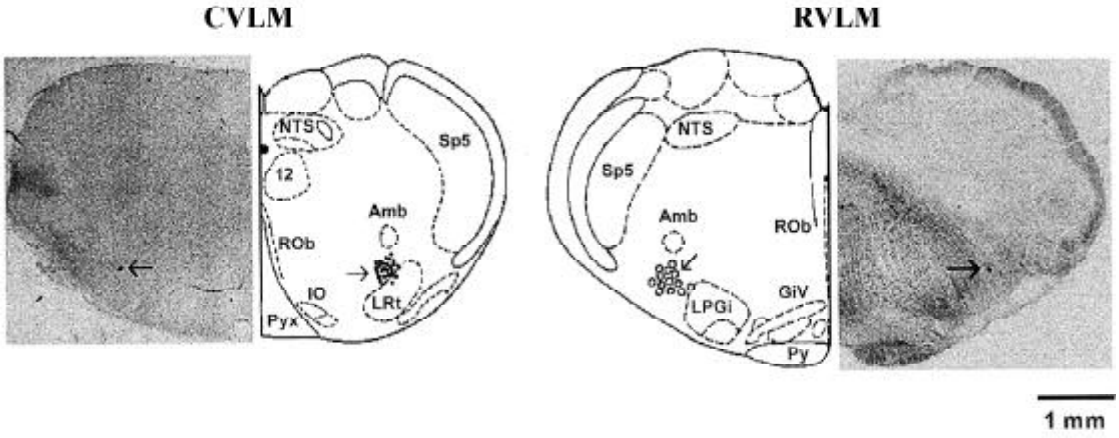

Fig 1. Localization of the CVLM injection sites and the RVLM recording sites. The CVLM injection sites of drugs (arrow-pointed black circles, ●) and the RVLM recording sites of barosensitive neurons (arrow-pointed open circles, ○) were plotted on two coronal sections through medulla 0-0.3 mm and 2.4-2.8 mm rostral to the obex, respectively. In the raw thinning 50 μm thick sections of brain stem, arrow-pointed spots mark a microinjection site (CVLM) and a unit recording site (RVLM). 12, hypoglossal nucleus; Amb, nucleus ambiguous; IO, inferior olive; LPGi, lateral paragigantocelluar nucleus; LRt, lateral reticular nucleus; Rob, raphe obscurus nucleus; Py, pyramidal tract; Pyx, pyramidal decussation, NTS, nucleus solitary tract; Sp5, spinal trigemina nucleus.

ceptor sensitivity were expressed as percent reduction from the magnitude of baroreflex sensitivity in the control period[9].

RESULTS

Effects of microinjection of CNQX into the CVLM on MAP, HR, and depressor response to aortic nerve stimulation In 9 rats, prior to CNQX injection, baseline values were (104±8) mmHg for MAP, (405±9) beats per minute (bpm) for HR, and (-30±2) mmHg for the depressor responses to electrical stimulation of aortic nerve. Bilateral microinjection of CNQX (200 pmol in 100 nL) into the CVLM produced significant (P<0.01) increases in MAP and HR by (15±7) mmHg and (17±8) bpm, respectively. These changes occurred immediately (within 1 min) after CNQX, and persisted for about 10 min. However the depressor responses to aortic nerve stimulation were significantly (P<0.05) attenuated to (–16±3) mmHg and (–24±3) mmHg 10 and 20 min after CNQX, respectively (Fig 2). The typical changes in depressor responses by aortic nerve stimulation before, 10 min and 20 min after the CVLM administration of CNQX are illustrated in Fig 3. In other 4 rats, bilateral microinjection of 100 nL PBS into the CVLM did not significantly (P>0.05) affect baseline BP, HR and the depressor response to aortic nerve stimulation (P>0.05). In above 13 animals, prior injection of *L*-glu (5 nmol in 100 nL) into the CVLM produced a significant decrease in MAP and HR [(-22±6) mmHg and (-21±5) bpm, P<0.01]. The effects of bilateral microinjection of CNQX, *L*-glu and PBS into the CVLM on MAP and HR are shown in Tab 1.

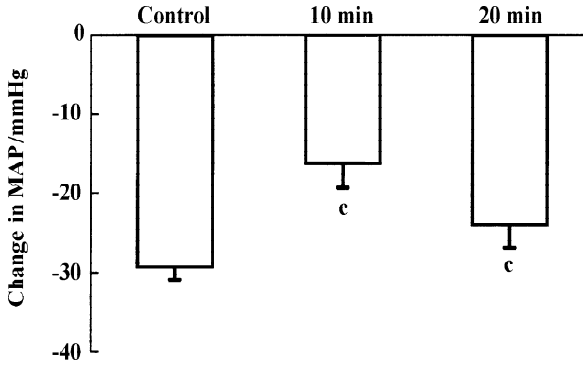

Fig 2. Depressor responses evoked by stimulation of aortic nerve during the control period, and 10 and 20 min after bilateral injection of CNQX (200 pmol for each side) into the CVLM. CNQX injection into the CVLM partially abolished the aortic baroreflex. ^cP<0.01 *vs* control.

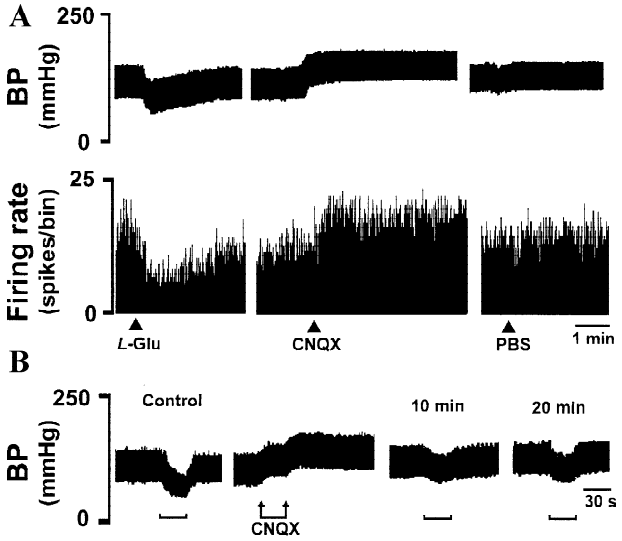

Fig 3. Effects of microinjection of CNQX into the CVLM on cardiovascular activities. A: microinjection site was functionally verified by the depressor response and neuronal inhibitory response to *L*-glu (5 nmol). Unilateral injection of CNQX (200 pmol) but not PBS (100 nL) into the CVLM produced the marked increases of both blood pressure and the firing rate of the RVLM barosensitive neuron. Bin width=1 s; B: the depressor responses evoked by stimulation of aortic nerve were partially abolished 10 and 20 min after bilateral microinjection of CNQX (200 pmol for each side) into the CVLM. Bars indicate 30-s stimulation periods.

Tab 1. Effects of *L*-glu, CNQX, and PBS injected into the CVLM on the cardiovascular activities. Mean±SD. ^cP <0.01 *vs* pretreatment level.

Groups	*n*	MAP/ mmHg	HR/ bpm	Firing rate of neurons/ spike· s^{-1}
Pre-*L*-glu	13	104±7	405±9	13±4
Post-*L*-glu		82±4^c	384±12^c	11±4^c
Pre-CNQX		104±8	405±9	13±4
Post-CNQX	9	119±7^c	422±12^c	16±5^c
Pre PBS	4	104±6	405±9	14±5
Post-PBS		106±6	404±11	14±5

1) Neurons indicate the RVLM barosensitive neurons.

Effects of microinjection of CNQX into the CVLM on the RVLM barosensitive neuronal activities Totally 13 neurons [resting firing rate (13.0±4.3) spikes/s, range from 6 to 25 spikes/s] in the RVLM were identified as the barosensitive neurons (left part of Fig 4). The extracellular recordings were kept stable

for about 10 min. Microinjection of 5 nmol *L*-glu into the CVLM significantly ($P<0.01$) decreased MAP and the firing rate of 13 barosensitive neurons in the RVLM by (19±6) mmHg and (2.5±1.5) spikes/s, respectively (Fig 3). In 9 units, unilateral microinjection of CNQX (200 pmol in 100 nL) into the CVLM produced a significant ($P<0.01$) increase of MAP and the firing rate of barosensitive neurons by (14±5) mmHg and (2.9± 2.2) spikes/s (Fig 3). The barosensitivity of the RVLM barosensitive neurons was observed 5 min after local injection of CNQX within the CVLM. The inhibitory responses to aortic nerve stimulation were attenuated by 39 %±23 % ($P<0.01$), the magnitude of changes varied from 0 (in 2 units) to 66 %. The cardiac-related rhythmicity in the firing of units activity was attenuated by 38 %±24 % ($P<0.01$), the magnitude of attenuation varied from 0 (in 2 units) to 72 %. The inhibitory responses of units evoked by BP elevation were reduced by 31 %±24 % ($P<0.01$), the magnitude of attenuation varied from 0 (in 3 units) to 56 % (Fig 4). Microinjection of PBS ($n=4$) showed no detectable effects on the baseline firing rate or the baroreflex activation responses of the RVLM barosensitive neurons (Fig 3). A summary of the changes in the firing rate of 13 RVLM barosensitive neurons flowering the CVLM treatments of *L*-glu, CNQX and PBS is presented in Tab 1.

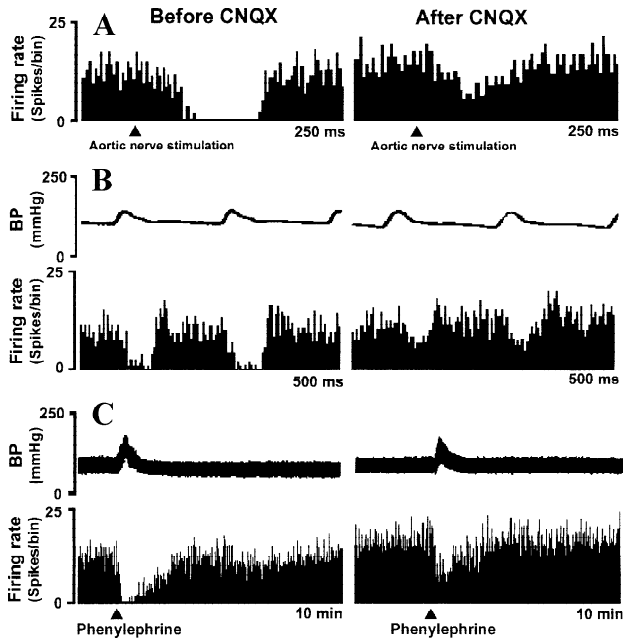

Fig 4. Responses of the RVLM neuronal barosensitivity before (the left panels) and after (the right panels) injection of CNQX (200 pmol) into the CVLM. A: Effect of CNQX on the inhibition of spikes evoked by stimulation of aortic nerve (300 sweeps). Bin width=2 ms; B: Effect of CNQX on cardiac cycle-related rhythm of neuron (200 sweeps). Bin width=2 ms; C: Effect of CNQX on neuronal inhibition induced by elevated BP. Bin width=1s.

DISCUSSION

The principal finding in the present study is that local injection of CNQX (a selective antagonist of non-NMDA receptors[15]) into the CVLM not only significantly increased baseline BP, HR, and the firing rate of the RVLM barosensitive neurons, but also partially blocked the baroreflex.

In the present study, we investigated the effect of non-NMDA receptors within the CVLM on the baroreflex. As the aortic nerve of rat is a pure baroreceptor nerve that contains few, or no, chemosensory or nociceptive afferent fibers[12,13], the depressor responses to aortic nerve stimulation together with the barosensitivity of barosensitive neurons in the RVLM were used as indices of change in the baroreceptor reflex[7,12]. Previous studies suggest that many RVLM barosensitive neurons receive a direct synaptic (GABAergic) input from neurons in CVLM[5], and that the RVLM barosensitive neurons are a heterogeneous group of neurons that exert nonuniform control over regional sympathetic vasomotor pathways[4]. Thus the activity of the single RVLM barosensitive neurons is likely to be more sensitive index with which to evaluate the effects of manipulating the activity of the CVLM neurons[7,9].

The CVLM is now recognized as an important region for the reflex control of BP[1,2,16]. The previous studies showed that baroreflex might be mainly mediated by NMDA receptors[7,8]. For example, selective blockade of NMDA receptors within the CVLM completely abolished synaptically mediated depressor responses evoked by aortic nerve stimulation but not those elicited by *L*-glu, kainic acid, or quisqualic acid injected at the same site[8]. In the present study, however, local administration of non-NMDA receptor antagonist CNQX within the CVLM significantly attenuated the depressor response to aortic nerve stimulation and the RVLM neuronal barosensitivity. These results provide strong evidence that the CVLM non-NMDA receptors were involved, at least partially, in the transmission of the baroreceptor reflex. In addition, microinjection of CNQX into the CVLM increased baseline BP, HR and the firing rate of the RVLM barosensitive neurons. These results clearly suggest that the CVLM non-NMDA re-

ceptors are involved in the maintenance of the tonic excitatory cardiovascular activity. However, the finding of Jung R *et al*[6] showed that NMDA receptors but not non-NMDA receptors within the CVLM were involved in maintaining baseline BP. Methodological differences, including anesthetics (pentobarbitone +urethane *vs* urethane), breathing (artificially *vs* spontaneously) and microinjected sites within the CVLM may contribute to the differences between the present and earlier findings. Our results are consistent with the findings of Miyawaki T *et al*[15] that the selective blockade of non-NMDA receptors within the CVLM attenuated inhibitory response of the RVLM barosensitive neuron to aortic nerve stimulation. These results have identified a synaptic non-NMDA receptor in the CVLM whose activation may be necessary for the elicitation of baroreflex, but we provide little information as to the identity of the endogenously released neurotransmitter that binds to non-NMDA receptors. It is possible that the endogenous ligand of non-NMDA receptors might mediate baroreflex information transfer across synaptic junctions in the CVLM. Confirmation of this suggestion requires further investigation.

Notably, our results also showed that both the depressor response evoked by aortic nerve stimulation and barosensitivity of the RVLM neurons were not completely abolished after administration of CNQX within the CVLM. Histological analysis revealed that the sites of injection localized within the rostral part of CVLM. This area of the CVLM has been termed "rostral CVLM" or "obex-ventrolateral medulla" in the previous studies and has been shown to contain sympathoinhibitory baroreflex interneurons[1,5]. The findings of the present and previous studies[6,9] showed that injection of CNQX into the CVLM partially blocked the baroreflex supported the idea that many RVLM barosensitive neurons might still receive baroreceptor information mediated via other receptors in the CVLM. Although the design of the present study is not appropriate to address the question whether the baroreflex is mainly mediated by non-NMDA receptors or by the other receptors. Importantly, previous studies showed that NMDA receptors in the CVLM played a critical role in the transmission of baroreceptor information[7, 8,17]. Therefore, the present work and previous studies supported the hypothesis that both NMDA and non-NMDA receptors within the CVLM might be responsible for transmission of baroreceptor information.

In summary, the present study demonstrates the existence of the CVLM neurons that are tonically activated by endogenous ligand acting on non-NMDA receptors. Furthermore, these data show that many of these neurons receive baroreceptor information through non-NMDA receptor and then, in turn, transmit it to the RVLM barosensitive neurons. It is strongly suggested that the CVLM non-NMDA receptors are involved, at least partially, in the transmission of baroreflex.

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