Contribution of AMPA/kainate receptors in the rostral ventrolateral medulla to the hypotensive and sympathoinhibitory effects of clonidine

Wei-Zhong Wang,1,2 Li-Gang Wang,1 Lie Gao,2 and Wei Wang2

1Department of Physiology, Second Military Medical University, Shanghai, China; and 2Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, Nebraska

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Wang W-Z, Wang L-G, Gao L, Wang W. Contribution of AMPA/kainate receptors in the rostral ventrolateral medulla to the hypotensive and sympathoinhibitory effects of clonidine. Am J Physiol Regul Integr Comp Physiol 293: R1232–R1238, 2007. First published June 20, 2007; doi:10.1152/ajpregu.00233.2007.—The depressor and sympathoinhibitory effect of the imidazoline drug clonidine is reported to be associated with functional states of the central glutamate receptors. The rostral ventrolateral medulla (RVLM) has been recognized as a specific target area for mediating the central depressor mechanism of clonidine. The objective of this study was to determine the role of the glutamate receptor subtype α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor within the RVLM in clonidine-induced depressor and sympathoinhibitory action in anesthetized normotensive rats. Unilateral microinjection of 200 pmol of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a potent AMPA/kainate receptor antagonist, into the RVLM completely abolished the pressor action evoked by clonidine (5 pmol) without affecting the pressor action of N-methyl-D-aspartate (20 pmol). Pretreatment with intra-RVLM injection of CNQX (20 and 200 pmol) dose-dependently attenuated the reduction in blood pressure (BP), heart rate (HR), and renal sympathetic nerve activity (RSNA) elicited by intra-RVLM clonidine (5 nmol) or intravenous clonidine (10 μg/kg), while 2 pmol of CNQX did not alter clonidine-induced cardiovascular action. Furthermore, the decreases in BP, HR, and RSNA evoked by intravenous clonidine (10 μg/kg) or intra-RVLM clonidine (5 nmol) were reversed when CNQX (20 and 200 pmol) was subsequently injected into the RVLM. In conclusion, these data show that blockade of AMPA/kainate receptors in the RVLM significantly antagonizes decreases in BP, HR, and sympathetic activity induced by clonidine, suggesting that the AMPA/kainate receptors within the RVLM contribute to the depressor and sympathoinhibitory effect of clonidine.

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**General surgery.** Rats were anesthetized with urethane (800 mg/kg, ip) and α-chloralose (40 mg/kg, ip). The trachea was cannulated, and the rats were paralyzed with pancuronium bromide (1 mg/kg, iv; 0.1 mg/kg thereafter as needed) and ventilated artificially with room air supplemented with 100% oxygen. The left common carotid artery was cannulated, and the BP was measured with a pressure transducer (model PT300; Grass Instruments, Quincy, MA) for measurement of mean arterial pressure (MAP). Heart rate (HR) was derived from the BP pulse using a Powerlab model 16S (AD instruments, Colorado Springs, CO). The femoral vein was cannulated for intravenous injections. Rats were placed in a stereotaxic frame (Stoelting, Chicago, IL), and the dorsal surface of the medulla was surgically exposed by occipital craniotomy and partial cerebellotomy. Suplemental doses of α-chloralose (20 mg/kg, iv) were administered to maintain an appropriate level of anesthesia. Depth of anesthesia was gauged by the stability of BP and HR and the absence of a pressor response to paw pinch. Body temperature was maintained at ~37°C by an animal temperature controller (ATC1000, World Precision Instruments).

**Recording of renal sympathetic nerve activity.** The left renal sympathetic nerves were exposed, identified and dissected free of the surrounding connective tissue, and placed on a pair of platinum-iridium recording electrodes. Both the nerve and the electrodes were covered with a fast-setting silicone (Wacker Sil-Gel). The signal was amplified (band pass 100–1,000 Hz) with a preamplifier (model P 18D, Grass Instruments). The amplified discharge was monitored on a storage oscilloscope (model 121 N; Tektronix, Beaverton, OR), imported to a computer system with other parameters, and then stored on disk until analyzed. Respective noise levels were subtracted from the nerve recording data before percent changes from baseline were calculated. Integrated RSNA was normalized as 100% baseline in the control period.

**Microinjections into the RVLM.** Microinjections were made from four-barrel micropipettes with total tip diameters of 20–50 μm and performed by a four-channel pressure injector (PM2000B, World Precision Instruments). The injections were made over 10–20 s, and the 100-nl injection volume was measured by observing the movement of the fluid meniscus along a reticle in a microscope. The coordinates for RVLM were 2.3–2.8 mm rostral to the obex, 1.8–2.0 mm lateral to the midline, and 3.0–3.4 mm below the dorsal surface of the medulla. Chemical identification of the RVLM was based on obtaining a pressor response elicited by microinjection of L-glutamate (5 nmol) at the start of each experiment, as in our previous study (36). The time interval between bilateral injections was within a 3-min period. At the end of the experiments, 100 nl of 2% pontamine sky blue were injected for marking the injection sites for subsequent histological identification.

**Injected drugs.** All chemicals including clonidine, L-glutamate, NMDA receptor agonist NMDA, AMPA/kainate receptor agonist AMPA, and AMPA/kainate receptor antagonist CNQX were obtained from Sigma Chemical. Clonidine, L-glutamate, NMDA, and AMPA were directly dissolved in artificial cerebrospinal fluid (aCSF) (in mM: 133.3 NaCl, 3.4 KCl, 1.3 CaCl₂, 1.2 MgCl₂, 0.6 NaH₂PO₄, 32.0 NaHCO₃, and 3.4 glucose, pH 7.4). CNQX was initially dissolved in 0.1 M phosphate buffer solution and then diluted in aCSF to the final concentration. CNQX was chosen as the antagonist for AMPA/kainate receptors based on its specificity to block the respective receptor agonist (12). The doses of injected clonidine and CNQX into the RVLM were based on previous studies (6, 22). In addition, clonidine for intravenous injection (10 μg/kg) was dissolved in 0.9% normal saline.

**Experimental groups and protocols.** First, a total of seven rats were used to confirm that 200 pmol of CNQX unilaterally injected into the RVLM can adequately and selectively block AMPA/kainate receptors without affecting NMDA receptors. We observed the cardiovascular effects of NMDA receptor or AMPA/kainate receptor activation by injection of their responding agonist, NMDA (20 pmol) or AMPA (5 pmol), respectively, into the RVLM 5 min after intra-RVLM injection of 200 pmol of CNQX. The interval between repeated injections of NMDA or AMPA into the same site was at least 60 min. Second, six groups of rats (n = 4–7 each) were used to investigate the effects of intra-RVLM pretreatment with AMPA/kainate receptor blockade on clonidine actions. Five minutes after unilateral injection of 100 nl of vehicle (n = 4) or three doses of CNQX (2, 20, and 200 pmol; n = 6 each), we observed the responses of BP, HR, and RSNA to injection of 5 nmol of clonidine into the same site, and the responses were continuously recorded for 60 min after intra-RVLM clonidine. Furthermore, we examined the responses (60 min) of cardiovascular values to intravenously injected clonidine (10 μg/kg) 5 min after bilateral microinjection of 100 nl of vehicle (n = 5) or three doses of CNQX (2, 20, and 200 pmol; n = 6 each) 20 min after unilateral injection of clonidine (5 nmol). We also determined the cardiovascular responses of bilateral injection of 100 nl of vehicle (n = 3) or 200 pmol of CNQX (n = 6) into the RVLM 20 min after intravenously applied clonidine (10 μg/kg). In the above experiments, the cardiovascular values of BP, HR, and RSNA at the 10-min interval after injection of clonidine were collected for further statistical analysis.

**Histological analysis.** At the end of the experiment, the rat was given a lethal injection of pentobarbital sodium (100 mg/kg, iv) and perfused with 10% formaldehyde solution (100 ml) intracardially. The brain stem was then quickly removed and fixed in 10% buffered Formalin. Frozen 50-μm coronal sections were made on a freezing microtome and mounted on slides. The dye spot for injection sites was identified and plotted on standardized sections according to the atlas of Paxinos and Watson (24). Data were excluded if the injection sites were not located in the RVLM. Figure 1 shows the locations of the centers of microinjection sites in the RVLM. Microinjection sites were located in the rostral medulla, just ventromedial to the compact portion of nucleus ambiguous, similar to the sites we have published previously (36). This area lies at the caudal end of the facial nucleus, which is known as the pressor area of the RVLM.

**Statistical analysis.** All values were expressed as means ± SE. The changes in integrated RSNA after treatments were evaluated as percent changes from control because of the variability in baseline RSNA in each animal. The averaged value of MAP, HR, and RSNA was calculated every 10 min after each treatment. Student’s t-test (paired or unpaired) was used for comparing the baseline data and the difference between pre- and postinjection. Statistical comparisons between different groups at corresponding time points were made by analysis of variance (ANOVA) followed by Newman-Keuls test. These analyses were performed by software (SigmaStat 3.5). Differences were considered to be significant at P < 0.05.

**RESULTS**

Effects of 200 pmol of CNQX on BP, HR, and RSNA response to the RVLM NMDA or AMPA/kainate receptor agonists. A total of seven rats [baseline MAP and HR: 92 ± 4 mmHg and 381 ± 16 beats/min (bpm), respectively] were used to determine whether unilateral injection of 200 pmol of CNQX into the RVLM adequately blocks AMPA/kainate receptors without affecting NMDA receptors. Figure 2A shows the original tracings of effects of pretreatment with CNQX (200 pmol) on the pressor action induced by AMPA or NMDA injected into the RVLM unilaterally. Unilateral injection of 20 pmol of NMDA or 5 pmol of AMPA into the RVLM markedly (P < 0.01) increased BP by 34 ± 4 or 28 ± 4 mmHg.
respectively. The maximal changes in cardiovascular values after NMDA or AMPA injection were reached within 2–5 min and gradually returned to control level. Unilateral injection of 200 pmol of CNQX did not significantly alter basal BP but completely abolished increases in BP evoked by intra-RVLM AMPA injection. However, prior injection of the same dose of CNQX failed to affect the NMDA-induced increase in BP. The agonist-evoked changes in BP before and after pretreatment with CNQX are shown in Fig. 2B.

Effects of pretreatments with CNQX on clonidine-induced cardiovascular responses. A total of 34 rats (baseline MAP and HR: 96 ± 4 mmHg and 376 ± 17 bpm, respectively) were used to determine the role of the AMPA/kainate receptors in the RVLM in modulating the effect of clonidine. Figure 3 shows original tracings of BP, HR, and RSNA response to clonidine microinjected unilaterally into the RVLM 5 min after pretreatment with aCSF or CNQX. Unilateral injection of clonidine (5 nmol) into the RVLM following an equal volume (100 nl) of aCSF caused gradual reductions in BP, HR, and RSNA. Notably, the maximal decreases in BP, HR, and RSNA after RVLM clonidine were reached within 20 min and gradually returned to preinjection values within 40–60 min. Although intra-RVLM injection of CNQX had no effect on basal cardiovascular values, CNQX at higher doses (20 and 200 pmol) significantly attenuated the decreases in BP, HR, and RSNA elicited by intra-RVLM clonidine in a dose-dependent manner. Surprisingly, in two rats, intra-RVLM clonidine-induced reductions in BP and RSNA were converted to a pressor and sympathoexcitatory effect after pretreatment with 200 pmol of CNQX (Fig. 3B). At the lowest dose of CNQX (2 pmol), the magnitudes of reductions in BP, HR, and RSNA evoked by clonidine were not significantly changed compared with aCSF pretreatment. The time course of changes in BP, HR, and RSNA evoked by intra-RVLM clonidine after pretreatment with CNQX or aCSF are shown in Fig. 4. Furthermore, we investigated the effects of AMPA/kainate receptor blockade in the RVLM on the cardiovascular actions of systemic clonidine (10 μg/kg) in 12 rats. Intravenously injected clonidine transiently increased BP and then gradually decreased BP, HR, and RSNA. The transient pressor action by peripheral α-adrenoceptor activation of intravenous clonidine produced rapid and strong decreases in HR and RSNA by arterial baroreflex activation (30). The maximal decreases in BP, HR, and RSNA by intravenous clonidine were reached within 20 min and then returned to control levels within 40–60 min. The magnitudes of reductions in BP, HR, and RSNA evoked by intravenously injected clonidine were significantly lower in the bilateral RVLM pretreated with 200 pmol of CNQX than in the RVLM pretreated with 100 nl of aCSF (Fig. 5). We also noted that, compared with intra-RVLM clonidine, the CNQX-induced (200 pmol) decreased responses of MAP (−3 ± 3 vs. −9 ± 3 mmHg), HR (−2 ± 5 vs. −15 ± 5 bpm), and RSNA (−9 ± 5 vs. −21 ± 5%) to intravenous clonidine were slightly lower.
Effects of subsequent CNQX injection into the RVLM on clonidine-induced cardiovascular responses. A total of 32 rats (baseline MAP and HR: 96 ± 5 mmHg and 375 ± 16 bpm, respectively) were used to obtain further evidence to support the role of RVLM AMPA/kainate receptors in the effect of clonidine. Figure 6 shows original tracings of BP, HR, and RSNA responses to subsequent injection of CNQX into the RVLM 20 min after intra-RVLM or intravenous injection of clonidine. At the higher doses (20 and 200 pmol), subsequent injection of CNQX into the RVLM dose dependently reversed BP and RSNA 20 min after intra-RVLM clonidine (5 nmol) injection. The increases in BP and RSNA evoked by subsequent CNQX injection persisted for at least 20 min. Only at the highest dose did CNQX reverse clonidine-induced decreased HR. Interestingly, the highest dose (200 pmol) of CNQX injected after clonidine produced a strong excitatory effect on RSNA in three rats that significantly exceeded the baseline value. Subsequent injection of the lowest dose of CNQX (2 pmol) did not modify RVLM clonidine action compared with an equal volume of aCSF. The time course of changes in BP, HR, and RSNA elicited by subsequent injection of CNQX or aCSF after intra-RVLM clonidine is shown in Fig. 7. Similar to when clonidine was unilaterally injected into the RVLM, potent elevations in BP, HR, and RSNA were produced by...
bilateral injection of CNQX (200 pmol, n = 6) but not 100 nl of vehicle (n = 3) 20 min after intravenous injection of clonidine (10 µg/kg) (mean data not shown).

DISCUSSION

The principal findings in this work are that pretreatment with injection of CNQX into the RVLM prevented the reductions in BP, HR, and RSNA evoked by RVLM injection of clonidine as well as by intravenous injection of clonidine. Furthermore, the reductions in BP, HR, and RSNA evoked by intra-RVLM or intravenous clonidine were significantly reversed by subsequent injection of CNQX into the RVLM. These results suggest that the AMPA/kainate receptors in the RVLM play an important role in modulating the central mechanism responsible for clonidine-induced cardiovascular and sympathetic inhibition.

It is well known that the glutamatergic system within the RVLM plays an important role in cardiovascular regulation (5, 29). Clonidine presynaptically modulates glutamate release in the RVLM but also attenuates the cardiovascu lar responses evoked by RVLM NMDA receptor activation (20, 31). Recently, results from our laboratory (36, 37) and other laboratories (38) suggest that blockade of NMDA receptors in the RVLM significantly attenuates the decrease in BP and inhibition of RVLM presympathetic neurons induced by imidazoline-like drugs (clonidine and rilmenidine) in hypertensive or normotensive rats. These previous studies mainly focused on the role of NMDA receptors in modulating the effects of imidazoline-like drugs. It has been reported that, compared with NMDA receptors, the RVLM AMPA/kainate receptors may play a major role in control of cardiovascular function (1).

In this work, we observed the effects of blockade of the AMPA/kainate receptors in the RVLM on clonidine-induced decreases in BP, HR, and RSNA. At a high dose, CNQX is a potent AMPA/kainate receptor antagonist that also blocks the glycine modulatory site on the NMDA receptor complex (19). The present data showed that prior injection of 200 pmol of CNQX into the RVLM completely abolished the pressor action induced by AMPA (5 pmol) but had no effect on that of NMDA (20 pmol), suggesting that the dosage of CNQX (200 pmol) can selectively and adequately block AMPA receptors without affecting NMDA receptors. Although we did not further test the effect of CNQX on kainate receptors, a previous study (17) reported that a similar dose of CNQX did block the pressor action induced by kainate (4.7 pmol). Taken together, we believe that these studies indicate that 200 pmol of CNQX can adequately block the AMPA/kainate receptors without affecting NMDA receptors within the RVLM. In addition, the present data showed that injection of CNQX (2–200 pmol) into the RVLM had no effect on basal BP, HR, and RSNA. The fact that we only took ~5 min to observe the effect of intra-RVLM CNQX may be a limitation. A longer period of time (60 min) for the control test of CNQX alone would be useful to determine its effects and for comparison with the clonidine actions. However, it has been demonstrated that, in the normal state, injection of glutamate receptor antagonists into the RVLM does not affect baseline cardiovascular activity (10, 16). It appears that excitatory amino acid receptors in the RVLM do not participate in generation of tonic cardiovascular activity. Interestingly, it has been suggested that, in normal baseline, the balance between excitatory and inhibitory influences may prevent the change in BP following simultaneous removal of these two influences by glutamate antagonists (29). Following removal of inhibitory input to the RVLM or in the hypertensive state, injection of glutamate receptor antagonists into the RVLM significantly decreases basal BP (13, 14).

Similar to previous studies (6, 7), we found that clonidine injected unilaterally into the RVLM produced long-lasting hypotensive, bradycardiac, and sympathoinhibitory effects. Importantly, the decreases in BP, HR, and RSNA evoked by clonidine injected into the RVLM were significantly attenuated after pretreatment with CNQX in a dose-dependent manner.

![Fig. 6](image-url) Representative tracings showing the responses of BP, MAP, HR, and inter-RSNA to subsequent injection of CNQX (200 pmol) into the RVLM 20 min after intra-RVLM clonidine (A) and intravenous clonidine (10 µg/kg) (B). The rapid decreases in HR and RSNA were produced by arterial baroreflex activation, which was induced by peripheral vasoconstriction of intravenous clonidine.

![Fig. 7](image-url) Time course (60 min) showing the changes in MAP (top), HR (middle), and RSNA (bottom) induced by subsequent injection of CNQX or aCSF (100 nl) following clonidine (5 nmol) injected unilaterally into the RVLM. Results are presented as means ± SE; n = no. of rats in each group. *P < 0.05 vs. aCSF or 2 pmol of CNQX; #P < 0.05 vs. 20 pmol of CNQX at corresponding time points.
Although 2 pmol of CNQX did not affect the clonidine action, it is possible that this dosage of CNQX was too low to effectively block the AMPA/kainate receptors. Similar to RVLM injection of clonidine, we further verified that hypotensive, bradycardic, and sympathoinhibitory effects evoked by systemic clonidine were significantly attenuated by blockade of the AMPA/kainate receptors in the RVLM. We also found that, compared with the intra-RVLM clonidine group, the degree of CNQX-induced (200 pmol) attenuation of MAP, HR, and RSNA in response to intravenous clonidine was slightly lower. Although the RVLM is a major area involved in the central depressor mechanism of clonidine, the RVLM may be not the sole target area modulating the central effects of systemic clonidine. It has been reported that other central areas such as the locus ceruleus are also sensitive to clonidine (27). It is possible that, in addition to the RVLM, AMPA/kainate receptors in other areas contribute to intravenous clonidine. Taken together, these findings strongly suggest that the cardiovascular and sympathetically inhibition evoked by local RVLM or systemic clonidine depended on the functional state of the AMPA/kainate receptors in the RVLM. In support of this idea, we also determined whether the cardiovascular and sympathetic inhibition evoked by clonidine can be reversed by subsequent injection of CNQX into the RVLM. Subsequent injection of CNQX into the RVLM after intra-RVLM or intravenous injection of clonidine produced a strong excitatory effect on cardiovascular and sympathetic activity. Because blockade of the AMPA/kainate receptors in the RVLM did not affect the basal BP and RSNA, these data raised the suggestion that clonidine significantly changed the function of the RVLM AMPA/kainate receptors and then caused the changes in cardiovascular and sympathetic activity. Notably, these results are very similar to the role of NMDA receptors in the RVLM in mediating the cardiovascular inhibition evoked by the imidazoline-like drugs clonidine and rilmenidine (15, 35, 36, 38).

We speculate that both NMDA and AMPA/kainate receptors in the RVLM contribute to cardiovascular inhibition evoked by imidazoline-like drugs. In fact, the clonidine action was mostly abolished following the high-dose CNQX injection into the RVLM. The relative strength or importance of NMDA and AMPA/kainate receptors in modulating the central depressor mechanism is not clear from the present data. It has been suggested that, compared with NMDA receptors, AMPA/kainate receptors in the RVLM play a major role in controlling cardiovascular activity (1). This may be a reason why the RVLM CNQX seems to exhibit a stronger blockade effect on clonidine action. Another possibility is that there exist different distribution densities of NMDA or AMPA receptors in the subarea of the RVLM. Therefore, it is possible that the relative strength of NMDA or AMPA receptors in modulating clonidine action is dependent on the distribution density of NMDA or AMPA receptors in the RVLM. Confirmation of this hypothesis requires additional study.

Although the present study shows that the hypotensive and sympathoinhibitory effects of clonidine are closely related to the functional states of the AMPA/kainate receptors in the RVLM, its physiological significance and exact mechanism remain to be defined. Because CNQX and clonidine act on their specific receptors, the interaction between CNQX and clonidine probably is a physiological antagonism rather than a pharmacological antagonism. This interaction may occur at many levels such as presynaptic, postsynaptic, acting receptor, and intracellular signal conduction mechanism, etc. There are several possible mechanisms underlying the interaction between AMPA/kainate receptors and clonidine. First, it has been reported that clonidine effectively stimulates the spontaneous release of the sympathoinhibitory transmitter γ-aminobutyric acid (GABA) in the RVLM, which is associated with enhancement of glutamate release (31). It is well known that increased GABA within the RVLM produces a significant sympathoinhibition (4, 5, 18). Acute and chronic clonidine application is also suggested to enhance the GABA content in rat brain, and GABA receptor blockade can attenuate the hypotension elicited by clonidine (3, 15). Stimulation of glutamate receptors increases GABA release and facilitates the GABAergic synaptic activity (25). Furthermore, activation of AMPA/kainate receptors is able to trigger GABA release (25, 26). The view that the subsequent release of GABA is associated with activation of AMPA/kainate receptors may explain why the cardiovascular inhibition evoked by clonidine is converted to an excitation after RVLM injection of the highest dose of CNQX (200 pmol) in the present study. However, there is no direct evidence to clarify the interaction between the functional states of the RVLM GABA receptor and clonidine action. Second, it has been reported that the apparent desensitization caused by the dopamine D2 receptor agonist quinpirole is specific to certain sympathetic vasomotor pathways, including those that mediate the antihypertensive action of clonidine, rilmenidine, and α-methyldopa (32, 33). Therefore, it is not clear whether CNQX produces the desensitization of neuronal excitation in response to clonidine and subsequently attenuates the effects of clonidine. However, there is a significant difference between these two pretreatments, because the basal BP can be elevated by quinpirole but not by CNQX. Finally, it has been reported that the imidazoline compounds interact with a specific site (such as the phenycyclidine-binding site) on glutamate receptors (23). Zhang et al. (38) also suggest that a common binding site on the NMDA receptor recognized by NMDA and I1R agonist contributes to the central antihypertensive mechanism. The possibility is that, on AMPA/kainate receptors, there exists a similar binding site recognized by CNQX and clonidine.

Another important question is whether I1R or α2AR contributes to mediation of processing of the interaction between glutamate receptors and clonidine actions. Clonidine is a mixed agonist for I1R and α2AR, but their specific antagonists were not used in this work to further characterize which receptor is responsible for mediating the interaction between clonidine and AMPA/kainate receptors. It is reported that the effect of clonidine on the release of glutamate and GABA is prevented by the selective α2AR antagonist yohimbine (15). On the other hand, the hypotension induced by injection of the selective I1R agonist rilmenidine into the RVLM is abolished by an NMDA receptor antagonist (38). Considering the evidence, it is likely that clonidine action is dependent on the glutamatergic system via a mechanism of I1R or α2AR or both. The relationship between AMPA/kainate receptors and the effects of imidazoline-like drugs is more complex and awaits further investigation.

In summary, the present results show that the decreases in BP, HR, and RSNA evoked by clonidine are significantly prevented by blockade of AMPA/kainate receptors in the RVLM. It is concluded that AMPA/kainate receptors in the
RVLM contribute to clonidine-induced cardiovascular and sympathetic inhibition. However, because of the present data obtained from the normotensive rats, it is not known whether the same relationship exists in the hypertensive model. Some receptors, such as glutamate receptors in the brain, are upregulated in the hypertensive model, and therefore it is also important to determine the relationship between glutamate receptors and the effects of imidazoline-like drugs in the hypertensive state.

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