NMDA receptors in nucleus tractus solitarii are linked to soluble guanylate cyclase

Deoceleio A. Chianca, Jr., Li-Hsien Lin, Deidre Nitschke Dragon, and William T. Talman

1Department of Neurology, University of Iowa and Department of Veterans Affairs Medical Center, Iowa City, Iowa 52242; and 2Department of Biological Sciences/Núcleo de Pesquisas em Ciências Biológicas Universidade Federal de Ouro Preto, Minas Gerais, Brazil

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Chianca, Deoceleio A. Jr., Li-Hsien Lin, Deidre Nitschke Dragon, and William T. Talman. NMDA receptors in nucleus tractus solitarii are linked to soluble guanylate cyclase. Am J Physiol Heart Circ Physiol 286: H1521–H1527, 2004; 10.1152/ajpheart.00236.2003.—We sought to test the hypothesis that cardiovascular responses to activation of ionotropic, but not metabotropic, glutamate receptors in the nucleus tractus solitarii (NTS) depend on soluble guanylate cyclase (sGC) and that inhibition of sGC would attenuate baroreflex responses to changes in arterial pressure. In adult male Sprague-Dawley rats anesthetized with chloralose, the ionotropic receptor agonist N-methyl-D-aspartate (NMDA) and trans-α-amino-3-hydroxy-5-methylisoxazole-propionic acid (AMPA) and the metabotropic receptor agonist trans-1-amino-1,3-cyclopentane-dicarboxylic acid (ACPD) were microinjected into the NTS before and after microinjection of sGC inhibitors at the same site. Inhibition of sGC produced significant dose-dependent attenuation of cardiovascular responses to NMDA but did not alter responses produced by injection of AMPA or ACPD. Bilateral inhibition of sGC did not alter arterial pressure, nor did it attenuate baroreflex responses to pharmacologically induced changes in arterial pressure. This study links sGC with NMDA, but not AMPA or metabotropic, receptors in cardiovascular signal transduction through NTS.

baroreflex; ionotropic receptor

IT IS WELL ESTABLISHED that glutamate is released at excitatory synapses and acts as a neurotransmitter in the central nervous system (6, 57). On its release at central synapses, glutamate acts at excitatory amino acid receptors and mediates responses through phosphoinositide hydrolysis, intracellular calcium mobilization, and neuronal depolarization via activation of intracellular second messenger systems (21, 22, 63). Those receptors at which glutamate acts are classified by their agonist selectivity into ionotropic and metabotropic types. Ionotropic receptors are further classified into three types, N-methyl-D-aspartate (NMDA), kainic acid (KA), and trans-α-amino-3-hydroxy-5-methylisoxazole-propionic acid (AMPA) receptors (24, 56). Both ionotropic and metabotropic receptors have been implicated in transmitting cardiovascular reflex signals in the nucleus tractus solitarii (NTS) (14, 17, 48, 60), the primary site of termination of cardiovascular and visceralafferent fibers of the vagus and glossopharyngeal nerve (8–10, 28, 29).

Stimulation of baroreceptor afferents causes release of glutamate from transmitter stores in the NTS (20, 36, 54, 59). Blockade of glutamate receptors in the NTS also blocks baroreceptor and chemoreceptor reflexes (69, 77). Therefore, it is believed that glutamate plays a critical role in cardiovascular reflex transmission in the NTS (72, 74). Nitric oxide (NO) may also play an important role in transmission of cardiovascular reflex signals through the NTS (68). In support of that role, microinjection of NO donors or l-arginine, an NO precursor, into the NTS produces hypotension and bradycardia in anesthetized and conscious rats (39, 40, 49, 51, 73, 75). On the other hand, microinjection of Nω-nitro-l-arginine methyl ester, a nonselective nitric oxide synthase (NOS) inhibitor, evokes opposite results (23). A role for NO in cardiovascular regulation by the NTS is further supported by anatomic studies that demonstrate neuronal NOS (nNOS) in vagal afferent nerves and their terminals in the NTS (43) and transport of NOS along vagus nerve afferents (15). In addition to their independent roles in the NTS, NO and glutamate may also interact with each other to effect cardiovascular regulation (11, 34, 35, 41, 42, 52, 58, 70). For example, inhibition of NOS reduces effects elicited by glutamate microinjection (11). An interaction is also supported by immunohistochemical studies that show colocalization of nNOS and glutamate in some neurons of subnuclei in rat NTS (44).

NO activates soluble guanylate cyclase (sGC) (26, 55, 65), and in NTS neurons may participate in transmitting cardiovagal reflexes (39, 40). Studies also suggest that sGC may be linked to activation of ion channels such as those associated with NMDA receptors (16, 37). On the other hand, responses to metabotropic receptor activation may be mediated through G proteins rather than through sGC (56, 62, 66). Therefore, the aim of this study was to test the hypothesis that sGC is linked to activation of NMDA but not metabotropic receptors in NTS and that inhibition of sGC would block baroreflex responses elicited by changes in arterial pressure (AP).

MATERIALS AND METHODS

All protocols abided by principles defined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996) and were approved by the institutional animal care and use committees of the University of Iowa and the Iowa City Department of Veterans Affairs Medical Center. Every effort was made to minimize the number of animals used and to eliminate any potential pain or suffering.

Adult male Sprague-Dawley rats (275–340 g) were initially anesthetized with 2% halothane mixed with 100% oxygen. A total of 45 animals were used in these studies. Cannulas were implanted into a femoral artery and vein for measurement of AP and for injection of drugs, respectively. Halothane was then stopped, and anesthesia was maintained with intravenous chloralose (40–60 mg/kg to induce and
20 mg·kg\(^{-1}\)·h\(^{-1}\) to maintain anesthesia). Body temperature was held at 37°C by means of a heating pad connected to a YSI temperature controller (model 73A) equipped with a rectal temperature probe. The femoral arterial cannula was connected to a strain gauge transducer (Statham P23 Db) and thence to a low-level direct-current preamplifier in a Grass model 7D polygraph for measurement of pulsatile and mean AP (MAP). Heart rate (HR) was derived from the arterial pulse wave by a cardiotachometer.

Animals were placed in a Kopf stereotaxic frame. After a partial occipital craniotomy, the cerebellum was retracted to expose the dorsal surface of the brain stem at the level of the obex. Coordinates for injection sites were 0.4 mm anterior and 0.5 mm lateral to the calamus scriptorius and 0.4–0.5 mm ventral to the dorsal surface of medulla at that site. Triple-barrel micropipettes (ED50) for each agent (61, 69). To ensure reproducibility, we assessed responses to each agonist twice at 10-min intervals before challenging with ODQ, not with LY-83583, and limited doses of ODQ to 200 pmol (see RESULTS for explanation). Injections of artificial cerebrospinal fluid with alcohol or DMSO were used as controls for volume effects of the enzyme inhibitors. Responses to NMDA, AMPA, or ACPD were assessed twice before and 10, 20, and 30 min after injection of the inhibitor or vehicle.

AP and arterial baroreflexes were tested after bilateral injection of ODQ (200 pmol in 100 nl) into the NTS of animals anesthetized with chloralose. In three rats we assessed reflex bradycardic responses to pressor effects elicited by intravenous injection of phenylephrine (0.05–3 µg in 0.1–3 µl of saline) or reflex tachycardic responses to depressor effects elicited by intravenous injection of sodium nitroprusside (0.5–5 µg in 0.1–5 µl of saline). Data were analyzed by linear regression and compared with control data developed in similarly anesthetized, instrumented rats (n = 5) that had not received injections into the NTS.

Control responses to NMDA, AMPA, and ACPD injected at 10-min intervals were qualitatively the same, but the magnitude of responses to different agonists differed as did the magnitude of responses when agonists were injected after an inhibitor. Therefore, the influence of time on effects of varying doses was subjected to linear mixed-model analysis for repeated measures with appropriate post hoc analysis (Tukey, Bonferroni). Responses were normalized as a percentage of the control response and expressed as mean percent change ± SE. Results, expressed as absolute values of change at each time, were compared with baseline control results and were also analyzed by paired t-tests. A P value ≤0.05 was accepted as significant.

RESULTS

In initial studies we utilized the sGC inhibitor LY-83583, which is known to block sGC but may do so with less selectivity than that seen with ODQ (33, 50, 67). We found that cardiovascular effects elicited by NMDA were blocked by injection of LY-83583 into the NTS before injection of NMDA. Depressor responses to NMDA (1 pmol in 20 nl) were reduced by prior injection of 0.5 pmol of LY-83583 (control depressor response of 25 ± 3 mmHg before LY-83583 reduced to 14 ± 5 mmHg after LY-83583; n = 6) and reduced even further by prior microinjection of 1 pmol of LY-83583 (control depressor response of 19 ± 3 mmHg before LY-83583 reduced to 6 ± 2 mmHg after LY-83583, P = 0.01; n = 6). Similarly, HR (bradycardic) responses were significantly reduced by LY-83583. In contrast to the effects of injecting LY-83583, injection of vehicle did not significantly affect responses to NMDA in the same animals. Because LY-83583 can lead to generation of oxygen radicals and may nonselectively inhibit NOS as well as sGC (33, 50), we utilized ODQ, a more selective inhibitor of sGC, to assess differential dependence of ionotropic (NMDA and AMPA) vs. metabotropic (ACPD) receptor-mediated responses on sGC.

Microinjections of 3 pmol of ODQ into the NTS produced depressor and bradycardic responses as shown in Fig. 1. These responses were comparable to those reported in other studies (69, 71). Control responses to NMDA were reproducible. In five animals, MAP fell 28 ± 3 mmHg with the first injection of NMDA and 30 ± 4 mmHg 10 min later. HR fell 29 ± 8 beats/min after the first injection and 28 ± 8 beats/min 10 min later. These responses were inhibited in a dose-dependent manner by prior

![Fig. 1. Inhibition of soluble guanylate cyclase (sGC) by injection of 1H-1(2,4)-oxadiazolo[4,3-a]quinoxaline-1-one (ODQ) into the nucleus tractus solitarii (NTS) elicits dose-dependent reduction of depressor and bradycardic responses to injection of the ionotropic agonist N-methyl-D-aspartate (NMDA; 3 pmol) at the same site. Injection of ODQ (200 pmol) at the same site in the NTS after the second injection of NMDA 10, 20, and 30 min later (A). Injection of vehicle (not shown) did not affect responses to NMDA. Injection of 50 pmol of ODQ blocked responses to NMDA 10 min later, but responses to NMDA partially returned 20 min and 30 min after ODQ (B). bpm, Beats/min.](http://ajpheart.physiology.org/Downloadedfrom/10.1152/ajpheart.00333.2016)
injection of ODQ, but not vehicle, at the same site in NTS (Figs. 1 and 2). Microinjection of 200 pmol of ODQ into the NTS of five rats significantly reduced NMDA-induced depressor responses at 10, 20, and 30 min after ODQ injection (Figs. 1A, 2, and 3). Microinjection of 50 pmol of ODQ (Figs. 1B, 2, and 3) also significantly reduced the MAP response to NMDA at 10 and 20 min, but not at 30 min, after injection of ODQ in five rats. Microinjection of 25 pmol of ODQ (n = 3) tended to reduce MAP responses (P > 0.05 after post hoc adjustment) to NMDA at 10 min (Figs. 2 and 3), but not at 20 and 30 min, after the injection (Fig. 3). The return of NMDA responses at the latter two doses demonstrated that blockade of NMDA activation by ODQ was reversible. A 10-pmol dose of ODQ (n = 3) did not significantly affect depressor responses to NMDA at any time after injection of the inhibitor (Fig. 3).

A 200-pmol dose of ODQ also significantly decreased HR responses to NMDA injection at 10 and 20 min (Fig. 2). Although there was still some effect of ODQ at 30 min, that effect was not significant. The inhibitory effect of 50 pmol of ODQ on NMDA-induced HR responses was significant at 10 min, but not at 20 (P = 0.084) or 30 min, after its injection. Again, these data indicate that the inhibitory effect of ODQ on NMDA responses is reversible. Neither a 25-pmol nor a 10-pmol dose of ODQ significantly altered NMDA-induced HR responses (Fig. 2).

Microinjection of 75 pmol of ACPD into the NTS produced depressor and bradycardic responses that were not affected by prior injection of ODQ (Fig. 4). Microinjection of 50 pmol of ODQ in four rats (data not shown) or 200 pmol of ODQ in three rats did not affect ACPD-induced MAP or HR responses (Table 1). Because of the lack of response to these two high doses, we did not perform studies at the lower doses of 25 and 10 pmol as we had in the NMDA studies. Unilateral injection of ODQ (200 pmol) into the NTS of four rats likewise did not significantly affect cardiovascular responses elicited by injection of AMPA (210 fmol in 50 nl) at the same site (Table 1).

Bilateral injection of ODQ (200 pmol) into the NTS did not significantly affect AP or HR. MAP was 89 ± 6 mmHg before ODQ and 88 ± 6 mmHg after the inhibitor, and HR was 326 ± 14 bpm before ODQ and 317 ± 15 bpm after ODQ. Likewise, bilateral injection of ODQ into the NTS did not significantly alter arterial baroreflex responses to changes in AP. In five control animals, increases of arterial pressure evoked by intravenous injection of phenylephrine ranged from 8 to 50 mmHg and decreases ranged from 12 to 53 mmHg. In three animals treated with ODQ, pressor responses ranged from 14 to 53 mmHg and decreases ranged from 7 to 37 mmHg. Regression analysis revealed a slope (R

Fig. 2. Dose-related inhibition (expressed as % of basal response) of NMDA (3 pmol) 10 min after injection of ODQ at the same site in the NTS. Doses of 50 and 200 pmol of ODQ significantly (P < 0.05) reduced decreases in mean arterial pressure (MAP; *) and heart rate (HR; †). Lower doses had lesser (P > 0.05) effects.

Fig. 3. The degree and duration of inhibition of responses to NMDA were related to the dose of ODQ injected. Here data are presented as changes in MAP (ΔMAP) in response to injection of NMDA (3 pmol) 10, 20, and 30 min after injection of varying doses of ODQ (10–200 pmol) at the same site in the NTS. A 200-pmol dose of ODQ produced maximal inhibition of responses, and that inhibition persisted for the 30 min of the study. Lower doses (10, 25, and 50 pmol) elicited less inhibition and demonstrated reversibility of responses. *P < 0.05.

Fig. 4. Inhibition of sGC by injection of ODQ (200 pmol) into the NTS does not affect depressor or bradycardic responses to injection of the metabotropic agonist trans-txl-amino-1,3-cyclopentane-dicarboxylic acid (ACPD; 75 pmol) at the same site. ACPD, like NMDA, elicited reproducible depressor and bradycardic responses (left column) that were not affected 10, 20, or 30 min after ODQ (right 3 columns, respectively). Data from this experiment are representative of those from the entire group (n = 7) of animals that received 200 or 50 pmol of ODQ (see Table 1).
Table 1. ODQ (200 pmol) does not affect cardiovascular responses to ACPD and AMPA injected into the nucleus tractus solitarii

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Values are means ± SE. ODQ, 1H-(1,2,4)oxadiazolo[4,3-a]quinazoline-1-one; ACPD, trans-DL-amino-1,3-cyclopentane-dicarboxylic acid; AMPA, DL-α-amino-3-hydroxy-5-methylisoxazole-propionic acid; ∆MAP, change in mean arterial pressure; ∆HR, change in heart rate.

ODQ when the inhibitor was exposed to light, we did find that a 1-pmol dose of ODQ inhibited responses to NMDA under those conditions. Inhibition of responses at such a low dose of the unprotected compound and persistence of responses to 50-fold higher doses of the protected compound suggests that breakdown products of ODQ may inhibit responses to glutamate receptor agonists. It is not clear what those products might be or whether they maintain any specificity for inhibiting sGC, as does ODQ itself. One of our own earlier studies (71) would also seem to conflict with selective blockade of NMDA- but not AMPA-mediated responses. In that study, we showed that cardiovascular responses elicited by microinjection of NMDA or either of the other ionotropic agonists, AMPA or KA, were significantly reduced by the sGC inhibitor methylene blue. However, methylene blue has a number of actions, including generation of reactive oxygen species and inhibition of nNOS (1, 33, 50, 53), that are independent of its effects on sGC and could account for its broader inhibition than that seen with ODQ. Differences between studies could also be explained if coupling between glutamate receptors and formation of cGMP were not uniform between brain regions. Results from one study (18) that investigated NTS in brain stem slices contrast with the current results. In that latter study, a heme oxygenase inhibitor blocked some cellular electrophysiological effects elicited by either ionotropic or metabotropic glutamate receptor agonists whereas a nonselective NOS inhibitor, Nω-nitro-L-arginine, failed to block ACPD despite its blocking responses to ionotropic agonists. The authors conjectured that metabotropic receptors may be linked to heme oxygenase and carbon monoxide transduction pathways through sGC whereas ionotropic receptors may be linked more directly to sGC and NO transduction pathways. We acknowledge that our data do not exclude a link between glutamate and carbon monoxide pathways that themselves depend on sGC (27, 31).

Table 1. ODQ (200 pmol) does not affect cardiovascular responses to ACPD and AMPA injected into the nucleus tractus solitarii

The study by W. O'Doherty and colleagues (64) showed that the influence of AMPA, NMDA, and metabotropic receptor agonists in rat cerebellum differed as the animals developed. Cerebellar slices from neonatal animals responded with increased sGC to NMDA, but not AMPA or quisqualate, whereas slices from adult animals responded to all agonists. One study seemingly included the same experiments as we report here and found that ODQ injected into the NTS of adult Sprague-Dawley rats blocked cardiovascular responses to locally injected AMPA as well as NMDA (42). That study would seem to mitigate our findings; however, several differences between the two studies are noteworthy. First, the dose of ODQ used in the former study was 100- to 1,000-fold less than what we found to have any effect in blocking any glutamate agonist. That dose response difference raises the possibility that the ODQ may have differed between the two studies. In the present study we carefully followed handling instructions provided by the vendor's (Sigma) technical service department, who indicated that ODQ, a quinoxalin derivative, is extremely sensitive to light and will break down if exposed to light. It is not clear that similar precautions were taken in the earlier study in that the compound’s light sensitivity was not recognized until after that study had been published. Although we did not fully analyze the effects of ODQ when the inhibitor was exposed to light, we did find that a 1-pmol dose of ODQ inhibited responses to NMDA under those conditions. Inhibition of responses at such a low dose of the unprotected compound and persistence of responses to 50-fold higher doses of the protected compound suggests that breakdown products of ODQ may inhibit responses to glutamate receptor agonists. It is not clear what those products might be or whether they maintain any specificity for inhibiting sGC, as does ODQ itself. One of our own earlier studies (71) would also seem to conflict with selective blockade of NMDA- but not AMPA-mediated responses. In that study, we showed that cardiovascular responses elicited by microinjection of NMDA or either of the other ionotropic agonists, AMPA or KA, were significantly reduced by the sGC inhibitor methylene blue. However, methylene blue has a number of actions, including generation of reactive oxygen species and inhibition of nNOS (1, 33, 50, 53), that are independent of its effects on sGC and could account for its broader inhibition than that seen with ODQ. Differences between studies could also be explained if coupling between glutamate receptors and formation of cGMP were not uniform between brain regions. Results from one study (18) that investigated NTS in brain stem slices contrast with the current results. In that latter study, a heme oxygenase inhibitor blocked some cellular electrophysiological effects elicited by either ionotropic or metabotropic glutamate receptor agonists whereas a nonselective NOS inhibitor, Nω-nitro-L-arginine, failed to block ACPD despite its blocking responses to ionotropic agonists. The authors conjectured that metabotropic receptors may be linked to heme oxygenase and carbon monoxide transduction pathways through sGC whereas ionotropic receptors may be linked more directly to sGC and NO transduction pathways. We acknowledge that our data do not exclude a link between glutamate and carbon monoxide pathways that themselves depend on sGC (27, 31).

Although the present study did not address cellular mechanism(s) between activation of NMDA receptors and activation of sGC, one attractive possibility is that NO, or NO moieties, may be generated and act as an intermediate step. NO does lead to activation of sGC in various tissues, and some evidence suggests that formation of NO, or NO moieties, precedes activation of sGC after stimulation of glutamate receptors (4, 13, 32). There is indirect evidence supporting this mechanism. nNOS is found in the NTS as well as in other nuclei of the central nervous system (30). In addition, microinjection of S-nitrosocysteine into the NTS produces cardiovascular responses similar to those elicited by glutamate agonists (40). Taking the results of the current study together with these observations, it is reasonable to speculate that stimulation of NMDA receptors leads to formation of NO or NO moieties that activate sGC.

Before this study we speculated that NO might likewise participate in mediating responses to metabotropic receptor activation but those responses would not depend on sGC. Our own immunohistochemical studies supported the proposed close association between each of the glutamate receptor subtypes and nNOS (45–47). However, the current study suggests that glutamate receptors of different types link to NO-mediated signal transduction through different mechanisms and that NMDA receptors are more directly linked to sGC than are AMPA and metabotropic receptors.
Our data demonstrate that microinjections of ODQ into the NTS reduce NMDA-induced depressor and bradycardic responses in a dose-related manner. These results indicate that NMDA receptors act through sGC in transduction of central cardiovascular reflex signals in the NTS. The data are consistent with results from earlier studies that showed that another sGC inhibitor, methylene blue, blocks the responses to ionotropic, but not metabotropic, receptor agonists (39, 40, 71). Together, they support the hypothesis that sGC may participate in transmission of cardiovascular reflexes that involve activation of ionotropic receptors in the NTS. The potential role of sGC in cardiovascular reflexes in the NTS is particularly intriguing in light of its apparent link to neurotransmission or modulation by excitatory amino acids and by the novel neural messengers, NO- and related compounds. Evidence indicates that, in the central nervous system, NO is produced enzymatically in postsynaptic structures in response to activation of excitatory amino receptors and diffuses to act on neighboring cellular elements including presynaptic nerve endings and astrocytic processes (16, 25).

Microinjection into the NTS of agonists for each category of glutamate receptor produces depressor and bradycardic responses (14, 60, 69). Therefore, each receptor subtype may participate in cardiovascular control in the NTS. Although their physiological importance to the baroreceptor reflex has been debated (20), considerable evidence supports a more prominent role for AMPA receptors than NMDA receptors in baroreflex transmission (2, 3, 7, 19, 76). Consistent with that concept, the current study shows that interrupting NMDA signal transduction through sGC in the NTS does not significantly alter baroreflex function. Likewise, interrupting transmission through NMDA receptors in the NTS did not elicit hypertension that would be expected with interruption of the baroreflex arc at the level of the NTS (12).

Our findings show that sGC may participate in, but not be essential for, transmission of arterial baroreflexes, which depend on glutamate transmission through intact ionotropic receptors (60). The persistence of baroreflex responses that we found after blockade of sGC is consistent with the uninterrupted transmission via the AMPA and metabotropic class of glutamate receptors despite significant attenuation of responses to NMDA. In fact, it is well recognized that cardiovascular responses to injection of glutamate into the NTS can persist even when all ionotropic, but not metabotropic, receptors have been blocked in NTS (38, 69), and near-total blockade of baroreflexes only occurs when both NMDA and AMPA receptors are inhibited, not when there has been isolated blockade of either receptor type (60). Thus we predict that simultaneous inhibition of sGC, AMPA receptors, and metabotropic glutamate receptors will be needed to block the reflex and elicit hypertension.

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