Cardiovascular response to a group III mGluR agonist in NTS requires NMDA receptors

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Mueller, Patrick J., C. Michael Foley, Helen W. Vogl, Meredith Hay, and Eileen M. Hasser. Cardiovascular response to a group III mGluR agonist in NTS requires NMDA receptors. Am J Physiol Regul Integr Comp Physiol 289: R198–R208, 2005. First published March 24, 2005; doi:10.1152/ajpregu.00185.2004.—Previous studies have demonstrated that microinjection of the putative group III metabotropic glutamate receptor (mGluR) agonist, L(+)-2-amino-4-phosphonobutyric acid (L-AP4), into the nucleus tractus solitarius (NTS) produces depressor and sympathoinhibitory responses. These responses are significantly attenuated by a group III mGluR antagonist and may involve ionotropic glutamatergic transmission. Alternatively, a previous report in vitro suggests that preparations of L-AP4 may nonspecifically activate NMDA channels due to glycine contamination (Contractor A, Gereau RW, Green T, and Heinemann SF. Proc Natl Acad Sci USA 95: 8969–8974, 1998). Therefore, the present study tested whether responses to L-AP4 specifically require the N-methyl-D-aspartate (NMDA) receptor and whether they are due to actions at the glycine site on the NMDA channel. To test these possibilities in vivo, we performed unilateral microinjections of L-AP4, glycine, and selective antagonists into the NTS of urethane-anesthetized rats. L-AP4 (10 mM, 2 mM) microinjection of glycine (0.02–20 mM) failed to mimic increases in arterial pressure and sympathetic nerve activity; decreases in arterial pressure and sympathetic nerve activity, consistent with a net excitation of NTS neurons (34, 36, 54).

The precise mechanisms by which L-AP4 decreases in arterial pressure and sympathetic nerve activity when microinjected into the NTS are currently unknown but do appear to involve group III mGluRs, as responses to L-AP4 are attenuated by a group III mGluR antagonist (36, 54). One possibility is that microinjection of L-AP4 into the NTS may produce an increase in glutamate release, as has been shown in other brain regions (10). In this case, responses to L-AP4 would then be expected to require ionotropic glutamate receptors, although evidence for such a requirement is lacking. Alternatively, it is possible that responses to L-AP4 and other mGluR compounds may produce sympathoinhibition by direct or indirect actions at ionotropic glutamate receptors in the NTS. For example, Contractor and colleagues (7) have demonstrated that, under specific conditions in vitro, several mGluR compounds enhance the actions of N-methyl-D-aspartate (NMDA). These investigators proposed that in the absence of saturating levels of glycine, certain mGluR compounds (or glycine contaminants in these preparations) act at the glycine site on the NMDA channel to enhance NMDA-mediated excitatory transmission (7). Therefore, it is possible that L-AP4 produces sympathoinhibition by increasing NMDA-mediated transmission via actions at the glycine site on NMDA channels in the NTS.

To address these possibilities in vivo, we performed microinjections of L-AP4 into the NTS of anesthetized rats. Specifically, we determined whether responses to L-AP4 require NMDA or non-NMDA receptors. In addition, studies were done to test the hypothesis that L-AP4 produces cardiovascular responses in the NTS via direct or indirect interactions with NMDA channels. Our findings suggest that responses to L-AP4 require NMDA receptor activation and are independent of non-NMDA receptors. Additional experiments suggest that L-AP4 does not interact with the glycine site located on NMDA channels. Finally, we propose that responses to L-AP4 are due to an alteration in neurotransmitter release, possibly via group III mGluR-mediated production of carbon monoxide (CO; Refs. 34, 35).

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METHODS

Experimental preparation. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Missouri-Columbia. Adult, male Sprague-Dawley rats (285–385 g; n = 27) were anesthetized with urethane (1.2–1.5 g/kg ip) and supplemented (0.1–0.2 mg/kg iv) as necessary. Catheters were placed in the femoral artery and vein for measurement of arterial pressure and administration of drugs, respectively. Mean arterial pressure (MAP) was derived electronically using a low-pass filter. Heart rate (HR) was determined with a cardiotachometer triggered from the arterial pressure pulse. A tracheotomy was performed, and the rats were ventilated mechanically with room air supplemented with 100% O2. Body temperature was monitored with a rectal probe and maintained within normal limits with a heating pad. To record lumbar sympathetic nerve activity (LSNA), a midline laparotomy was performed, and a portion of the lumbar chain was located caudal to the left renal vein. Electrodes for LSNA consisted of two Teflon-insulated silver wires (0.005 in. diameter, 36 gauge; Medwire; Mt. Vernon, NY) threaded through Silastic tubing (0.025 in. inside diameter). The electrodes were placed around the isolated sympathetic chain and covered with polyvinylsiloxane gel (President, Coltène/Whaledent Inc; Mahwah, NJ), which was allowed to harden before closure. A wire was attached to the exterior skin to serve as a ground. Experiments were performed within a Faraday cage to decrease electrical noise.

LSNA was amplified 1,000 times using a Grass preamplifier (P511) and filtered using a high-pass frequency level of 30 Hz and a low-pass frequency level of 3 kHz. Compound action potentials were monitored with a Tektronix oscilloscope and a Grass M8 audio monitor. Sym pathetic nerve activity was rectified and integrated using a root mean square converter with a time constant of 28 ms. The rectified, integrated signal was then averaged electronically. To determine background noise, the residual signal from the nerve was recorded after the animal was euthanized. LSNA was defined as the amount of recorded nerve activity minus background noise. The LSNA responses to agonist injections were analyzed as a percentage of the control level of LSNA before each injection.

Microinjections. After animals were placed in a Kopf stereotaxic frame, the dorsal surface of the medulla was exposed by partial removal of the occipital bone and incision through the atlanto-occipital membrane. Multibarrel glass pipettes (5 or 7 barrel, outside tip diameter 50–80 μm) were positioned unilaterally into the NTS (0.5 mm rostral and 0.5 mm lateral to calamus scriptorius and 0.5 mm ventral to the dorsal surface of the medulla). L-AP4-sensitive sites in the NTS were confirmed functionally by depressor and sympathophy- hibitory responses to unilateral microinjection of 10 mM L-AP4. In some experiments, glycine was injected before L-AP4 to avoid possible confounding effects of L-AP4 on responses to glycine. All drugs were ejected unilaterally in 30-nl volumes in less than 3 s using a custom-built pressure microinjection system. The volume of drug delivered was monitored by the movement of the meniscus in each barrel using a 150× microscope with a calibrated reticle.

Protocols. The protocol used for antagonist experiments included a control response to agonist followed by at least a 5-min recovery period. The antagonist was then injected, followed 1 min later by a second agonist injection. Agonist injections were repeated as necessary until recovery of responses. The specific drugs and concentrations used in each protocol are listed in the RESULTS.

Calculation of glycine contamination. We accounted for possible glycine contamination in our preparations of L-AP4 by using the maximum amount of glycine contamination (1%) reported by Contractor et al. (7) for several samples of mGluR compounds (including D,L-AP4). Because we used 10 mM L-AP4 (Mw = 183.1) in the present study, we estimated that the maximum possible contamination from glycine (Mw = 75.1) in our preparation was 0.244 mM. Therefore, we microinjected varying concentrations of glycine (0.02–20 mM, 0.6–600 pmol in 30 nl) that were at least 10-fold lower and at least 100-fold higher than the expected level of glycine contamination. We hypothesized that if glycine contamination was responsible for the actions of L-AP4 in the NTS, then microinjection of glycine should mimic sympathophyhibitory responses produced by L-AP4.

To compare responses to L-AP4 and glycine, L-AP4-sensitive sites were first identified by unilateral microinjection of L-AP4 into the NTS. After a minimum of 5 min to establish recovery from L-AP4, four separate concentrations of glycine (0.02–20 mM, 30 nl each) were injected in a randomized manner at the same site from distinct pipette barrels. A minimum of 5 min was allowed between injections to allow baseline parameters to stabilize.

Since in the previous protocol, we microinjected L-AP4 first to identify L-AP4-sensitive sites in the NTS, it is possible that L-AP4 affected subsequent responses to glycine. Therefore, in a separate group of animals (n = 5), we microinjected glycine (20 mM) into the NTS before any other compound. Next, we microinjected L-AP4 in the same spot from a different barrel to confirm that the first glycine injection was made in an L-AP4-sensitive site. Finally, glycine was microinjected 5 min after the L-AP4 microinjection to determine whether L-AP4 microinjection affected responses to glycine.

Histology. In addition to functional identification of NTS injection sites with L-AP4, we marked injection sites in the NTS with 2% pontamine sky blue dye (30–45 nl) in a subset of animals (n = 10) used in these studies. After animals were overdosed with anesthesia, the brains were removed and placed in 10% phosphate-buffered formalin containing sucrose for a minimum of 1 wk. The medulla was frozen and sectioned into 40-μm coronal slices, which were mounted on microscope slides. The dye spot was localized using a 40× microscope. Anatomical landmarks were identified with the aid of a rat brain atlas (41).

Data analysis. All data are expressed as means ± SE. Data comparing levels of MAP, HR, or LSNA before and after agonist injections during control, antagonist administration, and recovery were analyzed by two-way ANOVA with repeated measures. Peak changes in MAP, HR, or LSNA in response to agonist injections during control, antagonist administration, and recovery were analyzed by one-way ANOVA with repeated measures. In addition, the effects of antagonist injections on baseline parameters were analyzed by a paired t-test. When ANOVA indicated a significant interaction, differences between individual means were assessed by a least significant difference (LSD) test (44). A probability of P < 0.05 was considered statistically significant. All statistical analyses were performed using a commercially available software package (SigmaStat, SPSS, Chicago, IL).

Drugs. L-AP4 and the general mGluR agonist, 1S, 3R-1-aminocy clopentane-1,3-dicarboxylic acid (ACPD) was obtained from Tocris Cookson (St. Louis, MO). Urethane, glycine, and strychnine were obtained from Sigma Chemical (St. Louis, MO). Strychnine was used for its ability to prevent glycine activation of inhibitory chloride channels in the NTS (2, 12, 28) without affecting the potentiation of NMDA receptor-mediated responses by glycine (26, 43). Thus by eliminating the inhibitory actions of glycine with strychnine, we would have expected to observe any excitatory actions of glycine in the NTS (decreases in MAP and SNA).

The following drugs were used for their reported selectivity for NMDA and non-NMDA receptors (9, 21, 32, 37): NMDA (25 μM) = selective NMDA agonist; D,L-2-amino-5-phosphonovaleric acid (AP-5, 10 mM) = selective NMDA antagonist; D,L-2-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid hydrobromide (AMPA) (5 μM) = selective non-NMDA receptor agonist; and 6-nitro-7-sulfamobenzoquinoline-2,3-dione (NBQX, 2 mM) = selective non-NMDA receptor antagonist. NMDA, AMPA, AP-5 and NBQX were purchased from Research Biochemicals International (Natick, NJ). All drugs were dissolved in water, 0.9% saline, or artificial cerebrospinal fluid (aCSF). With the exception of urethane, all drugs were filtered, and the pH was adjusted to 7.10–7.45 using sodium hydroxide or hydrochloric acid.
RESULTS

Verification of antagonist selectivity. Although AP-5 (10 mM) and NBQX (2 mM) have been used at similar concentrations in the NTS to produce selective blockade of NMDA and non-NMDA receptors, respectively (9, 21, 32, 37), we verified that these concentrations were also appropriate in our preparation. The selectivity of AP-5 is demonstrated in Fig. 1. Depressor, bradycardic and sympathoinhibitory responses were produced by unilateral microinjections of NMDA (25 μM, 30 nl, Fig. 1A), the non-NMDA agonist, AMPA (2 μM, 30 nl, Fig. 1B), or the general mGluR agonist ACPD (1 mM, 30 nl, Fig. 1C). Responses to NMDA, but not AMPA or ACPD, were attenuated significantly by microinjection of AP5 (10 mM, 30 nl).

The selectivity of NBQX (2 mM) is demonstrated in Fig. 2. As before, depressor, bradycardic, and sympathoinhibitory responses were produced by unilateral microinjections of NMDA (25 μM, 30 nl, Fig. 2A), the non-NMDA agonist, AMPA (2 μM, 30 nl, Fig. 2B), and the general mGluR agonist ACPD (1 mM, 30 nl, Fig. 2C). Responses to AMPA, but not NMDA or ACPD, were attenuated significantly by microinjection of NBQX (2 mM, 30 nl). Therefore, similar to previous studies (9, 21, 32, 37), we observed that at the concentrations used in this study, AP5 selectively blocked NMDA receptors, and NBQX selectively blocked non-NMDA receptors. In addition, these results are consistent with previous studies that demonstrate that responses to ACPD are unaffected by generalized excitatory amino acid antagonism with kynurenic acid (15, 40).

Protocol 1: L-AP4 and NMDA or non-NMDA receptor blockade. To determine whether responses to L-AP4 required NMDA or non-NMDA receptors, we tested responses to L-AP4 in the presence and absence of the NMDA antagonist AP-5 (10 mM, 30 nl, n = 6) or the non-NMDA antagonist, NBQX (2 mM, n = 7). Figure 3 illustrates results from one animal in which responses to L-AP4 were tested in the presence of AP-5 or NBQX. Unilateral microinjection of L-AP4 (10 mM, 30 nl) alone into the NTS produced decreases in MAP, HR, and LSNA. These responses were virtually abolished when AP-5, but not NBQX, was administered unilaterally 1 min before microinjection of L-AP4. Group data from these experiments are shown in Fig. 4. Before injection of L-AP4, resting MAP and HR were 93 ± 6 mmHg and 415 ± 23 beats per minute (bpm) for AP5 experiments and 88 ± 3 mmHg and 423 ± 30 bpm for NBQX studies. L-AP4 alone produced significant decreases in MAP, HR, and LSNA before both AP5 and NBQX (Fig. 4). Unilateral microinjection of AP-5 did not significantly alter baseline variables, whereas NBQX produced a small but significant increase in LSNA and a near-significant increase in MAP (P = 0.051) without affecting HR (Table 1). AP-5 abolished changes in MAP, HR, and LSNA produced by subsequent microinjection of L-AP4 (Fig. 4A), whereas NBQX had no significant effect on similar changes (Fig. 4B). Responses to L-AP4 recovered typically within 15 min (13 ± 2 bpm). These results suggest that responses to L-AP4 require NMDA receptor activation and are independent of non-NMDA receptors.

Protocol 2: comparison of L-AP4 and glycine. It is possible that glycine contamination in our preparations of L-AP4 (7) could elicit depressor responses by activating the glycine site on NMDA receptors. To test whether low concentrations of glycine, found in preparations of L-AP4, were capable of producing excitatory responses in the NTS, we microinjected increasing concentrations of glycine unilaterally at 10 μM, 30 nl. A: unilateral microinjections of NMDA (25 μM, 30 nl); B: the non-NMDA agonist, (±)-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate acid hydrobromide (AMPA; 2 μM, 30 nl); or C: the general metabotropic glutamate receptor (mGluR) agonist ACPD (1 mM, 30 nl) produced significant decreases in mean arterial pressure (MAP), heart rate (HR), and lumbar sympathetic nerve activity (LSNA), as determined by two-way ANOVA. Responses to NMDA, but not AMPA or ACPD, were attenuated significantly by microinjection of AP5. Rec represents recovery of response where applicable. *P < 0.05 compared with NMDA response in the presence of AP-5.
sitive sites in the NTS \((n = 7)\). Examples of NTS microinjections of 1-AP4 (10 mM) and glycine (20 mM) in an individual animal are shown in Fig. 5, A and B, respectively. Group data from these experiments are shown in Fig. 6. Control injections of 1-AP4 produced decreases in MAP, HR, and LSNA. However, despite the use of glycine concentrations ranging from 10-fold below to 100-fold higher than the calculated glycine contamination levels (0.2 mM, see METHODS), glycine did not produce depressor and sympathoinhibitory responses (Fig. 6). The highest dose of glycine produced a small, but significant, increase in MAP and LSNA (Fig. 6), consistent with inhibition of the NTS.

Because in the previous protocol, we microinjected 1-AP4 first to identify 1-AP4 sensitive sites in the NTS, it is possible that 1-AP4 affected subsequent responses to glycine. However, in a separate group of animals \((n = 5)\), microinjection of glycine (20 mM) into the NTS before any other compound produced responses similar to those above (ΔMAP 11 ± 3 mmHg, ΔHR 8 ± 3 bpm, and ΔLSNA 11 ± 2% of control). In addition, glycine responses 5 min after an injection of 1-AP4 were not statistically different than those before 1-AP4 (ΔMAP 9 ± 1 mmHg, ΔHR 10 ± 3 bpm, and ΔLSNA 10 ± 1% of control). These data suggest that prior microinjection of 1-AP4 had no effect on responses to subsequent injection of glycine.

**Protocol 3: effect of strychnine on responses to glycine.** It is possible that glycine may not mimic the actions of 1-AP4 due to simultaneous activation of inhibitory glycine receptors on chloride channels in the NTS (2, 12, 28). Thus, even if responses to 1-AP4 are mediated by direct actions of 1-AP4 at glycine-sensitive sites on NMDA channels, microinjection of glycine at 1-AP4-sensitive sites in the NTS may or may not mimic the effects of 1-AP4. To isolate possible excitatory actions of glycine at NMDA channels and to test whether we could unmask sympathoinhibitory responses to glycine, we tested responses to glycine (20 mM, 30 nl) in the presence and absence of the inhibitory glycine receptor antagonist, strychnine (3 mM, 30 nl; \(n = 7\)). Examples of NTS microinjections of 1-AP4, glycine alone, and glycine after strychnine in an individual animal are shown in Fig. 5, and group data are shown in Fig. 7. Glycine alone (20 mM, 30 nl; \(n = 9\)) produced small, but significant, increases in MAP, HR, and LSNA. Microinjection of strychnine alone produced a small but significant decrease in MAP but had no significant effects on HR or LSNA (Table 1). Increases in MAP, HR, and LSNA produced by glycine were abolished by strychnine (Fig. 7). Small and variable changes in MAP, HR, and LSNA to glycine were observed 1 min after strychnine pretreatment, such that only a small but significant decrease in LSNA was observed after strychnine. Responses to glycine were not different from control 11 min after strychnine. Importantly, responses to glycine even after blockade of inhibitory glycine receptors with strychnine were very modest compared with the sympathoinhibitory responses observed after microinjection of 1-AP4 (Fig. 7).

We also tested responses to a lower concentration of glycine (2 mM, \(n = 6\)) in the presence and absence of strychnine. As before, unilateral glycine (2 mM) produced small and variable changes in MAP (Δ3 ± 3 mmHg), HR (Δ2 ± 3 bpm) and LSNA (Δ3 ± 3%), which were not significant. Strychnine had no significant effect on baseline variables in these animals, nor did it affect responses to 2 mM glycine. Small changes in MAP (Δ−3 ± 1 mmHg), HR (Δ−11 ± 5 bpm), and LSNA (Δ−3 ± 1%) produced by glycine (2 mM) after strychnine were not significant. Therefore, as with the higher dose of glycine (20 mM), strychnine failed to unmask depressor and sympathoinhibitory responses to a lower concentration of glycine.

**Protocol 4: effect of strychnine on responses to 1-AP4.** Higher concentrations of glycine than those used in the present study (>80 mM) have been shown to produce sympathoinhibit-
These responses are strychnine-sensitive and appear to involve release of acetylcholine (49). It is possible that preparations of L-AP4 (i.e., glycine contaminants) may activate these strychnine-sensitive glycine receptors and produce sympathoinhibitory responses. Therefore, we tested whether our preparations of L-AP4 acted at glycine receptors that are strychnine-sensitive (Fig. 8). In these experiments, L-AP4 decreased MAP, HR, and LSN A (Fig. 8) and strychnine alone had no effect on baseline variables. One minute after strychnine, arterial pressure and heart rate responses to L-AP4 were unaffected (Fig. 8). Strychnine did produce a small but significant attenuation of LSN A responses to L-AP4 (Fig. 8). These data suggest responses to L-AP4 are mediated primarily by strychnine-insensitive receptors.

**Histology.** In all injections sites, injection of L-AP4 produced decreases in MAP, HR, and LSN A. Histological analysis of the injection sites marked with pontamine sky blue (n = 10) verified that the pipettes were within the NTS. Pipette tips were located within the intermediate NTS, lateral to the area postrema, and ~500 μm rostral to calamus scriptorius (Fig. 9).

**DISCUSSION**

The purpose of this study was to determine if the cardiovascular response produced by NTS microinjection of the group III mGluR agonist, L-AP4, requires ionotropic glutamate receptors, specifically the NMDA or non-NMDA subtype of excitatory amino acid receptor. In addition, we tested whether responses to L-AP4 were due to nonspecific activation of NMDA channels due to glycine contamination (7). Previous studies have reported that L-AP4 produces dose-dependent depressor, bradycardic, and sympathoinhibitory responses when microinjected unilaterally into the NTS and that these responses are attenuated by a group III mGluR antagonist (36, 54). These responses are consistent with excitation of NTS neurons that are involved in inhibition of sympathetic nervous system activity. Therefore, the results of the present study and others (36, 54) suggest collectively that responses to L-AP4 require both group III mGluRs and the NMDA subtype of excitatory amino acid receptor. In addition, these responses do not appear to be due to activation of non-NMDA receptors or by actions of L-AP4 at the glycine site on the NMDA channel. Although we cannot completely eliminate the possibility of nonspecific effects, these data are consistent with the hypothesis that L-AP4 acts selectively at group III mGluRs in the NTS and produces an increase in NMDA receptor-mediated transmission.

Responses to L-AP4 were abolished by NMDA but not non-NMDA receptor antagonism. The two simplest interpretations of these data are that 1) generalized activation of group III mGluRs produces an enhancement of NMDA receptor-mediated excitation of NTS neurons or 2) L-AP4 or contaminants in the preparation have nonselective effects at the NMDA channel. Possible nonspecific effects could occur at either the glycine site or the NMDA binding site, both of which need to be occupied for full-channel activation (5, 29, 43, 55). We suggest that an enhancement of NMDA receptor-mediated transmission (rather than nonspecific effects) is more likely. Several pieces of evidence support this suggestion. First, several studies have now shown that group III mGluRs are present in the NTS (4, 24, 25, 39). Second, L-AP4 has been shown previously to be a selective group III mGluR agonist in vitro (6, 17, 45) and in vivo, including studies performed in NTS (36, 54) where responses to L-AP4 are attenuated or abolished with specific group III mGluR antagonists. Third, according to Contractor et al. (7) and others (11), D,L-AP4 lacks agonist activity at the NMDA binding site on the NMDA channel. These data correlate well with data suggesting that L-AP4 is at best a weak agonist at NMDA receptors (8) and, in fact, appears to have antagonistic properties at NMDA receptors at millimolar concentrations (7, 11). Furthermore, L-AP4 does not appear to directly activate neurons that are sensitive to glutamate (1, 17, 20, 42).
blocks responses to L-AP4 but is without effect on responses to NMDA or AMPA (54). Fifth, as discussed in detail later in the Discussion, recent evidence indicates that L-AP4 but not NMDA produces its actions in NTS, at least in part, via the release of CO, which in turn may increase glutamate release (34, 35). These data suggest that CO production may be an intermediary step by which L-AP4 enhances NMDA-mediated transmission without directly activating the NMDA receptor (34, 35). Finally, the results of the present study, discussed below, suggest that the cardiovascular effects of L-AP4 microinjection in the NTS are not mediated by L-AP4 or glycine contaminants acting at the glycine site on NMDA receptors. Therefore, when all of the evidence is considered, we suggest, as has been shown in other brain regions (52), that L-AP4 acts at group III mGluRs to potentiate synaptic transmission in the NTS via an NMDA receptor-dependent mechanism.

Although our results suggest that responses to L-AP4 require activation of NMDA receptors, the data do not support the hypothesis that these responses are due to activation of NMDA channels by glycine contaminants in our preparation of L-AP4. Unlike L-AP4, microinjection of increasing concentrations of glycine into the NTS failed to produce depressor and sympathetic responses even though glycine was injected into the NTS in a range of concentrations that was 10-fold below and 100-fold above the levels of glycine contamination measured in mGluR compounds by Contractor et al. (7). Our data are consistent with effects of glycine in the NTS demonstrated previously (30, 31), and thus it appears that the predominant action of glycine at concentrations used in this study is mediated by activation of inhibitory glycine receptors which are strychnine-sensitive (2, 12, 28, 43).

Our data also do not support the hypothesis that L-AP4 itself acts directly at the glycine site on NMDA channels to produce the observed effects. Because the excitatory, potentiating effect of glycine at NMDA channels is insensitive to strychnine (26), prior injection of strychnine (to block the inhibitory effects of glycine) should unmask any excitatory action of glycine at the NMDA channel. Contractor and coworkers (7) reported that glycine has an EC50 that was 10-fold lower compared with L-AP4 for the glycine site on NMDA channels. Thus we tested two different concentrations of glycine (20 and 2 mM), the higher of which was twice the concentration of L-AP4 used in this study, and glycine still failed to mimic responses to L-AP4, even when tested in the presence of strychnine. These data reinforce our contention that neither direct effects of L-AP4 at the glycine site nor indirect effects due to glycine contamination can explain the depressor and sympathoinhibitory responses observed after microinjection of L-AP4 into the NTS.

A seemingly important question is whether responses to L-AP4 are blocked by a selective antagonist for the glycine site on NMDA receptors. However, blockade of either the glycine or NMDA binding site prevents NMDA channel activation (29, 43, 55). Because we have already demonstrated by our exper-

Table 1. Baseline changes in response to unilateral NTS microinjection of AP-5, NBQX, and strychnine

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<th>MAP, mmHg</th>
<th>HR, bpm</th>
<th>LSNA, %control</th>
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<tr>
<td>Control</td>
<td>92 ± 6</td>
<td>411 ± 23</td>
<td>100</td>
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<tr>
<td>1 min.</td>
<td>96 ± 6</td>
<td>415 ± 23</td>
<td>102 ± 1</td>
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<tr>
<td>NBQX</td>
<td>82 ± 3</td>
<td>422 ± 29</td>
<td>100</td>
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<tr>
<td>1 min.</td>
<td>87 ± 4</td>
<td>421 ± 28</td>
<td>103 ± 1*</td>
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<tr>
<td>Strychnine</td>
<td>82 ± 4</td>
<td>366 ± 20</td>
<td>100</td>
</tr>
<tr>
<td>1 min.</td>
<td>78 ± 3*</td>
<td>364 ± 19</td>
<td>98 ± 1</td>
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Values are mean ± SE. AP-5, 2-amino-5-phosphonovaleric acid; NBQX, 6-nitro-7-sulfamobenzoxquinoline-2,3-dione; MAP, mean arterial pressure; HR, heart rate; LSNA, lumbar sympathetic nerve activity; NTS, nucleus tractus solitarius. For AP-5, concentrations are 10 mM, 30 nl (n = 6); NBQX, 2 mM, 30 nl (n = 7); strychnine, 3 mM, 30 nl (n = 8). *P < 0.05 from control (predrug).
iments using the NMDA antagonist AP-5 that responses to L-AP4 require a functional NMDA channel, experiments using a selective glycine site antagonist would be expected to produce similar results whether L-AP4 preparations acted at the glycine site or not. Furthermore, preliminary data from our laboratory suggest that the general excitatory amino acid antagonist, kynurenate, also blocks responses to L-AP4 when both are microinjected sequentially into the NTS (13). Kynurenate blocks NMDA channels by binding to the glycine site on NMDA receptors (3), and our data suggest that antagonism of the NMDA channel (via the glycine site with kynurenate or the NMDA site with AP-5) results in functional antagonism of responses to L-AP4. Additionally, as we were unable to reproduce L-AP4-like responses with glycine under a variety of conditions, we believe that the data do not support a role for the glycine site mediating the actions of L-AP4. Finally, responses to L-AP4 were not blocked by selective doses of NBQX, a non-NMDA antagonist. These data suggest that our ability to block responses to L-AP4 with kynurenate (13) are due to blockade of NMDA receptors rather than non-NMDA receptors. Therefore, we believe that responses to L-AP4 are mediated via group III mGluRs, which interact and require NMDA receptors for the full expression of depressor and sympathoinhibitory actions of L-AP4.

Interestingly, much higher concentrations of glycine (80–1,000 mM) have been shown to produce decreases in arterial pressure and heart rate when microinjected into the NTS (51). These responses appear to involve an increased release of acetylcholine in the NTS, as responses are blocked by the muscarinic antagonist, atropine (49). Therefore, it was possible that in the present study, L-AP4 and/or glycine contaminants produced an increase in acetylcholine release to elicit decreases in arterial pressure and sympathetic nerve activity. This does not appear to be the case for several reasons. First, depressor and bradycardic responses to microinjection of glycine into the NTS have a threshold concentration starting at 80 mM (51), which is well above the estimated levels of contamination in mGluR compounds (7) and is well above the concentrations used in this study. Furthermore, these responses to much higher concentrations of glycine are totally abolished by strychnine and, more importantly, are insensitive to blockade of NMDA receptors with either kynurenate or AP-5 (51). Because sympathoinhibitory responses to L-AP4 in the present study were blocked by antagonism of NMDA receptors and affected little by strychnine, we suggest that distinct mechanisms are involved in the depressor and sympathoinhibitory responses produced by L-AP4 and higher concentrations of glycine used by other investigators.

The mechanism(s) by which L-AP4 produces depressor and sympathoinhibitory responses in the NTS have not been fully resolved; however, alterations in neurotransmitter release are quite possible. Clearly, there is a large body of evidence, primarily from in vitro studies, which suggests L-AP4 acts presynaptically to inhibit neurotransmitter release, and this evidence includes experimental preparations involving NTS neurons (4, 20, 22, 23, 38). Therefore, one might expect that L-AP4 would produce a decrease in glutamate release, a decrease in the excitation of NTS neurons, and thus result in sympathetic excitation and an increase in arterial pressure when microinjected into the NTS. On the contrary, previous studies (36, 54) clearly demonstrate that L-AP4, in a dose-dependent manner, produces depressor and sympathoinhibitory responses, consistent with a net excitation of NTS neurons. Although this appears to be a disparity with the above in vitro work, Viard and Sapru (54) have suggested that the depressor and sympathoinhibitory effects of L-AP4 could still be mediated by an inhibition of glutamate release. They suggest that L-AP4 could inhibit the release of glutamate from nerve terminals that innervate neurons with direct excitatory projections to the rostral ventrolateral medulla (53). Although intriguing,
in order for this mechanism to be consistent with our data, it would require selective withdrawal of glutamate-mediated NMDA receptor activation. Because we are unaware of any reports involving such a mechanism, we can neither support nor refute this possibility.

Alternatively, L-AP4 could produce a net excitation of NTS via decreasing GABA release directly, as it has been shown to inhibit GABAergic transmission (6, 16, 20), including attenuation of inhibitory postsynaptic currents in an NTS slice preparations (20). Thus removal of tonic GABAergic inhibitory transmission, either directly or indirectly, would produce a net excitation of NTS neurons and sympathoinhibition. However, microinjections of GABA antagonists into the NTS do not produce immediate and robust changes in arterial blood pressure (46) like those observed with L-AP4. In addition, as above, an NMDA-specific effect of L-AP4 on GABAergic transmission would be required to be consistent with our data. Thus further experiments are required to entertain this possibility.

On the basis of more recent studies, however, another possible explanation for the sympathoinhibitory effects of L-AP4 is via an enhancement of glutamatergic transmission. Although this hypothesis is supported by work in other brain regions demonstrating that L-AP4 enhances glutamate release via group III mGluR activation (10), it is an intriguing one, as an increase in glutamate release would be expected to activate both NMDA and non-NMDA receptors. In addition to the present study, there is evidence that excitatory responses to L-AP4 can be mediated by NMDA receptors (52). Furthermore, accumulating evidence suggests a causal link between group III mGluR activation, production of CO, enhanced glutamatergic transmission, and activation of NMDA receptors in the NTS (34, 35). For example, depressor responses to L-AP4 in the NTS are antagonized by zinc porphyrin compounds, suggesting they involve the production of CO (34). Additionally, NTS responses to hematin, a CO precursor, are attenuated by NMDA receptor antagonists (35). Finally, responses to NMDA are unaffected by inhibitors of CO production (35). Collectively, these data suggest a lack of direct interaction between L-AP4 and NMDA receptors. Therefore, we propose, as others have (35), that group III mGluR activation results in CO production, which in turn facilitates NMDA-mediated transmission without L-AP4 directly activating the NMDA receptor. By necessity, this would involve activation of NMDA receptors located on neurons that influence sympathoinhibition. Finally, it is possible that L-AP4 specifically affects NMDA receptor transduction systems. Clearly, additional studies are necessary to test these hypotheses and determine the precise interactions among group III mGluRs, CO production, and NMDA receptors in NTS.

In addition to L-AP4, other mGluR compounds have been suggested by Contractor and coworkers (7) to exert effects at the NMDA channel directly or through amino acid contamination. We and others have reported that two of these compounds...
pounds, the general mGluR agonist ACPD and the group I-selective agonist 3,5-dihydroxyphenylglycine (DHPG), produce decreases in MAP, HR, and LSNA when microinjected into NTS (14, 15, 27, 34, 36, 54). These responses are abolished by the general mGluR antagonist MCPG, suggesting that they are mediated by group I mGluRs. Unlike L-AP4; however, responses to ACPD and DHPG are not affected by kynurenate (15). These data alone suggest that these mGluR compounds produce their responses independent of the glycine site on NMDA channels. Furthermore, since kynurenate blocks both NMDA and non-NMDA receptors in NTS (5, 15, 33, 40, 48), it is not surprising that neither AP-5 nor NBQX had effects on responses to ACPD in the present study. Therefore, despite a significant effect demonstrated in vitro, it appears that these mGluR compounds exert their primary effects in the NTS in vivo by acting at mGluRs specifically rather than through excitatory amino acid or glycine sites on NMDA channels.

**Perspectives**

The effects of glycine observed in our study and that of Contractor and colleagues (7) highlight the importance of evaluating neurotransmitter interactions with a variety of experimental techniques. Unlike our in vivo preparation, the in vitro system utilized by Contractor et al. (7) contained only NMDA receptors expressed in cultured cells. This system allowed these investigators to examine the specific interaction of mGluR compounds with the NMDA receptor, and by design, did not include the influence of other receptor systems that may have more prominent effects in vivo. For example, in addition to NMDA receptors, mGluRs and inhibitory glycine receptors also are present in the NTS (2, 12, 24, 28, 43). According to our results, these receptors appear to have a greater impact in the overall response to L-AP4 and glycine, respectively, in the NTS compared with effects mediated through the glycine site on NMDA channels in cultured cells (7).

**Summary/Conclusions.** As shown in the present study and others (34, 36, 54), activation of group III mGluRs in the NTS produces decreases in arterial pressure, heart rate, and sympathetic nervous system activity. Therefore, despite evidence demonstrating inhibition of excitatory transmission by L-AP4 (6, 16, 20, 22, 42), generalized activation of group III mGluRs in the NTS appears to produce an increase in the excitability of NTS neurons involved in inhibition of sympathetic nervous system activity. The mechanism by which these effects occur appears to involve an increase in activity of ionotropic excitatory amino acid receptors, specifically, the NMDA receptor subtype. One possibility is that group III mGluRs affect excitatory or inhibitory transmitter release, possibly by the formation of CO, which ultimately produces activation of NMDA receptors (34, 35). Alternatively, group III mGluR transduction pathways may interact with common second messenger sys-

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**Fig. 8.** Effect of inhibitory glycine antagonism with strychnine (3 mM, 30 nl) on responses to unilateral microinjection of L-AP4 (10 mM, 30 nl; n = 5) into the NTS. L-AP4 produced significant decreases in MAP, HR, and LSNA, as examined by two-way ANOVA. Strychnine had no effect on MAP or HR responses but produced a slight but significant attenuation of LSNA responses to L-AP4. (*P < 0.05 compared with L-AP4 response in the presence of strychnine).
tems to enhance NMDA-mediated transmission. Finally, although mGluR compounds have been shown to have effects at the glycine site on NMDA channels under specific conditions in vitro (7), these effects do not appear to contribute to responses in the NTS, where the effects of other receptors such as mGluRs and inhibitory glycine receptors seem to predominate.

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