Blockade of hindbrain NMDA receptors containing NR2 subunits increases sucrose intake

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1Physiology Interdepartmental Graduate Program, Huck Institutes of Life Sciences and 2Department of Nutritional Sciences, College of Health and Human Development, The Pennsylvania State University, University Park, Pennsylvania; 3Department of Veterinary Comparative Anatomy Pharmacology and Physiology, Program in Neuroscience, Washington State University, Pullman, Washington; and 4School of Veterinary Medicine, Ross University, St. Kitts

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Gaurd DB, Swartz TD, Ritter RC, Burns GA, Covasa M. Blockade of hindbrain NMDA receptors containing NR2 subunits increases sucrose intake. Am J Physiol Integr Comp Physiol 296: R921–R928, 2009. First published February 4, 2009; doi:10.1152/ajpregu.90456.2008.—We have previously shown that blockade of N-methyl-D-aspartate (NMDA) receptors in the caudal brain stem delays satiation and increases food intake. NMDA receptors are heterodimers made up of distinct, but different, ion channel subunits. The NR2 subunits of the NMDA receptor contain the binding site for glutamate. About half of vagal afferents express immunoreactivity for NMDA NR2B subunit and about half of the NR2B expressing afferents also express NMDA NR2C or NR2D subunits. This suggests that increased food intake may be evoked by interference with glutamate binding to NMDA channels containing the NR2B subunit. To test this, we measured deprivation-induced intake of 15% sucrose solution following fourth ventricle and intranuclear of the solitary tract (intra-NTS) injections of Conantokin G (Con G; NR2B blocker), D-3-(2-carboxypiperazin-4-yl)-1-propenyl-1-phosphonic acid (D-CPPene; NR2B/2A blocker), and (±)-cis-1-(phenantheren-2yl-carbonyl)piperazin-2,3-dicarboxylic acid (PPDA; NR2D/C blocker). Fourth ventricular administration of Con G (5, 20, 40, 80 ng), D-CPPene (3.0, 6.25, 12.5, 25, 50, 100 ng), and PPDA (300, 400 ng) increased sucrose intake significantly compared with control. Likewise, injections of Con G (10 ng), D-CPPene (5 ng, 10 ng), and PPDA (0.5, 1.0, 2.5, 5.0 ng) directly into the NTS significantly increased sucrose intake. These results show that hindbrain injection of competitive NMDA receptors with selectivity or preference for the NMDA receptor NR2B or NR2C subunits increases food intake.

satiation; vagal afferents; glutamate

SEVERAL YEARS AGO WE OBSERVED that systemic injections of the noncompetitive N-methyl-D-aspartate (NMDA) antagonist (MK-801) increases intake of either solid or liquid foods when food intake is driven by food deprivation or palatability (11). Others demonstrated that MK-801 also increases the size of spontaneously initiated meals (32). More recent work from our laboratory revealed that lesions of the nucleus of the solitary tract (NTS) abolish increased food intake evoked by systemic MK-801 (51). Moreover, we found that nanoinjection of MK-801 (50), as well as the competitive NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (AP5) (29), directly into the NTS enhances food intake. These results strongly suggest that both endogenously released glutamate and hindbrain NMDA receptors participate in control of meal size.

Naturally occurring NMDA receptors are heterotetrameric ion channels. They are composed of NR1 subunits (a-h) representing eight splice variants from one gene (49), NR2 subunits (A–D) from four distinct genes (30, 31, 40, 41), and NR3 (A and B) subunits (48). NR1 and NR3 subunits bind glycine or d-serine, but not glutamate or other excitatory amino acids. NR1 is an obligatory constituent of all NMDA receptors (7, 38), but only NR2 subunits possess a binding site for excitatory amino acids. Therefore, functional NMDA-type glutamate receptors must include at least one NR2 subunit (7, 13, 27, 40, 53). While all NR2 subunits possess a glutamate binding site (2, 34), the various NR2 subunit types (A–D) confer distinct biophysical characteristics to the NMDA receptors in which they occur (5, 12, 39). Furthermore, NMDA receptors expressed in different areas of the nervous system exhibit distinct NR2 subunit phenotypes (10, 16–18, 33, 37, 41, 42, 52).

mRNA and immunoreactivity for all of the NR2 subunits has been detected in hindbrain nuclei (8, 17, 20, 44), including the NTS (21). Involvement of hindbrain NMDA receptor-expressing neurons in integration of viscerosensory information from the gastrointestinal tract is supported by the results of Berthoud et al. (8), who used an antibody that binds to multiple NR2 subunit types to demonstrate that 70% of NTS neurons activated by gastric distention or intestinal infusions are NR2 immunoreactive.

In addition to being expressed by NTS neuron cell bodies, NMDA receptor immunoreactivity is present presynaptically on vagal afferent fibers and terminals in the NTS (1). Moreover, PCR analysis by Slattery et al. (47) indicates that the vagal afferent cell bodies in the nodose ganglia express transcripts for all four NR2 subunits. Our own results indicate that virtually all vagal afferents express NMDA receptors as indicated by the presence of NMDA R1 immunoreactivity. However, we also find that NMDA NR2B, NR2C, and NR2D subunits are expressed by discrete subpopulations of vagal afferent neurons, with NR2C and NR2D subunits often being coexpressed with NR2B. Interestingly, NR2B or NR2C immunoreactivities are expressed by 50% or less of all nodose neurons, and only 14% of nodose ganglion neurons are immunoreactive for NR2D. In contrast to expression in the overall vagal afferent neuron population, NR2B is expressed by 98% of neurons.
of the afferents projecting to the gastric fundus and by > 80% of the afferents projecting to the gastric corpus and proximal duodenum (18). Thus the proportion of NR2B immunoreactive afferents innervating the upper gastrointestinal tract is roughly double the proportion in the vagal afferent population in general. These percentages suggest that NR2B may be expressed primarily by gastrointestinal vagal afferents. The possibility that NMDA receptors located on central vagal afferent terminals and fibers may participate in control of food intake is supported by recent results of Gillespie et al. (23), who demonstrated that intact central vagal afferent terminals were necessary for increased food intake evoked by NTS nanoinjection of MK-801.

In our previous work, we used MK-801 to examine the role of hindbrain NMDA receptors in control of food intake. However, MK-801 is a noncompetitive antagonist of all NMDA receptors, regardless of their NR2 subunit composition. To evaluate potential participation in control of food intake by hindbrain NMDA receptors that include specific NR2 subunit types, we made fourth ventricle or intra-NTS injections of three compounds that bind competitively and preferentially to specific NR2 subunit types. The compounds we have tested are Conantokin G (Con G), τ-3-(2-carboxypiperazin-4-yl)-1-propan-1-yl-phosphonic acid (τ-CPPene), and (±)-cis-1-(phenan-thren-2-yl-carbonyl)piperazine-2,3-dicarboxylic acid (PPDA). Con G is a 15-amino acid peptide, isolated from the venom of Conus geographus. It is a competitive antagonist selective for NMDA receptors containing the NR2B subunit (19). τ-CPPene is a competitive antagonist for receptors containing both NR2B and NR2A subunits (10), while PPDA appears to have some preference for antagonism of NR2C- and NR2D-containing NMDA receptors (22).

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 225–249 g at the start of the experiments, were housed individually in a temperature-controlled vivarium and adapted to a 12:12-h light-dark schedule (lights on at 0600). Rats had ad libitum access to water and standard rodent chow (Purina 5001) except during the experiments or overnight deprivation, as described below.

All protocols used were conducted in accordance with the National Institute of Health Guide for the Use of Laboratory Animals and approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

Drugs. Con G (VWR, West Chester, PA), a competitive antagonist of NMDA receptor with selectivity for the NR2B subunit; τ-CPPene (TOCRIS Bioscience, Ellisville, MO), a competitive antagonist of NMDA receptor NR2A/B subunits; or PPDA (TOCRIS), a competitive antagonist of NMDA receptor with some preferential binding to NR2C/D subunits were dissolved in 0.9% sterile saline in preparation for central administration. All rats received total volumes of 3.0 μl or 100 nl into the fourth ventricle or NTS, respectively. Sterile saline (0.9%) served as the vehicle control in all experiments.

Surgical procedures. Following overnight food deprivation, rats anesthetized with a mixture of ketamine (50 mg/kg), xylazine (5 mg/kg), and acepromazine (1 ml/kg body wt) were secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) and implanted with chronic guide cannulae (Plastic One, Roanoke, VA) directed at the fourth ventricle or NTS. For fourth ventricular injections, 24-gauge guide cannulae were implanted using the following coordinates: 2.0 mm from the occipital crest, 0.0 mm lateral to the midline, and 6.7 mm ventral to the dura level according to Paxinos et al. (43). The 31-gauge injectors used with these cannulae extended 0.5 mm beyond the guide cannula tips to access the intraventricular space. For NTS injections, rats were implanted with 26-gauge guide cannulae directed toward the caudo-medial NTS, using the following coordinates: 0.0 mm from the occipital crest, 0.8 mm lateral from the midline, and 7.9 mm ventral from the skull level. The 33-gauge injector used with these cannulae extended 0.5 mm beyond the guide cannula tips. Cannulae were secured to the skull by stainless-steel screws and methacrylate cement. All rats were allowed a minimum of 7 days postsurgical recovery period and were weighed daily until they returned to presurgical weight before testing began. The protocol employed here was similar to earlier reports by this laboratory (11). The location of the cannula tips was determined histologically. At the end of the experiments, rats were deeply anesthetized and transcardially perfused with 0.1 M phosphate buffered saline, followed by 4% formalin. Brains were removed and postfixed in formalin for 4 h, cryoprotected in 20% sucrose solution, and sectioned at 40 μm with a cryostat. The sections were stained with cresyl violet, mounted on slides and examined under the microscope. Location of each cannula tip was mapped according to the rat brain atlas of Paxinos and Watson (43). Only data from rats whose cannula placements were in the fourth ventricle or NTS were included in the statistical analysis (see Table 1).

Experimental procedure. Three separate groups of naïve rats (n = 65) were used in these experiments. The number of animals assigned per each treatment group is presented in Table 1. All rats were adapted to consume a 15% sucrose solution following an overnight fast until stable baselines were achieved (after ~3 tests). During testing, overnight food-deprived rats (16 h; 1700–900) were removed from their home cages, the obturators were pulled from their cannulae, and injectors were connected to a dispensing device. Rats with fourth ventricular cannulae received 3.0-μl intraventricular injections of either drug or saline, via a 31-gauge injector connected to a Hamilton dispenser that accommodates a 25-μl Hamilton syringe (World Precision Instruments, Sarasota, FL). Rats implanted with intraparenchymal cannulae were injected with 100 nl of either drug or saline, via a 33-gauge injector connected to a motorized 10-μl microsyringe (World Precision Instruments). The following drug doses were used per rat and per each administration: 1) fourth ventricle: Con G, 5, 20, 40, 80 ng; τ-CPPene, 3.0, 6.25, 12.5, 25, 50, 100 ng; PPDA, 300, 400 ng; and 2) NTS: Con G, 10 ng; τ-CPPene, 5 ng, 10 ng and PPDA, 0.5, 1.0, 2.5, 5.0 ng. Each injection was made over a period of 1 min, and the injector remained seated in the guide cannula for an additional 1 min. Subsequently, the injectors were removed, obturators were replaced, and the rats were returned to their home cages. Calibrated drinking tubes filled with 15% sucrose solution were immediately presented, and sucrose intake was measured to the nearest 0.1 ml every 5 min for 60 min, after which pelleted rat chow and water were returned. For intracerebroventricular experiments, each animal was tested twice with each dose of the drug that was bracketed by a saline injection. Tests were conducted every other day. A minimum of 3 days were interspersed between drug injections and until baseline was returned to predrug intake. The intake after each drug dose was

Table 1. Total number of rats used in the experiments and statistical analysis

<table>
<thead>
<tr>
<th>Drug</th>
<th>Fourth Ventricle</th>
<th>NTS</th>
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<tbody>
<tr>
<td>Con G</td>
<td>11/0</td>
<td>10/1*</td>
</tr>
<tr>
<td>τ-CPPene</td>
<td>9/3†</td>
<td>10/2†</td>
</tr>
<tr>
<td>PPDA</td>
<td>14/1*3†</td>
<td>11/3†</td>
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NTS, nucleus of the solitary tract; Con G, Conantokin G; D-CPPene, τ-3-(2-carboxypiperazin-4-yl)-1-propan-1-yl-phosphonic acid; PPDA, (±)-cis-1-(phenan-thren-2-yl-carbonyl)piperazine-2,3-dicarboxylic acid. Some rats did not complete the experiment due to *loss of cannula patency or †cannula misplacement.
averaged for each rat as were the intakes after the saline injections. Rats received each dose in a randomized order. For NTS experiments, with one exception (Con G, 10 ng was injected two times) each rat received one injection of each drug dose, which was again bracketed by saline control injections. All injections were separated by a minimum of 48 h during which no experimental manipulations occurred. Pre- and postdrug sucrose intake did not statistically differ indicating the absence of drug carry over effects.

Statistical analysis. Statistical comparisons between intakes for all rats were performed separately for each drug by two-way repeated-measures ANOVA, with dose and time as independent variables. Significant interactions were followed up by one-way ANOVA comparing intakes at each time point followed up by post hoc Bonferroni test. All intakes are expressed as means ± SE in milliliters. In all cases, a P value of <0.05 was considered significant. All analyses were made using PC-SAS (version 9.1.3; SAS Institute, Carey, NC).

RESULTS

Effects of fourth ventricular administration of Con G on 15% sucrose intake. Results of two-way repeated-measures ANOVA showed a significant main effect of Con G on 60-min cumulative sucrose intake [F(4, 28) = 189.76, P < 0.0001], time [F(11, 28) = 84.33, P < 0.0001], as well as significant dose × time interaction [F(1, 28) = 9.21, P = 0.0038]. As illustrated in Fig. 1, intracerebroventricular administration of Con G significantly increased 60-min 15% sucrose intake compared with saline injection following 20-ng (15.79 ± 0.7 ml; P = 0.047), 40-ng (16.05 ± 0.7 ml; P = 0.0031), and 80-ng (17.08 ± 0.7 ml; P < 0.0001) doses vs. saline (13.54 ± 0.6 ml). The effect of Con G on sucrose consumption was evident as early as 5 min postsucrose presentation and continued to be significant throughout the 60-min feeding period. Although the 5.0-ng dose increased sucrose intake during the first 30 min, it did not reach statistical significance (P > 0.05). At 60 min, the intake after 5.0 ng was almost identical to that after saline. There was also a significant difference in 60-min intake between the 5.0-ng dose and all other three doses tested (P = 0.029; P = 0.0053; P < 0.0001 for 20-, 40-, and 80-ng dose, respectively). No statistical difference in intake occurred at any time point between the 20-, 40-, or 80-ng dose of Con G (P > 0.05).

However, the intake following the 80-ng dose was higher compared with the intake after the 20- and 40-ng doses. Five-minute interval intake analysis found that the 20- and 80-ng doses increased 15% sucrose intake significantly within the first 5 min (20 ng: 7.88 ± 0.46 ml, P = 0.0026; 80 ng: 9.90 ± 0.42 ml, P < 0.0001) compared with saline (6.71 ± 0.22 ml). The 20-ng dose also increased sucrose intake significantly higher than saline in the second 5-min interval (20 ng: 4.10 ± 0.54 ml, P < 0.0001; saline: 2.46 ± 0.18 ml).

Effects of fourth ventricular administration of n-CPePene on 15% sucrose intake. Results of two-way repeated-measures ANOVA showed a significant main effect of n-CPePene on 60-min cumulative sucrose intake [F(6, 28) = 142.85, P < 0.0001], time [F(11, 28) = 83.89, P < 0.0001], as well as significant dose × time interaction [F(1, 28) = 4.64, P = 0.04]. As demonstrated in Fig. 2, all doses of n-CPePene tested significantly increased sucrose intake. However, for some doses, the effect was more pronounced at 30 min compared with 60 min. For example, cumulative 30-min sucrose intake was significantly increased (3 ng: 15.7 ± 1.2 ml; 6.25 ng: 16.5 ± 1.2 ml; 12.5 ng: 15.1 ± 1.1 ml; 25 ng: 16.0 ± 1.2 ml; 50 ng: 17.4 ± 1.2 ml; 100 ng: 18.3 ± 1.2 ml) compared with saline injection (12.3 ± 0.95 ml). For most doses, the effect of n-CPePene on sucrose consumption was evident beginning at 10 min postsucrose presentation and continued to be significant throughout the 60-min feeding period. This was evident from the analysis of 5-min interval intakes, showing that the 25-, 50-, and 100-ng doses increased 15% sucrose intake significantly within the first 5 min (25 ng: 9.13 ± 0.51 ml, P < 0.0001; 50 ng: 9.58 ± 0.86 ml, P = 0.0001; 100 ng: 9.05 ± 0.58 ml, P < 0.0023) compared with saline intake (7.06 ± 0.24 ml). Additionally, the 12.5-, 25-, and 100-ng doses increased sucrose intake significantly higher than saline in the second 5-min interval (12.5 ng: 4.64 ± 0.58 ml, P < 0.0001; 25 ng: 4.67 ± 0.50 ml, P = 0.0002; 100 ng: 5.37 ± 0.84, P < 0.0001, saline: 2.77 ± 0.18 ml). No significant differences were observed between doses of n-CPePene tested, although sucrose intake was greatly increased particularly after the highest (50 and 100 ng) doses.

Effects of fourth ventricular administration of PPDA on 15% sucrose intake. Results of two-way repeated-measures ANOVA showed a significant effect of PPDA on 60 min cumulative sucrose intake [F(7, 34) = 104.90, P < 0.0001], time [F(11, 34) = 64.27, P < 0.0001], as well as significant dose × time interaction [F(1, 34) = 17.95, P < 0.0001]. As demonstrated in Fig. 3, intracerebroventricular administration of both doses of PPDA significantly increased 60-min 15% sucrose intake (300 ng: 16.8 ± 0.7 ml, P < 0.0001; 400 ng: 16.7 ± 0.6 ml, P < 0.0001) compared with saline injection (13.2 ± 0.6 ml). The effect of PPDA on sucrose consumption was evident beginning at 10 min postsucrose presentation and continued to be significant throughout the 60-min feeding period. When 5-min bin intakes were analyzed, the 200-, 300-, and 400-ng doses increased 15% sucrose intake significantly within the first 5 min (200 ng: 8.68 ± 0.65 ml, P < 0.01; 80 ng: 9.69 ± 0.42 ml, P < 0.0001; 400 ng: 9.22 ± 0.72 ml, P < 0.0001) compared with saline (7.13 ± 0.18 ml).

Effects of NTS administration of Con-G on 15% sucrose intake. Results of two-way repeated-measures ANOVA showed a significant effect of Con G on 60-min cumulative sucrose intake [F(1, 24) = 174.24, P < 0.0001], time [F(11, 24) = 73.87, P < 0.0001], as well as significant dose × time
interaction \[ F(1, 24) = 34.68, P < 0.0001 \]. As demonstrated in Fig. 4, NTS administration of Con G (10 ng) significantly increased 60-min 15% sucrose intake compared with saline injection (13.8 \( \pm \) 0.6 vs. 17.8 \( \pm \) 0.6 ml; \( P < 0.0001 \)). The effect of Con G on sucrose consumption was evident beginning at 25 min postsucrose presentation and continued to be significant throughout the 60-min recording period. Five-minute interval bin intake analysis showed no significant differences between treatments (\( P > 0.05 \) for all doses).

**Effects of NTS administration of d-CPPene on 15% sucrose intake.** Results of two-way repeated-measures ANOVA showed a significant effect of d-CPPene on 60-min cumulative sucrose intake \[ F(2, 23) = 99.70, P < 0.0001 \], time \[ F(11, 23) = 37.23, P < 0.0001 \], as well as significant dose \( \times \) time interaction \[ F(1, 23) = 32.12, P < 0.0001 \]. As demonstrated in Fig. 5, NTS administration of d-CPPene (10 ng) significantly increased 60-min 15% sucrose intake compared with saline injection (10.5 \( \pm \) 0.7 vs. 15.1 \( \pm \) 0.9 ml, \( P = 0.0004 \)). The effect of d-CPPene on sucrose consumption was evident beginning at 30 min postsucrose presentation and continued to be significant throughout the 60-min feeding period. The 5-ng dose did not produce any significant effects on sucrose consumption. Analysis of individual 5-min interval intakes showed no significant differences between treatments (\( P > 0.05 \) for all doses).

**Effects of NTS administration of PPDA on 15% sucrose intake.** Results of two-way repeated-measures ANOVA showed a significant effect of PPDA on 60-min cumulative sucrose intake.
intake [$F(7, 28) = 170.74, P < 0.0001$], time [$F(11, 28) = 35.85, P < 0.0001$], as well as significant dose × time interaction [$F(1, 1973) = 0.59, P = 0.026$]. As demonstrated in Fig. 6, only one dose (1.0 ng) of PPDA injected into the NTS significantly increased 60-min 15% sucrose intake compared with saline injection (13.7 ± 0.9 ml vs. 17.1 ± 1.0 ml, $P < 0.0001$). The effect of this dose of PPDA on sucrose consumption was evident beginning at 15 min post-sucrose presentation and continued to be significant throughout the 60-min testing period. When individual bin intakes were analyzed, ANOVA showed a significant increase in sucrose intake only at 5 min post-drug administration (9.1 ± 0.8 ml vs. 7.2 ± 0.25 ml; $P < 0.001$). Although the 0.5-, 2.5-, and 5.0-ng dose slightly increased sucrose intake above saline level, it did not reach significance (0.5 ng: 14.4 ± 1.2 ml; 2.5 ng: 15.8 ± 1.4 ml; 5.0 ng: 15.1 ± 1.4 ml; all $P$ values > 0.05). The highest dose tested (15.0 ng) produced a significant suppression of overall cumulative sucrose intake compared with saline ($P < 0.0001$).

**DISCUSSION**

Our results reveal that fourth ventricle and NTS injections of the three different NMDA antagonists d-cyclophosphamide, Con G, and PPDA increase 15% sucrose intake in food-deprived rats. d-Cyclophosphamide, an NMDA receptor antagonist, strongly competes with glutamate for binding to the NR2B subunit, but also has affinity for other NR2 subunits (10). d-Cyclophosphamide has shown moderate selectivity for certain subunit combinations of NR2A and 2B (NR2A and 2B: approximately > 2C = 2D; $K_i = 0.11, 0.14, 1.5, 1.8$ am NR2A, NR2B, NR2C, and NR2D subunits, respectively) (28). Similarly, PPDA exhibits some preference for the NR2C subunit binding site, but also binds to other NR2 subunits (NR2C and 2D NR2C = 2D > 2A = 2B; $K_i = 0.096, 0.125, 0.31$, and 0.55 am for NR2C, NR2D, NR2B, and NR2A subunits, respectively) (22, 28). Therefore, increased food intake following d-cyclophosphamide or PPDA is consistent with our previous results showing that hindbrain administration of competitive, non-competitive, or pan-NMDA receptor antagonists increase food intake when delivered into the fourth ventricle or NTS parenchyma (15, 29).

In contrast to d-cyclophosphamide and PPDA, Con G binds with high affinity to the glutamate binding site on NR2B subunits and has little affinity for other NR2 subunit types (19). Although specific selectivity and affinity values are not fully determined (19), one study found Con G providing a $K_a$ of 1.9 am when it was bound to Nora/NR2B receptors, but was not active for Nora/NR2A receptors (45). This high potency of inhibition of Con G for NADIR subtype Rib/NR2B shows that this subunit combination offers the best configuration for overall binding within the four dominant NADIR combinations. Therefore, increased food intake following hindbrain Con G injection extends our previous results, suggesting that selectively antagonizing NMDA receptors containing NR2B subunit(s) is sufficient to increase food intake.

The cellular location of receptors that mediate increased food intake following hindbrain NMDA receptor antagonist administration is not known with certainty. Immunohistochemical observations suggest that subpopulations of neurons in the NTS express each of the four NR2 subunits (1, 3, 8, 24), including NR2B and NR2C. Therefore, it is possible that antagonist action at receptor on the cell bodies of NTS neurons, which process signals involved in control of food intake, mediate the increased food intake following our hindbrain injections of NMDA antagonists. NMDA receptor antagonists acting at somatic or dendritic postsynaptic sites might attenuate gastrointestinal satiation signals, thereby enhancing food intake. Involvement of hindbrain NMDA receptor expressing neurons in integration of viscerosensory information from the gastrointestinal tract is supported by the results of Berthoud et al. (8) who used an antiserum that did not discriminate between NMDA NR2A and NR2B subunits to demonstrate that 70% of NTS neurons activated by gastric distention or intestinal infusions express NMDA receptor immunoreactivity.

In addition to expression on NTS neuron soma and dendrites, NMDA immunoreactivity is also present on primary vagal afferent terminals and axons in the NTS (17). Moreover, Czaja et al. (18) reported that the immunoreactivity for the obligatory NR1 subunit is expressed by at least 98% of all vagal afferent neurons, indicating that virtually all vagal afferents express NMDA receptors. Czaja et al. (17) also found that while 57% of afferents in young and adolescent rats express NR2B immunoreactivity, while < 30% of all afferents in adult rats express NR2B. However, NR2B immunoreactivity is expressed by at least 90% of afferents innervating the stomach or duodenum in adult rats (18), suggesting that the majority of NR2B immunoreactive vagal afferents innervate the gastrointestinal tract. In this context, the increase in intake we observed following PPDA may be important. PPDA prefers glutamate binding sites on NR2C and NR2D containing NMDA receptors. However, results of Czaja et al. (18) indicate that NR2C also is somewhat more prevalent in afferents that innervate the stomach or duodenum. Czaja et al. (17) also observed that NR2C is predominantly coexpressed with NR2B in vagal afferents. Therefore, increased food intake following hindbrain administration of the NMDA receptor could be the result of these antagonists acting presynaptically on gastrointestinal vagal afferent terminals expressing heterometric NR2B and NR2C NMDA receptors.
Electrophysiological results suggest that transmitter release by primary somatosensory afferent terminals in the spinal cord can be modulated by presynaptic NMDA receptors in the terminals of these neurons in the spinal cord (36). Unfortunately, there are currently no functional experiments to link increased food intake evoked by NR2B or NR2C. Nevertheless, the behavioral experiments of Gillespie et al. (23) strongly suggest that NMDA receptors expressed on central vagal afferent axons or terminals may mediate increased food intake in response to hindbrain NMDA antagonist administration. While the traditional view of ionotropic glutamate receptors, including NMDA, is that they are located on postsynaptic cell bodies and dendrites, NMDA receptor subunit immunoreactivity has been localized to axon terminals at a variety of sites in the brain (24), including on vagal afferent terminals in the NTS (1). Furthermore, electrophysiological data indicate that presynaptic NMDA receptors modulate transmitter release from axon terminals in the visual cortex (35), entorhinal cortex (54), and from primary somatosensory afferent terminals in the spinal dorsal horn (4). Thus, it now appears that NMDA-type glutamate receptors, located on axon terminals, play an important role in determining the amount of transmitter released onto postsynaptic receptors. Intriguingly, NMDA receptor subunit message has been detected in the nodose ganglia by in situ hybridization (46) and PCR (47). Most recently, we demonstrated that NMDA NR1 subunit immunoreactivity is present in > 90% of vagal afferent cell bodies in the nodose ganglia (17), and others have demonstrated its presence on vagal afferent terminals in the NTS (1). Thus, NMDA receptors expressed by vagal afferents, and are presynaptically localized where they could modulate incoming primary afferent signaling. Increase in meal size relies upon intact vagal afferent innervation of the NTS (23), consistent with the interpretation that gastrointestinal feedback signals controlling meal size are attenuated when NMDA receptors on vagal afferents are antagonized. The efficacy of Con G in increasing food intake, considering that NR2B subunit expression is concentrated in vagal afferents innervating the upper gastrointestinal tract, is consistent with the hypothesis that these antagonists increase food intake by antagonizing presynaptic NR2B-containing NMDA receptors on the central terminals of gastrointestinal vagal afferents.

Although all three NMDA receptor antagonists examined in our current study exhibited a trend toward increasing food intake with increasing fourth ventricular dose, a significant dose effect relationship could not be demonstrated over the range of doses we used. NMDA receptors are expressed by many neurons throughout the brain, including the hindbrain (1, 6, 9, 25). Consequently, one might expect increasing dosage of the antagonists to be accompanied by an increase in side effects that could compete with sensory motor coordination and thereby inhibit food intake. We and others have previously reported such interfering side effects for MK-801 (15, 29). In addition, it is worth remembering that the caudal hindbrain contains circuitry that participates in both increasing and decreasing food intake. It remains possible that NMDA receptors in some hindbrain circuits enhance food intake, and that the observed increases with our antagonist injections represent a net effect of attenuating both orexigenic and satiation signals.

In previous experiments, we found that directly injecting the dorsal vagal complex or fourth ventricle with MK-801 or AP5 increased food intake, with the dorsal vagal complex requiring lower doses to increase food intake (29, 30). During previous experiments using MK-801, we found that injections of the antagonist that failed to increase food intake when injected into the fourth ventricle did increase food intake when injected into the dorsal vagal complex. Furthermore, dorsal vagal cannula placements from which MK-801 evoked increases in food intake were centered in the caudal portions of the medial NTS, surrounded by negative placements laterally and ventrally (50), suggesting a fairly circumscribed localization of MK-801 action. Similarly, we found that injecting the competitive NMDA receptor antagonist AP5 into the NTS increased food intake at dose levels less than one tenth of the minimally effective fourth ventricular dose, again suggesting an action of the NMDA antagonist in the tissue of the dorsal hindbrain. In our current set of experiments, PPDA increased food intake with intraventricular doses of Con G and D-CPPene were equivalent to those that increased food intake when they were injected directly into the dorsal vagal complex. It is conceivable that NMDA receptors outside of the dorsal vagal complex contribute to feeding in response to some NMDA receptor antagonists. However, this explanation is not consistent with our findings that three of the five NMDA receptor antagonists we have worked with are significantly more effective for increasing food intake when injected into the NTS than when injected into the fourth ventricle. In contrast to PPDA, AP5, and MK-801, effective ventricular doses of Con G and D-CPPene were more rapidly cleared or degraded after intraperitoneal injection than after ventricular injection, thereby reducing the length of time that the antagonist is available to compete with glutamate for its binding site. This may also explain a significant cumulative effect on sucrose intake when the antagonists were given into the NTS compared with intracerebroventricular injection. The increase in sucrose intake, after fourth ventricle NMDA blockade was immediate, i.e., within the first 5- and 10-min period. Since during this short time interval not a significant amount of ingested sucrose is emptied from the stomach, it is likely that gastric mechanoreceptors have a major contribution in generating afferent signals leading to glutamate release in the dorsal hindbrain. This would support our earlier findings indicating that MK-801 exerts its effects on feeding by interfering with gastric mechanoreceptive signaling (15). A variety of evidence indicates vesicular glutamate transporters and glutamate release from central vagal afferent terminals (14). After glutamate is released from the presynaptic terminal, it is taken up by transporters on glial cells and converted to glutamine. Glutamine is then transported out of the glia and taken up by neurons where it is converted back to glutamate (26). However, the possibility that, in addition to primary afferents-released glutamate, the NMDA antagonists may also block the action of glutamate coming from other sources, such as interneurons or areas outside the NTS, should also be considered. At present, we cannot offer a satisfying explanation for the apparent difference in Con G and D-CPPene behavior compared with the three other NMDA antagonists that increase food intake at low NTS doses.

In summary, our results indicate that hindbrain injection of competitive NMDA antagonists with selectivity or preference
for the NMDA receptor NR2B or NR2C subunits increase food intake. Prior work from our group suggests that a high proportion of vagal afferent terminals innervate the upper gastrointestinal tract express heteromeric NMDA receptors containing these subunits. Together with previously published work implicating central vagal afferent terminals in increased feeding evoked by hindbrain NMDA antagonist injection, our current results support our working hypothesis that NMDA receptors on gastrointestinal vagal afferent terminals participate in the control of food intake.

GRANTS

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