Lateral hypothalamic NMDA receptor subunits NR2A and/or NR2B mediate eating: immunohistochemical evidence

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1Lateral hypothalamic NMDA receptor subunits NR2A and/or NR2B mediate eating: immunohistochemical/behavioral evidence. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R880–R891, 1999.—Cells within the lateral hypothalamic area (LHA) are important in eating control. Glutamate or its analogs, kainic acid (KA) and N-methyl-D-aspartate (NMDA), elicit intense eating when microinjected there, and, conversely, LHA-administered NMDA receptor antagonists suppress deprivation- and NMDA-elicited eating. The subunit composition of LHA NMDA receptors (NMDA-Rs) mediating feeding, however, has not yet been determined. Identifying this is important, because distinct second messengers/modulators may be activated by NMDA-Rs with differing compositions. To begin to address this, we detected LHA NR2A and NR2B subunits by immunoblotting and NR2B subunits by immunohistochemistry using subunit-specific antibodies. To help determine whether NMDA-Rs mediating feeding might contain these subunits, we conducted behavioral studies using LHA-administered ifenprodil, an antagonist selective for NR2A- and/or NR2B-containing NMDA-Rs at the doses we used (0.001–100 nmol). Ifenprodil maximally suppressed NMDA- and deprivation-elicited feeding by 63 and 39%, respectively, but failed to suppress KA-elicited eating, suggesting its actions were behaviorally specific. Collectively, these results suggest that LHA NMDA-Rs, some of which contribute to feeding control, are composed of NR2A and/or NR2B subunits, and implicate NR2A- and/or NR2B-linked signal transduction in feeding behavior.

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ELECTRICAL STIMULATION OF the lateral hypothalamic area (LHA) can elicit marked eating in rats, and lesions within this area, including those sparing fibers of passage, render animals aphagic or hypophagic (see Ref. 2 for a review). Moreover, some LHA neurons respond selectively to the sight, smell, or taste of food in a hunger-dependent manner (35, 38), and some are also sensitive to iontophoretic application of glucose and free fatty acids (29, 30) and to glycemic fluctuations within the blood (13, 39). Accordingly, the LHA has been suggested as a primary integration site controlling feeding.

Glutamate receptors in the LHA, especially of the N-methyl-D-aspartate (NMDA) subtype, appear to play an important role in feeding control and body weight regulation. Specifically, acute LHA microinjections of glutamate or its receptor agonists, NMDA, kainic acid (KA), or 3,5-diaminopropionate, elicit intense transient eating in satiated rats in an anatomically specific manner (42, 44). Conversely, acute LHA injections of NMDA receptor (NMDA-R) antagonists can suppress nocturnal and/or deprivation-induced eating and chronic injections suppress daily food intake and cause weight loss (41, 43).

Although these findings suggest that NMDA-Rs may be involved in LHA mechanisms of feeding stimulation, the subunit composition of these receptors has not yet been investigated. Molecular studies have identified the NMDA-R subunits, NR1, NR2A-D, and XenNR1G (reviewed in Refs. 1 and 45). Native mammalian NMDA-R complexes are thought to contain at least one NR1 subunit and several NR2 subunits, because expression of NR1 with one or more NR2 subunits in recombinant systems generates NMDA-Rs with properties that closely resemble native receptors (37). The NR1, NR2A, and NR2B subunits are widely distributed in the diencephalon, whereas the NR2C and NR2D subunits are present in relatively low levels in this region (26, 33, 34). Thus the most abundant heterogeneous NMDA-Rs in the hypothalamus are likely to be NR1-NR2A-, NR1-NR2B-, or, possibly, NR1-NR2A-NR2B-containing NMDA-Rs.

The identification of NMDA-R subunit composition is important, in part, because different subunit combinations yield functional NMDA-Rs that vary markedly in their electrophysiological properties and sensitivities to modulation by intracellular messengers; many second messengers, moreover, are preferentially coupled to only one or a few subunits (45). Given the additional complexity that subunit diversity affords NMDA-Rs, it is likely that NMDA-Rs in the LHA can be coupled to one or more intracellular signaling mechanisms, depending on their precise subunit composition.

To begin determining the subunit composition of LHA NMDA-Rs involved in feeding control, we conducted behavioral, biochemical, and anatomic studies to identify and localize the NR2A and NR2B NMDA-R subunits in the LHA and to test for their involvement in feeding. We report here the identification and localization of the NR2A and NR2B subunits in the LHA and also provide the first in vivo evidence suggesting that
NR2A and/or NR2B subunits are present on functional LHA NMDA-Rs mediating feeding. Portions of these data have been presented in preliminary form (16).

MATERIALS AND METHODS

Materials

Chemicals and solutions for behavioral studies. Ifenprodil tartrate (FW 800.99) was purchased from Research Biochemicals International (Natick, MA) and was dissolved in dimethyl sulfoxide (DMSO), purchased from Sigma Chemical (St. Louis, MO), just before central injection. NMDA was also purchased from Sigma and was dissolved in artificial cerebrospinal fluid (aCSF) just before central injection. The aCSF was prepared using boiled, double-distilled water sealed in glass ampules and consisted of (in mM) 147 Na+, 1,54 Cl−, 3.0 K+, 1.2 Ca2+, and 0.9 Mg2+ at a pH of 7.3.

Antibodies. Affinity-purified rabbit polyclonal antibodies raised against the NR1, NR2A, and NR2B subunits and the NR2A and NR2B fusion proteins for preabsorption experiments were purchased from Chemicon International (Temecula, CA), and nonbiotinylated and biotinylated goat anti-rabbit IgG (heavy + light chains) were from Vector Laboratories (Burlingame, CA). Peroxidase-labeled goat anti-rabbit IgG was purchased from Bio-Rad (Richmond, CA).

Other materials for immunoblotting and immunohistochemistry. Compounds comprising the protease inhibitor cocktail, as well as BSA, diaminobenzidine (DAB), and normal goat serum were purchased from Sigma. Other materials included avidin-biotin-peroxidase complex (ABC) solution (Vector), bicinchoninic acid (BCA) protein assay reagents (Pierce, Rockford, IL), enhanced chemiluminescence (ECL) reagent and Hyperfilm-ECL (Amersham International, Buckinghamshire, UK), and Immobilon-P polyvinylidene fluoride (PVDF) membranes, 0.45-µm pore size (Millipore, Bedford, MA). All other materials were of appropriate grade and were purchased from local suppliers or Sigma.

Methods

Behavioral studies. Subjects. Adult male Sprague-Dawley rats (n = 55; 350–500 g), descended from Charles River animals, were single housed in a vivarium with a 12:12-h light-dark photoperiod and ad libitum access to food and water, unless otherwise stated. Animals in behavioral experiments were maintained and tested on a mash diet consisting of Purina rat chow (46%), sucrose (37%), and Carnation evaporated milk (17%). In experiments testing whether ifenprodil suppresses eating elicited by NMDA, animals were fed freshly prepared mash at least 1.5 h before testing to maximize satiety. All procedures were conducted in strict accordance to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996) and the University of California at Riverside Chancellor’s Committee on Laboratory Animal Care.

STEREOTAXIC SURGERY. Animals undergoing behavioral testing were stereotaxically implanted under Metofane anesthesia with chronic unilateral or bilateral 26-gauge stainless steel indwelling guide cannulae targeted toward the LHA. With the incisor bar 3.3 mm below the ear bars, stereotaxic coordinates were 6.1–6.4 mm anterior to the interaural line, 1.8 mm lateral to the midsagittal sinus, and 8.2 mm ventral to the surface of the skull (see Figs. 28 and 29 in Ref. 32). Cannulae were secured to the skull by six stainless steel hex screws and dental acrylic, and a plastic guard was placed around the exposed portion of the cannula. A 33-gauge stainless steel obturator was inserted into each cannula to maintain patency. Animals were allowed 5–7 days of postoperative recovery before testing, during which time they were repeatedly handled and mock-injected to adapt them to the testing procedures.

BEHAVIORAL TESTING. Tests consisted of either acute bilateral injections (1 injection per cannula in quick succession) or acute unilateral injections (2 injections given 10 min apart). Solutions were delivered through 33-gauge injectors that projected exactly 1.0 mm beyond the guide cannulas directly into the LHA. Doses of NMDA and KA were selected on the basis of their observed effectiveness in eliciting food intake in previous studies (see Refs. 42–44). Food intake was measured to the nearest 0.1 g by weighing each subject’s food bowl before and after injection and calculating the difference between the two weights. No spillage of food was observed during any experiment. In all cases, injection volumes were 0.3 µl; food intake was measured 0.5, 1, 2, and 4 h after the final injection; and animals received treatments in counterbalanced order during successive test days, with each animal receiving each treatment by the end of the experiment. Tests were separated by at least 1 day of recovery, during which no procedures were performed on the animals. All tests were conducted within the midportion of the light phase of the circadian cycle.

EXPERIMENT 1: DOES IFENPRODIL SUPPRESS EATING ELICITED BY NMDA? To determine whether NMDA-elicited eating can be attenuated by the NR2A/NR2B-selective NMDA receptor antagonist ifenprodil, three separate studies were conducted (each on a naive set of rats) employing multiple doses of ifenprodil. The animals were separated into three groups before testing to minimize the number of treatments/injections each animal would have to receive. In the first study, 13 animals (designated as group A) were unilaterally injected with ifenprodil (1, 10, or 100 nmol) or DMSO vehicle, followed 10 min later by a unilateral injection of NMDA (10 nmol) or aCSF vehicle. The second and third studies differed from the first in both the number of naive animals used and in the doses of ifenprodil employed: 100 pmol, 1 nmol, and 10 nmol were used in the second study (n = 14; group B), and 1, 10, and 100 pmol were used in the third study (n = 7; group C). Note that within each group, each animal received each dose selected for that group; these doses were given in counterbalanced order over the course of the experiment. Thus, at the conclusion of these studies, three sets of animals were injected with ifenprodil, at doses ranging from 1, 10, and 100 pmol to 1, 10, and 100 nmol, with the 100 pmol, 1 nmol, and 10 nmol doses tested in two of three studies to ensure replication of effects.

EXPERIMENT 2: DOES IFENPRODIL SUPPRESS EATING ELICITED BY KA? To determine whether the eating-suppressive effects of ifenprodil also generalized to eating elicited by KA, 10 naive rats were injected unilaterally with 100 pmol of ifenprodil or DMSO vehicle followed 10 min later by unilateral injection of either KA (1 nmol) or aCSF vehicle.

EXPERIMENT 3: DOES IFENPRODIL SUPPRESS EATING ELICITED BY FOOD DEPRIVATION? To investigate whether ifenprodil can affect natural eating elicited by food deprivation, 11 naive animals were food deprived for ~24 h and then given bilateral injections of either ifenprodil (100 pmol/side or 1 nmol/side) or DMSO vehicle. Ifenprodil was given bilaterally in this experiment so that it might block the presumed bilateral activation of the LHA caused by food deprivation. Freshly prepared food was returned 10 min after the injections, and food intake was measured, treating the time of final injection as postinjection time t = 0.
rats were killed by ether inhalation, and their brains were quickly removed without prior fixation and placed on ice. One-millimeter-thick coronal sections containing most of the LHA were blocked using a stainless steel matrix (Activational Systems), and the LHA was microdissected from it using ultrafine microdissecting spring scissors according to a method modified from Ref. 31. The microdissected portion was just lateral to the fornix and medial to the internal capsule and spanned much of the rostrocaudal extent of the LHA. Samples were similarly obtained from the frontal cortex (taking care to spare the corpus callosum) and cerebellar cortex. Portions of the liver from each subject were used as a negative control for NMDA-R subunit expression.

Micro dissected tissue was placed in ice-cold protease inhibitor cocktail [10 mM Tris·HCl (pH = 7.4), 5 mM EDTA, apro tinin (2.3 µg/ml), bacitracin (200 µg/ml), leupeptin (10 µg/ml), 0.2 mM PMSF, 1 mM benzamid ine, and 0.32 M sucrose], homogenized, and stored at −70°C until further processing.

PROTEIN DETERMINATION. Samples were centrifuged (17,900 g) for 20 min at 4°C to obtain crude tissue homogenates. Tissue pellets were resuspended in 125 mM Tris·HCl, 100 µl of1.5% Triton x-100, 0.5 mg/ml aprotinin (2.3 µg/ml), bacitracin (200 µg/ml), leupeptin (10 µg/ml), 0.2 mM PMSF, 1 mM benzamidine, and 0.32 M sucrose, homogenized, and stored at −70°C until further processing.

Table 1. Effects of ifenprodil on NMDA-elicited food intake in LHA of satiated rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Hours Postinjection</th>
<th>Vehicle only</th>
<th>NMDA only</th>
<th>1 pmol Ifenprodil + NMDA</th>
<th>10 pmol Ifenprodil + NMDA</th>
<th>100 pmol Ifenprodil + NMDA</th>
<th>1 nmol Ifenprodil + NMDA</th>
<th>10 nmol Ifenprodil + NMDA</th>
<th>100 nmol Ifenprodil + NMDA</th>
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<tr>
<td>A</td>
<td>0.5</td>
<td>1.4 ± 0.5</td>
<td>9.5 ± 1.6*</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>5.1 ± 1.0†</td>
<td>7.8 ± 1.5†</td>
<td>9.8 ± 2.5†</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.0 ± 0.5</td>
<td>11.9 ± 1.8*</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>7.8 ± 1.4†</td>
<td>8.2 ± 1.5†</td>
<td>12.3 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.9 ± 0.7</td>
<td>13.5 ± 1.9*</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>10.2 ± 1.7†</td>
<td>9.0 ± 1.6†</td>
<td>12.9 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4.0 ± 0.7</td>
<td>15.2 ± 1.5*</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>12.3 ± 1.9†</td>
<td>9.7 ± 1.5†</td>
<td>13.5 ± 3.0</td>
</tr>
<tr>
<td>B</td>
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<td>8.8 ± 2.5*</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
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<td>NT</td>
<td>NT</td>
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<td>7.7 ± 2.0†</td>
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<td>NT</td>
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<tr>
<td>C</td>
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<td>14.8 ± 2.8</td>
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<tr>
<td></td>
<td>1</td>
<td>0.6 ± 0.1</td>
<td>17.0 ± 1.5*</td>
<td>13.1 ± 2.9</td>
<td>15.3 ± 2.8</td>
<td>9.2 ± 2.8†</td>
<td>NT</td>
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<td>NT</td>
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<td></td>
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<td>15.6 ± 2.8</td>
<td>10.3 ± 2.6†</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

Food intake in grams (mean ± SE) as a result of lateral hypothalamic area (LHA) injections of either vehicle alone, N-methyl-D-aspartate (NMDA) alone, or NMDA in combination with 1 of 6 doses of ifenprodil. Data presented are from 3 studies, each using a naive set of subjects (groups A–C); n = 13, 14, and 7 for groups A–C, respectively. *Intakes as a result of NMDA alone are significantly greater than vehicle intakes at corresponding time points at P < 0.05 by t-tests for paired means. †Intakes are significantly smaller than intakes as a result of NMDA alone, as revealed by 2-way ANOVA across all doses for that group, followed by a post hoc Student-Newman-Keuls test (P < 0.05). NT denotes that a given treatment was not tested in that group. Note that some treatments were tested in more than 1 group.
1.5 h at RT with an affinity-purified, polyclonal antibody (Chemicon) targeted against the putative COOH terminus of the NR1 (1:100, 50 µg/500 µl), NR2A (1:600, 10 µg/500 µl), or NR2B (1:600, 10 µg/50 µl) protein subunits of the NMDA-R for 1.5–2 h at RT. Blots were then washed three or four times over 30 min in PBS containing 0.05% Tween and incubated with a peroxidase-labeled goat anti-rabbit IgG (1:1,000; Bio-Rad) for 1 h at RT. After more washes, the immunoreactive subunits were detected as dark bands on photographic film using ECL. Molecular weights were then estimated using the positions of the prestained molecular weight standards on each blot. All samples to be compared were loaded on the same gel and processed on the same blot to prevent slight differences in methodology from interfering with the comparisons between groups.

In separate experiments to confirm the specificity of the primary antibodies used, anti-rat NR2A or NR2B antibodies were preabsorbed with the antibodies’ respective fusion proteins used to immunize rabbits (Chemicon). The NR2A and NR2B fusion proteins were made using the deduced amino acid sequences at positions 1,253–1,391 or 984–1,104, respectively, of the putative COOH-terminal regions of these subunits (14). Each antibody was preabsorbed for 1 h at RT before incubating with blots. Antibody and fusion protein concentrations were, respectively, 0.5 µg/ml and 2.5 µg/ml (NR2A) and 0.33 µg/ml and 10 µg/ml (NR2B).

Immunohistochemical localization of NR2A and NR2B subunits, PERFUSION AND TISSUE PREPARATION. Adult male Sprague-Dawley rats (325–475 g, n = 8) were injected intraperitoneally with pentobarbital sodium (60 mg/kg) and then perfused through the ascending aorta using a peristaltic pump (Masterflex; Cole-Parmer Instruments, Vernon Hills, IL; flow rate = 25 ml/min), first with at least 100 ml of cold PBS (0.1 M sodium phosphate buffer in 0.9% saline) containing heparin (200 units/10 ml PBS), followed by a minimum of 300 ml of cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH = 7.4). After perfusion, brains were quickly removed and the hypothalami and surrounding areas were blocked. Blocks were then postfixed in the same fixative at 4°C for at least 24 h. Blocks were transferred to a 25% sucrose cryoprotectant solution for at least 24 h at 4°C and then cryoprotected by immersion in 4°C for at least 24 h. Blocks were sectioned on a Jung CM3000 cryostat (Leica Instruments) into 25-µm-thick sections and collected in cold PBS. Freely floating sections were then reacted with 0.1% hydrogen peroxide in PBS for 10 min at RT, rinsed for 10 min in PBS, and then incubated for 1 h in 5% normal goat serum and 1% BSA in 0.3% Triton-X-100 containing PBS (T-PBS).

IMMUNODETECTION. All sections (except those used as “no primary antibody” controls for background staining) were incubated for ~72 h at 4°C with a polyclonal, affinity-purified antibody targeted against the COOH terminus of the NR2A or NR2B subunits (Chemicon). These antibodies were initially tested at dilutions of 1:500, 1:1,000, 1:1,500, and 1:3,000. The 1:1,000 titer was selected as the optimal dilution for the anti-NR2A antibody. Primary antibodies were diluted in T-PBS containing 1% normal goat serum and 0.2% BSA. Sections were then rinsed in cold T-PBS for at least 10 min at RT and were incubated with a biotinylated polyclonal goat anti-rabbit IgG (1:200, Vector) in 0.1% T-PBS for 1 h at RT. All sections were then incubated with ABC solution (1:150) in T-PBS for 1 h at RT. Sections were rinsed in PBS for 10 min, transferred to a 0.05 M Tris-HCl solution containing 0.9% NaCl, and then incubated in a DAB solution (20 mg DAB/100 ml of 0.05 M Tris-HCl) for 5–10 min at RT. NiSO4 (0.15 g/100 ml Tris-HCl) was added to intensify the stain. Reactions were stopped by rinsing sections with cold Tris-HCl. All procedures were identical for the methodological controls, except that the T-PBS/serum/BSA solvent was used without primary antibody during the primary incubation step.

MOUNTING AND OBSERVATION. Sections were mounted onto gelatin/chromate-subbed slides and air dried. Sections were then dehydrated and cleared, and in some cases were counterstained with either neutral red or thionin. They were then placed under a coverslip using DPX mountant and examined using a Nikon Microphot-FXA microscope. Photomicrographs were taken using Kodak Ektachrome 64 color slide film and Kodak Technical Pan Film 5612.

RESULTS

Behavioral Results

Histology. Of the 55 animals tested, the brains of 49 were examined. The injection sites of 90% of these were within the LHA, consistent with the percentages in previous studies (41–44). The scores for animals with misplaced cannulas were eliminated from all data analyses. The appearance of the tissue (data not shown), was similar to that of subjects in previous studies, the histological photomicrographs of which have been published (44).

Experiment 1: Ifenprodil suppresses NMDA-elicited eating. The results from three separate experiments are summarized in Table 1. As shown in Table 1, and consistent with previous studies (42–44), LHA injection of 10 nmol of NMDA significantly stimulated food intake over vehicle baselines in each of the three groups, ranging from a low of 8.8 g to a high of 16.5 g within 30 min of injection, with the bulk of eating occurring during this period. Several animals were unresponsive to NMDA in group C, and these were screened out of all analyses.

As shown in Table 1, ifenprodil at 100 pmol, 1 nmol, and 10 nmol, but not higher or lower doses, consistently suppressed eating. For each group, two-way ANOVA (excluding vehicle-only scores) revealed significant effects of treatment on food intake (group A: F3,157 = 4.1, P = 0.007; group B: F3,159 = 13.4, P < 0.0001; group C: F3,99 = 6.9, P = 0.0003). Significant effects of time postinjection occurred only for group A (F3,183 = 3.8, P = 0.01), but the interaction between treatment and time postinjection for this group was not significant (P = 0.98). Neither the 100 pmol nor the 100 nmol doses of ifenprodil elicited eating behavior when injected alone (data not shown).

Because there were no significant differences in the eating elicited by NMDA alone in these three groups (F2,27 = 2.0, 1.2, 0.9, and 1.0 for all 0.5-, 1-, 2-, and 4-h NMDA-only scores; P > 0.1, 0.3, 0.4, and 0.3, respectively), the food intakes elicited by NMDA for 0.5, 1, 2, and 4 h postinjection were normalized across all three groups (see Methods) and were compared with the pooled, averaged intakes elicited by the combined ifenprodil and NMDA treatments at the same time points.

Figure 1 shows the results of this analysis. Specifically, as shown in Fig. 1A, one-way ANOVA of pooled percentage scores at 0.5 h postinjection yielded a significant effect of treatment (F6,99 = 9.0; P < 0.0001). Multiple comparisons revealed that 100 pmol, 1 nmol,
Figure 1B shows how these effects extended to later postinjection times. One-way ANOVAs revealed significant effects of treatment at 1 (F_{6,92} = 7.11; P < 0.0001), and 4 h postinjection (F_{6,92} = 3.83; P < 0.01). Post hoc comparisons revealed that 100 pmol of ifenprodil continued to significantly suppress NMDA-elicited eating at 1 (~63%) and 2 h (~64%) postinjection. The 1 and 10 nmol doses were also effective in suppressing NMDA-elicited eating at 1 h postinjection (~44% and ~60%, respectively) but not at 2 h postinjection. At 4 h postinjection no single dose was effective. In contrast to the suppressive effects of the doses above, the lowest doses of ifenprodil tested (1 and 10 pmol) as well as the highest dose (100 nmol) failed to suppress NMDA-elicited eating at any time postinjection (Fig. 1B).

Experiment 2: Ifenprodil fails to suppress KA-elicited eating. To determine whether ifenprodil's suppression of NMDA-elicited eating was behaviorally specific, we tested ifenprodil against eating elicited by another glutamate receptor agonist, KA. As shown in Fig. 2, and consistent with previous reports (42, 44), injection of KA at 1 nmol elicited eating that was statistically significant at 1–2 h postinjection. The new finding is that the 100 pmol dose of ifenprodil that was most effective in suppressing NMDA-elicited eating (expt. 1) did not suppress KA-elicited eating at any time point. Specifically, a two-way ANOVA revealed significant effects of treatment on food intake (F_{2,95} = 17.9; P < 0.0001) and of time postinjection (F_{3,95} = 4.1; P < 0.01), but not their interaction. Post hoc multiple comparisons revealed that 1 nmol KA, both with and without 100 pmol ifenprodil, elicited significant eating compared with vehicle (P < 0.05) and that ifenprodil pretreatment did not significantly reduce KA-elicited eating (Fig. 2).

Experiment 3: Ifenprodil suppresses food deprivation-elicited eating. To determine whether NR2A and/or NR2B NMDA-R subunits might participate in mediating natural eating behavior, animals deprived of food but not water for 24 h were tested with ifenprodil (100 pmol or 1 nmol). As shown in Fig. 3, animals ate as much as 16.1 g within 2 h postinjection. A two-way ANOVA revealed significant effects of treatment on food intake (F_{2,123} = 20.2; P < 0.0001) and of time postinjection (F_{3,123} = 14.8; P < 0.0001), but not their interaction. Multiple comparisons revealed that although 100 pmol of ifenprodil failed to suppress deprivation-elicited eating compared with vehicle (Fig. 3), 1 nmol of ifenprodil significantly suppressed deprivation-elicited eating by ~39% within 0.5 h postinjection (P < 0.05). At later time points, the suppression remained statistically significant, although the magnitudes of suppression attenuated with increasing postinjection interval (~31, 26, and 18% for 1, 2, and 4 h postinjection, respectively).

**Discussion.**

As shown in Fig. 4A, anti-rat NR1, NR2A, and NR2B antibodies each detected a major immunoreactive species at ~117, 186, and 184 kDa, respectively, in homogenates obtained from cortex and the LHA. Molecular mass estimates for these main bands pooled from

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**Figure 1. Trend of suppression by ifenprodil of eating elicited by N-methyl-D-aspartate (NMDA) in lateral hypothalamic area (LHA) in satiated rats.** This figure presents a combined summary of data obtained from 3 separate dose-response studies shown in Table 1. Across all 3 groups in Table 1, average intakes resulting from NMDA injection alone for 0.5, 1, 2, and 4 h postinjection were (mean ± SE) 12.6 ± 1.4, 12.9 ± 1.4, 13.8 ± 1.4, and 14.8 ± 1.2 g, respectively. Scores from animals eating in response to NMDA alone were combined, and mean values across 4 time points (0.5, 1, 2, and 4 h) were obtained. These scores were treated as 100%, and scores for animals eating in response to combined treatments of both ifenprodil (1, 10, and 100 pmol and 1, 10, and 100 nmol) and NMDA [hypothalamic NMDA receptors (15)] or to its effects at other targets (see Discussion).

**Methods**

Values for sample size in all experiments are shown in Table 1. For sample sizes of groups in Table 1, please refer to Methods.

**Experiment 1:** Ifenprodil supresses NMDA-elicited eating. To determine whether ifenprodil's suppression of NMDA-elicited eating was behaviorally specific, we tested ifenprodil against eating elicited by another glutamate receptor agonist, KA. As shown in Fig. 2, and consistent with previous reports (42, 44), injection of KA at 1 nmol elicited eating that was statistically significant at 1–2 h postinjection. The new finding is that the 100 pmol dose of ifenprodil that was most effective in suppressing NMDA-elicited eating (expt. 1) did not suppress KA-elicited eating at any time point. Specifically, a two-way ANOVA revealed significant effects of treatment on food intake (F_{2,95} = 17.9; P < 0.0001) and of time postinjection (F_{3,95} = 4.1; P < 0.01), but not their interaction. Post hoc multiple comparisons revealed that 1 nmol KA, both with and without 100 pmol ifenprodil, elicited significant eating compared with vehicle (P < 0.05) and that ifenprodil pretreatment did not significantly reduce KA-elicited eating (Fig. 2).

**Experiment 2:** Ifenprodil fails to suppress KA-elicited eating. To determine whether ifenprodil's suppression of NMDA-elicited eating was behaviorally specific, we tested ifenprodil against eating elicited by another glutamate receptor agonist, KA. As shown in Fig. 2, and consistent with previous reports (42, 44), injection of KA at 1 nmol elicited eating that was statistically significant at 1–2 h postinjection. The new finding is that the 100 pmol dose of ifenprodil that was most effective in suppressing NMDA-elicited eating (expt. 1) did not suppress KA-elicited eating at any time point. Specifically, a two-way ANOVA revealed significant effects of treatment on food intake (F_{2,95} = 17.9; P < 0.0001) and of time postinjection (F_{3,95} = 4.1; P < 0.01), but not their interaction. Post hoc multiple comparisons revealed that 1 nmol KA, both with and without 100 pmol ifenprodil, elicited significant eating compared with vehicle (P < 0.05) and that ifenprodil pretreatment did not significantly reduce KA-elicited eating (Fig. 2).

**Experiment 3:** Ifenprodil suppresses food deprivation-elicited eating. To determine whether NR2A and/or NR2B NMDA-R subunits might participate in mediating natural eating behavior, animals deprived of food but not water for 24 h were tested with ifenprodil (100 pmol or 1 nmol). As shown in Fig. 3, animals ate as much as 16.1 g within 2 h postinjection. A two-way ANOVA revealed significant effects of treatment on food intake (F_{2,123} = 20.2; P < 0.0001) and of time postinjection (F_{3,123} = 14.8; P < 0.0001), but not their interaction. Multiple comparisons revealed that although 100 pmol of ifenprodil failed to suppress deprivation-elicited eating compared with vehicle (Fig. 3), 1 nmol of ifenprodil significantly suppressed deprivation-elicited eating by ~39% within 0.5 h postinjection (P < 0.05). At later time points, the suppression remained statistically significant, although the magnitudes of suppression attenuated with increasing postinjection interval (~31, 26, and 18% for 1, 2, and 4 h postinjection, respectively).

**Discussion.**

As shown in Fig. 4A, anti-rat NR1, NR2A, and NR2B antibodies each detected a major immunoreactive species at ~117, 186, and 184 kDa, respectively, in homogenates obtained from cortex and the LHA. Molecular mass estimates for these main bands pooled from...
several experiments were 110.8 ± 4.9 kDa (n = 6), 182.6 ± 6.1 kDa (n = 6), and 180.9 ± 2.0 kDa (n = 5) for the NR1, NR2A, and NR2B subunits, respectively. To compare subunit expression levels in LHA with those in cortex, the same amount of protein homogenate (15 µg) from each region was loaded on the gels. NR1 expression was robust in both the LHA and cortex (Fig. 4A). In NR2A and NR2B immunoblots, LHA homogenates showed immunoreactivity that was moderate relative to those of cortex. Liver homogenates failed to show immunoreactivity to NR1, NR2A, or NR2B antibodies. Similar findings were obtained in four experiments examining cortex and LHA homogenates. Expression of NR2B in LHA was verified in homogenates from several rats (Fig. 4B; n = 7).

To examine the specificity of the NR2A and NR2B antibodies, blots containing homogenates of cortex, LHA or cerebellum and liver were immunoprobed with anti-rat NR2A or NR2B antibodies with and without previous absorption with the fusion protein used to produce them.
immunize rabbits. Figures 5 and 6 demonstrate that preabsorption of each antibody with its respective fusion protein blocked immunoreactivity at 193.2 kDa (NR2A) and at 182.6 kDa (NR2B), respectively. These data suggest that these bands are specific for NR2A and NR2B subunits. A lower molecular mass band at 148.5 kDa was sometimes observed in addition to the main immunoreactive species, depending on the lot of antibody used and the amount of protein loaded (Fig. 4). Preabsorption of the NR2B antibody also blocked this band (data not shown). Our data suggest that both bands on NR2B blots are specific for the NR2B subunit, with the heavier band representing the bulk of NR2B protein subunits. Snell and colleagues (40) have previously detected two NR2B immunoreactive bands in mouse hippocampus and HEK-293 cells transfected with NR2B cDNA as well as a third nonspecific band using the same anti-rat NR2B antibody. Finally, all lanes loaded with liver homogenates failed to show immunoreactivity to any of the subunit-specific antibodies (Figs. 4–6), and control blots processed without these antibodies showed no immunoreactivity at the molecular weights corresponding to NR2A or NR2B. In contrast, the NR2A subunit was detected in liver homogenates, with a molecular mass of 148.5 kDa (Fig. 4).
NR2B control blots (Fig. 6), the dark band at 49.2 kDa is probably due to nonspecific staining produced by the secondary antibody or ECL procedure.

Immunohistochemical Localization of NR2B NMDA-R Subunits in the LHA

As shown in Fig. 7, the polyclonal anti-NR2B antibody we used robustly labeled cells in and around the lateral hypothalamus. Cells displaying NR2B immunoreactivity were qualitatively identified as neurons by their close morphological resemblance to neurons previously identified as such within this region by a Golgi study (25). Specifically, many NR2B-immunoreactive cells were triangular or fusiform in shape, with somata that at times tapered into two or three dendritic trunks. Figure 7, inset, shows a high-magnification image of a good example of such a neuron. In contrast to the immunoreactivity observed in sections incubated with the anti-NR2B primary antibody, sections incubated without this antibody failed to show immunoreactivity (data not shown).

Given that the LHA is generally a cell-poor region, it is especially important that more than one dilution for a given primary antibody be tested, because it is likely that the combination of few cells and the possibility of too dilute a solution could yield false negatives. In our hands, the best titer for the anti-NR2B primary antibody used for immunohistochemistry was 1:1,000. Although immunoreactivity was observed in sections incubated with other titers (e.g., 1:500, 1:1,500), the 1:1,000 dilution yielded the best signal-to-noise ratio. Moreover, because of the paucity of cells within the parenchyma in this region, sections were lightly counterstained with thionin to increase the background for suitable photography. On the black-and-white images depicted in Fig. 7, this counterstain appears as stained cell nuclei that are slightly dark in the background, but the major label seen throughout the borders of the neurons profiled is NR2B immunoreactivity (a few of which are indicated by arrows in Fig. 7) and not the thionin counterstain. This was visually confirmed by light microscopy as well as by color photography (not shown), which allowed differentiation between brown (NR2B) and blue staining (cell nuclei). In addition to NR2B immunoreactivity within the LHA, immunoreactivity was also observed in the perifornical hypothalamus, paraventricular nucleus of the hypothalamus (PVN), the supraoptic nucleus, and in many nuclei of the thalamus (Khan, Curra´s, and Stanley, unpublished observations). The Western blot and immunohistochemical evidence for the presence of the NR2A and NR2B subunits is in agreement with previous studies examining subunit mRNAs and protein expression (6, 48).

In contrast to the NR2B-immunoreactivity we observed in frozen sections, no tested dilution (1:500, 1:1,000, 1:1,500, 1:3,000) of an affinity-purified polyclonal antibody targeted against the NR2A subunit revealed any staining. Although this may suggest that NR2A subunits are not present within the tissue examined, this seems unlikely given that areas such as the neocortex, well established to contain the NR2A subunit, also failed to show any staining and that use of the same lot of this antibody in our Western blot analysis of the LHA revealed a positive 183-kDa band corresponding to the NR2A subunit in the LHA on seven separate occasions. This also rules out the possibility that the lot of antibody was defective. Rather, it is more likely that the fixative in the immunohistochemistry procedures prevented the detection of staining in tissue sections, but not in the Western blotting procedures where fixation was not performed. Furthermore, an NR2A-specific fusion protein, when preabsorbed with the anti-NR2A antibody, abolished the NR2A signal on the immunoblot, suggesting that the antibody-antigen reac-
tion is highly specific. Similarly, the NR2B fusion protein data also suggests that the anti-NR2B antibody used was highly specific, arguing against the possibility that the positive results obtained with this antibody in frozen sections were artifactual.

**DISCUSSION**

The NR2A/NR2B subunit-selective antagonist ifenprodil significantly attenuated NMDA-elicited eating when injected within the LHA. This suppression suggests that feeding-related LHA NMDA-Rs may contain the NR2A and/or NR2B subunits. This possibility was supported by both immunoblotting and immunohistochemical evidence, in which affinity-purified polyclonal antibodies, each specific for one of the two subunits, reacted positively with ~180-kDa proteins within microdissected portions of the LHA, as well as with LHA cell bodies and neurites in situ.

2B or not 2B: which NMDA-R subunit is most likely to mediate ifenprodil’s feeding-suppressive effects? Ifenprodil, the chemical structure of which is shown in Fig. 2, is a phenylethanolamine derivative that noncompetitively antagonizes NMDA-Rs containing the NR2B subunit with a 400-fold higher affinity than those containing the NR2A subunit and has little or no effect on NR2C or NR2D subunit-containing NMDA-R complexes (Ref. 51; see Fig. 2 in Ref. 52). Despite ifenprodil’s preference for NR2B-containing NMDA-Rs over those containing NR2A, the concentrations of ifenprodil we injected might have affected NMDA-Rs containing either subunit. The 100 pmol dose most effective in attenuating NMDA-elicited eating was injected into the LHA as a ~330 µM solution. Although the concentration produced by 0.3 µl of this solution of ifenprodil at the relevant LHA receptors is certainly much lower, it might still have been greater than the reported 161 µM IC₅₀ value for ifenprodil’s low-affinity interaction with NR2A-containing NMDA-Rs (22). Although it is difficult to accurately extrapolate the in vivo binding of ifenprodil from these in vitro pharmacological data, we suggest that ifenprodil suppressed feeding primarily by acting as an antagonist of the NR2B subunit, with an additional possible contribution by antagonism of the NR2A subunit.

Was ifenprodil’s suppression of NMDA-elicited eating actually due to its antagonist actions on NMDA-Rs? This is an issue because ifenprodil may also act as an antagonist at voltage-dependent calcium channels (4), α₁-adrenergic receptors (5), and 5-HT₃ receptors (24), and also acts as a σ site ligand (50). Although actions at these sites cannot be ruled out in the present study, it is unlikely that antagonistic effects on 5-HT₃ or α₁-adrenergic receptors accounted for ifenprodil’s suppression of NMDA-elicited eating, because serotonin’s effects on food intake are widely documented as being inhibitory (see Ref. 23 for a review), as are those of α₁-adrenergic stimulation (8), and the identified locus for these effects is the PVN and not the LHA. Mediation by actions at other receptors or channels also appears unlikely because the dose of ifenprodil that was most effective in suppressing NMDA-elicited eating did not suppress eating elicited by KA. If the observed suppression of NMDA-elicited feeding were due to actions at other targets, then these same nonspecific interactions would likely have become manifest when ifenprodil was injected in conjunction with KA (i.e., KA-elicited eating should have been suppressed). Interestingly, we found that the highest dose of ifenprodil did not suppress NMDA-elicited eating. Although the reasons for this are unknown, one possibility is that the high concentration of ifenprodil may have blocked serotonin or α₁-adrenergic receptors after diffusion to the PVN; these effects could mask the suppressive effects of ifenprodil in the LHA.

Could ifenprodil have suppressed NMDA-elicited eating by debilitating the animals or by making them sick? This is unlikely because the highest dose of ifenprodil tested (100 nmol) did not suppress NMDA-elicited eating, which should have occurred had ifenprodil’s suppressive effects resulted from sensorimotor debilitation or malaise. More importantly, ifenprodil did not suppress eating elicited by LHA injection of KA, nor did the 100 pmol dose suppress food deprivation-elicited eating, demonstrating that ifenprodil-injected rats can exhibit robust feeding responses and are thus not debilitated. Furthermore, “carryover” effects of ifenprodil between tests or tissue damage from repeated ifenprodil injection should not have caused the observed feeding suppression, because all doses were injected in counterbalanced order. Collectively, these behavioral results suggest that ifenprodil’s actions were chemically specific to NMDA-Rs and behaviorally specific to eating.

We have previously shown that eating elicited by LHA injection of NMDA or by food deprivation can be attenuated by LHA injections of antagonists of the NMDA recognition site or the NMDA-R glycine and/or D-serine binding site (41, 43). The present finding that ifenprodil suppressed deprivation-elicited eating provides additional evidence for a physiological role of LHA NMDA-Rs in the control of food intake and further suggests that some LHA NMDA-Rs involved in the physiological control of food intake may contain NR2A and/or NR2B subunits.

This conclusion was supported by our immunoblotting detection of proteins of ~180 kDa within the LHA. That these are NR2A and NR2B subunits is supported by the antigenicity of the migrated bands for affinity-purified, polyclonal antibodies raised against each of these subunits. The antibody-antigen reactions were chemically specific, because 1) immunoreactive bands were absent from liver tissue and 2) from control assays without the primary antibodies, and 3) preabsorption of the anti-NR2A and anti-NR2B antibodies with their respective fusion proteins abolished immunoreactivity. Although the 183- and 181-kDa-molecular mass bands we identify as NR2A and NR2B, respectively, are similar to those reported by other laboratories (20, 27), there are also reported values of ~165 kDa for these subunits (18, 40), similar to that predicted from cDNA (14). This molecular mass discrepancy is likely due to posttranslational modifications of the subunits, includ-
ing N-linked or O-linked glycosylation and/or phosphor-
ylation, because deglycosylating agents have report-
redly shifted the electrophoretic mobilities of NR2A-
and/or NR2B-immunopositive bands from ~180 kDa to
~160 kDa (18, 27) and because these subunits have
several sites for N-glycosylation and/or phosphoryla-
tion (14). Our immunoblots also detected the NR1
subunit in the LHA, consistent with the reported
detection of NR1 mRNA in this region (47).

Petralia et al. (33) immunohistochemically identified
potential NR2A and/or NR2B subunits among several
areas of the rat brain, including the LHA. However,
their antibody could not distinguish between the NR2A
or NR2B subunits and cross-reacted to a certain extent
with NR2C and NR2D subunits. We now extend these
data to report immunohistochemical detection in the
LHA of the NR2B subunit using an affinity-purified
polyclonal antibody specific for this subunit. NR2B
immunoreactivity was typically observed within both
cell bodies and neurites, and the stained LHA cells had
somata that were generally triangular or fusiform,
with some somata tapering into two to three dendritic
trunks, typical of neurons characterized within this
region (25). These immunohistochemical results sug-
gest that the NR2B subunit is expressed by hypotha-
lamic neurons.

What are the precise subunit requirements for func-
tional NMDA-Rs, and how does varying subunit compo-
sition alter NMDA-R function? Electrophysiological
studies suggest that native NMDA-Rs contain the NR1
subunit and at least one member of the NR2 subtype.
Recombinant NMDA-R complexes have distinct subunit-
dependent functional signatures, including different
channel kinetics, affinities for agonists/antagonists,
and sensitivities to modulators (45). Functional charac-
teristics diagnostic of native NMDA-R function (re-
viewed in Ref. 49), including sensitivity to Mg2+
block, the slow onset/offset time courses of NMDA-R-mediated
currents predominately carried by Na+
and Ca2+, and the glycine and/or (putative) β-serine coagonist
requirement for receptor activation (also see Refs. 7
and 36); all vary characteristically among NMDA-Rs
with different subunit compositions. Electrophysiological
studies using recombinant NMDA-Rs reveal that
NR1/NR2A- and/or NR1/NR2B-containing NMDA-Rs
have a lower affinity for glutamate (14, 19; but see Ref. 21)
and are more sensitive to Mg2+ block (37) than
are NR1/NR2C-containing NMDA-Rs.

NMDA-R subunit composition can also influence
modulation by intracellular agents, many of which preferentially target one subunit over another. The NR2B subunit contains a site sensitive to phosphoryla-
tion by CaM kinase II (28), and the NR2A, NR2B, and
NR2D subunits are identified as targets of tyrosine
phosphorylation (3, 9, 20). That NR2A and NR2B
subunits are targets of tyrosine phosphorylation may be
of special interest, given our preliminary evidence
(17) that NMDA-elicited eating in the LHA is sup-
pressed by a protein tyrosine kinase inhibitor. Our
evidence that NR2A and/or NR2B subunits are likely to
be components of LHA NMDA-Rs mediating feeding is
consistent with a possible role for tyrosine phosphoryla-
tion in modulating the signal(s) transduced via these
receptors to trigger eating. To conclude, LHA NMDA-Rs
have been implicated in the control and regulation of
food intake and body weight, respectively (41, 43), and
AMP-dependent protein kinase (10–12) and putative
LHA tyrosine kinase(s) (17), both of which can poten-
tially modulate NMDA-R activity (20, 46), have also
been implicated in mechanisms of feeding control in or
near this region. We therefore speculate that tuning the
activity of LHA NMDA-Rs by altering their phosphory-
lation state may be a mechanism that ultimately
contributes to alterations in food intake and body
weight.

Perspectives

Research conducted during the last half of this
century has provided many insights concerning the
neurochemical controls of eating behavior. However,
little is known about the cellular mechanisms (both
biochemical and electrophysiological) that operate
within neural substrates controlling eating. A useful
step in identifying some of these mechanisms may be to
identify the precise subunit composition of those neu-
transmitter receptors implicated in the control of
eating. This may be important, in part, because differences
in the subunit composition of receptors can lead to
profound differences in their function, including the
precise means by which these receptors are coupled to
intracellular signal transduction systems. In this study,
we have used a combined biochemical, anatomic, and
behavioral approach to provide evidence for the pres-
ence of three subunits of the NMDA receptor in the
LHA (the NR1, NR2A, and NR2B subunits) and to
implicate two of them (the NR2A and/or NR2B
subunits) in the physiological control of food intake. These
three subunits have been shown by others (20, 28, 46)
to be preferentially coupled to particular intracellular
second messenger systems; at least two such systems
have been implicated in the control of LHA-mediated
eating (10–12, 17). This raises the intriguing possibil-
ity that these second messenger systems subserve
NMDA receptor-mediated signals controlling eating and
perhaps play a role in the neuronal plasticity that
is thought to underlie food-related learning within this
region (35).

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