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

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Khan, Arshad M., Margarita C. Currás, Jennifer Dao, Faizi A. J amal, Chuck A. Turkowski, Rishi K. Goel, Elizabeth R. Gillard, Stefany D. Wolfsohn, and B. Glenn Stanley. Lateral hypothalamic NMDA receptor subunits NR2A and/or NR2B mediate eating: immunochemical/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 and 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-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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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\(^+\), 154 Cl\(^-\), 3.0 K\(^+\), 1.2 Ca\(^2+\), and 0.9 Mg\(^2+\) 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, dianminobenzidine (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 food deprived for 24 h and then given bilateral, 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.
Food intake values were analyzed by a two-way general linear model ANOVA; one-way ANOVAs were performed only when two-way ANOVAs yielded significant results. Furthermore, where overall effects were significant, multiple comparisons were performed using the Student-Newman-Keuls test at an α of 0.05. For each data set, the mean value for NMDA-elicited eating was compared across the three studies by one-way ANOVA. Statistical analysis of the combined data set was as follows. Intakes resulting from injecting NMDA alone were compared across the three studies by one-way ANOVA. Because these scores were not significantly different, they were pooled, and a mean value for NMDA-elicited eating was obtained for each time point (0.5, 1, and 2 h postinjection). These means were assigned the value 100%, and the mean percent NMDA-elicited food intake was calculated for animals receiving ifenprodil treatments using the formula (eating elicited by ifenprodil with NMDA treatment/mean value for eating elicited by NMDA alone) × 100. Percentages obtained in this way for each dose of ifenprodil were compared by one-way ANOVA, and, when significant, these were followed by a Student-Newman-Keuls test.

Note that in this combined analysis, the percentage scores for a particular dose of ifenprodil were from all animals receiving that dose, regardless of the group they were in. Thus (please refer to Table 1) a comparison of the effects of all doses of ifenprodil, for example, at 0.5 h postinjection, utilized 1) the derived percentages from group C animals for the 1 and 10 pmol doses; 2) the percentages from group C and B animals for the 100 pmol dose; 3) the percentages from group B and A animals for the 1 and 10 nmol doses, and finally 4) the percentages of group A animals for the 100 nmol dose.

Western blot analysis of NR2A and NR2B subunits. Tissue harvest. Naïve adult, male Sprague-Dawley or Holtzman 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 microscissors and 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.

Microdissected tissue was placed in ice-cold protease inhibitor cocktail (10 mM Tris·HCl (pH = 7.4), 5 mM EDTA, 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.

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, Tenti-microliter aliquots of BSA (0.1–6.0 mg/ml) standards and of the homogenized samples were assayed by adding 2.0 ml of BCA. The tubes were then incubated in an oven at 67°C for 30 min. Color intensity was spectrophotometrically analyzed at a λ of 562 nm (visible light). From this assay, a standard curve was prepared from which the concentrations of protein in the tissue samples were derived using computer software (WindowChem, Berkland Software).

SDS-PAGE and Western blot analysis. Five- to twenty-microgram protein aliquots obtained using crude homogenates from each group were denatured with an equal volume of 2× sample buffer (pH 6.8) containing 125 mM Tris, 0.002% bromophenol blue, 10%β-mercaptoethanol, 40% glycerol, and 6% SDS for 20 min and subsequently stored at 4°C until use. Samples were loaded and run on 5–7% polyacrylamide gels and subjected to SDS-PAGE. Separated proteins were subsequently electrotransferred to PVDF blotting membranes in modified Towbin’s buffer for 2.5 h at room temperature (RT). Blots were blocked overnight at 4°C in PBS containing 0.05% Tween, 10% nonfat milk, and 1% BSA and then incubated for
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. PERFIGURION 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/101 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 hypothalamus 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 quick-frozen in powdered dry ice. Frozen 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 then 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: F3,187 = 4.1, P < 0.007; group B: F3,189 = 13.4, P < 0.0001; group C: F3,99 = 6.9, P = 0.0003). Significant effects of time postinjection occurred only for group A (F3,187 = 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 (F2,73 = 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 (F6,69 = 9.0; P < 0.0001). Multiple comparisons revealed that 100 pmol, 1 nmol,
and 10 nmol of ifenprodil significantly suppressed NMDA-elicited eating by 62.5, 56.4, and 58.9, respectively. In contrast, the 1 and 10 pmol and the 100 nmol doses of ifenprodil were ineffective. The ineffectiveness of the high dose (100 nmol) may be related to the biphasic nature of ifenprodil's antagonism on the NMDA-R (15) or to its effects at other targets (see Discussion).

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), 2 (F_{6,92} = 4.37; 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 161.1 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).

Western Blot Analysis of NMDA-R Subunits in the LHA

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

**Fig. 2.** Ifenprodil fails to suppress eating elicited by kainic acid (KA) in satiated rats. Mean ± SE cumulative food intake (in g) following injection of either vehicle (Veh), KA alone, or KA with ifenprodil is shown; n = 10 naive animals. Chemical structure depicted is that of ifenprodil (tartrate salt not shown). *Intakes significantly greater than those elicited by vehicle treatment alone, as revealed by one-way ANOVAs for each time point (P < 0.05). NS denotes that intakes resulting from KA alone or KA with ifenprodil are not significantly different from each other at any time point (P > 0.05).

**Fig. 3.** Ifenprodil (1 nmol) suppresses eating elicited by food deprivation. Mean ± SE cumulative food intake (in g) after bilateral injection of either vehicle, 100 pmol ifenprodil, or 1 nmol ifenprodil in food-deprived rats is shown; n = 11 naive animals. *Intake significantly smaller than those of rats injected with either vehicle or 100 pmol ifenprodil (P < 0.05) at matched time points, as revealed by 1-way ANOVAs for each time point. v Intakes significantly smaller than those of rats injected with either vehicle or 100 pmol ifenprodil (P < 0.05) at matched time points, as revealed by 1-way ANOVAs for each time point. Note that by 0.5 h postinjection, animals had food available to them for only 20 min (see Methods).
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, liver homogenates showed a moderately stained immunoreactive band at 193.2 kDa indicating the NR2A subunit. No staining was observed in the methodological control. In addition, liver homogenates failed to show NR2A immunoreactivity. Arrows indicate positions and sizes of molecular mass standards (in kDa): myosin (210), β-galactosidase (111), bovine serum albumin (84), and ovalbumin (46).

Fig. 4. Expression levels of NR1, NR2A, and NR2B subunits in lateral hypothalamus of ad libitum-fed rats. Crude homogenates obtained from microdissected cortex (Cor), liver (Liv), and lateral hypothalamus (LH) were loaded on a 7% polyacrylamide gel, resolved by SDS-PAGE, electrotransferred to polyvinylidene fluoride membranes, and immunoblotted using polyclonal antibodies specific for NR1, NR2A, or NR2B subunits. A: blots of 15-µg aliquots were immunoprobed using polyclonal antibodies specific for NR1, NR2A, or NR2B subunits. Lateral hypothalamus shows moderate expression of NR2A and NR2B subunits and robust expression of the NR1 subunit. However, expression levels of all 3 NMDA receptor (NMDA-R) subunits are lower in lateral hypothalamus relative to cerebral cortex. B: NR2B immunoblot of 20-µg protein homogenates of lateral hypothalamic tissue from 3 different rats. Arrows indicate molecular masses (in kDa) of immunoreactive bands estimated using prestained molecular mass standards. Each lateral hypothalamus lane represents homogenates obtained from a separate rat. No immunoreactivity for any of the subunits was detected in liver homogenates.

**Fig. 5. Specificity of NR2A antibody.** Homogenates of cortex, lateral hypothalamus, and liver containing 5, 20, and 20 µg protein, respectively, were loaded into separate lanes of a 6% acrylamide gel, subjected to SDS-PAGE, and analyzed by Western blotting. Blots were immunoprobbed with anti-NR2A antibody (left) or the same antibody preabsorbed with NR2A fusion protein (FP) (middle) or with no primary antibody (right). All 3 blots were incubated with peroxidase-labeled goat anti-rabbit IgG, and proteins were detected using electrochemiluminescence (ECL). Homogenates of cortex and lateral hypothalamus show a moderately stained immunoreactive band at 193.2 kDa indicative of NR2A subunit. Note that preabsorption of NR2A antibody with NR2A fusion protein abolishes immunoreactivity. No staining was observed in the methodological control. Arrows indicate positions and sizes of molecular mass standards (in kDa): myosin (210), β-galactosidase (111), bovine serum albumin (83), and ovalbumin (46).

**Fig. 6. Specificity of NR2B antibody.** Homogenates of cortex, cerebellum (CBM), and liver containing 5, 20, and 20 µg protein, respectively, were loaded into separate lanes of a 6% acrylamide gel, subjected to SDS-PAGE, and analyzed by Western blotting. Blots were immunoprobbed with anti-NR2B antibody (left) or the same antibody preabsorbed with NR2B fusion protein (middle) or with no primary antibody (right). All 3 blots were incubated with peroxidase-labeled goat anti-rabbit IgG, and proteins were detected using ECL. Homogenates of cortex show immunoreactivity at 182.6 kDa indicative of NR2B subunit. Adult cerebellum and liver homogenates show little or no staining, respectively. Note that preabsorption of the NR2B antibody with NR2B fusion protein abolishes immunoreactivity. No staining was observed in the methodological control. Arrows indicate positions and sizes of molecular mass standards (in kDa): myosin (202), β-galactosidase (116), and bovine serum albumin (84).
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 nuclei 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 we 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 phenoylethanolamine 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 ω-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 phosphorylation, because deglycosylating agents have reportedly 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 phosphorylation (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 suggest that the NR2B subunit is expressed by hypothalamic neurons.

What are the precise subunit requirements for functional NMDA-Rs, and how does varying subunit composition 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 characteristics diagnostic of native NMDA-R function (reviewed in Ref. 49), including sensitivity to Mg$^{2+}$ block, the slow onset/offset time courses of NMDA-R-mediated currents predominately carried by Na$^{+}$ and Ca$^{2+}$, and the glycine and/or (putative) δ-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 Mg$^{2+}$ 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 phosphorylation 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 suppressed 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 phosphorylation 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 cAMP-dependent protein kinase (10–12) and putative LHA tyrosine kinase(s) (17), both of which can potentially 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 phosphorylation 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 neurotransmitter 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 presence 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 possibility 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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