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ORIGINAL ARTICLE

Activation of orexin neurons through non-NMDA glutamate receptors evidenced by c-Fos immunohistochemistry

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Abstract Orexin neuropeptides participate in the regulation of feeding as well as the regulation and maintenance of wakefulness and the cognitive functions. Orexin A and B share a common precursor, prepro-orexin and neurons are localized in the lateral hypothalamus. Physiological studies showed that these neurons are regulated by glutamatergic innervations. We aimed to assess the effects of kainic acid as a potent agonist for non-NMDA glutamate receptors in the activation of orexin neurons. We also analyzed the effect of glutamate antagonist CNQX, injected prior to kainic acid, on this activation. Expression of c-Fos protein was used as a marker for neuronal activation. Dual immunohistochemical labeling was performed for prepro-orexin and c-Fos and the percentages of c-Fos-expressing orexin neurons were obtained for control, kainic acid, and CNQX groups. Kainic acid injection caused statistically significant increase in the number of c-Fos-positive neurons when compared to control group (62.69 and 36.31%, respectively). Activation of orexin neurons was blocked, in part, by CNQX (43.36%). In the light of these results, it is concluded that glutamate takes part in the regulation of orexin neurons and partially exerts its effects through non-NMDA glutamate receptors and that orexin neurons express functional non-NMDA receptors.

Keywords Prepro-orexin · Kainic acid · CNQX · Kainate · AMPA · Hypothalamus

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Introduction

Orexin neuropeptides, also known as hypocretins, were originally identified with their regulatory effects on feeding [1, 2] but following reports emphasized on their primary role in regulating and maintaining wakefulness and the cognitive functions [1, 3]. Orexins are also known to participate in the control of diverse systems, including the cardiovascular system, neuroendocrine system, and in energy expenditure. Orexin A and B are derived from a common precursor: prepro-orexin. The neurons expressing prepro-orexin mRNA or protein are localized in the lateral hypothalamus, especially concentrating in the perifornical area, posterior hypothalamic area, and to some extent in the dorsomedial hypothalamic nucleus [4, 5].

Glutamate is the major excitatory amino acid neurotransmitter in the hypothalamus [6, 7] and its receptor subunits are widely expressed in most hypothalamic nuclei as well as hypothalamic areas [8, 9]. Glutamatergic signaling is mediated through two major families of receptors: metabotropic and ionotropic receptor. Kainic acid is a common agonist for both AMPA and kainate receptors and has been used to investigate the physiological, pharmacological, and functional properties of non-NMDA receptors [10].

c-Fos protein is the product of a transiently expressed *c-fos* gene and immunohistochemical detection of c-Fos expression has commonly been used to distinguish activated neurons from non-activated ones in the central nervous system including hypothalamus [11].

The aim of the present study was to assess the effect of kainic acid, as the agonist for non-NMDA glutamate receptors, in activating the orexin neurons in the lateral hypothalamus using dual immunohistochemistry for orexin and c-Fos proteins. In order to determine the specificity of

this possible effect, a non-NMDA glutamate receptor antagonist, 6-cyano-7-nitroquinoxalone-2,3-dione (CNQX), was administered prior to kainic acid injection and any decrease in the number of c-Fos-positive orexin neurons due to the blocking effect of CNQX on kainic acid-induced neuronal activation was examined.

Materials and methods

All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Uludag University.

Animals

Adult female Sprague–Dawley rats were obtained from the Uludag University Laboratory Animal Breeding, Usage and Research Center at which the animals were kept in a light- and temperature-controlled facility (12:12 h light–dark cycle with lights on at 7:00 am) with food and water freely available. Animals were allocated into four groups ($n = 5$/group). First group received saline (ip) as the control group, second group received kainic acid (2.5 mg/kg, ip), third group received higher dosage of kainic acid (10 mg/kg, ip) while the fourth group received CNQX (1 mg/kg, ip) 15 min prior to kainic acid injection (2.5 mg/kg, ip). All injections were done between 9:00 and 11:00 am. Ninety minutes after the last injection, animals were deeply anesthetized and fixed by transcardial perfusion with 4% paraformaldehyde in 0.13 M Sorenson's phosphate buffer, pH 7.4 (400 ml/animal). Brains were removed and post-fixed overnight in the same fixative. Fifty-micrometer-thick serial sections throughout the hypothalamus were cut with a vibratome and collected into Tris–HCl buffer (0.05 M, pH 7.6). After thoroughly washing in Tris–HCL buffer the sections were kept in cryoprotectant solution at −20°C.

Prepro-orexin immunohistochemistry

Sections were equilibrated to room temperature, washed in Tris–HCL buffer and incubated in blocking buffer (10% horse serum, 0.2% triton X-100, and 0.1% sodium azide in Tris–HCl buffer) for 2 h in order to prevent non-specific binding. Following the blocking step, the sections were incubated in 1:250 dilution of anti-prepro-orexin antibody (Lot number: 0507005530; Chemicon International Inc., Temecula, CA) for 48 h at room temperature. Anti-prepro-orexin is an affinity purified rabbit polyclonal antibody raised against a 17 amino acid peptide mapping near the C-terminus of mouse prepro-orexin. After the primary antibody incubation the sections were washed in Tris–HCl buffer and incubated for 2 h in secondary antibody (Biotin-SP-conjugated donkey anti-rabbit IgG, 1:300; Jackson Immunoresearch Labs, West Grove, PA). Blocking buffer (above) was used for the dilutions of all the antibodies. Sections were then washed in Tris–HCl buffer and incubated in avidin–biotin complex (ABC Elite Kit, Vector Labs, Burlingame, CA), prepared according to the manufacturer's instructions, for 60 min. Following three washes, 5 min each, in Tris–HCl buffer, sections were stained with diaminobenzidine with the presence of hydrogen peroxide (50 mg and 5 µl/100 ml Tris–HCl buffer, respectively). Sections were then transferred into Tris–HCl buffer, washed, mounted onto glass slides, air-dried, and cover slipped with DPX.

Dual immunohistochemistry with c-Fos

c-Fos immunohistochemistry was performed prior to prepro-orexin immunohistochemistry. Before the primary antibody incubation for c-Fos, the sections were prepared as mentioned above. Sections were then incubated in rabbit anti-c-Fos antibody at a dilution of 1:20,000 (Oncogene, Cambridge, MA) overnight at room temperature. This step was followed by Tris–HCl buffer washes and secondary antibody incubation using biotin conjugated donkey anti-rabbit IgG (1:300, Jackson Immunoresearch Labs, West Grove, PA) for 2 h. Peroxidase-labeled avidin–biotin complex was structured with the secondary antibody using ABC Elite Standard Kit (Vector Labs, Burlingame, CA). The visualization of the immunocomplex was obtained with diaminobenzidine (25 mg) and nickel ammonium sulfate (2 g) in the presence of 2 µl hydrogen peroxide in 100 ml Tris–HCl buffer. Finally, the sections were thoroughly washed in Tris–HCl buffer before the initiation of the prepro-orexin immunohistochemistry as explained above. Double-stained sections were then transferred into Tris–HCl buffer, washed, mounted onto glass slides, air-dried, and cover slipped with DPX. Negative control experiments included omission of primary or secondary antibodies.

Analysis

Sections were analyzed and photographed with Olympus BX50 photomicroscope equipped with a digital camera (Olympus DP-71, CCD color camera, 1.5 million pixels), attached to a computer. Cell counting was manually performed bilaterally (using 40× objective) on four consecutive sections for each animal taken from the same coordinates according to the brain atlas by Paxinos and Watson [12]. Selected sections were about 350 µm apart from each other (between Bregma −2.40 and −3.48) and

included the lateral hypothalamic area, perifornical area, and the posterior hypothalamic area where the orexin neurons are concentrated. First the immunolabeled orexin neurons were identified and then the presence or the absence of c-Fos reaction in the neuron was recorded. Once the total number of orexin neurons and the number of dual-labeled neurons were obtained, percentages of c-Fos-positive orexin neurons over all the identified orexin neurons were calculated for each animal. In order to analyze any possible dose–response effect of kainic acid, the percentages of the activated neurons in two groups which either received 2.5 or 10 mg/kg kainic acid were compared using Mann–Whitney test. The percentages in the experimental groups of control vs kainic acid (2.5 mg/kg) or CNQX vs kainic acid (2.5 mg/kg) were statistically compared using one-way ANOVA followed by Student–Newman–Keuls test and the $P < 0.05$ was set as the statistical significance value.

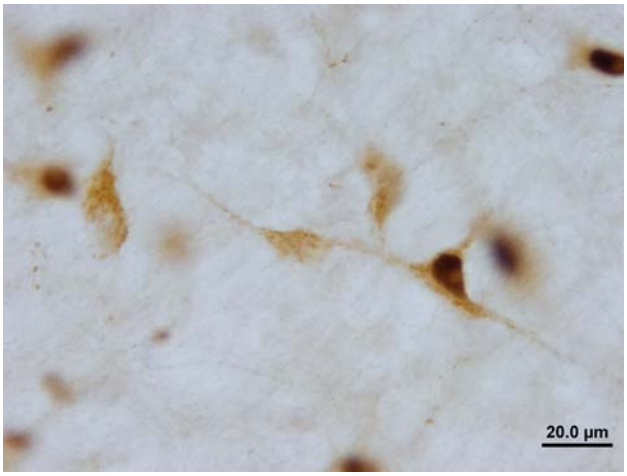

Fig. 2 Activated orexin neurons were identified by the presence of nuclear labeling in the prepro-orexin-positive neurons (neuron on the right). c-Fos-negative neurons were identified with cytoplasmic staining lacking the nuclear labeling (neuron on the left)

Results

Orexin neurons were identified by the presence of prepro-orexin immunoreaction in the cytoplasm revealed by the presence of a brown immunoprecipitation. The distribution pattern of the prepro-orexin-positive neurons was in agreement with the previous reports. Thus, the orexinergic neurons were localized in the lateral hypothalamus concentrating in the perifornical area and scattered neurons were detected spanning the posterior hypothalamic area (Fig. 1). c-Fos immunoreaction was localized in the nuclei with a dark brown-black chromogen reaction and the activated orexin neurons were differentiated by the presence of the nuclear staining from the inactivated neurons (Fig. 2). Injection of kainic acid-induced c-Fos expression in many hypothalamic neurons including the neurons in the lateral hypothalamus. Dual-immunohistochemistry for prepro-orexin and c-Fos revealed that orexin neurons express c-Fos protein in control (Fig. 3a) as well as in kainic acid-injected group (Fig. 3b). When analyzed, the obtained data showed that about 36% of the orexin neurons are c-Fos-positive in the control group. Following the kainic acid (2.5 mg/kg) administration the number of orexin-positive neurons increased to about 62%. Higher dose of kainic acid increased the number of c-Fos expressing neurons to about 68%. When two dosages of kainic acid were compared, Mann–Whitney test revealed no significant differences. Administration of CNQX prior to 2.5 mg/kg kainic acid injection, caused a partial reduction in the number of double-labeled neurons (Fig. 3c). Thus, the number of c-Fos-positive orexin neurons decreased to about 43% in the CNQX group. One-way ANOVA revealed significant changes among groups ($F = 34.851$, $P < 0.0001$). The Student–Newman–Keuls test showed that, about onefold increase in the number of activated orexin neurons in kainic acid group is statistically significant when compared to the control group ($P < 0.001$). Likewise the decrease in the number of c-Fos-positive orexin neurons in the CNQX group was proved to be significant when compared to kainic acid group ($P < 0.001$). Statistical analysis detected no significant differences between control and CNQX group. These data are summarized in Table 1 and Fig. 4.

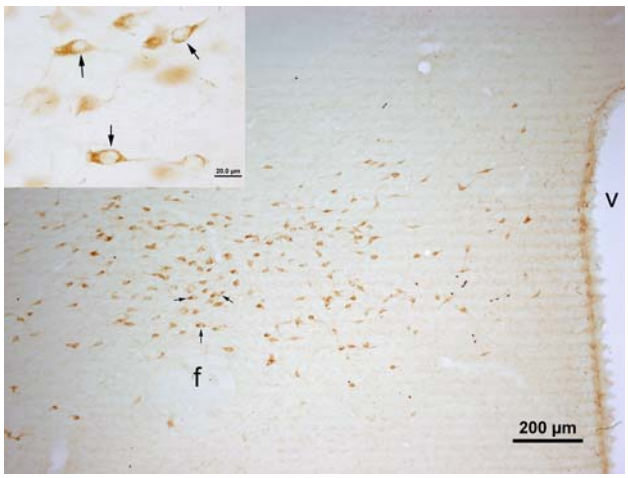

Fig. 1 Distribution of prepro-orexin-positive neurons in the hypothalamus. Note the preferential localization in the perifornical area (v: third ventricle, f: fornix). Higher magnification of the neurons (*arrows*) is given with the inlet figure

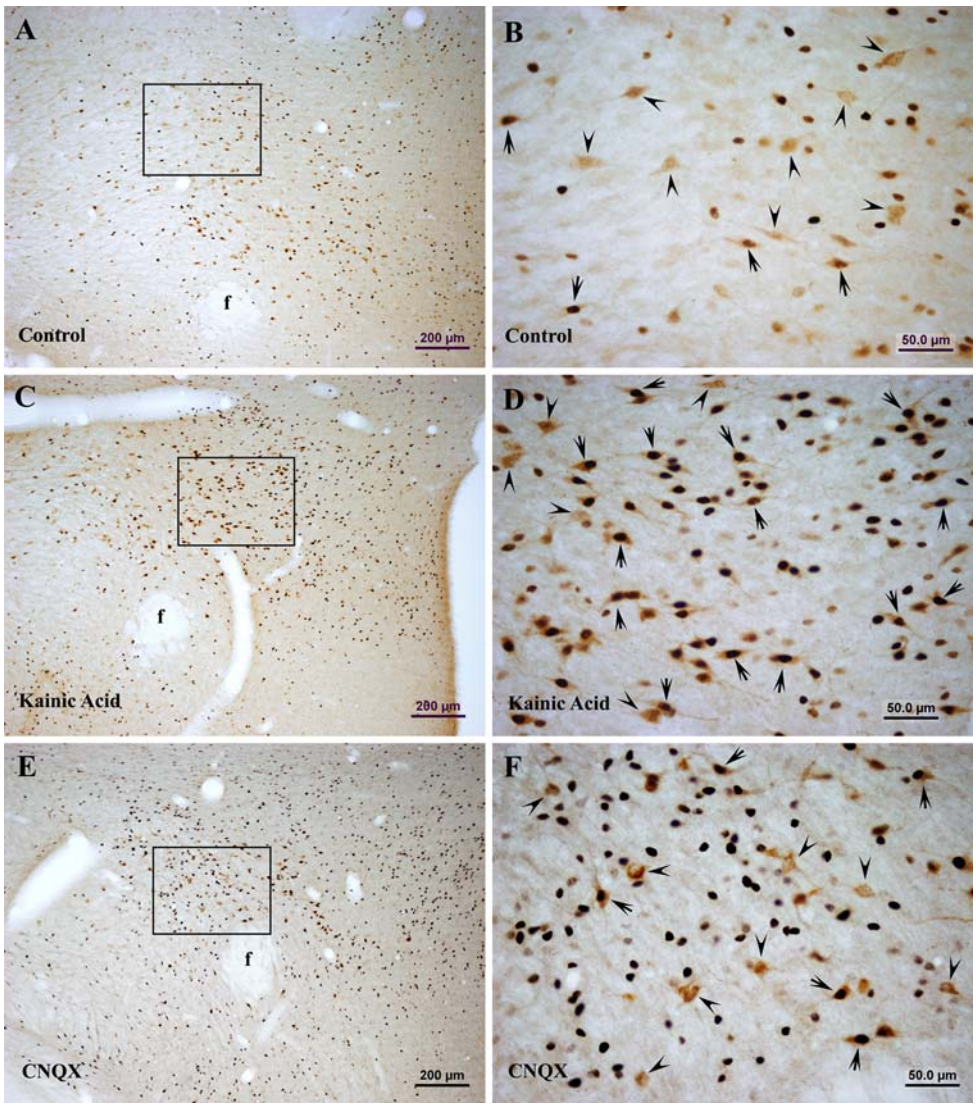

Fig. 3 Representative digital photographs taken from control (**a**, **b**), kainic acid (**c**, **d**), or CNQX (**e**, **f**) groups. Photographs are taken from the perifornical area for all groups. Photographs on the right (**b**, **d**, **f**) are taken at higher magnification of the representative area in boxes on the left photographs (**a**, **c**, **e**). *Arrows* indicate c-Fos-positive orexin neurons while *arrowheads* show c-Fos-negative orexin neurons

Table 1 c-Fos expression in orexin neurons in the hypothalamus

Groups	Mean number of orexin neurons counted per animal	Mean number of orexin neurons expressing c-Fos per animal	Mean percentage of orexin neurons expressing c-Fos per animal
Control	534 ± 48.46	196.6 ± 26.21	36.31 ± 2.05
Kainic acid (10 mg/kg)	463 ± 48.40	315.4 ± 38.35	68.28 ± 4.47[§]
Kainic acid (2.5 mg/kg)	473.6 ± 44.65	295.4 ± 25	62.69 ± 1.44***
CNQX	683 ± 110.16	309.6 ± 65.76	43.36 ± 3.12

Data are summarized as the mean ± SEM number or percentage of neurons in the hypothalamus ($n = 5$/each). When the effects of two different dosages of kainic acid compared (2.5 vs. 10 mg/kg) no statistically significant differences were detected ([§] Mann–Whitney test, $P > 0.5$)

ANOVA followed by Student–Newman–Keuls test revealed statistical significance for the percentages between kainic acid (2.5 mg/kg) and control groups, as well as between kainic acid (2.5 mg/kg) and CNQX groups (*** $P < 0.001$)

Discussion

The results of this study demonstrated that a subgroup of orexin neurons in the rat hypothalamus are stimulated and became active when challenged with kainic acid. In this study, immunohistochemical labeling for prepro-orexin revealed a similar distribution pattern of neuronal perikarya compared to the localization mosaic of prepro-orexin-expressing neurons [13]. It is well established in the literature that the projections from these neurons diffusely

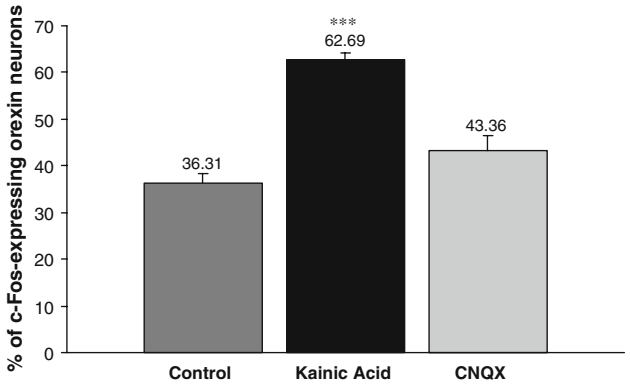

Fig. 4 Graphical representation of the quantitative data. Statistically significant chances were detected in the percentages of c-Fos-positive orexin neurons between the control and kainic acid (2.5 mg/kg) as well as between the kainic acid and CNQX groups (*** $P < 0.001$)

spread in many central nervous system areas emphasizing their diverse physiologic functions [5, 14].

The knowledge has now well established that glutamate is readily present and is the major excitatory amino acid neurotransmitter in the hypothalamus. Glutamate exerts its effects through ionotropic and/or metabotropic receptors. Different glutamate agonists have been used to dissect out the functional differences between NMDA and non-NMDA ionotropic glutamate receptor subfamilies. Based on their preferential binding of glutamate agonists, non-NMDA receptors are classified into α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate receptors [15, 16]. Kainic acid is a potent agonist working at the glutamate receptors of non-NMDA type. Ionotropic glutamate receptors, under most circumstances are formed as pentamers composed of several subunits [17, 18].

In the present study, the functional influences of kainic acid in activating the orexin neurons, which possibly express glutamate receptors, were assessed by c-Fos immunohistochemistry. In these experiments, the detection of the transient expression of c-Fos protein is used as a marker of neuronal activity. c-Fos, which is not expressed under basal conditions, is synthesized in response to stimulus-induced activation and c-Fos immunoreactivity could be detected within 60–90 min following the stimulus. Although a subgroup of orexin neurons was reported to express c-Fos in order to maintain the wakefulness and exhibit diurnal variation in activation [19, 20] as seen in the control group of this study, the number of activated neurons increases under different circumstances such as fasting [21], lactating [22], noxious stimulus [23], sleep deprivation [24] or following the administration of diverse substances including nicotine [25] or antipsychotic drugs [26]. Previous studies from our laboratory revealed that the oxytocinergic neurons express c-Fos, hence activate, following kainic acid administration [27]. Similarly the results presented above divulged that kainic acid (2.5 mg/kg) significantly increased the number of activated orexin neurons in the hypothalamus. About onefold increase in the number of these neurons support the presence of glutamatergic influence on the orexinergic system. Administration of higher dosage of kainic acid slightly increased the number of orexin neurons which express c-Fos but this increase was not statistically significant. Since no dose–response effect was detected we make further comparisons with the group receiving 2.5 mg/kg kainic acid, since 10 mg/kg dosage is mostly used for inducing status epilepticus in rats.

Lines of evidence emphasize the role of glutamate in the regulation of orexin neurons. Using hypothalamic slices taken from transgenic mice that express GFP-only in orexin neurons, Li et al. [28] as well as Yamanaka et al. [29] showed that the applications of glutamate agonists, AMPA or NMDA, stimulate orexin neurons, by recording the neuronal depolarization. Glutamatergic innervations further supported by histological demonstration of vesicular glutamate transporter 2 protein-containing axon terminals contacting orexin neuronal perikarya [28, 30]. These findings, suggesting the presence of functional non-NMDA receptors on orexin neurons, are in agreement with the results presented in this study. Thus, in order to activate orexin neurons kainic acid, a non-NMDA receptor agonist, possibly binds to functional glutamate receptors expressed in orexin neurons. Also in our study, the application of non-NMDA receptor antagonist CNQX prior to kainic acid decreased the number of activated orexin neurons compared to kainic acid injection alone. This finding further supports the idea that the functional glutamate receptors of non-NMDA type participate in the regulation of orexin neurons. Blocking of the glutamatergic excitatory synaptic activity by the application of CNQX, as reported by Li et al. [28], is in agreement with our findings. The important question then is whether the glutamatergic effects are directly through the receptors expressed by the orexin neurons or by an indirect mechanism involving glutamate-receptive interneurons. In order to support a direct mechanism in the glutamatergic regulation of the orexin neurons, it is necessary to reveal if the glutamate receptors are expressed in the orexin neurons. In situ hybridization studies showed the presence of glutamate receptor-expressing neurons in the lateral hypothalamus, including the perifornical area [8, 9]. This suggests that, at least some of these glutamate receptor-expressing neurons in the lateral hypothalamus may be orexin neurons or glutamate-receptive interneurons that are localized in close proximity to orexin neurons. Dual immunohistochemistry studies are underway in order to demonstrate the expression of ionotropic glutamate receptor subunits in orexin neurons, which also would reveal possible subunit combination in forming

functional receptors. Since it is known that kainic acid stimulates many neurons in the central nervous system, the activation effects of kainic acid on orexin neurons may also be mediated through non-NMDA receptors expressed in the neurons projecting to the orexinergic neurons by an indirect mechanism. The presence of vesicular glutamate transporter protein-immunoreactive axonal endings contacting the orexin neurons [30] suggests that the indirect mechanism, at least in part, is possible. Direct and/or indirect, the influence of a glutamate agonist on neuronal activation suggests that, glutamate-receptive orexin neurons, in turn may initiate orexinergic modulation in the central nervous system. The possible neurons innervated by the glutamate-activated orexin neurons include the neurons in the basal forebrain, brainstem, and/or thalamocortical areas in all of which orexinergic effects were demonstrated to regulate arousal [31–33]. It is also suggested that orexin neurons regulate neuroendocrine homeostasis. This influence is mostly through innervating other hypothalamic nuclei, including arcuate nucleus [13, 34]. Since orexin fibers cannot be demonstrated in the median eminence, it is suggested that orexin neurons indirectly influence pituitary functions by controlling the activity of neurons which regulate pituitary hormone secretion [34].

In conclusion, the results of the present study showed that the activation of orexin neurons by a glutamate agonist is possible, and suggested that this effect of kainic acid is mediated through non-NMDA glutamate receptors.

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