N-Methyl-d-aspartate receptor plasticity in kindling: Quantitative and qualitative alterations in the N-methyl-d-aspartate receptor–channel complex

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Communicated by Irving T. Diamond, July 24, 1989

ABSTRACT Kindling is an animal model of epilepsy and neuronal plasticity produced by periodic electrical stimulation of the brain. Electrophysiologic studies indicate that this phenomenon is associated with increased participation of N-methyl-D-aspartate (NMDA) receptors in excitatory synaptic transmission. Biochemical studies suggest that a change intrinsic to the NMDA receptor–channel complex may contribute to the increase in NMDA receptor-mediated synaptic transmission. We tested this idea by measuring the binding of 3-
[(+)-2-(carboxypiperazin-4-yl)](1,2-3H)propyl-1-phosphonic acid ([3H]CPP), [3H]glycine, and tritiated N-
[(1-thienyl)cyclohexyl]piperidine ([3H]TCP) to rat hippocampal membranes. In this preparation these ligands are selective for the NMDA receptor, the strychnine-insensitive glycine receptor, and the NMDA receptor-gated ion channel, respectively. Kindling increased the density of CPP, glycine, and TCP binding sites in hippocampal membranes by 47%, 42%, and 25%, respectively. No significant changes were detected in the affinity of these binding sites. Surprisingly, alterations in the glycine binding site were detected in animals sacrificed 1 month but not 1 day after the final kindling stimulation. Thus, delayed upregulation of the NMDA receptor–channel complex may be one molecular mechanism that maintains the long-lasting hyperexcitability of hippocampal neurons in kindled animals.

Kindling, a phenomenon produced by repeated administration of a focal electrical stimulus to the brain, is a widely studied animal model of epilepsy and neuronal plasticity. Initial stimulations result in little or no seizure response, but subsequent stimulations eventually result in intense limbic and clonic motor seizures (1). Once established, this hyperexcitable state may endure for the life of the animal.

Enhancement of excitatory synaptic transmission may be one mechanism underlying this enduring hyperexcitability. A recent study demonstrated that kindling is associated with increased synaptic activation of N-methyl-D-aspartate (NMDA) receptors in the hippocampal formation (2). Studies by our group indicate that kindling may bring this change about, at least in part, by increasing the sensitivity of hippocampal neurons to NMDA receptor agonists (3, 4). These results suggest that an alteration intrinsic to the NMDA receptor may contribute to the electrophysiologic (2, 3) and biochemical (4) findings.

NMDA receptor-mediated neurotransmission operates by opening a calcium-permeable channel. Activation of this channel is controlled by ligands that act at multiple sites on the receptor–channel complex (5–7). One of these sites is a strychnine-insensitive glycine binding site (8, 9). Glycine potentiates the opening of the cation channel by NMDA receptor agonists (8) and indeed may be required for their action (9). If an alteration in the NMDA receptor is present in kindled animals, then this alteration could reside in the recognition sites for either glycine or NMDA. To begin testing these possibilities, we measured ligand binding to the NMDA and glycine sites with 3-(+)-2-(carboxypiperazin-4-yl)](1,2-[3H]propyl-1-phosphonic acid ([3H]CPP) (a competitive NMDA antagonist) and [3H]glycine. Tritiated N-
[(1-thienyl)cyclohexyl]piperidine ([3H]TCP), an NMDA channel blocker whose binding is a reflection of channel activation, was used to measure NMDA and glycine activation of the channel and to quantify the number of NMDA receptor-gated channels (10–12).

METHODS

Kindling. Adult male Sprague-Dawley rats (Charles River Breeding Laboratories) underwent stereotaxic implantation of a bipolar electrode in the right amygdala under pentobarbital anesthesia (13). After a postoperative recovery period of at least 7 days, animals were stimulated twice daily (a 1-sec train of 1-msec biphasic rectangular pulses delivered at a frequency of 60 Hz) at an intensity 100 μA above the initial threshold afterdischarge. Stimulations were administered until the animals had exhibited at least three class 4 or class 5 seizures consisting of forelimb clonus, rearing, and/or falling (14). Control animals underwent electrode implantation but were not stimulated.

Dissection and Membrane Preparation. Rats were sacrificed either 24 hr or 28–32 days after the last electrically evoked seizure and the hippocampi were removed by blunt dissection. Each pair of hippocampi was homogenized with a Polytron (setting no. 6 for 20 sec) in 10 ml of an ice-cold 50 mM Tris acetate buffer (pH 7.7) that contained 10 mM EDTA. After centrifugation (23,000 × g for 20 min at 4°C), the membranes were washed by eight additional cycles of homogenization, resuspension in fresh buffer, and centrifugation. The membranes were frozen in a methanol bath chilled with solid CO2 and thawed at room temperature prior to the third and fourth centrifugations. Prior to the fifth centrifugation, the membranes were incubated for 15 min at 37°C. The fifth through ninth washes were performed with a 5 mM Tris acetate buffer (pH 7.2) that did not contain EDTA. Membranes were stored frozen after the third centrifugation (−70°C) until the day of the binding experiment. These steps were necessary to sufficiently reduce endogenous free amino acids (10).

[3H]Glycine Binding. [3H]Glycine binding was measured in 1 ml of 5 mM Tris acetate buffer (pH 7.2). Membranes were

Abbreviations: NMDA, N-methyl-D-aspartate; [3H]CPP, 3-(+)-2-(carboxypiperazin-4-yl)](1,2-[3H]propyl-1-phosphonic acid; [3H]-TCP, tritiated N-
[(1-thienyl)cyclohexyl]piperidine.

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incubated with the radioligand at 25°C for 40 min and sedimented by centrifugation at 12,000 × g for 15 min. Specific binding, defined as that component of total binding displaced by 1 mM nonradioactive glycine, accounted for 50% of total binding at a [3H]glycine concentration of 100 nM. Glycine binding was linear with respect to protein concentration and reached steady state by 25 min.

Saturation binding isotherms were constructed with eight concentrations of [3H]glycine ranging from 10 to 1000 nM. Membranes prepared from hippocampi of a single animal were sufficient to generate a complete binding isotherm with duplicate measurements at each concentration.

[^3H]CPP Binding. [^3H]CPP (NEN/DuPont) binding was measured in 1 ml of 50 mM Tris acetate buffer (pH 7.4). Membranes were incubated with the radioligand at 25°C for 40 min and were sedimented by centrifugation at 12,000 × g for 15 min. Specific binding, defined as that component of total binding displaced by 1 mM glutamate, accounted for 60% of total binding at a [^3H]CPP concentration of 40 nM.

Saturation binding isotherms were constructed with eight concentrations of [^3H]CPP ranging from 10 nM to 1000 nM. Membranes prepared from both hippocampi of a single animal were sufficient to generate a single binding isotherm with duplicate measurements at each concentration.

[^3H]TCP Binding. [^3H]TCP (NEN/DuPont) binding was measured under nonequilibrium and equilibrium conditions in different experiments. Nonequilibrium measurements of [^3H]TCP binding are sensitive to alterations in the kinetics of channel activation, the affinity of [^3H]TCP for its binding site, and the number of channel binding sites being activated (10–12). Equilibrium measurements can distinguish alterations in the affinity of [^3H]TCP for its binding site and alterations in the number of channel binding sites; equilibrium measurements are not affected by alterations in the kinetics of channel activation.

Nonequilibrium binding was measured in 1 ml of 5 mM Tris acetate buffer (pH 7.2). Membranes were incubated with 2.5 nM [^3H]TCP, 5 μM NMDA, and various concentrations of glycine for 16 min at 25°C. Specific binding was defined as that component of total binding displaced by 1.25 μM nonradioactive TCP. Reactions were terminated by vacuum filtration with a Skatron cell harvester. [^3H]TCP binding to filters was reduced by pretreatment with 0.075% polyethyl- enimine. After vacuum filtration, the filters were rinsed for 10 sec with ice-cold buffer.

Binding isotherms were generated under equilibrium conditions by using eight concentrations of [^3H]TCP (ranging from 0.6 to 60 nM), 3 μM glycine, and 30 μM NMDA; incubations were continued for 8 hr at 25°C to reach equilibrium. Specific binding was defined as that component of total binding displaced by 500 times excess nonradioactive TCP. Binding reactions were terminated as described above.

**Analysis of Radioligand Binding Data.** Measures of affinity (K_d) and number of binding sites (B_max) were obtained by using the curve-fitting program LIGAND (15). Initial seed values for the iterations were obtained from Scatchard analysis of the binding data. Measures of potency (EC_{50}) and efficacy for glycine stimulation of TCP binding were obtained by using a curve-fitting version of the Michaelis–Menten equation (16).

**RESULTS**

Significant increases in the number of CPP and glycine binding sites were detected in hippocampal membranes of kindled animals sacrificed 1 month after the last kindled seizure. The B_{max} of [^3H]CPP and [^3H]glycine binding increased 47% and 42%, respectively (Fig. 1 and Table 1) (P < 0.02 in each case). No statistically significant alterations in ligand affinity were detected.

![Fig. 1](image_url)

**FIG. 1.** Representative [^3H]CPP (A), [^3H]glycine (B), and [^3H]TCP (C) binding isotherms for control and kindled animals sacrificed 1 month after the last kindled seizure.

To determine whether the increase in glycine binding sites was associated with alterations in activation of the NMDA channel, the effects of glycine on [^3H]TCP binding were measured under nonequilibrium conditions. A 28% increase in the maximum effect of glycine on [^3H]TCP binding was found in hippocampal membranes isolated from animals sacrificed 1 month after the last evoked seizure (Fig. 2). The maximum rate of TCP binding in 5 μM NMDA and saturating concentrations of glycine was 109 ± 12 and 139 ± 15 fmol/mg of protein per 16 min for control and kindled animals, respectively (means ± SEM, n = 4; P < 0.02 by Student's t
RESULTS

To determine whether an alteration in affinity of[^3H]TCP for its binding site or an alteration in the number of[^3H]TCP binding sites contributed to these differences, ^[^3H]TCP binding isotherms were performed under equilibrium conditions. A significant increase (25%; P < 0.02) in the number of[^3H]TCP binding sites was found without an alteration in ligand affinity (Fig. 1 and Table 1).

To our surprise, no alterations in either the number or affinity of[^3H]glucose binding sites were detected in animals sacrificed 1 day after the last kindled seizure (Table 1).

DISCUSSION

These results show that kindling is associated with an increase in the density of hippocampal NMDA receptors as revealed by the binding of ligands to three distinct sites on the receptor-channel complex. Increases in receptor density appear to develop some time after establishment of the kindled state because no increase in glycine binding was detected 1 day after completion of kindling or in TCP binding 3 days after completion of kindling (32). These delayed alterations in the NMDA receptor stand in sharp contrast to a host of other kindling-induced, immediate but transient, changes in receptors (17-21).

It is not clear in what manner the NMDA receptor is altered. Increases in three major components of the NMDA receptor suggest an increase in the number of complete, functional NMDA receptor channel complexes. However, this interpretation does not explain our previous radiohistochemical findings documenting a small (7-11%) decrease in agonist ([^3H]glutamate) binding to the NMDA receptor in the hippocampus 1 month after completion of kindling by angular bundle stimulation (22). Our previous studies of other receptors suggest that the decreased site of kindling stimulation (amygdala versus angular bundle) or in the preparation (membranes versus slide-mounted sections for radiohistochemistry) will not explain this paradox (17-21). The recent identification of two classes or subtypes of NMDA receptors (23) suggests an alternative explanation.

Monaghan et al. (23) suggest that there may be different subtypes or conformations of the NMDA receptor; the fundamental difference between these subtypes is that one preferentially binds agonists while the other preferentially binds antagonists. The normal hippocampus apparently contains approximately equivalent numbers of agonist and antagonist preferring NMDA receptors. We suggest that kindling selectively upregulates the antagonist-preferring subtype of NMDA receptor as measured here with the antagonist[^3H]CPP. If correct, the study of kindling-induced changes in the NMDA receptor will provide an opportunity to elucidate the functional implications of these subtypes of NMDA receptors.

The idea that kindling produced qualitative as well as quantitative changes in the NMDA receptor channel complex is further supported by the observation that the increase in the number of accessible[^3H]TCP binding sites (0.4 pmol/mg of protein) is only about half that predicted by the increase in the number of[^3H]CPP binding sites (0.8 pmol/mg of protein) or glycine binding sites (1.1 pmol/mg of protein). This apparent change in the stoichiometry of the different components of the NMDA receptor might result from an increase in the number of complete receptor channel complexes, together with differential regulation of the agonist- and antagonist-preferring subtypes.

The present findings are not sufficient to explain establishment of the kindled state because animals are kindled before these changes occur. Rather, we suggest that these modifications of the NMDA receptor serve to maintain the hippocampus in a hyperexcitable state long after induction of kindling. Nevertheless, some NMDA receptor-mediated responses are enhanced during the early stages of kindling (3, 4). These effects must be brought about by some mechanism other than a change in receptor number.

NMDA receptor and channel blockers virtually abolish the development of kindling (24-27), indicating that activation of the NMDA receptor may be critical for this process. Activation of the NMDA receptor also appears to be necessary for other types of neuronal plasticity, such as long-term potentiation (28, 29), experience-dependent differentiation of the brain during the neonatal period (30), and certain forms of learning (31). However, kindling is the first form of neuronal plasticity in which it has been shown that not only is NMDA receptor activation essential for its development, but also that a transformation of the NMDA receptor occurs in the process. It will be interesting to determine whether this sort of “positive feedback” is unique to kindling or is
common to many forms of long-lasting experience-dependent plasticity.

We thank Ms. Carolyn Newman and Ms. Rena Wethington for assistance in preparation of this manuscript. This work was supported by Grants NS 16064, 17771, 24448, and 27311 from the National Institutes of Health and a grant from the Veterans Administration.