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Differential Roles of NR2A- and NR2B-Containing NMDA Receptors in Activity-Dependent Brain-Derived Neurotrophic Factor Gene Regulation and Limbic Epileptogenesis

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Fleeting activation of NMDA receptors (NMDARs) induces long-term modification of synaptic connections and refinement of neuronal circuits, which may underlie learning and memory and contribute to pathogenesis of a diversity of neurological diseases, including epilepsy. Here, we found that NR2A and NR2B subunit-containing NMDARs were coupled to distinct intracellular signaling, resulting in differential BDNF expression and extracellular signal-regulated kinase 1/2 (ERK1/2) activation. Selective activation of NR2A-containing NMDARs increased BDNF gene expression. Activation of NR2B-containing NMDARs led to ERK1/2 phosphorylation. Furthermore, selectively blocking NR2A-containing NMDARs impaired epileptogenesis and the development of mossy fiber sprouting in the kindling and pilocarpine rat models of limbic epilepsy, whereas inhibiting NR2B-containing NMDARs had no effects in epileptogenesis and mossy fiber sprouting. Interestingly, blocking either NR2A- or NR2B-containing NMDARs decreased status epilepticus-induced neuronal cell death. The specific requirement of NR2A and its downstream signaling for epileptogenesis implicates attractive new targets for the development of drugs that prevent epilepsy in patients with brain injury.

Key words: **epilepsy; NR2A; NR2B; BDNF; status epilepticus; kindling; hippocampus**

Introduction

Excitatory neurotransmission mediated by NMDA receptors (NMDARs) plays fundamental roles in both physiological and pathological processes in the mammalian CNS, as well as being a promising therapeutic target for human neurological diseases. Functional NMDARs are heteromeric complexes composed of at least one obligatory NR1 subunit and one or more NR2 or NR3 subunits. NR1 is expressed ubiquitously in the CNS. Other subunits show more limited expression. For example, NR2A and NR2B prevail in the adult cortex and hippocampus, whereas NR2C is highly enriched in cerebellum and NR2D in the brainstem (Monyer et al., 1994; Sheng et al., 1994; Cull-Candy et al., 2001). The functional diversity of NMDARs is rooted in their subunit diversity. Different subunit combinations yield functional NMDARs that vary markedly in their electrophysiological properties and sensitivities to modulation by intracellular messengers (Buller et al., 1994; Cull-Candy et al., 2001; Barria and Malinow, 2005).

Regulation of gene expression by NMDAR activation may be an important mechanism for neuronal development, synaptic plasticity, learning and memory, as well as the pathogenesis for a diversity of neurological disorders such as epileptogenesis. Pretreatment of rats with NMDAR antagonists at doses with minimal anticonvulsant effects prevents epileptogenesis in kindling, pilocarpine, and kainate models of limbic epilepsy (Ormandy et al., 1989; Stafstrom et al., 1993; Sutula et al., 1996; Rice and DeLorenzo, 1998). Conversely, activity-dependent BDNF expression is required for limbic epileptogenesis. Seizure activity increases BDNF expression and its receptor TrkB activation (Isackson et al., 1991; Danzer et al., 2004; He et al., 2004). Moreover, intracerebroventricular infusion of TrkB-Fc to scavenge endogenous BDNF slows the development of kindling (Binder et al., 1999). Conditional knock-out of the TrkB receptor itself prevents epileptogenesis in the kindling model (He et al., 2004). Together, these studies suggest that neuronal activity-dependent NMDAR activation and subsequent BDNF expression are crucial for limbic epileptogenesis.

In the present study, we examined the essential roles of NR2A- and NR2B-containing NMDARs in activity-dependent BDNF gene expression and limbic epileptogenesis. We found that NR2A and NR2B subunit-containing NMDARs were coupled to distinct intracellular signaling, resulting in differential BDNF expression and extracellular signal-regulated kinase 1/2 (ERK1/2)

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activation. Using kindling and pilocarpine rat models of limbic epilepsy, we found that activation of NR2A-, but not NR2B-, containing NMDARs was required for epileptogenesis and the development of mossy fiber sprouting, but both types of receptors were involved in seizure-induced neuronal cell death.

Materials and Methods

Primary neuronal culture. Primary hippocampal neurons were prepared from embryonic day 18 Sprague Dawley rats. Briefly, after dissection of the hippocampus, the tissue was rinsed twice in PBS (4°C) containing 6% glucose, minced, and then dissociated by trituration with fire-polished Pasteur pipettes in Neurobasal medium supplemented with B27 (Invitrogen, Carlsbad, CA), 0.5 mM L-glutamine (Invitrogen), and 20 mM HEPES, pH 7.4. Cells were centrifuged for 4 min at 800 × *g* and resuspended in Neurobasal medium supplemented with B27 and 0.5 mM L-glutamine. Cells were counted in the presence of trypan blue and plated in six-well plates precoated with 25 μg/ml poly-D-lysine (Sigma, St. Louis, MO). Fluorodeoxyuridine (10 μM; Sigma) was added 1–3 d after plating, and cells were fed twice weekly thereafter. All cells were grown at 37°C and in 5% CO_2. Most of the experiments were performed on 16–20 d *in vitro* cultures unless otherwise indicated.

Expression of recombinant NMDA receptors and whole-cell recording. Human embryonic kidney HEK293 cells were cotransfected with plasmids encoding enhanced green fluorescent protein (EGFP) (Clontech, Cambridge, UK), NR1, NR2A, or NR2B (gifts from Dr. Jianhong Luo, Zhejiang University, Hangzhou, Zhejiang, China) in a 1:1:3 ratio using the calcium phosphate method. The transfection was ended after 2–4 h by replacing the solution with fresh media [DMEM plus 10% fetal bovine serum (FBS)]. D,L-AP-5 (100 μM; Sigma) was added to prevent glutamate-induced excitotoxicity. Electrophysiological recordings were performed 48 h after transfection. During recording, cells were placed in a recording chamber at room temperature and continuously perfused with an extracellular solution containing the following: 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, and 20 μM glycine, pH 7.4. Patch electrodes (4–7 MΩ) were filled with the solution containing the following (in mM): 140 CsCl, 2 MgATP, 10 EGTA, and 10 HEPES, pH 7.3 (300–310 mOsm). Cells were clamped at −70 mV, and drugs were applied via a gravity-based perfusion system (Warner Instruments, Hamden, CT). NMDA at 100 μM (Sigma) was applied in the presence or absence of NR2A or NR2B antagonists at indicated concentrations.

DNA constructs and virus packaging. A cDNA encoding EGFP (Clontech) was amplified by PCR to generate 5′ *Xho*I and 3′ *Spe*I sites and inserted into the *Xho*I and *Spe*I sites of the noncytotoxic Semliki Forest virus (a mutant form of pSFV1 vector) [pSFV(pd)] vector (a gift from Dr. Kenneth Lundstrom, Basal, Switzerland) to produce pSFV(pd)–EGFP construct. The cDNAs encoding the carboxyl cytoplasmic tails of NR2A [838–1464 amino acids (aa)] and NR2B (839–1482 aa) were amplified by PCR to generate 5′ *Spe*I and 3′ *Not*I sites and inserted into the *Spe*I and *Not*I sites of the pSFV(pd)–EGFP vector to produce pSFV(pd)–NR2A^{tail}–EGFP and pSFV(pd)–NR2B^{tail}–EGFP constructs. The sequences of all constructs were verified by DNA sequencing.

Baby hamster kidney (BHK-21) cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) and 100 U/ml penicillin/streptomycin. Cultures were maintained at 37°C and 5% CO_2 in an incubator. *In vitro* transcribed RNA molecules from pSFV(pd)–EGFP, pSFV(pd)–NR2A^{tail}–EGFP, and pSFV(pd)–NR2B^{tail}–EGFP were cotransfected with pSFV–helper2 RNA (a gift from Dr. Kenneth Lundstrom) into BHK-21 cells by electroporation (GenePulserII; Bio-Rad, Hercules, CA). All virus production was performed at 31°C. Forty-eight hours after electroporation, virus stocks were harvested, filter sterilized, and activated with chymotrypsin (Invitrogen). The reaction was terminated with the trypsin inhibitor aprotinin (Invitrogen). Virus were concentrated by centrifugation for 4 h at 20,000 × *g* at 4°C and dissolved in PBS. Final virus titers ($\geq 10^{10}$ infectious units/ml) were determined by infection of BHK-21 cells with serial dilutions of virus stocks, followed by fluorescence microscopy examination at 3 d after infection. For viral delivery, neuronal cultures were incubated 30 min with activated virus diluted 200–500 times in culture media. Experiments were performed 16–20 h after infection.

Reverse transcription-PCR. For analysis the BDNF expression in neuronal cultures, the hippocampal neurons were pretreated with NMDAR antagonists for 15 min and then treated with bicuculline (50 μM; Tocris Cookson, Ballwin, MO) for 4 h and harvested with 1 ml Trizol Reagent (Invitrogen) at 4°C. For analysis of the BDNF expression *in vivo*, rats were killed by decapitation under an overdose anesthetic 3 h after status epilepticus (SE) induced by pilocarpine. Rat brains were removed rapidly, and hippocampi were dissected quickly on ice and homogenized in 1 ml of Trizol Reagent at 4°C. RNA was extracted according to the recommendations of the manufacturer, and the final RNA pellet was suspended in diethylpyrocarbonate-treated water. Reverse transcription (RT)-PCR was done using a kit from Promega (Madison, WI). The sequences of the primers were 5′-CATCCAGTTCCACCAGGT-3′, 5′-CCATGGGTCCGCACAGCT-3′ for BDNF and 5′-CCCCAATGTATCCGTTGTG-3′, 5′-CTCAGTGTAGCCCAGGATGC-3′ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In RT-PCR, quantification of band intensity was performed on PhosphorImager (Storm 860; Amersham Biosciences, Arlington Heights, IL) using Image-Quant 5.0 software, and all bands were normalized as percentage of control values.

In situ *hybridization.* Riboprobes were prepared from a BDNF cDNA insert (nucleotides 1–412) cloned in a Teasy vector (Promega). The tissue sections were fixed for 30 min with 4% formaldehyde in 0.1 M phosphate buffer, pH 7.2, and washed extensively with PBS. They were treated for 10 min at room temperature with 50 μg/ml proteinase K (Sigma) in PK buffer (5 mM EDTA and 50 mM Tris-HCl, pH 7.2) and reacted with acetic anhydride in 0.1 M RNase-free triethanolamine, pH 8.0. Afterward, the sections were incubated with 0.5–1 μg/ml probes in hybridization solution for 12–16 h at 62°C. Hybridization solution (2 ml/slide) contained 50% formamide, 5× SSC, 0.3 mg/ml yeast tRNA, 100 μg/ml heparin, 1× Denhardt's solution (0.02% BSA, 0.02% polyvinylpyrrolidone, and 0.02% Ficoll 400), 0.1% Tween 20, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 5 mM EDTA. After hybridization, the sections were sequentially washed in 1×, 1.5×, 2×, and 0.2× SSC for 10, 10, 80, and 60 min at 60, 60, 37, and 60°C sequentially. To remove mismatched cRNA hybrids, the sections were incubated with RNase A (0.1 μg/ml) in 2× SSC at 37°C for 30 min before they were washed by 0.2× SSC. The sections then were blocked with 20% heat-inactivated sheep serum in PBT (1× PBS, 2 mg/ml BSA, and 0.1% Triton X-100) for 3 h at room temperature and incubated with preabsorbed anti-digoxigenin antibody [coupled to alkaline phosphatase (AP)] diluted to a final concentration of 1:3000 with 20% sheep serum in PBT at 4°C overnight. Finally, the section were washed in PBT and AP buffer (100 mM Tris, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, and 0.1% Tween 20) and then visualized after reacting with nitroblue-tetrazolium-chloride (NBT)/5-bromo-4-chlor-indolyl-phosphate (BCIP) (1 μl of NBT and 3.5 μl of BCIP in 1 ml of AP buffer) in the dark for 8–10 h.

Western blot. Western blot was performed from cultured hippocampal neurons in six-well dishes (2×10^6 cells per well). The hippocampal neurons were pretreated with NMDAR antagonists for 15 min and then treated with bicuculline (50 μM) for 5 min as described previously (Hardingham et al., 2001). Cells were immediately rinsed, scraped into SDS-PAGE sample buffer, and processed for Western blot analysis using phosphorylated ERK1/2 (1:1000; Cell Signaling Technology, Beverly, MA) and ERK1/2 (1:1000; Cell Signaling Technology) antibodies. In Western blot, quantification of band intensity was performed on PhosphorImager (Storm 860; Amersham Biosciences) using Image-Quant 5.0 software, and all bands were normalized as percentage of control values.

Immunohistochemistry. The brain sections were embedded in 1% H_2O_2 for 30 min and washed twice by 1× PBS, incubated with 5% BSA for 60 min at room temperature to block nonspecific background staining, and then incubated with the primary antibody of phosphorylated ERK (1:200; Cell Signaling Technology) dissolved in 5% BSA overnight at 4°C. Afterward, the sections were incubated with the secondary antibody biotinylated anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA) for 30 min at room temperature and then washed by 1× PBS for three times. The sections were then reacted with avidin–biotin horseradish peroxidase solution (standard ABC Elite kit; Vector Laboratories)

for 30 min at room temperature and visualized with 0.003% H_2O_2 and 0.03% 3,3-diaminobenzidine tetrahydrochloride in 0.05 M Tris-HCl, pH 7.6.

Kindling procedure and anticonvulsant test. Adult male Sprague Dawley rats weighing 200–250 g were maintained on a 12 h light/dark cycle with *ad libitum* access to food and water. Procedures involving animals and their care were performed in accordance with the Animal Care and Use Committee of the Institute of Neuroscience. All efforts were made to minimize animal suffering. Under chloral hydrate (250 mg/kg, i.p.) anesthesia, bipolar electrode of stainless steel used for stimulation and recording was stereotaxically implanted in the left amygdala (2.8 mm posterior to bregma, 4.9 mm lateral to the midline, 8.6 mm below dura). Four screws were inserted into the skull through a drilled hole without piercing the dura. One of the screws (0.8 mm anterior to bregma, 3.0 mm right lateral to the midline) served as ground electrode; one served as reference (6.0 mm posterior to bregma, 3.0 mm right lateral to the midline) in the electroencephalographic (EEG) recording. Cannula was implanted into the right lateral ventricle (0.9 mm posterior to bregma, 1.4 mm lateral to the midline, and 4.0 mm below the skull surface) for drug infusion. Cannula, electrodes, and screws were fixed with a mixture of acrylic and dental cement. After a postoperative recovery period of at least 7 d, the electroencephalographic seizure threshold was determined by application of a 1 s train of 1 ms monophasic rectangular pulses at 62 Hz beginning at 50 μA. The 25 μA steps were administered at 2 min interval until an afterdischarge (AD) lasting at least 5 s was detected. Animals whose stimulation intensity was larger than 400 μA were excluded from this experiment. The intensity of AD threshold plus 100 μA was administered twice a day during the following days. The behavioral progression of kindling-induced seizures was scored according to Racine's standard classification (Racine et al., 1972): 0, no reaction; 1, stereotype mounting, eye blinking, and/or mild facial clonus; 2, head nodding and/or several facial clonus; 3, myoclonic jerks in the forelimbs; 4, clonic convulsions in the forelimbs with rearing; and 5, generalized clonic convulsions associated with loss of balance. Fully kindled was defined by the occurrence of three consecutive class 5 seizures. To examine the effect of NMDAR antagonists on kindling, rats were received intracerebroventricular injections of the NMDAR nonselective antagonist MK801 [(+)-5-methyl-10,11-dihydro-5*H*-dibenzo [a,d] cyclohepten-5,10-imine maleate] (50 nmol; Sigma), the NR2A subunit selective antagonist NVP-AAM077 ([(*R*)-[(*S*)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydro-quinoxalin-5-yl)-methyl]phosphonic acid) (2 nmol; synthesized as described by Auberson et al., 2002), or the NR2B subunit-selective antagonist ifenprodil (30 nmol; Sigma). All antagonists were dissolved in saline and infused in a total volume of 5 μl (1 μl/min), 30 min before each stimulation.

For the anticonvulsant test, a group of animals received twice a day stimulations without drug administration until the animals reached 10 constitutive class 5 seizures. Resting for 2 d, animals were acutely administered with saline, MK801 (50 nmol), NVP-AAM077 (2 and 4 nmol), or ifenprodil (30 nmol), respectively, by intracerebroventricular injection 30 min before stimulation to test whether they still reach class 4/5 seizures.

Pilocarpine-induced status epilepticus and behavioral monitoring. For the pilocarpine model, rats were implanted unilaterally with a 26-gauge stainless steel cannula into the right lateral ventricle (0.9 mm posterior to bregma, 1.4 mm lateral to the midline, and 4.0 mm below the skull surface) under chloral hydrate (250 mg/kg, i.p.) anesthesia. The cannula was anchored to the skull with four stainless steel screws. One of screws (0.8 mm anterior to bregma, 3.0 mm left lateral to the midline) served as a recording electrode for brain surface EEG, one served as ground electrode (6.0 mm posterior to bregma, 3.0 mm right lateral to the midline), and one served as reference (6.0 mm posterior to bregma, 3.0 mm left lateral to the midline) in EEG recording. Rats were given at least 7 d to recover before experimental procedures.

Pilocarpine hydrochloride (Sigma) dissolved in 0.9% saline was administered intraperitoneally at a dose of 340 mg/kg to rats. Control rats received an equivalent volume of saline. Scopolamine methylbromide (2 mg/kg, i.p.; Sigma) was injected 30 min before pilocarpine to suppress peripheral muscarinic cholinergic effects. Diazepam (4 mg/kg, i.p.; Sigma) was administered 2 h after the onset of SE, characterized by continual recurrent seizures (classes 3, 4, and 5), to terminate the seizure and standardize the duration of seizure activity. We determined the time of SE onset when rats first reached the class 4 seizure. Rats were monitored continuously for 8 h after pilocarpine injection. The seizure intensity and classification were evaluated according to Racine's classification (Racine et al., 1972) indicated in the kindling procedure. Only those rats that reached at least the class 4 seizures were considered in this study. To examine the effect of NMDAR antagonists on epilepsy, antagonists were infused intracerebroventricularly in a total volume of 5 μl (1 μl/min), 30 min before pilocarpine injection.

Rats in each group were divided into three parts. The aim of first part was to observe the effects of different antagonists on ERK1/2 phosphorylation and BDNF mRNA expression, in which rats were killed 3 h after SE. The aim of second part was to observe the effects of different antagonists on acute neuronal cell death, in which rats were killed 24 h after SE. The aim of third part was to observe the effects of antagonists on the epileptogenesis after SE, in which rats were continuously monitored 40 h/week (8 h/d, 5 d/week) to record spontaneous seizures by video camera from 7 d after SE. The monitoring of the spontaneous seizures was performed by a combination of video- and EEG-detection system. Observation continued up to 8 weeks after SE until rats killed to detect the mossy fiber sprouting in the inner molecular layer of dentate gyrus.

Timm staining. Timm staining is used to visualize mossy fiber reorganization in the inner molecular layer of dentate gyrus that accompanies epileptogenesis (Sutula et al., 1989; Cavazos et al., 1991). Rats surviving up to 8 weeks after SE or 10 times constitutive class 5 seizures in kindling were deeply anesthetized by an overdose chloral hydrate and perfused for 2 min with 0.9% NaCl, 5 min with sulfide solution, and 30 min with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The sulfide solution consisted of 1.2% $Na_2S \cdot 9H_2O$ and 1.0% NaH_2PO_4. Afterward, the brain was removed and postfixed overnight, immersed in 30% sucrose at 4°C for 2–3 d, and sectioned into frontal sections (30 μm) for histological analyses. The slides were immersed for 15 min in 100% alcohol, 2 min in 70% alcohol, 2 min in 50% alcohol, and 2 min in distilled water and then placed in a solution containing a 12:6:2:1 mixture of gum arabic (50% w/v), hydroquinone (5.67% w/v), citric acid–sodium citrate buffer (26% citric acid, w/v; 24% sodium citrate, w/v), and silver nitrate (17% w/v; all from Sigma) and developed for 45 min in dark. Timm staining was quantified by Image Pro-Plus software. Assessment of mossy fiber sprouting (Timm index) was obtained from the absolute value of the total area of Timm granules divided by total length of dentate gyrus (Watanabe et al., 1996; Sprengel et al., 1998). For each animal, the absolute value of the Timm index is the mean of the three sections. All procedures and analyses were done by an individual blinded to the treatment.

The Fluoro-Jade B and Nissl stainings. Degenerating neurons were detected with Nissl and Fluoro-Jade B stainings as described previously (Xiong et al., 2003). Briefly, rat brain slides were immersed for 3 min in 100% ethanol, 1 min in 70% ethanol, 1 min in distilled water, and then transferred to a solution containing 0.0004% Fluoro-Jade B (Histo-Chem, Jefferson, AR) and 0.1% acetic acid for 30 min. After three washes, the slides were coverslipped. Labeled sections were imaged with a confocal laser-scanning microscope (LSM510; Zeiss, Oberkochen, Germany) under green fluorescence. For Nissl staining, the slides were placed directly into 1:1 alcohol/chloroform for 30 min and rehydrated through 100, 95, 80, 70, 60, and 50 alcohol to distilled water. Afterward, the slides were stained in cresyl violet solution for 30 min and then dehydrated through distilled water to 50, 60, 70, 80, 95, and 100% alcohol, cleared in xylene. Finally, the slides were mounted with resinous medium and analyzed under a bright-field microscope (Nikon, Tokyo, Japan). Neuronal injury was assessed in CA1, CA3, and hilus in two pairs of two consecutive sections of dorsal hippocampus (at approximately −3.6 and −4.5 mm from bregma) using Image-Pro software 5.0. The number of Nissl-positive and Fluoro-Jade B-positive cells in each section were divided by the area sampled, respectively. The staining and data analyses were done by an individual blinded to the treatment.

Statistical analysis. All statistical analyses were performed using SPSS (Cary, NC) 13.0 software. Values are expressed as mean ± SEM. The data

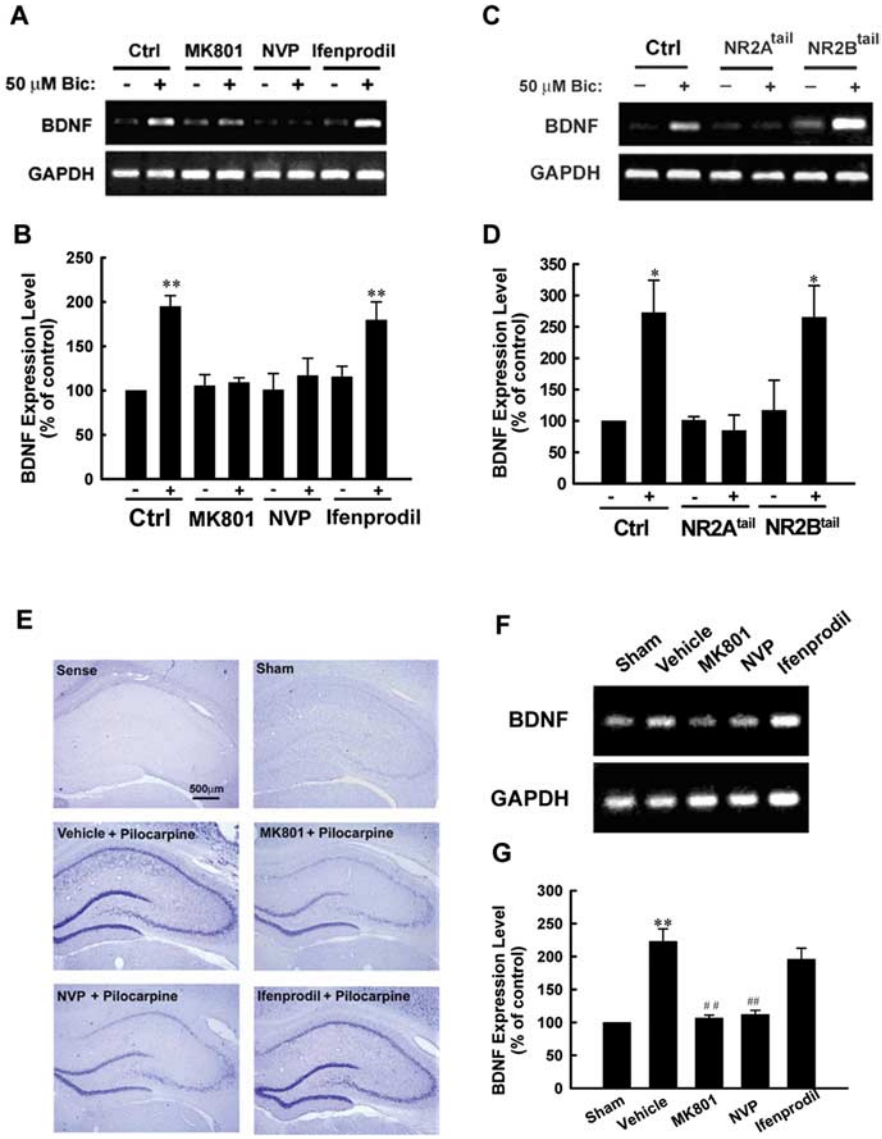

Figure 1. Differential roles of NR2A and NR2B subunits in activity-dependent BDNF mRNA expression. ***A***, Hippocampal neurons were incubated with control solution (Ctrl), MK801 (10 μM), NVP-AAM077 (NVP; 0.4 μM), or ifenprodil (3 μM) in the presence (+) or absence (−) of bicuculline (Bic; 50 μM) for 4 h, respectively. BDNF mRNA was then measured by RT-PCR. ***B***, The induction of BDNF mRNA was quantified by band density ratio of BDNF to GAPDH and then normalized to control values (without bicuculline treatment). BDNF mRNA was significantly increased after stimulation with bicuculline. Treatment with MK801 or NVP-AAM077, but not ifenprodil, blocked bicuculline-induced BDNF mRNA accumulation (data represent mean ± SEM; $n = 4$; $^{**}p < 0.005$ compared with control values). ***C***, Hippocampal neurons were infected with SFV(pd)–EGFP, SFV(pd)–NR2A^{tail}–EGFP, or SFV(pd)–NR2B^{tail}–EGFP for 16 h, and BDNF mRNA was then measured by RT-PCR in the presence (+) or absence (−) of bicuculline (50 μM) for 4 h. BDNF mRNA in neurons infected with SFV(pd)–EGFP or SFV(pd)–NR2B^{tail}–EGFP was markedly increased after bicuculline stimulation but not in neurons infected with SFV(pd)–NR2A^{tail}–EGFP. ***D***, The induction of BDNF mRNA was quantified by band density ratio of BDNF to GAPDH and then normalized to control values (without bicuculline treatment; data represent mean ± SEM; $n = 4$; $^{*}p < 0.05$ compared with control values). ***E***, *In situ* hybridization was used to evaluate the level of BDNF mRNA in rats killed 3 h after SE induced by pilocarpine. BDNF mRNA was increased in hippocampus of saline-treated rats. Treatment with MK801 (50 nmol) or NVP-AAM077 (2 nmol), but not ifenprodil (30 nmol), reduced BDNF mRNA accumulation. Scale bar, 500 μm. ***F***, RT-PCR analyses of BDNF mRNA level were performed in hippocampal homogenate from rats treated with vehicle or NMDAR antagonists 3 h after SE. ***G***, The induction of BDNF mRNA was quantified by band density ratio of BDNF to GAPDH and then normalized to control values (Sham; data represent mean ± SEM; $n = 4$; $^{**}p < 0.005$ compared with sham; $^{\#\#}p < 0.005$ compared with vehicle).

among the groups were compared using one-way ANOVA. Between-groups variance was determined by a Fisher's least significant difference *post hoc* test after ANOVA, except the study of percentage of rat with spontaneous seizure, which is examined by χ^2 test. Statistical significance was defined as $p < 0.05$.

Results

Differential roles of NR2A and NR2B subunits in activity-dependent BDNF mRNA expression

Stimulating cultured hippocampal neurons with the $GABA_A$ receptor antagonist bicuculline to elevate excitatory synaptic transmission markedly increased BDNF mRNA level. The nonselective NMDAR antagonist MK801 blocked bicuculline-induced BDNF mRNA expression, suggesting that synaptic NMDAR activation is required for activity-dependent BDNF expression (Hardingham et al., 2002). Mature hippocampal neurons express NR1 and both NR2A and NR2B but not other subunits (Tovar and Westbrook, 1999). To test whether NR2A and NR2B subunits differentially contribute to activity-dependent BDNF expression, we used two NR2 subunit-selective antagonists: NVP-AAM077 and ifenprodil. NVP-AAM077 is relatively selective for NR1/NR2A receptors at low concentrations (Auberson et al., 2002; Liu et al., 2004; Berberich et al., 2005). Ifenprodil is a selective inhibitor of NR1/NR2B-containing receptors (Williams, 1993; Priestley et al., 1995). We first tested the selectivity of these antagonists in the HEK293 cells expressing rat recombinant NR1/NR2A or NR1/NR2B receptors. We found that antagonist of NR2A-containing receptors (NVP-AAM077) at the concentration of 0.4 μM blocked nearly 90% of NR1/NR2A-mediated current evoked by 100 μM NMDA without significant effects on NR1/NR2B-mediated current. Ifenprodil at the concentration of 3 μM blocked NR1/NR2B current without any effect on NR1/NR2A current (supplemental Fig. 1*A–D*, available at www.jneurosci.org as supplemental material). Interestingly, application of NVP-AAM077 at 0.4 μM reduced bicuculline-induced BDNF mRNA expression in cultured hippocampal neurons. In contrast, treatment with the NR2B-selective antagonist ifenprodil at 3 μM did not affect bicuculline-induced BDNF mRNA expression (Fig. 1*A*,*B*), suggesting that NR2A-containing, but not NR2B-containing, NMDARs selectively contribute to activity-dependent BDNF expression.

To complement these pharmacological results, we used an acute gene delivery technique to express the cytoplasmic domain of NR2A or NR2B in hippocampal neurons to selectively interfere with their potential interaction with intracellular signaling molecules. The functional significance of NR2 subunit C termini has been highlighted by investigations in mice bearing C-terminally truncated mutations

(Sprengel et al., 1998; Sheng, 2001). These mice showed altered synaptic plasticity and behavior, suggesting that NR2A/NR2B subunit C termini bind to intracellular adaptor proteins and/or signaling molecules that determine the specificity of NR2A- and NR2B-dependent synaptic signaling pathways. To acutely interfere NR2A- and NR2B-dependent synaptic signaling, we used a Semliki Forest virus (SFV) to overexpress the carboxyl cytoplasmic tail (838–1464 aa) of NR2A or of NR2B (839–1482 aa) fused with EGFP in cultured hippocampal neurons. SFV confers several advantages over other gene delivery approaches, including easy and fast generation of recombinant viral particles, rapid and high-level transgene expression, and efficiently and preferentially infecting neurons rather than non-neuronal cells (Ehrengruber, 2002; Lundstrom et al., 2003). Electrophysiological recording showed that overexpression of NR2A^{tail}, NR2B^{tail}, or EGFP in cultured neurons did not affect of the NMDA-induced peak current, suggesting that these expressed peptides do not perturb NMDAR channel opening (supplemental Fig. 2*A–F*, available at www.jneurosci.org as supplemental material). Consistent with the results obtained by using subunit antagonists, expression of NR2A^{tail}, but not NR2B^{tail} or the EGFP alone, reduced bicuculline-induced BDNF mRNA expression (Fig. 1*C*,*D*).

Finally, additional experiments were performed to assess these antagonists on activity-dependent BDNF mRNA transcription *in vivo*. We first assessed the *in vivo* selectivity of these antagonists by studying the effects of these antagonists in NMDA receptor-dependent synaptic plasticity. Recent genetic and pharmacological studies suggested that NR2A and NR2B have differential roles in CA1 long-term potentiation (LTP) and long-term depression (LTD) (Liu et al., 2004; Massey et al., 2004). By infusing different doses of antagonists into cerebral ventricle of adult rats, we found that NVP-AAM077 (2 and 4 nmol) significantly attenuated LTP but did not affect LTD. In contrast, ifenprodil (30 nmol) inhibited LTD but had no significant effect on LTP (our unpublished data). These findings are consistent with the interpretation that 2 nmol of NVP-AAM077 is mainly inhibiting NR2A-containing NMDARs, whereas 30 nmol of ifenprodil is selective for NR2B subtype NMDARs *in vivo*. Three hours after pilocarpine-induced SE, BDNF mRNA was increased in the hippocampus as demonstrated by *in situ* hybridization and RT-PCR. A single injection of NVP-AAM077 (2 nmol), but not ifenprodil (30 nmol), inhibited SE-induced BDNF mRNA expression in hippocampus (Fig. 1*E–G*). Both of these *in vitro* and *in vivo* experiments indicated that NR2A, but not NR2B, subunit contributed to NMDAR-mediated BDNF expression.

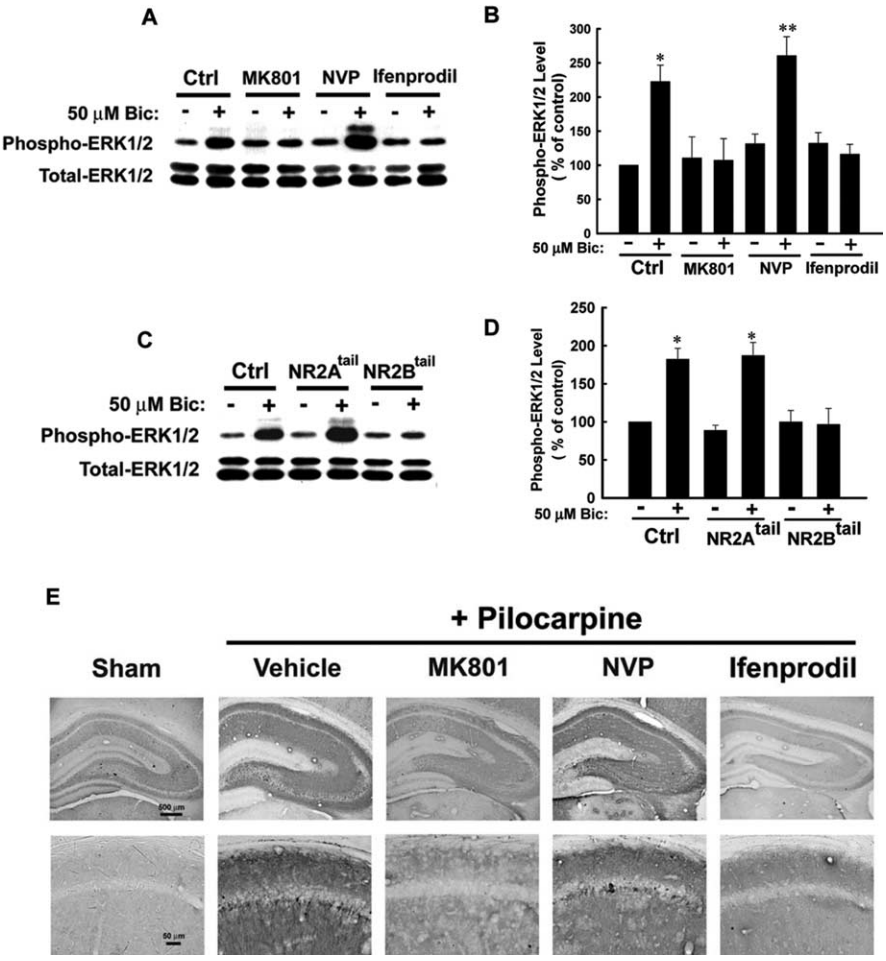

Figure 2. NR2B subunit, but not NR2A subunit, coupled to NMDAR-dependent ERK activation in hippocampal neuron. ***A***, Hippocampal neurons were treated with control solution (Ctrl), MK801 (10 μM), NVP-AAM077 (NVP; 0.4 μM), or ifenprodil (3 μM) in the presence (+) or absence (−) of bicuculline (Bic; 50 μM) for 5 min, respectively. The phosphorylated ERK1/2 was then measured by Western blotting. ***B***, The induction of ERK1/2 phosphorylation by bicuculline stimulation was quantified by measuring phosphorylated/total ERK1/2 protein band density ratio and normalizing to control values (without bicuculline treatment). Phosphorylated ERK1/2 expression significantly increased after stimulation. Treatment with MK801 or ifenprodil, but not NVP-AAM077, blocked bicuculline-induced ERK1/2 phosphorylation. Data represent mean ± SEM; $n = 8$; $^*p < 0.05$, $^{**}p < 0.005$ compared with control values. ***C***, Hippocampal neurons were infected with SFV(pd)–EGFP, SFV(pd)–NR2A^{tail}–EGFP, or SFV(pd)–NR2B^{tail}–EGFP, respectively for 16 h. Phosphorylated ERK1/2 was then measured by Western blot in the presence (+) or absence (−) of bicuculline (50 μM) for 5 min. ***D***, Phosphorylated ERK1/2 in neurons infected with SFV(pd)–EGFP or SFV(pd)–NR2A^{tail}–EGFP, but not SFV(pd)–NR2B^{tail}–EGFP, was markedly increased after stimulation (data represent mean ± SEM; $n = 4$; $^*p < 0.05$ compared with control values). ***E***, The phosphorylated ERK1/2 was measured by immunohistochemistry in rats that were perfused 3 h after SE. The high magnification of CA1 pyramidal neurons was shown in the bottom row. Phosphorylated ERK1/2 immunoreactivity increased in the hippocampus of saline-treated rats. Treatment with MK801 (50 nmol) or ifenprodil (30 nmol) reduced SE-induced phosphorylated ERK1/2 activation, whereas NVP-AAM077 (2 nmol) did not. Scale bars: top row, 500 μm; bottom row, 50 μm.

Differential roles of NR2A and NR2B in NMDAR-dependent ERK1/2 phosphorylation

The ERK1/2 pathway plays critical roles in cell proliferation, differentiation, and cell survival (Grewal et al., 1999; Impey et al., 1999; Agell et al., 2002; Cheung and Slack, 2004). Recent studies indicate that the NR2B subunit is coupled to ERK1/2 activation by Ras-guanylnucleotide releasing factor 1 (Krapivinsky et al., 2003). We thus investigated the relative contribution of NR2A and NR2B in NMDAR-mediated ERK1/2 activation. Selective activation of NMDARs in cultured neurons with 50 μM bicuculline resulted in increased phosphorylation of ERK1/2, which was prevented by ifenprodil but not by NVP-AAM077 (Fig. 2*A*,*B*). Consistent with the results obtained by using the antagonists, expression of NR2B^{tail}, but not NR2A^{tail} or the EGFP alone, re-

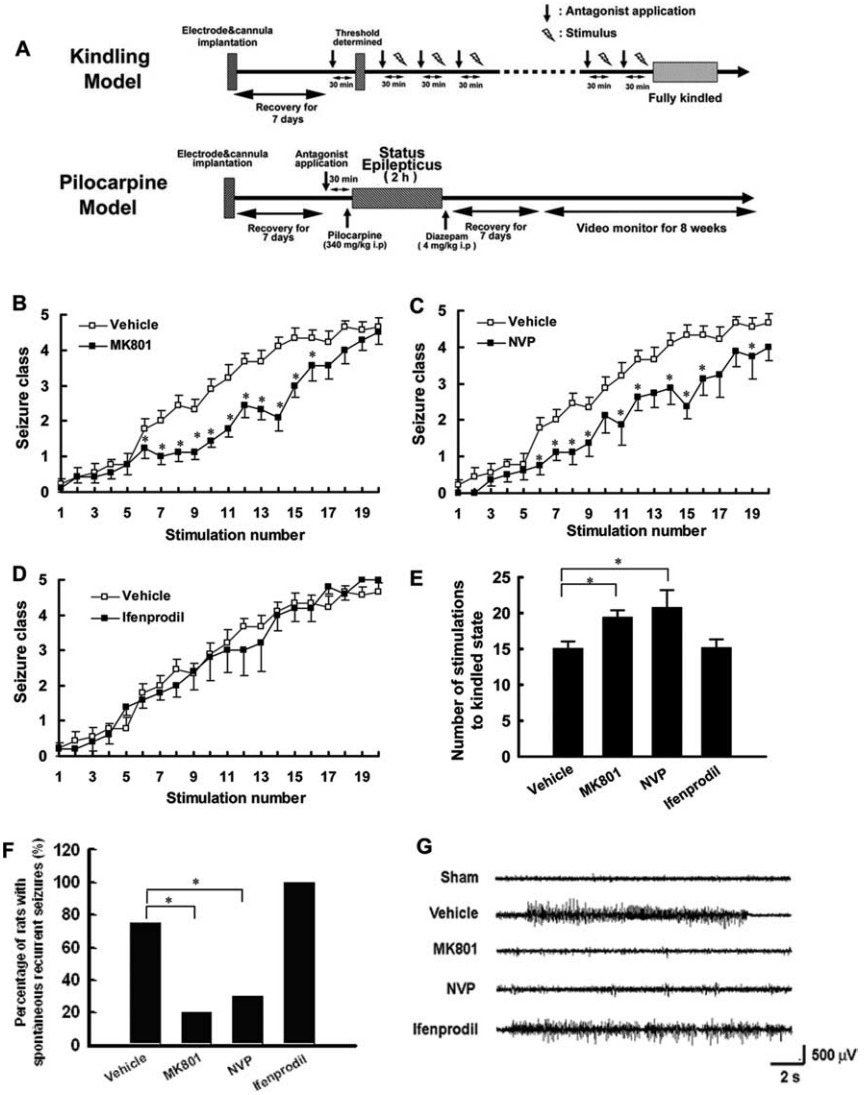

Figure 3. NR2A subunit-selective, but not NR2B subunit-selective, antagonist inhibited the epileptogenesis. ***A***, Schematic presentation of the protocol used in the kindling model and the pilocarpine model. MK801 (***B***; 50 nmol; $n = 9$) or NVP-AAM077 (NVP) (***C***; 2 nmol; $n = 8$), but not ifenprodil (***D***; 30 nmol; $n = 5$), suppressed the behavioral progression of kindling compared with rats treated with vehicle (saline; $n = 9$) (data represent the mean ± SEM; $^*p < 0.05$). ***E***, MK801 or NVP-AAM077, but not ifenprodil, increased the number of stimuli required to reach the kindled state compared with vehicle-treated animals (data represent the mean ± SEM; $^*p < 0.05$). ***F***, MK801 (50 nmol; $n = 10$) or NVP-AAM077 (2 nmol; $n = 12$), but not ifenprodil (30 nmol; $n = 9$), significantly decrease the incidence of spontaneous seizures after SE compared with vehicle-treated rats (saline; $n = 12$). χ^2 test was used to examine the significance of differences about the incidence of spontaneous seizure between groups ($^*p < 0.05$). ***G***, Typical EEGs were recorded, respectively, from vehicle-, MK801-, NVP-AAM077-, or ifenprodil-treated rats 8 weeks after SE.

duced bicuculline-induced ERK1/2 phosphorylation (Fig. 2*C*,*D*). Thus, NR2B, but not NR2A, appeared to be selectively coupled to ERK1/2 activation in cultured neurons.

This finding in cell cultures was further confirmed by examining the role of NR2A and NR2B in ERK1/2 activation *in vivo*. Using immunohistochemical staining, we found that hippocampal sections from rats exhibiting SE showed a marked increase in phosphorylated ERK1/2, which was attenuated by injection with MK801 or ifenprodil but not by NVP-AAM077 (Fig. 2*E*). Both of these *in vitro* and *in vivo* experiments indicated that NR2B subunit, but not NR2A subunit, contributed to ERK1/2 activation.

Selective effects of NR2A and NR2B antagonists on epileptogenesis

To determine whether the NR2A- and NR2B-containing NMDARs have distinct roles in epileptogenesis, we first examined the effect of subunit-selective antagonists on the progression of kindling of rats in response to repeated amygdala stimulation, as assessed by the number of electric stimuli required to evoke stereotypic behavioral seizures (Fig. 3*A*). Intracerebroventricular injection of the NMDAR antagonists had no effect on the intensity of afterdischarge threshold in the kindling (supplemental Fig. 3*D*, available at www.jneurosci.org as supplemental material). Intracerebroventricular injection of the nonselective NMDAR open-channel blocker MK801 30 min before daily stimulation to the rats impaired the rate of kindling compared with saline-injected rats (Fig. 3*B*). Consistent with previous studies (Sutula et al., 1996), these observations implicated the involvement of NMDARs in the progression of kindling. Interestingly, injection of NVP-AAM077 also significantly delayed the acquisition of kindling to a similar degree as that caused by MK801 injection (Fig. 3*C*). In contrast, injection of the NR2B subunit-selective antagonist ifenprodil had no effect on kindling (Fig. 3*D*). Injection of MK801 and NVP-AAM077 inhibited the behavioral seizure development as evident in the 19.5 ± 0.9 and 20.8 ± 2.4 stimulations, respectively, required to reach the kindled state (defined as three consecutive class 5 seizures; see Materials and Methods) compared with 15.1 ± 0.9 stimulations for saline-treated and 15.2 ± 1.1 stimulations for ifenprodil-treated animals (Fig. 3*E*). We noted that all three antagonists appeared to increase the duration of afterdischarge (supplemental Fig. 3*A–C*, available at www.jneurosci.org as supplemental material), consistent with the previous reports (Sutula et al., 1996), suggesting that there is no direct correlation between the duration of afterdischarge and the seizure behavior. These findings suggest that NR2A subunit, but not NR2B subunit, selectively contributes to kindling.

Another widely studied animal model of limbic epileptogenesis is pilocarpine-induced epilepsy. This model is characterized by the induction of an intense limbic and tonic–clonic status epilepticus, which lasts for hours, followed by acute neurodegeneration and by the onset of spontaneous seizures a few weeks later (Morimoto et al., 2004). To determine the effects of NR2A and NR2B subunit-selective antagonists on SE-induced epileptogenesis, the behavioral and electrographic seizures of drug-treated animals were continuously monitored. In rats injected with saline and ifenprodil, 75% (9 of 12) and 100% (9 of 9) of animals developed spontaneous seizure 8 weeks after SE, respectively (Fig. 3*F*). In contrast, in MK801- and NVP-AAM077-injected groups, 20% (2 of 10) and 33% (4 of 12) of animals developed spontaneous seizures, respectively (Fig. 3*F*). EEG recording confirmed the presence of epileptic discharge activities in the brain (Fig. 3*G*). In

the rats treated with MK801 and NVP-AAM077, the frequency of spontaneous seizure were also significantly lower than that of control group (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Thus, in both kindling and pilocarpine models, activation of NR2A-containing NMDARs is selectively required for epileptogenesis.

Absence of anticonvulsant effects of NR2A and NR2B antagonists

The above findings on the selective effects of NMDAR antagonists may be caused by acute anticonvulsant rather than anti-epileptogenetic actions, because MK801 has significant anticonvulsant effects in some animal models at high doses (McNamara et al., 1988). To exclude this possibility, it is essential to demonstrate that the dosage of antagonists used in our studies has no or minimal anticonvulsant effects. We found that MK801, NVP-AAM077, and ifenprodil all were ineffective in preventing the expression of kindled seizures (Fig. 4*A*) and modifying the duration of afterdischarge (Fig. 4*B*). Similarly, none of the antagonists affected the development of pilocarpine-induced SE, as measured by both EEG recording (Fig. 4*C*) and the latency to the onset of behavioral seizures (Fig. 4*D*). Thus, the doses of NMDAR antagonists used in the present study had no significant anticonvulsant effects.

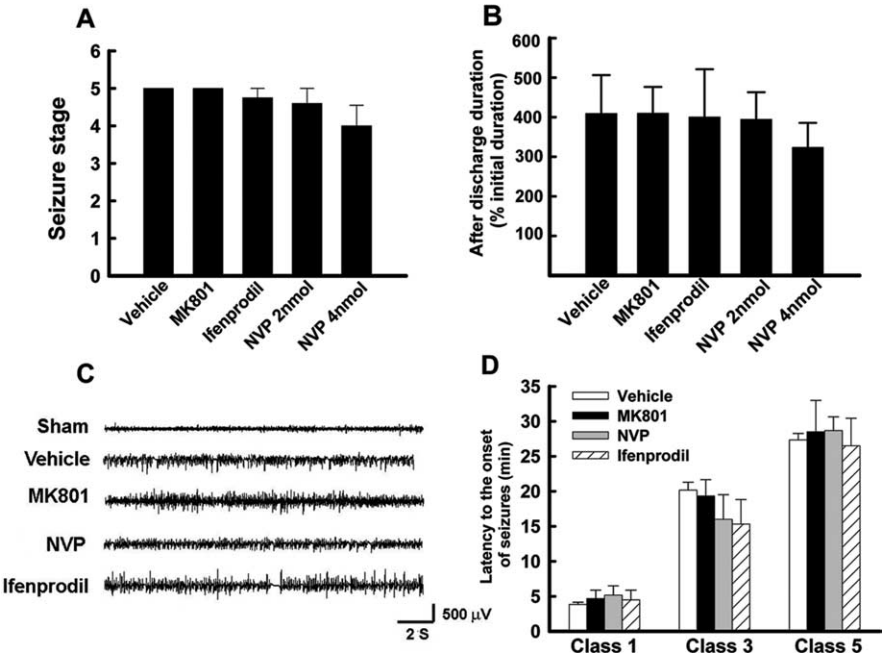

Figure 4. Absence of anticonvulsant effects of NMDAR antagonists. MK801 (50 nmol; $n = 4$), NVP-AAM077 (NVP) (2 nmol, $n = 5$; 4 nmol, $n = 5$), ifenprodil (30 nmol; $n = 4$), as well as the vehicle (saline; $n = 9$) did not affect the expression of kindled seizures (***A***) or the duration of afterdischarges (***B***). Data represent the mean ± SEM. ***C***, The typical EEGs of seizure observed during the SE induced by pilocarpine in different antagonist-treated animals. ***D***, The time of onset of class 1, 3, or 5 seizure after pilocarpine injection in vehicle-treated (saline; $n = 22$), MK801-treated (50 nmol; $n = 18$), NVP-AAM077-treated (2 nmol; $n = 21$), and ifenprodil-treated (30 nmol; $n = 18$) animals. Data represent the mean ± SEM.

Effects of NR2A and NR2B antagonists on SE-induced neuronal cell death

Seizure activity is often accompanied by neuronal cell death in human patients and animal models (Chang and Lowenstein, 2003; Henshall and Simon, 2005). To determine whether activation of NR2A- or NR2B-containing NMDARs are involved in SE-induced cell death, we examined the cell death in the hippocampus from rats 24 h after pilocarpine-induced SE. Extensive neuronal cell loss in the CA1, CA3, and hilus region was detected by Fluro-Jade B staining (Fig. 5*Aa*,*Ab*) and Nissl staining (Fig. 5*Ac*,*Ad*) of nearby sections. A single injection of MK801, NVP-AAM077, or ifenprodil all markedly reduced the severity of SE-induced cell death in CA1 and CA3 regions (Fig. 5*B*). Interestingly, none of the antagonists could protect the cells in the hilus, consistent with previous finding of MK801 in the kainate model (Brandt et al., 2003). Thus, in sharp contrast to their roles in epileptogenesis, both NR2A- and NR2B-containing NMDARs contribute to SE-induced neuronal cell death.

Selective effects of NR2A and NR2B antagonists on mossy fiber sprouting

Activation of NMDARs has been implicated in seizure-induced mossy fiber sprouting, which is also a feature of human limbic epilepsy (Sutula et al., 1989; Houser et al., 1990; Sutula et al., 1996). It is thus of interest to determine whether the selective impediment of epileptogenesis by NR2A antagonist is also reflected in seizure-induced mossy fiber sprouting, which normally occurs a few weeks after SE. As shown in Figure 6*A*, we found extensive Timm-stained mossy fiber sprouting in the supragranular region of the dentate gyrus of the hippocampus 8 weeks after pilocarpine-induced SE. The mean Timm index was high in both the saline- and ifenprodil-injected groups but significantly lower in MK801- and NVP-AAM077-treated groups (Fig. 6*A*,*B*). These findings suggested that activation of NR2A-containing NMDARs selectively contributed to SE-induced long-term alteration of neural circuitry. Similar results were obtained from the kindling model (Fig. 6*C*).

Discussion

The principal findings of this study are fivefold: (1) NR2A and NR2B subunit-containing NMDARs coupled to different intracellular signals, specifically, NR2A contributed to activity-dependent BDNF expression and NR2B activation resulted in ERK1/2 phosphorylation; (2) NR2A subunit-containing, but not NR2B subunit-containing, NMDAR activation was required for development of limbic epilepsy in the kindling and pilocarpine models; (3) NR2A subunit-containing, but not NR2B subunit-containing, NMDAR activation was required for seizure-induced development of mossy fiber sprouting; (4) both NR2A and NR2B subunits were involved in SE-induced neuronal death; and (5) there were no necessary correlations between neuroprotective and anti-epileptogenic effects of NMDAR antagonists.

NMDARs in the mammalian CNS are heteromeric assemblies composed of an NR1 subunit and at least one type of NR2 subunits with predominantly NR2A and NR2B subunits in adult rat hippocampus (Wenzel et al., 1997). NR2A- and NR2B-containing NMDARs differ in kinetic properties, sensitivity to various ligands, permeability to divalent ions, and interactions with intracellular proteins (Cull-Candy et al., 2001). Recent studies demonstrated that NR2A- and NR2B-containing NMDAR subtypes have distinct roles in long-term potentiation and long-term

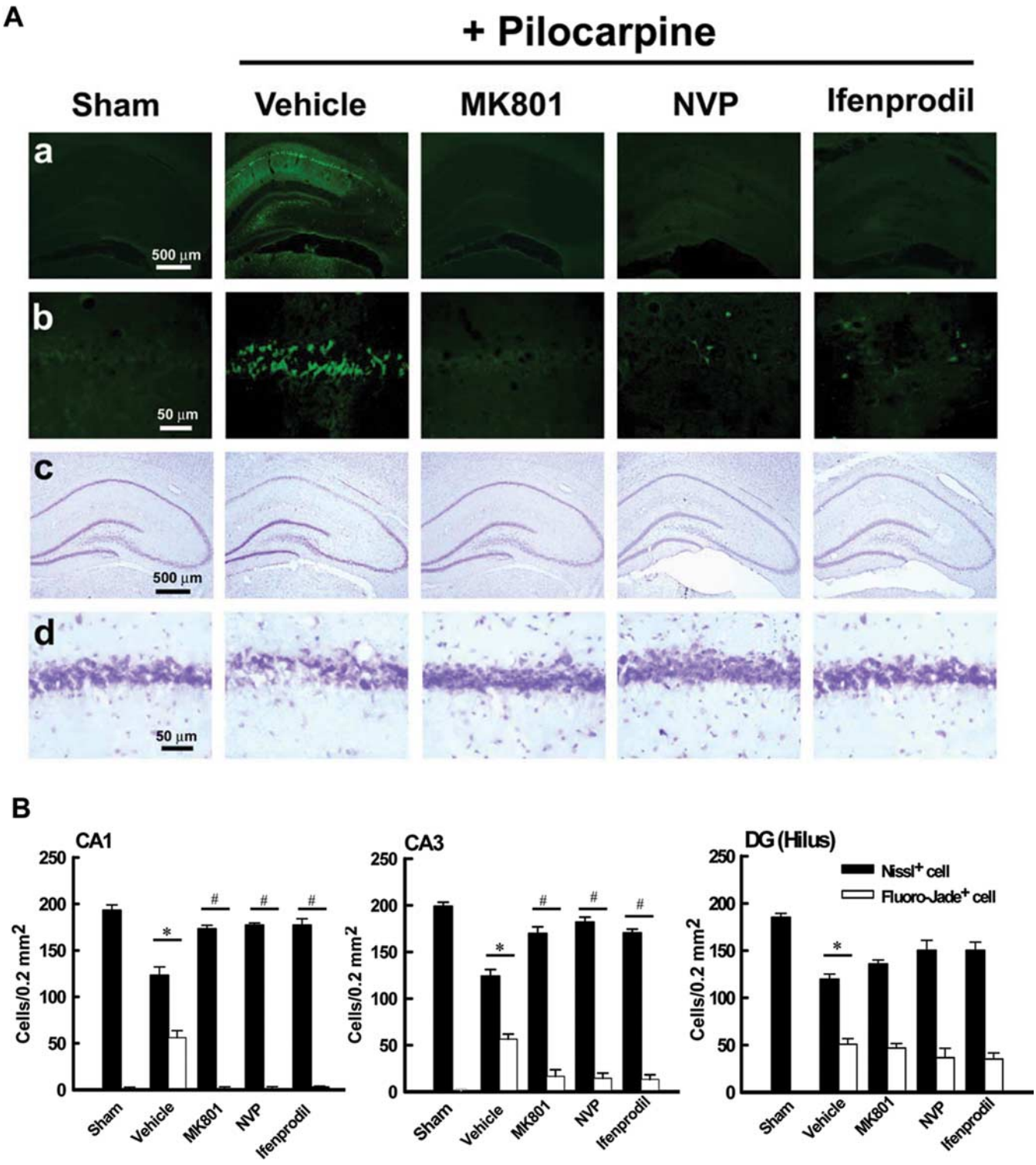

Figure 5. Effects of NMDAR antagonists on SE-induced neuronal cell death. ***A***, Twenty-four hours after pilocarpine injection, cell death in the dorsal hippocampus was measured by Fluoro-Jade B staining (***a***, ***b***) and Nissl staining (***c***, ***d***). The high magnification of CA1 pyramid neuron with Fluoro-Jade B staining was shown in ***b*** and Nissl staining in ***d***. A single injection of MK801 (50 nmol; $n = 8$), NVP-AAM077 (NVP) (2 nmol; $n = 9$), or ifenprodil (30 nmol; $n = 9$) had protective effects against SE-induced cell loss in CA1 and CA3 (***a***, ***c***). Scale bars: ***a***, ***c***, 500 μm; ***b***, ***d***, 50 μm. ***B***, Quantitative analysis of neuronal cell death (data represent mean ± SEM; $^{*}p < 0.05$ compared with sham; $^{\#}p < 0.05$ compared with vehicle).

depression in the hippocampus and cortex (Liu et al., 2004; Massey et al., 2004) using subtype-selective antagonists, although subsequent studies get variable conclusions (Berberich et al., 2005; Neyton and Paoletti, 2006). NVP-AAM007 is a potent NMDAR antagonist with modest selectivity for NR1/NR2A NMDARs (Frizelle et al., 2006), whereas ifenprodil displays a great selectivity for NR1/NR2B (Williams, 1993). Under the conditions used in this study, selectivity seems adequate. We found NR2A- and NR2B-containing NMDAR subtypes did activate different intracellular cascades and have differential roles in limbic epileptogenesis.

There are considerable evidences from experimental studies that NMDAR antagonists can inhibit epileptogenesis. The non-

selective antagonists MK801 and APV, at doses with minimal anticonvulsant activities, suppress the rate of kindling and inhibit the development of spontaneous seizures in both pilocarpine and kainate models (McNamara et al., 1988; Rice and DeLorenzo, 1998). The NR2A and NR2B subunits are the predominant NR2 subunits in the adult hippocampus and neocortex (Monyer et al., 1994; Sheng et al., 1994), the regions involved in limbic epilepsy. We hypothesized that NMDAR subtype-selective signaling contributes to limbic epileptogenesis and its associated neuropathology. Using NR2A and NR2B subunit-selective antagonists, we tested this hypothesis by quantifying epileptogenesis and mossy fiber sprouting in both kindling and pilocarpine models of limbic epilepsy. Our results implicate a potentially specific role of NR2A subunit in epileptogenesis and mossy fiber sprouting. This conclusion is based on the observations that the nonselective NMDAR antagonist MK801 and the NR2A relatively selective antagonist NVP-AAM077, but not the NR2B-selective antagonist, significantly delayed the development of kindling and inhibited SE-induced development of spontaneous seizures and mossy fiber sprouting. This conclusion is consistent with the finding of previous studies using NR2B-selective antagonists, which have no effects on epileptogenesis at doses with minimal anticonvulsant action (Yourick et al., 1999). Studies from NR2 mutant mice implicate that NR2A-specific signaling contributes to kindling and mossy fiber sprouting. There is a more than twofold increase in the number of stimulations required to achieve kindling criterion and significant decrease in the Timm staining of mossy fiber sprouting in NR2A C-terminal truncated mutant mice compared with wild-type mice (Sprengel et al., 1998), although the interpretation of this finding is complicated by the fact that the C-terminal truncation leads to impairment of the synaptic targeting of NR2A and developmental motor behavior deficits of the mutant mice (Sprengel et al., 1998).

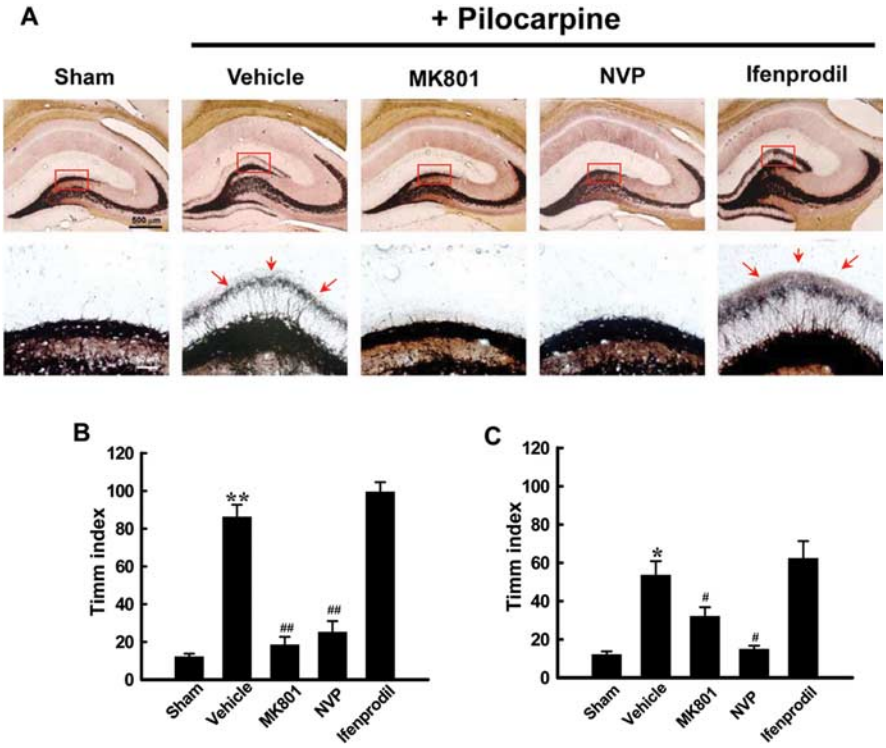

Figure 6. Selective effects of NR2A and NR2B antagonists on mossy fiber sprouting. ***A***, Timm staining for mossy fiber sprouting was measured 8 weeks after SE. Rats in the vehicle group (saline; $n = 12$) developed extensive mossy fiber sprouting in supragranular region of dentate gyrus 8 weeks after SE. Treatment with a single injection of MK801 (50 nmol; $n = 10$) or NVP-AAM077 (NVP) (2 nmol; $n = 12$), but not ifenprodil (30 nmol; $n = 9$), blocked SE-induced mossy fiber sprouting in supragranular region. The high magnification of dentate gyrus was shown in the bottom row. Arrowheads point to Timm granules in the supragranular region. Scale bars: top row, 500 μm; bottom row, 50 μm. Quantification of Timm granules in the supragranular region in the pilocarpine model (***B***) and the kindling model (***C***) (data represent mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.005$ compared with sham; $^{\#}p < 0.05$, $^{\#\#}p < 0.005$ compared with vehicle).

In the CNS, NMDAR-mediated signals are critical for the survival of developing neurons and for several forms of synaptic plasticity and learning and memory (Sheng and Kim, 2002). Conversely, NMDAR-mediated excitotoxicity has been implicated in a diversity of neurodegenerative disorders. The molecular basis for the dichotomy (death vs survival) of NMDAR signaling has been unclear. One hypothesis is that the location of NMDARs determines the nature of NMDAR signaling. Synaptic NMDAR activation promotes neuronal survival, whereas extrasynaptic NMDAR activation results in cell death (Sattler et al., 2000; Hardingham et al., 2002). NR2A is preferentially located at synaptic sites, and NR2B is enriched in extrasynaptic sites in mature neurons (Stocca and Vicini, 1998; Tovar and Westbrook, 1999). We found that the blockade of SE-induced neuronal death of the pyramidal neurons of hippocampus by either NR2A- or NR2B-selective antagonist suggests that both subtypes are involved in death-promoting signaling after abnormal NMDAR activation. Our finding is consistent with the results of previous studies using selective antagonists and mutant mice (Sprengel et al., 1998). NR2B-selective antagonists are neuroprotective in a diversity of animal models of neuronal injury (O'Mahony et al., 1998; Picconi et al., 2006), and NR2A C-terminal truncated mutant mice displayed a lesser sensitivity to focal ischemia than wild-type mice (Morikawa et al., 1998). Furthermore, Ser1232 phosphorylation of NR2A subunit catalyzed by cyclin-dependent kinase 5 was found to be involved in selective neuronal death in CA1 region of hippocampus induced by transient global ischemia (Morikawa et al., 1998; Wang et al., 2003). It seems plausible that the duration and magnitude of NMDAR stimulation determine the fates of neurons. Normal excitatory synaptic transmission-mediated NMDAR activation is required for synaptic plasticity, neuronal growth, and survival, whereas abnormal NMDAR activation causes excitotoxicity.

A number of studies have suggested that loss of neurons after status epilepticus or during kindling is causal for the increased seizure susceptibility or the development of spontaneous seizures, although evidence from the kindling model has been contradictory. In theory, the loss of neurons, especially GABAergic interneurons, would account nicely for the neuronal hyperexcitability in epilepsy (Sloviter, 1987; Morin et al., 1998; Cossart et al., 2001). In this study, although NR2A and NR2B antagonists offered similar degrees of neuroprotection, only NR2A antagonist prevented epileptogenesis. Interestingly, although NR2B antagonist provided great neuroprotection, more rats in the NR2B

antagonist-treated group developed epilepsy than the saline-treated group. These observations suggest that the anti-epileptogenetic effects of NMDAR antagonists could be dissociated from their neuroprotective effects. Dissociations between the development of limbic epilepsy and neuronal loss have also been reported in humans and other animal models. Seizures in some patients with limbic epilepsy were successfully controlled by partial removal of hippocampal tissue that exhibited without obvious neurodegeneration (Spencer and Spencer, 1994; Mathern et al., 1995). Kindling, a widely used animal model of limbic epilepsy, has no detectable neuronal cell loss. Rats experienced two priming kainic acid-induced short seizure episodes, and one kainic acid-induced sustained episodes of status epilepticus also displayed no detectable neuronal loss but did develop epilepsy (Zhang et al., 2002).

The initial seizures evoked by kindling or pilocarpine trigger a signaling cascade that culminates in expression of immediate early genes, transcription factors, and neurotrophic factors, thereby altering neuronal structures and the balance between excitation and inhibition in neural networks, resulting in epileptogenesis (DeLorenzo, 1998; Zagulska-Szymczak et al., 2001; Lukasiuk et al., 2003). Both NMDARs (Ormandy et al., 1989; Stafstrom et al., 1993; Sutula et al., 1996; Rice and DeLorenzo, 1998) and BDNF (Binder et al., 1999) have been implicated in animal models of limbic epilepsy. BDNF is a cAMP response element-target gene. Ca^{2+} influx from voltage-dependent calcium channel (Ghosh et al., 1994) or synaptic NMDAR (Hardingham et al., 2002) is sufficient to initiate BDNF expression. It is possible that seizure activity activates synaptic NMDARs and increases BDNF expression. Consistent with this possibility, Tongiorgi et al. (2004) found that pilocarpine-induced seizure activity increased BDNF expression and dendritic targeting. The seizure-induced dendritic targeting of BDNF mRNA is NMDAR dependent. However, Zafra et al. (1990) found that kainic acid-induced BDNF expression was not prevented by NMDAR antagonist. Kainic acid can directly activate kainate receptor and thus increase intracellular calcium, which may be sufficient to increase BDNF expression independent of NMDAR activation. Conversely, activation of kainate receptor will depolarize neurons and indirectly activate voltage-dependent calcium channel and, thus, increase BDNF expression. In our present work, we used a pilocarpine, but not a kainic acid, model. It is possible that pilocarpine indirectly increases excitatory synaptic transmission, activates synaptic NMDARs, and increases BDNF expression. The specific requirement of NR2A activation for epileptogenesis in the present work suggests that NR2A activates a distinct intracellular signal pathway that links BDNF expression and then contributes to the development of epilepsy. Because selectively scavenging BDNF by TrkB IgG inhibited kindling (Binder et al., 1999) and conditional deletion of BDNF receptor TrkB prevented epileptogenesis in the kindling model (He et al., 2004), selective activation of NR2A-containing NMDARs could be coupled to epileptogenesis by elevating BDNF expression. Interestingly, the appearance of epilepsy (Sankar et al., 2000) and increased BDNF expression (Danzer et al., 2004) as a consequence of SE is age dependent, which correlates well with age-dependent NR2A subunit expression (Takai et al., 2003). Our finding thus provides a molecular pathway linking glutamate receptor activation to limbic epileptogenesis and suggests selective targets for the development of pharmacological treatments that can prevent epilepsy.

References

Agell N, Bachs O, Rocamora N, Villalonga P (2002) Modulation of the Ras/Raf/MEK/ERK pathway by Ca^{2+}, and calmodulin. Cell Signal 14:649–654.

Auberson YP, Allgeier H, Bischoff S, Lingenhoehl K, Moretti R, Schmutz M (2002) 5-Phosphonomethylquinoxalinediones as competitive NMDA receptor antagonists with a preference for the human 1A/2A, rather than 1A/2B receptor composition. Bioorg Med Chem Lett 12:1099–1102.

Barria A, Malinow R (2005) NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMKII. Neuron 48:289–301.

Berberich S, Punnakkal P, Jensen V, Pawlak V, Seeburg PH, Hvalby O, Kohr G (2005) Lack of NMDA receptor subtype selectivity for hippocampal long-term potentiation. J Neurosci 25:6907–6910.

Binder DK, Routbort MJ, Ryan TE, Yancopoulos GD, McNamara JO (1999) Selective inhibition of kindling development by intraventricular administration of TrkB receptor body. J Neurosci 19:1424–1436.

Brandt C, Potschka H, Loscher W, Ebert U (2003) *N*-methyl-D-aspartate receptor blockade after status epilepticus protects against limbic brain damage but not against epilepsy in the kainate model of temporal lobe epilepsy. Neuroscience 118:727–740.

Buller AL, Larson HC, Schneider BE, Beaton JA, Morrisett RA, Monaghan DT (1994) The molecular basis of NMDA receptor subtypes: native receptor diversity is predicted by subunit composition. J Neurosci 14:5471–5484.

Cavazos JE, Golarai G, Sutula TP (1991) Mossy fiber synaptic reorganization induced by kindling: time course of development, progression, and permanence. J Neurosci 11:2795–2803.

Chang BS, Lowenstein DH (2003) Epilepsy. N Engl J Med 349:1257–1266.

Cheung EC, Slack RS (2004) Emerging role for ERK as a key regulator of neuronal apoptosis. Sci STKE 2004:PE45.

Cossart R, Dinocourt C, Hirsch JC, Merchan-Perez A, De Felipe J, Ben-Ari Y, Esclapez M, Bernard C (2001) Dendritic but not somatic GABAergic inhibition is decreased in experimental epilepsy. Nat Neurosci 4:52–62.

Cull-Candy S, Brickley S, Farrant M (2001) NMDA receptor subunits: diversity, development and disease. Curr Opin Neurobiol 11:327–335.

Danzer SC, He X, McNamara JO (2004) Ontogeny of seizure-induced increases in BDNF immunoreactivity and TrkB receptor activation in rat hippocampus. Hippocampus 14:345–355.

DeLorenzo RJM (1998) Long-term modulation of gene expression in epilepsy and other neurological diseases. The Neuroscientist 5:86–89.

Ehrengruber MU (2002) Alphaviral vectors for gene transfer into neurons. Mol Neurobiol 26:183–201.

Frizelle PA, Chen PE, Wyllie DJ (2006) Equilibrium constants for NVP-AAM077 acting at recombinant NR1/NR2A and NR1/NR2B NMDA receptors: implications for studies of synaptic transmission. Mol Pharmacol 70:1022–1032.

Ghosh A, Carnahan J, Greenberg ME (1994) Requirement for BDNF in activity-dependent survival of cortical neurons. Science 263:1618–1623.

Grewal SS, York RD, Stork PJ (1999) Extracellular-signal-regulated kinase signalling in neurons. Curr Opin Neurobiol 9:544–553.

Hardingham GE, Arnold FJ, Bading H (2001) A calcium microdomain near NMDA receptors: on switch for ERK-dependent synapse-to-nucleus communication. Nat Neurosci 4:565–566.

Hardingham GE, Fukunaga Y, Bading H (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci 5:405–414.

He XP, Kotloski R, Nef S, Luikart BW, Parada LF, McNamara JO (2004) Conditional deletion of TrkB but not BDNF prevents epileptogenesis in the kindling model. Neuron 43:31–42.

Henshall DC, Simon RP (2005) Epilepsy and apoptosis pathways. J Cereb Blood Flow Metab 25:1557–1572.

Houser CR, Miyashiro JE, Swartz BE, Walsh GO, Rich JR, Delgado-Escueta AV (1990) Altered patterns of dynorphin immunoreactivity suggest mossy fiber reorganization in human hippocampal epilepsy. J Neurosci 10:267–282.

Impey S, Obrietan K, Storm DR (1999) Making new connections: role of ERK/MAP kinase signaling in neuronal plasticity. Neuron 23:11–14.

Isackson PJ, Huntsman MM, Murray KD, Gall CM (1991) BDNF mRNA expression is increased in adult rat forebrain after limbic seizures: temporal patterns of induction distinct from NGF. Neuron 6:937–948.

Krapivinsky G, Krapivinsky L, Manasian Y, Ivanov A, Tyzio R, Pellegrino C, Ben-Ari Y, Clapham DE, Medina I (2003) The NMDA receptor is cou-

pled to the ERK pathway by a direct interaction between NR2B and RasGRF1. Neuron 40:775–784.

Liu L, Wong TP, Pozza MF, Lingenhoehl K, Wang Y, Sheng M, Auberson YP, Wang YT (2004) Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. Science 304:1021–1024.

Lukasiuk K, Kontula L, Pitkanen A (2003) cDNA profiling of epileptogenesis in the rat brain. Eur J Neurosci 17:271–279.

Lundstrom K, Abenavoli A, Malgaroli A, Ehrengruber MU (2003) Novel Semliki Forest virus vectors with reduced cytotoxicity and temperature sensitivity for long-term enhancement of transgene expression. Mol Ther 7:202–209.

Massey PV, Johnson BE, Moult PR, Auberson YP, Brown MW, Molnar E, Collingridge GL, Bashir ZI (2004) Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. J Neurosci 24:7821–7828.

Mathern GW, Pretorius JK, Babb TL (1995) Quantified patterns of mossy fiber sprouting and neuron densities in hippocampal and lesional seizures. J Neurosurg 82:211–219.

McNamara JO, Russell RD, Rigsbee L, Bonhaus DW (1988) Anticonvulsant and antiepileptogenic actions of MK-801 in the kindling and electroshock models. Neuropharmacology 27:563–568.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12:529–540.

Morikawa E, Mori H, Kiyama Y, Mishina M, Asano T, Kirino T (1998) Attenuation of focal ischemic brain injury in mice deficient in the epsilon1 (NR2A) subunit of NMDA receptor. J Neurosci 18:9727–9732.

Morimoto K, Fahnestock M, Racine RJ (2004) Kindling and status epilepticus models of epilepsy: rewiring the brain. Prog Neurobiol 73:1–60.

Morin F, Beaulieu C, Lacaille JC (1998) Selective loss of GABA neurons in area CA1 of the rat hippocampus after intraventricular kainate. Epilepsy Res 32:363–369.

Neyton J, Paoletti P (2006) Relating NMDA receptor function to receptor subunit composition: limitations of the pharmacological approach. J Neurosci 26:1331–1333.

O'Mahony S, Harkany T, Rensink AA, Abraham I, De Jong GI, Varga JL, Zarandi M, Penke B, Nyakas C, Luiten PG, Leonard BE (1998) Beta-amyloid-induced cholinergic denervation correlates with enhanced nitric oxide synthase activity in rat cerebral cortex: reversal by NMDA receptor blockade. Brain Res Bull 45:405–411.

Ormandy GC, Jope RS, Snead III OC (1989) Anticonvulsant actions of MK-801 on the lithium-pilocarpine model of status epilepticus in rats. Exp Neurol 106:172–180.

Picconi B, Tortiglione A, Barone I, Centonze D, Gardoni F, Gubellini P, Bonsi P, Pisani A, Bernardi G, Di Luca M, Calabresi P (2006) NR2B subunit exerts a critical role in postischemic synaptic plasticity. Stroke 37:1895–1901.

Priestley T, Laughton P, Myers J, Le Bourdelles B, Kerby J, Whiting PJ (1995) Pharmacological properties of recombinant human *N*-methyl-D-aspartate receptors comprising NR1a/NR2A and NR1a/NR2B subunit assemblies expressed in permanently transfected mouse fibroblast cells. Mol Pharmacol 48:841–848.

Racine R, Okujava V, Chipashvili S (1972) Modification of seizure activity by electrical stimulation. 3. Mechanisms. Electroencephalogr Clin Neurophysiol 32:295–299.

Rice AC, DeLorenzo RJ (1998) NMDA receptor activation during status epilepticus is required for the development of epilepsy. Brain Res 782:240–247.

Sankar R, Shin D, Mazarati AM, Liu H, Katsumori H, Lezama R, Wasterlain CG (2000) Epileptogenesis after status epilepticus reflects age- and model-dependent plasticity. Ann Neurol 48:580–589.

Sattler R, Xiong Z, Lu WY, MacDonald JF, Tymianski M (2000) Distinct roles of synaptic and extrasynaptic NMDA receptors in excitotoxicity. J Neurosci 20:22–33.

Sheng M (2001) Molecular organization of the postsynaptic specialization. Proc Natl Acad Sci USA 98:7058–7061.

Sheng M, Kim MJ (2002) Postsynaptic signaling and plasticity mechanisms. Science 298:776–780.

Sheng M, Cummings J, Roldan LA, Jan YN, Jan LY (1994) Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. Nature 368:144–147.

Sloviter RS (1987) Decreased hippocampal inhibition and a selective loss of interneurons in experimental epilepsy. Science 235:73–76.

Spencer DD, Spencer SS (1994) Hippocampal resections and the use of human tissue in defining temporal lobe epilepsy syndromes. Hippocampus 4:243–249.

Sprengel R, Suchanek B, Amico C, Brusa R, Burnashev N, Rozov A, Hvalby O, Jensen V, Paulsen O, Andersen P, Kim JJ, Thompson RF, Sun W, Webster LC, Grant SG, Eilers J, Konnerth A, Li J, McNamara JO, Seeburg PH (1998) Importance of the intracellular domain of NR2 subunits for NMDA receptor function in vivo. Cell 92:279–289.

Stafstrom CE, Holmes GL, Thompson JL (1993) MK801 pretreatment reduces kainic acid-induced spontaneous seizures in prepubescent rats. Epilepsy Res 14:41–48.

Stocca G, Vicini S (1998) Increased contribution of NR2A subunit to synaptic NMDA receptors in developing rat cortical neurons. J Physiol (Lond) 507:13–24.

Sutula T, Cascino G, Cavazos J, Parada I, Ramirez L (1989) Mossy fiber synaptic reorganization in the epileptic human temporal lobe. Ann Neurol 26:321–330.

Sutula T, Koch J, Golarai G, Watanabe Y, McNamara JO (1996) NMDA receptor dependence of kindling and mossy fiber sprouting: evidence that the NMDA receptor regulates patterning of hippocampal circuits in the adult brain. J Neurosci 16:7398–7406.

Takai H, Katayama K, Uetsuka K, Nakayama H, Doi K (2003) Distribution of *N*-methyl-D-aspartate receptors (NMDARs) in the developing rat brain. Exp Mol Pathol 75:89–94.

Tongiorgi E, Armellin M, Giulianini PG, Bregola G, Zucchini S, Paradiso B, Steward O, Cattaneo A, Simonato M (2004) Brain-derived neurotrophic factor mRNA and protein are targeted to discrete dendritic laminas by events that trigger epileptogenesis. J Neurosci 24:6842–6852.

Tovar KR, Westbrook GL (1999) The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses *in vitro*. J Neurosci 19:4180–4188.

Wang J, Liu S, Fu Y, Wang JH, Lu Y (2003) Cdk5 activation induces hippocampal CA1 cell death by directly phosphorylating NMDA receptors. Nat Neurosci 6:1039–1047.

Watanabe Y, Johnson RS, Butler LS, Binder DK, Spiegelman BM, Papaioannou VE, McNamara JO (1996) Null mutation of c-fos impairs structural and functional plasticities in the kindling model of epilepsy. J Neurosci 16:3827–3836.

Wenzel A, Fritschy JM, Mohler H, Benke D (1997) NMDA receptor heterogeneity during postnatal development of the rat brain: differential expression of the NR2A, NR2B, and NR2C subunit proteins. J Neurochem 68:469–478.

Williams K (1993) Ifenprodil discriminates subtypes of the *N*-methyl-D-aspartate receptor: selectivity and mechanisms at recombinant heteromeric receptors. Mol Pharmacol 44:851–859.

Xiong ZQ, Qian W, Suzuki K, McNamara JO (2003) Formation of complement membrane attack complex in mammalian cerebral cortex evokes seizures and neurodegeneration. J Neurosci 23:955–960.

Yourick DL, Repasi RT, Rittase WB, Staten LD, Meyerhoff JL (1999) Ifenprodil and arcaine alter amygdala-kindling development. Eur J Pharmacol 371:147–152.

Zafra F, Hengerer B, Leibrock J, Thoenen H, Lindholm D (1990) Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. EMBO J 9:3545–3550.

Zagulska-Szymczak S, Filipkowski RK, Kaczmarek L (2001) Kainate-induced genes in the hippocampus: lessons from expression patterns. Neurochem Int 38:485–501.

Zhang X, Cui SS, Wallace AE, Hannesson DK, Schmued LC, Saucier DM, Honer WG, Corcoran ME (2002) Relations between brain pathology and temporal lobe epilepsy. J Neurosci 22:6052–6061.