Plasticity of Both Excitatory and Inhibitory Synapses Is Associated With Seizures Induced by Removal of Chronic Blockade of Activity in Cultured Hippocampus

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Bausch, Suzanne B., Shujin He, Yelena Petrova, Xiao-Min Wang, and James O. McNamara. Plasticity of both excitatory and inhibitory synapses is associated with seizures induced by removal of chronic blockade of activity in cultured hippocampus. J Neurophysiol 96: 2151–2167, 2006. First published June 21, 2006; doi:10.1152/jn.00355.2006. One factor common to many neurological insults that can lead to acquired epilepsy is a loss of afferent neuronal input. Neuronal activity is one cellular mechanism implicated in transducing deafferentation into epileptogenesis. Therefore the effects of chronic activity blockade on seizure susceptibility and its underlying mechanisms were examined in organotypic hippocampal slice cultures treated chronically with the sodium channel blocker, tetrodotoxin (TTX), or the N-methyl-D-aspartate receptor (NMDAR) antagonist, D-2-amino-5-phosphonovaleric acid (D-APV). Granule cell field potential recordings in physiological buffer revealed spontaneous electrographic seizures in 83% of TTX-, 9% of D-APV-, but 0% of vehicle-treated cultures. TTX-induced seizures were not associated with membrane property alterations that would elicit granule cell hyperexcitability. Seizures were blocked by glutamate receptor antagonists, suggesting that plasticity in excitatory synaptic circuits contributed to seizures. The morphology of granule cells and their mossy fiber axons remained largely unchanged, and the number of synapses onto granule cells measured immunohistochemically was not increased in TTX- or D-APV-treated cultures. However, voltage-clamp recordings revealed that miniature excitatory postsynaptic current frequency and kinetics were increased and miniature inhibitory postsynaptic current kinetics were decreased in D-APV- and TTX-treated cultures compared with vehicle. Changes were more profound and qualitatively different in TTX- compared with D-APV-treated cultures, consistent with the dramatic effects of TTX treatment on seizure expression. We propose that chronic blockade of action potentials by TTX induces homeostatic responses including plasticity of both excitatory and inhibitory synapses. Removal of TTX unmasks the impact of these synaptic plasticities on local circuit excitability, resulting in spontaneous seizures.

INTRODUCTION

Temporal lobe epilepsy can be acquired by otherwise healthy individuals as a consequence of brain insults such as injury, ischemia, or neurodegenerative disease. Development of acquired epilepsy is typically delayed by months or years from initial neural damage. One factor common to many neurological insults that can lead to acquired epilepsy is a loss of neuronal input to select populations of neurons due to neuronal death or transection or hypoactivity of afferent pathways. In support of a causal role for loss of action potential driven neuronal input in epilepsy, visually deprived infants and children often present interictal spikes in occipital cortex (Kellaway 1989). Hearing loss during the sensitive period (2–3 wk postnatal) in rats leads to a permanent susceptibility to sound-triggered seizures (Pierson and Swann 1988) and chronic blockade of activity with tetrodotoxin (TTX) in the developing hippocampus produces chronic focal epilepsy in vivo (Galvan et al. 2000) and prolonged epileptiform activity in vitro (Niesen and Ge 1999).

The mechanisms underlying abnormal brain function after loss of presynaptic input remain speculative. However, action potential-driven activity from presynaptic neurons (Goodman and Shatz 1993; Katz and Shatz 1996) and activation of N-methyl-D-aspartate receptors (NMDARs) (Bear et al. 1990; Cline et al. 1987; Lin and Constantine-Paton 1998) regulate synaptogenesis during development and play a crucial role in synaptic plasticity (Bear et al. 1990; Bliss and Collingridge 1993; Cline et al. 1987; Cotman et al. 1995; Lin and Constantine-Paton 1998). Moreover, blockade of action-potential-driven activity in developing circuits can cause synaptic reorganization (Lin and Constantine-Paton 1998; McKinney et al. 1999) and alterations at individual synapses (Liao et al. 1999; Rao and Craig 1997), and synaptic reorganization and aberrant synapse formation are thought to contribute to pathophysiology associated with temporal lobe epilepsy. Therefore we hypothesized that loss of action-potential-driven activity or NMDAR function would induce synaptic plasticity and promote seizures. The possible similarities and differences between each type of blockade also were examined because diminished NMDAR activation has been proposed to play a contributory role in alterations associated with chronic blockade of action potential-driven activity (Rao and Craig 1997).

Hippocampal dysfunction is thought to contribute to acquired epilepsy because the hippocampus is a seizure-prone structure (Green 1964), hippocampal damage occurs after traumatic brain injury (Diaz-Arrastia et al. 2000; Marks et al. 1995; Mathern et al. 1994; Schuh et al. 1998), and surgical removal of the hippocampus often cures acquired epilepsy. Thus we...
examined the effects of loss of activity or NMDAR function in an in vitro hippocampal model of temporal lobe epilepsy. Cultures of organotypic hippocampal slices experience trauma, cell loss, rearrangements in excitatory circuitry, abnormal excitatory activity, and a latent period much like that seen after brain insult (Bausch and McNamara 2000, 2004). Slice cultures were treated chronically with the sodium channel blocker, TTX, to block activity or the antagonist, D(-)-2-amino-5-phosphonovaleric acid (D-APV), to block NMDAR activation. Electrographic seizures were documented using field potential recordings and synaptic plasticity was verified using electrophysiological and morphological analyses. Experiments were performed in the dentate gyrus because alterations in this region are thought to be pivotal in limbic epileptogenesis and seizure expression (Behr et al. 1996, 1998; Collins et al. 1983).

Portions of this manuscript were presented previously in abstract form (Bausch and McNamara 2001a,b; He et al. 2005a,b).

METHODS

Organotypic hippocampal slice cultures

Slice cultures were prepared using the method of Stoppini et al. (1991) as described previously (Bausch and McNamara 2000). All treatment of animals was according to National Institutes of Health and institutional guidelines. Briefly, postnatal day 11 Sprague-Dawley rat pups (Zivic-Miller, Zenplo, PA; Taconic, Germantown, NY) were anesthetized with pentobarbital and decapitated. The brains were removed, and hippocampi were cut into 400-μm transverse sections using a McIlwain tissue chopper and placed into Gey’s balanced salt solution (GBSS) containing (in mM) 137 NaCl, 5 KCl, 0.25 MgSO4, 1.5 CaCl2, 1.05 MgCl2, 0.84 NaH2PO4, 0.22 KH2PO4, 2.7 NaHCO3, and 5.6 glucose] supplemented with 6.5 mg/ml glucose. The middle four to six slices of each hippocampus (with the entorhinal cortex removed) were placed onto tissue culture membrane inserts (Millipore, Bedford, MA) in a tissue culture dish containing medium consisting of 50% minimum essential medium, 25% Hank’s buffered salt solution, 25% heat-inactivated horse serum, 0.5% GlutaMax, 10 mM HEPES [all from Gibco BRL (Invitrogen, Carlsbad, CA)] and 6.5 mg/ml glucose (pH 7.2). Cultures were maintained at 37°C under room air with 5% CO2, and medium was changed three times per week. Cultures were treated with TTX (1 μM; Sigma) or D-APV (50 μM; Tocris Cookson) diluted in medium. Drug efficacy under tissue culture condition was confirmed with perforant path-evoked field potential recordings in the dentate granule cell layer of acute rat hippocampal slices using TTX (1 μM) and D-APV (50 μM) incubated in media at 37°C for 4 days (1 day longer than between media changes; data not shown). Vehicle-treated cultures were treated similarly, but drugs were omitted. Treatment began at the stated days in vitro (DIV) and continued throughout the culture period, unless stated otherwise. Vehicle- and drug-treated cultures were always studied concurrently under identical experimental conditions. All slice cultures were used to document neuronal survival. Only cultures that displayed bright, well-defined cell layers were utilized for electrophysiological recordings and anatomical labeling.

Electrophysiological recording in hippocampal slice cultures

Recordings in slice cultures were conducted as described previously (Bausch and McNamara 2000). Briefly, a portion of the tissue culture insert membrane containing a single cultured slice was placed into a submerged recording chamber mounted to a Zeiss Axioskop microscope. Slice cultures were superfused (2–3 ml/min) with a recording buffer composed of (in mM) 120 NaCl, 3.5 KCl, 1.3 MgSO4, 2.5 CaCl2, 1.25 NaH2PO4, 25.6 NaHCO3, and 10 glucose, equilibrated with 95% O2/5% CO2. Unless stated otherwise, TTX, D-APV, bicuculline methiodide (BMI), or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM; Tocris Cookson) were applied by bath superfusion. Recording pipettes were filled with 3–4 M NaCl for extracellular recordings or with (in mM) 125 K-gluconate, 13, KCl, 10 HEPES, 10 EGTA, and 2 MgATP (pH 7.2 with KOH) for whole cell recordings. Data were collected using Axopatch 1D or NEX-1 recording amplifiers for extracellular recordings, Multiclamp 700A (2 kHz analog filter) amplifiers for whole cell recordings and pCLAMP or Axotape software (Axon Instruments, Union City, CA).

For extracellular field potential recordings, slice cultures treated with TTX or D-APV were placed into recording buffer containing TTX or D-APV, respectively, until beginning the experimental recording. Field potentials were recorded at 32–34°C in the suprapyramidal blade of the dentate granule cell layer and were deemed acceptable if hilar stimulation (0.3-ms square pulse, 0.03 Hz, 50–150 μA) using a bipolar concentric electrode (MCE-100X) and Grass stimulator elicited an action potential that immediately followed the stimulus artifact with a response threshold ≤150 μA (Bausch and McNamara 2000). Neither the amplitude of the spike nor the shape of the waveform was used as criterion for acceptable recordings.

Whole cell recordings of dentate granule cells from the suprapyramidal blade of the granule cell layer were conducted after washout of treatment drugs (≥20 min) and were performed at room temperature (−28°C) to minimize the likelihood of seizures during washout of TTX. Cultures displaying profound rhythmic excitatory postsynaptic activity consistent with seizures were omitted. Current clamp data were collected within 2 min of establishing whole cell configuration. The resting membrane potential (RMP) was documented using Multiclamp software. Input resistance (Rinf) was calculated with pCLAMP software using the linear portion of a current-voltage plot of the change in membrane voltage in response to a series of 450-μs 25-pA steps. Action potential properties were determined by generating a series of 450-μs 25-pA steps. Action potential threshold was determined as the first current step that elicited an action potential.

For voltage-clamp experiments, membrane potential was clamped to −70 mV and recordings were excluded if the RMP was more positive than −50 mV. Recordings of miniature excitatory postsynaptic currents (mEPSCs) were conducted for 2.5 min in the presence of TTX (1 μM) and BMI (10 μM). Recordings of AMPA/KA receptor-modified mEPSCs were conducted for 2.5 min after subsequent addition of D-APV (50 μM) to recording buffer containing TTX (1 μM) and BMI (10 μM). Recordings of miniature inhibitory postsynaptic currents (mIPSCs) were conducted for 2.5 min in the presence of TTX (1 μM), CNQX (10 μM), and D-APV (50 μM). PSCs were analyzed and cumulative probability distributions were plotted using MiniAnalysis software (Synaptosoft, Fort Lee, NJ).

Toluidine blue stain

Cultures were stained with Toluidine blue as described previously (Bausch and McNamara 2004). Vehicle-, D-APV-, and TTX-treated cultures were always stained concurrently under identical experimental conditions. Briefly, cultures were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4), permeabilized with 0.5% Triton X-100, stained with 0.5% Toluidine blue, treated with 70% ethanol, then dehydrated in a series of ethanol containing 0.2% glacial acetic acid, mounted onto subbed glass slides, dehydrated, cleared and coverslipped. Slice cultures were assessed coded numbers to permit a blind analysis. Cell layers were scored subjectively as: 3, many prominently stained neurons; 2, sparser number of stained neurons; 1, very sparse number of scattered stained neurons; 0, no stained neurons in cell layer using an Axiovert 135 microscope at ×40 magnification as described previously (Routbort et al. 1999). Neurons were differentiated from glia based on their larger nuclear size. CA3c was defined as the CA3 pyramid cell layer located between the blades of
the dentate granule cell layer. CA3a/b was defined as the CA3 pyramidal cell layer excluding the CA3c region.

Neurobiotin

Individual neurons were filled with neurobiotin using whole cell recording techniques and visualized as described previously (Bausch and McNamara 2000). Neurobiotin (0.4 or 0.5% Vector, Burlingame, CA) was added to the pipette solution immediately prior to use. After 20–45 min diffusion of the neurobiotin-containing solution into the granule cell, cultures were fixed overnight with 4% paraformaldehyde in PB, removed from the insert membrane, sunk in 30% sucrose in 0.1 M PBS [PB containing 0.15 M NaCl and 2.7 mM KCl (pH 7.4),] and stored at −70°C. Briefly, thawed cultures were treated with PBS containing 10% methanol and 0.6% H2O2, blocked with PBS containing 2% bovine serum albumin (BSA), and 0.75% Triton X-100 and incubated in ABC elite (Vector) diluted in PBS containing 2% BSA and 0.1% Triton X-100 according to kit instructions overnight at 4°C. Cultures were then treated with 0.05% 3,3'-diaminobenzidine (DAB, Sigma), 0.028% CoCl2, 0.02% nickel ammonium sulfate and 0.00075% H2O2 in PBS until staining was evident. Cultures were then mounted onto subbed glass slides, dehydrated, cleared, and coverslipped. Three-dimensional (3D) camera lucida reconstructions were drawn manually and analyzed using a Zeiss Axioskop microscope, ×63 oil objective, motorized stage, z-axis focus encoder and Neurolucida software (MicroBrightField, Colchester, Vermont). Length measurements were documented in 3D and regions for length measurements were defined as: molecular layer, supragranular regions of the dentate gyrus; granule cell layer, the tightly packed layer of granule cell somata; hilus, the region confined between the blades of the granule cell layer excluding CA3c pyramidal cell somata and proximal dendrites; CA3, included CA3a-c pyramidal cell layers and proximal dendrites. Branch points, ends and boutons were marked during digital reconstructions. Branch points were defined as points of process bifurcation. Ends were defined as points of termination. Boutons were defined as a thickening of at least twice the width of the adjacent axon. Bouton area was measured in 2D.

Synaptophysin immunohistochemistry

Slice cultures were fixed with 4% paraformaldehyde in 0.1 M PB for 20 min, removed from the membrane and processed for immunohistochemistry. All steps were performed at room temperature unless stated otherwise. Slice cultures were pretreated with 70% ethanol, 100% methanol, and 70% ethanol, followed by 0.1 M PB and 0.1 M PBS. Next, slice cultures were treated with 7% streptavidin in PBS followed by 7% biotin in PBS. Finally, cultures were blocked with PBS containing 2% gelatin and 10% normal goat serum for 1 h at 37°C. Slice cultures were then incubated with a mouse monoclonal anti-MAP2 antibody (IgG, clone HM-2 ascites; Sigma) diluted 1:1,000 to label dendrites, and a mouse monoclonal anti-synaptophysin antibody (IgM, MAB328 ascites; Chemicon, Temecula, CA) diluted 1:500 to label presynaptic terminals for 1 h at room temperature followed by 36 h at 4°C. All antibodies were diluted in PBS containing 2% BSA, 10% normal goat serum, and 0.1% Triton X-100. Slice cultures were then processed as follows: rinsed with PBS containing 0.1% Triton X-100; incubated in biotinylated goat anti-mouse IgM (Jackson Immuno Research, West Grove, PA) diluted 1:3,000 and Alexa 488-conjugated goat anti-mouse IgG diluted 1:1,000 (Molecular Probes, Eugene, OR) in diluent for 1 h; rinsed; incubated in Alexa 555-conjugated streptavidin (Molecular Probes) diluted 1:1,000 in PBS containing 1% BSA and 0.1% Triton X-100 for 1 h; and rinsed. Slice cultures were then mounted onto subbed glass slides and coverslipped with Vectashield mounting media (Vector, Burlingame, CA).

Images of the dentate molecular layer, granule cell layer and hilus were collected in a single frame using a Zeiss Pascal LSM5 confocal microscope, ×63 oil objective and multi-track scanning with an Argon laser and 405/488/543 nm excitation, 505- to 530-nm band-pass emission and 560-nm long-pass emission filters. Z-series reconstructions were compiled from 7 to 14 consecutive optical sections (2,048 × 2,048 pixels; 0.1 μm/pixel) with a z-axis interval of 0.3 μm. Parameters were established to minimize photobleaching and eliminate labeling in control slice cultures in which primary antibodies were omitted. Quantitative analyses were performed with TIFFany image processing software (Caffeine Software) and custom analyzes programs. To estimate synaptic contacts in the dentate molecular layer, granule cell layer and hilus, the number of pixels containing synaptophysin immunoreactivity was normalized to the number of pixels exhibiting MAP2 immunoreactivity in each region. To more precisely document synapse number and distribution on individual granule cell dendrites, synaptophysin-immunoreactive (IR) puncta directly apposed to MAP2-IR primary apical granule cell dendrites were identified and placed into 10-μm bins. Dendritic length was measured from somata to end of the MAP2-positive dendrites using z-series reconstructions. Only those dendrites that could be clearly attributed to a single granule cell were used for further analyses; usually three to four granule cell dendrites/slice culture. After 3D reconstructions to identify dendrites, synaptophysin clusters were quantified from individual 2D z-stack images. Synaptophysin clusters were counted only once, even if they appeared in more than one sequential image. Synaptophysin clusters were all ~1 μm².

Statistics

Investigators were blinded to experimental groupings for all data analyses. Parametric data were represented as means ± SE. Nonparametric data were represented as medians. Most statistical analyses were performed with Sigma Stat software (SPSS, Chicago, IL). Data fitting a nonparametric distribution were tested for significance using the Kruskal-Wallis ANOVA by ranks test with Dunn’s post hoc comparison when comparing multiple groups or a t-test when comparing two experimental groups. Proportions were tested for significance using a Fisher exact test (≤5 observations in any group). Significance was defined as P ≤ 0.05. Cumulative probability distributions were tested for significance with a two-tailed Kolmogorov-Smirnov test using MiniAnalysis software; significance was defined as P = 0.025

RESULTS

Hippocampal slice cultures exhibit a latent period followed by onset of seizures

One hallmark of acquired epilepsy is a latent period after initial insult and subsequent emergence of seizures. Our previous work suggested that preparation of organotypic hippocampal slice cultures resulted in recurrent seizures but only after a seizure-free latent period. Using granule cell layer field potential recordings, no electrographic seizures were detected at 3–5 DIV, but after 40–60 DIV, a single spontaneous electrographic seizure was detected in 22% of cultures in physiological buffer and 75% of cultures during wash-in of the GABA_A receptor antagonist, BMI (Bausch and McNamara 2000). To more precisely define the latent period and provide a baseline for comparison with D-APV- and TTX-treated cultures, we first conducted field potential recordings of electrographic seizures in vehicle-treated cultures. The recording temperature was raised from 27–29°C (Bausch and McNamara 2000) to a more physiologically relevant temperature of 32–
34°C because recording at 28°C can exert powerful anti-seizure effects (Traynelis and Dingledine 1988). Dentate granule cell layer field potential recordings were conducted for ~30 min in physiological recording buffer followed by 45 min in the presence of BMI. Spontaneous electrographic seizures were defined as the abrupt onset of a burst of rhythmic activity lasting ≥3 s during which the waveforms evolved over time and terminated abruptly (Figs. 1 and 2) (Bausch and McNamara 2000).

**PHYSIOLOGICAL RECORDING BUFFER.** In physiological buffer, no spontaneous seizures were observed in 3–30 DIV vehicle-treated cultures (n = 27), despite raising the temperature to 32–34°C (Table 1). However, one culture did exhibit a cluster of very low-amplitude spikes (Fig. 1A).

**RECORDING BUFFER CONTAINING BMI.** In the presence of BMI, a progressive increase in the proportion of cultures exhibiting electrographic seizures was observed as a function of time in culture; 0% (0/5) of cultures at 3–5 DIV, 67% (4/6) at 10–13 DIV, and 100% (16/16) at ≥17 DIV (different from 3–5 DIV; Fisher exact test, P < 0.001; Table 1, Fig. 2A). Electrographic seizures were composed of spikes superimposed on a short-duration positive field potential shift riding on a long-duration negative shift in baseline potential (Fig. 2A) and similar to our previous findings, displayed both “tonic” and “clonic” phases (data not shown). However, in contrast to the single seizure observed during wash-in of BMI in our previous study, application of BMI to slice cultures induced multiple, recurrent seizures (Fig. 3A). Durations of subsequent seizures decreased

**FIG. 2.** Electrographic seizures were observed in the dentate granule cell layer of vehicle-, d-APV-, and TTX-treated hippocampal slice cultures after blockade of GABA_A receptors. Extracellular field potentials were recorded in the suprapyramidal blade of the dentate granule cell layer of hippocampal slice cultures (17–21 DIV) in the presence of BMI (10 μM) as described in METHODS. Slice cultures were treated chronically with vehicle (A), d-APV (50 μM, B), or TTX (1 μM, C). A1: trace from a representative recording shows 1 episode of the transient high-frequency spiking observed in the granule cell layer in 11 of 11 vehicle-treated slice cultures. A2: expanded time scale of A1 reveals rhythmic spiking with an abrupt onset superimposed on a positive field potential shift. B1: trace from a representative recording shows 1 episode of the transient high-frequency spiking observed in the granule cell layer in 10 of 11 d-APV-treated slice cultures. B2: expanded time scale of B1 reveals rhythmic spiking with an abrupt onset superimposed on a positive field potential shift. C1: trace from a representative recording shows 1 episode of the transient high-frequency spiking observed in the granule cell layer in 3 of 6 TTX-treated slice cultures. C2: expanded time scale of C1 reveals a slightly different pattern of spiking than shown in A2 or B2. All spiking patterns were classified as seizures. Horizontal scale bar in A1 applies to A1, B1, and C1; bar in A2 applies to A2, B2, and C2. Vertical scale bar in A2 applies to all traces.

**TABLE 1.** Seizure incidence was increased in TTX- but not d-APV- treated hippocampal slice cultures

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<th>Treatment</th>
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<td>d-APV</td>
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<td>TTX</td>
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Extracellular field potentials were recorded in the suprapyramidal blade of the dentate granule cell layer of hippocampal slice cultures treated with vehicle, d-2-amino-5-phosphonovaleric acid (d-APV) (50 μM) or TTX (1 μM). Recordings were conducted in physiological recording buffer [artificial cerebrospinal fluid (ACSF)] or in the presence of the GABA_A receptor antagonist, BMI (10 μM), as described in METHODS. Seizures were defined as described in the text and shown in Figs. 1 and 2. Seizure incidence; cultures displaying seizures/total cultures investigated. Seizure incidence induced by BMI in TTX-treated cultures is listed as not determined (ND) because BMI-induced seizures could not be differentiated from the continuation of seizure activity begun in physiological buffer in a subset of cultures. * different than vehicle; Fisher exact test. P < 0.01.

**FIG. 1.** Electrographic seizures were observed in the dentate granule cell layer of TTX- and d-2-amino-5-phosphonovaleric acid (d-APV)-treated hippocampal slice cultures in physiological buffer. Extracellular field potentials were recorded in the suprapyramidal blade of the dentate granule cell layer of hippocampal slice cultures [17–21 days in vitro (DIV)] in physiological recording buffer as described in METHODS. Slice cultures were treated chronically with vehicle (A), d-APV (50 μM, B), or TTX (1 μM, C). A1: trace illustrates the transient low-amplitude spiking observed in the granule cell layer in 1 of 11 vehicle-treated slice cultures. A2: expanded time scale of A1 reveals rhythmic spiking with an abrupt onset closely spaced simple spikes. The very low spike amplitude suggests that activity originated from a population of neurons. Because seizures (by definition) require population activity, the pattern shown in A was not classified as a seizure. B1: trace from a representative recording illustrates the abrupt onset of transient high-frequency spiking observed in the granule cell layer in 1 of 11 d-APV slice cultures. B2: expanded time scale of B1 reveals a rhythmic pattern of closely spaced simple spikes superimposed on a flat baseline. The larger spike amplitude than that shown in A2 suggests that activity originated from a population of neurons. Thus this pattern was classified as a seizure. C1 and C2: traces from representative recordings show the high-frequency spiking observed in the granule cell layer in 11 of 11 d-APV slice cultures. C3: expanded time scale of C2 reveals rhythmic spiking with an abrupt onset superimposed on a positive field potential shift. This pattern was classified as a seizure. Vertical scale bar in A1 applies to all traces.
significantly as a function of seizure number (Fig. 3B). Differences between our present observations and previous findings are likely due to the increased recording temperature. Because all vehicle-treated hippocampal slice cultures exhibited recurrent seizures in response to acute suppression of GABAergic transmission with BMI at 17–21 DIV, subsequent experiments investigating the effects of activity and NMDAR blockade on the emergence of recurrent seizures and synaptic plasticity were conducted at 17–21 DIV.

Prolonged exposure to TTX, but not D-APV, exerted profound neurotoxic effects

Sodium-dependant action potentials and NMDAR activation are important for neuronal survival during development (Allen and Iversen 1990; Gould et al. 1994; Ikonomidou et al. 1999; Olney et al. 1989). Because neuronal toxicity could complicate the interpretation of results from subsequent experiments, D-APV and TTX treatment paradigms were first refined to maximize neuronal survival. Hippocampal slice cultures were treated with vehicle, D-APV or TTX beginning at 0, 1, 2, or 3 DIV and stained with Toluidine blue at 9–11 DIV (Fig. 4A). Vehicle-treated cultures displayed well-defined, intact principal cell layers (Fig. 4, Table 2). Compared with vehicle, cultures treated with D-APV beginning at 0 DIV displayed slight loss in the CA1 and CA3b pyramidal cell layers but no loss in the granule cell layer. Delaying D-APV treatment until

FIG. 3. Electrographic seizure durations were increased in D-APV-treated hippocampal slice cultures. Extracellular field potentials were recorded in the suprapyramidal blade of the dentate granule cell layer of hippocampal slice cultures (17–21 DIV) treated chronically with vehicle or D-APV (50 μM). Recordings were conducted in the presence of BMI (10 μM) as described in METHODS. Seizures were defined as described in the text and shown in Fig. 2. A and B: slice cultures treated with D-APV (n = 11) showed no significant change in seizure number compared with vehicle (n = 12, Mann-Whitney rank sum test, P > 0.05). Line represents the median. B: analyses of seizure duration vs. recurrent seizure number revealed: 1) decreased seizure duration as a function of recurrent seizure number (*, different from 1st seizure, P < 0.05), 2) increased seizure duration in D-APV compared with vehicle (#, P < 0.05), 3) but no interaction between 1 and 2 (P > 0.05); 2-way ANOVA, Dunn’s post hoc comparison; vehicle, n = 12; D-APV, n = 10.

FIG. 4. Prolonged exposure to TTX, but not D-APV, exerted profound neurotoxic effects on hippocampal neurons. A: hippocampal slice cultures were treated with vehicle, D-APV (50 μM), or TTX (1 μM) beginning at 0, 1, 2, or 3 DIV, then fixed and stained with Toluidine blue at 9–11 DIV as described in METHODS. B: Toluidine blue staining revealed a normal distribution of pyramidal cells and dentate granule cells in the hippocampus of vehicle-treated cultures. When D-APV was included in the media from the time of plating (0 DIV), slight cell loss was noted in CA1 and CA3b. No principal cell loss was observed when D-APV treatment began at 1 DIV. When TTX was included in the media from the time of plating (0 DIV), profound cell loss was noted in the granule cell layer as well as in the CA1, CA3b, CA3c pyramidal cell layers. When TTX treatment was started at 1 or 2 DIV, cell loss was noted in the granule cell and CA1 pyramidal cell layers. The best preservation of cell layers was observed when TTX treatment began at 3 DIV, although a significant loss in the CA1 pyramidal cell layer was still noted. C: higher resolution images of the dentate gyrus also suggest a loss of hilar neurons in TTX-treated cultures. dgc, dentate granule cell layer. Scale bar in B, vehicle is for all panels in B; in C, vehicle is for all panels in C, 400 μm.
1 DIV, significantly improved survival (Fig. 4, Table 2). In contrast, slice cultures treated with TTX beginning at 0 DIV showed profound loss in most principal cell layers. Progressively delaying treatment with TTX until 3 DIV significantly improved survival, although loss was still apparent in the CA1 pyramidal cell layer (Fig. 4, Table 2). To minimize potential confounds caused by neuronal death, cultures subsequently were treated with D-APV or TTX beginning at 0–1 and 3 DIV, respectively.

**TTX, but not D-APV increased the proportion of cultures exhibiting recurrent seizures in physiological recording buffer**

Next we examined the effects of D-APV or TTX on seizure expression. Extracellular field potentials were recorded in hippocampal slice cultures during and immediately following withdrawal of the NMDAR antagonist, D-APV or the sodium channel blocker, TTX, as described for vehicle-treated slice cultures.

**D-APV-TREATED CULTURES.** In physiological recording buffer, the incidence of spontaneous electrographic seizures involving granule cells was not significantly altered in D-APV-treated cultures when compared with vehicle. During washout of D-APV, only a single culture exhibited a spontaneous electrographic seizure involving granule cells (Table 1), a seizure composed of negative spikes superimposed on a relatively flat baseline (Fig. 1B).

A pattern similar to vehicle-treated cultures was observed in D-APV treated cultures following addition of BMI (Fig. 2, A and B, Table 1). The pronounced negative shift in baseline potential associated with the electrographic seizure depicted in Fig. 2B occurred in 50% of D-APV-treated slice cultures, which was not significantly different from similar negative shifts observed in 25% of vehicle-treated cultures (Fisher exact test, \( P > 0.05 \)). The sole significant difference between vehicle and D-APV-treated cultures was a small but significant increase in the duration of recurrent seizures relative to that of the initial seizure recorded (Fig. 3B). Together these findings reveal that chronic NMDA receptor blockade had a minimal effect on emergence of seizures in the hippocampal slice culture model.

**TTX-TREATED CULTURES.** During washout of TTX with physiological recording buffer, 83% of cultures treated with TTX exhibited a single prolonged spontaneous seizure involving granule cells (Table 1). This stands in stark contrast to 0% of vehicle- and 9% of D-APV-treated slice cultures exhibiting seizures under similar conditions (Fisher exact test, \( P < 0.01 \)). Interestingly, spontaneous seizures in TTX-treated cultures in physiological buffer were similar to seizures seen in vehicle- and D-APV-treated cultures in the presence of BMI (compare Fig. 1C with Fig. 2, A and B). That is, seizures in TTX-treated cultures were composed of spikes superimposed on a short-duration positive field potential shift riding on a longer-duration negative shift in baseline potential (Fig. 1C) and displayed both “tonic” and “clonic” phases (data not shown).

Application of BMI subsequent to recordings in physiological buffer elicited variable responses in TTX-treated slice cultures. In three TTX-treated cultures, BMI had little effect; a continuation of seizure activity begun in physiological buffer was observed (data not shown). In two TTX-treated cultures, recurrent electrographic seizures were seen, which resembled BMI-induced seizures in vehicle- or D-APV-treated slice cultures (Fig. 2). In one TTX-treated slice culture, application of BMI elicited a single seizure followed by high-frequency epileptiform bursts, which persisted for the duration of the recording (data not shown). For all BMI-induced seizures in TTX treated cultures, inter-spike interval appeared longer than in vehicle-treated cultures (compare Fig. 2, A with C).

Incubation of cultured slices in TTX for 24 h immediately prior to recordings was not sufficient to trigger the striking increase in occurrence of spontaneous seizures in physiological buffer. That is, when 17–21 DIV cultures were treated with TTX for 24 h immediately prior to recordings (\( n = 4 \)), no spontaneous electrographic seizures were observed during washout of TTX with physiological buffer and BMI-induced seizures were indistinguishable from vehicle-treated controls (data not shown).

**Exacerbation of seizures was associated with plasticity of both excitatory and inhibitory synapses**

Next we examined the potential mechanisms contributing to the facilitation of seizures induced by NMDAR or activity blockade in slice cultures. Chronic NMDA receptor and/or activity blockade can change intrinsic neuronal membrane properties (Desai et al. 1999; Niesen and Ge 1999), induce

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**Table 2. Prolonged exposure to TTX, but not D-APV, exerted profound neurotoxic effects on hippocampal neurons**

<table>
<thead>
<tr>
<th>Treatment from DIV</th>
<th>Treatment</th>
<th>( n )</th>
<th>CA1</th>
<th>CA2</th>
<th>CA3a</th>
<th>CA3b</th>
<th>CA3c</th>
<th>dgc</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Vehicle</td>
<td>55–61</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>Vehicle</td>
<td>9–13</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Vehicle</td>
<td>19–20</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>Vehicle</td>
<td>18–19</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>0</td>
<td>D-APV</td>
<td>32</td>
<td>3(^*)</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>D-APV</td>
<td>16</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>0</td>
<td>TTX</td>
<td>33</td>
<td>1(^*)</td>
<td>3</td>
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<td>3</td>
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<tr>
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<td>TTX</td>
<td>14</td>
<td>1(^*)</td>
<td>3</td>
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<td>3</td>
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<td>3</td>
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<tr>
<td>2</td>
<td>TTX</td>
<td>14</td>
<td>2(^*)</td>
<td>3</td>
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<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>TTX</td>
<td>19</td>
<td>2(^*)</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

Slice cultures were treated with vehicle. D-APV (50 \( \mu \)M) or TTX (1 \( \mu \)M) beginning at 0, 1, 2, or 3 days in vitro (DIV) (see Fig. 4A), then fixed and stained with Toluidine blue at 9–11 DIV as described in METHODS. Cell layers were scored as: 3, many prominently stained neurons; 2, sparser number of neurons; 1, very sparse number of scattered neurons; 0, no neurons in cell layer. CA3a was defined as the pyramidal cell layer located between the blades of the granule cell layer; CA3a/b was defined as the CA3 pyramidal cell layer excluding CA3c. Representative slice cultures are shown in Fig. 4B. \(^*\), different than vehicle; \(^#\), different than TTX at 0 DIV; Kruskal-Wallis ANOVA by Ranks, Dunn’s post hoc comparison \( P < 0.05 \).
Seizures, Synaptic Plasticity, and Activity Blockade

**TABLE 3. Action potential properties were altered in granule cells from TTX- but not d-APV- treated hippocampal slice cultures**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>RMP, mV</th>
<th>R_in, mΩ</th>
<th>AP Threshold, mV</th>
<th>AP at Threshold</th>
<th>AP at 200 pA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>40</td>
<td>−59.5 ± 0.49</td>
<td>139.8 ± 9.19</td>
<td>−37.7 ± 0.34</td>
<td>2.0</td>
<td>5.5</td>
</tr>
<tr>
<td>d-APV</td>
<td>11</td>
<td>−59.1 ± 0.51</td>
<td>146.9 ± 6.94</td>
<td>−38.3 ± 0.26</td>
<td>2.0</td>
<td>6.0</td>
</tr>
<tr>
<td>TTX</td>
<td>43</td>
<td>−60.6 ± 0.47</td>
<td>133.5 ± 7.87</td>
<td>−34.2 ± 0.41**</td>
<td>2.0</td>
<td>2.0**</td>
</tr>
</tbody>
</table>

The resting membrane potential (RMP), input resistance (R_in), action potential (AP) threshold and the number of AP elicited at threshold and after a 200-pA input were measured within 2 min of establishing whole cell configuration using current-clamp recordings as described in METHODS. *Significant difference than vehicle and d-APV; **P < 0.01, ANOVA on Ranks with Dunn's post hoc comparison. Any or all of these possibilities could contribute to the exacerbation of seizures seen in hippocampal slice cultures treated chronically with d-APV or TTX.

**MEMBRANE PROPERTIES.** Granule cell membrane properties were examined using whole cell current-clamp recordings. Granule cells in APV-treated cultures exhibited no significant alterations in resting membrane potential, input resistance or action potential properties compared with vehicle (Table 3). Likewise, in TTX-treated cultures, granule cell membrane potential, input resistance, and number of action potentials elicited at threshold were unchanged. However, the action potential threshold was significantly more positive and the potential threshold was significantly more positive and the number of action potentials elicited by a 200-pA input was reduced by 71% in TTX- compared with vehicle-treated cultures (Table 3). Such a change should render granule cells less likely to fire action potentials in TTX-treated cultures and thus cannot account for the increased propensity to exhibit recurrent seizures.

**SEIZURE SUSCEPTIBILITY AND GLUTAMATERGIC TRANSMISSION.** To begin to examine whether plasticity of excitatory pathways contributed to expression of seizures in the slice culture model, recording conditions revealing seizures were repeated in the presence of the NMDA receptor antagonist, d-APV, and the AMPA/Kainate receptor antagonist, CNX, to block fast glutamatergic transmission (see Table 4 schematic). Antagonists should eliminate seizures if excitatory glutamatergic pathways are required for seizure expression. For vehicle- and d-APV-treated cultures, no spontaneous electrographic seizures were detected when recordings were conducted for 30 min in ACSF, followed by 45 min in ACSF containing BMI, APV, and CNX. However, spontaneous seizures did occur during washout of APV and CNX with ACSF containing BMI (Table 4), which documents the capability of these cultures to generate seizures. Likewise, for TTX-treated cultures, no spontaneous seizures were observed when recordings were conducted for 45 min in ACSF containing APV and CNX, but seizures were noted during washout of APV and CNX with ACSF (Table 4). Together, these data demonstrate that seizures in vehicle-, d-APV-, and TTX-treated hippocampal slice cultures required glutamatergic transmission. These findings further suggest that plasticity of excitatory glutamatergic synapses or inhibitory control of glutamatergic circuits are potential mechanisms underlying the enhanced propensity to exhibit seizures in TTX- compared with d-APV- or vehicle-treated slice cultures and the increase in seizure duration in d-APV- compared with vehicle-treated cultures.

**AXONAL AND DENDRITIC REARRANGEMENTS.** Given the dependence of seizures on glutamatergic transmission and the ability of activity and NMDAR blockade to elicit synaptic reorganization (Lin and Constantine-Paton 1998; McKinney et al. 1999), we next asked whether TTX-treated cultures exhibited more extensive axonal or dendritic sprouting of dentate granule cells in comparison to vehicle- and APV-treated cultures. Granule cell mossy fiber axons were chosen because mossy fiber sprouting into the supragranular layer of the dentate gyrus is associated with temporal lobe epilepsy in humans and animal models and hypothesized to contribute to the expression of limbic seizures (Cronin and Dudek 1988; de Lanerolle et al. 1989; Houser et al. 1990; Mello et al. 1992; Sloviter 1992; Sutula et al. 1988, 1989; Tauck and Nadler 1985). Quantitative analysis of individual granule cells filled with neurobiotin revealed no significant change in mossy fiber morphology to suggest axonal sprouting. Total and layer-specific mossy fiber length, branch points, ends, and boutons were similar in vehicle-, d-APV-, and TTX-treated cultures (Table 5, Fig. 5). Likewise, granule cell dendrite length, branch points, ends, or basilar dendrites were similar in d-APV-, and TTX- treated cultures.

**TABLE 4. Seizures were dependent on glutamatergic transmission**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>30 min</th>
<th>45 min</th>
<th>Vehicle ACSF</th>
<th>BMI/APV/CNQX BMI</th>
<th>BMI ACSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTX</td>
<td>ACSF/AVP/CNQX</td>
<td>ACSF</td>
<td>45 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-APV</td>
<td>ND</td>
<td>0/5</td>
<td>0/5*</td>
<td>4/5</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>ND</td>
<td>0/5</td>
<td>0/5**</td>
<td>5/5</td>
<td></td>
</tr>
<tr>
<td>TTX</td>
<td>0/6*</td>
<td>0/5</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Extracellular field potentials were recorded in the suprapyramidal blade of the dentate granule cell layer of hippocampal slice cultures treated with vehicle, d-APV (50 μM) or TTX (1 μM). Recordings were conducted in physiological recording buffer (ACSF); in the presence of the glutamate receptor antagonists, d-APV (50 μM) and 6-cyano-7-nitroquinolinoxide-2,3-dione (CNX, 10 μM) (ACSF+APV/CNQX); in the presence of the GABA<sub>A</sub> receptor antagonist, BMI (10 μM) (BMI); or in the presence of BMI (10 μM). d-APV (50 μM) and CNX (10 μM) (BMI+APV/CNQX) as shown in the schematic and described in METHODS. Seizures were defined as described in the text and shown in Figs. 1 and 2. Seizure incidence: cultures displaying seizures/total cultures investigated. ND, not determined. *significant difference than washout of APV/CNQX: Fisher exact test, **P < 0.05; **P < 0.01.
cultures when compared with vehicle. In fact, total dendritic length was shorter in TTX- than vehicle- or D-APV-treated cultures. These findings suggest that sprouting of mossy fiber axons or dendrites of the granule cells did not contribute to increased propensity to exhibit seizures in the TTX- compared with APV- and vehicle-treated cultures or the increase in seizure duration in D-APV- compared with vehicle-treated cultures.

SYNAPTIC ALTERATIONS. Anatomy. Sprouting of neurons other than the granule cells could contribute to increased excitability in D-APV- and TTX-treated slice cultures. Therefore we next documented synaptic contacts onto granule cells using double-immunofluorescent labeling. Dendrites were identified using an antibody generated against microtubule associated protein 2 (MAP2), and presynaptic terminals were labeled with an antibody generated against the synaptic vesicle protein, synaptophysin. Initial analyses of synaptophysin immunoreactivity showed synaptic contacts in the molecular layer, granule cell layer, and hilus in vehicle-treated slice cultures. In D-APV-treated slice cultures, levels of synaptophysin immunoreactivity were similar to vehicle in the molecular layer, granule cell layer and hilus (Fig. 6A). Likewise, in TTX-treated cultures synaptophysin immunoreactivity was similar to controls in the granule cell layer and hilus. However, a significant reduction in synaptophysin immunoreactivity was noted in the molecular layer of TTX-treated slice cultures compared with vehicle (Fig. 6A).

To examine these findings further, the number of synaptic contacts onto individual granule cell dendrites was quantified. Individual granule cells were identified morphologically and the number of synaptophysin-immunoreactive (IR) puncta directly apposed to the entire length of a primary MAP2-IR dendrite was counted. Single granule cell dendrites in vehicle-treated hippocampal slice cultures exhibited 1.25 ± 0.20 syn-
aptophysin-IR contacts/micrometer of dendrite (n = 6 dendrites). Contacts were distributed preferentially near somata but also were present in more distal dendrites (Fig. 6, B and C). In cultures treated with n-APV, the density (0.98 ± 0.09 synaptophysin-IR contacts/micrometer; n = 8 dendrites) and distribution of synaptic contacts onto individual granule cells were not significantly altered compared with vehicle, although there was trend toward increased synaptic contacts near the somata (Fig. 6, B and C). In contrast, in TTX-treated slice cultures, the density of synaptophysin-IR contacts/micrometer of dendrite (0.70 ± 0.06; n = 5 dendrites) was significantly decreased (P < 0.05, ANOVA with Holm-Sidak post hoc comparison) and the distribution was shifted significantly closer to somata. Few synaptic contacts were observed in distal granule cell dendrites in TTX-treated cultures (Fig. 6, B and C). These data suggest a decrease in the number of synapses onto the apical dendritic tree of individual granule cells in TTX- compared with vehicle or n-APV-treated slice cultures. These findings in turn raised the question as to whether the reduction reflected excitatory and/or inhibitory synapses.

**Physiology.** To determine whether functional alterations were detectable in excitatory and/or inhibitory synapses, mEPSCs and mIPSCs, respectively, were recorded in individual granule cells using whole cell voltage-clamp recordings. Miniature EPSCs were recorded first because antagonists of glutamatergic transmission eliminated seizures in vehicle-, n-APV-, and TTX-treated cultures. Compiled means showed few significant differences in mEPSC measurements in d-
APV- or TTX-treated cultures compared with vehicle-treated controls (Table 6). However, distribution analyses revealed that mEPSCs were significantly increased in frequency (Fig. 7B), peak amplitude (Fig. 7A and C), area (A and D), rise time (A and E), and decay time (A and F) in d-APV-treated cultures compared with vehicle. These changes primarily involved NMDA-type glutamate receptors because results were no longer significant after subsequent addition of the NMDAR antagonist, d-APV, (Fig. 7, B–F, insets). In TTX-treated slice cultures, mEPSCs in granule cells were also significantly increased in frequency (Fig. 7B), peak amplitude (A and C), area (A and D), rise time (A and E), and decay time (A and F) compared with vehicle. For most measures, these increases were far greater in TTX- than d-APV-treated slice cultures (Fig. 7, A–F), which paralleled the much more dramatic increases in seizure expression in TTX-treated cultures. Moreover, in TTX-treated cultures, increases in mEPSCs primarily involved AMPA/KA type glutamate receptors because results were similar after subsequent addition of the NMDAR antagonist, d-APV, (Fig. 7, B–F, insets). These findings show that changes in mEPSCs were both quantitatively and qualitatively different in d-APV and TTX-treated cultures and suggest that different mechanisms of excitatory synaptic plasticity contributed to the dramatic increases in seizure propensity in TTX-treated cultures and the minor increases in seizure duration in d-APV-treated cultures.

Decreased inhibitory control of excitatory transmission also could contribute to exacerbation of seizures in d-APV- and TTX-treated slice cultures. In d-APV-treated cultures, there was no change in mIPSC frequency (Fig. 8B) or peak amplitude (A and C) compared with vehicle-treated cultures. However, mIPSC area (Fig. 8, A and D), rise time (A and E), and decay time (A and F) were significantly, albeit modestly, reduced compared with vehicle. In contrast, in TTX-treated slice cultures, mIPSC frequency (Fig. 8B) was significantly increased, and peak amplitude was modestly yet significantly reduced compared with controls (Fig. 8, A and C). Significant decreases in mIPSC area (Fig. 8, A and D), rise time (A and E), and decay time (A and F) were more robust. For most measures, alterations were significantly greater in TTX- than d-APV-treated slice cultures (Fig. 8, A–F), consistent with the dramatic increases in seizure expression in TTX-treated cultures in physiological recording buffer compared with no change in seizure incidence in d-APV-treated cultures. These findings show that changes in mIPSCs were quantitatively different in d-APV and TTX-treated cultures and suggest that the dramatic reduction in mIPSC kinetics in TTX- compared with vehicle- or d-APV-treated cultures may have contributed to the profound increases in the propensity of TTX-treated cultures to exhibit seizures in physiological recording buffer. However, the presence of mIPSCs, increased mIPSC frequency and seizure-inducing effects of BMI in a subset of TTX-treated cultures suggest that decreased mIPSC amplitude and kinetics alone are not sufficient for the increased propensity of seizures in TTX-treated cultures.

**DISCUSSION**

This study examined the effects of chronic activity blockade on the propensity of hippocampal slice cultures to exhibit seizures upon removal of activity blockers. Electrophysiological and morphological analyses of organotypic hippocampal slice cultures treated chronically with TTX or d-APV were performed. The following principal findings emerged. Chronic treatment with TTX induced a striking increase in the propensity of dentate granule cells to exhibit seizures upon removal of TTX when compared with vehicle treatment. By contrast, d-APV treatment induced only a minimal increase in the duration of seizures and only when seizures were induced by the GABA receptor antagonist, BMI. Seizures were eliminated by inclusion of CNQX and APV in the recording buffer, implying that glutamatergic synaptic transmission was required for expression of seizures. Immunocytochemical analyses revealed a significant reduction of synapticophysin-IR contacts/micrometer of granule cell apical dendrite and a shift in distribution closer to somata in TTX- compared with vehicle- or d-APV-treated cultures, raising the possibility that synaptic plasticity contributed to the increased excitability. Whole cell voltage-clamp recordings in granule cells revealed striking increases in mEPSC frequency, amplitude, and kinetics in
FIG. 7. Miniature excitatory postsynaptic currents (EPSCs) in dentate granule cells were enhanced in D-APV- and TTX-treated hippocampal slice cultures. Miniature EPSCs in individual dentate granule cells were recorded in the suprapyramidal blade of the dentate granule cell layer of hippocampal slice cultures (17–21 DIV) treated with vehicle, D-APV (50 μM), or TTX (1 μM) as described in METHODS. A—F: recordings of mEPSCs were conducted in the presence of TTX (1 μM) and BMI (10 μM). B—F, insets: recordings of AMPA/KA receptor-mediated mEPSCs were recorded after subsequent addition of D-APV (50 μM) to recording buffer containing TTX (1 μM) and BMI (10 μM). A1: representative mEPSCs illustrate the increase in peak amplitude, area, rise time, and decay time observed in granule cells from D-APV- and TTX-treated slice cultures. A2: representative mEPSCs scaled to the same amplitude document that alterations in mEPSCs from granule cells in D-APV- and TTX-treated slice cultures were not due solely to increases in peak mEPSC amplitude. Cumulative histograms show that the frequency (B), peak amplitude (C), area (D), rise time (E), and decay time (F) of mEPSCs were significantly increased in D-APV- and TTX-treated cultures when compared with vehicle; these increases were significantly smaller in D-APV- than TTX-treated slice cultures. [Significant difference between D-APV- and TTX-treated cultures is apparent when scale is reduced (not shown)]. Similar results were observed in frequency (B, inset), peak amplitude (C, inset), area (D, inset), rise time (E, inset), and decay time (F, inset) of AMPA/KA-receptor-mediated mEPSCs. However, the rise time (E, inset) and decay time (F, inset) of AMPA/KA-receptor-mediated mEPSCs were similar in D-APV- and TTX-treated slice cultures. Data from all cells were pooled to generate the cumulative probability plots. P values were obtained using a 2-tailed Kolmogorov-Smirnov test to assess significant differences between the cumulative probability plots; significance was defined as P ≤ 0.025. Legend in A1 applies to A–F; the number of slice cultures is indicated in parentheses.
TTX- compared with vehicle-treated cultures; changes were expressed by AMPA, but not NMDA, receptors. Recordings also revealed an increase in mIPSC frequency but significant reductions in mIPSC amplitude and kinetics in TTX-compared with D-APV- or vehicle-treated cultures. By contrast, although mEPSC frequency, amplitude, and kinetics were also increased in D-APV- compared with vehicle-treated cultures, these increases were significantly smaller and qualitatively different from TTX-treated cultures in that changes were expressed by NMDA, but not AMPA, receptors. No alterations in membrane
properties or axonal or dendritic sprouting were detected in TTX- or D-APV- compared with vehicle-treated cultures. Taken together, these findings demonstrate that chronic treatment of organotypic hippocampal cultures with TTX increased the propensity of granule cells to express seizures and that plasticity of both excitatory and inhibitory synapses contributed to this propensity. These findings further demonstrate that chronic treatment with D-APV led to far less synaptic plasticity and was not sufficient to increase the propensity of granule cells to express seizures.

Comparison with previous studies investigating the effects of NMDAR or action potential driven activity blockade on seizures and synaptic plasticity

Much of our data are consistent with individual findings from previous studies investigating the effects of NMDAR or activity blockade on seizures and synaptic plasticity in the developing or epileptic brain. However, this is the first study to document a relationship between functional individual synapse alterations and seizure propensity and the first to show opposite effects on glutamate-mediated excitation and GABA-mediated inhibition in the seizure-resistant dentate granule cells following chronic loss of activity or NMDAR activation.

CHRONIC BLOCKADE OF NMDAR ACTIVATION. Whereas brief inhibition of NMDA receptor activation prevents epileptogenesis in some models, chronic inhibition of NMDA receptor activation paradoxically increases neuronal excitability and can result in epileptic seizures. Numerous studies have shown that brief NMDAR blockade selectively during induced seizures prevents epileptogenesis in both the kindling and pilocarpine models in adult rats in vivo (see Dingledine et al. 1990; Loscher 1998; McNamara et al. 1990; Meldrum 1994; Rice and DeLorenzo 1998; Sutula 1991; Sutula et al. 1996). By contrast, abrupt withdrawal of NMDAR antagonists after chronic treatment of otherwise normal developing and adult rats increases susceptibility to seizures (Sircar et al. 1994; Tandon et al. 1996; Yang et al. 2001). Likewise chronic blockade of NMDAR further increased propensity or severity of seizures in animal models and even humans with epilepsy (Loscher 1998; Sveinbjornsdottir et al. 1993). Although the magnitude of the increased seizure expression induced by D-APV was much less than that induced by TTX, our findings are nonetheless consistent with other studies demonstrating increased excitability induced by withdrawal of NMDAR antagonists after chronic treatment.

The present study provides insight into the cellular mechanisms underlying the increased excitability after chronic inhibition of NMDAR. Chronic treatment with D-APV produced only small changes in mIPSCs and changes in seizure duration occurred only when GABAergic transmission was blocked, suggesting that alterations in GABAergic transmission did not contribute to the changes in seizure expression in D-APV-treated cultures in the current study. The major changes in D-APV- compared with vehicle-treated cultures, other than seizure duration, were increases in mEPSC frequency, amplitude, and kinetics. Changes in mEPSC amplitude and kinetics were mediated by NMDA, but not AMPA, receptors, consistent with previous reports that D-APV treatment increased postsynaptic NMDAR clusters (Liao et al. 1999; O’Brien et al. 1998; Rao and Craig 1997), but had no effect (O’Brien et al. 1998; Rao and Craig 1997) or decreased AMPAR clusters (Liao et al. 1999). Other possibilities that could underlie changes in amplitude and kinetics include alterations in glutamate receptor subunit composition or posttranslational modifications in existing receptors. The increase in mEPSC frequency is similar to previous findings in CA1 pyramidal cells after chronic NMDAR blockade (Luhtti et al. 2001) and could reflect increased presynaptic glutamate release sites, release rates, release probability or activation of previously silent synapses. Increased afferent synapses is an unlikely possibility because, consistent with previous morphological findings in hippocampus (Gomperts et al. 2000; Kossel et al. 1997; Rao and Craig 1997), synaptogenesis and axonal and dendritic sprouting were not significantly increased in granule cells from D-APV- compared with vehicle-treated cultures. However, because mEPSC amplitude also was increased, these interpretations may be confounded by enhanced detectability of mEPSCs.

CHRONIC BLOCKADE OF ACTION POTENTIAL MEDIATED ACTIVITY. Recordings from acute hippocampal slices prepared from a site of chronic infusion of TTX in vivo revealed spontaneous epileptiform discharges in the CA3 pyramidial cell layer during acute TTX withdrawal (Galvan et al. 2003). Acute withdrawal after chronic application of TTX to hippocampal slice cultures also caused abnormal spontaneous epileptiform discharges in CA1 pyramidal cells (Niesen and Ge 1999). Epileptiform discharges in both studies were blocked with glutamate receptor antagonists (Galvan et al. 2003; Niesen and Ge 1999). Our results extend these previous findings by documenting the expression of glutamatergic transmission-dependent electrographic seizures involving the normally seizure-resistant granule cells.

Our examination of the mechanisms underlying the TTX induction of spontaneous seizures revealed significant reductions in mIPSC amplitude and kinetics together with striking increases in mEPSC amplitude and kinetics in TTX- compared with vehicle-treated cultures. Chronic treatment of hippocampal cultures with TTX has been shown previously to increase mEPSC amplitude (Burrone et al. 2002; Murthy et al. 2001) and increase the number of synaptic AMPAR (O’Brien et al. 1998) and NMDAR clusters (Rao and Craig 1997). Co-incubation of NMDA with TTX partially reversed the TTX-induced upregulation of NMDAR clusters (Rao and Craig 1997), suggestive of a role for decreased NMDAR function in mediating the effects of activity blockade with TTX. However, our findings showed that increased mEPSC amplitudes in TTX-treated cultures were mediated predominantly by AMPAR. These and other qualitative and quantitative differences in the effects of TTX and D-APV in our study are not consistent with the idea that diminished NMDAR activation plays a dominant role in mediating the effects of chronic blockade of action-potential-driven activity in dentate granule cells. However, the dramatic increases in AMPAR-mediated transmission may have masked more subtle effects on NMDAR.

We also documented increases in mIPSC and mEPSC frequency. Increased mEPSC frequency has been reported previously in hippocampal cultures treated chronically with TTX. Increased frequency was associated with increased neurotransmitter release probability and presynaptic vesicle pool size.
(Burrone et al. 2002; Murthy et al. 2001), which could partially account for our increases in mPSC frequency in TTX-treated cultures. Increased PSC frequency is unlikely to be due to increased afferent synapses. No significant increases in synaptic alterations after chronic activity blockade were present and contributed to the occurrence of seizures. Chronic blockade of action potential-driven activity has been shown previously to reduce afterhyperpolarizations and increase T-type calcium currents in CA1 pyramidal cells (Niesen and Ge 1999) and increase the intrinsic excitability of cortical pyramidal neurons via alterations in the balance of inward and outward currents (Desai et al. 1999).

Despite similarities between our study and others in hippocampus, our findings stand in sharp contrast to results obtained after chronic activity blockade with TTX in undercut sensorimotor cortex. Activity blockade from the time of injury decreased the incidence of evoked and spontaneous epileptiform events (Graber and Prince 1999). The timing of activity blockade was critical, because activity blockade beginning on the 3rd but not the 4th day after initial injury prevented emergence of epileptiform events (Graber and Prince 2004). Because we initiated treatment of our cultures with TTX beginning on the 3rd day after slice preparation, disparities are not likely due to differences in the timing of TTX treatment relative to injury. However, disparities may be due to differences between hippocampus and cortex or developmental and adult time points. Arguing against brain region-specific effects, chronic activity blockade in visual cortex during development decreased GABA expression and GABA-mediated inhibition (Benevento et al. 1998; Turrigiano et al. 1998; Wierenga et al. 2005). These results from visual cortex during development are consistent with our findings in hippocampal slice cultures and would likely exacerbate rather than reduce epileptiform events, although differences between sensorimotor and visual cortex cannot be ruled out. In sum, it appears that differences in the effects of TTX found by Graber and Prince (2004) and the present study may be due to use of developing (present study) versus adult (Graber and Prince 2004) brain.

Potential mechanisms underlying plasticities after action-potential-driven activity blockade

Previous reports have suggested that effects of chronic activity blockade could be mediated in part by decreased brain-derived neurotrophic factor (BDNF) function. In cortex, chronic blockade of action potential driven activity with TTX decreased expression of BDNF (Bozzi 1995; Castren et al. 1992; Zafra et al. 1991) and GABA (Benevento et al. 1995; Hendry and Jones 1986), reduced the frequency and total inhibitory current of spontaneous IPSCs (Rutherford 1997), and increased AMPAR-mediated mEPSC amplitude (Rutherford 1997). Co-incubation of BDNF with TTX blocked TTX-induced effects on mEPSC amplitude and GABA expression and inhibition. Blockade of endogenous BDNF function also mimicked the effects of TTX (Rutherford et al. 1998). Therefore decreased expression of BDNF may have contributed to synaptic alterations after chronic TTX treatment in hippocampal slice cultures. Neurotrophins and their receptors also play an important role in neuronal survival during development (Levi-Montalcini 1987; Lindsay et al. 1994). Thus the profound neurotoxic effects caused by prolonged exposure to TTX in our study also may have been mediated by BDNF.

Relevance to temporal lobe epilepsy

One popular hypothesis as to the cause of hyperexcitability coincident with epileptogenesis and emergence of seizures is a concurrent increase in glutamate-mediated excitation and decrease in GABA-mediated inhibition. Moreover, electrophysiological studies have documented altered NMDAR properties in granule cells from kindled rats (Kohr et al. 1993; Mody et al. 1988), and anatomical studies have shown up-regulation of both NMDAR and AMPAR in the dentate gyrus of resected human tissue (Babb et al. 1996; Mathern et al. 1999) and in the rat KA model (Babb et al. 1996; Mikuni et al. 1998, 1999) of temporal lobe epilepsy, suggesting that increased glutamate receptor number and/or function may contribute to seizure expression. Thus the homeostatic increases in AMPAR-mediated excitatory transmission coupled with alterations in inhibitory control seen in TTX-treated cultures could lead to the emergence of seizures when TTX was removed. The increases in NMDAR-mediated excitatory transmission observed in d-APV-treated cultures would serve to prolong EPSC duration and lead to the modest increases in BMI-induced seizure duration when d-APV was removed. The neurotoxicity caused by treatment with TTX also may play a role in the loss of synapses and increased seizure expression and is the topic of future investigation. However, whether the increases in seizure expression in our study resulted from pro-epileptogenic or transient effects is unclear. In support of a pro-epileptogenic effect, chronic NMDAR blockade during development increased kindling-induced seizure expression during adulthood (Gorter et al. 1991) and increased audiogenic seizure susceptibility for ≤6 mo after withdrawal (Yang et al. 2001). Conversely, chronic NMDAR blockade during development did not affect susceptibility to flurothyl- or PTZ-induced seizures in adulthood (Sircar et al. 1994; Tandon et al. 1996), suggesting that alterations induced by NMDAR blockade may be transient. Last, whether chronic treatment with TTX is the best model to investigate the mechanisms that contribute to the epilepsies that develop after hypoactivity or loss of afferent pathways in vivo remains a topic of debate. Treatment with TTX causes both pre- and postsynaptic activity blockade, whereas deafferentation usually results only in the loss of presynaptic activity. Furthermore, spontaneous fusion of synaptic vesicles and resultant mEPSCs and mPSCs occur in the presence of TTX, but mPSCs are eliminated after deafferenta-
tion. Nevertheless, blockade of activity with TTX does reproduce part of the consequences of deafferentation and thus may shed light on some of the mechanisms that underlie the effects of loss of afferent activity in both developmental and pathological conditions.

Summary

Excessive neuronal activity and NMDAR activation are thought to play important roles in the pathophysiology associated with traumatic brain injury and epilepsy. However, chronic loss of activity and NMDAR activation also can promote homeostatic responses that could enhance seizure expression. Our findings provide important new information on the effects of chronic loss of activity and NMDAR activation on seizure expression and synaptic plasticity and the role of synaptic plasticity on seizures. This information will help provide a rational basis for the development of therapies designed to disrupt epileptogenesis and the emergence of seizures.

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