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Rapid hippocampal network adaptation to recurring synchronous activity – a role for calcineurin

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Abstract

Neuronal networks are thought to gradually adapt to altered neuronal activity over many hours and days. For instance, when activity is increased by suppressing synaptic inhibition, excitatory synaptic transmission is reduced. The underlying compensatory cellular and molecular mechanisms are thought to contribute in important ways to maintaining normal network operations. Seizures, due to their massive and highly synchronized discharging, probably challenge the adaptive properties of neurons, especially when seizures are frequent and intense – a condition common in early childhood. In the experiments reported here, we used hippocampal slice cultures to explore the effects that recurring seizure-like activity had on the developing hippocampus. We found that developing networks adapted rapidly to recurring synchronized activity in that the duration of seizure-like events was reduced by 42% after 4 h of activity. At the same time, the frequency of spontaneous excitatory postsynaptic currents in pyramidal cells, the expression of biochemical biomarkers for glutamatergic synapses and the branching of pyramidal cell dendrites were all dramatically reduced. Experiments also showed that the reduction in *N*-methyl-D-aspartate receptor subunits and postsynaptic density protein 95 expression were *N*-methyl-D-aspartate receptor-dependent. To explore calcium signaling mechanisms in network adaptation, we tested inhibitors of calcineurin, a protein phosphatase known to play roles in synaptic plasticity and activity-dependent dendrite remodeling. We found that FK506 was able to prevent all of the electrophysiological, biochemical, and anatomical changes produced by synchronized network activity. Our results showed that hippocampal pyramidal cells and their networks adapted rapidly to intense synchronized activity and that calcineurin played an important role in the underlying processes.

Keywords

dendrite; development; epilepsy; FK506; memory

Introduction

Neuronal activity is known to play critical roles in the development and plasticity of the central nervous system. Recently, concepts of Hebbian synaptic plasticity have been supplemented by studies of homeostatic plasticity. The latter emphasizes the adaptive properties of neurons in that they are able to gradually adjust their firing and synaptic

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properties in response to long-term changes in network excitability (Turrigiano & Nelson, 2004; Turrigiano, 2012). For instance, chronic increases of neuronal excitability can lead over time to significant decreases in the amplitude of spontaneous excitatory synaptic events (Turrigiano et al., 1998; Watt et al., 2000; Leslie et al., 2001; Perez-Otano & Ehlers, 2005). Such adaptive changes are thought to be essential as they should act to stabilize neuronal networks and thus allow neuronal activity to be maintained within the physiological range required for proper network operations.

However, the intense synchronized network discharging that characterizes seizures may pose unique challenges to the adaptive properties of the central nervous system – particularly if seizures occur frequently, a condition that characterizes severe childhood epilepsy (Camfield & Camfield, 2002). Several laboratories have employed experimentally accessible *in-vitro* models to probe the effects that recurring seizures have on hippocampal neurons (Thompson et al., 1996; Debanne et al., 2006; Swann et al., 2007a; Berdichevsky et al., 2012). In this regard, dendritic abnormalities including a reduction in branching have been reported in both humans and an animal model of epilepsy (Swann et al., 2000; Nishimura et al., 2011), and similar dendritic abnormalities have been observed in an *in-vitro* model of developmental epilepsy (Nishimura et al., 2008).

The temporal characteristics of such seizure-induced simplification of dendritic arbors are unknown but could provide important clues to underlying mechanisms. Calcium influx through *N*-methyl-D-aspartate (NMDA) receptors and voltage-dependent calcium channels is known to be an important contributor to dendrite growth and synaptic plasticity (Cline, 2001; Wong & Ghosh, 2002; Lohmann et al., 2002; Malenka & Bear, 2004; Espinosa et al., 2009; Gambrill & Barria, 2011). Thus, it seems possible that excessive calcium influx produced by synchronous network activity during development could induce unique adaptive mechanisms to limit hyperexcitability. One calcium-dependent signaling molecule that is regulated by neuronal activity and capable of acting directly on the dendritic cytoskeleton is the Ca^{2+} /calmodulin-dependent serine/threonine phosphatase, calcineurin (Winder & Sweatt, 2001; Malenka & Bear, 2004; Sarmiere & Bamburg, 2004). Increased calcineurin activity has been observed in several animal models of epilepsy (Kurz et al., 2001; Kurz et al., 2003; Sanchez et al., 2005).

Thus, calcium signaling through calcineurin is one potential molecular mechanism that developing hippocampal neurons could utilize in response to intense synchronized network activity. Moreover, given calcineurin's role in the expression of long-term depression (LTD), such compensatory changes could take place more rapidly than commonly reported in homeostatic plasticity (Mulkey et al., 1993; Winder & Sweatt, 2001; Zhou et al., 2004). In the experiments reported here, we describe a surprisingly rapid form of network compensation in response to intense spontaneous network discharging that is prevented by pharmacological inhibition of calcineurin.

Materials and methods

Animals

Rats were used in the majority of the experiments reported here. The exceptions were studies of dendrite morphology, in which Thy1GFP-M transgenic mice (Feng et al., 2000) were used. In these mice, the expression of green fluorescent protein (GFP) in CA1 hippocampal pyramidal cells facilitated the examination of alterations in dendrite microanatomy. The maintenance of animals and surgical procedures were approved by the Baylor College of Medicine institutional animal care committee and were in keeping with guidelines established by the National Institutes of Health.

Hippocampal culture preparation

Slice cultures of the hippocampus were made as previously described (Stoppini et al., 1991). Briefly, the day of birth was designated as postnatal day 0 and transverse hippocampal slice cultures were prepared from 5- or 6-day-old Wistar rats (Harlan, Indianapolis, IN, USA) or Thy1GFP-M mice. Animals were deeply anesthetized with isoflurane vapors, and the forebrain was removed and submerged in cooled Leibovitz's L-15 culture media (Invitrogen, Carlsbad, CA, USA). The hippocampi were dissected free of adhering tissue and placed on a Teflon stage of a custom-built mechanical tissue chopper, with a small amount of L-15. Transverse (375 μ m) slices were cut and placed on prewarmed Millicell (Millipore, Billerica, MA, USA) membrane inserts soaked in normal culture media consisting of 98% Neurobasal A (Invitrogen), 2% B-27 (Invitrogen), and 0.5 mM glutamine (Sigma, St Louis, MO, USA). Three to five slices were put on each insert and arranged such that they did not contact one another. The entorhinal cortex was purposefully removed during dissections and not included in slice culture preparations. The Millicell membranes were then placed into 35 mm Falcon Petri dishes or six-well plates and placed in a water-jacketed incubator at 37 °C with 5% CO₂ atmosphere. The culture media were changed at 2 day intervals. Following 3 days *in vitro*, designated slices were treated with 100 μ M bicuculline methobromide (Sigma) for varying periods of time (from 1 to 48 h). In some experiments, slice cultures were pretreated with 7.5 or 15 μ M FK506 (Sigma), 25 or 50 μ M d-APV (Tocris, Bristol, UK), 100 μ M cyclosporine A (Tocris), 25 nM tetrodotoxin (TTX) (Alomone Labs, Jerusalem, Israel) for 1 h and then co-treated with bicuculline (Bic) for an additional 4 h. Control sister cultures were handled identically with the exception that they were treated with the vehicle used.

Electrophysiology

Field potential recordings were made from slice cultures on Millicell inserts in a custom-built interface recording chamber. Recordings were made in warmed (32 °C), oxygenated (95% O₂, 5% CO₂) artificial cerebrospinal fluid (ACSF) containing (in mM): 1.5 CaCl₂, 1.5 MgSO₄, 122.75 NaCl, 3.5 KCl, 10 glucose, 26 NaHCO₃ and 1.25 NaH₂PO₄. The chamber was constantly perfused at a rate of 1 mL/min. Recording electrodes (4-6 M Ω) were pulled from borosilicate glass and filled with ACSF. Recordings were obtained from the stratum pyramidale of CA3 and/or CA1 utilizing an Axoprobe 1A amplifier (Molecular Devices, Sunnyvale, CA, USA). Data were filtered at 5 kHz and sampled at 10 kHz by a Digidata 1322A (Molecular Devices).

To assay alterations in network excitability under various experimental paradigms, field potential recordings were made for at least 20 min once slice cultures were in the recording chamber. Once a drug treatment took place in the incubator, the drug was not washed from the slice before recordings commenced. Instead, the same drug concentration was included in the ACSF recording chamber perfusate. The only exception to this was the concentration of Bic, which was 100 μ M in the incubator and 50 μ M in the chamber ACSF. The higher concentration was used in the incubator to circumvent any breakdown during prolonged treatments in the incubator.

For assays of excitability, 10 min segments of recordings were analyzed. To accomplish this, synchronized discharging was detected by a custom MATLAB graphical user interface utilizing PeakFinder as the peak detection algorithm (PeakFinder.m, Mathworks file ID: 25500). The detected peaks (i.e. network discharges) were superimposed on the electrophysiological recordings and any peaks erroneously detected were manually eliminated. The resultant data were segregated into bursts by a user-determined index to indicate the maximum intraburst interval before an additional algorithm assigned the

detected peaks as a new discharge event. The mean burst duration, burst frequency, and percent time bursting were calculated for each slice.

Whole-cell recordings of spontaneous excitatory postsynaptic currents (sEPSCs) were obtained from submerged membrane inserts perfused with ACSF supplemented with 2.5 mM MgCl₂, 2.5 mM CaCl₂ and 50 μM Bic. Bic was used to eliminate spontaneous GABA_A receptor-mediated events and the increased concentration of divalent cations was employed to suppress neuronal excitability and limit the occurrence of synchronized network activity. An internal electrode solution consisting of (in mM): 130 CsMeSO₄, 10 HEPES, 12 BAPTA, 4 Mg-ATP, 10 CsCl, 0.3 Na-GTP, and 5 QX-314 (pH 7.3) was used to fill 1.5 mm borosilicate glass electrodes fabricated to produce a final tip diameter of 2.0–2.5 μm and resistance of 2.5–6.0 MΩ using a Brown–Flaming patch pipette puller (Sutter, Novato, CA, USA). CA1 pyramidal cell bodies were visualized on a fixed-stage Axoskop II microscope (Zeiss, Oberkochen, Germany) equipped with IR/DIC optics. Pipette resistances and capacitances were negated prior to accessing the internal milieu. Once accessed, neurons were voltage clamped at -70 mV and the internal electrode solution was allowed to dialyze through the cell for 7–10 min prior to data acquisition, which was preformed with an Axopatch 200B amplifier (Molecular Devices) interfaced with pClamp10 (Molecular Devices). Data from neurons with initial resting membrane potentials more negative than -50 mV and initial series resistances less than 20 MΩ were collected for 10 min. Data were first low pass filtered (5 kHz) prior to digitization (10 kHz) with a 1440 series Digidata (Molecular Devices). Offline analysis of sEPSCs was performed using Axograph X (Berkeley, CA, USA) following a template-based event detection algorithm (τ_{rise}, 0.5 ms; τ_{decay}, 3 ms). Events that exceeded three times the SD of the noise were retained and graphed using OriginPro Version 8 (OriginLab, Northampton, MA, USA).

Quantitative immunoblotting

The methods used have been described previously (Swann et al. 2007a). Briefly, four or five slices were pooled from a treatment group, snap frozen on dry ice and later homogenized by sonication. Protein concentrations were determined by the BCA assay. Equal quantities of protein (10 μg) from each sample were resolved by polyacrylamide gel electrophoresis. The primary antibodies used were: NMDA receptor 2A (NR2A) (1 : 1000, Millipore), postsynaptic density protein 95 (PSD95) (1 : 250 000, Millipore), and NMDA receptor 1 (NR1) (1 : 1000, Millipore). Secondary antibodies were conjugated to horseradish peroxidase and visualization of immunoreactive bands was produced by enhanced chemiluminescence and then exposure to Kodak Biomax film. After digitization, the band intensity was quantified with Image J Image software. Blots were also probed with antibodies to α -actin (1 : 10 000, Sigma), which served as a loading control. The band intensity was normalized to control values on the same blot. In experiments where drug treatments occurred, two controls were used: untreated slices and vehicle-treated slices. In these instances, results were normalized to bands from untreated control slices. This permitted statistical comparison of treatment groups to vehicle-treated controls.

Biolistic-mediated transfections

At 24 h after slice culture preparation, CAG promoter-driven eGFP vectors were transfected into individual neurons using a Helios gene-gun system (BioRad, Hercules, CA, USA). Briefly, 50 μg of plasmid DNA was precipitated onto 25 mg of gold particles (1.6 μm in diameter). The particles were coated on the inside of Gold-Coat tubing (BioRad), which was cut into small cartridges and loaded into the gene gun following the manufacturer's suggested protocol. On the day of transfection, helium gas (120 psi) was used to accelerate the gold particles into the slices and cultures were then returned to the CO₂ incubator until 4 days *in vitro*, when experiments were undertaken.

Neuron tracing and analysis of dendrite anatomy

Slice cultures prepared from GFP-transfected rat slices or GFP-M mice were fixed overnight in 4% paraformaldehyde/4% sucrose-buffered saline at 4 °C. Thereafter, they were washed with 1× phosphate-buffered saline and mounted on slides. CA1 neurons that had strong GFP fluorescence throughout their dendritic trees and were well isolated from other GFP neurons were randomly selected and imaged with a confocal microscope. Basilar dendritic arbors were reconstructed digitally from the image stacks using Neurolucida software (MicroBrightField, Colchester, VT, USA). It was not possible to reconstruct CA1 apical dendrites from GFP-M mice due to the large number of fluorescent processes in the apical dendritic layer. Confocal imaging was accomplished using a FluoView FV300 confocal laser scanning microscope on a BX50WI fixed-stage upright microscope equipped with an FV5-ZM stepper motor and FluoView software (Olympus, Melville, NY, USA). GFP images were acquired via excitation with an argon laser (488 nm line), 505–525 nm bandpass emission filter set, and 20× UPlanApo objective (numerical aperture, 0.8; Olympus) using the appropriate manufacturer-suggested confocal apertures. Steps in the Z-axis were in 2 µm increments and Kalman accumulation averaging of 3 was used. Maximum projection images were generated with FluoView software. All Neurolucida reconstructions were conducted in a blinded manner. Quantitative analysis of the traced data was performed using Neuroexplorer software (MicroBrightField). Numerical data (total dendritic length and branch points), such as geometric means and SEMs, were then calculated for each treatment group and for the control group. Each type of experiment was repeated on three separate occasions and results combined for the final analysis.

Statistics

OriginPro Version 8.0 (OriginLab) was employed for statistical analysis and for the construction of histograms and graphs. The data were analyzed statistically using a two-tailed t-test when comparing two normally distributed groups or the non-parametric two-sample Kolmogorov–Smirnov test when comparing data that were not drawn from a normal distribution as determined by the Shapiro–Wilk test for normality. Otherwise, a one-way or two-way ANOVA followed by a Fisher's LSD or a Scheffe posthoc test to correct for multiple comparisons was used. All data are presented as the mean ± SEM.

Results

Hippocampal networks rapidly adapt to synchronous neuronal activity

In keeping with concepts of homeostatic plasticity, previous studies in our laboratory have shown that prolonged (7 days) treatment of slice cultures with GABA_A receptor antagonists dramatically reduces the ability of hippocampal networks to synchronously discharge (Swann et al., 2007a). However, given the speed at which synaptic plasticity, such as long-term potentiation and LTD, alters synaptic physiology (Malenka & Bear, 2004), we wondered whether hippocampal network activity could be altered by shorter periods of epileptiform activity. Thus, slices were prepared from postnatal day 5–6 Wistar rat pups and maintained *in vitro* in Neurobasal A media for 3 days. At 4 days *in vitro*, randomly selected slices were switched to media (Neurobasal A) containing Bic (100 µM) for an additional 1, 4, 24 or 48 h prior to undertaking electrophysiological recordings (see time line in Fig. 1A). When untreated control slices were transferred to the recording chamber and perfused with normal ACSF, no spontaneous synchronized network activity was observed (data not shown). However, when Bic was applied to the perfusate, network disinhibition was achieved and robust spontaneous network activity was observed (Con 4h + Bic, Fig. 1B). To quantify the resulting synchronous network activity, the duration of these events was measured as the difference between the peaks of the primary and the last after discharge (see arrows, Fig. 1B1).

When compared with control slices, prior treatment with Bic for 4 h attenuated network excitability in response to Bic in the chamber perfusate (Bic 4h + Bic, Fig. 1B). Selected events outlined by boxes B1 and B2 are shown below at a faster time base to further illustrate the reduction in burst duration. On average, Bic treatments of 4 h reduced the duration of network discharging by 40% compared with control sister cultures that were only treated with Bic once in the recording chamber (Fig. 1C: Con 4h vs. Bic 4h: $t_{11} = 2.63$, $P = 0.05$). Both 24 h (Con 24h vs. Bic 24h: $t_{12} = 3.31$, $P = 0.005$) and 48 h (Con 48h vs. Bic 48h: $t_{11} = 5.28$, $P = 0.005$) of Bic treatment also reduced network excitability. When data were compared across hours in culture and treatments, a two-way ANOVA showed a significance Bic treatment effect on the duration of the network discharges (Fig. 1C, $F_{1,51} = 38.18$, $P = 0.005$). Additional analysis showed that the frequency of synchronous discharging was unchanged with time in Bic (data not shown). Thus, the percentage of the total time that slices spent bursting was significantly decreased after 4, 24 and 48 h of Bic treatment (Fig. 1D, $F_{1,51} = 17.65$, $P = 0.005$).

Network adaptation is accompanied by a decrease in the excitatory synaptic drive onto individual pyramidal cells

The recordings in Fig. 1 were from the CA3 subfield. However, recordings in acute hippocampal slices have routinely shown that network activity in area CA3 can drive similar activity in area CA1 through Schaffer collateral axons. This also appears to be the case in slice cultures, e.g. Fig. 2A shows simultaneous recordings from areas CA3 and CA1 where network activity occurs synchronously in both subfields. Given this level of synchrony, it is perhaps not surprising that neurons in both subfields undergo a similar degree of network adaptation following chronic Bic treatment. For instance, Fig. 2B shows that, following 4 h of Bic treatment, although CA3 and CA1 subfields still fire in synchrony, the duration of discharges in both subfields is reduced compared with recording from control slices that are only acutely exposed to Bic in the recording chamber (Fig. 2A).

To begin to explore the potential mechanisms underlying this form of network adaptation, we conducted whole-cell recordings from CA1 pyramidal cells and analyzed spontaneous excitatory synaptic currents as a cellular biomarker for the levels of spontaneous network activity impinging on these neurons. Although the amplitudes of sEPSCs were unchanged in our recordings (Con 4h vs. Bic 4h: $t_{16} = 0.54$, NS), the frequency of sEPSCs was routinely decreased following 4 h of Bic treatment (Fig. 2C). This is illustrated by the rightward shift in cumulative probability plots (Fig. 2D) and a 50% decrease in the frequency of sEPSCs (Con 4h vs. Bic 4h: $D = 0.62$, $P = 0.05$). This suggests that network adaptation is produced at least in part by a decrease in the excitatory synaptic drive onto individual CA1 hippocampal pyramidal cells.

Rapid reductions in postsynaptic protein expression

During early postnatal development, the expression of many glutamatergic synaptic markers and postsynaptic scaffolding proteins gradually increases over time before reaching adult levels of expression (Hestrin, 1992; Sans et al., 2000; Liu et al., 2004). This increase is prevented by 7 days of synchronized network activity (Swann et al., 2007a). Here we examined the effects of seizure-like activity on the expression of synaptic proteins after 1, 4, 24, and 48 h of Bic treatment as a mechanism contributing to the rapid reduction of bursting phenotype observed in Fig. 1. To do this, we quantified alterations in the expression of the NMDA receptor subunit NR2A and the scaffolding protein PSD95, both biochemical markers of glutamatergic synapses. Following 4 h of network disinhibition, the expression of NR2A was significantly decreased compared with that of untreated sister cultures (4 h, Con vs. Bic: $t_{10} = 2.54$, $P = 0.05$). Continued network hyperactivity maintained the reduced NR2A protein expression at 24 h ($t_{10} = 3.25$, $P = 0.005$) and 48 h ($t_{10} = 3.48$, $P = 0.05$).

Similar effects were observed in the expression of PSD95. When data were compared across hours in culture and treatments, a two-way ANOVA showed a significant Bic treatment effect (NR2A: $F_{1,46} = 32.06$, $P = 0.001$; PSD95: $F_{1,54} = 33.03$, $P = 0.001$). These results suggest that the network adaptation illustrated in Fig. 1 may be mediated at least in part by alterations in the number of functional synaptic inputs, and/or the molecular makeup of each glutamatergic synapse.

Given the speed at which changes in synaptic protein expression occurred in Fig. 3 and the robust seizure-like activity recorded from slice cultures (e.g. Figs 1 and 2), it seemed likely that the biochemical changes observed might be dependent on neuronal activity. To test this hypothesis, slice cultures were co-treated with Bic and the sodium channel antagonist TTX, which reliably blocks action potential generation and thus seizure-like network activity. Alterations in the expression of three synaptic proteins (PSD95, NR2A, and NR1) were assessed. Hippocampal slice cultures were treated for 4 h with a combination of Bic (100 μ M) and TTX (25 nM) or each drug alone. Inhibiting neuronal activity with TTX completely blocked the ability of Bic-induced network activity to suppress the expression of these three synaptic proteins (Fig. 4A) (PSD95: Bic vs. TTX + Bic: $t_{10} = -5.95$, $P = 0.001$; NR2A: Bic vs. TTX + Bic: $t_{10} = -5.16$, $P = 0.001$; NR1: Bic vs. TTX + Bic: $t_{10} = -5.53$, $P = 0.001$). The application of TTX alone for 4 h did not impact the expression of these proteins except for NR2A, the expression of which was slightly increased (Fig. 4A) (V-Con vs. TTX: $t_{10} = -2.52$, $P = 0.01$). These data support the notion that the downregulation in the expression of these synaptic proteins is an activity-dependent process.

We next examined whether the effects of a 4 h Bic treatment were NMDA receptor-dependent as the prolonged (7 days) effects of Bic treatment are prevented by the NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (Swann et al., 2007a). The results in Fig. 4B show that, like TTX, d-APV (25 μ M) prevented the downregulation of PSD95, NR2A and NR1. These results support the conclusion that Bic produces abnormal neuronal activity – most likely synchronized network discharges – that leads to an NMDA receptor-dependent downregulation in glutamatergic synaptic protein expression.

A rapid reduction in dendritic arbor branching

As mentioned previously, one possible explanation for the disinhibition-induced reduction in the frequency of sEPSCs and reductions in synaptic protein expression shown in Figs 2-4 is that synchronized network activity in some way reduces the number of excitatory synapses in slice cultures. Previous studies from tissue removed during epilepsy surgery to control seizures have shown that epilepsy is often associated with reduced dendritic arbors and spine density. In *in-vivo* and *in-vitro* epilepsy models, reductions in the length and branching complexity of the dendrites of hippocampal pyramidal cells have also been observed (Isokawa & Levesque, 1991; Jiang et al., 1998; Swann et al., 2000). As dendrites are the major site of excitatory synaptic contact on hippocampal pyramidal cells, it seemed possible that the rapid loss of synaptic proteins coincident with network adaptation might in some way be explained by alterations in dendrite morphology.

To examine this possibility, slice cultures were biolistically transfected with GFP plasmid-coated gold beads and allowed to grow in control media for 3 days prior to Bic treatment. At 4 h after initiating the treatment, slices were fixed. Individual CA1 pyramidal cells were then confocally imaged and the basilar and apical dendritic arbors reconstructed. Results from three separate experiments showed that the number of branches in basilar dendritic arbors was decreased by 26.7% after Bic treatment (Con, 19.9 ± 1.5 vs. Bic, 14.6 ± 1.3 : $t_{16} = 2.7$, $P = 0.05$), whereas those in apical dendrites decreased by 31.7% (Con, 24.3 ± 2.4 vs. Bic, 16.6 ± 1.8 : $t_{15} = 2.6$, $P = 0.05$). Thus, it is possible that the rapid alterations in network

excitability illustrated in Fig. 1 result at least in part from a decrease in synapse number as a consequence of the surprisingly rapid reduction in dendritic branching.

Inhibiting calcineurin prevents the rapid anatomical changes in hippocampal dendrites

Activity-dependent alterations in intracellular calcium signaling have important and well-recognized roles in dendrite growth as well as in a variety of forms of synaptic plasticity (Wong & Ghosh, 2002; Sin et al., 2002; Lohmann et al., 2005; Konur & Ghosh, 2005; Wang & Zhang, 2012). Movement of calcium through NMDA receptors as well as voltage-dependent calcium channels provides the major source of cytoplasmic calcium that is thought to promote the highly orchestrated morphological and physiological changes that underlie neuron development and plasticity (Sin et al., 2002; van Zundert et al., 2004; Bhattacharyya et al., 2009; Sturgill et al., 2009). One secondary signaling molecule that plays a particularly important role in neurite growth and synaptic plasticity is the Ca^{2+} /calmodulin-dependent serine/threonine phosphatase, calcineurin (Mulkey et al., 1993; Bhattacharyya et al., 2009). In terms of activity-dependent alterations in dendrite anatomy, a number of recent studies have implicated calcineurin in mediating alterations of the actin cytoskeleton, including dendritic spine morphology, dendrite spine collapse and swelling of dendrites (Gomez et al., 2002; Zhou et al., 2004; Wang et al., 2005; Zeng et al., 2007; Li et al., 2011). On this basis, we undertook experiments to determine whether calcineurin plays a role in the rapid morphological changes that we observed in disinhibited slices. To accomplish this we used the calcineurin inhibitor FK506.

Slice cultures were prepared at postnatal day 5 or 6 and, in this instance, GFP-M mice expressing GFP in subpopulations of CA1 pyramidal cells were used. The use of these mice facilitated our studies, as many more pyramidal cells express GFP in developing slice cultures from these mice than are available for analysis using biolistic methods. Figure 5A shows an image of the basilar dendrites of three CA1 pyramidal cells expressing GFP from one slice culture and overlaid (in orange) with the computer reconstruction of the dendrites of one of these cells. With these methods in hand, slices were treated with Bic at 4 days *in vitro* for 4 h and immediately fixed. Separate groups of slices were co-treated with Bic and FK506. Controls consisted of untreated slices and a group treated with FK506 alone. The results in Fig. 5B show that, just as in rat slice cultures, Bic treatment resulted in a significant decrease in the number of branches in basilar dendrites in slices from GFP-M mice (Con vs. Bic: $t_{47} = 2.0$, $P = 0.05$). FK506 alone did not alter the number of dendritic branches in basilar dendrites compared with control values. However, when FK506 was combined with Bic, the effect of disinhibition on dendrite arbor branching complexity was abolished. The dendrite branch number in this group differed from that of Bic alone (Bic vs. FK + Bic: $t_{35} = -3.2$, $P = 0.01$) but was not significantly different from the control and FK506 groups. Similar results were obtained when alterations in dendrite length were analyzed. The total dendrite arbor length was significantly reduced by Bic treatment (Con vs. Bic: $t_{47} = 2.2$, $P = 0.05$). FK506 alone increased the length of dendrites over that of the control group (Con vs. FK: $t_{26} = -2.1$, $P = 0.05$). This result is consistent with previous reports showing that, under baseline conditions, calcineurin regulates dendrite maturation by suppressing growth (Schwartz et al., 2009). However, FK506 abolished the effects of disinhibition on dendrite length (Bic vs. FK + Bic: $t_{35} = -4.3$, $P = 0.001$). Despite the increase in length induced by FK506, the dendrite length in the FK506 plus Bic group did not differ from that of FK506 alone, supporting the conclusion that inhibiting the activity of calcineurin blocked the effects of synchronous network activity on developing hippocampal dendrites.

Additional analysis was undertaken to examine the interaction of Bic and FK506 treatments on dendritic arbors. In Fig. 5C, a Sholl analysis was undertaken where virtual concentric spheres, centered on the soma of a neuron, were drawn at 10 μm intervals and the number of

dendrite branches that intersected each sphere was counted. Results showed that, when compared with control pyramidal cells, Bic-treated cells had fewer dendrite branch crossings at nearly all distances as they projected into the basilar dendritic layer. FK506 reversed these effects of Bic (FK + Bic). In this case, far more dendrite crossings were observed in the FK506-treated group than in pyramidal cells treated with Bic alone. For clarity, the Sholl analysis for the FK506-only group is not plotted as it did not differ from that of FK + Bic (see also Fig. 5B) and overlapped in this graph. A two-way ANOVA showed a significant effect of drug treatment, with the Bic-treated group differing from both the control and FK + Bic (Fig. 5C: Con vs. Bic: $F_{1,1272} = 32.2$, $P = 0.001$; Bic vs. FK + Bic: $F_{1,971} = 66.9$, $P = 0.001$). These data support the hypothesis that inhibiting calcineurin blocks the effects of Bic on dendritic arbor length and branching complexity.

Inhibiting calcineurin prevents the biochemical alterations in synaptic protein expression

As glutamatergic synapses are primarily localized to the surface of hippocampal pyramidal cell dendrites, it seems reasonable to suspect that the suppression of glutamatergic synaptic protein expression reported in Figs 3 and 4 is due, at least in part, to a decrease in dendritic branch number and length. It then follows that, if FK506 prevents the alterations in dendrite anatomy due to disinhibition, FK506 should then also prevent the downregulation in glutamatergic synaptic proteins. Indeed, the results in Fig. 6 show that 4 h of Bic-induced disinhibition in rat slice cultures robustly reduced the expression of PSD95, NR2A and NR1 compared with controls. In every case, FK506 prevented the decrease in protein expression induced by Bic (PSD95: Bic vs. FK + Bic: $t_{14} = -7.26$, $P = 0.001$; NR2A: Bic vs. FK + Bic: $t_{14} = -7.05$, $P = 0.001$; NR1: Bic vs. FK + Bic: $t_{14} = -5.86$, $P = 0.001$). In the case of NR2A, FK506 alone produced a small (12%) increase in synaptic protein expression compared with that of vehicle-treated slice cultures ($P = 0.04$). In addition to FK506, we also examined the effects of another calcineurin inhibitor, cyclosporine A. The results obtained were very similar to those of FK506 in that, for all three synaptic proteins, cyclosporine A prevented the Bic-mediated decrease. Treatment with cyclosporine A alone did not significantly increase the expression of any of the proteins examined.

Inhibiting calcineurin prevents network adaptation to synchronous activity

Based on the effects that calcineurin inhibitors have on the anatomical and biochemical changes resulting from disinhibition, we reasoned that if the adaptation of network activity shown in Fig. 1 resulted from these alterations in anatomy and biochemistry then FK506 should prevent the network adaptation observed by 4 h of network hyperexcitability illustrated in Fig. 1. To examine this possibility, slice cultures from rats were previously treated for 4 h with Bic, FK506 alone or a combination of the two drugs. Individual traces in Fig. 7A and the data summarized in Fig. 7B show that 4 h treatment with Bic reduced the duration of synchronous network activity (Con vs. Bic: $t_{11} = 5.85$, $P = 0.001$) and the percentage of time that slices were engaged in synchronous activity (Con vs. Bic: $t_{11} = 2.51$, $P = 0.05$). More to the point of these experiments is the effect of FK506. Alone, the drug had no effect on network activity. However, co-treatment with Bic and FK506 prevented the network adaptation that occurs with Bic treatment alone (Bic vs. FK + Bic: $t_{12} = -3.81$, $P = 0.001$).

Discussion

The experiments reported here were all performed in hippocampal slice cultures taken from immature (5-6 days old) rats or mice and studied during the first week in culture. Pyramidal cell dendrites in these cultures add new branches and grow in length, and biochemical markers of synaptic maturation reach developmental milestones much as they do *in vivo* (De Simoni et al., 2003; Swann et al., 2007a; Swann et al., 2007b; Nishimura et al., 2008;

Nishimura et al., 2011). Importantly, at the same time, recurrent excitatory networks have developed sufficiently to produce robust synchronous network discharges when pharmacologically disinhibited (Gomez-Di Cesare et al., 1997). Thus, seizure-like events can be induced by disinhibition at a time when hippocampal neurons are growing.

Using this experimental system, we report rapid alterations in network excitability in response to brief periods of synchronous network activity – a previously unappreciated phenomenon. Electrophysiological recordings consistently showed that the duration of network discharging induced by disinhibition is reduced as early as 4 h after treatment onset. At this time, the frequency of excitatory postsynaptic currents in CA1 hippocampal pyramidal cells is decreased consistent with a decrease in overall network excitability. In addition, the expression of glutamate receptor subunit NR2A and the scaffolding protein PSD95 also rapidly declines as does the branching complexity of pyramidal cell dendrites. As this form of network adaptation was found to be dependent on neuronal activity (blocked by TTX) and to be NMDA receptor-dependent, a role for calcium signaling was anticipated. Results from additional experiments showed that pharmacological inhibition of the calcium-dependent protein phosphatase, calcineurin, prevented the alterations in synaptic protein expression and reduction in dendritic arbor branching. Suppressing calcineurin activity also prevented network adaptation when monitored electrophysiologically.

Mechanisms of rapid network adaptation to synchronous activity

The precise cellular and molecular mechanisms responsible for the network adaptation reported here are not known at this time. However, a large number of studies of homeostatic plasticity published over the past decade may provide some insight (for review see Turrigiano, 2012). Experiments from many laboratories have reported alterations in synaptic transmission or intrinsic excitability in dissociated neurons following chronic perturbations in activity. In terms of models of disinhibition, suppression of excitatory synaptic transmission has been observed (Turrigiano et al., 1998; Qiu et al., 2012). As such, previous experiments from our laboratory have produced similar results, in that synchronous network discharging over 7 days results in a decrease in the amplitude and frequency of mEPSCs and a reduction in glutamate receptor subunit expression, which could help to explain the electrophysiological findings (Swann et al., 2007a). The observations reported here, in which a few hours of epileptiform activity reduce glutamatergic synaptic protein expression and decrease sEPSC frequency (Figs 2 and 3), and that is similar to those that occur after 7 days of increased activity, call into question whether the fundamental mechanisms responsible for homeostatic plasticity and the type of neuronal adaptation that we have observed are the same. One of the major differences between homeostatic plasticity experimental paradigms of disinhibition and our studies in slice cultures is the intensity of the neuronal activity. It seems entirely possible that these network-synchronized events – due to their intensity and frequency (Figs 1 and 2) – could induce adaptive mechanisms quite different from those when increased activity is more temporally distributed (Turrigiano et al., 1998).

One alternative mechanism to homeostatic plasticity would be that intense synchronized network activity injures developing dendrites and this could lead to branch elimination (Zeng et al., 2007; Guo et al., 2012). One argument against this possibility is that, when very intense neuronal activity occurs, large amounts of glutamate are released that lead to excitotoxic injury and neuronal death, and at these times dendrites swell and form beads along their length (Al-Noori & Swann, 2000; Oliva et al., 2002; Zeng et al., 2007; Hoskison et al., 2007). However, we have not observed dendritic beading in disinhibited slice cultures. Moreover, detailed cell counts have shown that pyramidal cells do not die in response to disinhibition even after 1 week of treatment (Swann et al., 2007a). This leads us to conclude that developing dendrites are not overtly injured by synchronous network activity.

During early development, and at the times investigated here, synaptogenesis leading to the establishment of mature spines has yet to occur. Thus, the predominant sites of synaptic inputs are thought to be filopodia and dendritic shafts (Fiala et al., 1998). Indeed, previous studies have shown that, as dendrites grow during development, they are constantly adding and retracting nascent branches (Dailey & Smith, 1996; Wu et al., 1999). According to the “synaptotropic model” of dendrite growth, nascent branch formation is thought to be guided by the formation of excitatory synapses. For instance, when a vector containing PSD95 fused to GFP is introduced into neurons within the developing zebrafish optic tectum, nascent dendritic branches are formed at sites of PSD95 aggregation; this would suggest that new branch addition is directed by the formation of synapses (Niell et al., 2004). In relation to the network adaptation reported here, recurring synchronous activity rapidly reduced the expression levels of not only PSD95 but also the NMDA receptor subunits NR2A and NR1 (Figs 3, 4 and 6). The decreased bioavailability of these synaptic proteins would be expected to limit the number of synapses present and could prevent dendrite branch formation, thus shifting the balance of branch addition and retraction to favor retraction.

However, given the speed of the effects reported here, it seems possible that recurring synchronized network discharges may induce Hebbian-like mechanisms. Indeed, previous studies have shown that synchronous epileptiform activity can induce either long-term potentiation or NMDA receptor-dependent LTD of glutamate synapses in the hippocampus depending upon the age and severity of seizure activity (Smith & Swann, 1999; Bains et al., 1999; Abegg et al., 2004; Debanne et al., 2006). One argument for an LTD-like mechanism in explaining the results reported here is that LTD has been shown to reduce the frequency and amplitude of mEPSCs following a rapid internalization of glutamate receptors, many of which are targeted for degradation (Ehlers, 2003; Colledge et al., 2003; Citri et al., 2009). Such a mechanism could help to explain the reduction in sEPSC frequency and rapid loss of NR2A and PSD95 expression reported in Figs 2 and 3. In addition, LTD has been shown to be accompanied by rapid alterations in dendrite morphology (Zhou et al., 2004; He et al., 2011). These effects are primarily on the microanatomy of dendrite spine where spines have been shown to shrink or collapse. However, seizures and their accompanying network discharging may induce more extreme anatomical changes that could be manifested as branch retraction as opposed to spine collapse. This may be the case early in life, when dendrites are growing and possess few spines.

Calcineurin's role in network adaptation

Some forms of LTD are well-known to be dependent on calcineurin activity (Mulkey et al., 1993). As the biochemical, electrophysiological, and anatomical alterations produced by disinhibition are all antagonized by the calcineurin antagonist FK506, this gives credence to the idea that the mechanisms underlying this form of neuronal and network adaptation may share underlying mechanisms with LTD. As mentioned earlier, repetitive orthodromic activation that induces LTD has been shown to induce calcineurin-dependent reductions in dendritic spine volume and AMPA receptor endocytosis (Zhou et al., 2004; Bhattacharyya et al., 2009; Pontrello et al., 2012). In addition, by anchoring to the postsynaptic density via A kinase anchoring protein 79/150, calcineurin's proximity to synaptically active substrates such as AMPA and NMDA receptors and voltage-gated calcium and potassium channels provides additional means to quickly regulate neuronal excitability and, in turn, network excitability (Lieberman & Mody, 1994; Krupp et al., 1996; Lai et al., 1999; Krupp et al., 2002; Oliveria et al., 2007; Schwartz et al., 2009; Oliveria et al., 2012). For instance, striatal enriched protein tyrosine phosphatase is a well-characterized substrate for calcineurin (Baumgartel & Mansuy, 2012). One important substrate for striatal enriched protein tyrosine phosphatase is the NMDA receptor, which upon dephosphorylation is internalized. Thus, it is plausible that the calcineurin-dependent loss of NMDA receptors following synchronized

network activity reported here is mediated by a calcineurin to the striatal enriched protein tyrosine phosphatase cascade. In addition, calcineurin signaling to slingshot has been shown to initiate the actin depolymerization protein cofilin (Niwa et al., 2002; Endo et al., 2003; Wang et al., 2005; Sun et al., 2010). This is thought to reduce the membrane surface area and destabilize f-actin filaments, and potentially could provide a complementary mechanism of regulating dendrite morphology during development.

One caveat relating to the use of the drugs FK506 and cyclosporine to suppress calcineurin activity is that it is possible that these compounds may also have “off-target” effects in that they may act on other molecules, e.g. other phosphatases, to produce their effects. Although it is difficult to completely eliminate this possibility, the concentrations of these drugs that were used in our experiments are thought to selectively suppress the activity of calcineurin.

Several studies have shown that calcineurin activity is increased in neurons after seizures (Kurz et al., 2001; Sanchez et al., 2005). Thus, it seems possible that, during seizures, excessive calcineurin signaling within the developing dendrites may limit dendrite growth or even alter dendrite dynamics to favor branch retraction. Consistent with this contention are results showing that inhibiting calcineurin activity during development increases dendritic branching in the visual system of living *Xenopus* tadpoles similar to the effects of FK506 reported here (Fig. 5) (Schwartz et al., 2009). Thus, a plausible explanation for our results is that seizures in early life increase calcineurin activity, and this in turn contributes to the loss of dendrite branching over time. These early events would be expected to lead to a decrease in the number of glutamatergic synapses and a reduction in network excitability.

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Abbreviations

ACSF	artificial cerebrospinal fluid
Bic	bicuculline
GFP	green fluorescent protein
LTD	long-term depression
NMDA	<i>N</i> -methyl-D-aspartate
NR1	<i>N</i> -methyl-D-aspartate receptor 1
NR2A	<i>N</i> -methyl-D-aspartate receptor 2A
PSD95	postsynaptic density protein 95
sEPSC	spontaneous excitatory postsynaptic current
TTX	tetrodotoxin

Fig. 1.

Hippocampal networks adapt rapidly to recurring synchronized activity. (A) Time line for experiments. Cultured hippocampal slices were grown in Neurobasal A medium for 3 days. Thereafter, a subset of slices was treated with Bic (100 μ M) for the times indicated. At each time, slices were transferred to the recording chamber that was being perfused with Bic-containing ACSF (referred to as +Bic) and electrophysiological recordings from area CA3. (B) Prolonged recurring synchronized activity was recorded in both control slices (Con 4h + Bic) and slices treated previously with Bic (Bic 4h + Bic). However, network discharges were not as prolonged in slices treated previously with Bic. (B1 and B2) Events outlined by boxes in the slow time base recordings in B are shown at a faster time base. Arrows above the event in B1 indicate the times of the initial and last discharge of the event and were used to measure event duration. (C) Time-dependent alterations in network discharge durations in response to acute Bic treatment in control slices and slices previously treated with Bic. Over time, prior Bic treatment resulted in significant decreases in discharge duration compared with that in control slices (two-way ANOVA; $F_{1,51} = 37.18$, $P = 0.001$). (D) Prior Bic treatment also reduced the percentage of time that slices were engaged in synchronized

activity (two-way ANOVA; $F_{1,51} = 17.6$, $P = 0.001$). Comparison of the results at 1, 4, 24 and 48 h was significant (one-way ANOVA; Fisher's LSD posthoc test for multiple comparisons). $*P = 0.05$, $**P = 0.01$, $***P = 0.001$ ($n = 5-9$ at each time point).

Fig. 2.

Network discharging is synchronized across hippocampal subfields and is concomitant with a reduction in the frequency of sEPSCs in CA1 pyramidal cells. (A) Simultaneous field potential recordings from areas CA3 and CA1 demonstrate the synchrony of network activity. (B) This synchrony is present after prolonged (4 h) treatment with Bic although, in accordance with Fig. 1, the duration of network discharging is reduced in both subfields. (C) Given the network adaptation in area CA1, recordings from individual CA1 pyramidal cells revealed a decrease in the frequency of sEPSCs that produces (D) a shift in cumulative probability plots towards longer interevent intervals (Kolmogorov–Smirnov test, $P = 0.001$) and on average a 50% decrease in sEPSC frequency. Alterations in sEPSC amplitude were not observed. * $P = 0.05$ ($n = 8-10$).

Fig. 3.

Disinhibition results in a marked reduction in glutamatergic synaptic protein expression. Both NR2A (A) and PSD95 (B) expression are rapidly and markedly reduced within hours of initiating Bic treatment. Thereafter, expression levels remains low. All data were normalized to expression levels at t = 0 in control slices on the same blots. Results of representative western blots are shown below each graph. C, Control; B, Bic at 1, 4, 24 and 48 h in culture. -Actin served as a loading control. A two-way ANOVA revealed the effects of Bic treatment for both NR2A ($F_{1,47} = 32.1$, $P = 0.001$) and PSD95 ($F_{1,55} = 33.0$, $P = 0.001$). Comparison of Bic-treated and control slices at the same points in time: * $P = 0.05$, ** $P = 0.01$, *** $P = 0.001$ (n = 6-7).

Fig. 4.

Alterations in glutamatergic synaptic protein expression are dependent on neuronal activity and are also NMDA receptor-dependent. (A) Following 4 h of Bic treatment, the expression of the synaptic proteins PSD95, NR2A, and NR1 were all significantly reduced. However, co-treatment of sister cultures with the sodium channel antagonist TTX (TTX + Bic) prevented the Bic-induced reduction in the expression of these synaptic proteins. TTX alone negligibly altered the concentration of PSD95 and NR1 and increased the concentration of NR2A compared with vehicle treated (d-water) sister control cultures (V-Con). (B) Treatment with the NMDA competitive receptor antagonist, 2-amino-5-phosphonopentanoic acid, also prevented the effects of network bursting on synaptic protein expression. Bic treatment (4 h) consistently reduced the expression of the synaptic proteins PSD95, NR2A and NR1. This reduction was prevented by co-treatment with 2-amino-5-phosphonopentanoic acid, whereas treatment of the antagonist alone did not significantly alter the protein expression. All data are normalized to expression levels in control untreated slices. *** $P < 0.001$ ($n = 6-7$).

Fig. 5.

The calcineurin inhibitor, FK506, prevents Bic-induced reductions in dendritic arborization. (A) Image of CA1 hippocampal pyramidal cells in a slice culture taken from a GFP-M mouse. Superimposed on the image of one neuron is the computer reconstruction of its basilar dendrites (orange). Branch points are denoted by orange dots. For further clarity, this dendrite is shown to the right in A. (B) Bar graphs comparing basilar dendrite length and number of branch points in pyramidal cells. Treatment with Bic (4 h) produced a significant decrease in both branch number and dendrite length. This effect was abolished by co-treatment with FK506. FK506 alone increased dendrite length, probably reflecting calcineurin's ability to partially suppress ongoing dendrite growth. (C) Sholl analysis. Left: Sholl analysis where the number of dendritic branches that intersect equally spaced

concentric spheres were counted. Cross-sections of four spheres at 50 μm intervals from the soma are shown. Right: Bic reduces the number of branch intersections at nearly all distances from the soma. FK506 blocks these effects of disinhibition on dendritic arbors. For clarity, data for FK506-only treatment are not plotted but did not differ from the FK + Bic plot. Whereas Bic reduced both the length and number of dendrite branches, FK506 prevented these effects. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ ($n=13-25$). For Sholl analysis, a two-way ANOVA with posthoc Scheffe test showed: Con vs. Bic: $F_{1,1272} = 32.2$, $P < 0.001$; Bic vs. FK + Bic: $F_{1,971} = 66.9$, $P < 0.001$; Con vs. FK + Bic: $F_{1,949} = 12.1$, $P < 0.001$). Scale bar in A = 100 μm .

Fig. 6.

The calcineurin inhibitors FK506 and cyclosporine A prevent the Bic-induced reductions in glutamatergic postsynaptic protein expression in slice cultures from rats. (A) After 4 h of Bic-induced disinhibition, the expression of PSD95, NR2A and NR1 are reduced. These effects are abolished by co-treatment with FK506 (7.5 μ M). FK506 alone appeared to increase NR2A expression slightly but this effect was not significant. Images below each graph are results from representative western blots. (B) Cyclosporine A also blocked the effects of Bic on synaptic protein expression. All data are normalized to expression levels in control untreated slices. V-Con are control sister cultures that were treated with the vehicles used to dissolve drugs: dimethyl sulfoxide for FK506 and cyclosporine A and d-water for Bic. *** P < 0.001 (n = 8-10).

Fig. 7.

The calcineurin inhibitor FK506 abolishes network adaptation induced by 4 h of recurring synchronous activity in slice cultures from rats. (A) Representative recordings from area CA3 under four experimental conditions. Bic treatment (4 h) resulted in a decrease in the duration of synchronized network discharging. This effect was prevented by co-treatment with FK506 (15 μ M). (B) Graphs summarizing the results of recordings from multiple experiments where both the burst duration and percentage of time that slices were engaged in network bursting were quantified. When comparing Bic-treated slices with all other groups: * P 0.05, *** P 0.005 (n = 5-9 slice cultures).