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Pharmacologically induced calcium oscillations protect neurons from increases in cytosolic calcium after trauma

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Abstract

Increases in cytosolic calcium ($[Ca^{2+}]_i$) following mechanical injury are often considered a major contributing factor to the cellular sequelae in traumatic brain injury (TBI). However, very little is known on how developmental changes may affect the calcium signaling in mechanically injured neurons. One key feature in the developing brain that may directly impact its sensitivity to stretch is the reduced inhibition which results in spontaneous $[Ca^{2+}]_i$ oscillations. In this study, we examined the mechanism of stretch-induced $[Ca^{2+}]_i$ transients in 18-days *in vitro* (DIV) neurons exhibiting bicuculline-induced $[Ca^{2+}]_i$ oscillations. We used an *in vitro* model of mechanical trauma to apply a defined uniaxial strain to cultured cortical neurons and used increases in $[Ca^{2+}]_i$ as a measure of the neuronal response to the stretch insult. We found that stretch-induced increases in $[Ca^{2+}]_i$ in 18-DIV neurons were inhibited by pretreatment with either the NMDA receptor antagonist, APV [D(-)-2-Amino-5-phosphonopentanoic acid], or by depolymerizing the actin cytoskeleton prior to stretch. Blocking synaptic NMDA receptors prior to stretch significantly attenuated most of the $[Ca^{2+}]_i$ transient. In comparison, cultures with pharmacologically induced $[Ca^{2+}]_i$ oscillations showed a substantially reduced $[Ca^{2+}]_i$ peak after stretch. We provide evidence showing that a contributing factor to this mechanical desensitization from induced $[Ca^{2+}]_i$ oscillations is the PKC-mediated uncoupling of NMDA receptors (NMDARs) from spectrin, an actin-associated protein, thereby rendering neurons insensitive to stretch. These results provide novel insights into how the $[Ca^{2+}]_i$ response to stretch is initiated, and how reduced inhibition – a feature of the developing brain – may affect the sensitivity of the immature brain to trauma.

Keywords: calcium oscillations, NMDA, spectrin, traumatic brain injury.

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Traumatic brain injury (TBI) is a widespread health problem, affecting nearly 1.4 million people annually (Centers for Disease Control 1998). In the past decade, there has been an increasing effort to describe the mechanism(s) of force transduction for neurons during TBI, with a focus on how these mechanisms can lead to alterations in ionic homeostasis and, in turn, cell dysfunction/death. Factors leading to direct alterations in cytosolic calcium ($[Ca^{2+}]_i$) have received the greatest attention, because of the contributing role that $[Ca^{2+}]_i$ changes can play in determining cell fate (Tymianski and Tator 1996). Neurons typically respond to traumatic mechanical insults with an immediate transient rise in $[Ca^{2+}]_i$ (Cargill and Thibault 1996; LaPlaca and Thibault 1998; Weber *et al*. 1999; Geddes and Cargill 2001; Lusardi *et al*. 2004). Several initiating mechanism(s) of stretch-induced $[Ca^{2+}]_i$ transients have been reported, including direct membrane damage (Geddes *et al*. 2003) and flux through NMDA receptors (NMDARs; Regan and Choi 1994; LaPlaca and Thibault 1998).

NMDARs have previously been shown to be mechanically activated (Paoletti and Ascher 1994), and are therefore a

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Abbreviations used: APV, D(-)-2-Amino-5-phosphonopentanoic acid; $[Ca^{2+}]_i$, cytosolic calcium; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DIV, days *in vitro*; NMDAR, NMDA receptors; OI, oscillation index; TBI, traumatic brain injury; TPA, 12-O-tetradecanoyl phorbol-13-acetate; TTX, tetrodotoxin.

likely source of the $[Ca^{2+}]_i$ increases that can occur following TBI. Previous studies show that both *in vitro* and *in vivo* traumatic injury causes a reduction in the voltage-dependent magnesium block of NMDARs (Zhang *et al*. 1996; Furukawa *et al*. 2000), a factor that may significantly enhance excitotoxic damage in the acute period following injury. Studies also indicate that blocking NMDARs prior to stretch injury can dramatically reduce $[Ca^{2+}]_i$ increases following injury (Regan and Choi 1994; LaPlaca and Thibault 1998; Lusardi *et al*. 2004), and that a sustained block of this receptor can result in protection from cell death (Shapira *et al*. 1990; Dempsey *et al*. 2000; Rao *et al*. 2001).

Although these data indicate an important role for the stretch activation of NMDARs in TBI, little is know on how alterations in the expression and function of receptors, ion channels and ion pumps that naturally occur during brain ontogeny may play a role in how neurons respond to stretch (Cherubini *et al*. 1991; Wenzel *et al*. 1997; Li *et al*. 1998; Rivera *et al*. 1999; Tovar and Westbrook 1999; Li *et al*. 2002). One key feature in the developing brain that may directly impact the sensitivity of the NMDAR is the incidence of $[Ca^{2+}]_i$ oscillations that occur during development of the cortex. Synchronous oscillations in $[Ca^{2+}]_i$ occur spontaneously during development both *in vitro* and *in vivo* as a result of depolarizing GABAergic function (Garaschuk *et al*. 2000; Numakawa *et al*. 2002; Opitz *et al*. 2002). Furthermore, at synapses that become established in the absence of GABA-mediated inhibition, spontaneous neurotransmitter release activates NMDARs, AMPA receptors, and voltage-gated calcium channels (Garaschuk *et al*. 2000; Liljelund *et al*. 2000; Numakawa *et al*. 2002; Opitz *et al*. 2002). In the mature brain, $[Ca^{2+}]_i$ oscillations disappear with the establishment of the chloride gradient by using membrane exchangers and extruders to maintain low levels of internal chloride (Garaschuk *et al*. 2000; Khazipov *et al*. 2004).

In this study, we examine key regulating features of mechanically induced $[Ca^{2+}]_i$ transients. We hypothesized that neurons responded to mechanical stretch with synaptic NMDAR activation as a result of the linkage of NMDAR with the actin cytoskeleton. We further hypothesized that pharmacological inhibition of GABAergic activity would induce $[Ca^{2+}]_i$ oscillations and reduce the sensitivity of neurons to stretch-induced $[Ca^{2+}]_i$ transients as a result of the mechanical uncoupling of synaptic NMDARs from the actin cytoskeleton. Mechanical injury was simulated using an *in vitro* model of TBI and mechanical sensitivity was measured by the magnitude of immediate stretch-induced $[Ca^{2+}]_i$ changes. We found that the stretch-induced $[Ca^{2+}]_i$ transient was predominantly initiated by activation of NMDARs; these neurons were preferentially located at or around synapses. Intriguingly, we could protect neurons from the stretch-induced $[Ca^{2+}]_i$ transients by pharmacologically inducing oscillations using an array of manipulations. We used co-immunoprecipitation to show the NMDAR link to the cytoskeleton was uncoupled when oscillations were induced in cultured neurons. Moreover, we show that $[Ca^{2+}]_i$ oscillations resulted in phosphorylation of NMDARs, consistent with our co-immunoprecipation data. Therefore, our data indicate the stretch protection of $[Ca^{2+}]_i$ oscillations occurs as a result of PKC-mediated phosphorylation of the NR1 subunit of NMDARs and subsequent disruption of its linkage with spectrin.

Materials and methods

Cell culture

All chemicals were purchased from Sigma (St Louis, MO, USA), unless otherwise noted. All animal procedures were performed in accordance with the University of Pennsylvania Institutional Animal Care and Use Committee. Primary cortical neuronal cultures were isolated from day 18 embryonic rats. Briefly, pregnant CD®IGS rats (Charles River Laboratories Inc., Wilmington, MA, USA) were anesthetized with CO_2 and killed by cervical dislocation. Embryos were surgically removed by Caesarian section and the cortices were isolated in sterile calcium- and magnesium-free Hank's balanced salt solution (Invitrogen, Carlsbad, CA, USA). Cortices were incubated in trypsin (0.3 mg/mL) and Dnase I (0.2 mg/mL; Roche Molecular Biochemicals, Indianapolis, IN) for 20 min at 37°C, and then triturated in soybean trypsin inhibitor (1 mg/mL; Invitrogen). Soybean trypsin inhibitor was removed via centrifugation and neurons were re-suspended in plating media (neurobasal with B-27; Invitrogen), 0.5 mM L-glutamine, 25 μM L-glutamic acid and 1% penicillin/streptomycin (Biowhittaker Molecular Apparatus, Rockland, ME, USA). The cell suspension was consecutively passed through 400-, 60- and 28-μm filters. Neurons were then seeded at either 1.0×10^6 or 0.5×10^6 cells/mL on silicone substrates [pretreated with poly-L-lysine (5 μg/mL) overnight at 20°C]. Four days after plating, media was replaced with feeding media (neurobasal with B-27 and 0.5 mM L-glutamine). Cells were incubated at 37°C, 5% CO_2 for 18 days *in vitro* (DIV) before experimentation. Media was completely replaced twice per week.

$[Ca^{2+}]_i$ measurement

The calcium-sensitive fluorescent dye, fura-2AM (Molecular Probes, Eugene, OR, USA), was used to determine transient alterations in $[Ca^{2+}]_i$. Neurons were incubated in 5 μM fura-2AM for 40 min at 37°C, 5% CO_2 and then cells were rinsed three times with saline (120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM HEPES, 15 mM glucose). Cells were excited alternately at 340 and 380 nm and an image of the emission from each excitation wavelength was collected using a Hamamatsu digital camera (Universal Imaging, West Chester, PA, USA) every 3 s. In all experiments, 2 min of baseline data were recorded prior to stretch and $[Ca^{2+}]_i$ was monitored continuously for 5 min following stretch. The emission intensity images were post-processed to calculate changes in $[Ca^{2+}]_i$ in regions of interest in the images using MetaFluor software from Universal Imaging. All cells within the field of view were analyzed for each experiment. The average number of cells imaged per field of view was 105 ± 3, and ranged

from 50 to 152. At least four experimental wells from at least two separate neuronal isolations were used for each experimental condition. All experiments were performed at room temperature.

$[Ca^{2+}]_i$ oscillations were pharmacologically manipulated by blocking $GABA_A$ receptors with 50 μM (+)-bicuculline, sodium channels with 1 μM tetrodotoxin (TTX), NMDARs with 10 μM (+)-MK-801 hydrogen maleate (MK801) or 25 μm D(-)-2-amino-5-phosphonopentanoic acid (APV), AMPA receptors with 2 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and voltage-gated calcium channels with 10 μM nimodipine. GABA function was modulated by decreasing external chloride, thereby reducing the chloride gradient. Neurons were treated with either saline containing 91 mM Cl (80.6 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 19.7 mM Na_2SO_4, 25 mM HEPES, 15 mM glucose) or normal saline containing 130 mM chloride. Synaptic NMDARs were blocked using a technique developed by Hardingham *et al*. (2002). $[Ca^{2+}]_i$ oscillations were induced with bicuculline and then NMDARs that were activated as a result of reduced GABA inhibition were blocked with MK801, an irreversible, open channel blocker. Bicuculline and MK801 were then rinsed away so that only bicuculline-activated (synaptic) NMDARs were inhibited. Throughout this study, we will term these blocked NMDARs as synaptic NMDARs. However, it should be noted that the blocked NMDARs may also include NMDARs that were already in the open state prior to bicuculline administration, as well as those NMDARs that are located nearby to the synapse. Unless otherwise noted, neurons were treated with drugs for 5 min prior to stretch and drugs remained on cells throughout stretch experiment.

The actin cytoskeleton was polymerized or depolymerized by treating neurons with either 2 μM jasplakinolide or 5 μM latrunculin A (Molecular Probes), respectively, for 40 min prior to testing. Latrunculin-induced actin depolymerization was verified by staining neurons with 5 units/mL Alexa Fluor® 488 phalloidin. Calmodulin and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) were inhibited by treating neurons for 40 min prior to an experiment with 10 μM W-7, hydrochloride [N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, HCl; EMD Biosciences, San Diego, CA, USA] and 2 μM KN-93, respectively. Protein kinase C was either inhibited or activated by pretreating neurons for 40 min with 3 μM Ro32–0432 (EMD Biosciences) or 100 nM 12-O-tetradecanoyl phorbol-13-acetate (TPA), respectively.

In vitro model of TBI

We used a custom-designed apparatus to controllably stretch cultured neurons once at a defined strain magnitude and rate of stretch. The level (50%) and duration (25 ms) of the single stretch was intended to simulate the severe deformations that occur during traumatic injury, and are within the range of conditions we have used in past studies of *in vitro* TBI (Geddes and Cargill 2001; Lusardi *et al*. 2004). In our custom system, neurons cultured on silicone substrates were placed directly on a stainless steel plate and covered with a top plate to form a sealed chamber as previously described (Smith *et al*. 1999; Wolf *et al*. 2001). Increasing the pressure within the sealed chamber causes the compliant silicone membrane to stretch, in turn applying a stretch to the cultured neurons. We designed the supporting stainless steel plate to allow only a portion of the population (a 6 mm × 18 mm rectangular region) to be exposed to a defined stretch, to ensure that the cultures are uniformly stretched along only one axis. We used a stretch level for these studies (average pressure = 4–5 psi; rise time = 15 ms) that corresponded to a uniaxial strain field of 55 ± 1%. At this level of mechanical injury, cells remain attached to the membrane following stretch and show no overt signs of tearing or lysis. The device is placed on a Nikon TE300 microscope and cells are imaged immediately before and after stretch.

Data analysis

A combined measure of the frequency and magnitude of $[Ca^{2+}]_i$ oscillations – termed the oscillation index [OI; previously used by Nunez *et al*. (1996)] – was used to quantify inhibitory function. The OI was defined as an average absolute value of the change in fura-2 ratio at every ratioed time point, as shown in eqn 1, where t is time point, n = total number of time points, and R is the fura-2 ratio (340/380 nm). This measure can be considered an average of the rates of change in the fura-2 ratio over the monitoring period. Neurons with an OI less than 0.02 were considered not oscillating. Alternatively, an OI greater than 0.05 represented significant fluctuations in the fura-2 ratio that were representative of an oscillating neuron.

$$\mathrm{OI} = \frac{\sum_{t=0}^{n} \mathrm{abs}(R_{t+1} - R_t)}{n} \quad (1)$$

$[Ca^{2+}]_i$ was calibrated according to eqn 2 where R is the recorded fura-2 ratio, K_D is the dissociation constant of fura-2 (140 nM), R_{min} is the minimum fura-2 ratio, R_{max} is the maximum fura-2 ratio, and Q is the ratio of the 380 nm intensity upon attaining R_{min} to that upon attaining R_{max} (Grynkiewicz *et al*. 1985). To determine R_{max}, neurons were treated with the calcium ionophore, A-23187 (100 μM; Molecular Probes). $[Ca^{2+}]_i$ was then reduced with 1 mM BAPTA AM (Molecular Probes) diluted in calcium-free saline and R_{min} was determined. R_{min}, R_{max} and Q were determined experimentally to be 0.18, 14.6 and 9.04, respectively.

$$[Ca^{2+}]_i = K_D Q \frac{R - R_{min}}{R_{max} - R} \quad (2)$$

Normalized $[Ca^{2+}]_i$ after stretch was assessed for all cells individually and defined as the average $[Ca^{2+}]_i$ over the first minute after stretch divided by the baseline $[Ca^{2+}]_i$ prior to stretch. Neurons exhibiting normalized $[Ca^{2+}]_i$ after stretch greater than 2, representing increases of more than 100% in $[Ca^{2+}]_i$, were considered to 'respond' to stretch. Data are presented as means ± SEM. Unpaired two-tailed Student's t-tests were performed in order to assess statistical significance and p-values less than 0.05 were considered significant.

Immunoprecipitation

We used a co-immunoprecipitation procedure to examine the association between spectrin and NMDAR in control and oscillating neurons. Experimental cells were treated with 50 μM bicuculline methiodide for 5 min before harvesting. Cortical neurons (DIV 18) were washed three times with saline and harvested in lysis buffer [10 mM HEPES, pH 7.4, 200 mM NaCl, 30 mM EDTA, 0.5% TritonX-100, Complete mini protease inhibitor (Roche Molecular Biochemicals) and 1% phosphatase inhibitor]. Cell lysates were centrifuged at 16 000 *g* for 15 min at +4°C. Supernatants were collected and immediately transferred into Spin-X tubes (Costar,

Corning, NY, USA) containing immobilized protein-G agarose (Pierce, Rockford, IL, USA). After proteins were pre-cleared with protein-G agarose 1 h at +4°C, they were incubated with 15 μg NR1 antibody (Chemicon, Temecula, CA, USA) overnight at +4°C. Proteins were then transferred into new Spin-X tubes containing immobilized protein-G and incubated for 2 h at room temperature. The proteins bound to the agarose beads were washed four times with lysis buffer and then were eluted with 2 × NuPAGE loading buffer (Invitrogen) at 75°C for 10 min. Eluted proteins were stored at −80°C.

Western blotting

Proteins immunoprecipitated with NR1 antibody were analyzed by western immunoblot. The eluted protein complex was heated at 75°C for 5 min after the addition of 0.05% β-mercaptoethanol. Equal volumes of proteins were loaded in 3–8% Tris-Acetate gels (Invitrogen) and were resolved at 150 V for 1 h 30 min. After gel electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen) at a constant voltage of 10 V overnight (12 h) in 1 × transfer buffer (Invitrogen) containing 20% methanol. Membranes were blocked in 5% dry milk containing 20 mM Tris-HCL (pH 7.4), 1.5 M NaCl, 0.1% Tween-20. The blots were then incubated with primary antibodies for the intact form of spectrin (1 : 500 dilution; clone FOD009; Chemicon) or NR1 (1 : 200). Following incubation with primary antibodies overnight at +4°C, blots were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1 : 2000; Jackson Immuno-Research, West Grove, PA, USA) for 2 h at room temperature. Enhanced chemiluminescence (Perkin Elmer, Boston, MA, USA) reagents were used to visualize the immunoreactivity on X-ray film. Protein bands were quantified with a computer assisted two-dimensional densitometric scanning (Kodak 1D Image Analysis Software, Eastman Kodak Company, Rochester, NY, USA). The densitometric readings of spectrin proteins were normalized to their corresponding NR1 bands.

We examined two possible mechanisms for spectrin dissociating from the NMDARs when $[Ca^{2+}]_i$ oscillations occur in neurons: the breakdown of spectrin and the phosphorylation of NR1 at Ser896. To confirm bicuculline has no effect on the proteolysis of spectrin upon dissociation from NR1, a second spectrin antibody (1 : 1000; 240/235E; Chemicon) that detects both the intact form of the native protein (~240 kDa) and calpain cleavage products of spectrin (~145 kDa) was also used (Neumar *et al*. 2003; Williams *et al*. 2003). NR1 phosphorylation was evaluated with the phosphor-NR1 (Ser896) antibody (1 : 350; Cell Signaling Technology, Beverly, MA, USA).

Results

Mechanical injury *in vitro* causes $[Ca^{2+}]_i$ influx through synaptic NMDARs

After rapid mechanical stretch injury to 18-DIV neurons, we observed an immediate rise in $[Ca^{2+}]_i$ that was 3.3 ± 0.1 times greater than baseline levels of $[Ca^{2+}]_i$ prior to stretch (50 ± 2 nM). There was no immediate or delayed detachment of any neurons from the substrate following stretch, and the general morphological appearance of the neurons appeared normal in the acute period following stretch injury. The measured $[Ca^{2+}]_i$ response was transient, reaching a peak within 30 s of the stretch injury and slowly decaying back to baseline within 5 min following stretch injury. On average, 54 ± 2% of neurons showed a change in $[Ca^{2+}]_i$ that was more than double the baseline $[Ca^{2+}]_i$, and were classified as 'responding neurons' (see Materials and methods). It should be noted that the per cent of neurons responding to stretch varied across different cortical isolations, so all experiments were performed across at least two isolations and compared only with other experiments performed over the same period.

In past studies, investigators have found that such an immediate rise in $[Ca^{2+}]_i$ occurred from several different mechanisms, including alterations in plasma membrane permeability (Geddes *et al*. 2003), stretch activation of sodium channels (Wolf *et al*. 2001), or opening of various voltage-gated or receptor-mediated calcium channels (Regan and Choi 1994; LaPlaca and Thibault 1998; Samii *et al*. 1999; Lusardi *et al*. 2004). We did not detect any significant uptake of carboxyfluorescein (a normally impermeant fluorescent molecule, MW = 380 Da) following mechanical injury at the stretch level used in this study (data not shown), suggesting that the immediate changes in $[Ca^{2+}]_i$ were not as a result of changes in the non-specific membrane permeability. Rather, we observed that NMDAR activation was the principal mechanism underlying changes in $[Ca^{2+}]_i$ following stretch. Pretreatment of cultures with APV significantly reduced stretch-induced $[Ca^{2+}]_i$ changes ($p < 0.001$), and dramatically reduced the per cent of neurons responding to stretch from 49 ± 3 to 4 ± 2% (Fig. 1). We then proceeded to examine the location of NMDARs that were responsible for the observed changes in $[Ca^{2+}]_i$ after mechanical injury. Synaptic NMDARs were blocked using an established method to stimulate and then antagonize synaptic NMDARs (Hardingham *et al*. 2002; see Materials and methods). We verified that extrasynaptic NMDARs were still active by measuring the $[Ca^{2+}]_i$ response to 100 μM NMDA in the presence of the synaptic NMDAR block. The average $[Ca^{2+}]_i$ response was reduced from 7.2 ± 0.8 to 3.2 ± 0.3, but was still evident, suggesting that extrasynaptic NMDARs remained unblocked. Blocking synaptic NMDARs prior to mechanical stretch injury caused the $[Ca^{2+}]_i$ change to be significantly attenuated ($p < 0.001$), with only 19 ± 3% of neurons responding to stretch injury (Fig. 1). We further verified that NMDARs were directly activated by stretch by pretreating neurons with a cocktail of CNQX, TTX and nimodipine, to block AMPA receptors and voltage-gated sodium and calcium channels, respectively. $[Ca^{2+}]_i$ transients persisted in the presence of this cocktail and were only attenuated with the addition of APV ($p < 0.001$) or blockade of synaptic NMDAR ($p < 0.001$; Fig. 1b).

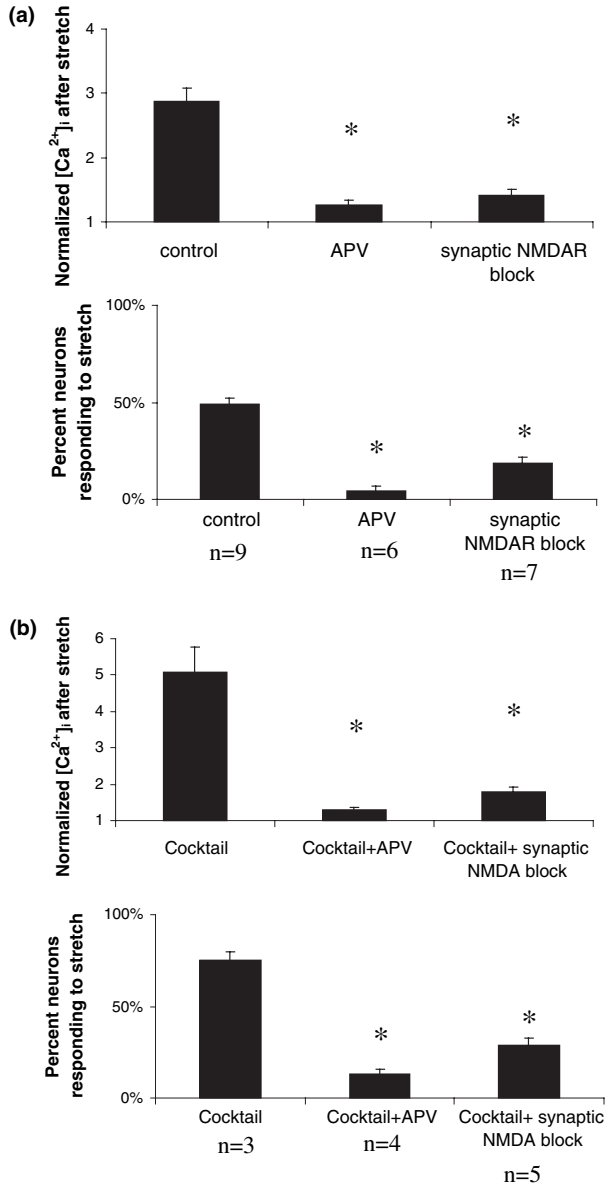

Fig. 1 The $[Ca^{2+}]_i$ response to stretch in 18-DIV neurons is initiated by NMDAR activation. (a) Both the normalized magnitude of the $[Ca^{2+}]_i$ stretch response and the per cent of neurons responding to stretch were almost completely attenuated in the presence of the NMDAR antagonist, 25 μm APV. Interestingly, blocking only the synaptic component of NMDARs also dramatically reduced the $[Ca^{2+}]_i$ transient after stretch to levels near those of a complete NMDAR block; $^*p < 0.001$ versus control. (b) Significant $[Ca^{2+}]_i$ transients persisted when neurons were pretreated with a cocktail containing CNQX, TTX and nimodipine and were only blocked by treatment with either APV or antagonizing synaptic NMDARs. Between three and seven experimental wells were used for each experiment, as indicated on the graphs.

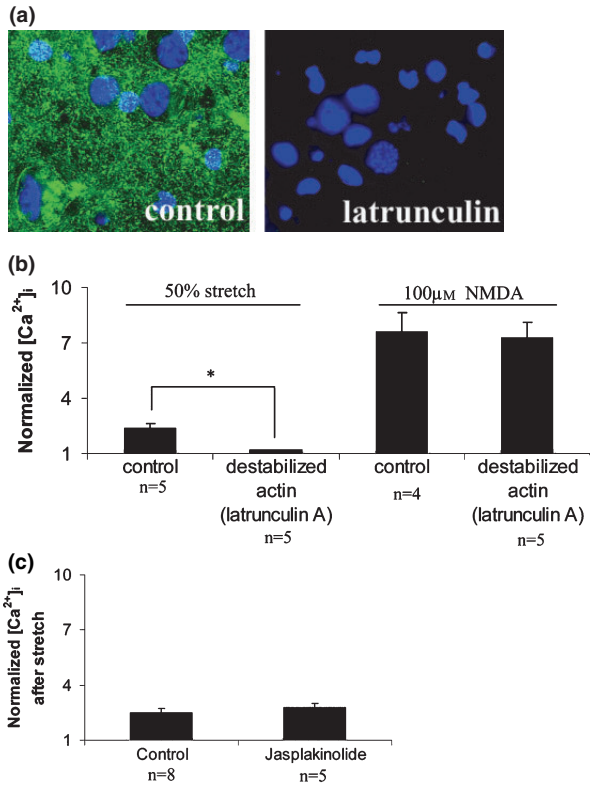

Fig. 2 An intact actin cytoskeleton is necessary for neurons to respond to stretch with increases in $[Ca^{2+}]_i$. (a) Phalloidin staining (green) shows that treatment with 5 μM latrunculin A for 40 min completely depolymerized the actin cytoskeleton. Hoechst positive nuclei (blue) are also shown. (b) The $[Ca^{2+}]_i$ response to stretch to 18-DIV neurons is nearly eliminated when the actin cytoskeleton was depolymerized with 5 μM latrunculin A although the $[Ca^{2+}]_i$ response to NMDA is unaffected by actin depolymerization; $^*p < 0.001$ versus control. (c) Jasplakinolide (2 μM), an actin stabilizer, has no effect on stretch-induced $[Ca^{2+}]_i$ transients.

A large fraction of NMDAR subunits (NR1, NR2A and NR2B) are indirectly tethered to the actin cytoskeleton (Kornau *et al*. 1995; Wyszynski *et al*. 1997; Wechsler and Teichberg 1998). We therefore hypothesized that stretch sensitivity of neurons via NMDARs was because of the linkages between NMDARs and actin, mediated by spectrin and α-actinin (Ehlers *et al*. 1996; Wyszynski *et al*. 1997; Wechsler and Teichberg 1998; Zhang *et al*. 1998; Krupp *et al*. 1999). To test this hypothesis, we depolymerized actin with 5 μM latrunculin A (see representative images in Fig. 2a) and then stretch injured the cultured neurons. Indeed, the stretch response in latrunculin A-treated neurons was significantly reduced to levels not different from a complete block ($p < 0.001$; Fig. 2b). NMDARs were still functional after manipulating the actin cytoskeleton, as a 100 μM NMDA stimulation produced an increase in $[Ca^{2+}]_i$ in latrunculin A-treated cultures that was not significantly different from untreated cultures ($p > 0.05$; Fig. 2b). Alternatively, stabilizing the actin cytoskeleton with 2 μM jasplakinolide did not alter the $[Ca^{2+}]_i$ response to stretch

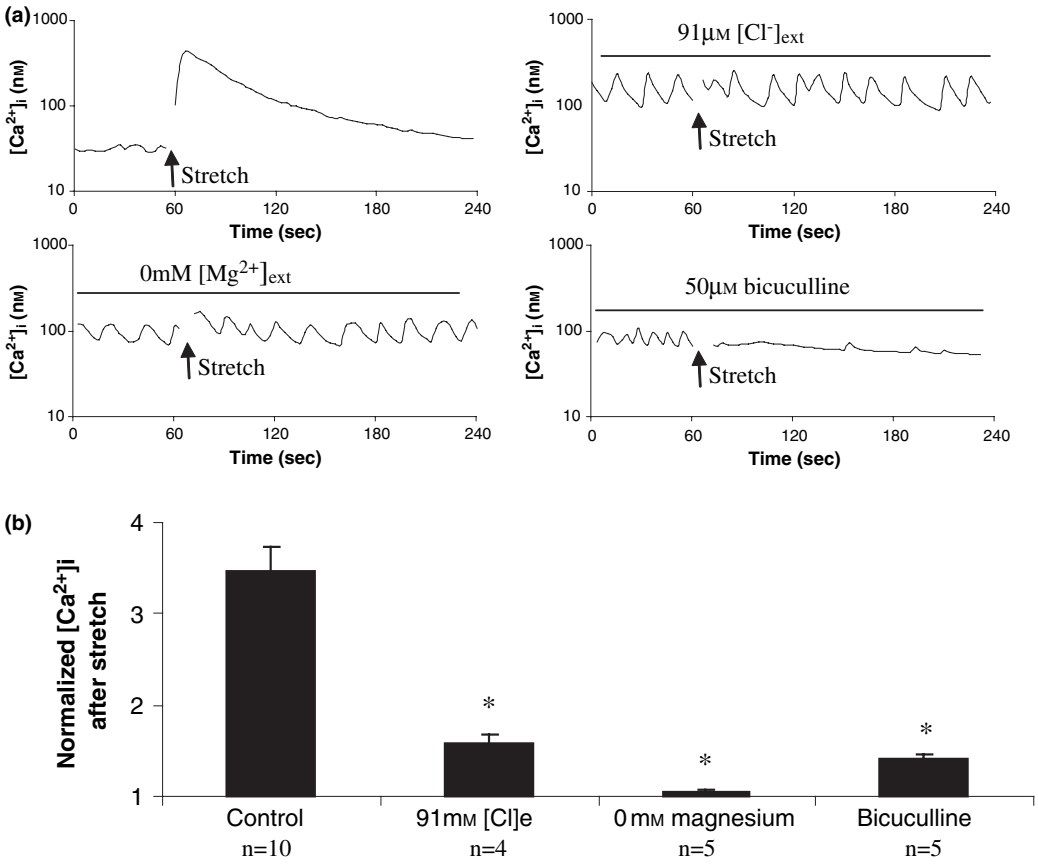

Fig. 3 Pharmacologically induced $[Ca^{2+}]_i$ oscillations protect neurons from stretch-induced $[Ca^{2+}]_i$ transients. (a) Representative $[Ca^{2+}]_i$ responses to stretch are shown for 18-DIV neurons exhibiting either steady baseline $[Ca^{2+}]_i$ or spontaneous oscillations in $[Ca^{2+}]_i$, as induced by either reduced external chloride, 0 mM magnesium or 50 μM bicuculline. (b) The normalized $[Ca^{2+}]_i$ after stretch is summarized for neurons exhibiting oscillations induced by 91 mM external chloride, 0 mM magnesium or 50 μM bicuculline. In each of the three conditions described above, inducing oscillations dramatically reduced the $[Ca^{2+}]_i$ response to stretch; $^*p < 0.001$ versus untreated control neurons.

(Fig. 2c). These data suggest the intact actin cytoskeleton is necessary for transferring the applied stretch to NMDARs.

Activating $[Ca^{2+}]_i$ oscillations consistently diminishes the response to mechanical stretch

We proceeded to test whether triggering $[Ca^{2+}]_i$ oscillations in 18-DIV neurons would reduce the stretch-induced alteration in $[Ca^{2+}]_i$. We first altered GABA inhibition; the chloride gradient was reduced by reducing the external chloride concentration from 130 to 91 mM, thereby mimicking conditions present early in development (Rivera *et al.* 1999). Representative $[Ca^{2+}]_i$ transients are shown for 18-DIV neurons stretched in normal saline or 91 mM external chloride in Fig. 3(a). Decreasing external chloride from 130 to 91 mM significantly enhanced $[Ca^{2+}]_i$ oscillations among 18-DIV neuronal cultures, with the per cent of oscillating neurons increasing from 28 to 62% ($p < 0.001$). In cultures where the oscillations were triggered using this low chloride condition, the normalized $[Ca^{2+}]_i$ response to stretch was reduced from 3.5 ± 0.3 to 1.6 ± 0.1 ($p < 0.001$; Fig. 3b).

$[Ca^{2+}]_i$ oscillations were also induced by relieving the magnesium block of NMDARs (Robinson *et al.* 1993; Nunez *et al.* 1996; Wang and Gruenstein 1997). Normal media containing 0.8 mM magnesium (consistent with standard neurobasal media) was replaced with a media containing 0 mM magnesium. This manipulation served to unmask any spontaneous glutamate released onto primarily synaptic NMDARs, as NMDARs located synaptically would now respond directly to tonic glutamate release upon relief of the magnesium block. After replacing the media with a media containing 0 mM magnesium, the per cent of neurons exhibiting $[Ca^{2+}]_i$ oscillations increased from 14 to 42%. Once again, this oscillatory behavior reduced the response of the cortical neurons to mechanical stretch injury, now to a level where there was no detectable response to the stretch insult ($p < 0.001$; Fig. 3).

Finally, we employed an additional method used in the past to trigger $[Ca^{2+}]_i$ oscillations by blocking $GABA_A$-mediated inhibition in older neurons (Badea *et al.* 2001). Blocking inhibitory activity by using the $GABA_A$ receptor

antagonist (50 μM bicuculline) triggered oscillations in nearly 60% of all neurons. Bicuculline-induced oscillations were nearly identical to those induced by zero magnesium. This induced oscillation behavior also demonstrated a significant reduction in the stretch response, with the average peak change in $[Ca^{2+}]_i$ reduced by nearly 60% ($p < 0.001$; Fig. 3). Therefore, using three distinct experimental techniques, triggering $[Ca^{2+}]_i$ oscillations always reduced the stretch response. It is worth noting, however, that not all experimental manipulations reduced the stretch response. Activating voltage-gated calcium channels with saline containing 10 mM KCl transiently increased the baseline $[Ca^{2+}]_i$, but did not induce oscillations and the $[Ca^{2+}]_i$ response to stretch (1.6 ± 0.1) did not differ significantly from untreated neurons (1.5 ± 0.1, $p = 0.22$).

Targeting the source of reduced sensitivity to stretch from $[Ca^{2+}]_i$ oscillations: role of the actin cytoskeleton

After establishing that the appearance of $[Ca^{2+}]_i$ oscillations coincides with a decrease in the calcium influx following stretch, we assessed the mechanisms of bicuculline-induced $[Ca^{2+}]_i$ oscillations in order to examine the putative mechanism responsible for the stretch desensitization. Consistent with past data (Robinson *et al*. 1993; Bacci *et al*. 1999; Opitz *et al*. 2002), bicuculline-induced $[Ca^{2+}]_i$ oscillations were entirely eliminated with 1 μM TTX (Fig. 4a). Bicuculline-induced oscillations were reduced with 10 μM MK801 (Fig. 4b) and eliminated with 2 μM CNQX (Fig. 4c). Elevations in $[Ca^{2+}]_i$ during oscillations are from both voltage-gated calcium channels and NMDARs, as the oscillations were eliminated with a combination treatment of 10 μM nimodipine and 10 μM MK801 (Fig. 4d).

We therefore tested whether activation of sodium channels or synaptic NMDARs conferred the stretch protection onto oscillating neurons. Pretreatment with TTX did not significantly alter the stretch-induced $[Ca^{2+}]_i$ transient in non-oscillating (control) cultures compared with untreated neurons (Fig. 5). The presence of TTX partially restored the $[Ca^{2+}]_i$ response to stretch in bicuculline-treated neurons ($p < 0.001$, compared with bicuculline alone). However, neurons pretreated with bicuculline (even in the absence of $[Ca^{2+}]_i$ oscillations because of TTX) still alone exhibited a reduction in the $[Ca^{2+}]_i$ response to stretch, compared with neurons treated with TTX ($p < 0.001$, Fig. 5). This suggested that the transient oscillations in baseline $[Ca^{2+}]_i$ were not the sole source of bicuculline-mediated protection, but likely played a role in the protection afforded to bicuculline-treated neurons.

We proceeded to examine if bicuculline-induced oscillations selectively altered synaptic NMDARs that were responsible for initiating stretch-activated $[Ca^{2+}]_i$ transients, thereby causing the neurons to be insensitive to stretch. Synaptic NMDARs were blocked by pretreating with bicuculline, adding MK801, and then rinsing off all drugs.

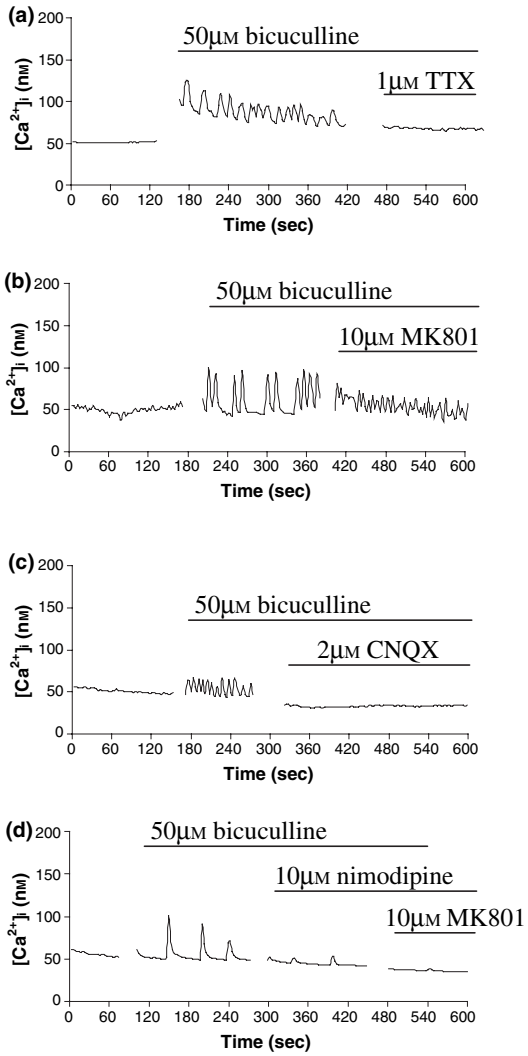

Fig. 4 Bicuculline-induced $[Ca^{2+}]_i$ oscillations in 18-DIV neurons are caused by unmasking spontaneous glutamatergic neurotransmission. (a) TTX (1 μM) completely blocks $[Ca^{2+}]_i$ oscillations. (b) Blocking NMDAR with 10 μM MK801 reduces, but does not eliminate, oscillations. (c) CNQX (2 μM), an AMPA receptor antagonist, completely blocks bicuculline-induced $[Ca^{2+}]_i$ oscillations. (d) $[Ca^{2+}]_i$ oscillations are reduced with 10 μM nimodipine and completely blocked with a combination of nimodipine and MK801.

The temporary bicuculline treatment did not alter spontaneous $[Ca^{2+}]_i$ oscillations as there were no significant differences between the baseline OIs of untreated neurons (OI = 0.025 ± 0.001) compared with those with synaptic NMDARs blocked (OI = 0.023 ± 0.001). The incidence of bicuculline-induced $[Ca^{2+}]_i$ oscillations in neurons with synaptic NMDARs blocked (OI = 0.055 ± 0.003) was significantly more than untreated cultures and less than normal bicuculline-induced $[Ca^{2+}]_i$ oscillations (OI = 0.137 ± 0.006). Inhibiting synaptic NMDARs in bicuculline-treated cultures showed no significant differences in the

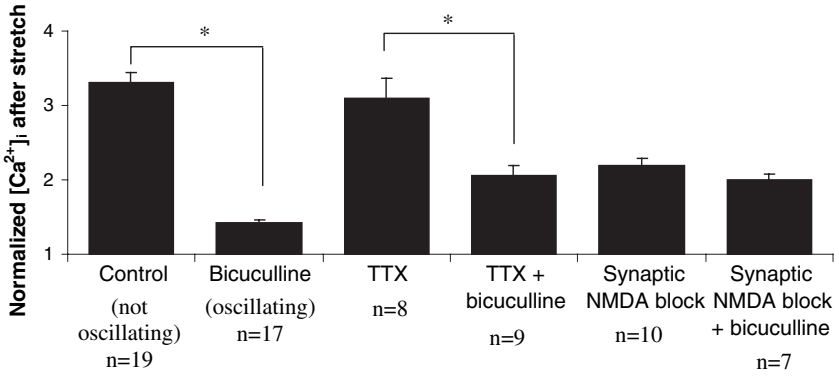

Fig. 5 $[Ca^{2+}]_i$ response to stretch in bicuculline-treated neurons (18 DIV) is only partially restored when $[Ca^{2+}]_i$ oscillations are inhibited with the sodium channel antagonist TTX. TTX did not affect the $[Ca^{2+}]_i$ response to stretch, relative to control, but did enhance the $[Ca^{2+}]_i$ response to stretch, compared with neurons treated with only bicuculline. Bicuculline-mediated protection of neurons is no longer evident when synaptic NMDARs are blocked prior to stretch. The normalized peak $[Ca^{2+}]_i$ after stretch was significantly reduced with bicuculline compared with control neurons. However, bicuculline pretreatment did not alter the normalized peak $[Ca^{2+}]_i$ after stretch when synaptic NMDARs were inhibited; $^*p < 0.001$.

$[Ca^{2+}]_i$ response to stretch compared to similar cultures without functional synaptic NMDARs (Fig. 5). These data suggested that the induced oscillations reduced the stretch activation of synaptic NMDARs.

Next, we determined whether $[Ca^{2+}]_i$ oscillations were disrupting the linkage of NMDARs to the actin cytoskeleton and thereby rendering oscillating neurons insensitive to stretch. Two separate factors were considered that could contribute to this alteration in stretch sensitivity. Calcium influx could either depolymerize the local actin network and untether NMDARs from the structural cytoskeleton, or the calcium influx could disrupt the interaction of NMDARs with actin binding proteins. Although depolymerization of actin does eliminate the stretch sensitivity of NMDARs in cultures that were not oscillating (see Fig. 2), we did not see a significant return of the stretch response when the actin network was stabilized with 2 μM jasplakinolide in oscillating cultures (data not shown). We also verified that treatment with bicuculline did not cause actin depolymerization as evidenced by no observable differences in phalloidin staining between untreated and bicuculline treated cultures (data not shown). We therefore focused on how $[Ca^{2+}]_i$ oscillations altered the linkage of NMDARs to the actin network. One commonly studied binding site of α-actinin and spectrin to the subunit is a domain that also competitively binds the calcium-associated proteins, calmodulin and CaMKII (Ehlers *et al*. 1996; Wyszynski *et al*. 1997; Wechsler and Teichberg 1998; Krupp *et al*. 1999; Leonard *et al*. 2002). Although inhibition of both calmodulin and CaMKII may provide a means to protect the tethering of the NR1 subunit to the actin cytoskeleton, the stretch protection conferred to bicuculline-treated neurons persisted even when both calmodulin and CaMKII were inhibited with 10 μM W-7 and 2 μM KN-93, respectively (Fig. 6).

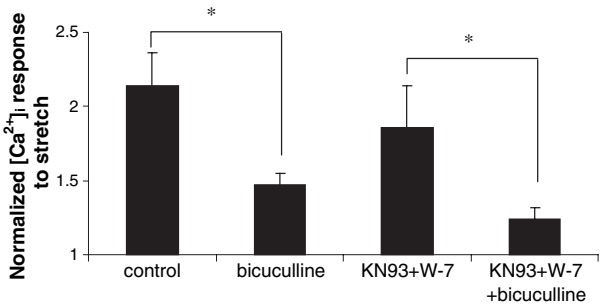

Fig. 6 Calmodulin and CaMKII did not appear to be the source of stretch protection to 18-DIV neurons undergoing bicuculline-induced $[Ca^{2+}]_i$ oscillations. Inhibiting calmodulin and CaMKII with 10 μM W-7 and 2 μM KN-93, respectively, did not significantly alter the reduced stretch-induced $[Ca^{2+}]_i$ transients in neurons undergoing bicuculline-induced $[Ca^{2+}]_i$ oscillations; $^*p < 0.001$.

We next investigated whether the bicuculline-induced $[Ca^{2+}]_i$ oscillations protected neurons from stretch-induced $[Ca^{2+}]_i$ responses because of a phosphorylation-mediated disruption in the linkage between spectrin and NR1. The linkage between spectrin and NR1 is calcium-dependent and is disrupted via PKA- and PKC-mediated phosphorylation of the NR1 subunit, in addition to calmodulin (Wechsler and Teichberg 1998). We therefore studied whether inhibiting PKC in bicuculline-treated neurons would eliminate the stretch protection conferred to oscillating neurons. Although the stretch response was fully restored in neurons treated with the PKC inhibitor Ro32–0432 (3 μM) (Fig. 7a), the treatment protocol also eliminated bicuculline-induced $[Ca^{2+}]_i$ oscillations, possibly because of the role of PKC isoforms in mediating presynaptic glutamate release (Coffey *et al*. 1993). Alternatively, we found that activating PKC by pretreating neurons with 100 nM TPA dramatically reduced the $[Ca^{2+}]_i$

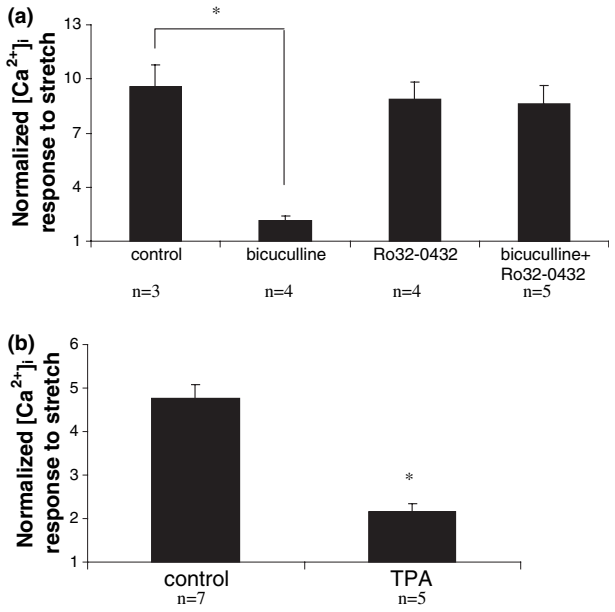

Fig. 7 Inhibiting PKC eliminates stretch protection afforded to bicuculline-treated neurons. (a) Treatment with 3 μM Ro32–0432, a selective cell permeable PKC inhibitor, has no effect on the $[Ca^{2+}]_i$ transient after stretch in untreated neurons, but restores the $[Ca^{2+}]_i$ transient in bicuculline-treated neurons. (b) PKC activation in 18-DIV neurons significantly reduces the normalized $[Ca^{2+}]_i$ response to stretch compared with untreated control neurons; $^*p < 0.001$.

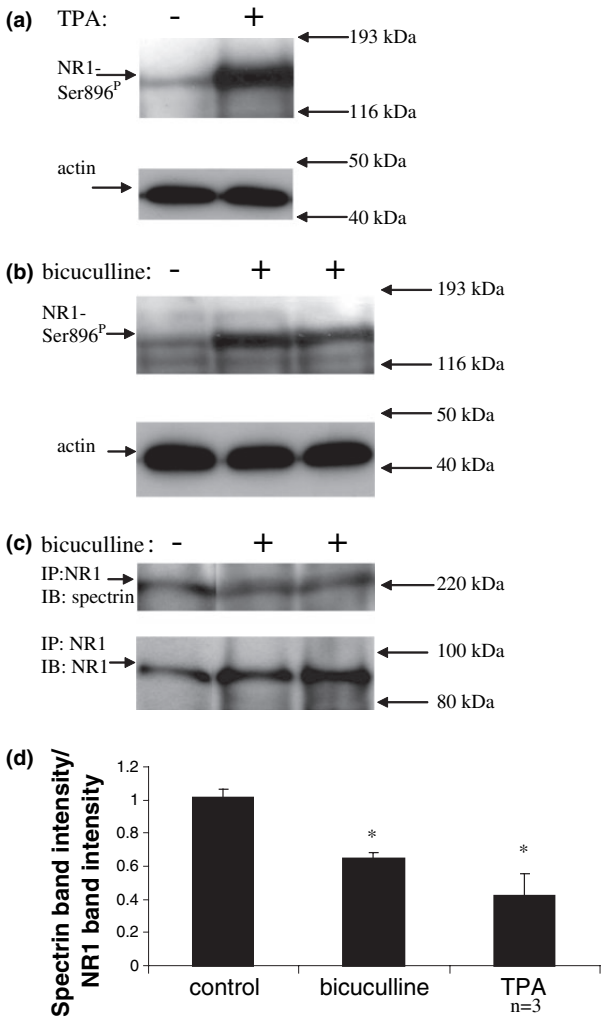

Fig. 8 (a) Phosphorylation of the NR1 NMDAR subunit at Ser896 is driven by PKC-mediated phosphorylation, as evidenced by the increased band intensity in TPA-treated neurons. Actin-labeled loading controls are also shown. (b) Bicuculline treatment to 18-DIV neurons for 5 min enhances NR1 phosphorylation at Ser896. Actin-labeled loading controls are also shown. (c) Blots show spectrin and NR1 staining for protein samples that were immunoprecipitated with NR1. (d) Data was quantified by normalizing the spectrin band intensity with the total NR1 band intensity. Bicuculline significantly disrupts the linkage between NR1 and spectrin; $^*p < 0.001$.

response to stretch (Fig. 7b) and did not significantly change the appearance of calcium oscillations following bicuculline treatment. We do not attribute this change in the TPA-treated neurons as being because of modification of the NMDARs, as the $[Ca^{2+}]_i$ response to 100 μM NMDA was not significantly different for untreated neurons (6.2 ± 1.5) compared with TPA-treated neurons (4.4 ± 0.9).

Finally, we assessed how bicuculline or TPA pretreatment altered NR1 phosphorylation and linkage to spectrin. We verified that TPA treatment phosphorylated NR1 subunits by staining for NR1 phosphorylated at Ser896, a PKC-dependent site (Tingley *et al.* 1997). Indeed, we found that activating PKC with 100 nM TPA for 5 min enhanced NR1 phosphorylation at Ser896, with no change in total actin content, as illustrated in the representative western blots shown in Fig. 8(a). Similarly, Fig. 8(b) shows representative western blots for phosphorylated NR1 where bicuculline-treated neurons exhibited more intense protein bands than untreated controls. We did not observe any spectrin-mediated breakdown products as a result of bicuculline treatment (data not shown). We identified the interactions between NR1 and spectrin using co-immunoprecipitation techniques. Figure 8(c) shows representative immunoblots for untreated and bicuculline-treated neurons. These data were quantified in Fig. 8(d). We found a significant decrease in immunostaining for spectrin in NR1 immunoprecipitated samples treated with both bicuculline and TPA ($p < 0.001$). Therefore, these data suggest that bicuculline-induced $[Ca^{2+}]_i$ oscillations phosphorylate the NR1 subunit via PKC at Ser896, thereby disrupting binding between NMDARs and actin binding proteins and rendering neurons insensitive to stretch.

Discussion

At the level of traumatic mechanical injury examined in this study, we found that the calcium influx through NMDARs is

responsible for the majority of the immediate increase in $[Ca^{2+}]_i$ following injury to 18-DIV cortical neurons in culture. Moreover, we showed that a large fraction of this calcium influx occurs through synaptically localized NMDARs. Depolymerizing the actin network nearly abolished the stretch-induced change in $[Ca^{2+}]_i$, suggesting that an interaction between an intact actin network and the NMDAR was necessary for NMDAR stretch sensitivity. Interestingly, we found that neurons showing $[Ca^{2+}]_i$ oscillations exhibited a substantially reduced $[Ca^{2+}]_i$ peak following *in vitro* traumatic injury. Thus, we investigated factors that could underlie a mechanical uncoupling of NMDARs from actin in oscillating neurons. We did not observe a marked change in the mechanical desensitization when we inhibited calmodulin or CaMKII, two calcium sensitive proteins that can compete with actin-associated proteins in binding to the NR1 subunit, and therefore uncouple NMDARs from the actin network. Rather, we found that bicuculline-induced PKC-mediated phosphorylation of NR1 receptors untethered spectrin from NR1 subunits. We believe that the uncoupling of spectrin from NMDARs, that occurred by PKC-mediated phosphorylation of NR1 subunits, consequently reduced the stretch activation of NMDARs.

Previous studies have shown NMDAR activation immediately following trauma (Regan and Choi 1994; LaPlaca and Thibault 1998; Lusardi *et al*. 2004). However, to our knowledge, this is the first study to show that activation of synaptically localized NMDARs was responsible for the majority of the $[Ca^{2+}]_i$ response. We believe NMDARs were activated by stretch as a result of the indirect linkage with the actin cytoskeleton via α-actinin and spectrin, as actin depolymerization eliminated the $[Ca^{2+}]_i$ response, similar to NMDAR antagonists. The indirect tethering of NMDARs to the cytoskeleton via synaptically localized proteins such as PSD-95, SAP-102, and PSD-93 may explain the enhanced sensitivity of synaptic NMDARs to mechanical forces (Kornau *et al*. 1995; Muller *et al*. 1996; Naisbitt *et al*. 1999; Passafaro *et al*. 1999). Although AMPA receptors are also linked to the cytoskeleton, via RIL which binds to α-actinin (Schulz *et al*. 2004), we do not believe AMPA receptor activation contributed to the stretch-induced $[Ca^{2+}]_i$ transient, because a significant $[Ca^{2+}]_i$ transient persisted when neurons were blocked with a cocktail antagonizing AMPA receptors and voltage-gated calcium and sodium channels.

An important finding of this study was that oscillating cortical neurons were largely resistant to stretch-induced $[Ca^{2+}]_i$ transients. The change in $[Ca^{2+}]_i$ following stretch injury was consistently attenuated when $[Ca^{2+}]_i$ oscillations were pharmacologically induced with either low external chloride, zero magnesium or $GABA_A$ antagonists. Interestingly, the protection conferred to neurons undergoing pharmacologically induced oscillations persisted despite the fact that $GABA_A$ receptors were either inhibited with bicuculline or impaired because of the reduced chloride gradient. At first glance, the protective effects of limiting GABA inhibitory function may be unexpected as GABA is thought to be beneficial in the adult brain following trauma by suppressing the excitotoxic insult (O'Dell *et al*. 2000). However, previous studies have shown that GABA can exacerbate the neuronal response to injury by reverting to an excitatory neurotransmitter after trauma, primarily as a result of increases in internal chloride mediated by a down-regulation of chloride extruders (van den Pol *et al*. 1996; Nabekura *et al*. 2002; Toyoda *et al*. 2003). In this study, we do not believe that the bicuculline-mediated protection was due to GABA transitioning into an excitatory neurotransmitter because the changes in $[Ca^{2+}]_i$ would likely precede changes in internal chloride as a result of the large calcium gradient present in cells. Rather, we show that limiting the activity of GABA can play an additional role prior to injury, by conditioning mechanically sensitive NMDARs in cortical neurons.

The molecular basis for the reduced stretch sensitivity of neurons exhibiting $[Ca^{2+}]_i$ oscillations appears linked, in part, to the tethering of NMDARs to the actin network. Both NR1 and NR2B subunits of NMDARs are linked to actin via actin-binding proteins including α-actinin and spectrin in a calcium-sensitive manner (Ehlers *et al*. 1996; Wyszynski *et al*. 1997; Wechsler and Teichberg 1998; Zhang *et al*. 1998; Krupp *et al*. 1999). We expected that inhibiting the interaction between one or more of the NMDAR subunits would modulate the mechanical sensitivity of the receptor. We initially focused on inhibiting calcium-activated calmodulin and CaMKII as both molecules have been shown to disrupt the binding between NR1 and both α-actinin and spectrin (Ehlers *et al*. 1996; Wechsler and Teichberg 1998; Zhang *et al*. 1998). However, we found that inhibition of calmodulin and CaMKII did not restore the stretch response in oscillating neurons. Alternatively, we showed that bicuculline treatment untethered NR1 from spectrin. Spectrin is normally bound to the NR1 subunit but this link can be disrupted by either calmodulin or PKA- or PKC-mediated phosphorylation of NR1 (Wechsler and Teichberg 1998). As calmodulin inhibition did not restore the stretch response of oscillating neurons, we hypothesized and confirmed that bicuculline treatment resulted in PKC-mediated phosphorylation of NR1 and consequent disruption of the spectrin–NR1 link. Moreover, we found that we could restore the $[Ca^{2+}]_i$ transient in bicuculline-treated neurons by inhibiting PKC. The specific PKC isoforms that were translocated to the membrane by bicuculline-induced $[Ca^{2+}]_i$ oscillations were not investigated in this study but likely are calcium-sensitive (α, β1, β2, γ), which were shown previously to be activated within 30 min of glutamate treatment (Siniscalchi *et al*. 2005). Figure 9 shows a cartoon of our proposed model of how $[Ca^{2+}]_i$ oscillations protect neurons from stretch-induced

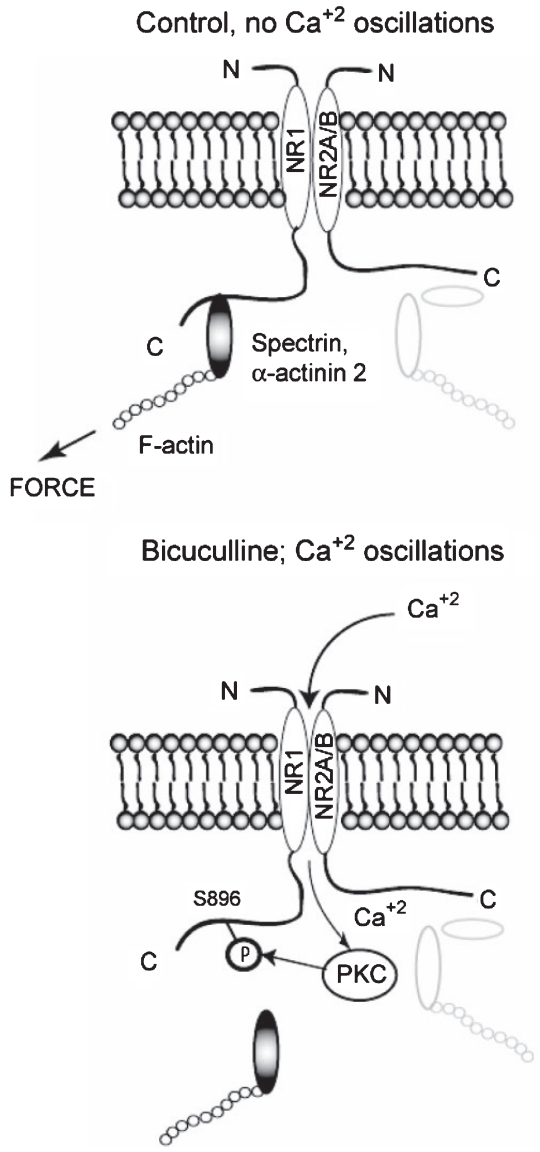

Fig. 9 Illustration of mechanism by which neurons undergoing spontaneous $[Ca^{2+}]_i$ oscillations are protected from stretch-induced $[Ca^{2+}]_i$ responses via the untethering of the NR1 subunit of NMDAR from the actin cytoskeleton.

$[Ca^{2+}]_i$ transients. We believe that the calcium entry through NMDARs and voltage-gated calcium channels as a result of AMPA receptor depolarization, activates PKC. PKC then phosphorylates NR1 at Ser896 and disrupts the linkage between spectrin and NR1, thereby rendering neurons insensitive to stretch.

In summary, we present evidence that $[Ca^{2+}]_i$ oscillations, a physiological condition that occurs during development of the cortex, can perturb the sensitivity of a subpopulation of NMDARs. We proposed that the mechanism for this mechanical desensitization is centered on the local untethering of NMDARs from the actin cytoskeleton via spectrin. These results provide novel insights into how the $[Ca^{2+}]_i$ response to stretch is initiated and may help to explain the reduced sensitivity of the immature brain to trauma.

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