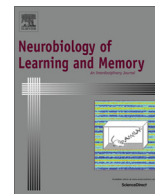




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Long-term depression-associated signaling is required for an *in vitro* model of NMDA receptor-dependent synapse pruning

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ABSTRACT

Activity-dependent pruning of synaptic contacts plays a critical role in shaping neuronal circuitry in response to the environment during postnatal brain development. Although there is compelling evidence that shrinkage of dendritic spines coincides with synaptic long-term depression (LTD), and that LTD is accompanied by synapse loss, whether NMDA receptor (NMDAR)-dependent LTD is a required step in the progression toward synapse pruning is still unknown. Using repeated applications of NMDA to induce LTD in dissociated rat neuronal cultures, we found that synapse density, as measured by colocalization of fluorescent markers for pre- and postsynaptic structures, was decreased irrespective of the presynaptic marker used, post-treatment recovery time, and the dendritic location of synapses. Consistent with previous studies, we found that synapse loss could occur without apparent net spine loss or cell death. Furthermore, synapse loss was unlikely to require direct contact with microglia, as the number of these cells was minimal in our culture preparations. Supporting a model by which NMDAR-LTD is required for synapse loss, the effect of NMDA on fluorescence colocalization was prevented by phosphatase and caspase inhibitors. In addition, gene transcription and protein translation also appeared to be required for loss of putative synapses. These data support the idea that NMDAR-dependent LTD is a required step in synapse pruning and contribute to our understanding of the basic mechanisms of this developmental process.

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1. Introduction

In many brain regions, synapse number initially increases and subsequently decreases over the course of postnatal development (Rakic, Bourgeois, Eckenhoff, Zecevic, & Goldman-Rakic, 1986). These changes in synapse number represent a period of time during which the rate of synapse formation exceeds that of pruning, followed by a net loss of synapses as the rate of pruning overtakes that of synapse formation (Alvarez & Sabatini, 2007; Chen & Regehr, 2000; Glantz, Gilmore, Hamer, Lieberman, & Jarskog, 2007; Holtmaat et al., 2005; Huttenlocher, 1979). With increasing age, the turnover rate of synapses, usually inferred from dendritic spine instability, declines in several brain regions (Elston, Oga, & Fujita, 2009; Holtmaat et al., 2005; Qiao et al., 2016). The loss of synapses on a large scale may be one way neuronal circuits are

sculpted in response to experience. During early postnatal and adolescent development, this experience- and activity-dependent process is required for the refinement and proper functioning of neuronal circuits; disruption of synapse pruning can lead to dysfunction underlying some neurodevelopmental and psychiatric disorders (Kang et al., 2012; Kim et al., 2013; Penzes, Buonanno, Passafaro, Sala, & Sweet, 2013). For example, many disorders in the autism spectrum are thought to be caused by pruning deficits (Auerbach, Osterweil, & Bear, 2011; Pfeiffer et al., 2010; Tang et al., 2014). Conversely, schizophrenia is often accompanied by excessive pruning of synaptic connections, particularly in prefrontal cortical areas (Calabrò, Drago, Sidoti, Serretti, & Crisafulli, 2015; Glantz & Lewis, 2000; McGlashan & Hoffman, 2000; Selemon & Zecevic, 2015). In addition, a gene recently identified as a risk factor for schizophrenia, *C4*, encoding complement protein C4, has also been linked to synapse elimination in the lateral geniculate nucleus (Sekar et al., 2016).

Supporting the idea that synapse pruning requires neuronal activity, and that it is not simply due to a lack of neuronal activity,

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is evidence showing that loss of dendritic spines and functional connections is often greater with more activity in the form of visual experience (Elston et al., 2009; Reiter & Stryker, 1988; Rittenhouse, Shouval, Paradiso, & Bear, 1999). Although the precise mechanisms underlying activity-dependent synapse elimination in the developing brain remain unknown, the idea that repeated synapse weakening by long-term depression (LTD) is a trigger for this synapse loss has been strengthened with experimental support (Bastrikova, Gardner, Reece, Jeromin, & Dudek, 2008; Becker, Wierenga, Fonseca, Bonhoeffer, & Nägerl, 2008; Coleman et al., 2010; Wiegert & Oertner, 2013; Yoon, Smith, Heynen, Neve, & Bear, 2009). Interestingly, although spine shrinkage accompanies LTD, the two phenomena can be dissociated, suggesting that the same initiating events (i.e., NMDA receptor activation) can trigger both distinct signaling pathways (He, Lee, Song, Kanold, & Lee, 2011; Oh, Hill, & Zito, 2013; Zhou, Homma, & Poo, 2004). Similarly, spine loss does not always accompany synapse loss, suggesting that the two processes might occur through independent mechanisms (Bastrikova et al., 2008; Becker et al., 2008). Some evidence suggests that synapses on the smallest spines are most susceptible to separation (Bastrikova et al., 2008) (but see Wiegert & Oertner, 2013).

NMDA receptor-dependent LTD (NMDAR-LTD) can be induced in a variety of experimental models, including *in vivo*, acutely prepared hippocampal slices, hippocampal slices maintained in cultures, as well as dissociated cortical neurons (Bastrikova et al., 2008; Carroll, Lissin, von Zastrow, Nicoll, & Malenka, 1999; Dudek & Bear, 1992; Heynen, Abraham, & Bear, 1996). Most typically, LTD is induced with low frequency (0.5–3 Hz) afferent stimulation (Dudek & Bear, 1992), but it can also be induced with application of NMDA (chemLTD) (Kameyama, Lee, Bear, & Haganir, 1998; Lee, Kameyama, Haganir, & Bear, 1998). NMDAR-dependent LTD requires serine-threonine phosphatase and caspase activity (Li et al., 2010; Mulkey, Herron, & Malenka, 1993), as well as mRNA and/or protein synthesis (Kauderer & Kandel, 2000; Sajikumar & Frey, 2003), however it is distinct from mGluR-dependent LTD, which requires protein synthesis but not necessarily phosphatase activity (Casimiro et al., 2011; Fitzjohn, Kingston, Lodge, & Collingridge, 1999; Huber, Kayser, & Bear, 2000; Huber, Roder, & Bear, 2001). Both types of synaptic depression have been shown to cause dendritic spine shrinkage/loss (He et al., 2011; Hsieh et al., 2006; Hu et al., 2014; Nägerl, Eberhorn, Cambridge, & Bonhoeffer, 2004; Oh et al., 2013; Wiegert & Oertner, 2013; Zhou et al., 2004), axonal bouton shrinkage/retraction (Becker et al., 2008), and/or changes in miniature synaptic currents (Carroll et al., 1999; Casimiro et al., 2011; Pfeiffer et al., 2010). The process of experimentally-induced synapse loss can take place over a wide range of timeframes, on the order of minutes/hours (Bastrikova et al., 2008; Becker et al., 2008) to days/weeks (Egashira et al., 2010; Hasegawa, Sakuragi, Tominaga-Yoshino, & Ogura, 2015; Kamikubo et al., 2006; Shinoda, Kamikubo, Egashira, Tominaga-Yoshino, & Ogura, 2005; Shinoda, Tanaka, Tominaga-Yoshino, & Ogura, 2010; Wiegert & Oertner, 2013). We hypothesized that the specific signaling cascades that are required for NMDAR-LTD can initiate synapse loss (separation of pre- and post-synaptic structures). This is an important point, as LTD-like mechanisms offer the advantage of synapse specificity, similar to that of LTP (Dudek & Bear, 1992). Interestingly, biasing plasticity toward LTP with active CaMKII reduces synaptic contact turnover (Pratt, Taft, Burbea, & Turrigiano, 2008), suggesting that LTP and LTD counter each other in terms of synapse stability.

To begin to address whether LTD is a mechanistic prerequisite for synapse pruning, we established an *in vitro* model of LTD-induced synapse loss by activation of NMDA receptors. We also investigated whether pharmacological inhibition of LTD-related

signaling can prevent synapse loss in a model of synapse pruning in cultured rat cortical neurons.

2. Materials and methods

2.1. Animal use

The protocols for animal use in this study were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the institution's Animal Care and Use Committee.

2.2. Reagents

The following reagents were used in this study: N-methyl-D-aspartic acid (Sigma, M-3262), FK506 (Tocris, 3631), calyculin A (LC Labs, C-3987), okadaic acid (LC Labs, O-5857), foscicic (Santa Cruz Biotechnology, sc-202160), actinomycin D (Sigma, A-9415), anisomycin (Sigma, A-5862), cycloheximide (Sigma), Z-DEVD-FMK (Tocris, 2166), natural mouse laminin (Life Technologies, 23017-015), fetal bovine serum (HyClone, SH30910), poly-D-lysine (Sigma, P6407), DME medium (Life Technologies, 11995-065), Neurobasal medium (Life Technologies, 21103-049), B27 Supplement (Life Technologies, 17504-044), GlutaMAX (Life Technologies, 35050-061), 5-fluoro-2'-deoxyuridine (FUDR; Sigma, F0503), uridine (Sigma, U3003), Hanks Balanced Salt Solution (HBSS; Invitrogen 14025076 and 14175079), bovine serum albumin (BSA; Sigma, A7030), DMSO (Sigma, D2650), paraformaldehyde (Electron Microscopy Sciences, 157-8), propidium iodide (Sigma, P-4170), and Prolong Gold Antifade Mounting Medium (Invitrogen, P36934).

Foscicic, cycloheximide, and NMDA were dissolved in water. Calyculin A, okadaic acid, FK506, Z-DEVD-FMK, actinomycin D and anisomycin were dissolved in DMSO. The final concentration of DMSO was no more than 0.1%. All of the above were prepared as concentrated stock solutions, stored at -20°C , diluted to their indicated final concentrations in culture medium, and allowed to equilibrate in a 37°C incubator for 1 h before each experiment. A modified Sindbis virus encapsulating a gene for eGFP was prepared by NIEHS Viral Vector Core Laboratory and stored at -80°C as concentrated stocks until ready to use (Jeromin, Yuan, Frick, Pfaffinger, & Johnston, 2003).

2.3. Preparation of cultured neurons

Mixed neuronal cultures were prepared from embryonic day 18 Sprague-Dawley rat brains. Hippocampal and cortical tissue pieces were mechanically disrupted by gentle trituration in HBSS, washed, and resuspended in HBSS. Dissociated neurons were plated at low-density ($\sim 80,000$) on poly-D-lysine (25 $\mu\text{g}/\text{ml}$) and laminin (2 mg/ml)-coated 12 mm glass coverslips or MatTek dishes in DMEM with 10% fetal bovine serum and grown at 37°C . Half of the medium was replaced 3–4 days later, and then every 3–4 days with serum-free Neurobasal medium plus 2% NS21 made in-house (Chen et al., 2008) or B27 supplement (Life Technologies; in a limited number of experiments), and 1% GlutaMAX. FUDR was added at 4 days *in vitro* (DIV) to inhibit proliferation of non-neuronal cells. Microglia cultures prepared as described by Harry, Tyler, d'Hellencourt, Tilson, and Maier (2002) were generously provided by C. McPherson.

2.4. Electrophysiological recordings

Whole-cell patch-clamp recordings were performed on either unlabeled neurons or those expressing eGFP. Neurons were

perfused at 1 ml min⁻¹ at room temperature in ACSF consisting of 124 mM NaCl, 2.5 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 17 mM D-glucose, 0.5 mM picrotoxin and 0.001 mM TTX. Patch electrodes (3–7 MΩ) were filled with 120 mM potassium gluconate, 10 mM KCl, 3 mM MgCl₂, 0.5 mM EGTA, 40 mM HEPES, 2 mM Na₂ATP, 0.3 mM NaGTP, with pH adjusted to 7.2 by NaOH. Neurons were voltage clamped at -60 mV and mEPSCs recorded. mEPSCs were detected and analyzed using the Mini Analysis program (version 6.0.7, Synaptosoft). Series resistance was monitored throughout the experiments and data from those cells with >20% change were excluded from the analyses. Baseline recordings were acquired for 3 min from cultured neurons on coverslips (18–22 DIV), followed by 20 μM NMDA with 20 μM glycine in artificial cerebrospinal fluid (ACSF) for 8 min (Beattie et al., 2000). Ten minutes after washout, miniature EPSCs were recorded for 3 additional minutes from the same cell for comparison to the baseline recordings.

2.5. Viral infection

Dissociated cultures were infected with modified Sindbis-eGFP (Jeromin et al., 2003) at 18–20 DIV, as this range was long enough for the neurons to develop mature spines (Boyer, Schikorski, & Stevens, 1998; Dailey & Smith, 1996; Papa, Bundman, Greenberger, & Segal, 1995) (Fig. 1A and B). Cells on coverslips were treated with virus diluted in conditioned media according to a series of pilot studies in which distribution of neurons expressing GFP was compared and the chosen working dilution resulted in neuronal GFP expression across roughly 25–50% of the coverslip by 16–20 h post-infection, the time of the experiments. Antibodies against eGFP were used in addition to maximize fluorescent labeling of dendritic spines (described below).

2.6. Immunocytochemistry

Staining of putative synapses was generally performed as described previously (Glynn & McAllister, 2006). Two or four hours following experimental treatment, cells were fixed in warm 4% paraformaldehyde (PFA)/2.5% sucrose in phosphate-buffered saline (PBS) for 10 min and made permeable with 0.2% Triton X-100 in PBS for 5 min. After blocking in 5% BSA/PBS for 30 min at room temperature, cells were stained overnight at 4 °C with antibodies diluted in blocking buffer, washed 3 times in PBS, stained with the corresponding AlexaFluor 488, 568, or 633 secondary antibodies for 1 h at room temperature, and washed 3 times in PBS before mounting on slides with Prolong Gold Antifade Mounting Medium (Invitrogen). The following primary antibodies were used at the respective dilutions: chicken anti-GFP (1:40,000, Gene-Tex, GTX13970), rabbit anti-synapsin 1 (1:1000, Millipore, AB1543P), rabbit anti-bassoon (1:500, Cell Signaling, D63B6), mouse anti-PSD95 (1:250, NeuroMab, 75-028), chicken anti-MAP2 (1:2000, Abcam, ab5392), rabbit anti-Iba1 (1:1000, Wako). AlexaFluor goat anti-mouse 488, goat anti-rabbit 568, and goat anti-chicken 633 secondary antibodies (Invitrogen) were used at 1:500 dilutions. To confirm specificity of staining, omission of primary antibody was used as a negative control and showed only negligible staining.

2.7. Imaging and analysis

Pyramidal neurons with a spiny appearance, in contrast to the non-spiny type with relatively unbranched dendrites that are thought to belong to GABAergic neurons (Benson, Watkins, Steward, & Banker, 1994), were chosen specifically to study putative excitatory glutamatergic synapses. Fluorescent images from

neuronal cultures were acquired with a Zeiss LSM 510-UV meta confocal microscope (Carl Zeiss Inc, Oberkochen, Germany) using either a Plan-NEOFLUAR 40X/1.3 Oil DIC or a Plan-APOCHROMAT 63X/1.4 Oil DIC objective. Confocal images were acquired and then an optical zoom of 4 was applied for viewing synapses in high-resolution z-stacks. Z-stack images were collapsed into 2D maximum intensity projections before analyzing fluorescence of dendrites using Zen 2010 (Zeiss) for cropping, MetaMorph software (Molecular Devices) for counting, and ImageJ (NIH) for display adjustment. PSD-95- or GFP-labeled spines visible in the green channel were counted manually, followed by visualization of red puncta (synapsin- or bassoon-labeled presynaptic terminals) that were directly adjacent or otherwise contacting spines or the dendritic shafts. Thus, putative synapses were defined as colocalized presynaptic synapsin or bassoon (red), and postsynaptic GFP or PSD-95 (green) fluorescence. Synapses were counted along 20-μm traced segments of dendrites, with proximal dendritic synapses measured 20–50 μm from the soma, and distal synapses defined as those >100 μm from the soma. Note that fluorescence colocalization here indicates only that the two structures (pre- and postsynaptic) are closer than can be resolved in our conditions. Mean numbers of synapses in each condition were normalized to the appropriate vehicle- or mock-treated controls within experiments, then grouped and averaged across experiments. A minimum of three neurons per condition was imaged in each experiment. No attempt was made to quantify shape or size of spines. Slides were coded to ensure blind acquisition and analysis of images.

In some experiments, neurons were treated with MitoTracker Red (Invitrogen, 1 nM in DMSO) to reveal mitochondria, and were imaged using the structured illumination technique on a Zeiss Elyra PS.1 super-resolution microscope (Carl Zeiss Inc, Oberkochen, Germany). A Plan-APOCHROMAT 63X/1.4 Oil DIC objective was used to collect Z-stack images, which were subsequently processed with the SIM processing module of Zeiss Zen 2012.

2.8. Experimental design

To investigate the signaling mechanisms underlying synapse pruning, we first sought to establish an appropriate *in vitro* model of LTD that also induces synaptic elimination. Three-week-old cultured neurons exhibit highly-branched dendrite morphology, with many fully decorated with mature spines, thus providing an ideal timeframe for imaging synapses (Boyer et al., 1998; Dailey & Smith, 1996; Papa et al., 1995). Neurons fixed after virus-eGFP infection, and labeled with validated markers for pre- and postsynaptic structures were used as a measure of synapse density (Micheva, Busse, Weiler, O'Rourke, & Smith, 2010) (Fig. 1A). For example, staining with antibodies raised against synapsin in the presynaptic terminal of Neuron A (red) is apposed to the GFP-labeled spines and dendrite of postsynaptic Neuron B (green) (Fig. 1B). Consistent with many studies that established the pharmacological induction of LTD (chemLTD) in cultured neurons (Beattie et al., 2000; Carroll et al., 1999; Ehlers, 2000; Lee, Liu, Wang, & Sheng, 2002; Lu et al., 2001; Snyder et al., 2005), we replicated a previous report that a brief exposure to NMDA can reduce the frequency of miniature excitatory synaptic currents (Beattie et al., 2000) (Fig. 1C–F), which is one indicator of synaptic depression. This NMDA treatment induced a reduction in mEPSC frequency but not amplitude (Fig. 1D: Mini frequency recordings from $n = 10$ cells, 3 biological samples, and shown as a percentage of the pre-drug baseline; frequency $70.94 \pm 8.89\%$, $p = 0.0097$; amplitude $111.5 \pm 10.6\%$, $p = 0.305$), consistent with previous reports for chemLTD in culture preparations (Beattie et al., 2000; Lissin, Carroll, Nicoll, Malenka, & von Zastrow, 1999). Input resistances were unchanged, ruling out overt breakdown of the

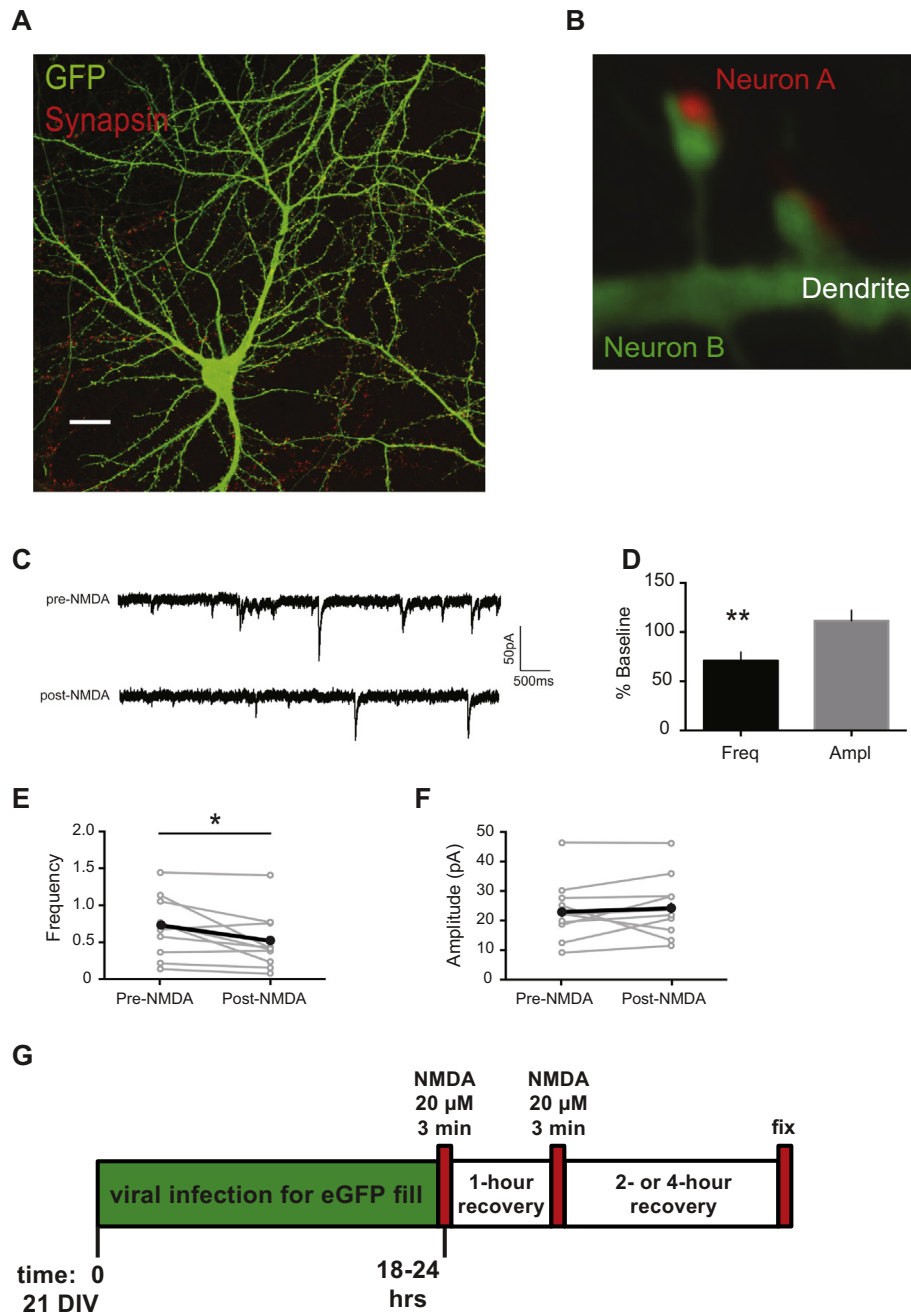


Fig. 1. Experimental design. (A) Dissociated neuronal cultures at 18–22 days DIV have fully-branched dendrites studded with mature spines that can be visualized with fluorescent markers using confocal microscopy, and the changes in synapse density can then be quantified. Scale bar, 20 μ m. (B) High magnification confocal images of dendrites with spiny synapses. Synapsin antibody staining (red) in the presynaptic terminal of Neuron A is apposed to the GFP-labeled spines and dendrite (green) of postsynaptic Neuron B. NMDA treatment of dissociated neuronal cultures causes a decrease in frequency of miniature EPSCs. (C) Representative traces from mEPSCs recorded from cultured neurons pre- and post-NMDA treatment. (D) Data from cells, normalized to baseline, after NMDA treatment shows a decrease in mEPSC frequency, but no change in amplitude ($n = 10$ cells, 3 biological samples). Paired analyses of mEPSC recordings from individual cells pre-NMDA and post-NMDA treatments: (E) frequency and (F) amplitude. Some points/error bars are obscured by overlying symbols with similar values. $p_{\text{freq}} = 0.031$. (G) Schematic diagram of experimental timeline showing that dissociated neurons were cultured for 21 days, infected with a modified Sindbis-eGFP to fill some of the cells, treated twice with the LTD-inducing drug, NMDA (20 μ M for 3 min), allowed to recover two or four hours, and fixed. Cells were then stained for synapse markers (immunofluorescence), imaged, and putative synapse numbers determined. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

membrane ($112.92 \pm 7.65\%$, $p = 0.38$). Thus, brief, low-dose NMDA application to our mixed cortical cultures reduces excitatory transmission as reported previously (Fig. 1E and F: mean \pm SEM of paired analyses of recordings from individual cells pre-NMDA and post-NMDA treatments; frequency_{pre} 0.71 ± 0.13 , amplitude_{pre} 23.33 ± 3.28 pA; frequency_{post} 0.50 ± 0.12 , amplitude_{post} 24.66 ± 3.35 pA; $n = 10$ cells; $*p_{\text{freq}} = 0.031$). Although this treatment included

added glycine, together with the NMDA in ACSF, we note that the Neurobasal media used in the imaging experiments contains 0.4 mM glycine. Therefore, additional glycine was not included in subsequent experiments. To summarize, our experimental design entailed stimulating neurons at \sim 21 DIV with NMDA to induce synaptic depression and determining the number of putative synapses on neurons expressing GFP (Fig. 1G).

2.9. Pharmacological treatments

To pharmacologically induce LTD (chemLTD) for subsequent image analysis of GFP-expressing neurons, cultures on coverslips (~21 DIV) were either mock-treated with culture medium only, or treated with 20 μ M NMDA dissolved in culture medium, which was removed after 3 min. Cells were then returned to NMDA-free conditioned medium for 1 h and were subjected to a second treatment, unless indicated (Fig. 1G). Cells were exposed to pharmacological inhibitors 10–45 min prior to and during NMDA addition. In cases where inhibitors were dissolved in DMSO as vehicle, the vehicle alone was used in the control medium. To inhibit protein phosphatase activity, cultures were pre-incubated with serine-threonine phosphatase inhibitors at concentrations selected from published reports, and only those concentrations that did not cause overt neuronal toxicity (blebbing), as assessed in low magnification images, or detachment (floating cells), were used in further studies: calyculin A (CA, 1–5 nM, 10 min prior to NMDA), okadaic acid (OA, 1–20 nM, 10 min prior to NMDA), fostriecin (fos, 10–100 nM, 10 min prior to NMDA), or FK506 (10–100 nM, 20 min prior to NMDA). To inhibit mRNA and protein synthesis, we used actinomycin D (actino, 10 nM–1 μ M, 20 min prior to NMDA) for transcription inhibition, and anisomycin (aniso, 1–20 μ M, 20 min prior to NMDA), or cycloheximide (CHX, 60 μ M, 20 min prior to NMDA) for translation inhibition. Similarly, for caspase-3 inhibition, cultures were incubated with z-DEVD-FMK (DEVD, 10 μ M, 45 min prior to NMDA), as described (Erturk, Wang, & Sheng, 2014). Enzyme activity was not measured. Neuronal cell death was assessed by incubating cultured neurons for 30 min to 1 h at 37 °C in propidium iodide (PI; 5 μ g/ml)-containing media before imaging and analysis (Lau, Cui, & Tymianski, 2007). PI uptake by nuclei was visualized at 20 \times magnification using a Zeiss Axio-Observer Z1 epi-fluorescence microscope. Images were then imported into MetaMorph image analysis software (Molecular Devices) where the 'Count Nuclei' application was used to count the total number of PI-positive cells.

2.10. Statistical analysis

All experiments were performed on at least three independent neuronal cultures (biological samples) prepared from separate litters of pups. Error bars represent standard errors of the means (SEM). Statistical evaluations were performed using either unpaired student *t*-tests, or one-way analyses of variance (ANOVAs) followed by appropriate between-group comparisons (Graphpad Prism 6 and InStat, San Diego, CA). All levels of significance represent two-tailed values. Significance was placed at $p < 0.05$.

3. Results

3.1. NMDA induces synapse loss in culture

Repeated periods of LTD-inducing stimulation can be accompanied by synapse loss (Bastrikova et al., 2008; Shinoda et al., 2010), and bath application of NMDA can induce synaptic depression in slices and in culture preparations (Atkins, Davare, Oh, Derkach, & Soderling, 2005; Beattie et al., 2000; Carroll et al., 1999; He et al., 2011; Kameyama et al., 1998; Kamikubo et al., 2006; Lee et al., 1998; Lu et al., 2001), as well as a rapid loss of dendritic spines (Halpain, Hipolito, & Saffer, 1998). We therefore tested whether repeated applications of NMDA would influence the number of synaptic connections in dissociated cultures by determining the density of fluorescently-labeled presynaptic terminals (synapsin Micheva, Busse, Weiler, O'Rourke, & Smith, 2010) co-localized with dendritic spines of fluorescently-labeled neurons (GFP Jeromin

et al., 2003; Fig. 2A). We found that brief applications of NMDA (20 μ M for 3 min) induced a lasting decrease in the number of putative synaptic contacts (Fig. 2B1). Two applications of NMDA were required for reliable detection of synapse loss, though, as one application induced a much smaller loss that was less consistent across individual dendrites (Fig. 2B2). The reduction in synapse density was apparent at two hours post-NMDA treatment and did not reverse or progress further at four hours post-treatment (ANOVA; two hours: $F_{(2, 17)} = 10.38$; $p = 0.0011$; $n = 20$ biological samples; four hours: $F_{(2, 9)} = 14.81$; $p = 0.0014$; $n = 12$ biological samples); or normalized to same day mock-treatment branches in control coverslips (ANOVA; two hours: $F_{(2, 222)} = 34.15$; $p < 0.0001$; $n = 225$ dendritic segments; four hours: $F_{(2, 277)} = 38.92$; $p < 0.0001$; $n = 280$ dendritic segments). Thus, we chose to focus our efforts to measure synapse changes on the two-hour post-recovery timeframe.

The effect of repeated NMDA applications on synapse density was not significantly different between proximal and distal dendrites (Fig. 2C1: 20–50 μ m, or >100 μ m from the cell body, respectively), indicating that synapse loss does not appear to differ in these two dendritic locales (Fig. 2C2: unpaired *t*-test; control (CTRL) vs. NMDA, proximal: $70.29 \pm 3.10\%$, $n = 16$ –21 dendritic segments; distal, $78.10 \pm 5.47\%$, $n = 15$ –16 dendritic segments).

Presynaptic and postsynaptic markers are generally stable in the early phases of NMDA-induced LTD (Halpain et al., 1998), and our use of eGFP-filled neurons was intended to avoid confounding degradation of specific proteins after NMDA treatment (Colledge et al., 2003). Nevertheless, to confirm our findings using an alternative method of labeling synaptic contacts, we stained for the presynaptic protein, bassoon, and the postsynaptic protein, PSD-95 (Fig. 3A1 and A2). We found that similar to our observations using synapsin/GFP to label synapses, two treatments with NMDA induced a significant loss of putative synaptic contacts measured with bassoon as the presynaptic and GFP as the postsynaptic labels, or with synapsin together with PSD-95 (Fig. 3A3; synapse density normalized to CTRL, GFP/bassoon: $69.95 \pm 4.11\%$, $n = 29$ –32 dendritic segments, $p < 0.0001$; PSD-95/synapsin: $61.72 \pm 3.11\%$, $n = 24$ –37 dendritic segments, $p < 0.0001$). We conclude that protein degradation of pre- or postsynaptic markers is unlikely to underlie our observations.

To address possible concerns that repeated exposure to NMDA renders neurons vulnerable to excitotoxic cell death (Choi, 1992), we stained the cultures with propidium iodide (PI), a marker of disrupted cell membranes. Two hours following two applications of NMDA, we found no difference in the number of neurons staining positive for PI, compared with mock-treated controls (unpaired *t*-test, CTRL: 100%, NMDA: $100.8 \pm 7.94\%$, $n = 4$ –5 biological samples, $p = 0.91$). Even when cultures were assessed 24 h after drug treatment, no significant difference in PI staining was observed (unpaired *t*-test, CTRL: 100%, NMDA: $89.2 \pm 24.3\%$, $n = 4$ –5 biological samples, $p = 0.63$). Consistent with previous reports (Li et al., 2010; Shehata, Matsumura, Okubo-Suzuki, Ohkawa, & Inokuchi, 2012), these data confirm that the synapse losses we observed were not a result of excitotoxicity induced by NMDA. Finally, to determine whether the virus and/or the eGFP made the neurons more susceptible to NMDA-induced cell death, we looked at neuronal cultures infected with Sindbis-eGFP that were PI-positive. Again, we found no significant changes in PI uptake between the mock-treated neurons and those treated twice with NMDA at two hours after NMDA application (unpaired *t*-test, CTRL: 100%, NMDA: $128.5 \pm 18.7\%$, $n = 3$ biological samples, $p = 0.20$) and 24 h post-NMDA treatment (CTRL: 100%, NMDA: $106.3 \pm 18.3\%$, $n = 3$ biological samples, $p = 0.70$). Together these data indicate that two brief applications of NMDA, of the same concentration and duration that can induce LTD, induce loss of putative synapses

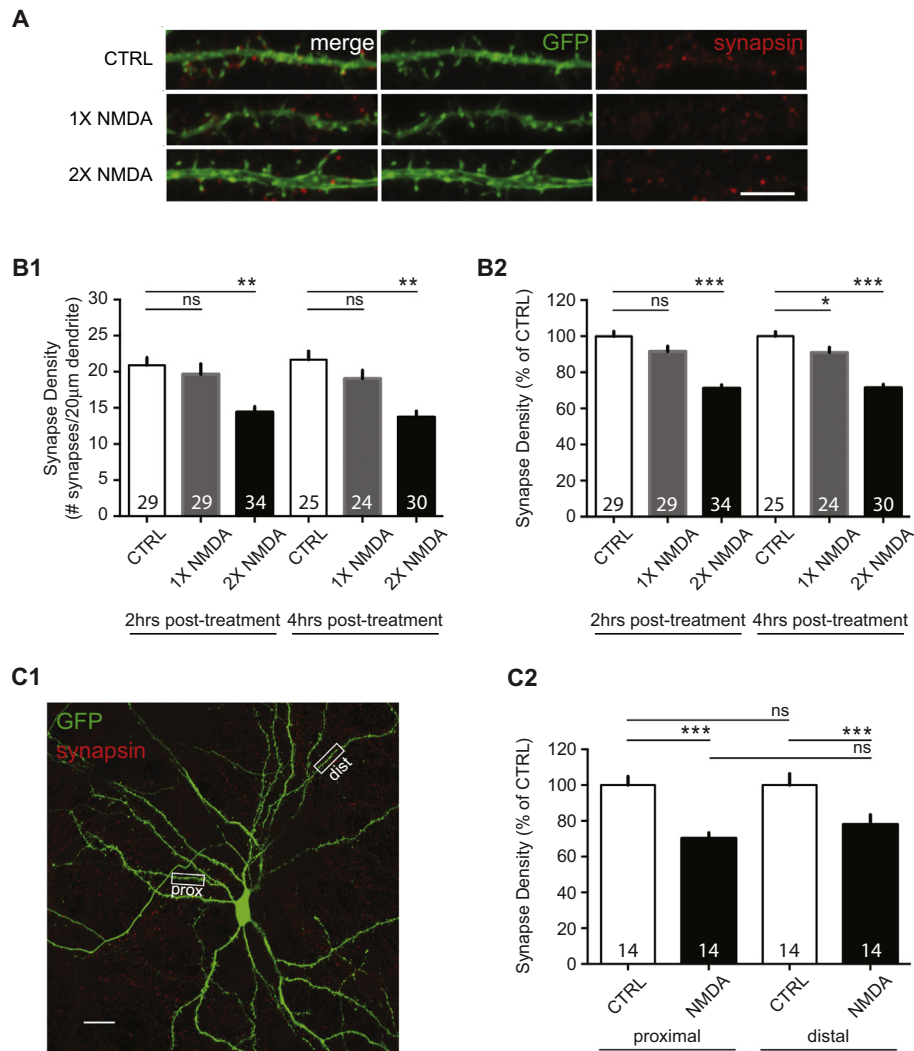


Fig. 2. NMDA-induced LTD results in synapse elimination. (A) Confocal images of neurons after treatments with media alone (CTRL), or one (1× NMDA) or two (2× NMDA) treatments with NMDA showing fluorescent immunoreactive staining of the presynaptic bouton (red, synapsin) and postsynaptic spine (green, GFP) in 21 DIV cultured dissociated neurons. Scale bar, 5 µm. (B1) Synapse loss occurs reliably after two NMDA treatments in dissociated neurons. Quantification of synapse density (averaged means of # of synapses/20 µm dendrite) in mock-treated (CTRL), once-treated (1× NMDA), and twice-treated (2× NMDA) cultured cells. (B2) Quantification of normalized synapse density (% of CTRL) in mock-treated (CTRL), once-treated (1× NMDA), and twice-treated (2× NMDA) cultured cells. Data show that synapse density changes at 2 h or 4 h post-NMDA treatment are similar. N = at least three independent biological samples. Values within bars represent sample sizes (# neurons). Error bars represent SEM. Significance from control (CTRL): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant. (C1) NMDA-mediated synapse loss occurs similarly at both proximal and distal synapses. Representative confocal image of a GFP-expressing neuron, with 20 µm-boxed regions marking examples of proximal (20–50 µm from the soma) and distal (>100 µm from the soma) dendritic areas used in analyses shown in (C2). Scale bar, 20 µm. (C2) Quantification of normalized synapse density for proximal and distal synapses in mock-treated (CTRL) and treated (NMDA) cells. Data are averaged means of synapses from at least three biological samples. Values within bars represent sample sizes (# neurons). Error bars represent SEM. Significance: *** $p < 0.001$; ns, not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lasting at least four hours that is unlikely to be attributed to protein (label) degradation, dendritic location, or neuronal death.

Because microglia have been proposed to be critical for synapse pruning (Schafer et al., 2012; Stevens et al., 2007), we sought to determine whether this cell type could contribute to the synapse loss we observe after NMDA treatment. In our cultures, cells are grown for four days in serum-containing media, which supports glial proliferation during the critical early stages of neuronal development. Subsequent FUDR exposure and feeding with serum-free Neurobasal media arrests further glial growth. To first determine whether microglia appear within proximity of the dendrites we assessed, we stained our cultures using an antibody for a microglia-specific protein ionized calcium binding adaptor molecule 1 (Iba-1) (Kraft, McPherson, & Harry, 2016). Cultures plated with $\approx 80,000$ neurons/coverslip contained only rare Iba-1-

stained cells (in the range 8–28/coverslip in three biological samples), and fewer in contact with eGFP-expressing neurons (Fig. 3B1). A microglia-enriched culture served as a positive control for the antibody (Fig. 3B2). Because microglia did not appear in sufficient numbers to contact the synapses we sampled, we attempted no further study on the topic.

3.2. Relationship between spine shrinkage and synapse loss

NMDA receptor-dependent LTD has long been known to be accompanied by spine shrinkage, and NMDA can induce spine collapse (Graber, Maiti, & Halpain, 2004; Halpain et al., 1998; Oh et al., 2013; Zhou et al., 2004). Because synaptic contacts consist of a dense network of extracellular matrix molecules, a simple shrinkage or even complete collapse of a spine may, or may not result in a

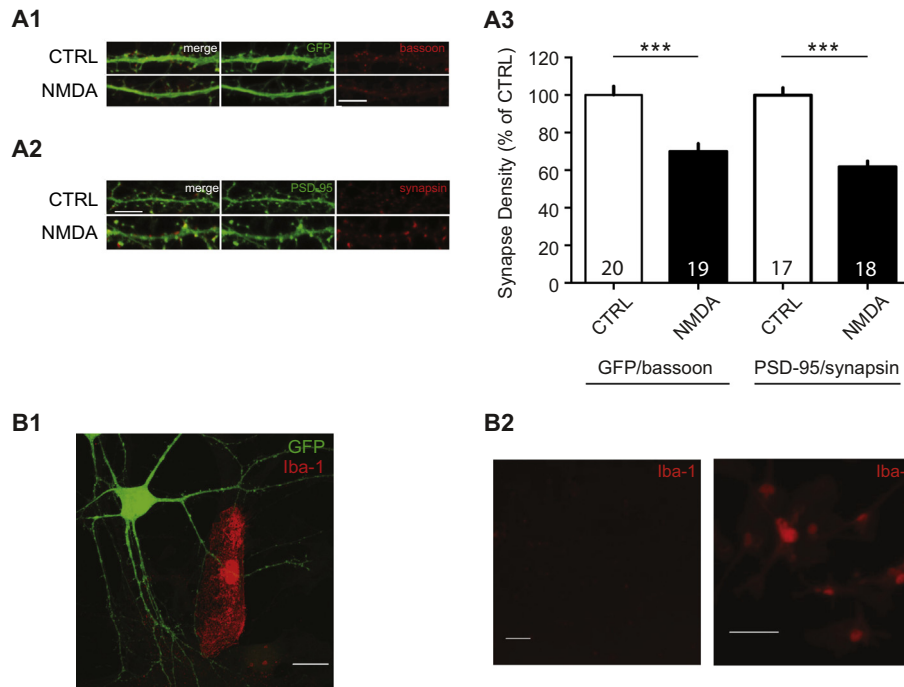


Fig. 3. NMDA-induced synapse loss is not likely to be due to loss of synapsin or GFP. NMDA-induced synapse loss is still observed using an alternative presynaptic marker, bassoon (A1), or postsynaptic marker, PSD-95 (A2). Confocal images after 0 (CTRL) and 2 NMDA treatments showing fluorescent immunoreactive staining using alternative markers of (A1) the presynaptic bouton (red, bassoon), and (A2) the postsynaptic spine, PSD-95. Scale bar, 5 μ m. (A3) Quantification of normalized synapse density from neurons stained with alternative antibody combinations, anti-GFP/anti-bassoon, and anti-PSD95/anti-synapsin. Data are averaged means of synapses from at least three independent experiments. Values within bars represent sample sizes (# neurons). Error bars represent SEM. Significance from control (CTRL): *** $p < 0.001$. (B1) Synapse pruning is unlikely to be a result of microglia action. Example image of an Iba1-positive microglial cell contacting a GFP-expressing neuron. Scale bar, 20 μ m. (B2) Relative Iba-1-immunoreactivity in neuronal cultures using our neuron culture protocol (left), and microglia-enriched cultures grown in serum-containing media (Iba1-positive control, right). Scale bars, 50 μ m.

loss of contact with a presynaptic terminal. In many cases, synapse loss may occur without spine shrinkage, and vice versa (Bastrikova et al., 2008). To test then whether the degree of spine shrinkage or loss was correlated with the synapse loss reported here, we compared the amount of synapse loss to the amount of spine loss in individual experiments. This analysis also would address whether spine shrinkage below the level of detection is a likely explanation for our observation of an apparent synapse loss. We found that, consistent with previous findings, NMDA treatment resulted in modest net spine loss (Fig. 4A1: CTRL, 16.76 ± 0.48 ; NMDA, 15.11 ± 0.52 ; $n = 16$ biological samples, $p = 0.025$). However, a linear regression analysis indicated that there was no correlation between spine loss and synapse loss (Fig. 4A2: linear regression, $R^2 = 0.014$; $p = 0.636$). Thus, in many cases synapse loss could occur without a corresponding loss of spines.

To determine whether we could infer that a separation of synaptic structures had indeed occurred after repeated NMDA treatment, we sought to assess the frequency of unconnected synaptic partners and instances of 'unpaired' spines. Although each dendritic spine is generally thought to contain a synapse (Alvarez & Sabatini, 2007; Nimchinsky, Sabatini, & Svoboda, 2002), some studies have reported spines with and without presynaptic partners (Arellano, Espinosa, Fairén, Yuste, & DeFelipe, 2007; Portera-Cailliau, Pan, & Yuste, 2003), and we also observe instances of the same. Indeed, in our culture preparation and staining conditions, over 30% of spines had no apparent presynaptic partner within the resolution of our imaging capability (i.e. fluorescence appearing co-localized; Fig. 4B1). However, nearly 60% of these unpaired spines had synapsin-positive puncta within 2 μ m of the center of the spine head, possibly indicative of undetected synaptic structure or recently separated contact. We found that NMDA treatment results in a significant increase in the number of

unpaired spines, as might be expected if synaptic structures were separated without overt loss of spines (Fig. 4B2: CTRL, $34.66 \pm 1.71\%$; NMDA, $44.45 \pm 1.78\%$, $n = 16$ biological samples, $p = 0.0004$). However, NMDA treatment caused no change in the percentage of these unpaired spines with synapsin-positive puncta nearby (Fig. 4B3: CTRL, $59.06 \pm 3.82\%$; NMDA, $61.95 \pm 4.11\%$; $n = 16$ biological samples, $p = 0.61$). We conclude from these data that two NMDA treatments result in a net synapse loss that cannot be accounted for by spine loss alone. This loss appears to be reflected by an increase in the number of spines without presynaptic partners.

3.3. Role of LTD-related signaling in NMDA-induced synapse loss

Depression of synaptic responses and NMDA-induced spine loss have been shown to be prevented by inhibitors of protein phosphatases, consistent with the finding that glutamatergic synaptic proteins are dephosphorylated by serine-threonine protein phosphatases PP1, PP2A, and PP2B, resulting in AMPA receptor internalization (Beattie et al., 2000; Delgado et al., 2007; Halpain et al., 1998; Lee, Barbarosie, Kameyama, Bear, & Huganir, 2000; Lee et al., 1998; Mauna, Miyamae, Pulli, & Thiels, 2011; Morishita et al., 2001; Mulkey, Endo, Shenolikar, & Malenka, 1994; Mulkey et al., 1993; Oh, Derkach, Guire, & Soderling, 2006; Thiels, Norman, Barrionuevo, & Klann, 1998). We therefore tested whether phosphatase activity was critical for synapse loss induced by NMDA in our cultures. To block protein phosphatase activity, cultures were pre-incubated for 10–20 min with inhibitors of the serine/threonine-specific phosphatases PP1/PP2A and PP2B. Cultures were then treated simultaneously with NMDA and one of the phosphatase inhibitors, calyculin A (CA, 1 nM), okadaic acid (OA, 5 nM), fostriecin (fos, 100 nM), or FK506 (100 nM), or inhibitor

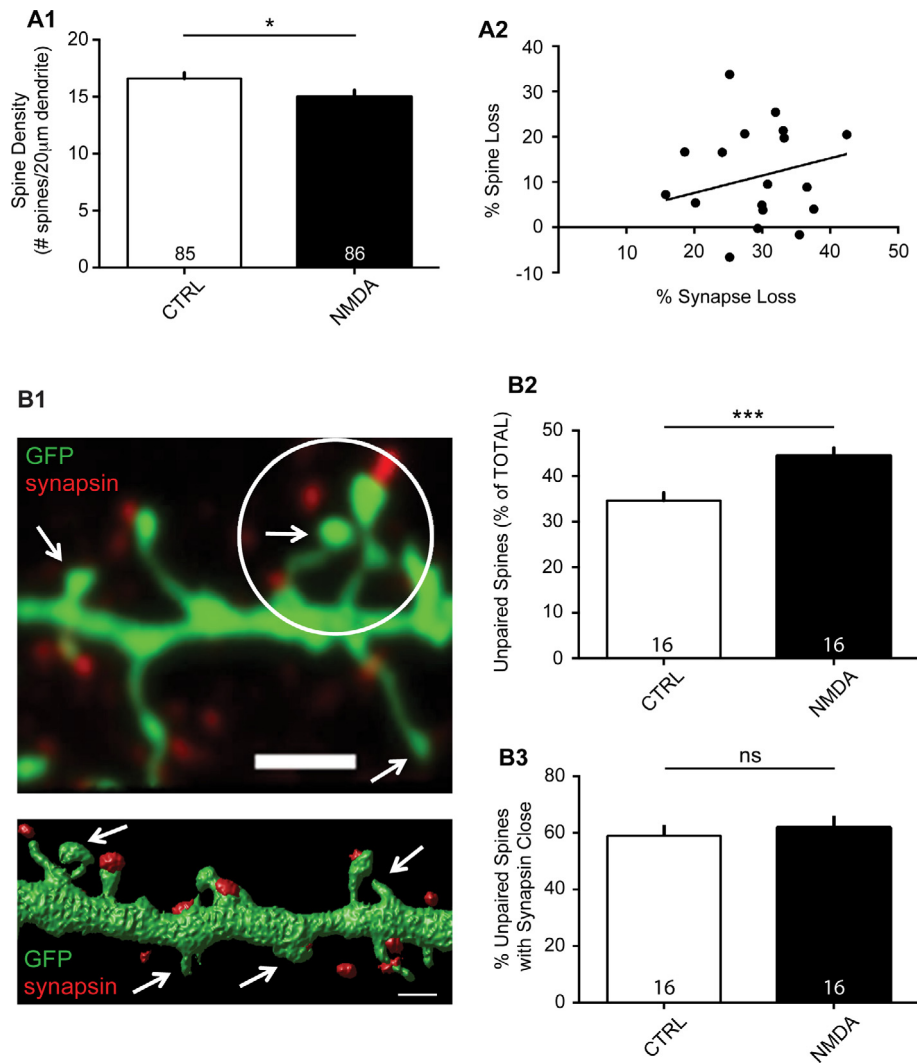


Fig. 4. NMDA treatment results in spine loss. (A1) Data are averaged means of spine density (# spines/20 μm dendrite) in cultured neurons after mock or NMDA treatment from 16 independent experiments. Values within bars represent sample sizes (# neurons). Error bars represent SEM. Significance from control (CTRL): * $p = 0.025$. (A2) Spine loss is not correlated with putative synapse loss. Data are averaged means of spine and synapse numbers, expressed as % spine loss and % synapse loss, in cultured neurons after mock or NMDA treatment from 16 independent experiments. $R^2 = 0.014$; $p = 0.636$. (B1) Confocal image (top; scale bar, 2 μm) and surface rendering (bottom; scale bar, 1 μm) of spiny dendritic sections (GFP-expressing) with punctate antibody staining to endogenous synapsin protein. Arrows indicate examples of 'unpaired spines'. Circle marks a 2- μm radius region of interest for an 'unpaired spine with synapsin close'. Center of the white circle is on an unpaired spine head center; any unpaired synapsin within the circle denotes 'synapsin close'. (B2) NMDA treatment increases the number of unpaired spines (without synapsin, as a % of the total number of spines; *** $p = 0.0004$), but not (B3) unpaired/nonsynaptic spines with synapsin close (ns, not significant; $p = 0.61$). Values within bars represent sample sizes (# neurons). Error bars represent SEM. Significance from control (CTRL): *** $p < 0.001$; ns, not significant.

alone. After washout and a one-hour recovery period, cells received a second treatment and were fixed two hours later. We found that the NMDA-induced reduction in putative synapses was significantly attenuated in all cases (Fig. 5A: CTRL, $100.0 \pm 4.42\%$; NMDA, $62.68 \pm 2.92\%$; 1 nM CA, $89.08 \pm 6.29\%$; NMDA + CA, $99.90 \pm 4.46\%$; ANOVA, $F_{(3, 156)} = 20.21$, $p < 0.0001$; $n = 3\text{--}5$ biological samples; Fig. 5B: CTRL, $100.0 \pm 4.87\%$; NMDA, $68.36 \pm 3.29\%$; 100 nM Fos, $92.01 \pm 5.50\%$; NMDA + Fos, $112.30 \pm 6.85\%$; ANOVA, $F_{(3, 122)} = 13.95$, $p < 0.0001$; $n = 3\text{--}4$ biological samples; Fig. 5C: CTRL, $100.0 \pm 3.28\%$; NMDA, $73.04 \pm 2.29\%$; 5 nM OA, $79.77 \pm 5.77\%$; NMDA + OA, $108.90 \pm 3.93\%$; ANOVA, $F_{(3, 197)} = 22.39$, $p < 0.0001$; $n = 3\text{--}5$ biological samples; Fig. 5D: CTRL, $100.0 \pm 3.59\%$; NMDA, $71.74 \pm 2.77\%$; 100 nM FK506, $100.66 \pm 4.16\%$; NMDA + FK506, $96.64 \pm 3.63\%$; ANOVA, $F_{(3, 238)} = 16.32$, $p < 0.0001$; $n = 7$ biological samples). These results strongly suggest that PP1/PP2A and PP2B activity, which are critical for NMDAR-dependent LTD, are also necessary for NMDA-mediated synapse pruning. Interestingly, okadaic acid alone consistently induced a loss of synaptic contacts,

which was apparently mitigated by the NMDA treatments (Fig. 5C). Thus, it remains unknown whether okadaic acid-induced synapse loss is prevented by NMDA treatment, or whether NMDA-induced loss is prevented by okadaic acid. In summary, our findings are strongly suggestive of a role for protein phosphatases in NMDA-induced reductions in synapse density *in vitro*, and support the idea that NMDA-LTD may be a trigger for synapse pruning *in vivo*.

Caspases have recently been found not only to function in apoptosis, but also are required for LTD (Jiao & Li, 2011; Li et al., 2010; Snigdha, Smith, Prieto, & Cotman, 2012). In addition, we found that MitoTracker Red labeled substantial numbers of mitochondria in live eGFP-filled dendrites, as imaged with structured illumination microscopy (3D-SIM) (Fig. 6A). We therefore reasoned that if LTD is required for synapse pruning, then inhibition of caspase-3 activity should similarly inhibit synapse loss induced with NMDA treatment. Thus, we asked whether blockade of LTD with a caspase inhibitor also occludes NMDA-induced pruning. We found that

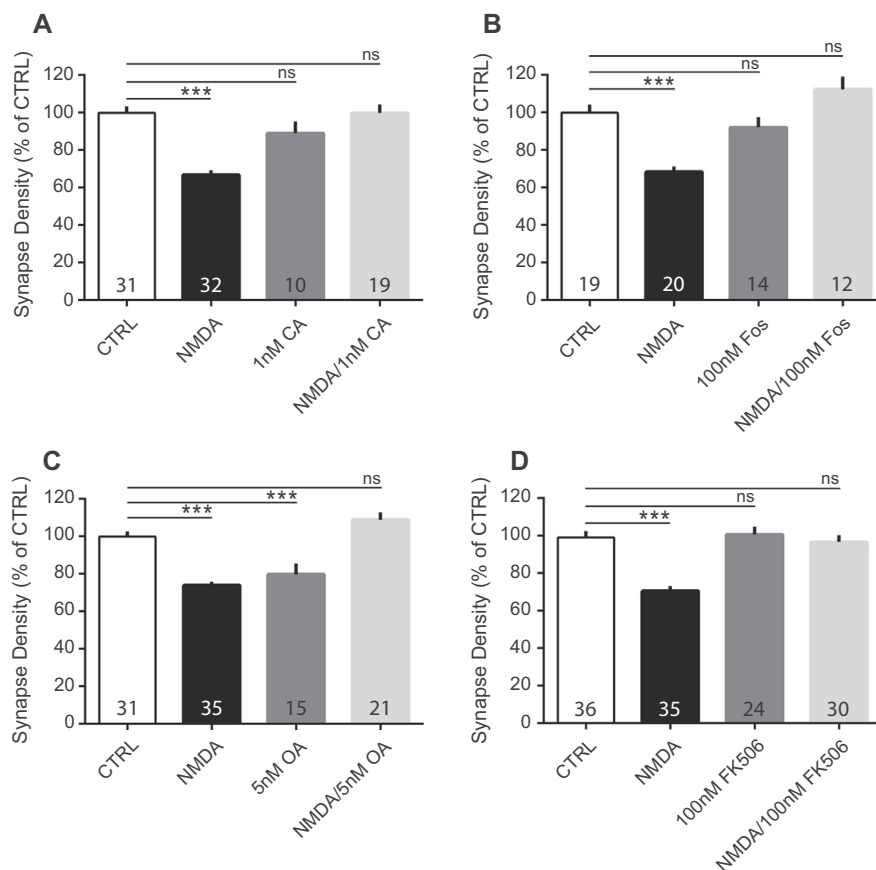


Fig. 5. Phosphatase inhibitors prevent NMDA-induced synapse pruning. Cultured neurons were treated twice with NMDA (20 μ M for 3 min) alone, NMDA plus a serine-threonine phosphatase inhibitor (CA, fos, OA, or FK506), inhibitor alone, or vehicle-/mock-treated, and processed for immunocytochemistry after 2 h. Quantification of the effects of (A) CA, (B) fos, (C) OA, or (D) FK-506 on NMDA-induced changes in synapse density from neurons stained with GFP and synapsin. Data are averaged means of synapses from at least three biological samples. Values within bars represent sample sizes (# neurons). Error bars represent SEM. Significance from control (CTRL): *** $p < 0.001$; ns, not significant.

10 μ M z-DEVD-FMK, a caspase-3 inhibitor, applied prior to and during NMDA treatment of cultured neurons prevented NMDA-induced synapse loss (Fig. 6B: CTRL, $100.0 \pm 5.28\%$; NMDA, $73.48 \pm 4.18\%$; 10 μ M DEVD, $94.86 \pm 4.84\%$; NMDA + DEVD, $97.56 \pm 4.81\%$; ANOVA, $F_{(3, 151)} = 6.613$, $p = 0.0003$; $n = 3$ biological samples). The caspase-3 inhibitor alone had no significant effect on synapse number. Interestingly, although one group found no effect of pan-caspase inhibitor, z-VAD-FMK, on NMDA-mediated F-actin loss in spines (Graber et al., 2004), Erturk et al. reported that inhibition of caspase-3 activity prevented spine shrinkage from NMDA-induced LTD (Erturk et al., 2014). Overall, these data suggest that synapse loss occurred in a caspase-dependent manner, further supporting a model by which synapse pruning is triggered by NMDAR-LTD.

3.4. Role of transcription and translation in synapse loss

To examine the importance of new protein or mRNA synthesis in the mechanisms underlying synapse pruning, we tested cultures that were treated with NMDA and protein synthesis inhibitors. Protein synthesis has been shown to be important for LTD in several studies (Huber et al., 2000; Manahan-Vaughan, Kulla, & Frey, 2000; Sajikumar & Frey, 2003), but whether or not translation is required for synapse pruning in cortical neurons has not been investigated. To block protein synthesis activity, cultures were pre-incubated 20 min with inhibitors at concentrations that did not cause neuronal toxicity in pilot experiments. Cells were then treated simultaneously with NMDA and one of two protein

synthesis inhibitors that use different mechanisms to block peptide elongation (anisomycin (aniso), 2 μ M; or cycloheximide (CHX), 60 μ M). We found that the reduction of synaptic contacts in NMDA-treated cultures appeared to be prevented by application of either anisomycin or cycloheximide. Interestingly though, as in the case with OA, treatment of cells with protein synthesis inhibitors alone reduced synapse numbers as well (Fig. 7A1: CTRL, $100.0 \pm 3.19\%$; NMDA, $66.20 \pm 2.85\%$; Aniso, $84.69 \pm 4.14\%$; NMDA + Aniso, $101.90 \pm 5.68\%$; ANOVA, $F_{(3, 203)} = 19.11$, $p < 0.0001$; $n = 4-6$ biological samples; Fig. 7A2: CTRL, $100.0 \pm 4.96\%$; NMDA, $64.93 \pm 3.95\%$; CHX, $62.19 \pm 4.68\%$; NMDA + CHX, $92.46 \pm 6.35\%$; ANOVA, $F_{(3, 107)} = 14.28$, $p < 0.0001$; $n = 3$ biological samples).

To determine whether synapse loss requires synthesis of new mRNA, or whether existing pools of mRNA are sufficient, we tested whether the transcription inhibitor actinomycin D (actino, 10 nM) prevented NMDA-induced synapse pruning. Indeed, we found that the reduction of synaptic contacts in NMDA-treated cultures is prevented by the application of actinomycin D (Fig. 7B: CTRL, $100.0 \pm 3.34\%$; NMDA, $66.06 \pm 3.29\%$; actino, $102.60 \pm 5.32\%$; NMDA + actino, $97.73 \pm 5.55\%$; ANOVA, $F_{(3, 159)} = 13.36$, $p < 0.0001$; $n = 4-5$ biological samples). These data indicate that gene transcription, which may be required for LTD, is also required for synapse loss (Kauderer & Kandel, 2000) (but see Hu et al., 2014; Manahan-Vaughan et al., 2000). Further, because this drug treatment did not cause synapse loss on its own, these data suggest that effects of protein synthesis inhibitors on synapse number are independent of new mRNA synthesis.

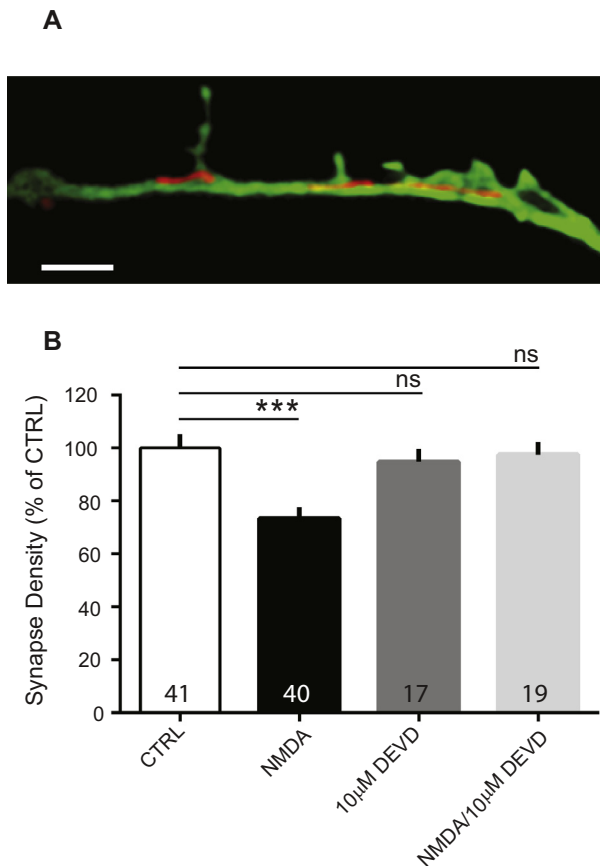


Fig. 6. Caspase-3 inhibition blocks NMDA-induced synapse elimination. (A) 3-D SIM image of MitoTracker Red-stained mitochondria in a dendrite from a GFP-expressing cultured neuron. Scale bar, 2 μ m. (B) Cultured neurons were treated twice with NMDA (20 μ M for 3 min) alone, NMDA plus caspase-3 inhibitor, z-DEVD-FMK (DEVD), DEVD alone, or vehicle-treated, and processed for immunocytochemistry after 2 h. Quantification of the effect of DEVD on NMDA-induced changes in synapse density from neurons stained with GFP and synapsin. Data are averaged means of synapses from three biological samples. Values within bars represent sample sizes (# neurons). Error bars represent SEM. Significance from control (CTRL): *** $p < 0.001$.

4. Discussion

Synapse numbers increase during early postnatal development in most mammals, and are thought to decrease with neuronal activity during experience (Yang, Pan, & Gan, 2009). Despite the growing knowledge of the molecules important for synapse pruning (Adelson et al., 2014; Bian, Miao, He, Qiu, & Yu, 2015; Bochner et al., 2014; Datwani et al., 2009; Hu et al., 2014; Kehoe et al., 2014; Orefice, Shih, Xu, Waterhouse, & Xu, 2016; Pielarski et al., 2013; Sekar et al., 2016; Stevens et al., 2007; Talantova et al., 2013; Woolfrey et al., 2009), the mechanisms by which neuronal activity regulates this process in relation to LTD in cortical structures remains unknown. Several models involving neuronal activity have been proposed, including competition for growth factors or other molecules (Bian et al., 2015), heterosynaptic-type interactions, where highly active synapses drive pruning at the expense of unused synapses (Buffelli et al., 2003; Liu, Fields, Fitzgerald, Festoff, & Nelson, 1994), homeostatic-type mechanisms (Lee et al., 2011), and homosynaptic depression of synaptic transmission, such as in LTD (Elston et al., 2009; Wiegert & Oertner, 2013). Alternative, but not mutually exclusive, models for synapse loss posit that microglia or astrocytes consume synaptic components (Baharini, Song, Diez, & Hanayama, 2015; Chung, Allen, &

Eroglu, 2015; Chung et al., 2013; Ji, Akgul, Wollmuth, & Tsirka, 2013; Kettenmann, Kirchhoff, & Verkhratsky, 2013; Mishra, Kim, Shin, & Thayer, 2012; Paolicelli et al., 2011; Schafer et al., 2012; Stevens et al., 2007) (but see Cheng et al., 2010). Also unknown is whether the mechanisms underlying developmental loss of synapses at the neuromuscular junction are similar to those in cortical structures (Sanes & Lichtman, 2001). Although we cannot rule out the idea that some of these other mechanisms play a role in cortical synapse elimination, our study adds to the evidence that LTD-inducing stimulation can also induce synapse pruning.

Perhaps the best evidence to date that LTD-like mechanisms are required for synapse pruning in the central nervous system is that decreases in synapse number are impaired by genetic manipulations that also inhibit LTD. Some examples include knockout of components of the major histocompatibility complex (MHC1), where LTD and/or ocular dominance plasticity are also impaired (Adelson et al., 2014; Datwani et al., 2009; Djurisic et al., 2013; Huh et al., 2000; Lee et al., 2014), and deletion of different subunits of NMDARs, which results in increases in synapse number (Adesnik, Li, Daring, Pleasure, & Nicoll, 2008; Fiuza & González-González, 2013; Kehoe et al., 2014; Ohno et al., 2010; Roberts et al., 2009) (but see Attardo, Fitzgerald, & Schnitzer, 2015). However, whether these manipulations impact development of synapses or whether they directly prevent synapse pruning is often ambiguous (Glynn et al., 2011; Goda & Davis, 2003; Park et al., 2011).

Here, we tested whether signaling pathways more traditionally associated with synaptic plasticity, particularly those closely linked to LTD, also were required for synapse pruning. To do this, we first sought to establish a model of LTD *in vitro* that also induced synaptic elimination. We found that repeated, but not single, applications of NMDA induced reliable loss of putative synaptic contacts, consistent with previous findings showing that repeated electrically-induced LTD was accompanied by synapse elimination in slice cultures (Bastrikova et al., 2008; Becker et al., 2008; Wiegert & Oertner, 2013). The loss of synaptic structures was apparent at two hours after drug application and was not dependent on position in relation to the cell soma or on degradation of pre- or post-synaptic markers. Also, although NMDA is known to be excitotoxic to neurons at high doses and extended exposures (Karpova et al., 2013; Qiu et al., 2013), NMDA treatment ($2 \times 20 \mu$ M for 3 min) did not cause a significant increase in cellular death, as measured by propidium iodide staining. Moreover, although we cannot rule out a role in the pruning process for astrocytes, or factors released from non-neuronal cells, we found that microglia were too sparse in our cultures to play a role requiring their direct contact with individual synapses. Thus, our model is suitable for testing our hypothesis that signaling pathways required for LTD are also required for synapse pruning.

4.1. Role of LTD-linked mechanisms

We do note that both NMDA-LTD and the putative synapse loss reported here require protein phosphatase activity and caspase activity, indicating that this model of synapse elimination has some mechanistic similarities with typical LTD. For example, LTD and glutamate receptor dephosphorylation and/or internalization are blocked by phosphatase and caspase inhibitors (Delgado et al., 2007; Li et al., 2010; Mulkey et al., 1993). Moreover, inhibition of caspase-3 prevents NMDA-induced spine shrinkage (Erturk et al., 2014).

Parsing the contributions of phosphatases PP1 and/or PP2A, PP4/5/6 by inhibitors calyculin A, fostriecin, or okadaic acid is challenging, as these drugs have broad, overlapping specificities and inhibit enzyme activity at different IC_{50} values (Beaumont, Zhong, Fletcher, Froemke, & Zucker, 2001; Favre, Turowski, &

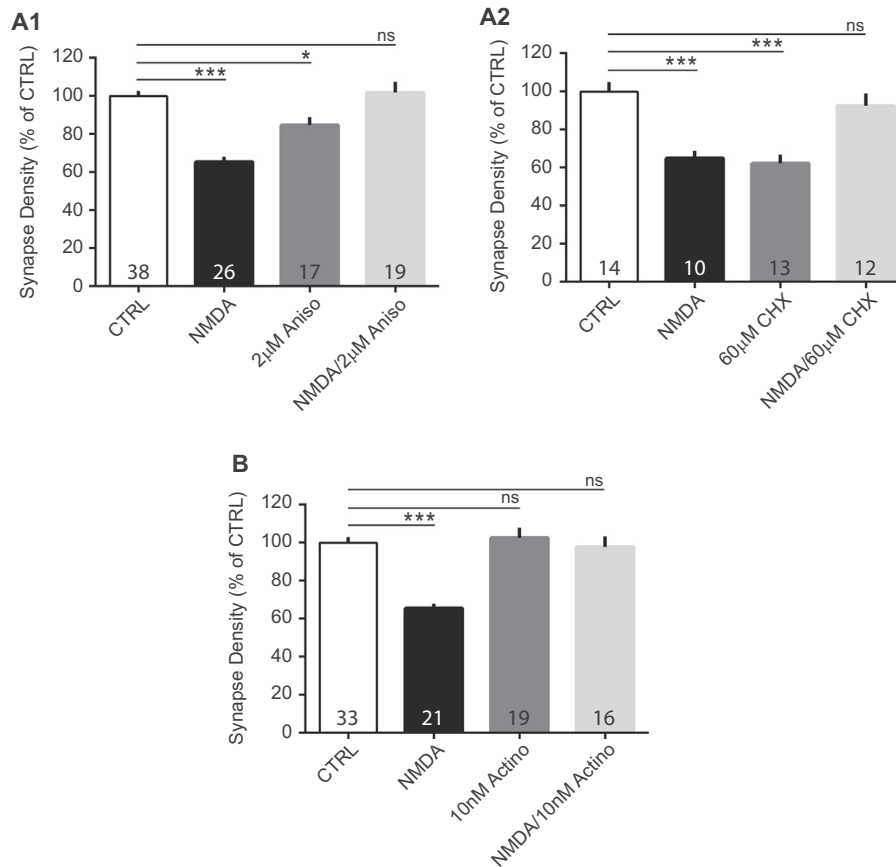


Fig. 7. Protein synthesis and gene transcription inhibition prevent NMDA-mediated synapse loss. Cultured neurons were treated twice with NMDA (20 μ M for 3 min) alone, NMDA plus a protein synthesis or transcription inhibitor, inhibitor alone, or vehicle-/mock-treated, and processed for immunocytochemistry after 2 h. Quantification of the effects of (A1) anisomycin (aniso), (A2) cycloheximide (CHX), or (B) actinomycin D (actino) on NMDA-induced changes in synapse density from neurons stained with GFP and synapsin. Data are averaged means of synapses from at least three biological samples. Values within bars represent sample sizes (# neurons). Error bars represent SEM. Significance from control (CTRL): * $p < 0.05$, *** $p < 0.001$.

Hemmings, 1997; Shepcke, Gauss, & Chamberlin, 1997). Although work in other neuronal systems found no inhibitory effects of CA and OA on NMDA-LTD (Beattie et al., 2000; Kameyama et al., 1998), the concentrations used here (CA, 1 nM; OA, 5 nM; fos, 100 nM) were of varying magnitudes lower, and likely impacted the phosphatases that were blocked. Future studies such as those using selective knockout of the individual phosphatases may be able to distinguish between PP1/PP2A on the basis of their differential sensitivity to concentration, cellular permeation properties, timing of activity, and subcellular location (Allen, Ouimet, & Greengard, 1997; Bordelon et al., 2005; Slupe, Merrill, & Strack, 2011). Interestingly, although FK506 blocks NMDA-induced AMPAR internalization in dissociated cultures (Beattie et al., 2000; Kameyama et al., 1998), FK506 treatment alone may actually increase dendritic spine density and complexity of branching (Rozkaine, Hyman, & Spiess-Jones, 2011). Additionally, there may be interactions between PP1, PP2A, and PP2B (Winder & Sweatt, 2001). Future studies testing whether AMPAR internalization, which is required for LTD and ocular dominance plasticity (Yoon et al., 2009), is required for pruning will be essential for determining whether the actual 'readout' of LTD is also critical for this type of synapse loss.

The role of protein synthesis in LTD clearly lacks consensus; several studies have shown no effect of protein synthesis inhibitors on LTD induced with low-frequency stimulation (Huber et al., 2000; Xiong et al., 2006), whereas others have observed inhibitor sensitivity (Kauderer & Kandel, 2000; Linden, 1996; Sajikumar & Frey, 2003). Interestingly, anisomycin has been reported to activate

the p38 MAPK pathway to induce LTD (Xiong et al., 2006), an effect not replicated with cycloheximide. Thus, the NMDA-mimicking effect of anisomycin or cycloheximide pretreatment on synapse loss preclude our making any definitive conclusions about the requirement for protein synthesis in synapse pruning (although see similar results reported with anisomycin at the neuromuscular junction (McCann, Nguyen, Santo Neto, & Lichtman, 2007)). Our results do raise the intriguing possibility that proteins made from newly synthesized mRNA drive pruning, whereas proteins made from existing pools of mRNA are required for synapse maintenance. Supporting this idea are data from 'tagging' experiments under conditions of protein synthesis inhibition suggesting that some plasticity-related proteins/mRNA are limited (Fonseca, Vabulas, Hartl, Bonhoeffer, & Nägerl, 2006). Here though, we show that when all synapses are activated with NMDA, some synaptic contacts are lost, but that NMDA action oddly protects against loss due to protein synthesis inhibition. Our experiments cannot address whether a synapse-specific LTD-type mechanism, or an LTP-at-the-expense-of-others type mechanism, is responsible. Supporting the latter, though, is evidence that (post-synaptic) neuronal activity can lead to synaptic weakening after several hours (Bukalo, Campanac, Hoffman, & Fields, 2013), although no LTP is induced to compete in a way hypothesized by this model. Interestingly, several different miRNAs related to LTD are induced with NMDA treatment and can be regulated locally in dendrites (Hu et al., 2014). Our LTD-inducing treatment also was unlikely to have engaged the signaling molecule Jacob, which is recruited to the nucleus under primarily LTP-inducing stimulation (Behnisch

et al., 2011). In either case, local protein synthesis, possibly involving regulation by phosphatases, may be serving a critical role in maintaining synapses (Liu-Yesucevitz et al., 2011).

Several possible mechanisms for NMDA-mediated synapse pruning remain to be tested. For example, in a study of synapse elimination in *C. elegans*, and in rat cortical cultures, E3 ubiquitin ligases play a role (Ding, Chao, Wang, & Shen, 2007; Helton, Otsuka, Lee, Mu, & Ehlers, 2008). In addition, extracellular proteases could be involved in pruning (Liu, Fields, Festoff, & Nelson, 1994), either serving in a signaling capacity such as in a culture model of the developing neuromuscular junction (Jia, Li, Dunlap, & Nelson, 1999), or as a way of breaking down the extracellular matrix in the synaptic cleft by matrix metalloproteinases (Huntley, 2012). Related to extracellular matrix is Sema5B, which when proteolytically processed, can induce synapse pruning (O'Connor et al., 2009). Furthermore, evidence of the effects of environmental exposure on synaptic remodeling (e.g. bisphenol A and other endocrine disruptors of brain function) is mounting (Hajszan & Leranth, 2010). Our model of NMDA-induced synapse pruning easily can be applied to study the role of these possible mechanisms.

4.2. Role of spine loss in synapse pruning

Dendritic spines, and likely synapses on them, are dynamic throughout the brain and their numbers can be manipulated by environmental enrichment or NMDAR blockade, depending on the brain region (Attardo et al., 2015; Bian et al., 2015). However, it is important to distinguish between the mechanisms required for functional weakening (i.e. LTD; AMPA receptor internalization) and those related to structure (i.e. spine shrinkage, spine loss, and separation of pre- and post-synaptic structures), as any or all of these processes may occur as a result of LTD initiation by NMDARs or mGluRs (Hasegawa et al., 2015). Strong evidence has been presented that LTD induction can cause spine shrinkage (Calabrese, Saffin, & Halpain, 2014; Nägerl et al., 2004; Oh et al., 2013; Stein, Gray, & Zito, 2015), although the signaling required for LTD can be dissociated from those required for spine shrinkage (Zhou et al., 2004). Likewise, although it is assumed that spine shrinkage would lead to eventual loss of spines resulting in synapse loss, examples of LTD-related synapse separation have been observed to occur in the absence of spine loss or shrinkage (Bastrikova et al., 2008; Becker et al., 2008). Our data showing little correlation between spine loss and synapse loss are also in agreement with this idea (Fig. 4A2). Consistent also with this dissociation between spine shrinkage and synapse loss is our observation that the percentage of 'unpaired' spines increased with NMDA treatment (Fig. 4B2). These data are suggestive of presynaptic terminals disconnecting from spines that remained intact. Alternatively, we cannot rule out that NMDA treatment resulted in the appearance of new spines lacking presynaptic partners, but we note that many of these 'unpaired' spines were structurally mature in that they had no resemblance to filopodia, arguing against this possibility. Because as yet no markers of recently separated synaptic structures exist to measure this process directly, we therefore conclude that spine shrinkage/loss is unlikely to be a requisite step in synapse loss, but that LTD may still be an initiating event.

4.3. Synapse loss in disease

These studies were performed at three weeks in culture, and so the results are most relevant to developing cortical structures and developmental disorders such as those discussed above. Nevertheless, synapse elimination may also have relevance to normal brain function in adulthood and to age- and disease-related cognitive decline. For example, dendritic spine remodeling, including elimi-

nation, can occur after fear conditioning, extinction, and reconditioning in layer V neurons of mouse frontal association cortex (Lai, Franke, & Gan, 2012). Synapse loss by stress hormones also is well documented (Tata, Marciano, & Anderson, 2006), but whether its mechanism is related to LTD is unknown. In addition, this study may have implications in diseases such as Alzheimer's disease (AD) and Parkinson's disease, where synapse loss is profound (Helton et al., 2008; Koffie et al., 2009). Phosphatase inhibition blocks synapse loss in a mouse model of AD Cavallucci et al. (2013), so it is conceivable that NMDARs and LTD-like mechanisms underlie AD-related synapse loss as well (Li et al., 2009; Wei et al., 2009) (but see also Hong et al., 2016). We therefore look forward to a deeper understanding of the possible role for LTD in mechanisms underlying activity-dependent synapse pruning in both developmental and disease contexts.

Contributions

S.M.D. and M.A.H. conceived and designed the studies. S.M.D. and M.A.H. wrote the manuscript. M.A.H., and M.Z. conducted experiments and analyzed data. C.J.T. designed analyses of confocal imaging data. S.M.D. supervised the project.

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