Postsynaptic, not presynaptic NMDA receptors are required for spike-timing-dependent LTD induction

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Long-term depression (LTD) between cortical layer 4 spiny stellate cells and layer 2/3 pyramidal cells requires the activation of NMDA receptors (NMDARs). In young rodents, this form of LTD has been repeatedly reported to require presynaptic NMDARs for its induction. Here we show that at this synapse in the somatosensory cortex of 2- to 3-week-old rats and mice, postsynaptic, not presynaptic NMDARs are required for LTD induction. First, we find no evidence for functional NMDARs in L4 neuron axons using two-photon laser scanning microscopy and two-photon glutamate uncaging. Second, we find that genetic deletion of postsynaptic, but not presynaptic NMDARs prevents LTD induction. Finally, the pharmacology of the NMDAR requirement is consistent with a nonionic signaling mechanism.

There is mounting evidence that NMDARs can be expressed presynaptically, as well as postsynaptically. Presynaptic NMDAR activation is thought to enhance neurotransmitter release and to be essential for the induction of spike-timing-dependent forms of long-term depression (t-LTD) in a number of brain regions. Consistent with presynaptic expression of NMDARs, bath application of NMDAR agonists and antagonists increases and decreases, respectively, the frequency of miniature postsynaptic currents in several neuronal cell types. However, the activation of postsynaptic NMDARs can result in neuronal depolarizations that spread passively into the axon, opening voltage-gated calcium channels (VGCCs) and resulting in increased vesicular release. In several neuronal cell types where presynaptic NMDAR activity has been reported, including cerebellar stellate cells, cerebellar basket cells, hippocampal CA3 pyramidal cells and cortical layer (L) 5 cells, attempts to image NMDAR activation in axons using calcium-sensitive fluorescent dyes and local NMDAR agonist application have met with mixed results: some papers report calcium excursions while others do not. Presynaptic NMDARs have also been implicated in certain forms of plasticity, with strong, though indirect, evidence for their involvement in the induction of t-LTD, which entails the repeated pairing of a postsynaptic action potential (AP) with a presynaptic AP that follows within a few tens of milliseconds. In particular, t-LTD has been extensively studied at the cortical synapse between layer 4 spiny stellate cells and layer 2/3 pyramidal cells. This form of long-term plasticity disappears in rodents older than about 3 weeks and is thought to be involved in the formation of receptive fields in visual, auditory and somatosensory cortex. Recently, t-LTD has been extended or restored in visual cortex of older animals by dark rearing or placing animals in the dark for several days. This reestablishment of t-LTD was prevented by genetically downregulating NMDARs in the presynaptic neurons.

At the L4–L2/3 synapse, bath application of the NMDAR antagonist d-2-amino-5-phosphonopentanoic acid (d-AP5) blocks induction of t-LTD, but blocking only postsynaptic NMDAR current with intracellular MK-801 (ref. 12) does not. This implies that the relevant NMDARs are located in the presynaptic membrane. Indeed, inclusion of MK-801 in presynaptic L4 neurons is reported to block the induction of t-LTD. We attempted to image NMDA-mediated calcium signals in the presynaptic L4 axons elicited by focal, two-photon uncaging of 4-methoxy-7-nitroindolinyl-caged-l-glutamate (MNI-glutamate) onto presynaptic varicosities in L2/3 of rodent somatosensory cortex but found no evidence for functional presynaptic NMDARs. By genetically deleting NMDARs in either the pre- or postsynaptic neurons, we found that the relevant NMDARs for inducing t-LTD at this synapse were postsynaptic, not presynaptic. Our pharmacological experiments indicate that only glutamate binding, and not glycine or d-serine binding nor ionic flux, is required for t-LTD induction, a result similar to those in recent reports for LTD at hippocampal synapses between Schaffer collaterals and CA1 pyramidal cells.

RESULTS
No evidence for presynaptic NMDARs in L4 axons

To study the signaling mechanism of presynaptic NMDARs in L4 spiny stellate cell axons for t-LTD induction, we attempted to measure presynaptic NMDAR activity using two-photon laser uncaging (2PLU) of MNI-glutamate and calcium measurements with two-photon laser scanning microscopy (2PLSM). L4 neurons in acute slices containing barrel cortex from juvenile rats were patch clamped and filled with the calcium-sensitive fluorescein dye Fluor5F (200 μM) and the calcium-insensitive fluorescein dye Alexa 594 (10 μM) and simultaneously imaged with 840-nm laser light. In the absence of extracellular Mg2+, 2PLU of MNI-glutamate (2.5 mM) with 0.5 ms pulses of 720-nm light next to dendritic spines led to large increases in calcium through NMDARs (Fig. 1a,b). The uncaging laser power was adjusted by the depth in the slice to levels that were previously determined to cause ~30% photobleaching of the Alexa 594 signal.
Figure 1 2PLU of MNI-glutamate and calcium influx in L4 neuronal axonal varicosities and dendritic spines. (a) Left, 2PFSM image of a dendritic spine and shaft from a L4 neuron showing line scan position (dashed line) and uncaging spot (yellow circle). Right, fluorescence transients of the spine and dendrite evoked with a 0.5-ms uncaging pulse of MNI-glutamate. (b) Average fluorescence transient (ΔG/R) from the spine in a (green trace, mean ± s.e.m.) interleaved with blank trials (no uncaging laser pulse, black trace). (c) Summary of calcium signals in dendritic spines with and without 2PLU (n = 19 spines from 7 cells from 7 slices from 3 animals, mean ± s.e.m. uncaging: 148 ± 15, blank: 0.37 ± 0.36; Wilcoxon rank-sum test z = 3.803, *P = 3.8 × 10−6). (d) Left, 2PFSM image of axonal varicosity showing the line scan position (dashed line) and uncaging spots (yellow circles). Right, fluorescence transients in axon varicosity evoked with two 2-ms uncaging pulses. (e) Average fluorescence transients of interleaved traces with and without 2PLU (n = 44 varicosities from 6 cells from 6 slices from 3 animals, mean ± s.e.m. uncaging: 0.16 ± 0.13, blank: 0.38 ± 0.17; Wilcoxon rank-sum test z = 0.566, P = 0.57; ns, not significant).

Calcium signals were quantified by the change in green fluorescence relative to the red fluorescence (ΔG/R, Fig. 1b). In every spine tested, large increases in calcium signals were seen in response to glutamate uncaging (Fig. 1b,c). Interleaved trials without the uncaging laser pulse showed no change in the ΔG/R signal (Fig. 1b,c). In the same neurons, the axon was traced toward the pial surface of the slice and varicosities, presumed boutons, were chosen to test for presynaptic NMDARs by uncaging glutamate. The radial distances of the varicosities chosen ranged from 36 to 380 μm from the somata (mean distance: 163.9 μm, s.d.: 89.6 μm, N = 44 varicosities). Two uncaging pulses, one on each side of an axonal varicosity, each 2 ms long, were used to release glutamate (Fig. 1d). No change in ΔG/R signal was observed in any of the axonal varicosities when uncaging, and there was no difference in the ΔG/R signal in interleaved trials without uncaging laser pulses (Fig. 1e,f; P = 0.57).

Sensitivity to detect single calcium channels

If there is just one NMDAR in the presynaptic structure, calcium influx may be difficult to detect and our failure to observe a calcium signal in response to glutamate uncaging onto axonal varicosities could result from an insufficient sensitivity of our microscope. Calcium signals arising from single NMDARs have been observed in dendritic spines22; however, dendritic spines may be structurally unique in their ability to prevent calcium diffusion, enhancing detection of small calcium transients with fluorescent dyes.23 Because we did not observe NMDAR calcium signals in the L4 axonal varicosities, we tested the sensitivity of our microscope to detect calcium transients in axonal varicosities by adapting the technique used by Sabatini and Svoboda23 to count VGCCs activated by APs.

Assuming that an axonal varicosity contains N VGCCs that open independently with a probability Popen the number of channels that are opened by an AP follows the binomial distribution. Measurement of the rate of failures for VGCCs to open, p(0) = (1 − Popen), and measurement of the coefficient of variation due to VGCC activity, CV2 = (1 − Popen)/Npopen can be used to estimate the two parameters N and Popen (ref. 24). We measured calcium transients in response to APs in neighboring axonal varicosities of L4 neurons (Fig. 2a). In control conditions, there were no failures in axonal varicosities (Fig. 2b).

Figure 2 Counting voltage-gated calcium channels in axonal varicosities from an L4 neuron. (a) Left, 2PFSM image of two axon varicosities. Right top, AP evoked by somatic current injection. Middle, example line scan through both varicosities. Bottom, quantified fluorescence from varicosity 1 (black) and 2 (red). Time of baseline measurement, open circle; of peak measurement, filled circle. (b) ΔG/R measurements from varicosity 1 and 2 (black and red, respectively) just after the action potential (filled symbols) and the baseline signal (open symbols) before and during application of 1 μM α-conotoxin MVIIIIC. (c) Top, AP evoked by somatic current injection. Bottom, average ± s.e.m. AP-evoked ΔG/R signal in each varicosity before and after α-conotoxin MVIIIIC application. (d) In α-conotoxin MVIIIIC, average of all trials (black) and of trials representing failures of calcium channel opening (blue) in varicosity 2 were not failures of AP propagation as they did not fail in varicosity 1 (blue traces). (e) Measuring the variance (red) of the fluorescent calcium signal (fu, fluorescence units) and comparing it to the variance (blue trace) predicted from the dark noise and shot noise calculated from the mean fluorescent signal in varicosity 2 (black, bottom). (f) Variance versus mean fluorescence plot. Dashed line is the background, dark noise; blue symbols are shot noise measurements at different mean fluorescent intensities, and the variance-mean relationship of each varicosity is measured over 6 ms just before and just after the action potential (mean ± s.d.: varicosity 1, black; varicosity 2, red). The variance due to calcium channels is the difference between the measured and predicted variance. (g) Estimated number of channels (left) and probability of channel opening (right) in 15 boutons using this technique (mean ± s.d.).
Adding 1 μM α-conotoxin MVIIIC to the bath to block the majority of N-, P- and Q-type calcium channels\(^ {25} \) decreased the size of the calcium signals and led to instances of failure of channels to open (Fig. 2b,c). Imaging two varicosities simultaneously ensured that a trial identified as a failure in one varicosity did not result from AP failure because the calcium signal in the second varicosity did not fail (Fig. 2d). Trials were counted as failures if the calcium signal measured just after the action potential was less than 2 s.d. from the baseline calcium signal measured just before the action potential. As described previously\(^ {24,25} \), failures are accompanied by a slow rise in ΔG/R signal, which results from diffusion of calcium-bound dye from neighboring structures. The coefficient of variation of channel opening was estimated from the fluorescence traces by measuring the mean fluorescent signal and the trial-to-trial variance \((\text{Fig. 2e, f})\). The variance due to VGCC activity was isolated by subtracting the dark noise and photon shot noise from the total variance \((\text{Fig. 2e, f})\). In the example shown in Figure 2, the estimated number of VGCCs contributing to the AP-evoked calcium signal in the presence of α-conotoxin MVIIIC was 2.55 channels in varicosity 1 and 0.79 channels in varicosity 2, and the probabilities of channel opening during an action potential were 0.76 and 0.79, respectively. Figure 2g shows estimates of \(N \) (1.8 ± 1.2, mean ± s.d.) and \(p \) (0.83 ± 0.11, mean ± s.d.) from 15 varicosities. Because the estimation of the number of failures using a criteria of 2 s.d. above the baseline noise is arbitrary, we also estimated the number of failures using either 1 or 3 s.d. above the baseline noise as a cutoff for inclusion as a failure. This led to estimates of 3.0 ± 2.25 channels with a \(p_{\text{open}} \) of 0.72 ± 0.21 for 1 s.d. cutoff \((N = 11)\) and 1.3 ± 0.96 channels with a \(p_{\text{open}} \) of 0.87 ± 0.10 for 3 s.d. cutoff \((N = 18)\).

Axonal boutons in cortical neurons are thought to contain N-, P/Q- and R-type high-voltage-activated calcium channels\(^ {4,26} \), which have a calcium conductance between 5 and 10 pS (ref. 26), and during an AP the channels are expected to be open for <1 ms (ref. 27). NMDARs have a comparable calcium conductance (~10% of 50 pS; refs. 28, 29), but are expected to be open for >10 ms (ref. 30). Because we have the sensitivity to detect calcium arising from a single VGCC in an axonal varicosity, we should be able to detect calcium from single NMDARs.

**t-LTD at L4–L2/3 synapses**

Although we did not find evidence for NMDAR calcium influx in the axons of L4 neurons in response to 2PLU, we repeated the t-LTD experiments that have suggested the existence of presynaptic NMDARs. Figure 3a shows an example time course of excitatory postsynaptic potentials (EPSPs) recorded in artificial cerebrospinal fluid (ACSF) from a L2/3 neuron while stimulating in L4 at 0.1 Hz. After a baseline period, L4 stimulation was paired with a postsynaptic AP 25 ms before L4 stimulation (a protocol shown to induce t-LTD\(^ {16} \)). This pairing was repeated 100 times at 0.2 Hz, and the EPSP was monitored for ~60 min after pairing. In ACSF, this protocol led to t-LTD (EPSP slope reduced to 47 ± 9% after pairing, mean ± s.e.m.; \(n = 15\), \(P = 1.8 \times 10^{-4}\)). As others have reported\(^ {12,13} \), blocking NMDARs with 50 μM D-AP5 prevented t-LTD (EPSP slope 101 ± 6% after pairing; \(n = 10\), \(P = 0.56\)) but including 1 mM MK-801 in the internal pipette solution did not block t-LTD (EPSP slope 62 ± 7% after pairing, \(n = 20\), \(P = 1.3 \times 10^{-4}\); Fig. 3b,c).

**NMDAR pharmacology of t-LTD at L4–L2/3 synapses**

Evidence has recently emerged that the function of NMDARs in generating hippocampal LTD depends on glutamate binding the receptor but is independent both of binding of glycine or d-serine and of ion flux through the receptor’s channel\(^ {19,20} \). t-LTD at the L4–L2/3 synapse is consistent with this mechanism, as D-AP5, a competitive antagonist at the glutamate binding site, prevented t-LTD, but the open channel blocker MK-801 did not. Figure 4 shows summary experiments for t-LTD induction in the presence of several other NMDAR antagonists. 3-((R)-2-Carboxypropylazin-4-yl)-propyl-1-phosphonic acid (R-CPP) (10 μM, Fig. 4a), a competitive antagonist at the glutamate binding site, blocked both NMDAR current recorded in voltage clamp experiments and t-LTD (EPSP slope 99 ± 6% after pairing, \(n = 7\), \(P = 0.69\)). Bath application of MK-801 (100 μM, Fig. 4b) blocked NMDAR currents, but did not block t-LTD (EPSP slope 58 ± 9% after pairing, \(n = 12\), \(P = 9.8 \times 10^{-4}\)). The NMDAR glycine-site competitive antagonists 7-chlorokynurenate (7-Ck) (100 μM, Fig. 4c) and 5,7-dichlorokynurenate (5,7-DCK) (50 μM, Fig. 4d) both blocked NMDAR currents, but did not block t-LTD (EPSP slope in 7-Ck 76 ± 3%, \(n = 11\), \(P = 9.8 \times 10^{-4}\); EPSP slope in 5,7-DCK 77 ± 9%, \(n = 7\),...
Figure 4 Pharmacology of t-LTD. (a–d) Left, initial EPSP slope measurements before (Pre) and after (Post) t-LTD induction protocol; middle, time course of blockade of the NMDA EPSC recorded at +60 mV by the addition of the NMDAR antagonist followed by the addition of o-AP5; inset, example traces in control (black), drug (color) and o-AP5 (red); right, summary of NMDAR EPSC amplitudes in the antagonist and with the addition of o-AP5. Error bars represent s.e.m. (a) Effects of R-CPPP (10 μM; t-LTD: 100 ± 6%, n = 7 cells from 5 slices from 3 animals, Wilcoxon sign-rank test z = 0.423, P = 0.69; ns, not significant; NMDAR EPSCs: n = 10 pathways from 5 cells from 5 slices from 1 animal, Wilcoxon sign-rank test z = 2.752, P = 0.002; inset scale bars 50 ms and 200 pA). (b) Extracellular MK-801 (eMK-801, 100 μM; t-LTD: 58 ± 9%, n = 12 cells from 9 slices from 5 animals, Wilcoxon sign-rank test z = 2.942, *P = 9.8 × 10−4; NMDAR EPSCs: n = 8 pathways from 5 cells from 5 slices from 1 animal, Wilcoxon sign-rank test z = 2.451, P = 0.0078; inset scale bars 50 ms and 100 pA). (c) 7-CK (100 μM; t-LTD: 76 ± 3%, n = 11 cells from 6 slices from 3 animals, Wilcoxon sign-rank test z = 2.889, *P = 9.8 × 10−4; NMDAR EPSCs: n = 7 pathways from 4 cells from 4 slices from 1 animal, Wilcoxon sign-rank test z = 2.282, P = 0.016; inset scale bars 50 ms and 100 pA). (d) 5.7-DCCK (50 μM; t-LTD: 77 ± 9%, n = 7 cells from 5 slices from 1 animal, Wilcoxon sign-rank test z = 2.113, *P = 0.031; NMDAR EPSCs: n = 7 pathways from 4 cells from 4 slices from 1 animal, Wilcoxon sign-rank test z = 2.282, P = 0.016; inset scale bars 50 ms and 25 pA).

P = 0.031). This pharmacological profile of t-LTD is consistent with activation of NMDARs that is dependent on glutamate binding but not ion flux.

Genetic deletion of postsynaptic NMDARs

Because blocking NMDAR ion flux globally using extracellular MK-801 or glycine-site antagonists did not block t-LTD induction, the same result obtained with intracellular MK-801 in the postsynaptic cell cannot be used to exclude either pre- or postsynaptic NMDARs as the relevant receptors. To determine if postsynaptic NMDARs receptors are involved in t-LTD, we deleted NMDARs in a sparse subset of L2/3 neurons by injecting an adeno-associated virus (AAV) that expresses Cre and EGFP into the ventricles of E15–16 mouse embryos homozygous for a floxP-flanked Grin1 gene (Grin1loxPloxP)31. Cre expression in the cells of these mice ablates the NMDAR GluN1 subunit that is required for functional channels to traffic to the membrane32,33.

Figure 5a shows an example 2PLSM image of sparse infection of L2/3 neurons in barrel cortex from a Grin1loxPloxP mouse by AAV–Cre-EGFP injection at E16 with two neighboring neurons, one GFP+ and one GFP−, filled with Alexa 594 from the patch pipettes.

The cells were voltage-clamped and L4 was stimulated to evoke synaptic currents. While holding at −75 mV, both GFP+ and GFP− cells showed large, rapid, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX)-sensitive inward currents (Fig. 5b). o-AP5-sensitive NMDAR currents were measured at a holding potential of +40 mV and were only present in GFP− neurons (Fig. 5b,c).

We next tested whether t-LTD could be induced in GFP+ and GFP− cells (Fig. 5d,e). t-LTD could be induced in GFP− neurons (EPSP...
slopes 53 ± 7% after t-LTD pairing, n = 19, P = 1.9 × 10−5) whereas it was absent in GFP+ neurons (EPSP slope 109 ± 14% after pairing, n = 20, P = 0.55). These results indicate that activation of postsynaptic NMDARs is required for t-LTD. Combined with our pharmacological results (Figs. 3 and 4), we suggest that t-LTD requires postsynaptic NMDAR signaling but not ion flux through the NMDAR channel.

**Genetic deletion of presynaptic NMDARs**

Although our inability to observe NMDAR-mediated calcium transients in axon varicosities suggests the absence of presynaptic NMDARs, we nonetheless tested whether t-LTD could be induced when NMDARs were deleted in the presynaptic L4 neurons. To preferentially target the viral infection to L4 neurons, we injected AAV-Cre-EGFP in Grin1fx/fx mice at E14. While this led to sparse labeling of L4 neurons, it was difficult to find connected pairs of GFP+ L4 and GFP− L2/3 neurons. To circumvent this difficulty, we crossed the Grin1fx/fx mice with Ai32 mice, which have channelrhodopsin2(H134R) (ChR2) preceded by a loxP-flanked stop cassette34. In the resulting double homozygous mice, ChR2 expression should be restricted to those neurons in which NMDARs are deleted.

**Figure 6a** shows an example 2PLSM image of a L2/3 neuron filled with Alex 594 and GFP+ L4 neurons in an Ai32;Grin1fx/fx mouse that was infected with AAV1-Cre-EGFP in Grin1fx/fx mice at E14. NMDA receptor currents were present in GFP− neurons, but absent in 8 of 9 neighboring GFP+ neurons (Fig. 6b). EPSPs recorded in L2/3 neurons could be evoked by brief (1–5 ms) flashes of 473-nm laser light that was focused over L4, and t-LTD was tested by pairing postsynaptic action potentials with laser flashes. t-LTD was present in Ai32 mice (Fig. 6c, EPSP slope 85 ± 12%, n = 12, P = 0.012) and could be evoked in the presence of 5,7-DCK (Fig. 6c, EPSP slope 68 ± 13%, n = 8, P = 0.023). t-LTD was intact in double homozygous Ai32;Grin1fx/fx mice (Fig. 6d, EPSP slope 54 ± 6%, n = 9, P = 0.0039) and in A×G mice with intracellular MK-801 (MK-801; 56 ± 8%, n = 7 cells from 7 slices from 5 animals, Wilcoxon sign-rank test z = 2.282, *P = 0.016). Error bars represent s.e.m.

We attempted to detect functional NMDARs in L4 neuronal axons by measuring calcium transients in response to 2PLU. In contrast to robust calcium signals evoked in dendritic spines, no responses were seen in axon varicosities. Revisiting the pharmacology of t-LTD at this synapse that led to the suggestion of presynaptic NMDARs, we found that glutamate binding to NMDARs was required but ion flux through the channels was not, a result similar to recent findings in hippocampal t-LTD19,20. Because nonionic signaling through NMDARs was not blocked by MK-801, the insensitivity of t-LTD induction to postsynaptic intracellular MK-801 does not implicate a presynaptic location of the relevant receptors. We found that genetic deletion of NMDARs in postsynaptic L2/3 neurons prevented t-LTD. Conversely, deletion of NMDARs in the presynaptic L4 neurons in conjunction with expression of ChR2 to stimulate only presynaptic, NMDAR-lacking neurons did not prevent t-LTD.

**Explanation of lack of calcium imaging results**

We tested the sensitivity of our microscope and could detect calcium excursions from single VGCCs activated by APs in the presynaptic neuron. Because the calcium conductance of VGCCs is similar to the calcium conductance of NMDARs and the open time of NMDARs is 10 times that of AP-gated VGCCs, we should have been able to detect calcium influx through NMDARs if they were in the axonal structures. It has been suggested that, in layer 5 neurons of visual cortex, the expression of presynaptic NMDARs is specific to the target cell type10. Because we did not identify the postsynaptic cell in our axonal uncaging experiments, it is possible that we could have missed the boutons in L2/3 region that express NMDARs in our 44 recordings. However, t-LTD that has been attributed to presynaptic NMDARs in this region seems to be fairly ubiquitous. Another possibility for our lack of detection of calcium signals could be that the presynaptic NMDARs contain NR3A subunits37 that have a lower calcium permeability and less voltage-dependent Mg2+ block38 than more standard NMDARs. Kunz et al.39 suggested that these receptors could function by depolarizing the terminals, which would lead to activation of VGCCs. If this were the case, we would still expect to see calcium excursions because we are able to detect calcium signals from single VGCCs. However, our pharmacological and genetic manipulations suggest that it is the postsynaptic NMDARs acting through a nonionic signaling mechanism that is required for t-LTD, which is consistent with the lack of axonal calcium signals in response to glutamate uncaging.

**DISCUSSION**

NMDARs are required for many forms of plasticity in the brain. In addition to their function in the postsynaptic membrane, NMDARs have been suggested to function in presynaptic axonal membranes in cortex10,12-18, cerebellum2,35, hippocampus8 and striatum36.

**Inconsistencies with the literature**

Axonal NMDARs have been identified in a number of cultured neuronal preparations by both calcium indicators and electrical
In cerebellar stellate and basket cells, the lack of NMDAR calcium signals in response to NMDAR agonists applied either iontophoretically or by 2PLU led to the proposal that somato-dendritic NMDARs could be causing axonal release through the electrotonic spread of depolarization1-7. In cortical layer 5 neurons, NMDAR agonists did not affect axonal excitability and did not cause calcium increases, and the antagonist did not affect AP calcium signaling11 (but see ref. 10).

Nonionic signaling through NMDARs has been described in several preparations19,20,43–46. Recent work in hippocampus has indicated that nonionic signaling through NMDARs is essential in the induction of LTD19,20, and the downstream signaling mechanism has been proposed either to be through p38 MAPK activity or to be a direct interaction with the C-terminal domains of NMDAR subunits and protein phosphatase-1 (PP1) and Ca2+/calmodulin-dependent protein kinase II (CAMKKII)46. Future studies will be required to dissect the nature of the NMDAR signal at the L4–L2/3 synapse. t-LTD requires a postsynaptic AP that back-propagates through the dendritic arbor of the L2/3 neuron, providing a source for calcium; blocking of this source by VGCC antagonists or chelation by BAPTA prevents t-LTD induction12,13. Additionally, metabotropic glutamate receptor activation of phospholipase C leads to inositol-1,4,5-trisphosphate production, which induces release of calcium from internal stores that also seems to be required for t-LTD12. The NMDAR signal could be required in concert with these other signals to generate endocannabinoids, although some experiments suggest that NMDAR signaling is downstream of endocannabinoid release12. Consistent with this view, Min and Nevian47 suggested that endocannabinoid release activates astrocytes to release glutamate, which they interpreted to be the source of glutamate for signaling to presynaptic NMDARs. However, postsynaptic NMDARs could be responding to this astrocytic release of glutamate if that is the important signal for t-LTD.

Rodriguez-Moreno et al.17 have reported that t-LTD at the L4–L2/3 synapse is disrupted when 1 mM intracellular MK-801 is introduced selectively to the presynaptic neuron and suggest that ion flux through presynaptic NMDARs is required for t-LTD induction. These results are at odds with both our calcium measurements and our genetic deletion of presynaptic NMDARs. Although it is possible that millimolar concentrations of MK-801 block t-LTD via off-target effects48, we have no evidence to support this and, as such, we have no explanation for the contradictory results.

t-LTD at the L4–L2/3 synapse is developmentally regulated1-3. In the visual cortex, for example, t-LTD in animals older than about 3 weeks becomes sensitive to postsynaptic MK801 (ref. 3). In addition, when animals are dark reared or, as adults, returned to darkness for a period of days, t-LTD induction in the visual cortex reverts to a mechanism that is eliminated by genetic knockdown of L4 neuronal NMDARs15. The explanation for the differences between our results and those in the visual cortex15 is unclear but may depend on the use of different cortical regions or developmental stages. In the somatosensory cortex of P14–21 animals, we find no evidence for functional NMDAR expression in the axons of L4 neurons and we find that knocking out NMDARs in postsynaptic but not presynaptic neurons prevents t-LTD induction.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

B.C.C. and C.E.J. designed the experiments and wrote the manuscript. B.C.C. conducted and analyzed the experiments.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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28. Jahr, C.E. & Stevens, C.F. Calcium permeability of the N-methyl-d-aspartate receptor channel in hippocampal neurons in culture. Proc. Natl. Acad. Sci. USA 90, 11573–11577 (1993).

29. Burnashev, N., Zhou, Z., Neher, E. & Sakmann, B. Fractional calcium currents through recombinant GluR channels of the NMDA, AMPA and kainate receptor subtypes. J. Physiol. (Lond.) 485, 403–418 (1995).

30. Lester, R.A., Clements, J.D., Westbrook, G.L. & Jahr, C.E. Channel kinetics determine the time course of NMDA receptor-mediated synaptic currents. Nature 346, 565–567 (1990).

31. Adesnik, H., Li, G., During, M.J., Pleasure, S.J. & Nicoll, R.A. NMDA receptors inhibit synapse unslilencing during brain development. Proc. Natl. Acad. Sci. USA 105, 5597–5602 (2008).

32. Fukaya, M., Kato, A., Lovett, C., Tonegawa, S. & Watanabe, M. Retention of NMDA receptor NR2 subunits in the lumen of endoplasmic reticulum in targeted NR1 knockout mice. Proc. Natl. Acad. Sci. USA 100, 4855–4860 (2003).

33. Tsien, J.Z., Huerta, P.T. & Tonegawa, S. The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. Cell 87, 1327–1338 (1996).

34. Madisen, L. et al. A toolbox of Cre-dependent optogenetic transgenic mice for light-induced activation and silencing. Nat. Neurosci. 15, 793–802 (2012).

35. Casado, M., Isope, P. & Ascher, P. Involvement of presynaptic N-methyl-d-aspartate receptors in cerebellar long-term depression. Neuron 33, 123–130 (2002).

36. Park, H., Popescu, A. & Poo, M.M. Essential role of presynaptic NMDA receptors in activity-dependent BDNF secretion and corticostriatal LTP. Neuron 84, 1009–1022 (2014).

37. Larsen, R.S. et al. NR3A-containing NMDARs promote neurotransmitter release and spike timing-dependent plasticity. Nat. Neurosci. 14, 338–344 (2011).

38. Sasaki, Y.F. et al. Characterization and comparison of the NR3A subunit of the NMDA receptor in recombinant systems and primary cortical neurons. J. Neurophysiol. 87, 2052–2063 (2002).

39. Kunz, P.A., Roberts, A.C. & Philpot, B.D. Presynaptic NMDA receptor mechanisms for enhancing spontaneous neurotransmitter release. J. Neurosci. 33, 7762–7769 (2013).

40. Fiszman, M.L. et al. NMDA receptors increase the size of GABAergic terminals and enhance GABA release. J. Neurosci. 25, 2024–2031 (2005).

41. Wang, P.Y., Petralia, R.S., Wang, Y.X., Wenthold, R.J. & Brenowitz, S.D. Functional NMDA receptors at aonal growth cones of young hippocampal neurons. J. Neurosci. 31, 9289–9297 (2011).

42. Clark, B.A. & Cull-Candy, S.G. Activity-dependent recruitment of extrasynaptic NMDA receptor activation at an AMPA receptor-only synapse. J. Neurosci. 22, 4428–4436 (2002).

43. Vissel, B., Knupp, J.J., Heinemann, S.F. & Westbrook, G.L. A use-dependent tyrosine dephosphorylation of NMDA receptors is independent of ion flux. Nat. Neurosci. 4, 587–596 (2001).

44. Krapivinsky, G. et al. The NMDA receptor is coupled to the ERK pathway by a direct interaction between NR2B and RasGRF1. Neuron 40, 775–784 (2003).

45. Weilinger, N.L. et al. Metabotropic NMDA receptor signaling couples Src family kinases to pannexin-1 during excitotoxicity. Nat. Neurosci. 19, 432–442 (2016).

46. Aow, J., Dore, K. & Malinow, R. Conformational signaling required for synaptic plasticity by the NMDA receptor complex. Proc. Natl. Acad. Sci. USA 112, 14711–14716 (2015).

47. Min, R. & Nevian, T. Astrocyte signaling controls spike timing-dependent depression at neocortical synapses. Nat. Neurosci. 15, 746–753 (2012).

48. Rothman, S. Non-competitive N-methyl-d-aspartate antagonists affect multiple ionic currents. J. Pharmacol. Exp. Ther. 246, 137–142 (1988).
ONLINE METHODS

Animals. Unweaned juvenile (postnatal day 14–21) Sprague Dawley rats of either sex were used in most experiments. Animals were anesthetized with isoflurane and decapitated, and the brain was rapidly removed into ice-cold ACSF consisting of (in mM) 119 NaCl, 2.5 KCl, 1 NaH2PO4, 25 NaHCO3, 1 MgCl2, 2 CaCl2, 10 glucose, 3 sodium pyruvate, 1.3 sodium ascorbate, equilibrated with 95% O2 and 5% CO2 (chemicals from Sigma). The brain was blocked ~35° from the coronal plane to leave the barrel columns intact 49, and 300-µm-thick slices were prepared with a Leica 1200 Vibratome. Slices were incubated in 37 °C ACSF for 30 min and then kept at room temperature (22–25 °C) until use. All experiments were performed at 32–35 °C. Animal handling and procedures followed OHSU IACUC approved protocols.

In vitro viral injection. For sparse genetic manipulation of NMDARs and ChR2, Cre-EGFP was delivered intraventricularly using a Picospritzer. AAV was injected at either embryonic day 15–16, for preferential expression in L4 neurons, or embryonic day 14, for preferential expression in L2/3 neurons, or embryonic day 15–16, for preferential expression in L4 neurons.

Electrophysiology. t-LTD experiments were performed using whole-cell current clamp recordings in L2/3 neurons while stimulating with a glass electrode in a visually identified L4 barrel. Internal pipette solutions consisted of 128 potassium gluconate, 10 NaCl, 10 HEPES, 4 MgCl2, 14 sodium phosphocreatine, 4 ATP, 0.4 GTP, 0.5 EGTA, pH adjusted to 7.3 with KOH. Synaptic strength was monitored by stimulating at 0.1 Hz, and EPSP slope was measured. t-LTD was induced by 100 pairings at 0.2 Hz of an action potential in the postsynaptic L2/3 neuron generated by brief current injections (1–3 nA, 0.5–3 ms) and L4 stimulation, 25 ms after the postsynaptic action potential. Recordings in which the membrane voltage changed or membrane resistance changed by more than 20% were discarded.

ChR2-expressing L4 neurons from Ai32 and Ai32;Grin1lox/lox mice were stimulated with brief (1–5 ms) flashes of 473-nm laser focused through the objective over GFP + L4 neurons while recording from L2/3 neurons. t-LTD was induced with the same protocol as in experiments using electrical stimulation.

Voltage-clamp recordings to assay NMDAR current and pharmacology were performed using a cesium-based internal pipette solution with cesium gluconate and CsOH replacing potassium gluconate and KOH, respectively. AMPAR currents were recorded at −75 mV and isolated by subtraction with traces recorded in 5 µM NBQX and 50 µM picrotoxin. NMDAR currents were measured at +40 mV in picrotoxin and NBQX and isolated by subtraction of traces in 50 µM D-AP5, 10 µM R-CPP, 100 µM MK-801, 100 µM 7-CK, or 50 µM 5,7-DCK (drugs from Tocris). Reported membrane potentials have not been corrected for the pipette junction potential.

Two-photon laser scanning microscopy. Imaging was performed on a custom two-photon laser scanning microscope using an Olympus BX-51 microscope and an Olympus 60× 1.0 NA water-immersion objective. Two Ti:sapphire lasers (Coherent Chameleon) were used for imaging (840 nm) and uncaging MN-glutamate (720 nm). Line scans were collected at 500 Hz and monitored in both epi- and transfluorescence pathways using photomultipliers (HH224PA-40 or H10770PA-40, Hamamatsu) placed after a 565-nm dichroic mirror and 525/50 and 620/60 bandpass filters (Chroma and Semrock). ScanImage software was used for microscope control and data acquisition 49. Physiology data was collected at 10 kHz and filtered at 4 kHz using a Multiclamp 700B amplifier (Molecular Devices). Pipettes were wrapped with Parafilm to reduce capacitance and had open tip resistances ranging from 2.5 to 5 MΩ.

Calcium imaging. Cells were filled with K+-based internal solution containing the red fluorescent dye Alexa 594 (10 µM) and the green fluorescent, calcium-sensitive dye Fluo-5F (200 µM) through the patch electrode for >15 min before imaging. Fluorescent dyes were purchased from Life Technologies. In glutamate uncaging experiments (Fig. 1), fluorescence was measured as the average ΔG/R signal over 20 ms at the peak of the calcium signal in control traces and the same time window was used to analyze interleaved traces without uncaging glutamate. To isolate NMDAR Ca2+ signals, NBQX (5 µM), tetrodotoxin (1 µM) and D-serine (10 µM) were added to ACSF that contained 3 mM Ca2+ but no added Mg2+. For calcium channel counting experiments (Fig. 2), we adapted the method used by Sabatini and Svoboda 44 to count calcium channels in dendritic spines. For action potential calcium signals (Fig. 2), baseline ΔG/R signal was measured as the average of 6 ms before the action potential, and the peak ΔG/R signal was measured as the average of 6 ms after the action potential. Failures of calcium channels to open were counted if the peak signal was within 2 (or 1 or 3) s.d. from the baseline signal. The mean and variance of the green fluorescent signal was calculated for each time point, and the predicted variance, σ2predicted was estimated as the sum of the background (shutter closed) variance and photon shot noise (measured using a dye-filled pipette). The variance due to calcium channel activity was then calculated σ2calc = σ2total − σ2predicted, and the coefficient of variation of the calcium signal due to calcium channel activity can be calculated as CV2calc = σ2calc/mean2. The two measurements, failure rate for channels opening and CV2calc, can then be used to solve for the two parameters N and popen.

Statistical analysis. Unless otherwise noted, values reported are mean ± s.e.m. For paired data, one-way comparisons were made using the nonparametric Wilcoxon sign-rank test. For unpaired data, one-way comparisons were made using the nonparametric Mann-Whitney U test. Data collection and analysis were not performed blind to the conditions of the experiments. Randomization was not used. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those generally employed in the field.

Data availability. All relevant data are available from the authors.

A Supplementary Methods Checklist is available.

49. Agmon, A. & Connors, B.W. Thalamocortical responses of mouse somatosensory (barrel) cortex in vitro. Neuroscience 41, 365–379 (1991).
50. Polologruto, T.A., Sabatini, B.L. & Svoboda, K. ScanImage: flexible software for operating laser scanning microscopes. Biomed. Eng. Online 2, 13 (2003).