Presynaptic GluN2D receptors detect glutamate spillover and regulate cerebellar GABA release

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Summary

Glutamate directly activates NMDA receptors on presynaptic inhibitory interneurons and enhances GABA release, altering the excitatory-inhibitory balance within a neuronal circuit. However which class of NMDA receptors are involved in the detection of glutamate spillover is not known. GluN2D subunit-containing NMDA receptors are ideal candidates as they exhibit a high affinity for glutamate. We now show that cerebellar stellate cells express both GluN2B and GluN2D NMDA receptor subunits. Genetic deletion of GluN2D subunits prevented a physiologically relevant stimulation-induced, lasting increase in GABA release from stellate cells (inhibitory LTP, I-LTP). NMDA receptors are tetramers composed of two GluN1 subunits associated to either two identical subunits (di-heteromeric receptors) or to two different subunits (tri-heteromeric receptors). To determine whether tri-heteromeric GluN2B/2D NMDA receptors mediate I-LTP we tested the prediction that deletion of GluN2D converts tri-heteromeric GluN2B/2D to di-heteromeric GluN2B NMDA receptors. We find that prolonged stimulation rescued I-LTP in GluN2D knockout mice, and this was abolished by GluN2B receptor blockers that failed to prevent I-LTP in wild type mice. Therefore NMDA receptors that contain both GluN2D and GluN2B mediate the induction of I-LTP. Because these receptors are not present in the soma and dendrites, presynaptic tri-heteromeric GluN2B/2D NMDA receptors in inhibitory interneurons are likely to mediate the cross-talk between excitatory and inhibitory transmission.
INTRODUCTION

Inhibitory synaptic transmission sets the excitability of principal neurons and therefore activity-dependent changes in inhibitory drive are critical for most forms of experience-dependent learning (Marsicano et al., 2002; Nugent et al., 2007; Sceifo et al., 2008; Ehrlich et al., 2009; Chao et al., 2010; Woodin and Maffei, 2011; Levelt and Hübener, 2012). Growing evidence supports the idea that glutamate is a major regulator of inhibitory transmission (McBain and Kauer, 2009; Castillo et al., 2011). Cross-talk between excitatory and inhibitory presynaptic terminals offers a powerful means to alter the excitatory/inhibitory balance and so modify the output of neuronal circuitry. Such studies highlight the importance of understanding how glutamate communicates with inhibitory neurons to regulate the release of the inhibitory transmitter GABA.

Glutamate modulates inhibitory transmission through diverse mechanisms (Gaiarsa et al., 2002; Castillo et al., 2011). The best understood presynaptic modification of the strength of inhibitory synapses involves the release of the retrograde signals, endocannabinoids, NO and BDNF, which is triggered by the activation of metabotropic glutamate receptors and NMDA receptors in postsynaptic neurons (Castillo et al., 2011). However glutamate can also directly activate NMDA receptors on presynaptic GABAergic interneurons to regulate GABA release. This second form of presynaptic plasticity has been commonly observed in several brain regions (Duguid and Smart, 2004; Lien et al., 2006; Liu et al., 2007; Lachamp et al., 2009). We have previously shown that glutamate released from parallel fibers (PF) activates NMDA receptors in stellate cells (cerebellar inhibitory interneurons) to induce a lasting increase in GABA release probability (I-LTP) (Liu and Lachamp, 2006). The change is triggered by activation of NMDA receptors in presynaptic stellate neurons, but not by the retrograde signals, NO and endocannabinoids. This modulation enhances both evoked and spontaneous inhibitory transmission and markedly alters the activity of the cerebellar inhibitory network (Lachamp et al., 2009). One critical feature of this form of presynaptic plasticity is the heterosynaptic nature, requiring NMDA receptors on presynaptic inhibitory interneurons to detect glutamate released from nearby excitatory inputs. However, the molecular identity of the NMDA receptors responsible for the induction of I-LTP and their subcellular localization are not known.

Although NMDA receptors are localized in the postsynaptic membrane (Petralia et al., 2009), anatomical evidence also supports the existence of NMDA receptors in the presynaptic
terminals of neurons in the neocortex, hippocampus, spinal cord, amygdala and cerebellum (Rodríguez-Moreno et al., 2010). NMDA receptor immunolabeling has been found in presynaptic axons of many GABAergic neurons, including in the axon terminals of cerebellar interneurons (Petralia et al., 1994; Paquet and Smith, 2000) and activation of these receptors elevates calcium levels in axons (Shin and Linden, 2005; Rossi et al., 2011) (but also see (Clark and Cull-Candy, 2002; Christie and Jahr, 2008)). If these presynaptic NMDA receptors are activated by glutamate spillover from parallel fiber terminals to regulate GABA release, they would need to be able to detect a low concentration of glutamate. One strong candidate is the GluN2D (or NR2D) subunit of NMDA receptors, which is predominantly expressed in GABAergic interneurons, including cerebellar stellate cells (Akazawa et al., 1994; Monyer et al., 1994; Thompson et al., 2000). NMDA receptors that contain GluN2D subunits exhibit a higher glutamate affinity, lower Mg$^{2+}$ blockade, and prolonged decay time constant after a brief application of agonist, relative to GluN2A and GluN2B-containing receptors (Misra et al., 2000; Cull-Candy et al., 2001; Siegler Retchless et al., 2012). These unique features enable the receptors to detect a spillover of glutamate from nearby excitatory terminals and give rise to a sustained glutamatergic activation signal. We therefore tested the hypothesis that GluN2D-containing receptors in stellate cell induce I-LTP.

Using genetic and pharmacological approaches we found that both GluN2B and GluN2D receptors are expressed in the dendrites and somata of stellate cells. Our experiments show that genetic deletion of GluN2D abolished the parallel fiber stimulation-induced I-LTP in stellate cells. Unexpectedly this was rescued by prolonging parallel fiber stimulation duration and thus the presence of GluN2D in NMDA receptors lowered the threshold for induction of I-LTP. Although GluN2B receptor inhibitors prevented such I-LTP in mutant mice, they failed to attenuate I-LTP in wild type mice, indicating that these NMDA receptors contain both GluN2D and GluN2B subunits. Pharmacological inhibition of tri-heteromeric GluN2B/2D NMDA receptors prevented the induction of I-LTP in wild type mice. We conclude that presynaptic GluN2B/2D NMDA receptors are required for the induction of I-LTP in wild type mice. Incorporation of GluN2D in an NMDA receptor therefore allows a physiologically relevant activity, such as occurs during sensory stimulation, to modulate GABA release.
Cerebellar slice preparation and electrophysiology. Cerebellar slices were prepared as previously described (Liu and Cull-Candy, 2000; Dubois et al., 2012). Briefly, P7 to P10 and P18 to P40 wild type (The Jackson Laboratory) and GluN2D knockout (Ikeda et al., 1995) mice on a C57BL/6J background were decapitated and the cerebellum was isolated. Horizontal or sagittal slices (400 µm) were cut from the cerebellum using a vibratome (Leica VT1200) in an ice cold artificial CSF (containing in mM: 81.2 NaCl, 2.4 KCl, 23.4 NaHCO₃, 1.4 NaH₂PO₄, 6.7 MgCl₂, 0.5 CaCl₂, 23.3 glucose, 69.9 sucrose, pH 7.4). Slices were then maintained in aCSF (in mM: 125 NaCl, 2.5 KCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 1 MgCl₂, 2 CaCl₂, 25 glucose, pH 7.4) saturated with 95% O₂, 5% CO₂ at room temperature for at least 30 min before recording. Experimental procedures were in accordance with the Louisiana State University Health Sciences Center and Penn State University guidelines for care and use of laboratory animals (IACUC).

Whole cell patch clamp recordings were made from cerebellar stellate cells in an O₂/CO₂-saturated aCSF. Stellate cells were identified by their location in the outer two thirds of the molecular layer and by the presence of spontaneous action potentials in the cell-attached mode. Analog signals were filtered at 2 or 10 kHz and digitized at 10 kHz (Multiclamp 700A, Axon Instruments). Series resistance was monitored throughout the recordings and if this changed by more than 20%, the recordings were discarded. All synaptic and dendritic currents were recorded at near physiological temperature (33 - 37°C; except for the data in Fig 1) while single channel currents in outside-out patches were recorded at room temperature to reduce noise.

Long-term potentiation of inhibitory transmission (I-LTP). Miniature inhibitory synaptic currents (mIPSCs) were recorded in stellate cells. Cells were voltage clamped at -30 mV in the presence of 0.5 µM TTX in aCSF, using borosilicate electrodes (6-8 MΩ) filled with a low chloride pipette solution (in mM: 120 Cs acetate, 0.4 MgCl₂, 0.1 CaCl₂, 2.5 MgATP, 0.4 NaGTP, 1.5 NaATP, 10 Cs-EGTA, 5 QX-314 and 10 HEPES, pH 7.3). After obtaining a stable recording for at least 15 minutes, TTX was washed out for 20 min. Parallel fibers were then stimulated with a parallel bipolar electrode (150 µm spacing) that was placed across the molecular layer about 200 µm from the recording electrode. The strength of the stimulation ranged from 5 to 50 V with a duration of 200 µs and was adjusted to evoke NMDA receptor currents at +40 mV in response to a single burst stimulation (4 stimuli at 100 Hz). I-LTP was then induced using 5 or 15 repeated trains of burst stimulation (repeated every second) while the postsynaptic cell was voltage-clamped at -60 mV. These are physiological relevant stimulation protocols since...
sensory stimulation in vivo evokes a burst of action potentials at ~80 Hz in granule cells (Chadderton et al., 2004; Wilms and Häusser, 2015), the axons of which innervate stellate cells. Axonal calcium transients in stellate cells elicited by activation of presynaptic NMDA receptors reach a peak value at ~400 ms after glutamate uncaging and remain high at 800 ms (Fig 5, Fig 2Ad; (Rossi et al., 2011). Thus repeated stimulation of PFs with a 960 ms inter-train interval is expected to lead to a sustained increase in presynaptic calcium levels. Immediately after parallel fiber stimulation TTX was re-introduced into the aCSF and recordings of mIPSCs were started within 2 minutes.

Dendritic NMDA receptor currents. Dendritic NMDA receptor-mediated currents were recorded using a Cs-based pipette solution (in mM: 140 CsCl, 2 NaCl, 10 HEPES, 4 Mg-ATP, 5 QX314, 5 tetraethylammonium (TEA), and 10 CsEGTA, pH 7.3). A single train of parallel fiber stimulation has been shown to induce a spillover of glutamate and activate extrasynaptic NMDA receptors (Carter and Regehr, 2000; Clark and Cull-Candy, 2002; Sun and Liu, 2007). Therefore we evoked NMDA receptor currents by stimulating parallel fibers using a monopolar electrode (4-8 MΩ, filled with aCSF) with a single train of 4 depolarizations at 100 Hz. This allowed us to compare our results to other studies that have characterized NMDA receptors properties using a monopolar stimulating electrode (Carter and Regehr, 2000; Clark and Cull-Candy, 2002; Sun and Liu, 2007). The typical stimulation intensity was 20 V with a duration of 200 µs. Recordings were obtained at +40 mV to relieve the magnesium block of NMDA receptors and the PF stimulation protocol was repeated every 30 s. Gabazine (SR-95531, 10 µM) and NBQX (5 µM) were included in the aCSF to block GABA<sub>A</sub> and AMPA receptors, respectively. The evoked currents were recorded for 15 min to obtain a stable baseline before application of NMDA receptor inhibitors.

Dendritic AMPA currents and paired pulse ratio. AMPA receptor-mediated EPSCs were recorded using the Cs-based internal solution and evoked by stimulating PFs with a monopolar stimulating electrode in the presence of 10 µM CPP (to block NMDA receptor currents). AMPA currents were recorded at -60 mV following 2 consecutive PF stimulations (50 Hz) repeated every 3 seconds. Typical stimulation intensity was 2 to 5 V with a duration of 200 µs. The paired-pulse ratio of average EPSCs (typically 100 to 150 events) at the PF-stellate cell synapse was calculated as the ratio of the amplitude of the second EPSC (EPSC<sub>2</sub>) over the first (EPSC<sub>1</sub>).
**Somatic NMDA receptor currents in outside-out patches.** Recordings were obtained in a magnesium-free aCSF that contained 0.5 µM TTX, 5 µM NBQX, 10 µM SR-95531 and 0.5 µM strychnine. Somatic outside out patches were voltage-clamped at -60 mV using borosilicate electrodes (7-15 MΩ) filled with a cesium fluoride-based pipette solution (in mM: 95 CsF, 35 CsCl, 2 NaCl, 1 CaCl₂, 4 MgATP, 10 Cs-EGTA, 1 QX-314, 5 TEA and 10 HEPES, pH 7.3). If recordings did not contain any spontaneous channel openings, the aCSF was supplemented with 10 µM glycine and 10 µM NMDA. Currents at 0 or -100 mV were also recorded to ensure that the reversal potential was 0 mV. The NMDA-evoked currents were recorded for 10 min to obtain a stable baseline (control period) and then in the presence of a NMDA receptor inhibitor for at least 10 min. Following washout of each inhibitor, NMDA-evoked currents returned to control levels (% change: CPP, 4 ±10%, n = 16; Ifenprodil, -16 ± 12%, n = 5; PPDA, -5 ± 8%; n = 7, P > 0.05 for all drugs, washout vs control). For analysis, current traces were re-sampled at 10 kHz and filtered at 1 kHz. Events of more than 0.6 pA and lasting more than 2 filter rise times (332 µs) were then selected over a 1 minute period and charge transfer was calculated.

**Data analysis.** Clampfit 9.0 (Axon Instruments) was used for the analysis of dendritic NMDA receptor currents and mIPSCs. The average dendritic NMDAR-mediated current was obtained by aligning all the traces to the stimulus artifact (typically 10 events). The inhibition of NMDA receptor currents by antagonists was calculated from the amplitude and integral of EPSCs (charge transfer) before and after the addition of an inhibitor. The decay time constant was obtained by fitting the decay phase of the EPSCs with a two-exponential function. Miniature IPSCs were selected using an event detection template. The average frequency and amplitude of mIPSCs were calculated over periods of 5 minutes. The group data in the I-LTP experiment in wild type mice induced by 5 and 15 trains of PF stimulation includes several cells from our previous published results (Lachamp et al., 2009), since no difference was observed between the two data sets. During the induction of I-LTP, the PF stimulation-evoked currents were recorded at -60 mV in postsynaptic stellate cells. This current consisted of a fast component mediated via AMPA receptors and a slow component. The latter is mediated by NMDA receptors because it was blocked by 10 µM CPP as we have shown previously (Fig 6C in Liu and Lachamp, 2006). Since the decay time constant of the fast component of the current was 8 ms (7.5 ± 0.9 ms, n=7, not shown), we determined the charge transfer of the slow component of the current 8 to 500 ms after the last of the 4 stimuli. Because the amplitude of dendritic NMDA receptor-mediated currents evoked by local glutamate uncaging is 5 fold greater than axonal
NMDA receptor currents (Fig 3C in Rossi et al, 2012), the currents recorded during PF stimulation were largely mediated by somato-dendritic receptors.

No statistical method was used to predetermine sample sizes, but they are similar to previous studies (Liu and Cull-Candy, 2000; Sun and Liu, 2007; Lachamp et al., 2009). Data sets were obtained from at least 3 different litters and animals from either sex were assigned randomly to the different experimental conditions. All values are expressed as mean ± SEM and a $P$ value < 0.05 was considered as significant. All tests were performed on primary data (not normalized). Normality and equality of the variances were assessed and statistical tests were chosen accordingly. These mostly included one-way or two-way ANOVA with repeated measurements (except for figure 11), and Tukey post hoc procedures were applied when needed. For detailed statistical analysis, see the statistical tables 1 and 2.
RESULTS

Stellate cells express functional GluN2D and GluN2B receptors.

We first determined whether functional GluN2D-containing receptors are present in the dendrites of stellate cells. While NMDA receptors are not present at the synapse, high frequency stimulation of parallel fibers triggers a glutamate spillover, which can evoke currents mediated by NMDA receptors. These receptors are presumably located on dendrites but at extrasynaptic sites (Carter and Regehr, 2000; Clark and Cull-Candy, 2002; Sun and Liu, 2007). Because both recombinant and native di-GluN2D receptors display a slow decay time when characterized at room temperature (Misra et al., 2000), we measured dendritic NMDA receptor currents in response to a train of parallel fiber stimulation at 100 Hz (4 stimuli) in wild type and GluN2D knockout mice (Ikeda et al., 1995). We found that deletion of GluN2D subunits accelerated the decay kinetics of dendritic NMDA receptor currents (Fig 1A). The decay time constant of NMDA receptor currents decreased from 338 ± 30 ms (n = 18) in wild-type to 203 ± 23 ms (n = 12; P < 0.002; Fig 1B) in GluN2D KO mice. This this is in agreement with the presence of NMDA receptors that contain GluN2D subunits (Cull-Candy et al., 2001). The total charge transfer of NMDA-currents decreased by ~60% in mutant mice (Fig 1D). Furthermore 0.1 µM PPDA, which blocks GluN2D-containing NMDA receptors, reduced the NMDA-evoked currents in outside-out patches from wild type stellate cells by 57 ± 6 % (n = 5; P < 0.01; Fig 1E and F). These results indicate that GluN2D-containing receptors mediate the dendritic and somatic NMDA receptor currents in stellate cells.

We next investigated the contribution of GluN2B-containing NMDA receptors to dendritic NMDA receptor currents in wild type mice. At 33-37°C 3 µM Ifenprodil inhibited the amplitude of dendritic NMDA receptor currents by 32 ± 6 % (pre 427 ± 103; Ifenprodil 291 ± 89 pA; n = 7; P < 0.001) and reduced the charge transfer by 20 ± 7 % (P < 0.001). Thus GluN2B receptors are present in the dendrites of stellate cells, consistent with a report that GluN2B but not GluN2A-containing NMDA receptors mediate dendritic NMDA receptor currents in rat stellate/basket cells (Bidoret et al., 2015). Ifenprodil also prolonged the decay time constant in wild type mice from 163 ± 10 ms to 201 ± 15 ms (Fig 2A and B; P < 0.05), consistent with the presence of GluN2D-containing NMDA receptors. Furthermore we found that 3 µM ifenprodil inhibited NMDA-evoked currents in outside-out patches from wild type stellate cells by 47 ± 9 % (n = 5; P < 0.01; Fig 2C and 2D). A marked increase in the blockade of NMDA receptor currents by...
ifenprodil in GluN2D KO mice to ~90% inhibition, compared to WT mice indicates that somatic NMDA receptor currents are largely mediated by GluN2B and GluN2D receptors.

**Deletion of GluN2D subunits prevents I-LTP.**

We next investigated the role of the GluN2D subunit in the induction of I-LTP. We recorded mIPSCs from stellate cells to monitor spontaneous GABA release and then stimulated parallel fibers (PFs) to evoke glutamate release. *In vivo* studies show that sensory stimulation evokes a burst of 3-4 action potentials at ~80 Hz in rats (Chadderton et al., 2004). We therefore stimulated PFs using 5 trains of 4 depolarizations at 100 Hz and found that this induced a lasting increase in mIPSC frequency of 55 ± 23 % (pre-stimulation 1.3 ± 0.4; post-stimulation 2.0 ± 0.6 Hz; n = 8; P < 0.001) in most neurons tested (7 of 8 cells), consistent with our previous observation (Lachamp et al., 2009). The mIPSC amplitude was not altered (pre-stim 22.7 ± 0.8; post-stim 20.2 ± 1.8 pA), indicating an increase in spontaneous GABA release from stellate cells in wild type mice (Fig 3A, C-E). However we found that this threshold stimulation protocol failed to produce a sustained increase in mIPSC frequency in all 6 stellate cells tested from GluN2D KO mice (pre-stim 1.5 ± 0.5; post-stim 1.3 ± 0.4 Hz) and the amplitude remained unaltered (pre-stim 32 ± 4; post-stim 32 ± 3 pA; Fig 3B-E).

To test whether this change was due to reduced glutamate release from PFs in mutant mice, we stimulated PFs with two consecutive stimuli and EPSCs were recorded at -60 mV in the presence of CPP (10 µM) and picrotoxin (100 µM) to block NMDA and GABA receptors, respectively. The paired pulse ratio of evoked EPSCs did not change in the GluN2D knockout mice (WT 1.9 ± 0.2, n = 7; GluN2D KO 2.0 ± 0.2, n = 6; Fig 4A), indicating that the probability of glutamate release from PFs in mutant mice was not altered. Furthermore, mIPSC frequency was not altered in GluN2D knockout mice (WT 2.2 ± 0.3 Hz, n = 49; GluN2D KO 2.1 ± 0.3 Hz, n = 36; Fig 4B), suggesting that basal spontaneous GABA release was also unaffected. Thus deletion of GluN2D subunits prevents the NMDA receptor-dependent, lasting increase in GABA release induced using a threshold stimulation protocol.

**GluN2D sets a low threshold for induction of I-LTP**

Tri-heteromeric receptors are composed of two GluN1 and two distinct GluN2 subunits and are thought to be the majority of the native NMDA receptors in the hippocampus (Rauner and Köhr,
NMDA receptors that contain both GluN2B and GluN2D subunits have been described in cerebellar Golgi cells and substantia nigra dopaminergic neurons (Brickley et al., 2003; Jones and Gibb, 2005; Brothwell et al., 2008). While NMDA receptors containing two GluN2D subunits have low channel conductance and opening probability, tri-heteromeric GluN2B/2D receptors exhibit distinct biophysical characteristics, with additional large 50 pS conductance channel openings and low sensitivity to Mg$^{2+}$ blockade (Misra et al., 2000; Huang and Gibb, 2014). Thus these receptors can be activated at more hyperpolarized potentials than di-heteromeric GluN2B (di-GluN2B) receptors and generate a larger current than di-heteromeric GluN2D (di-GluN2D) receptors. Prominent GluN2A/B staining was observed in the axonal terminals of cerebellar basket cells (Petralia et al., 1994). This raises the possibility that tri-GluN2B/2D NMDA receptors could be present in axons and induce I-LTP in wild type mice.

Deletion of GluN2D would convert a tri-heteromeric GluN2B/2D NMDAR to di-GluN2B receptor. Because GluN2D-Rs have a higher affinity for glutamate than GluN2B-Rs, we predicted that prolonging PF stimulation would increase the likelihood of activation of low affinity NMDARs by spillover glutamate and thus rescue I-LTP in GluN2D knockout mice. To test this possibility we increased the PF stimulation to 15 trains.

As we have shown previously, 15 trains of PF stimulation induced a sustained increase in the frequency of mIPSCs (74 ± 14 %; pre-stim 4.2 ± 1.0; post-stim 7.8 ± 2.4 Hz; n = 8; P < 0.001) with little effect on the amplitude (pre-stim 39 ± 6, post-stim 35 ± 5 pA; Fig 5A) in wild type mice (Lachamp et al., 2009). In contrast to the 5 train stimulation, this protocol successfully induced a sustained increase in the frequency of mIPSCs in GluN2D knockout mice (Fig 5B, C and E). The frequency of mIPSCs increased by 91 ± 25 % (pre-stim 1.3 ± 0.4; post-stim 2.2 ± 0.6 Hz; n = 9; P < 0.05; Fig 5B and C) over a period of 30 min relative to control without changing the mIPSC amplitude (pre-stim 24.2 ± 4.4; post-stim 24.1 ± 4.4 pA, Fig 5B and D). Thus the presence of GluN2D in NMDA receptors lowered the threshold for induction of I-LTP.

We next investigated the possibility that tri-GluN2B/2D NMDA receptors induce I-LTP in stellate cells from wild type mice. Deletion of GluN2D is predicted to alter the tri-GluN2B/2D receptors to di-GluN2B receptors which contain two GluN2B subunits. Ifenprodil at 3 µM has been shown to inhibit di-GluN2B receptors, but not tri-GluN2B/2D NMDA receptors (Brickley et al., 2003). Application of ifenprodil (3 µM) did not block the 15 train PF-stimulation-induced increase in mIPSC frequency (95 ± 37 %; pre-stim 1.2 ± 0.3; post-stim 2.4 ± 0.8 Hz; n = 6; P < 0.05; Fig 6A, C and E) and did not alter the basal mIPSC frequency (Fig 4D) in wild type mice.
Thus di-GluN2B receptors are not necessary for the induction of I-LTP. We therefore determined whether activation of di-GluN2B receptors triggered I-LTP in GluN2D knockout mice. In contrast to wild type mice, application of the selective antagonists ifenprodil (3 µM) and RO 04-5595 (5 µM) during a 15 train PF stimulation protocol completely prevented the induction of I-LTP (pre-stim 2.2 ± 0.8; post-stim 1.6 ± 0.6 Hz; Fig 6B-E) in mutant mice. These results indicate that activation of di-GluN2B receptors induces a lasting increase in GABA release only in mutant mice but not in wild type mice (Fig 6E). Therefore tri-GluN2B/2D receptors in wild type mice are responsible for the induction of I-LTP.

Pharmacological blockade of the PF-stimulation-induced lasting increase in GABA release.

Our results so far suggest that NMDA receptors that are responsible for I-LTP contain both GluN2B and GluN2D subunits. NMDA receptors are located in both the dendrites and axon terminals of stellate cells. We have previously shown that neither the release of NO or endocannabinoids nor a Ca rise in postsynaptic stellate cells are required for I-LTP (Lachamp et al., 2009). Thus postsynaptic NMDAR activation is unlikely to trigger I-LTP. Axonal NMDARs can be activated by glutamate (Duguid et al., 2007; Rossi et al., 2011), and therefore are strong candidates for induction of I-LTP. It has also been shown that activation of somato-dendritic NMDA receptors can lead to depolarization of axon terminals of stellate cells and enhances GABA release (Christie and Jahr, 2008). Thus I-LTP can potentially be induced either by direct activation of axonal receptors or by activation of somato-dendritic receptors. The latter model makes two predictions. First inhibition of somato-dendritic receptors should prevent I-LTP. Our results, however, show that GluN2B blockers inhibited dendritic NMDA receptors, but failed to block I-LTP (Fig 2E and 6A). Second inhibitors that block I-LTP should also inhibit NMDA receptor currents in the soma or dendrites. To address this issue we tested the ability of two inhibitors to block tri-GluN2B/2D-Rs and determined whether these inhibitors prevented the induction of I-LTP.

First, PPDA exhibits a moderate preference for GluN2C and GluN2D over GluN2A and GluN2B recombinant receptors. Thus PPDA at 0.1 µM is reported to preferentially block GluN2C/D-containing NMDA receptors (Feng et al., 2004). To determine whether 0.1 µM PPDA inhibits native GluN2D-containing receptors we took advantage of the well characterized expression of
tri-GluN2B/2D (and di-GluN2B) receptors in P7-10 Golgi cells, and di-GluN2D receptors in Purkinje cells (Misra et al., 2000; Brickley et al., 2003). We excised outside-out patches from somata of these neurons and evoked NMDAR currents by application of NMDA and glycine. Renzi et al (2007) have shown that ~25% of Purkinje cell patches also have large conductance NMDA receptor currents due to GluN2A and 2B receptors. However such currents were not detected in our patches and therefore NMDA receptor currents in these patches were mainly mediated by di-GluN2D receptors. We found that 0.1 µM PPDA inhibited NMDA-evoked currents in outside-out patches excised from Golgi cells (-45 ± 13 %; n = 5; P < 0.01; Fig 7B and D). PPDA also reduced the somatic single channel currents mediated via di-GluN2D NMDA receptors in Purkinje cells (-59 ± 13 %; n = 5; P < 0.05; Fig 7C and D). In contrast PPDA at 0.1 µM did not inhibit somatic currents mediated by di-GluN2B receptors in stellate cells from P18 GluN2D knockout mice (3 ± 9 %; n= 6; P > 0.05; Fig 7A and D; the predicted inhibition of recombinant di-GluN2B receptors is ~15-20%). Thus 0.1 µM PPDA inhibits both tri-GluN2B/2D and di-GluN2D receptors, but not di-GluN2B receptors (Fig 7D) in cerebellar neurons. When 0.1 µM PPDA was applied during a 15 train PF stimulation protocol, PF stimulation failed to enhance GABA release (mIPSC frequency: pre-stim 4.2 ± 1.1; post-stim 4.0 ± 1.0 Hz; n = 6; Fig 7E and F). Application of PPDA alone did not alter the frequency of mIPSCs (Fig 4D). This result is consistent with the idea that GluN2D receptors are critically involved in the induction of I-LTP in stellate cells.

Second, a low concentration of CPPene has been shown to inhibit recombinant tri-GluN2B/2D receptors (IC50 = 0.06 µM), di-GluN2B receptors (IC50 = 0.14 µM), but not di-GluN2D receptors (IC50 = 1.8 µM) (Buller and Monaghan, 1997). We next tested whether 0.2 µM CPP, the parent compound of D-CPPene, blocked tri-GluN2B/2D, but not di-GluN2D, and determined its effects on di-GluN2B receptors in neurons. CPP at 0.2 µM did not inhibit somatic GluN2B NMDA receptor currents in stellate cells from GluN2D knockout mice (4 ± 6 %; Fig 8A and D), which could be due to a reduced inhibitory potency of CPP relative to CPPene (Lowe et al., 1990). Application of 0.2 µM CPP reversibly inhibited NMDA-evoked currents mediated via tri-GluN2B/2D NMDA receptors in outside-out patches from the somata of Golgi cells (-54 ± 8 %; n = 7; P < 0.01; washout: -6 ±12%; n = 5; Fig 8B and D), but did not block NMDA receptor currents in patches from Purkinje cells (7 ± 9 %; n = 6; P > 0.05; Fig 8C and D). Thus in cerebellar neurons CPP at 0.2 µM blocks tri-GluN2B/2D NMDA receptors rather than di-GluN2D or di-GluN2B receptors.
If tri-GluN2B/2D NMDA receptors are involved in I-LTP induction, a low concentration of CPP should block I-LTP. Indeed we found that application of 0.2 µM CPP during PF stimulation prevented the induction of I-LTP in wild type mice (pre-stim 1.1 ± 0.4; post-stim 0.8 ± 0.3 Hz; n = 8; P > 0.05; Fig 9A-B), but did not modify basal GABA release (Fig 4D). This is consistent with the idea that tri-GluN2B/2D receptors are required to induce I-LTP in stellate cells.

Our results show a low concentration of CPP and PPDA can block tri-GluN2B/2D receptors, but they are likely to also inhibit recombinant GluN2A and GluN2C receptors, respectively (Feng et al, 2004; Buller and Monaghan, 1997). Because ifenprodil blocked I-LTP in GluN2D KO mice, GluN2A and GluN2C receptors are unlikely to be involved in the induction of I-LTP. The effect of a low concentration of CPP on other NMDA receptor subtypes remains to be determined.

Do I-LTP inhibitors also block somato-dendritic NMDA receptor currents?

The dendritic model in which activation of dendritic NMDA receptors is necessary for the induction of presynaptic I-LTP predicts that inhibitors that block I-LTP should also inhibit NMDA receptor currents in the soma or dendrites. We therefore determined the effect of a low concentration of CPP on somato-dendritic NMDA receptor currents in wild type mice.

Application of CPP at 0.2 µM did not block the NMDA receptor current in somatic patches from stellate cells (1 ± 4 %; n= 5; Fig 10A and B). Because a low concentration of CPP inhibits NMDA-evoked currents in outside-out patches from cerebellar Golgi cells that express tri-GluN2B/2D receptors, these receptors are unlikely to be present in the somata of stellate cells.

We then stimulated parallel fibers at 100 Hz (4 stimuli) to activate dendritic NMDA receptors at 33-37°C and found that 0.2 µM CPP had no effect on the amplitude of dendritic NMDA receptor currents (pre 463 ± 85; CPP 412 ± 79 pA; n = 5) or total charge transfer (3 ± 3 %). The decay time also remained unaltered (pre 219 ± 17; CPP 234 ± 22 ms; Fig 10C-F). Thus NMDA receptors that are sensitive to a low concentration of CPP, including tri-GluN2B/2D receptors, are absent from the somata and dendrites of stellate cells. The IC50 of CPP for NMDAR subtypes may differ between somatic and dendritic receptors, which were activated by application of NMDA and spillover glutamate, respectively. We therefore compared the effects of 0.2 µM CPP on I-LTP with that on dendritic NMDA receptors, since in the dendritic model, activation of dendritic NMDA receptor is responsible for the induction of I-LTP. Because 0.2 µM
CPP completely prevented the induction of I-LTP, but failed to block dendritic currents (Fig 9A and B), the tri-GluN2B/2D receptors that are responsible for the induction of I-LTP are unlikely to be located in the somata and dendrites of stellate cells. Consistent with this idea, we found no correlation between the degree of I-LTP and the total charge transfer of the slow component of the dendritic currents recorded at -60 mV during PF stimulation (Fig 11). The latter is presumably mediated in large part by postsynaptic GluN2D-containing receptors due to their lower Mg$^{2+}$ blockade compared to GluN2B receptors (Lachamp et al., 2009). These results are not in agreement with the prediction of a model in which activation of dendritic NMDA receptors induces I-LTP. Therefore NMDA receptors located in axons rather than in dendrites are most likely to induce I-LTP.
Discussion

Our results address a fundamental issue concerning synaptic cross-talk: what types of presynaptic NMDA receptors in inhibitory interneurons sense glutamate release and modulate GABA release? We found that tri-heteromeric GluN2B/2D receptors are responsible for the induction of I-LTP and these receptors are absent in dendrites. This result indicates that GluN2D-containing NMDA receptors at the presynaptic terminals sense the low concentration of glutamate that spills over from neighboring excitatory inputs. These receptors thus mediate the cross-talk between excitatory and inhibitory transmission.

Which type of NMDA receptors induce I-LTP? The subunit composition of NMDA receptors controls a number of biophysical properties of the channel, as well as their subcellular localization (Cull-Candy et al., 2001; Petralia et al., 2009; Yuan et al., 2009; Siegler Retchless et al., 2012) and therefore endows them with different roles in regulating synaptic transmission. Postsynaptic NMDA receptors that contain GluN2A and GluN2B subunits are important for the induction of long-term plasticity at excitatory synapses (Wyllie et al., 2013). Many GABAergic interneurons express high levels of GluN2D mRNA (Akazawa et al., 1994; Monyer et al., 1994; Thompson et al., 2000). However the role of GluN2D receptors in inhibitory neurons is not known. GluN2D has a high glutamate binding affinity, and therefore may detect a low concentration of glutamate that has spilled over from nearby excitatory inputs. In vivo studies show that somatosensory stimulation evokes a short burst of action potentials in parallel fiber inputs to stellate cells (Chadderton et al., 2004). Our finding that deletion of GluN2D abolished the I-LTP induced by a few trains of PF stimulation, reveals that GluN2D receptors are critical for triggering a lasting increase in GABA release from inhibitory interneurons. Using a combined pharmacological and genetic approach, we identify these receptors as tri-GluN2B/2D receptors and the presence of GluN2D lowers the threshold of induction of I-LTP. This result is in agreement with previous observations that a NMDA-evoked enhancement in mIPSC frequency displayed a low sensitivity to ifenprodil and Mg$^{2+}$ blockade, a characteristic of tri-GluN2B/2D receptors (Glitsch and Marty, 1999; Rossi et al., 2011; Huang and Gibb, 2014). Such tri-GluN2B/2D receptors are also present in substantia nigra dopaminergic neurons (Jones and Gibb, 2005; Brothwell et al., 2008) and may be involved in the regulation of neurotransmitter release from these cells. Indeed deletion of GluN2D subunits reduces dopamine levels in the prefrontal cortex and hippocampus (Miyamoto et al., 2002) and alters locomotor activity (Hagino...
et al., 2010). Therefore GluN2D receptors are likely to play a major role in the glutamate-dependent modulation of neurotransmitter release.

Where are the tri-GluN2B/2D receptors located? We found that a low concentration of CPP completely abolished I-LTP, but had no effect on somato-dendritic NMDA receptor currents. In contrast, ifenprodil partially blocked somato-dendritic NMDA receptors, but failed to prevent I-LTP. Therefore activation of dendritic NMDA receptors is not required for the induction of I-LTP and thus NMDA receptors responsible for I-LTP are most likely present in axons. Burst stimulation of parallel fibers can also activate NMDA receptors in the dendrites (Carter and Regehr, 2000; Clark and Cull-Candy, 2002; Sun and Liu, 2007), producing a sustained depolarization (Carter and Regehr, 2000), which activates calcium channels in axons and enhances GABA release from stellate cells (Christie and Jahr, 2008; Pugh and Jahr, 2011). However our results do not support the idea that an NMDA receptor-dependent somato-dendritic depolarization electrotonically spreads to the axon to induce I-LTP. Although NMDA receptors can act as both metabotropic and ionotropic receptors (Nabavi et al., 2013), we have previously shown that calcium entry via NMDA receptors is required for the induction of I-LTP (Liu and Lachamp, 2006), thus a metabotropic action of somato-dendritic NMDARs is unlikely to trigger I-LTP. Our results suggest that spillover glutamate acts on NMDA receptors at the presynaptic terminals of GABAergic interneurons to induce I-LTP, which is supported by a variety of other reports. First, glutamate uncaging at axonal locations in stellate cells evoked a calcium transient in the axon terminals via NMDA receptor activation (Rossi et al., 2011). Second, glutamate release from isolated Purkinje cells induces an NMDA receptor-dependent increase in GABA release from presynaptic terminals (Duguid et al., 2007). Third, GluN2A/B immunoreactivity is present in basket cell terminals (Petralia et al., 1994) in the cerebellum. Fourth, functional NMDA channel currents with high and low conductance, a characteristic of tri-GluN2B/2D receptors (Brickley et al., 2003; Jones and Gibb, 2005), have been detected in the enlarged varicosities of cultured stellate/basket cell axon terminals following NMDA treatment (Fiszman et al., 2005). Thus GluN2D-containing NMDA receptors at presynaptic sites may modulate GABA release in other neurons since GluN1 subunits have been found in the axon terminals of other GABAergic interneurons and GluN2D mRNA is present in many inhibitory interneurons (Akazawa et al., 1994; Monyer et al., 1994; Paquet and Smith, 2000; Thompson et al., 2000).
Glutamate acts on NMDA receptors in presynaptic and postsynaptic neurons to regulate GABA release via distinct mechanisms. It is known that glutamate receptors in postsynaptic neurons can trigger the release of retrograde signals that modulate GABA release (Castillo et al., 2011). While a metabotropic glutamate receptor-evoked release of endocannabinoids suppresses GABA release, NMDA receptor activation promotes an NO-dependent enhancement of GABA secretion (Marsicano et al., 2002; Chevaleyre and Castillo, 2003; Chevaleyre et al., 2007; Nugent et al., 2007; Adermark and Lovinger, 2009; Jiang et al., 2010). However I-LTP induction in stellate cells is not induced by retrograde signals because we have previously shown that loading postsynaptic stellate cells with BAPTA or the use of NOS inhibitors failed to prevent I-LTP (Lachamp et al, 2009). Here we found that tri-GluN2B/2D NMDA receptors in presynaptic inhibitory interneurons detect the spillover of glutamate and lead to an enhancement of GABA release. The ability to modulate GABA release in the cerebellum is critical for the physiological functioning of this brain region because an increase in GABA release from stellate/basket cells alters motor coordination and has also been implicated in neurological disorders such as episodic ataxia (Herson et al., 2003). Our finding that activation of presynaptic tri-heteromeric GluN2D/2B receptors induces I-LTP opens the possibility for selectively modulating GABA release without affecting plasticity at those excitatory synapses that require postsynaptic NMDARs which contain GluN2A and GluN2B subunits (Wyllie et al., 2013).
Figure legends

**Figure 1.** NMDA receptors that contain GluN2D subunits are expressed in the soma and dendrites of stellate cells. A. Dendritic NMDA receptor currents were evoked by a single burst stimulation of PFs at 100 Hz (4 stimuli) and recorded at +40 mV at room temperature. NMDA receptor currents recorded in stellate cells from GluN2D KO mice exhibited a faster decay time compared to wild type mice, as shown in the normalized currents (GluN2 KO, grey trace; wild-type, black trace). B. Summary of the decay time constants of NMDA receptor currents recorded in stellate cells from WT and GluN2D knockout mice. Deletion of GluN2D accelerated the decay time of evoked dendritic NMDA receptor currents. C. The amplitude of dendritic NMDA receptor-mediated currents. D. The charge transfer of dendritic NMDA receptor-mediated currents, which was calculated as the time integral of NMDA receptor currents, was reduced in GluN2D knockout mice. Thus NMDA receptors that contain GluN2D subunits are present in cerebellar stellate cells. Open circles represent data from individual cells. E. Outside-out somatic patches were excised from stellate cells from wild type mice and NMDA receptor currents were recorded during the application of 10 µM NMDA and 10 µM glycine. PPDA (0.1 µM) were added to the superfusion medium to block GluN2D-containing NMDA receptors. F. PPDA reduced the charge transfer of somatic NMDAR currents. *, P < 0.05; **, P < 0.01.

**Figure 2.** GluN2B-containing receptors mediated somato-dendritic NMDA receptor currents in stellate cells. A. Dendritic NMDA receptor currents were evoked by a train of PF stimulation at 100 Hz (4 stimuli) at 33-37°C. Currents were recorded at +40 mV in stellate cells before (black traces) and during (colored traces) superfusion of an NMDA receptor blocker, ifenprodil (3 µM), a di-GluN2B receptor antagonist. Ifenprodil blocked dendritic NMDA receptor currents, suggesting that NMDA receptors that contain GluN2B subunits mediate the dendritic NMDA receptor current. B. Summary of the effects of ifenprodil (3 µM) on the amplitude, charge transfer and decay time of NMDA receptor currents at near-physiological temperature (open circles indicate data from individual experiments). C. Ifenprodil also blocked NMDA receptor currents in outside-out somatic patches excised from stellate cells from wild type mice (left) and thus GluN2B receptors mediate NMDA receptor currents. In GluN2D KO mice (right) ifenprodil nearly abolished the NMDA-evoked currents from stellate cells. F. The total charge transfer was markedly reduced in wild-type and abolished in GluN2D KO mice. NMDA receptor currents are mediated by a pure population of di-GluN2B NMDA receptors in mutant mice. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 3. Genetic deletion of GluN2D subunits abolished I-LTP induced by threshold PF stimulation. A - B. mIPSCs were recorded in stellate cells in the presence of TTX and PFs were stimulated with 5 trains of 4 pulses at 100 Hz (5x) after washing out TTX for 20 min. Left, representative traces of mIPSCs recorded at -30 mV (outward currents) before (top) and after (bottom) PF stimulation. Center, corresponding time course of mIPSC frequency (top) and amplitude (bottom). Right, group data shown as mean ± SEM showing the time course of mIPSC frequency (top) and amplitude (bottom) following PF stimulation in wild type (A, open symbols) and in GluN2D KO mice (B, grey symbols, for comparison the light gray area shows the mean ± SEM of mIPSC frequency after PF stimulation in wild type mice). Insert: representative recordings at -60 mV during PF stimulation in stellate cells. Five trains of PF stimulation were sufficient to trigger I-LTP in stellate cells from wild type mice (n = 8), but failed to induce a change in mIPSCs in GluN2D KO mice (n = 6). Therefore NMDA receptors that contain GluN2D subunits mediate the induction of I-LTP. C - D. Group data showing mIPSC frequency (C) and amplitude (D) of individual cells before (15 min average before TTX washout) and after (average 15 to 30 min after stimulation) PF stimulation in wild type (left) and GluN2D KO (right) mice. Only mIPSC frequency from wild type animals increased following 5 trains of PF stimulation. E. Group data showing that 5 trains of PF stimulation increased mIPSC frequency in individual cells from wild type, but not GluN2D KO mice. *, P < 0.05, **, P < 0.01.

Figure 4. NMDA receptors do not regulate basal spontaneous GABA release in cerebellar stellate cells. A. Top, Representative recordings of EPSCs evoked by parallel fiber stimulation with two consecutive stimuli in wild type (left) and GluN2D knockout mice (right). Bottom, the paired-pulse ratio of EPSCs at the PF-stellate cell synapse (the ratio of amplitude of EPSC2 / EPSC1) was not modified in GluN2D KO mice (wild-type n = 7, GluN2D KO n = 6), indicating that the release probability of glutamate from parallel fibers was not altered. B. Cumulative distribution of mIPSC frequency (left) and amplitude (right) in wild type (solid line) and GluN2D KO mice (dashed line). Miniature IPSC frequency was not altered in GluN2D KO mice (n = 36) compared to wild type mice (n = 49). Thus deletion of GluN2D did not change basal spontaneous GABA release from stellate cells. C. Representative traces of mIPSCs (outward currents) in wild type (left) and GluN2D KO (right) mice. The bottom traces are enlargements of regions designated by the dashed lines. D. NMDA receptor blockers did not alter basal mIPSC frequency recorded at -60 mV using a CsCl-based pipette solution in wild type stellate cells. After obtaining a stable baseline in the presence of 0.5 µM TTX and 5 µM NBQX to isolate
mIPSCs, PPDA (left, 0.1 µM, n = 5), Ifenprodil (center, 3 µM, n = 5) or CPP (right, 0.2 µM, n = 5) were added to the aCSF for at least 20 min.

**Figure 5. Genetic deletion of GluN2D subunits increased the threshold of induction of I-LTP.** A prolonged stimulation protocol, 15 trains of parallel fiber (PF) stimulation, was used to induce an increase in mIPSC frequency (I-LTP) in both wild type and GluN2D knockout mice. A - B. **left**, representative traces of mIPSCs (outward currents) before (top) and after (bottom) PF stimulation; **Center**, time course of mIPSC frequency (top) and amplitude (bottom); **Right**, group data (mean ± SEM) showing the time course of mIPSC frequency (top) and amplitude (bottom); insert, representative traces during PF stimulation. **C - D.** In wild type mice, 15 trains of PF stimulation induced a lasting increase in mIPSC frequency without any change in the amplitude (n = 8), suggesting an increase in spontaneous GABA release from stellate cells (I-LTP). In GluN2D KO mice this stimulation protocol also induced a sustained increase in mIPSCs frequency without a change in the amplitude (n = 9). **E.** Group data showing that 15 trains of PF stimulation was able to rescue I-LTP in GluN2D KO mice. Therefore incorporation of GluN2D subunits in NMDA receptors in wild type mice lowers the threshold for the induction of I-LTP. *, P < 0.05.

**Figure 6. Activation of tri-GluN2B/2D NMDA receptors, but not di-GluN2B receptors, induced I-LTP in wild type mice.** A 15 train parallel fiber (PF) stimulation protocol was used to induce an increase in mIPSC frequency (I-LTP) in wild type and GluN2D knockout mice. **A - B. left**, representative traces of mIPSCs (outward currents) before (top) and after (bottom) PF stimulation; **Center**, time course of mIPSC frequency (top) and amplitude (bottom); **Right**, group data (mean ± SEM) showing the time course of mIPSCs frequency (circles) and amplitude (triangles). **A. left** and **center**, Ifenprodil (3 µM), a di-GluN2B antagonist, was applied during a 15 train PF stimulation protocol and failed to prevent I-LTP in wild type mice. **Right**, summary of the effect of ifenprodil on the long-lasting increase in mIPSC frequency induced by 15 trains of PF stimulation (n = 6). Light gray area shows the mean ± SEM of mIPSC frequency after PF stimulation without GluN2B antagonist in wild type mice (from Fig 5A) for comparison. **B.** In GluN2D KO animals, I-LTP was blocked by GluN2B antagonists (3 µM ifenprodil or 5 µM RO 04-5595, n = 8). For comparison the light gray area shows the mean ± SEM of mIPSC frequency after PF stimulation without GluN2B antagonist in GluN2D KO mice (from Fig 5B). **C - D.** Mean mIPSC frequency (**C.**) and amplitude (**D.**) in individual cells following 15 trains of PF stimulation in wild type and mutant mice. **E.** Summary of the effects of GluN2B antagonists on
the long-lasting increase in mIPSC frequency induced by 15 trains of PF stimulation. GluN2B antagonists effectively blocked I-LTP only in GluN2D KO mice, suggesting that activation of di-GluN2B receptors induced a lasting increase in GABA release in mutant mice. Ifenprodil failed to prevent I-LTP in wild type mice and thus NMDA receptors that contain both GluN2D and GluN2B subunits are responsible for the induction of I-LTP in wild type mice. *, $P < 0.05$.

Figure 7. A low concentration of PPDA inhibited GluN2D-containing NMDA receptors and blocked I-LTP. A-D. Assessment of the selectivity of 0.1 µM PPDA on native NMDAR subtypes in somatic patches from (A) stellate cells (GluN2D KO), (B) Golgi cells (WT) and (C) Purkinje cells (WT). In wild type P8-P10 mice, Golgi cells are known to have a mixed population of di-GluN2B and tri-GluN2B/2D receptors (Brickley et al., 2003) whereas mainly di-GluN2D receptors are present in Purkinje cells (Renzi et al., 2007). D. PPDA (0.1 µM) reduced the charge transfer of NMDA receptor currents in both Purkinje cells ($n = 5$) and Golgi cells ($n = 5$), but not in stellate cells from GluN2D KO mice ($n = 6$). Thus 0.1 µM PPDA inhibits GluN2D-containing NMDA receptors but not di-GluN2B NMDA receptors. E. Application of 0.1 µM PPDA during TTX washout and during the PF stimulation in wild type animals. Left, representative traces of mIPSCs before (top) and after (bottom) PF stimulation. Center, time course of mIPSC frequency (top) and amplitude (bottom) from the corresponding recordings on the left. Right, group data (mean ± SEM) showing the time course of mIPSC frequency (circles) and amplitude (triangles). Light gray area shows the mean ± SEM of mIPSC frequency after PF stimulation without antagonist for comparison (Fig 5A). F. Application of 0.1 µM PPDA during PF stimulation abolished I-LTP ($n = 7$). For drug effects: *, $P < 0.05$, **, $P < 0.01$, for the interaction between drugs and cell types #, $P < 0.05$.

Figure 8. A low concentration of CPP inhibited tri-GluN2B/2D receptors, but not di-GluN2B or di-GluN2D receptors. A - C. The effects of 0.2 µM CPP on native NMDA receptors containing the GluN2B and/or GluN2D subunits was assessed in outside-out patches excised from the somata of 3 types of neurons. NMDA receptor currents in somatic patches from (A) stellate cells (P18-21 GluN2D KO), (B) Golgi cells and (C) Purkinje cells were evoked by bath application of 10 µM NMDA and 10 µM glycine. 0.2 µM CPP blocked NMDA receptor currents in outside out patches from Golgi cells. D. Group data shows that 0.2 µM CPP reduced the charge transfer of NMDA receptor currents in patches from Golgi cells ($n = 7$) but not from Purkinje cells ($n = 6$) or stellate cells from GluN2D KO mice ($n = 6$). These results demonstrate that a low concentration of CPP blocked tri-GluN2B/2D receptors but not di-GluN2B or di-GluN2D.
Figure 9. A low concentration of CPP prevented I-LTP in wild type mice. To test the effects of 0.2 µM CPP on the induction of I-LTP, 0.2 µM CPP was applied during TTX washout and during the 15 train PF stimulation in wild type mice (see figure 4). A. Left and Center, representative mIPSC traces and time course of mIPSC frequency and amplitude from a typical recording. Right, group data (mean ± SEM) showing that CPP at 0.2 µM prevented the PF stimulation-induced increase in GABA release (n = 8). For comparison the light gray area shows the mean ± SEM of mIPSC frequency after PF stimulation without antagonist found in Fig 5A. B. The frequency of mIPSCs was not altered following PF stimulation (15 min before TTX washout and 15-30 min after PF stimulation). This supports the idea that activation of tri-GluN2B/2D NMDA receptors is required for the induction of I-LTP.

Figure 10. A low concentration of CPP did not inhibit somato-dendritic NMDA receptor currents in stellate cells. A-B: Outside-out somatic patches were excised from stellate cells from wild type mice and NMDA receptor currents were recorded during the application of 10 µM NMDA and 10 µM glycine. After establishment of a stable baseline, CPP (0.2 µM) that blocks tri-GluN2B/2D was added to the superfusion medium. B. Group data showing that CPP had no effect, suggesting that tri-GluN2B/2D NMDA receptors are not expressed in the somata of stellate cells. C. Dendritic NMDA receptor currents were evoked by a train of PF stimulation at 100 Hz (4 stimuli). Currents were recorded at +40 mV in stellate cells before (black traces) and during (red traces) superfusion of an NMDA receptor blocker. CPP (0.2 µM) did not reduce the PF-evoked NMDA receptor current. D - F. CPP (0.2 µM) did not alter the amplitude, charge transfer and decay time of dendritic NMDA receptor currents (open circles indicate data from individual experiments). In wild type mice, NMDARs that are sensitive to a low concentration of CPP, including tri-GluN2B/2D receptors, are not present on the soma of stellate cells. CPP prevented I-LTP but did not inhibit dendritic currents therefore I-LTP, which requires tri-GluN2B/2D receptors (Fig 5 and 6), is not triggered by a somato-dendritic depolarization.

Figure 11. The induction of I-LTP is independent of dendritic currents evoked by PF stimulation and of the initial mIPSC frequency. A. During the induction of I-LTP, postsynaptic currents evoked by PF stimulation were recorded at -60 mV. The total charge transfer of the slow component of the inward current was measured starting at 8 ms after the last stimulus during each train (see Methods section). This is shown as the red area in the representative
example (truncated at 135 ms). Individual values are represented by open circles and the median value is shown as a horizontal bar. There was no significant difference between groups (see statistical table). B. Plot of the change in mIPSC frequency after PF stimulation in all individual cells as a function of the total charge transfer of postsynaptic currents evoked by PF stimulation. Regression line is represented in blue. Together these results suggest that the induction of I-LTP was not correlated with the magnitude of the charge transfer of the postsynaptic currents recorded during PF stimulation. C. Plot of the change in mIPSC frequency (%) after PF stimulation (I-LTP) in all cells as a function of initial mIPSC frequency (n = 59). There was no correlation between the magnitude of I-LTP and the initial mIPSC frequency. D. Summary of individual mIPSC frequency for each experimental condition.
References


A. Normalized current traces (Room temp.)

B. Decay time constant (ms)

C. Dendritic NMDA current amplitude (pA)

D. Charge transfer (pAs)

E. Wild type

F. % change in charge transfer
A. Wild type

B. Dendritic NMDA current amplitude (pA)

C. % change in charge transfer

D. Physiol. Temp.
A. Wild type

Before stimulation

After 5 trains of PF stimulation

B. GluN2D KO

Before stimulation

After 5 trains of PF stimulation

C. 

**

*

NS

D. 

NS

NS

NS

E. 

% change in mIPSC frequency
A. WT GluN2D KO

B. Cumulative fraction (%)

C. Wild type

GluN2D KO

D. NS

mIPSC frequency (Hz)

mIPSC amplitude (pA)

mIPSC frequency (Hz)
A. Wild type
Before stimulation
After 15 trains of PF stimulation

B. GluN2D KO
Before stimulation
After 15 trains of PF stimulation

C. % change in mIPSC amplitude (%)
Before After
GluN2D KO

D. % change in mIPSC frequency (%)
Before After
GluN2D KO

E. % change in mIPSC amplitude (μA)/frequency (Hz)
WT GluN2D KO

TTX washout
mIPSC frequency (Hz)
Rs (MΩ)
mIPSC amplitude (pA)

Time (min)
50 ms 1 nA
Dubois et al., Figure 6

A. Wild type
Before stimulation

After 15 trains of PF stimulation

50 pA

5 s

50 pA

5 s

B. GluN2D KO
Before stimulation

After 15 trains of PF stimulation

25 pA

5 s

25 pA

5 s

C. 
WT GluN2D KO

* 
NS

D. 
WT GluN2D KO

NS

NS

E. 
Control
GluN2B antagonists

% change in mIPSC frequency

% change in mIPSC amplitude

GluN2B antag

WT GluN2D KO

Control
GluN2B antag
A. Stellate cells (GluN2D KO)  
Control  
Before stimulation  
After 15 trains of PF stimulation  

B. Golgi cells (Wild type)  
Control  

C. Purkinje cells (Wild type)  
Control  

D. % change in charge transfer

E. Wild type  
Before stimulation  
After 15 trains of PF stimulation  

F. % change in mIPSC frequency  

GluN2D KO  
Wild type  

PPDA  
0.1 μM  
No drug  
PPDA 0.1 μM
A. Stellate cells (GluN2D KO) Control

B. Golgi cells (Wild type) Control

C. Purkinje cells (Wild type) Control

D. % change in charge transfer

- SC KO
- GC
- PC

#
A. Wild type

Before stimulation

After 15 trains of PF stimulation

B. mIPSC frequency (Hz)

Before + CPP

Af

After + CPP

WT
Dubois et al., Figure 10

A. Stellate cells (Wild type)

Control

+ 0.2 μM CPP

200 ms

3 pA

3 pA

200 ms

0.2 μM CPP

Physiol. Temp.

100 pA

500 ms

B. % change in charge transfer

CPP

0

-50

-100

C. Dendritic NMDA current amplitude (pA)

NS

Ctrl

CPP

D. Decay time constant (ms)

Ctrl

CPP

E. Charge transfer (pA)

Ctrl

CPP

F. Decay time constant (ms)

Ctrl

CPP
A. Charge transfer (pC) vs. time (50 ms).

B. Charge transfer (pA s) vs. mIPSC frequency (% change).

C. Initial mIPSC frequency (Hz) vs. mIPSC frequency (% change).

D. Initial mIPSC frequency (Hz) vs. GluN2B antag concentration.
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RM, Repeated Measurements; bold P values indicate a significant effect (P<0.05)
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<th>Analysis</th>
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RM, Repeated Measurements; bold P values indicate a significant effect (P<0.05)