# EXPERIENCE-DEPENDENT REGULATION OF PRESYNAPTIC NMDA RECEPTORS (PRENMDARS) AND THEIR ROLE IN NEUROTRANSMISSION AND SYNAPTIC PLASTICITY

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## ABSTRACT

**REBEKAH CORLEW: Experience-dependent regulation of presynaptic NMDA receptors (preNMDARs) and their role in neurotransmission and synaptic plasticity**
**(Under the direction of Benjamin D. Philpot)**

Many aspects of synaptic development, plasticity, and neurotransmission are critically influenced by NMDA-type glutamate receptors (NMDARs). Moreover, dysfunction of NMDARs has been implicated in a broad array of neurological disorders, including schizophrenia, stroke, epilepsy, and neuropathic pain. Though NMDARs are classically thought to be postsynaptic, recent evidence demonstrates that presynaptic NMDARs (preNMDARs) also exist and have critical roles in synapse function and plasticity. One of the most fascinating areas of research for postsynaptic NMDARs is how they are modified with development and experience and how their changing roles in synaptic transmission and synaptic plasticity change with sensory driven activity. Only a small number of studies have suggested that preNMDARs are modified with experience and a mechanism for this change has been speculative at best. Also highly speculative is the question of how preNMDARs are able to function tonically. In this dissertation I hope to satisfy both queries with one solution. I will attempt to explain how preNMDARs are tonically active and how this can explain their developmental and possibly experience-dependent modifications.

Chapter 1 reviews the current knowledge of the role of preNMDARs in synaptic transmission and plasticity, in the neocortex, and a discussion of the prevalence, function, and development of these receptors. Chapter 2 provides the first evidence of developmental control of preNMDAR expression, function, and role in synaptic plasticity. Chapter 3 answers the most perplexing question that plagues the study of preNMDARs "how do they overcome their $Mg^{2+}$ block to be tonically active?" Here, evidence is presented that preNMDARs contain the novel subunit NR3A which is substantially less $Mg^{2+}$ sensitive allowing preNMDARs to be tonically active. Thus, the developmental loss of NR3A would cause the preNMDAR to loose its tonic activity though not its ability to enhance spontaneous release in $Mg^{2+}$ free solution. Chapter 4 suggests that preNMDARs may not be subject only to developmental control but may also be modified by experience. Chapter 5 explores how altered visual experience modifies preNMDARs at different points in development. Chapter 6 discusses how these findings will contribute to the study of preNMDARs, clinical outcomes of this research, and possible future directions.

## DEDICATION

I would like to dedicate this dissertation primarily to Deric John Corlew. Deric has been my support, cheerleader, voice of reason, and shoulder to cry on from the moment I became a neuroscientist as a wide-eyed undergraduate. He makes me laugh at myself, question myself, and he is the only person that can convince me to acknowledge my accomplishments. He has so directly supported the work in this dissertation that he should be awarded a PhD in Neuroscience. I would also like to acknowledge Whitman John Corlew. Though his birth actually severely impeded the progression of this dissertation, he is by far the most amazing, significant thing, that I have ever produced. Watching him develop in his first year has been more fascinating than any experiment. I want to thank my mother, Susan Michelle Sisk, who is my best friend and therapist, who listens to me lament every time an experiment doesn't turn out, and then is always excited for me when I figure it out. I owe to my dad, Oscar Kelt Sisk, my scientific thirst for knowledge and the creativity that has carried me through my first ten years in neuroscience. I love you all and thank you all very much.

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Chapter 5 Larsen R contributed to Figure 5.1B. Phend K and Weinberg R contributed to figure 5.2.

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| | |
|---|---|
| ACSF | artificial cerebrospinal fluid |
| AMPAR | AMPA-type glutamate receptors |
| AP | action potential |
| APV | (2R)-amino-5-phosphonovaleric acid |
| BSA | bovine serum albumin |
| CaMKII | calcium/calmodulin kinase II |
| CB1 | cannabinoid type 1 |
| CICR | calcium induced calcium release |
| CNS | central nervous system |
| CV | coefficient of variation |
| DR | dark-reared |
| EM | electron microscopy |
| EPSC | excitatory postsynaptic current |
| EPSP | excitatory postsynaptic potential |
| DAB | diaminobenzidine |
| GABAAR | GABAA receptor |
| GABAR | GABA receptor |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethan |
| I-V | current-voltage |
| iMK801 | internal MK801 |
| KO | knockout mouse |
| LOVD | late-onset visual deprivation |

| | |
|---|---|
| LSM | lysed synaptosomal membrane |
| L | layer |
| LTD | long-term depression |
| LTP | long-term potentiation |
| MD | monocular deprivation |
| mEPSC | miniature excitatory post synaptic currents |
| mGluR | metabotropic glutamate receptor |
| mIPSC | miniature inhibitory post-synaptic current |
| MK801 | (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate |
| NMDAR | NMDA-type glutamate receptors |
| NR | normally-reared |
| NR1 | NMDA-type glutamate receptor subunit 1 |
| NR2A | NMDA-type glutamate receptor subunit 2A |
| NR2B | NMDA-type glutamate receptor subunit 2B |
| NR2C | NMDA-type glutamate receptor subunit 2C |
| NR2D | NMDA-type glutamate receptor subunit 2C |
| NR3A | NMDA-type glutamate receptor subunit 3A |
| NR3B | NMDA-type glutamate receptor subunit 3B |
| NO | nitric oxide |
| OD | ocular dominance |
| OE | overexpresser mouse |
| P | postnatal day |
| PB | phosphate-buffer |

| | |
|---|---|
| PBS | phosphate-buffered saline |
| PNS | post-nuclear supernatant |
| PPF | Paired pulse facilitation |
| preNMDAR | presynaptic NMDAR |
| PSD | postsynaptic density |
| PSD-95 | postsynaptic density protein-95 |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| sEPSCs | spontaneous excitatory postsynaptic currents |
| STD | short-term depression |
| STDP | spike timing-dependent plasticity |
| S1 | primary somatosensory cortex |
| tLTD | spike timing-dependent LTD |
| TR | tube-reared |
| TTX | tetrodotoxin |
| VSCC | voltage-sensitive calcium channel |
| V1 | primary visual cortex |
| WT | wild-type mouse |

# Chapter 1:

# Presynaptic NMDA Receptors: Newly Appreciated Roles in Cortical Synaptic Function and Plasticity

**This chapter was published with Daniel J. Brasier, Daniel E. Feldman, and Benjamin D. Philpot in *The Neuroscientist* (2008) Dec; 14(6): 609-25**

## 1.1 Overview

NMDA-type receptors (NMDARs) are ionotropic glutamate receptors that act as non-specific cation channels which are permeable to sodium, calcium, and potassium. These receptors are hetero-tetrameric transmembrane proteins which contain two NR1 (NMDA receptor 1) and two NR2 or NR3 subunits (Dingledine et al., 1999). NMDARs exhibit a voltage-dependent block by extracellular magnesium, causing them to be outwardly rectifying; however, this block varies in strength depending on the type of NR2 or NR3 subunit(s) expressed (Monyer et al., 1992; Cull-Candy et al., 2001; Sasaki et al., 2002) NMDARs were first discovered as postsynaptic receptors at glutamatergic synapses, and have since been shown to be involved in many aspects of synaptic transmission, dendritic integration, synaptic and neuronal maturation, and plasticity throughout the brain. Given the diverse roles of NMDARs, it is not surprising that NMDAR dysfunction is thought to contribute to neurological and psychiatric disorders, including neurodegenerative conditions, stroke, epilepsy,

neuropathic pain, and schizophrenia (Meldrum, 1994; Cull-Candy et al., 2001; Kristiansen et al., 2007; Lau and Zukin, 2007) . As a result, NMDARs are targets for many therapeutic drugs (Chen and Lipton, 2006). Recently, NMDARs have joined the list of ionotropic receptors that can also function presynaptically (briefly discussed below). Presynaptic NMDARs (preNMDARs) may be molecularly and pharmacologically distinct from classical postsynaptic NMDARs. PreNMDARs share some functions with postsynaptic NMDARs such as involvement in long-term synaptic depression (LTD) (although by a distinct mechanism than postsynaptic NMDARs). PreNMDARs also have some novel functions such as regulation of presynaptic release probability and short-term plasticity. It is possible that some of the cellular- and network-level functions previously attributed to postsynaptic NMDARs are, in fact, mediated by preNMDARs.

Here we summarize the growing understanding of cortical preNMDARs. In an effort to elucidate the possible mechanisms by which preNMDARs modulate synaptic function, we briefly compare cortical preNMDAR activity with preNMDAR function at other central synapses (see Table 1.1 and *How do preNMDARs regulate release?*). We argue that without knowing the specific contribution of preNMDARs versus postsynaptic NMDARs to neurotransmission and plasticity, it is impossible to understand NMDAR regulation of cortical development, or to rationally design pharmacotherapies for NMDAR-related diseases. Specific involvement of preNMDAR in diseases is now being considered, including in epilepsy (Yang et al., 2006) and fetal alcohol spectrum disorder (Valenzuela et al., 2008).

**Table 1.** Effects of Endogenous or Exogenous preNMDAR Activation on Synapses

| CNS Region | Synaptic Projection | NR2 Subunit | Spontaneous Release | Evoked Release | Plasticity | Ages | References |
|---|---|---|---|---|---|---|---|
| Visual cortex (V1) | L4 to L2/3 | nd | Promotes | nd | req. for tLTD | P7–20; lost @ P23 | (Corlew and others 2007; Li and Han 2007) |
| | Ex. inputs to L4 pyr. | nd | Promotes | nd | nd | P7–20; lost @ P23 | (Corlew and others 2007) |
| | L5 pyr. to L5 pyr. | NR2B | Promotes | Promotes | req. for tLTD | P7–21; lost @ P23 | (Sjöström and others 2003; Corlew and others 2007) |
| S1 | L4 to L2/3 | NR2B | Promotes | Promotes | req. for tLTD | P13–21 | (Bender and others 2006; Brasier and Feldman 2008; Rodríguez-Moreno and Paulsen 2008) |
| Entorhinal cortex | Ex. inputs to L2 pyr. | NR2B | Promotes | No change | nd | nd | (Berretta and Jones 1996; Woodhall and others 2001) |
| | Ex. inputs to L5 pyr. | NR2B | Promotes | nd | nd | Lost by 5 months | (Woodhall and others 2001; Yang and others 2006) |
| Hippocampus | CA3 to CA1 | NR2D/NR2B | Promote (<P5) | Promotes | nd | NR2D loss at P5 | (Mameli and others 2005; Suarez and others 2005; Suarez and Solis 2006) |
| | Ex. inputs to granule cells | NR2B | Promotes | nd | nd | P10–22 | (Jourdain and others 2007) |
| Lat. Amygdala | Cortical inputs | nd | nd | nd | req. for LTP | P21–28 | (Humeau and others 2003) |
| Cerebellum | Parallel fiber to Purkinje | nd | nd | Suppresses | req. for LTD | P18–26 | (Casado and others 2000, 2002) |
| | Interneuron to Purkinje | nd | Promotes | Promotes | nd | P11–14 | (Glitsch and Marty 1999; Duguid and Smart 2004) |
| Spinal cord | Substance P afferents | nd | Directly triggers release | | nd | Adult | (Liu and others 1997) |
| | Dorsal horn ex. afferents | nd | nd | Suppresses | nd | P6–12 | (Bardoni and others 2004) |

preNMDAR = presynaptic NMDA receptor; LTD = long-term depression; tLTD = spike timing-dependent LTD; CNS = central nervous system; Ex = excitatory (glutamatergic); pyr = pyramidal neurons; L = cortical layer; Lat = lateral; nd = no data; req = required; S1 = primary somatosensory cortex; Spont = spontaneous.

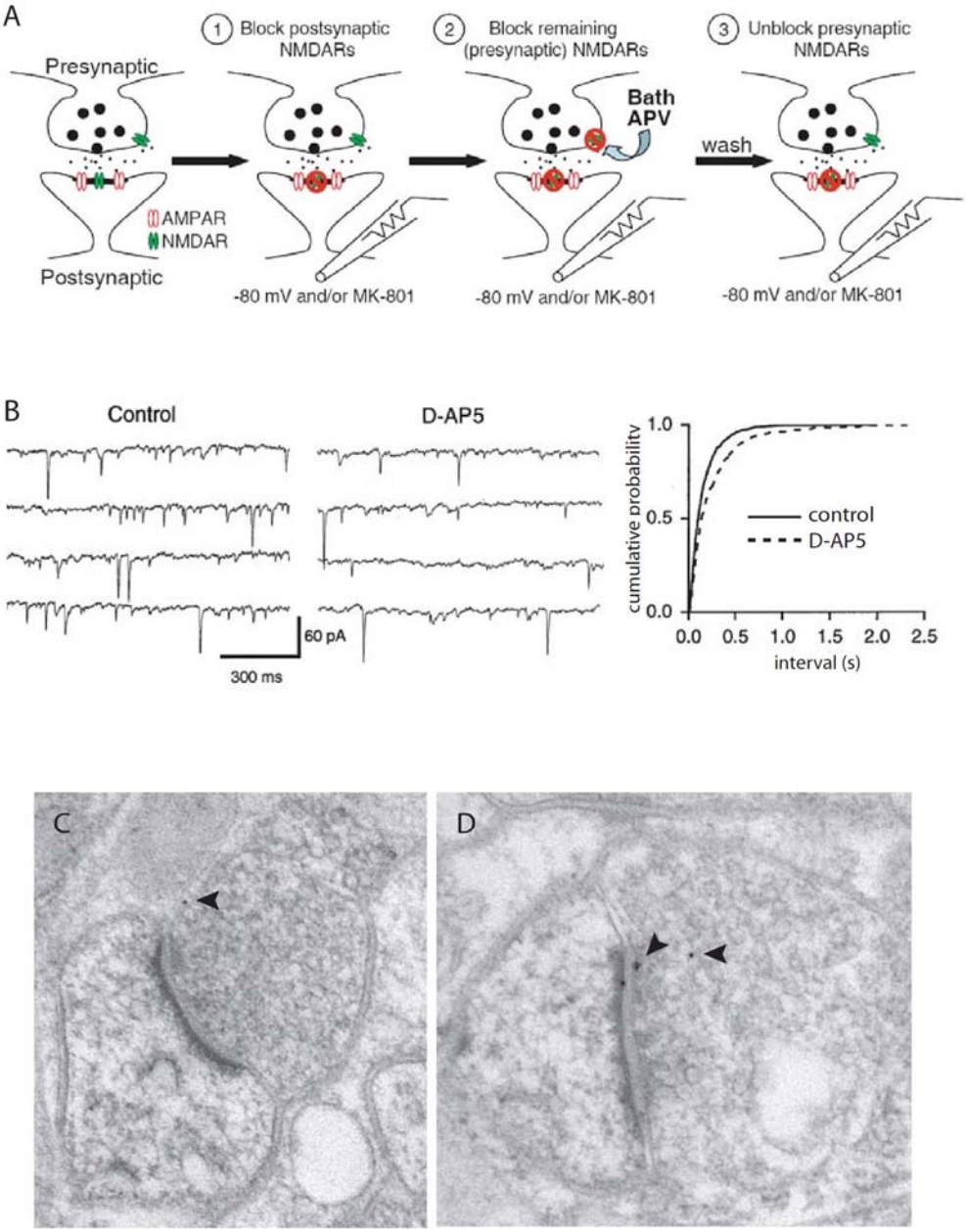
## 1.2 Synapse regulation by presynaptic ligand-gated ion channels

Many neurotransmitters activate presynaptic receptors that modulate presynaptic function. Some of these presynaptic receptors are metabotropic receptors (e.g., $GABA_B$ receptors and mGluRs) that can modulate neurotransmitter release via second messenger systems, affect presynaptic voltage-sensitive calcium channels (VSCCs), and/or directly alter the release machinery itself. Other presynaptic receptors are ligand-gated ion channels, including $GABA_A$ receptors, glycine receptors, kainate receptors, and most recently, NMDARs (MacDermott et al., 1999; Khakh and Henderson, 2000; Engelman and MacDermott, 2004; Pinheiro and Mulle, 2008). Particularly well characterized among these are the presynaptic kainate receptors, which are present at several synapse classes in neocortex and hippocampus (Contractor et al., 2001; Lauri et al., 2006; Sun and Dobrunz, 2006; Pinheiro et al., 2007) and the presynaptic glycine receptors, which are present at the Calyx of Held in the auditory brainstem (Turecek and Trussell, 2001; Trussell, 2002; Awatramani et al., 2005). Activation of presynaptic ionotropic receptors generally enhances synaptic release, while presynaptic metabotropic receptors typically decrease release probability (Vitten and Isaacson, 2001; Pinheiro and Mulle, 2008), although this trend is not universal (Casado et al., 2000; Casado et al., 2002; Bardoni et al., 2004). Heterogeneity in presynaptic receptor expression across synapses, including preNMDAR expression, contributes to the diversity of synapse function and plasticity in the central nervous system (CNS).

## 1.3 Tools for identifying preNMDARs

While postsynaptic NMDARs were first observed in 1963 (Curtis and Watkins, 1963), the possibility that preNMDARs exist and that they can regulate synaptic transmission has arisen relatively recently. There are likely several reasons for the lag in the identification of preNMDARs. First, preNMDARs appear to be less prevalent than postsynaptic NMDARs. Second, preNMDAR functions appear to be most pronounced during a small window of early development. Third, and perhaps most significant, recent technological advances have allowed for the selective blockade of postsynaptic NMDARs allowing physiological effects of preNMDARs to be measured.

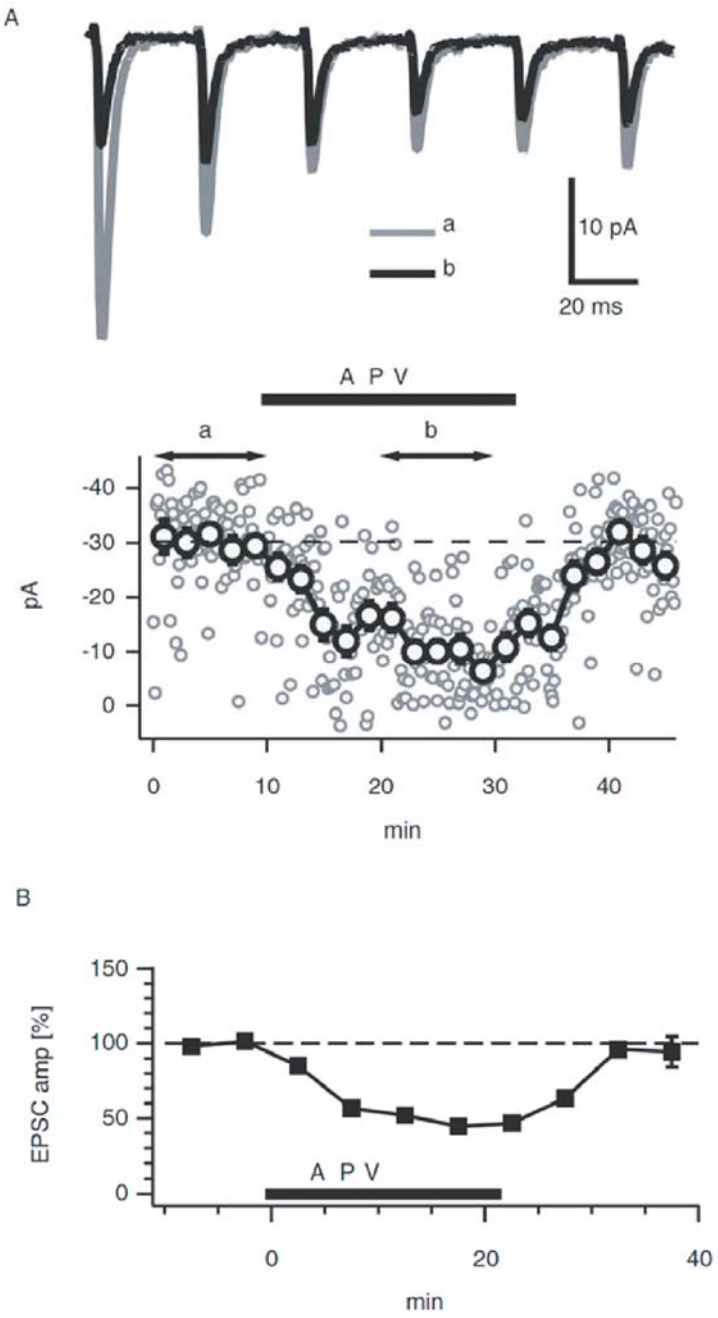
How have preNMDARs been identified thus far? Two general approaches for detecting the presence / function of preNMDARs have been used. The most common approach has been measuring the electrophysiological effect of blocking preNMDARs on spontaneous neurotransmitter release frequency. If preNMDARs on terminals function to increase spontaneous release frequency onto a postsynaptic cell, then blocking those receptors should transiently decrease the frequency of these events, recorded as miniature excitatory postsynaptic currents (mEPSCs). Provided that postsynaptic NMDARs are first blocked, the subsequent effects can be attributed to preNMDARs (Berretta and Jones, 1996; Woodhall et al., 2001; Sjostrom et al., 2003; Bender et al., 2006a; Corlew et al., 2007; Brasier and Feldman, 2008) (Fig. 1.1A & B). This technique has been by widely used and accepted for the detection of preNMDARs that affect the frequency of spontaneous release. However, there are two major




**<u>Figure 1.1</u> Physiological and anatomical methods to detect preNMDARs.** **(A)** Schematic of mEPSC recording paradigm (Corlew 2007). **(B)** Example (reproduced with permission from Beretta and Jones 1996) to illustrate the approach. After first blocking postsynaptic NMDARs, subsequent bath application of the NMDAR antagonist of D-APV (2-amino-5 phosphonopentanoic acid) reduces the frequency of sEPSCs measured in L2 entorhinal cortex neurons. **(C & D)** Example of anatomical evidence for preNMDARs in L2/3 of the rodent neocortex suggested by postembedding immunogold for an antibody that recognizes both NR2A and NR2B. (Figure is courtesy R. Weinberg, University of North Carolina.) Presynaptic labeling with 10 nm gold particles is indicated by arrowheads.

criticisms to using this approach. Although postsynaptic NMDARs can be ruled out, the relevant NMDARs are only presumed to be presynaptic, but could in fact be located on another non-postsynaptic structure. Given the rapid change in spontaneous release, however, the most parsimonious explanation is that they are located on presynaptic terminals. The second criticism is that even if preNMDARs affect spontaneous release, they may not necessarily play a role in action potential-evoked release. To test this, some groups have begun looking at changes in the rate of short-term synaptic depression (or facilitation) to a train of stimuli as a read-out of evoked release probability, because the rate of short-term synaptic depression is, typically, inversely related to release probability. To assess a preNMDAR role in evoked transmitter release, postsynaptic NMDARs can first be blocked, and then changes in the rate of synaptic depression at a specific class of synapses can be measured in response to NMDAR application. As above, the postsynaptic NMDARs are first blocked so any change in the rate of synaptic depression is likely due to preNMDARs (Fig. 1.2).

In addition to electrophysiological approaches, immuno-peroxidase and immunogold electron microscopy (EM) has been used to identify preNMDARs. Immuno-peroxidase staining is a very sensitive assay in which an enzymatic reaction increases the size of the reporter signal for maximum detection (Fig. 1.8B), and several studies have used this approach to positively identify preNMDARs (Aoki et al., 1994; DeBiasi et al., 1996; Charton et al., 1999).




**<u>Figure 1.2</u> PreNMDARs enhance action potential-evoked neurotransmission.** Evidence that preNMDARs modulate evoked transmitter release between pairs of L5 pyramidal neurons in primary visual cortex. **(A)** With postsynaptic NMDARs blocked by voltage-clamping the postsynaptic neuron at -90mV, preNMDAR blockade decreases EPSC amplitude and short-term synaptic depression. Sample recording showing averaged responses before **(a)** and during **(b)** application of APV. **(B)** APV wash-in reversibly decreased responses to 30 Hz spiking between pairs of L5 neurons recorded in voltage-clamp at -90 mV. Reproduced with permission from Sjöström and others (2003).

Unfortunately the increase in signal with the immuno-peroxidase EM approach comes at the cost of precise subcellular localization within the presynaptic terminal. A more precise localization method is to use gold particles for a secondary antibody (Fig. 1.1C, D). Few studies have examined the putative expression of preNMDARs using immunogold electron microscopy (Aoki et al., 2003), but evidence is accumulating that preNMDARs have a precise localization to presynaptic membranes, where they are well-positioned to alter neurotransmitter release (Jourdain et al., 2007). The relatively limited identification of preNMDARs could be due to several factors, including a low prevalence of preNMDARs compared to postsynaptic NMDARs, age- or region-specific expression of preNMDARs (discussed below), or to the simple fact that very few studies have bothered to examine the possibility that preNMDARs exist.

## 1.4 Physiological evidence for preNMDARs in cerebral cortex

Physiological evidence that preNMDARs regulate spontaneous synaptic transmission has been found at a wide array of cortical synapses. The first evidence was at glutamatergic synapses in layer 2 (L2) of entorhinal cortex, where bath application of the NMDAR antagonist APV decreased the frequency of spontaneous mEPSCs, even when postsynaptic NMDARs had been previously blocked intracellularly. This suggested that preNMDARs normally act to enhance spontaneous neurotransmitter release (Berretta and Jones, 1996) (Fig. 1.1B). Subsequently, NMDAR agonists were shown to enhance mEPSC frequency in both L2 and L5 of entorhinal cortex. This effect was blocked by the

NR2B subunit-specific antagonist ifenprodil, suggesting that the relevant preNMDARs contain NR2B (Woodhall et al., 2001). Similar effects of preNMDARs were subsequently observed at excitatory synapses in L2/3, L4, and L5 of the rodent V1 (Sjostrom et al., 2003; Corlew et al., 2007; Li and Han, 2007; Li et al., 2008), CA1 of the hippocampus (Mameli et al., 2005), dentate gyrus (Jourdain et al., 2007), entorhinal cortex (Yang et al., 2006), and in L2/3 of S1 (Bender et al., 2006b; Brasier and Feldman, 2008). In addition to the aforementioned studies in the neocortex and hippocampus, there is also compelling evidence for preNMDARs function in the spinal cord (Liu et al., 1997; Bardoni et al., 2004), the cerebellum (Glitsch and Marty, 1999; Casado et al., 2000; Casado et al., 2002; Duguid and Smart, 2004) reviewed in (Duguid and Sjostrom, 2006), and amygdala (Humeau et al., 2003). Therefore, there is an abundance of evidence that preNMDARs function widely throughout the central nervous system and may qualify as a general mechanism of modulating spontaneous neurotransmitter release.

In addition to affecting spontaneous release, it is now clear that preNMDARs also modulate evoked (action potential-driven) transmitter release. For example, blockade of presumptive preNMDARs decreases unitary synaptic responses between L5 pyramidal cells in V1 (Sjostrom et al., 2003) (Fig. 1.2) and at synapses between L4 and L2/3 pyramids (L4-L2/3 synapses) in S1 (Bender et al., 2006b; Brasier and Feldman, 2008). Paired pulse facilitation (PPF) increases and coefficient of variation $(CV)^{-2}$ decreases during this effect, reflecting decreased presynaptic release probability. These experiments were performed

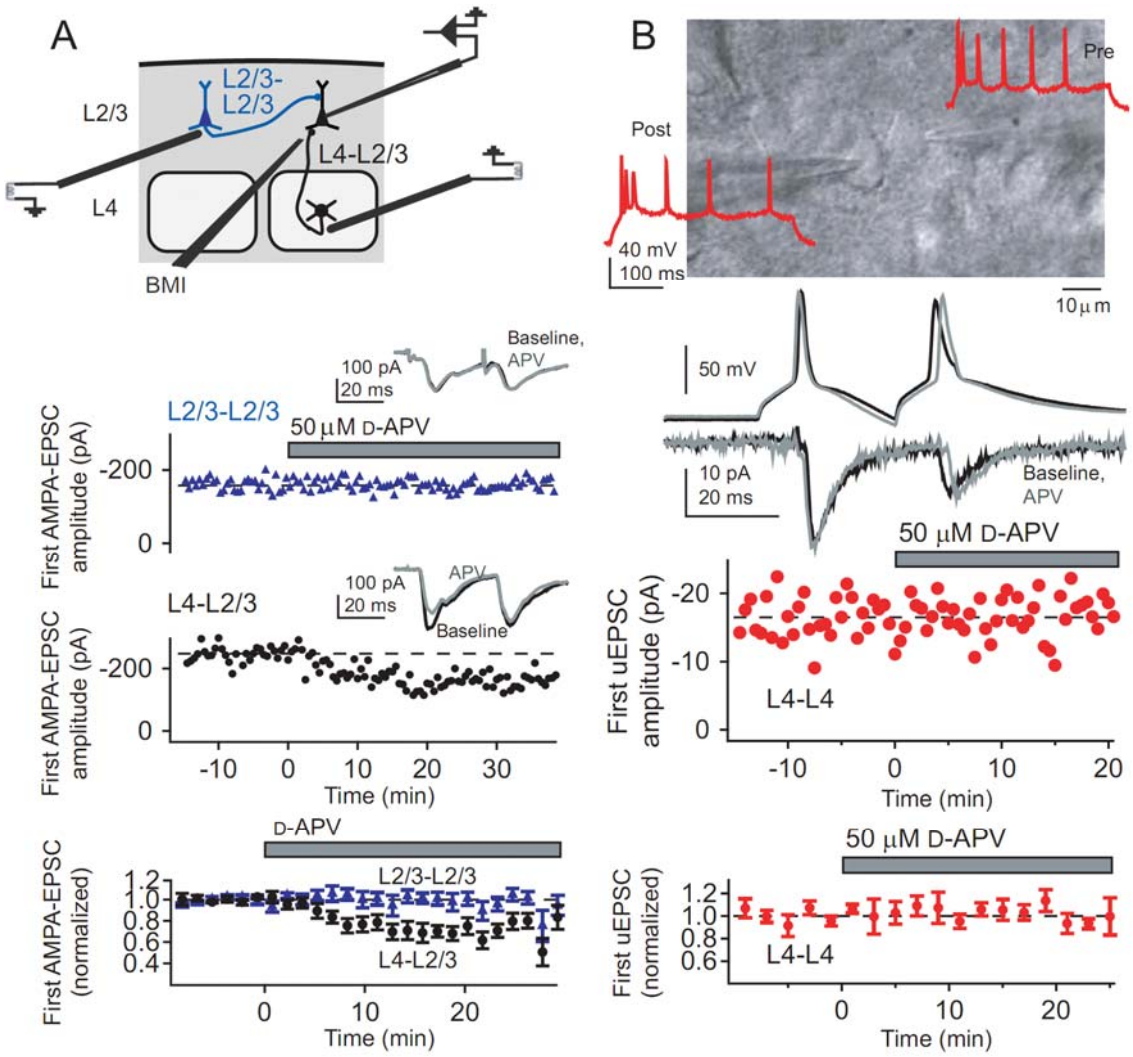
with postsynaptic NMDARs blocked, indicating that the relevant NMDARs that regulate evoked release are non-postsynaptic, presumably preNMDARs. PreNMDAR modulation of evoked release is only evident during high-frequency burst stimulation in L5 of primary visual cortex, but occurs with sparse stimulation (two action potentials at 33 ms inter-spike interval) at L4-L2/3 synapses in S1 (Sjostrom et al., 2003; Brasier and Feldman, 2008). These data indicate that preNMDARs are active during conditions of modest synaptic activity in acute slices, and this endogenous activation of preNMDARs helps to maintain higher probabilities of evoked neurotransmitter release, compared to release probability when preNMDARs are blocked. Thus, preNMDARs enhance the probability of both spontaneous and evoked neurotransmitter release.

### 1.5 Anatomical evidence for presynaptic NMDARs in cerebral cortex

The physiological findings described above indicate that non-postsynaptic, presumably presynaptic, NMDARs exist, which influence transmitter release. While presynaptic localization of NMDARs is the simplest explanation for the effects on presynaptic transmitter release, it remains possible that the relevant receptors are actually on another nearby neuron or glial cell, which signals to the presynaptic terminal via an unknown mechanism. Thus, an important line of evidence indicating that putative preNMDARs are actually localized on presynaptic terminals comes from electron microscopy (EM) studies indicating that NMDAR subunits are physically present at cortical presynaptic terminals (Fig. 1.1C, D). In 1994, Aoki and colleagues observed immuno-peroxidase

labeling of the NMDAR subunit NR1 in axons and presynaptic terminals in V1 from postnatal day (P) 30 rats (Aoki et al., 1994). Sparse labeling for the NR2B subunit has also been observed in presynaptic terminals from neocortex of 5-6 month-old rats (DeBiasi et al., 1996; Charton et al., 1999). Using immunogold labeling, NMDAR protein has also been found in presynaptic terminals of the hippocampal dentate gyrus (Jourdain et al., 2007). Together these studies confirm the presence of NMDAR protein at presynaptic cortical terminals. In addition to the neocortex (Aoki et al., 1994; DeBiasi et al., 1996; Charton et al., 1999; Fujisawa and Aoki, 2003; Corlew et al., 2007) and hippocampus (Siegel et al., 1994; Charton et al., 1999; Jourdain et al., 2007), preNMDARs have also been observed using EM in the spinal cord (Liu et al., 1994; Lu et al., 2005) and amygdale (Farb et al., 1995; Pickel et al., 2006).

In EM studies, NMDAR immuno-staining in presynaptic terminals is often sparse, and substantially less intense than postsynaptic staining. This suggests a relatively low number of preNMDARs, at least in rats > P25, when most EM studies have been performed. (See *Developmental regulation of preNMDARs*, below, for evidence that preNMDAR expression is substantially higher in young animals.) Another reason for sparse staining may be selective expression of preNMDARs at specific classes of excitatory terminals. Consistent with this idea, physiological evidence reveals functional preNMDARs at ascending L4-L2/3 synapses onto L2/3 pyramidal cells in S1, but not at horizontal, L2/3-L2/3 synapses onto the same postsynaptic neurons, or on local L4-L4 synapses made



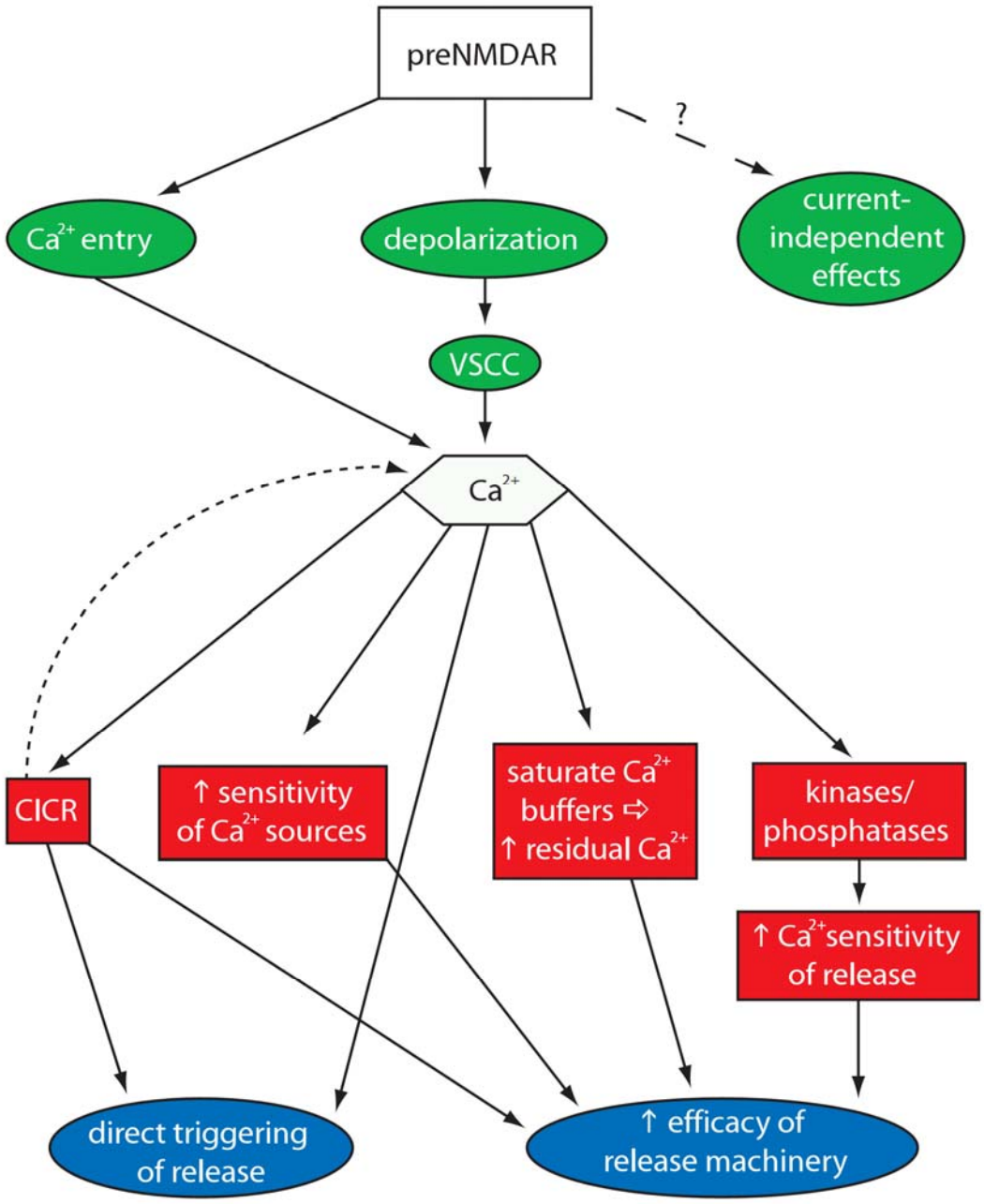

**<u>Figure 1.3</u> Synapse-specific expression of preNMDARs.** PreNMDARs are located on layer (L) 4-L2/3 synapses, but not on L2/3-L2/3 synapses or on L4-L4 synapses in the rodent somatosensory cortex. **(A)** Recording set-up for these experiments. Top: Representative experiment testing the effect of D-APV on AMPA-EPSCs evoked on the L2/3 cross-columnar pathway (L2/3-L2/3) and on L4-L2/3 inputs to the same postsynaptic cell. Amplitude of the first AMPA-EPSC on each pathway. Insets: Pairs of AMPA-EPSCs before (black) and during (grey) D-APV application. Bottom: Mean effect of D-APV on first AMPA-EPSC amplitude for L2/3-L2/3 inputs (triangles) and simultaneously measured L4-L2/3 inputs (circles). Bars represent population means (* = $p < 0.05$). **(B)** Differential interference contrast image of example synaptically coupled L4 excitatory cells, with regular-spiking pattern for these cells. Postsynaptic EPSCs elicited by a pair of presynaptic spikes before (black) and after (grey) 50µM D-APV application for the regular-spiking pair above. Lack of effect of D-APV on amplitude of the first uEPSC for one representative cell pair. Mean effect of D-APV application on first uEPSC amplitude. Reproduced with permission from Brasier and Feldman 2008.

by the same presynaptic neurons (Brasier and Feldman, 2008) (Fig. 1.3). This input- and target-specific expression of preNMDARs parallels synapse-specific expression of other presynaptic metabotropic and ionotropic receptors (Scanziani et al., 1998; Sun and Dobrunz, 2006) and may be a general mechanism for establishing synapse-selective release properties. The molecular mechanisms by which preNMDARs and other presynaptic receptors are targeted to a specific subset of terminals are not known, and there appear to be differences in preNMDAR expression between cortical regions (Corlew et al., 2007; Brasier and Feldman, 2008).

### 1.6 How do preNMDARs regulate release?

NMDARs generate depolarization and are permanent to calcium, either of which could enhance synaptic release probability via calcium-mediated signaling pathways. Which of these pathways is most relevant, and what downstream signaling events lead to modulation of release, are not known. Many potential mechanisms exist, and it is even possible that preNMDARs signal through an unknown, non-voltage dependent, non-calcium dependent mechanism. Here we consider several broad signaling motifs that may be involved in preNMDAR-mediated enhancement of transmitter release (Fig. 1.4).

*Direct depolarization of the terminal*. PreNMDARs may act by causing depolarization of the presynaptic terminal. For example, at the Calyx of Held, activation of presynaptic ionotropic glycine receptors causes subthreshold depolarization of the terminal, which results in modest calcium influx through




**<u>Figure 1.4</u> Schematic of possible mechanisms for preNMDAR-enhancement of neurotransmitter release.** There are three potential signals (green) emanating directly from the preNMDAR that could modulate release: direct $Ca^{2+}$ entry, depolarization leading to the opening of VSCCs, and current-independent effects. The two ultimate mechanisms (blue) for expression of increased release are direct triggering of release by $Ca^{2+}$ signals, or indirect signaling that changes the probability of release. Signaling to one of these ends could be carried out directly by $Ca^{2+}$, or by several different intermediates (red): an amplified $Ca^{2+}$ signal through calcium-induced calcium release (CICR), kinases/phosphatases that alter future $Ca^{2+}$ entry, or increased $Ca^{2+}$ -sensitivity of release machinery.

VSCCs, which in turn increases the probability of release to a subsequent action potential (Awatramani et al., 2005). Such depolarization of the presynaptic terminal may be a general mechanism by which presynaptic ionotropic receptors, including preNMDARs, enhance release probability (Engelman and MacDermott, 2004).

*Calcium influx through preNMDARs*. Calcium influx through preNMDARs, rather than depolarization, could be the initial trigger for modulation of release. Recently, preNMDAR enhancement of spontaneous miniature inhibitory post-synaptic current (mIPSC) frequency at synapses onto cerebellar Purkinje cells has been shown to be independent of VSCCs, consistent with a direct effect of calcium through preNMDARs (Glitsch, 2008). However, whether cortical preNMDARs act independent of VSCCs remains unknown.

The end result of either preNMDAR-mediated depolarization of the terminal or direct calcium entry would be an increase in presynaptic $Ca^{2+}$. This $Ca^{2+}$ may then act directly on release machinery, for example by activating synaptotagmin and triggering vesicle fusion. Alternatively, $Ca^{2+}$ may act to increase release probability via one of several more indirect pathways. First, $Ca^{2+}$ may enhance the function of other calcium sources to facilitate subsequent transmitter release. For example, increasing the association of presynaptic vesicle proteins with VSCCs could prime transmitter-containing vesicles so that subsequent VSCC activation more efficiently triggers transmitter release (Kim and Catterall, 1997; Catterall, 1998). Second, the $Ca^{2+}$ signal could act by partially saturating endogenous calcium buffers, which can lead to an increase in

free calcium when VSCCs open during a subsequent action potential (Felmy et al., 2003). Third, many protein kinases and phosphatases depend on calcium and could be activated by preNMDARs to modify function of various presynaptic ion channels or biochemical pathways, even including modifications of the release machinery itself. In support of this idea, postsynaptic NR2B-containing NMDARs have been found to closely associate with calcium/calmodulin-dependent protein kinase II (Strack et al., 2000); if such association exists for preNMDARs, calcium-dependent kinase activity would be even more plausible. Indeed, preNMDARs at the cerebellar parallel fiber to Purkinje synapse have been proposed to activate presynaptic nitric oxide (NO) synthase and trigger an anterograde NO signal which controls LTD at this synapse (Casado et al., 2000; Casado et al., 2002) although whether the relevant NMDARs are truly presynaptic has been questioned (Shin and Linden, 2005). Finally, indirect signaling may occur through $Ca^{2+}$ release from internal stores. Evidence for this pathway is found in presynaptic inhibitory terminals onto cerebellar Purkinje cells, where calcium influx through preNMDARs triggers calcium-induced calcium release (CICR) via presynaptic ryanodine receptors, resulting in depolarization-induced potentiation of inhibition (Duguid and Smart, 2004). For a summary of preNMDAR action in the central nervous system (see Table 1.1).

The dizzying array of possible mechanisms by which preNMDARs might alter neurotransmission at different central synapses highlights the diverse roles they could play. Which putative mechanisms are involved in their function at different synapses is likely to be an important question over the next few years.

Whether similar mechanisms act at different synapses or whether each class of preNMDAR-expressing synapse has a unique set of mechanisms that underlie the enhancement of transmitter release is a crucial question to understanding the general principles that regulate synaptic transmission.

## 1.7 Endogenous activation of preNMDARs

To conduct ionic current, most NMDAR subtypes require glutamate binding, binding of glycine (or D-serine) at the glycine site, and depolarization to relieve voltage-dependent magnesium block. How these requirements are met for preNMDARs is not yet clear. The first issue is what is the source of glutamate that activates preNMDARs? Cortical preNMDARs contain the NR2B subunit (Woodhall et al., 2001; Sjostrom et al., 2003; Yang et al., 2006; Brasier and Feldman, 2008; Li et al., 2008), which confers high affinity for glutamate (Laurie and Seeburg, 1994; Priestley et al., 1995). This opens the possibility that low ambient levels of glutamate might tonically activate cortical preNMDARs. Such tonic activation has been observed for postsynaptic NMDARs in slice preparations (Sah et al., 1989; Cavelier and Attwell, 2005; Le Meur et al., 2007), but see (Herman and Jahr, 2007). Tonic activation of preNMDARs by ambient glutamate is indicated by studies in which NMDAR antagonists decreased mEPSC frequency in slices in which all action potential (AP) activity was blocked by tetrodotoxin TTX (Berretta and Jones, 1996; Woodhall et al., 2001; Sjostrom et al., 2003; Corlew et al., 2007; Li and Han, 2007; Li et al., 2008). These findings demonstrate that ambient glutamate, present in slices without action

potential-evoked release, is sufficient to functionally activate preNMDARs. Notably, the ability of preNMDARs to modify mEPSC frequency is apparent at physiological temperatures, but is absent at room temperature unless glutamate concentration is enhanced by high-frequency synaptic activity or blockade of excitatory amino acid transporters (Cavelier and Attwell, 2005; Bender et al., 2006b; Brasier and Feldman, 2008). Importantly, preNMDAR enhancement of release is not saturated by ambient glutamate because application of NMDAR agonists enhances mEPSC frequency (Woodhall et al., 2001; Brasier and Feldman, 2008). These data suggest that activity-dependent changes in local glutamate concentration might dynamically regulate preNMDAR activation, and therefore release probability.

The cellular source of glutamate for preNMDARs remains unclear. PreNMDARs only contribute to evoked release at L5 pyramidal cell synapses when the presynaptic cell fires bursts, suggesting that preNMDARs act as autoreceptors for glutamate that builds up with release from the presynaptic terminal (Sjostrom et al., 2003). As such, preNMDARs may help maintain a high release probability in the face of continuous firing. Presynaptic kainate receptors act in a similar autocrine fashion to depolarize terminals and enhance transmitter release (Sun and Dobrunz, 2006). Unlike presynaptic kainate receptors, whose effect on release gradually increases with subsequent spikes within a burst, preNMDARs affect transmitter release even during the first action potential in a burst (Sjostrom et al., 2003). This difference may owe to the comparatively low affinity for glutamate of presynaptic kainate receptors versus NR2B-containing

NMDARs (Pinheiro et al., 2007). Glutamate for preNMDAR activation can also arise from postsynaptic dendritic release of glutamate, which allows preNMDARs to modulate GABAergic synapses in the cerebellum (Duguid and Smart, 2004).

Glia may also play a key role in dynamically regulating glutamate concentration for preNMDAR activation. Astrocytes have recently been shown to contain glutamate vesicles which undergo exocytosis when stimulated by prostaglandins, tumor necrosis factor-alpha, or mGluR agonists (Bezzi et al., 1998; Bezzi et al., 2001; Bezzi et al., 2004). In the hippocampal perforant path to granule cell synapse, whole-cell stimulation of synaptically associated astrocytes increases mEPSC frequency (Jourdain et al., 2007) (Fig 1.5*A*). This effect was prevented when astrocytic exocytosis was blocked or when preNMDARs were blocked with NR2B-selective antagonists. Furthermore, immuno EM revealed NR2B-containing preNMDARs in the extrasynaptic portion of excitatory perforant path terminals, closely apposed to the vesicles of synaptically associated astrocytes (Jourdain et al., 2007) (Fig. 1.5*B*). These findings strongly suggest that dynamic glial release of glutamate contributes to activation of preNMDARs. It will be vital to test whether other preNMDARs (including in neocortex) are regulated by a similar mechanism.

A second issue is whether preNMDARs are functionally regulated by availability of glycine or other ligands at the glycine binding site. Glycine binding is required for preNMDAR function, because blockade of the glycine binding site fully prevents the effects of preNMDARs on mEPSCs in primary visual cortex.

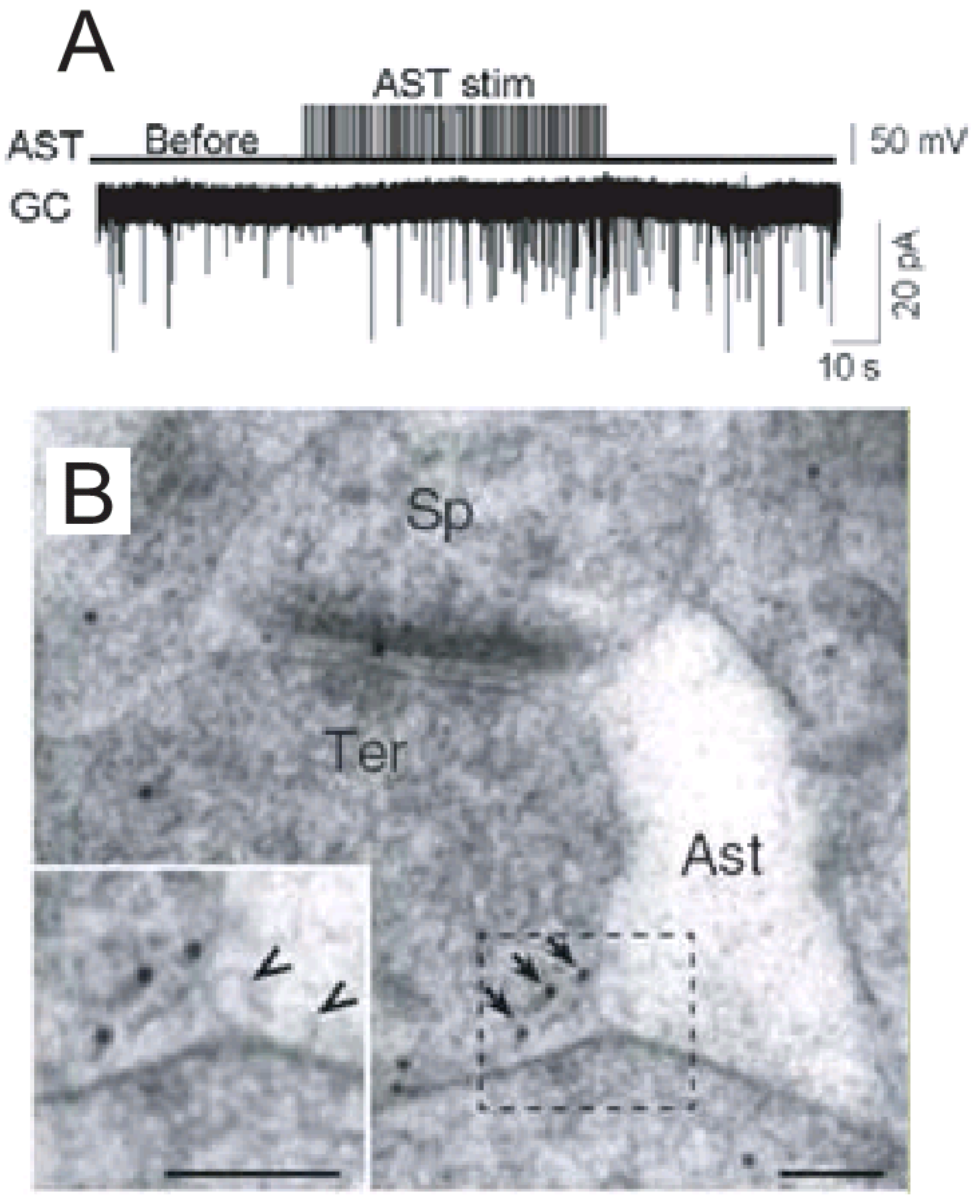



**Figure 1.5 Glutamate released from astrocytes can regulate neurotransmitter release. (A)** Electrical stimulation (stim) of an astrocyte (AST) increases miniature excitatory synaptic currents recorded in a granule cell (GC) in the dentate gyrus of the hippocampus. **(B)** Electron micrographs showing NR2B gold particles in extrasynaptic membranes (arrows) of nerve terminals (Ter) making asymmetric synapses with dendritic spines (Sp) in the dentate molecular layer and an associated astrocytic process (Ast). Inset shows at higher magnification NR2B particles apposed to an astrocytic process that contains synaptic like microvesicles (arrowheads). Scale bars, 100 nm. Reproduced with permission from Jourdain and others (2007).

However, exogenous application of D-serine (a selective agonist for the glycine site) does not enhance mEPSC frequency (Awobuluyi et al., 2007). Thus, unlike the postsynaptic NMDAR, the glycine site on preNMDARs may be saturated under physiological conditions, which may be due in part to the relatively high affinity NR2B-containing and possibly NR3A-containing NMDARs have for glycine (Kew et al., 1998). In addition to glycine and D-serine, preNMDAR function is modulated by other amino acids. Taurine, an endogenous analogue of glycine, has been found to increase the preNMDAR-mediated enhancement of Schaffer collateral fiber volley amplitude without affecting the postsynaptic NMDAR-mediated enhancement of field EPSC slope (Suarez and Solis, 2006).

A third issue is the source of depolarization for preNMDAR activation. The fact that preNMDAR blockade alters mEPSC frequency when spikes are blocked by TTX (Berretta and Jones, 1996; Sjostrom et al., 2003; Corlew et al., 2007; Li and Han, 2007; Brasier and Feldman, 2008; Li et al., 2008) indicates that preNMDARs actively enhance release even when depolarization from sodium spikes is absent. This could indicate that (i) resting potential in terminals is sufficiently depolarized to partially relieve $Mg^{2+}$ block of preNMDARs, (ii) preNMDARs may exhibit less voltage-dependence than classical postsynaptic NMDARs, or (iii) preNMDAR modulation of release may not require current flow. Although a relatively depolarized resting potential is a possibility because the presynaptic terminal is a small, high input resistance compartment that would be readily depolarized by small local excitatory currents, this idea is speculative and, at least in the Calyx of Held terminals where it can be measured, the resting

membrane potential is close to -80mV (Duguid and Smart, 2004; Awatramani et al., 2005). It is possible that preNMDARs lack a voltage-activated component, as this has been observed in some receptor subtypes. In particular, heteromeric NMDAR channels containing the obligatory NR1 subunit with either NR2C, NR2D, NR3A, or NR3B subunits (perhaps in addition to NR2B subunits) would be expected to exhibit less basal magnesium block compared to postsynaptic NMDARs composed primarily of NR1 with NR2A and/or NR2B (Monyer et al., 1992; Cull-Candy et al., 2001; Sasaki et al., 2002). Notably, preNMDARs in CA1 hippocampus of very young (<P5) rats have been suggested to contain NR2D subunits (Mameli et al., 2005). Thus, determining the molecular composition of preNMDARs will be crucially important to understanding their voltage dependence and their function under physiological conditions.

The function of preNMDARs might also be subject to neuromodulation. In hippocampal slices from <P5 rats, the excitatory neurosteroid pregnenolone sulfate increases mEPSCs frequency. This effect is blocked by bath application of NMDAR antagonists, including the NR2C/NR2D-selective antagonist PPDA, but not by selective blockade of postsynaptic NMDARs. An endogenous pregnenolone sulfate-like neurosteroid was found to be released by postsynaptic depolarization and to enhance preNMDAR function (Mameli et al., 2005), raising the possibility that preNMDARs may be an important site of neuromodulatory control of release probability and network excitability.

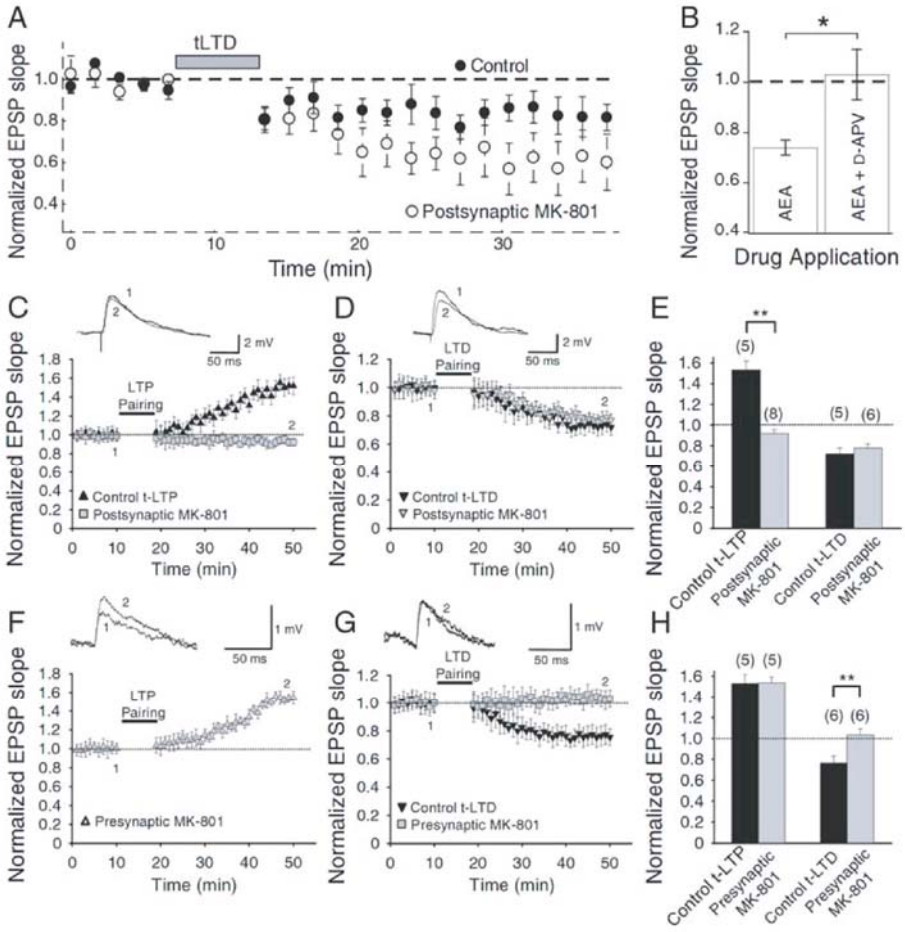
## 1.8 Role in LTD

NMDARs are required for many forms of synaptic plasticity, including long-term potentiation (LTP) and depression (LTD) (Bliss and Collingridge, 1993; Collingridge and Bliss, 1995; Malenka and Bear, 2004). While postsynaptic NMDARs are well established to trigger induction of classical forms of LTP and LTD, as best defined at CA3-CA1 excitatory synapses in hippocampus, recent studies indicate that preNMDARs, rather than postsynaptic NMDARs, mediate at least one prominent form of plasticity, spike timing-dependent LTD (tLTD), at some synapses.

Spike timing-dependent plasticity (STDP) is a physiologically realistic form of bidirectional synaptic plasticity in which LTP or LTD is induced in response to the precise timing between presynaptic spikes (and the excitatory postsynaptic potentials (EPSPs) they elicit) and postsynaptic spikes (Magee and Johnston, 1997; Markram et al., 1997; Dan and Poo, 2006). Spike timing-dependent LTP (tLTP) is induced when presynaptic spikes precede postsynaptic spikes by < 20 ms, while tLTD is induced when presynaptic spikes follow postsynaptic spikes by up to 20-50 ms. STDP occurs throughout the neocortex (Egger et al., 1999; Feldman, 2000; Sjöström et al., 2001; Froemke and Dan, 2002) and has functional properties that may underlie development, plasticity, and competition within sensory maps. In particular, tLTD may underlie deprivation- and experience-induced weakening of sensory responses during receptive field plasticity (Sjöström et al., 2001; Allen et al., 2003; Celikel et al., 2004; Dan and Poo, 2006).

NMDAR activation is necessary for most forms of cortical STDP. At several synapses in young rodents, including L4-L2/3 synapses in somatosensory and visual cortex, and synapses between L5 pyramidal neurons in visual cortex, the induction of tLTD has been found to specifically require preNMDARs, but not postsynaptic NMDARs (Sjostrom et al., 2003; Bender et al., 2006b; Corlew et al., 2007; Rodriguez-Moreno and Paulsen, 2008) (Fig. 1.6). This was elegantly shown at unitary L4-L2/3 synapses in mouse S1, using dual whole-cell recordings of synaptically connected L4 and L2/3 neurons. Postsynaptic or presynaptic NMDA receptors were selectively blocked by internal application of the NMDAR pore blocker MK-801 into the pre- or postsynaptic neuron. tLTD was completely blocked by presynaptic MK-801, but was unaffected by postsynaptic MK-801. This indicates that LTD involved presynaptic, but not postsynaptic, NMDARs, and rules out the possibility that glial NMDARs are required in tLTD (Rodriguez-Moreno and Paulsen, 2008).

PreNMDAR-dependent tLTD requires postsynaptic calcium elevation and, in the S1 at least, activation of postsynaptic group I mGluRs, and is expressed as a reduction in the probability of neurotransmitter release, implicating a retrograde signal (Bender et al., 2006b; Nevian and Sakmann, 2006). This retrograde signal involves the well-established endocannabinoid retrograde signaling pathway, which mediates many forms of short- and long-term synaptic plasticity (Chevaleyre et al., 2006), because postsynaptic synthesis of endocannabinoids and activation of presynaptic cannabinoid type 1 (CB1) receptors are required for tLTD (Sjostrom et al., 2003; Bender et al., 2006b).




**<u>Figure 1.6</u> Induction of long-term depression (LTD) requires preNMDARs. (A & B)** Extracellularly evoked L4–L2/3 excitatory postsynaptic currents (EPSPs) in somatosensory cortex (S1). **(A)** In L4–L2/3 S1 connections, robust timing-dependent LTD can be induced in control experiments (closed circles) by repetitively pairing postsynaptic action potentials followed closely by EPSPs (pairing indicated by gray bar). LTD is observed even when postsynaptic NMDARs are blocked with iMK-801 (open circles), but not when APV (2-amino-5-phosphonopentanoic acid) is bath applied (not shown). **(B)** Bath-applying the endocannabinoid agonist AEA (arachidonyl ethanolamide) induces a lasting LTD at L4–L2/3 synapses (indicated by a reduction of EPSP slope, normalized to baseline). Prior blockade of pre- and postsynaptic NMDARs with bath-applied D-APV blocks this AEA-induced LTD (no reduction in EPSP slope from baseline), whereas postsynaptic NMDAR blockade with MK-801 does not block AEA-induced LTD (not shown). **(C–H)**, Synaptically coupled L4–L2/3 excitatory pairs in the S1. **(C)** iMK-801 (gray) completely blocks induction of timing-dependent long-term potentiation (tLTP), while control (no iMK-801) neurons (black) exhibit normal LTP. Inset, EPSP before (1) and 30 min after (2) the LTP pairing protocol. **(D)** Postsynaptic MK-801 did not block tLTD. **(E)** Summary of **(C & D). (F)** During paired recordings, presynaptic MK-801 did not block the induction of tLTP. **(G)** Presynaptic MK-801 completely blocks tLTD. **(H)** Summary of **(F & G).** Control tLTP refers to values obtained using extracellular stimulation. **(E & H)** The numbers of slices are shown in parentheses. All error bars are S.E.M. * = $p < 0.05$, ** = $p < 0.01$. Reproduced with permission from **(A & B)** Bender (2006), and **(C & H)** Rodríguez-Moreno and Paulsen (2008).

Thus, tLTD at these synapses involves both preNMDAR and CB1 signaling (Sjostrom et al., 2003; Bender et al., 2006b; Nevian and Sakmann, 2006).

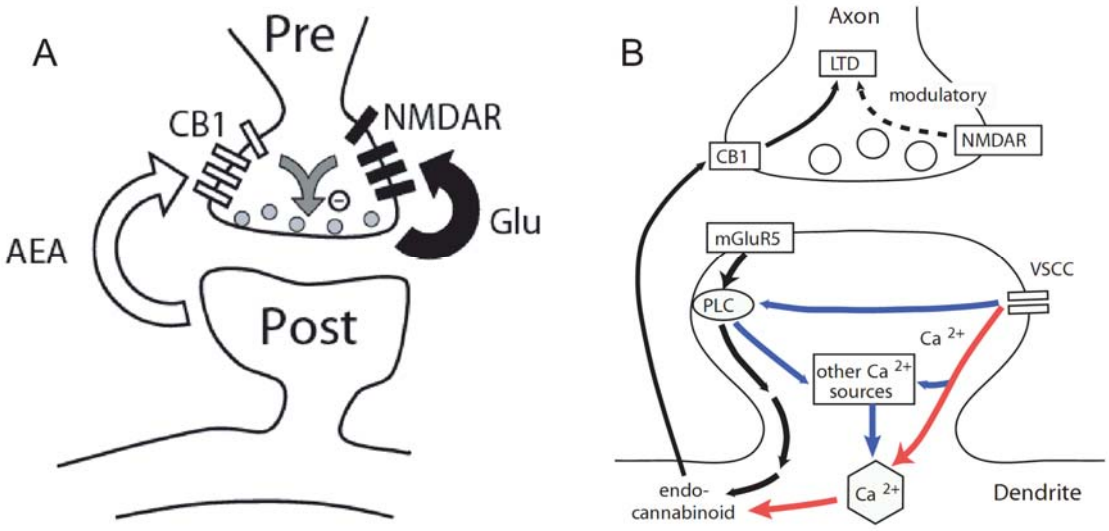
Such CB1- and preNMDAR-dependent, presynaptically expressed tLTD is common in neocortex, at least during early postnatal development (Corlew et al., 2007), but is not universal (Froemke et al., 2005). Currently, there is no explanation for why some forms of LTD are preNMDAR-dependent while others are postsynaptic NMDAR-dependent. In addition to tLTD, it is tempting to speculate that more forms of preNMDAR-dependent plasticity exist, but have not been discovered due to assumptions (based on analogy to CA3-CA1 synapses in hippocampus) that postsynaptic NMDARs generally mediate plasticity. The prevalence of multiple, distinct forms of LTD throughout the brain, including preNMDAR-CB1 LTD, and the recent discovery of preNMDAR-dependent LTP in the amygdale (Humeau et al., 2003), indicate that plasticity at CA3-CA1 synapses may not be canonical and underscore the need to test specifically for pre- versus post-synaptic NMDAR involvement in specific forms of synaptic plasticity.

### 1.9 Role of preNMDARs in CB1/preNMDAR-dependent LTD

How might preNMDARs contribute to tLTD? Coincidence detection for STDP, and for Hebbian plasticity generally, is widely posited to be performed by postsynaptic NMDARs (Dan and Poo, 2006). In one standard STDP model, presynaptically released glutamate strongly activates postsynaptic NMDAR currents when release is rapidly followed by depolarization from postsynaptic

spikes, leading to calcium influx that is supralinear compared to glutamate release or postsynaptic spikes alone. This supralinear calcium is thought to drive LTP induction. In contrast, post-leading-pre spike order drives sublinear calcium influx, leading to LTD (Shouval and Perrone, 1995; Koester and Sakmann, 1998). While this model may hold at synapses that exhibit postsynaptic NMDAR-dependent STDP, calcium from postsynaptic NMDARs is not required to drive tLTD at synapses which exhibit preNMDAR/CB1-dependent tLTD (Sjostrom et al., 2003; Bender et al., 2006b; Duguid and Sjostrom, 2006; Corlew et al., 2007; Corlew et al., 2008). Thus, at these synapses, postsynaptic NMDARs cannot perform coincidence detection for tLTD, and the induction of tLTD must involve separate coincidence detection mechanisms than those for tLTP (Bender et al., 2006b; Nevian and Sakmann, 2006). Induction of tLTD at these synapses may involve preNMDARs and CB1 receptors (Sjostrom et al., 2003; Duguid and Sjostrom, 2006).

Whether and how preNMDARs contribute to coincidence detection for preNMDAR-dependent, CB1-dependent tLTD is debated. In one hypothesis, presynaptic spikes provide glutamate and depolarization to activate preNMDARs, postsynaptic spikes evoke endocannabinoid release to activate CB1 receptors, and coincident activation of preNMDARs and CB1 receptors triggers tLTD (Sjostrom et al., 2003; Duguid and Sjostrom, 2006) (Fig. 1.7*A*). Consistent with this model, exogenous cannabinoid agonists induce tLTD at synapses between L5 pyramidal cells in visual cortex, and this effect is blocked when preNMDARs




**<u>Figure 1.7</u> Two models showing preNMDAR involvement in the coincidence detection of spike timing-dependent LTD. (A)** It has been proposed in L5 of visual cortex (Sjostrom et al., 2003) that postsynaptic action potentials trigger the release of endocannabinoids in a mechanism similar to short-term depression caused during depolarization-induced suppression of inhibition. When presynaptic activity follows this cannabinoid release in a short time window, it coincidently activates preNMDARs and presynaptic CB1 receptors to trigger LTD induction. Reproduced with permission from Sjöström and others (2003). **(B)** Schematic for a possible postsynaptic coincidence detector for CB1-mediated tLTD. Black, pathway for mGluR-dependent cannabinoid synthesis. Red, pathway for VSCC- and calcium-dependent cannabinoid synthesis. Purple, potential synergistic pathways that increase cannabinoid production in response to appropriately timed pre- and postsynaptic spikes. Presynaptic NMDARs play a modulatory role in LTD in this hypothesis. Schematic based on data in Bender and others 2006.

are blocked by APV, indicating that coincident activation of CB1 receptors and preNMDARs is required for this form of synapse weakening. Also consistent with this model, prolonging the half-life of endogenously released cannabinoids broadens the timing window for tLTD within the STDP rule (Sjostrom et al., 2003). However, a potential difficulty with this model is that endocannabinoid synthesis, release, and retrograde signaling must occur with ~10 ms precision, which may be faster than possible for endocannabinoid signaling (Heinbockel et al., 2005). Another difficulty is that, at least at L4-L2/3 synapses in S1, tLTD can be induced even when preNMDARs are blocked during post-leading-pre spike pairing, indicating that these receptors do not participate in coincidence detection for tLTD (Bender et al., 2006a).

An alternative hypothesis is that preNMDARs do not contribute to millisecond-scale coincidence detection of pre- and postsynaptic activity during tLTD induction, but rather act on a slower time scale to modulate tLTD induction. In this model, rapid coincidence detection for tLTD occurs through a separate, postsynaptic mechanism. One such mechanism is suggested by findings at L4-L2/3 synapses in S1, where tLTD requires postsynaptic VSCCs and group I mGluRs, both of which are upstream of endocannabinoid synthesis (Bender et al., 2006b) (Fig. 1.7*B*). Calcium and mGluR activation are known to drive endocannabinoid synthesis, and joint activation of these pathways greatly facilitates endocannabinoid synthesis and cannabinoid-dependent plasticity (Hashimotodani et al., 2005; Chevaleyre et al., 2006). According to this model, presynaptic spikes provide glutamate to activate mGluRs, postsynaptic spikes

drive calcium entry through VSCCs, and appropriately timed, near-coincident activation of these two pathways leads to synergistic endocannabinoid synthesis and release. The retrograde endocannabinoid signal then instructs the presynaptic terminal to express LTD. While acute blockade of preNMDARs during post-pre spike pairing does not block tLTD at this synapse, persistent blockade of preNMDARs for many minutes prior to pairing does block tLTD (Bender et al., 2006b). This suggests that preNMDAR activity is required on long time scales for tLTD induction, but not for rapid coincidence detection during pairing. This model does not predict strong synapse specificity for tLTD, since post-pre pairing at one synapse would generate cannabinoid signals that could diffuse retrogradely to neighboring synapses and drive heterosynaptic LTD. In contrast, the model proposed by Sjöström et al. (2003) predicts that only those presynaptic terminals which are active within milliseconds of postsynaptic activation would experience coincident activation of preNMDARs and CB1 receptors, and would undergo LTD. Future studies need to distinguish between these distinct tLTD models.

## 1.10 Developmental regulation of preNMDARs role in neurotransmission and plasticity

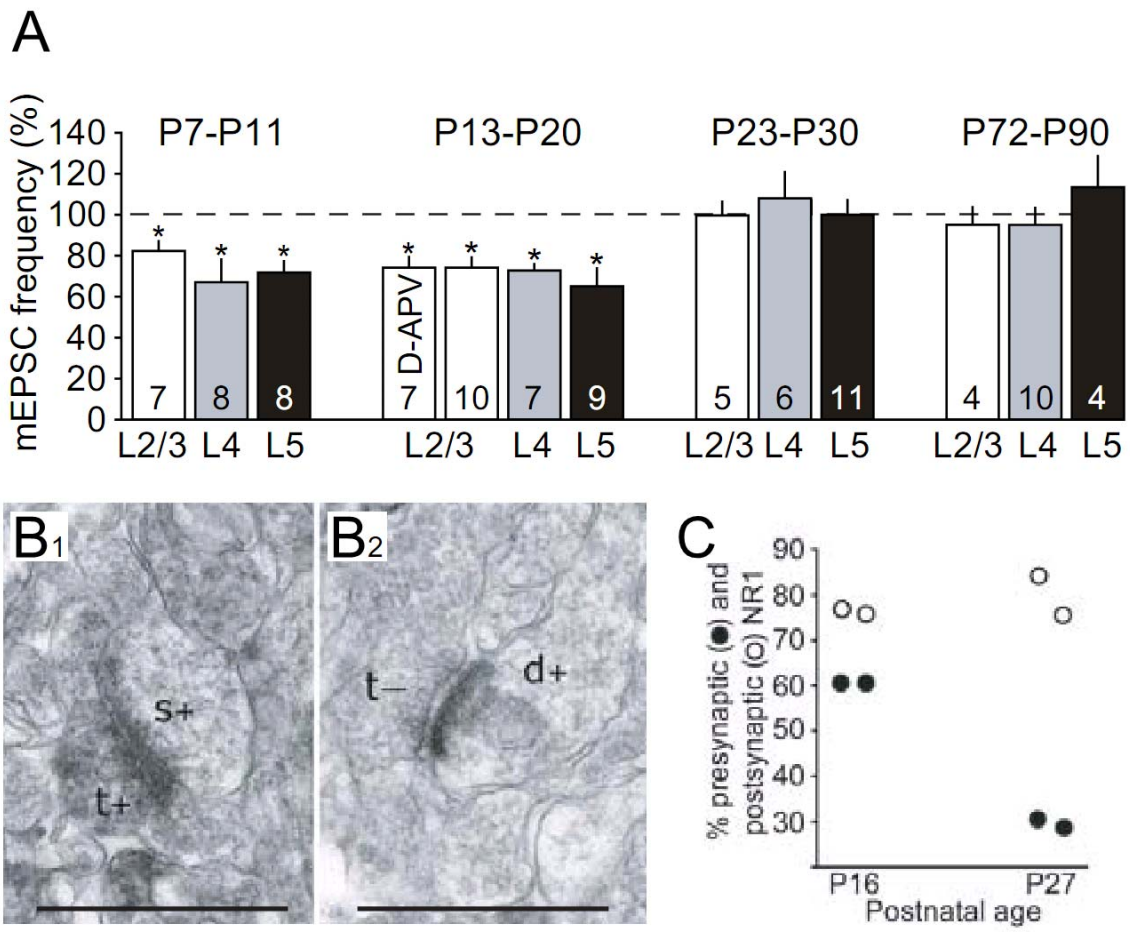
Despite the large body of evidence reviewed above, preNMDARs are not found at all synapses, in all brain areas, in all studies. Some explanation is offered by a growing body of evidence indicating that the function of preNMDARs decreases dramatically over development. One of the first suggestions that

preNMDARs are regulated over development came from a study in the CA1 region of the hippocampus, where the neuromodulatory effects of pregnenolone sulfate on preNMDARs was observed only during a brief window of postnatal rodent development (<P5) (Mameli et al., 2005). While this effect might arise at any point along the neuromodulatory pathway, it was hypothesized that the loss of function arose from a decline in preNMDAR function due to a developmental loss of NR2D-containing preNMDARs. The presence of NR2D permits NMDARs to function at hyperpolarized potentials and, thus, could account for the ability of preNMDARs to be tonically active in the absence of strong depolarization (Mameli et al., 2005). The developmental loss of NR2D might then increase voltage-dependence of preNMDARs, effectively disabling their function. In support of this interpretation, while preNMDARs may facilitate tonic transmitter release at the CA3-CA1 synapse only in young (<P5) mice (Mameli et al., 2005) they may continue to alter evoked transmitter release at older ages (Suarez et al., 2005; Suarez and Solis, 2006). Thus, with a change in the subunit composition of preNMDARs, their ability to participate in spontaneous and evoked transmitter release might change.

Several studies now indicate that preNMDAR function attenuates with development, although the developmental timing of this attenuation varies in different regions of the brain. For example, unlike the early loss of preNMDAR function observed in the hippocampus (Mameli et al., 2005), preNMDAR function in entorhinal cortex L5 pyramidal neurons is not lost until late in development (Yang et al., 2006). Specifically, preNMDARs enhance the frequency of

spontaneous excitatory postsynaptic currents (sEPSCs) at 5 weeks of age in the entorhinal cortex, but this effect is absent by 5 months (Yang et al., 2006). Although the time course for the developmental loss of preNMDARs is different between the hippocampus and entorhinal cortex, a change in preNMDAR subunit composition may underlie the loss of preNMDAR function in both regions (Mameli et al., 2005; Yang et al., 2006). To examine this possibility in the entorhinal cortex, the investigators took advantage of the observation that preNMDARs, but not postsynaptic NMDARs, are thought to contain the NR2B subunit in L5 pyramidal neurons at 5 weeks of age. Accordingly, the NR2B agonist Ro25-6981 could effectively reduce the frequency of sEPSCs at 5 weeks of age but not at 5 months of age, indicating that NR2B-containing preNMDARs were no longer contributing to the spontaneous release probability.

A profound developmental reduction in preNMDAR functions has been observed in V1 (Corlew et al., 2007), suggesting that this might be a general feature of preNMDAR expression. In the mouse primary visual cortex, the ability of preNMDARs to enhance spontaneous release probability onto cells in L2/3, L4, and L5 is completely lost at 3 weeks of age (Fig. 1.8). Consistent with this observation, there is a dramatic reduction in the anatomical expression of terminals containing preNMDARs; while roughly 60% of presynaptic terminals contain the obligatory NMDAR subunit NR1 in L2/3 asymmetric (excitatory) synapses at P16, only 30% do so at P27. This will be explored further in Chapter 2. While there are several possible explanations for the total loss of preNMDAR




**<u>Figure 1.8</u> Evidence for a developmental reduction in preNMDAR functions.** **(A)** D,L-APV (or D-APV where indicated) strongly reduced AMPAR-mediated mEPSC frequency from baseline in L2/3, L4, and L5 pyramidal cells in the visual cortex of mice at P7-20 but not older mice. Sample sizes are given within the bars. **(B)** Immuno-electon microscopy for the obligatory NR1 subunit of the NMDAR reveals a developmental decrease in presynaptic, but not postsynaptic, NR1. Electron micrograph in L2/3 of visual cortex of a **($B_1$)** P16 mouse, demonstrating an NR1-positive terminal (t+) forming a synapse onto a NR1 positive spine (s+) and **($B_2$)** from a P27 mouse, demonstrating an unlabeled terminal (t-) forming a synapse onto a labeled dendrite (d+). Scale bar 250 nm. **(C)** Scatter plot from four mice (2 at each age) quantifying the selective loss of presynaptic, but not postsynaptic, NR1 over development. Reproduced with permission from Corlew and others (2007).

functions in spontaneous release without the complete loss of expression at P27, one parsimonious explanation is that the reduction in preNMDAR expression is also coupled to a change in preNMDAR subunit composition. Such an observation would be consistent with the mechanisms suggested for the developmental loss of preNMDARs in entorhinal cortex and hippocampus. Moreover, a role for preNMDARs in supporting *evoked* neurotransmitter release in the adult neocortex has not been tested to date.

What is the physiological outcome of a developmental decrease in preNMDARs? Neurotransmitter release probability decreases significantly during early development, causing some synapses to switch with age from displaying a depressing response with pairs or bursts of stimulation to a facilitating response (Bolshakov and Siegelbaum, 1995; Choi and Lovinger, 1997; Pouzat and Hestrin, 1997; Reyes and Sakmann, 1999; Dekay et al., 2006). The timing for these observed changes in presynaptic function coincides roughly with the developmental decrease in preNMDAR function in the hippocampus (Mameli et al., 2005), entorhinal cortex (Yang et al., 2006), and visual cortex (Corlew et al., 2007). Therefore, a reduction in preNMDAR function might contribute to a developmental decrease in release probability, which may be a general property of early circuit formation. Synaptic terminals may require a high release probability to facilitate synapse formation during early synaptogenesis (Rumpel et al., 2004). When synaptic connections become more stable, the need for that high release probability may decrease as connections can be strengthened and weakened by changes on the postsynaptic side.

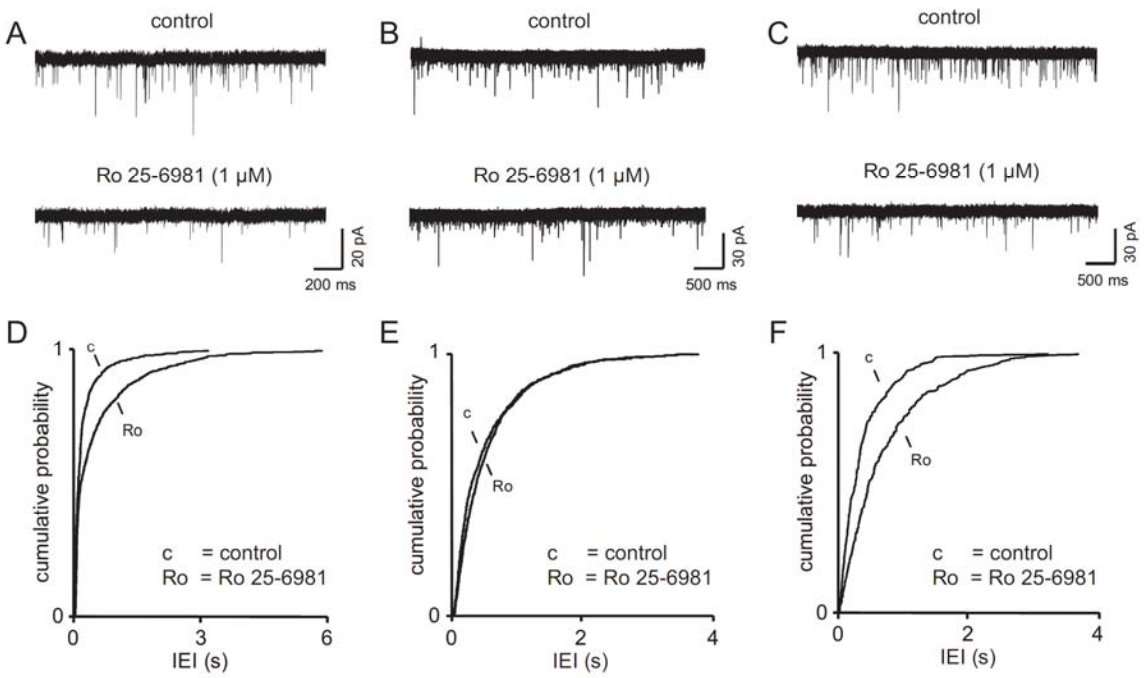
As presynaptic and postsynaptic receptor properties change with development, synaptic plasticity mechanisms may be forced to adapt to these changes (Yasuda et al., 2003; Frenkel and Bear, 2004; Nosyreva and Huber, 2005; Yashiro et al., 2005; He et al., 2006; Jo et al., 2006). The mechanisms of synaptic plasticity cannot be studied without reference to development: even the pre- versus postsynaptic locus induction and expression of LTP and LTD can vary with development (Nosyreva and Huber, 2005; Corlew et al., 2007; Crozier et al., 2007). As preNMDARs are lost during development of the L4-L2/3 pathway in visual cortex, there is a developmental switch from the involvement of preNMDARs to postsynaptic NMDARs in LTD (Corlew et al., 2007). Thus, while preNMDARs can contribute to the properties of neurotransmission and act as coincidence detectors for tLTD induction during early life, the role of postsynaptic NMDARs in tLTD increases as that of preNMDARs diminishes.

### 1.11 Activity-dependent and disease-induced changes in preNMDARs

While postsynaptic NMDAR expression and function are sensitive to experience-driven changes in neural activity levels (Carmignoto and Vicini, 1992; Hestrin, 1992; Monyer et al., 1994; Philpot et al., 2001a), it was only recently that similar activity dependence has been observed for preNMDARs. For example, in cultured cerebellar GABAergic neurons, where preNMDARs may act as heteroreceptors for glutamate to increase GABA release, the developmental loss of preNMDARs can be accelerated by treatment with NMDA (Fiszman et al., 2005). While there may be several mechanisms that underlie this activity-

dependent change in preNMDAR function, it is possible that changes in activity levels may alter the subunit composition of preNMDARs and/or their expression at the synapse (due to differential receptor trafficking). In support of this idea, pharmacological blockade of NMDARs *in vivo* rapidly increases the presence of presynaptic NR2A subunits, and decreases that of NR2B subunits, in both postsynaptic spines and presynaptic terminals of adult rat visual cortex (Aoki et al., 1994; Fujisawa and Aoki, 2003). The time scale (<30 min) of these changes suggest an activity-dependent modification in preNMDAR trafficking, and changes in activity levels may uniquely alter the trafficking of the various NMDAR subtypes.

If modulating activity *in vivo* similarly alters expression and function of preNMDARs, then changes in preNMDAR function could occur during, or contribute to, neurological disorders such as epilepsy that involve large-scale changes in network activity and excitation. Chronic changes in neural activity levels in epileptic patients have been shown to affect NMDAR function and expression (Dalby and Mody, 2001; Avanzini and Franceschetti, 2003; Morimoto et al., 2004). However, few studies have considered the possibility that preNMDARs may be affected. One recent study in rodents indicates that epileptic activity could affect the function of preNMDARs. Specifically, preNMDARs were reinstated, or their normal developmental down-regulation prevented, in the adult entorhinal cortex following 2-4 weeks of lithium-pilocarpine treatment to induce seizures (Yang et al., 2006). In these mice, the recurrent seizure activity recovered a high frequency of sEPSCs that could be




**<u>Figure 1.9</u> Developmental and activity-dependent regulation of preNMDAR functions.** Presynaptic NR2B-containing receptors enhance spontaneous release in the entorhinal cortex of young rats and epileptic adults, but not in normal adults. **(A-C)** Voltage-clamp recordings of sEPSCs in a layer 5 neurons in a slice from **(A)** a 4-week-old rat, **(B)** a 5-month-old rat, and **(C)** and a 5-month-old epileptic rat. Blockade of NR2B receptors with Ro 25-6981 decreases the frequency of sEPSCs only in young and epileptic rats. Postsynaptic NMDARs are blocked by intracellular MK-801. **(D-F)** Corresponding pooled data for inter-event interval of the sEPSCs from control and Ro 25-6981 recordings. Reproduced with permission from Yang and others (2006).

decreased by the NR2B-specific antagonist Ro 25-6981. Littermate controls with no seizures had no increase in sEPSCs, and the frequency of sEPSCs in these control mice was not affected by Ro 25- 6981 application (Yang et al., 2006) (Fig. 1.9). These data indicate that the ability of preNMDARs to enhance spontaneous transmitter release was enhanced in a seizure model, although it has not yet been determined whether this increase was causal to, or a consequence of, increased neural activity. In support of the idea that preNMDARs may be involved in some forms of epilepsy, it is interesting to note that gabapentin, a drug prescribed to treat epilepsy, may act to decrease neurotransmitter release via preNMDARs (Suarez et al., 2005).

# Chapter 2:

# Developmental switch in the contribution of presynaptic NMDA receptors (preNMDARs) to long-term depression (tLTD)

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## 2.1 Introduction

NMDA receptor (NMDAR) activation is required for many forms of learning and memory as well as sensory system receptive field plasticity, yet the relative contribution of pre- and postsynaptic NMDARs over cortical development remains unknown. Here we demonstrate a rapid developmental loss of functional presynaptic NMDARs (preNMDARs) in the neocortex. PreNMDARs enhance neurotransmitter release at synapses onto visual cortex pyramidal cells in young mice (< postnatal day 20; P20), but they have no apparent effect after the onset of the critical period for receptive field plasticity (>P21). Immuno-electron microscopy revealed that the loss of preNMDAR function is due in part to a 50% reduction in the prevalence of preNMDARs. Coincident with the observed loss of preNMDAR function, there is an abrupt change in the mechanisms of timing-dependent long-term depression (tLTD). Induction of tLTD before the onset of the critical period requires activation of pre but not postsynaptic NMDARs, while the induction of tLTD in older mice requires

activation of postsynaptic NMDARs though the requirement of pre NMDARs at this stage is unknown. By demonstrating that both pre- and postsynaptic NMDARs contribute to the induction of synaptic plasticity, and that their relative roles shift over development, our findings define a novel, and perhaps general, property of synaptic plasticity in emerging cortical circuits.

Synaptic connections in sensory cortices such as the primary visual cortex are initially sculpted in an experience-independent manner, allowing rough cortical maps and receptive field properties to emerge in the absence of sensory experience (Rakic, 1977; Mower et al., 1985; Stryker and Harris, 1986; Horton and Hocking, 1996; Crowley and Katz, 1999; Feller and Scanziani, 2005). This is followed by a relatively brief "critical period" of postnatal life where receptive field properties are additionally refined in an experience-dependent manner (Fox and Zahs, 1994; Berardi et al., 2000; Sengpiel and Kind, 2002; Hensch, 2004). Activation of the NMDA-type glutamate receptors (NMDARs) is required for many experience-dependent forms of plasticity as well as some forms of activity-dependent plasticity that do not rely on sensory experience (Bear and Rittenhouse, 1999; Iwasato et al., 2000; Malenka and Bear, 2004). Thus, an important goal is to establish the precise role of NMDARs in key forms of cortical plasticity, and whether this changes over development.

NMDARs were traditionally thought to exert their influences postsynaptically, and their presynaptic existence has been largely ignored. A surprising finding is that NMDARs are anatomically expressed presynaptically (Aoki et al., 1994; Charton et al., 1999) and these presynaptic receptors are

involved in both neurotransmission and plasticity (Berretta and Jones, 1996; Woodhall et al., 2001; Casado et al., 2002; Humeau et al., 2003; Bardoni et al., 2004; Duguid and Smart, 2004; Mameli et al., 2005; Duguid and Sjöström, 2006; Lien et al., 2006; Yang et al., 2006). For example, tLTD between layer (L) 5 pyramidal cells in the visual cortex requires the simultaneous activation of preNMDARs and cannabinoid receptors (Sjöström et al., 2003). This tLTD is expressed presynaptically as a reduction in the probability of neurotransmitter release. PreNMDARs are also involved in the induction of tLTD at L2/3 in barrel cortex and visual cortex of young rodents (Bender et al., 2006b; Li and Han, 2006). While growing evidence suggests an important role for preNMDARs early in development (Lien et al., 2006), it remains unknown whether preNMDARs are regulated in a laminar or developmental fashion.

Here we examined the laminar and developmental profile of preNMDAR function in the visual cortex, a well-studied model for sensory map plasticity (Fox and Zahs, 1994; Katz and Shatz, 1996; Bear and Rittenhouse, 1999; Berardi et al., 2000; Sengpiel and Kind, 2002; Hensch, 2004; Taha and Stryker, 2005; Hofer et al., 2006b). We demonstrate that while preNMDARs function to enhance spontaneous neurotransmission onto pyramidal neurons in L2/3, L4, and L5 early in development, there is an abrupt loss of this function at the onset of the critical period. Furthermore, pre- but not postsynaptic NMDARs are required for the induction of tLTD at the L4-L2/3 synapse during the pre-critical period. At the onset of the critical period, however, a dramatic loss of

preNMDARs appears to trigger the emergence of a postsynaptic requirement of NMDARs for tLTD though a remaining presynaptic requirement was not tested.

## 2.2 Materials and Methods

*Subjects.* C57BL/6 mice were purchased from Charles River laboratories and used between P7 and P84. Mice were maintained on a 12:12 light:dark cycle and fed *ad libitum*. All experiments were performed under the animal care guidelines for Tufts University School of Medicine, the University of Virginia, and the University of North Carolina at Chapel Hill.

*Cortical slice preparation:* Mice were anesthetized with pentobarbital sodium (40 mg/kg, i.p.) and decapitated upon disappearance of corneal reflexes. Brains were rapidly removed and immersed in ice-cold dissection buffer (composition in mM: 87 NaCl, 2.5 KCl, 1.25 $NaH_2PO_4$, 25 $NaHCO_3$, 75 sucrose, 10 dextrose, 1.3 ascorbic acid, 7 $MgCl_2$, and 0.5 $CaCl_2$) bubbled with 95% $O_2$ and 5% $CO_2$. The visual cortex was rapidly dissected and 350 μM coronal slices were prepared using a vibrating microtome (Leica VT100S). Slices were allowed to recover for 20 min in a submersion chamber at 35°C filled with warmed ACSF (124 mM NaCl, 3 mM KCl, 1.25 mM $Na_2PO_4$, 26 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 20 mM D-glucose, saturated with 95% $O_2$, 5% $CO_2$; ~315 mOsm and pH ~7.25) and then kept at room temperature until use. For recordings, visual cortex slices were placed in a submersion chamber, maintained at 30°C, and perfused with oxygenated ACSF.

*Voltage-clamp recordings.* Patch pipettes were pulled from thick-walled borosilicate glass with open tip resistances of 3-6 MΩ when filled with one of two internal solutions either containing (in mM) 102 cesium gluconate, 5 TEA-chloride, 3.7 NaCl, 20 HEPES, 0.3 sodium guanosine triphosphate, 4 magnesium adenosine triphosphate, 0.2 EGTA, 10 BAPTA, and 5 QX-314 chloride (Alomone Labs, Jerusalem, Israel) or containing 20 KCl, 100 (K)Gluconate, 10 HEPES, 4 (Mg)ATP, 0.3 (Na)GTP, 10 (Na)Phosphocreatine with pH adjusted to 7.25 and osmolarity adjusted to ~300 mOsm with sucrose or dd$H_2O$. Cells were voltage-clamped in the whole-cell configuration using a patch-clamp amplifier (Multiclamp 700A; Molecular Devices), and data were acquired and analyzed using pCLAMP 9.2 software (Molecular Devices). For current-voltage (I-V) curves, voltage was adjusted for the 16mV junction potential, which was empirically determined for the internal solution. Changes in series resistance were monitored throughout the experiment by giving a test pulse and measuring the amplitude of the capacitive current. Only cells with series resistance < 30 MΩ were included for analysis. No series resistance compensation was applied. Input resistance was monitored throughout the experiment by measuring the amplitude of the steady-state current, filtered at 2 kHz, evoked from a test pulse. Only cells with <30% change in $R_{input}$, $R_{series}$, and $I_{holding,}$ or <100 pA change for $I_{holding}$ were included for analysis. EPSCs were evoked from a stimulating electrode (two-conductor cluster electrodes with 75 μM tip separation, FHC Inc., Bowdoin, ME.). NMDAR currents were pharmacologically isolated by modifying the standard ACSF to

contain 4 mM $Mg^{2+}$, 4 mM $Ca^{2+}$, 1 μM glycine, 50 μM picrotoxin, and 40 μM DNQX or 20 μM CNQX.

***mEPSC recordings*** Excitatory miniature excitatory postsynaptic currents (mEPSCs) were recorded in the presence of blockers for voltage-gated sodium channels (tetrodotoxin, TTX; 200 nM) and $GABA_A$ receptors (picrotoxin; 50 μM) as well as 1 μM glycine. We recorded AMPA receptor-mediated mEPSCs at negative holding potentials (-80 mV) to block the postsynaptic NMDAR currents, and measured mEPSC amplitude and frequency before, during, and after bath application of the NMDAR antagonist D-APV (50μM) or D,L-APV (100 μM). Comparisons were made for (1) the last 4 minutes of a 10 minute baseline period, (2) the last 4 minutes of an 8-10 minute window following application of 100 μM D,L-APV, and (3) the last 4 minutes following drug washout lasting 15-20 minutes. We also performed experiments of the same duration without drug application as a demonstration of the stability of the recordings. Events were identified by their rapid rise time (<3 ms), and were detected using an automatic template detection program (pCLAMP, Molecular Devices) (Clements and Bekkers, 1997). The detection threshold remained constant for the duration of each experiment. All events were manually verified, and only events with a monotonic rise time and exponential decay were included in the analysis. Normalized frequency and amplitudes were used for mEPSC data analysis. Over 150 events, with an average of ~700 events, were analyzed for each data point for each cell.

*tLTD induction and short-term plasticity* The internal recording solutions used for these experiments consisted of (in mM): 100 (K)Gluconate, 20 KCl, 4 (Mg)ATP, 10 phosphocreatine, 0.3 GTP, 10 Hepes, with pH adjusted to 7.25 and osmolarity adjusted to 290-295 mOsm. Some solutions also contained 0.4% biocytin and/or MK-801 (0.5-1 mM). Picrotoxin (50 μM) was included in the bath solution for a subset of recordings. In the younger age group experiments recorded in the presence or absence of picrotoxin were combined, as they yielded similar results, consistent with previous observations that the properties of spike timing-dependent plasticity are similar with and without the blockade of inhibition (Feldman, 2000; Froemke and Dan, 2002). In the P23-30 age group however, tLTD could only be induced in the presence of picrotoxin to block inhibition. Therefore, at this age group experiments with and without picrotoxin were kept separate. L2/3 pyramidal cells were recorded in current-clamp, and weak stimulation was delivered to L4, which makes a particularly strong and vertically organized projection to L2/3 (Burkhalter, 1989). Extracellular stimulation produced a monophasic and fixed latency response, which we interpreted as predominately consisting as coming from L4 or other vertical inputs. We feel that it is unlikely that we are activating local axon collaterals, as we never evoked antidromic action potentials at the low stimulation intensities used in these studies. Baseline stimulation was delivered once every 15-18 seconds. After a 10-15 minute stable baseline, action potential (AP) and excitatory postsynaptic potential (EPSP) pairings were delivered 75-100 times (@ ~0.2Hz) with a postsynaptic action potential produced by brief (<5 ms) depolarization followed 5-

25 ms later by an EPSP evoked by L4 stimulation. After pairing, and in a few instances after an additional 5 minute period without stimulation, stimulation was delivered for 30 minutes at baseline frequencies. Short-term plasticity (i.e. the rate of synaptic depression) was compared before and after pairing by stimulating 6 pulses at 30 Hz.

*Synaptic depression analysis* A change in the amount of synaptic depression between the baseline responses and the responses after the induction of tLTD was quantified using a short-term depression (STD) index described by Sjöström et al. (2003). The change in the amount of synaptic depression observed in a train of six EPSPs evoked at 30 Hz was compared between the average responses in the first 10 minutes (baseline) and the last 10 minutes (post-induction). Net amplitudes for each EPSP were used. The STD index is equal to the ratio (post-induction/baseline) of $EPSP_1$ – the average ratios of $EPSP_{2\text{-}6,}$ all normalized to the ratio of $EPSP_1$.

*Electron microscopy (EM)* Mice were given an overdose of Nembutal, and perfused transcardially with heparinized Tyrode solution for 3 minutes, followed by a mixture of 4% paraformaldehyde and 0.5% gluteraldehyde, for 15 minutes. Fixatives were dissolved in 0.1M phosphate buffer (PB) at pH 7.4. After perfusions, brains were kept within the skull and postfixed overnight in the same fixative. A vibrating microtome was used to cut 60 µM sections coronally through the visual cortex. Sections were treated with 1% $NaBH_4$ to terminate the cross-linking actions of the fixatives, and stored free-floating at 4°C in 0.01M phosphate buffered saline (PBS) containing 0.05% sodium azide. To prepare slices for pre-

embedding immunocytochemistry, sections were rinsed and treated in 1% bovine serum albumin (BSA) in PBS for 30 minutes, and they were incubated in a 1 µg/ml dilution of polyclonal rabbit anti-NR1 (Chemicon, CA) in PBS with 1% BSA and 0.05% $NaN_3$, for three days at room temperature. The sections were then rinsed and incubated in a biotinylated goat anti-rabbit (Vector Labs, CA) for 2 hours, followed by 2 hours incubation in HRP-conjugated avidin-biotin complex (ABC; Vector Labs, CA). Immunoreactivity was visualized using diaminobenzidine (DAB; 0.05%) and $H_2O_2$ (0.001%). Deletion of primary antibodies eliminated all specific staining discernible at the EM level.

Immunostained sections were fixed with 2% glutaraldehyde in PB for 10 minutes, followed by 1% osmium tetroxide for 1 hour. Sections were then dehydrated in a series of alcohols, infiltrated with liquid resin (Embed 812, EMS, PA), and placed between two sheets of clear acetate (Aclar; Ted Pella, CA). The resin was allowed to polymerize overnight at 60°C. Sections were drawn with the aid of camera lucida, and the areas to be analyzed by EM were cut and placed on flat surfaces of Beem capsule caps. These capsules then were filled with resin and left in a 60°C oven, until the resin in the capsule polymerized. Trapezoids that contained a strip of cortex from the pial surface to the white matter were prepared, and ultrathin sections were cut using an ultramicrotome (Leica UMC). Ultrathin sections were oriented near-parallel to the surface of vibratome sections, maximizing the area of tissue-EPON interface where most of the antibody penetration can be expected and allowing reliable laminar analysis. Sections were examined on a JEOL JEM1010 microscope.

To determine cortical layer borders on the resin-embedded sections, capsule-embedded sections were drawn using camera lucida, and certain landmarks, such as the border of cell-sparse layer 1, and the position of tissue within the resin block, were marked. The drawings from each block were then compared to ultrathin sections obtained from those blocks, and the position of L2/3 was determined within the resin trapezoid. For quantitative EM analysis, the synapse was used as the main counting unit. From each immunostained brain, at least 50 adjacent but non-overlapping images were captured at 10,000X magnification, using a 16Mpixel CCD camera (SIA). The images were examined at 35- 60,000X final magnification using Image-Pro Express software. Systematic sweeps were used to locate synapses on each image. Then, the experimenter judged if the pre- or postsynaptic elements of the synapse contained any label, and evaluated the type of synaptic contact (symmetric or asymmetric). A second experimenter who was blinded to the experimental conditions reevaluated the images to confirm the quantitative analysis.

Identification of synapses and the DAB label was performed with the following considerations. A synaptic terminal was identified by the presence of at least one synaptic vesicle in contact with a plasma membrane, at least three or more vesicles within the same profile, and the parallel alignment of the postsynaptic plasma membrane with that of the terminal. A postsynaptic density was deemed DAB positive, if it contained an accumulation of black DAB chromagen at the postsynaptic membrane. The chromagen accumulation was darker and irregularly shaped in contrast to the unlabeled postsynaptic densities.

The criterion to classify a presynaptic terminal as DAB positive was the presence of any discernible black DAB accumulation anywhere in the terminal. Typically, DAB accumulation either uniformly filled the terminal, or appeared as patches of label attached to the presynaptic membrane or nonsynaptic membranes. From the counts of synapses that are unlabeled or displayed labeling in the presynaptic terminal, or at the postsynaptic density, we calculated (1) the prevalence of presynaptic labeling (N presynaptic label x100 / N all synapses), (2) the prevalence of postsynaptic labeling (N postsynaptic label x 100 / N all synapses), and (3) the ratio of presynaptic / postsynaptic label. The number of synapses that were encountered in each brain ranged from 159-420. The difference in the number of synapses examined from each brain was an outcome of the presence of different number of synapses within a predetermined number of images captured from each brain. Every synapse from all images was included in the analysis. Two brains each at P16 and P27 were analyzed. The experimenter analyzing the data was blind to both the age of the animal and to the expected outcome.

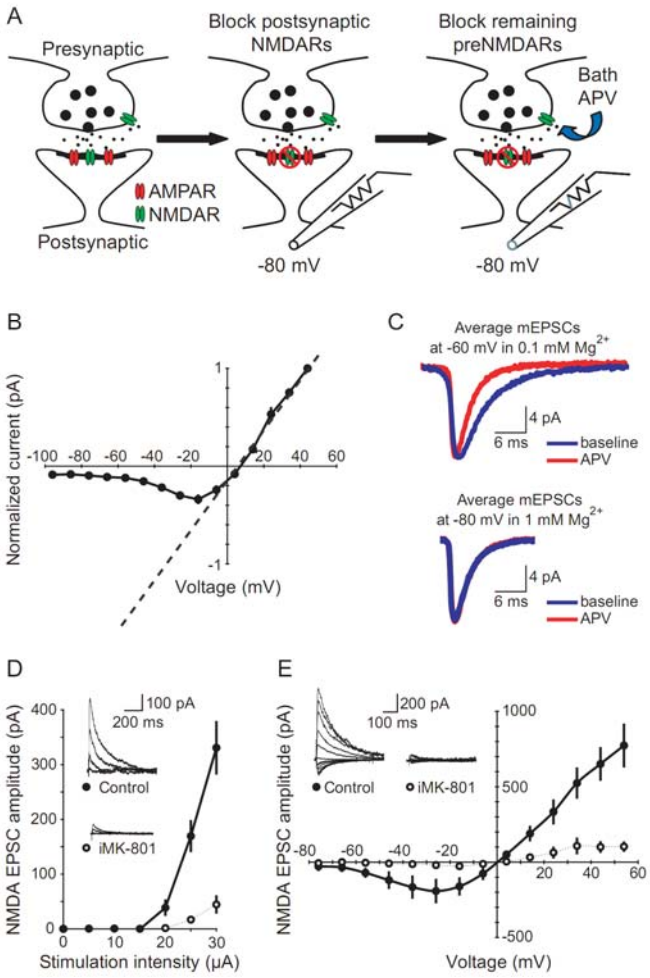
*Pharmacological agents* purchased from Sigma (St. Louis, MO).

*Statistics* Means are reported as ±S.E.M, unless specified otherwise. The nonparametric Kruskal-Wallis test was used to determine statistical significance of cumulative probability histograms. Student's t tests for were used at the $p < 0.05$ significance level, except where significance was corrected to $p < 0.0045$ for multiple comparisons using the Bonferroni-Dunn method (Fig. 2.3).

## 2.3 Results

### 2.3.1 Postsynaptic hyperpolarization or MK-801 block postsynaptic NMDARs

To probe for the presence of functional presynaptic NMDARs (preNMDARs) in visual cortical pyramidal cells, we used an established protocol in slices from mice aged P7-P84 (Berretta and Jones, 1996; Sjöström et al., 2003; Bender et al., 2006b; Yang et al., 2006). In this strategy, postsynaptic NMDARs are first blocked with strong hyperpolarization. Then, the subsequent effects of bath applying the NMDAR antagonist D,L-APV (100 μM) are measured to reveal the effect that the remaining, presumably presynaptic, NMDARs have on spontaneous neurotransmitter release (Fig. 2.1A). To validate this approach, we first pharmacologically isolated NMDAR-mediated EPSCs and examined the current-voltage (I-V) relationship at the L4-L2/3 synapse. L4 stimulation was adjusted to evoke ~100 pA response at +40 mV. The synaptically evoked NMDAR currents exhibited strong rectification generated from $Mg^{2+}$ block (Mayer et al., 1984; Nowak et al., 1984), showing that <4% of the current remained at -80 mV compared to what would be expected from a linear I-V relationship (Fig. 2.1B). Although strong hyperpolarization clearly blocks evoked NMDAR currents, we also wanted to verify that it blocked NMDAR currents driven by spontaneous neurotransmitter release. Our findings demonstrate that D,L-APV has no effect on the amplitude or kinetics of mEPSCs recorded at -80 mV in the presence of TTX (200 nM) (Fig. 2.1C), suggesting that the NMDAR component of the mEPSCs was either absent or so small that is was below the detection level. To demonstrate that functional postsynaptic NMDARs can be detected in




**<u>Figure 2.1</u> Postsynaptic NMDAR function can be blocked with hyperpolarization or MK-801 in the postsynaptic recording pipette (iMK-801). (A)** Model explaining t experimental protocol for detecting functional preNMDARs. After blocking postsynaptic NMDARs with hyperpolarization, the role of preNMDARs on synaptic transmission can be tested by blocking the remaining preNMDARs with bath application of D,L-APV. **(B)** Normalized current-voltage (I-V) relationship at the L4→L2/3 synapse of pharmacologically isolated NMDAR-mediated excitatory postsynaptic currents (EPSCs). L4 stimulation was adjusted to evoke a ~100 pA response at +40 mV. Dotted line is a fit to the linear portion of the I-V relationship. Note the strong block of NMDAR currents by hyperpolarization (n=11, average age of animals ~P24). **(C)** Top: D,L-APV blocks the NMDAR component of mEPSCs recorded at -60 mV in 0.1 mM $Mg^{2+}$. Bottom: D,L-APV has no postsynaptic effect on the amplitude or kinetics of mEPSCs recorded at -80 mV in 1 mM $Mg^{2+}$, suggesting that the mEPSC currents are mediated by AMPA receptors and that the NMDAR component is non-existent. **(D)** Synaptic input-output (I-O) relationship for L2/3 cells recorded with (open circles) and without (filled circles) iMK-801. Inset: traces for control and iMK-801 I-O curves. Note that iMK-801 blocks more than 96% of the NMDAR current evoked at 20 μA, which is our average stimulation intensity. **(E)** Synaptic I-V relationship for the same cells shown in **(D)** showing that, with iMK-801 and a stimulation of 30 μA, the NMDAR current is completely blocked (n=4) compared to control (n=5) at -65mV corrected for a junction potential measured at 16mV. Inset: traces for the I-V recordings.

mEPSC recordings in the absence of $Mg^{2+}$ block, we showed that D,L-APV blocked a long duration current recorded at -60 mV in low $Mg^{2+}$ (0.1mM)(Fig. 2.1C). These observations demonstrate that hyperpolarization is an effective means for blocking postsynaptic NMDAR currents.

Postsynaptic NMDARs can also be blocked by including 0.5-1 mM MK-801 in the postsynaptic recording pipette (iMK-801) (Berretta and Jones, 1996; Bender et al., 2006b). Notably, the postsynaptic block of NMDARs with iMK-801 does not act by spillover into the extracellular medium (see (Bender et al., 2006b) and results in Fig. 2.5). By examining the input output (I-O) relationship of L2/3 cells recorded at +40 mV with and without iMK-801, we demonstrate that iMK-801 blocks more than 96% of the NMDAR current evoked at 20 μA, which was our average stimulation intensity for further experiments utilizing this technique. On average we evoked 38±12.8 pA responses under control conditions (n=6) but only 1.4±1.8 pA responses with iMK-801 (n=7; p=0.0076), similar to the >90% block of NMDAR currents that has been observed previously using iMK-801 (Berretta and Jones, 1996; Bender et al., 2006b). With iMK-801 and using a stimulation intensity of 30 μA, the NMDAR current measured at -65mV is completely blocked (0.22±5.42 pA; n=4) compared to control (-35.13±22.78 pA; n=5) (Fig. 2.1E).

### 2.3.2 PreNMDARs facilitate spontaneous neurotransmitter release in the visual cortex of young mice (<P20) but not older mice (>P21)

To probe for functional preNMDARs in L2/3 at P16, we first blocked postsynaptic NMDARs with hyperpolarization to -80 mV. We then measured the effects of bath applying D,L-APV on the frequency and amplitude of mEPSCs recorded in TTX. mEPSCs are evoked by spontaneous neurotransmitter release, and their frequency typically depends on presynaptic properties, whilst their amplitude depends on postsynaptic properties. Under these conditions, D,L-APV reversibly attenuated the frequency of mEPSCs in L2/3 pyramidal cells (Fig. 2.2A-C ; p=0.002, Kruskal-Wallis test), without affecting their amplitude or kinetics (Fig. 2.1C and 2.2C). Notably, control cells recorded for the same duration, but without D,L-APV application, showed no change in mEPSC frequency or amplitude (Fig. 2.2D). In a subset of experiments D-APV was used to confirm that the decrease in frequency was due to a block of NMDARs and not an effect of L-APV on mGluRs (Thoreson and Ulphani, 1995; Lieske and Ramirez, 2006). Taken together these observations indicate that preNMDARs tonically enhance the frequency of spontaneous release in young animals.

The observation of preNMDARs in L2/3 visual cortex of young mice prompted us to establish whether there are laminar or developmental differences in their function. We chose to assay preNMDARs in 4 age groups: before eye-opening (P7-P11), after eye-opening (P13-P20), during the peak of the critical period for ocular dominance plasticity (P21-P27)(Gordon and Stryker, 1996), and in adulthood (P74-P84). We found that the reduction in mEPSC frequency by

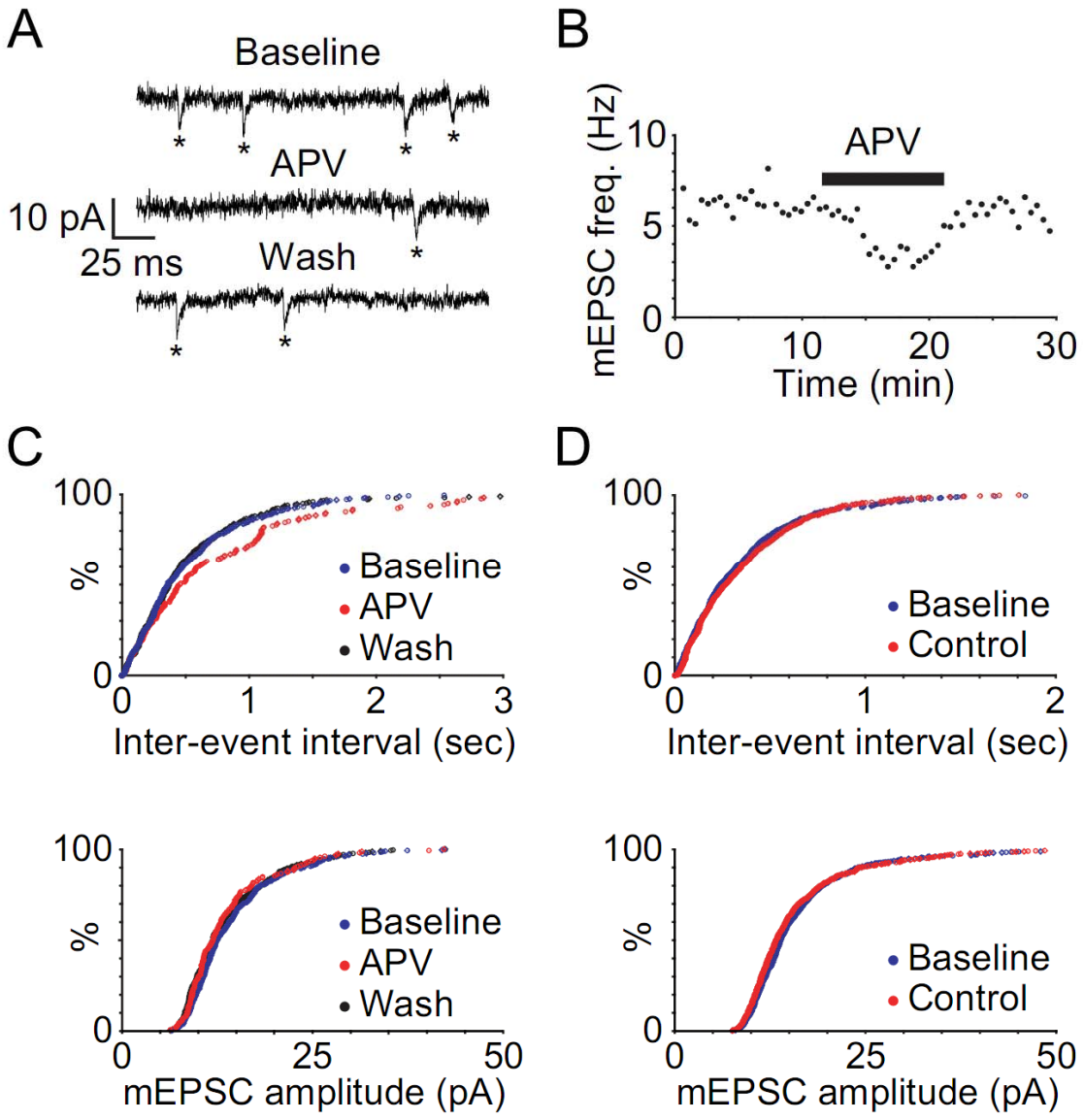



**Figure 2.2 PreNMDARs tonically increase the probability of neurotransmitter release onto L2/3 pyramidal cells in the mouse visual cortex.** **(A)** Example recording from a L2/3 pyramidal cell from a P16 mouse demonstrating that 100 μM D,L-APV reversibly reduces mEPSC frequency. Events are indicated by "∗". **(B)** Example experiment demonstrating that the reduction in mEPSC frequency by D,L-APV is reversible. **(C)** Cumulative probability histograms from a L2/3 pyramidal cell at P16 demonstrating D,L-APV application reversibly increases mEPSC inter-event interval without affecting amplitude. **(D)** Representative data from a single cell demonstrating that neither inter-event interval nor amplitude changed in the absence of D,L-APV treatment. The control cell was recorded for the same duration as experiments in which D,L-APV was applied.

D,L-APV was not limited to L2/3 at P16, as D,L-APV similarly reduced mEPSC frequency in L2/3, L4, and L5 in mice from P7-11 and P13-P20 (Fig. 2.3A and Table 2.1; p's < 0.0045, paired t-tests with a Bonferroni correction for 11 separate tests). However, there was a striking absence of an effect by D,L-APV in L2/3 and L5 in older mice (P21-P27 and P74-P84; Fig. 2.3A and Table 2.1; p's > 0.05), arguing that functional preNMDARs are lost. Several lines of evidence indicate that the reduction in mEPSC frequency in young mice was due to a presynaptic change in neurotransmitter release and was not mediated by a postsynaptic mechanism (e.g. rundown or reduced postsynaptic AMPA receptor number). (1) The reduction in mEPSC frequency by D,L-APV was reversible (Fig. 2.2B, 2.3B; frequency in D or D,L-APV = 75.3 $\pm$ 5.0 % baseline, p=0.0003; frequency after D,L-APV washout = 99.6 $\pm$ 5.0 % baseline, p=0.93 n=15). (2) We did not observe a significant reduction in mEPSC frequency in control cells that were recorded for a similar duration (Fig. 2.3B). (3) There was no significant change in mEPSC amplitude or kinetics by D,L-APV application in all lamina at all ages studied (Fig. 2.2D, Fig. 2.3C,D; Table 2.1). Collectively, our data show that there is a developmental loss in the ability of preNMDARs to facilitate neurotransmitter release in the visual cortex of young mice.

**Table 1. Data from mEPSC recordings (means ± SEM)**

| | ~P9 (P7–P11) | | | ~P16 (P13–P20) | | | ~P26 (P23–P30) | | | ~P80 (P72–P90) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | L2/3 | L4 | L5 | L2/3 | L4 | L5 | L2/3 | L4 | L5 | L2/3 | L4 | L5 |
| Sample size | 7 | 8 | 8 | 17 | 7 | 9 | 5 | 6 | 11 | 4 | 10 | 4 |
| $I_{holding}$ (pA) | −46.7 ± 24.4 | −28.0 ± 8.1 | −26.5 ± 4.6 | −60.0 ± 8.9 | −51.2 ± 8.2 | −80.7 ± 19.4 | −45.4 ± 5.7 | −81.2 ± 25.3 | −152.6 ± 37 | −55.8 ± 13 | −98.9 ± 21.3 | −145.2 ± 77 |
| $R_{input}$ (MΩ) | 428 ± 57 | 359 ± 57 | 249 ± 53 | 210.2 ± 15.3 | 230 ± 33 | 143 ± 24 | 117 ± 13 | 159 ± 36 | 113 ± 14 | 161 ± 42 | 142 ± 16 | 166 ± 65 |
| $R_{series}$(MΩ) | 16.6 ± 0.4 | 21.6 ± 1.8 | 19.1 ± 2.3 | 20.0 ± 0.9 | 22.8 ± 1.7 | 17.8 ± 1.2 | 20.7 ± 2.4 | 26.8 ± 2.9 | 20.5 ± 1.4 | 19.2 ± 1.2 | 25.8 ± 1.8 | 21.6 ± 3.5 |
| Amplitude (pA) baseline | 22.3 ± 0.8 | 19.5 ± 1.4 | 17.1 ± 1.4 | 16.1 ± 0.4 | 13.1 ± 0.8 | 14.4 ± 0.7 | 14.5 ± 1.6 | 17.3 ± 2.2 | 16.1 ± 1.2 | 19.4 ± 1.8 | 12.6 ± 0.9 | 14.0 ± 1.6 |
| Amplitude (pA) in APV | 21.5 ± 0.9 | 19.5 ± 1.6 | 14.7 ± 1.0 | 15.5 ± 0.5 | 12.9 ± 0.7 | 13.6 ± 0.6 | 14.4 ± 1.3 | 16.3 ± 1.4 | 15.7 ± 1.1 | 17.2 ± 1.4 | 12.2 ± 0.7 | 16.0 ± 2.5 |
| Frequency (Hz) baseline | 1.4 ± 0.6 | 1.4 ± 0.4 | 0.4 ± 0.1 | 4.0 ± 0.6 | 4.6 ± 0.7 | 3.1 ± 0.7 | 4.1 ± 1.1 | 11.4 ± 4.2 | 1.6 ± 0.4 | 4.7 ± 2.4 | 3.8 ± 0.9 | 1.9 ± 0.6 |
| Frequency (Hz) in APV | 1.2 ± 0.5 | 1.0 ± 0.3 | 0.3 ± 0.1 | 3.0 ± 0.5 | 3.3 ± 0.5 | 1.9 ± 0.4 | 4.1 ± 1.0 | 12.2 ± 4.7 | 1.8 ± 0.4 | 4.6 ± 2.4 | 3.6 ± 0.9 | 2.0 ± 0.5 |
| $\tau_{Decay}$ (ms) baseline | 3.7 ± 0.0.3 | 5.7 ± 1.3 | 5.0 ± 0.04 | 4.0 ± 0.2 | 4.5 ± 0.2 | 5.1 ± 0.3 | 4.7 ± 0.2 | 4.3 ± 0.6 | 5.8 ± 0.3 | 7.6 ± 2.8 | 5.5 ± 0.8 | 4.3 ± 0.4 |
| $\tau_{Decay}$ (ms) in APV | 3.8 ± 0.3 | 5.2 ± 0.5 | 5.6 ± 0.7 | 4.3 ± 0.1 | 4.6 ± 0.2 | 5.4 ± 0.3 | 4.5 ± 0.3 | 4.7 ± 0.6 | 6.2 ± 0.4 | 7.4 ± 2.2 | 5.4 ± 0.4 | 4.5 ± 0.6 |
| Rise time (ms) baseline | 1.0 ± 0.1 | 1.2 ± 0.2 | 1.3 ± 0.1 | 1.2 ± 0.1 | 1.4 ± 0.1 | 2.0 ± 0.1 | 1.6 ± 0.1 | 1.0 ± 0.3 | 1.6 ± 0.1 | 1.6 ± 0.4 | 1.3 ± 0.1 | 1.6 ± 0.3 |
| Rise time (ms) in APV | 1.0 ± 0.1 | 1.3 ± 0.2 | 1.4 ± 0.2 | 1.2 ± 0.1 | 1.4 ± 0.1 | 2.1 ± 0.2 | 1.6 ± 0.1 | 1.0 ± 0.2 | 1.6 ± 0.1 | 1.6 ± 0.5 | 1.3 ± 0.1 | 1.4 ± 0.2 |
| Area (pA · ms) baseline | 69.4 ± 5.1 | 75.5 ± 11.5 | 61.6 ± 6.8 | 55.2 ± 3.2 | 48.4 ± 2.9 | 66.9 ± 3.2 | 56.6 ± 8.4 | 47.4 ± 8.5 | 71.2 ± 5.5 | 81.1 ± 23.1 | 40.8 ± 3.8 | 54.5 ± 5.0 |
| Area (pA · ms) in APV | 67.2 ± 5.2 | 77.3 ± 13.6 | 56.0 ± 5.4 | 51.9 ± 3.2 | 46.8 ± 2.9 | 64.9 ± 3.6 | 55.6 ± 6.1 | 45.3 ± 6.7 | 69.5 ± 4.9 | 72.4 ± 20.2 | 39.2 ± 3.2 | 60.1 ± 4.2 |

**Table 2. Data for immuno-EM labeling of NR1**

| Animal # | Age | Number of synaptic terminals examined | Number of synapses with presynaptic NR1 | Number of unlabeled presynaptic terminals | Number of synapses with NR1 at the postsynaptic density | Percentage of synapses with NR1 at the presynaptic terminal | Percentage of synapses with NR1 at the postsynaptic density | Ratio of presynaptic/ postsynaptic NR1 labeling |
|---|---|---|---|---|---|---|---|---|
| 1 | P16 | 240 | 145 | 95 | 185 | 60.42 | 77.08 | 0.7838 |
| 2 | P16 | 420 | 254 | 166 | 319 | 60.48 | 75.95 | 0.7962 |
| 3 | P27 | 254 | 78 | 176 | 214 | 30.71 | 84.25 | 0.3644 |
| 4 | P27 | 159 | 46 | 113 | 120 | 28.93 | 75.47 | 0.3833 |

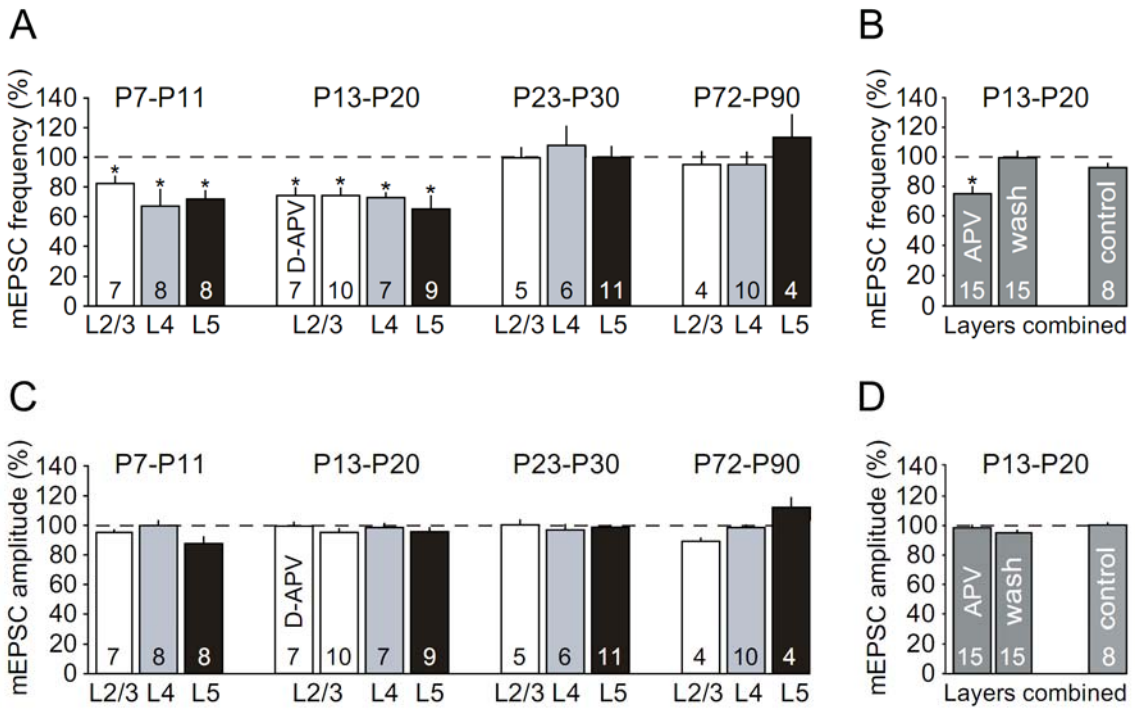
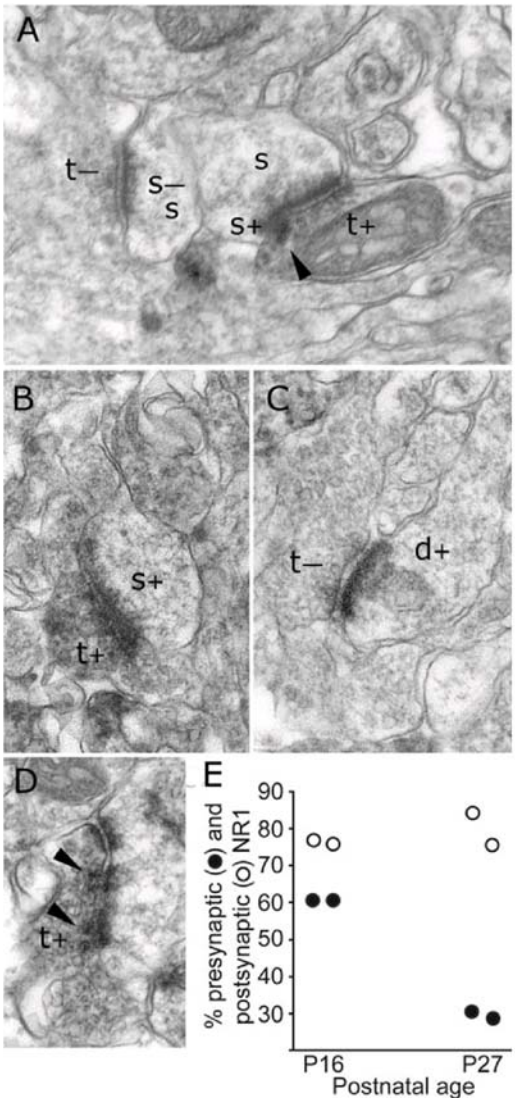



**Figure 2.3 Developmental loss of functional preNMDARs in visual cortex.** **(A)** Averaged data demonstrating that D,L-APV strongly reduced mEPSC frequency in L2/3, L4, and L5 pyramidal cells in mice aged P7-P11 and P13-P20, suggesting the presence of functional preNMDARs. D,L-APV had no effect in L2/3, L4, or L5 cells in mice aged P21-P27 or P74-P84. D-APV was used in a subset of experiments at P13-20 in layer 2/3 and showed the same effect on mEPSC frequency. **(B)** The reduction in mEPSC frequency was reversed with washout of either D,L-APV or D-APV. No significant rundown in mEPSC frequency was observed in control cells not exposed to D,L-APV but recorded for a similar duration. **(C & D)** No significant changes in mEPSC amplitude were observed in any of the groups. "*" in **(A & B)** indicate significance of $p<0.0045$ corrected for multiple tests using the Bonferroni-Dunn method, and sample sizes are given within the bars. The mEPSC frequency and amplitude in **(A-B)** were normalized to the averaged baseline values before D,L-APV application.

### 2.3.3 Developmental reduction in presynaptic / postsynaptic NR1

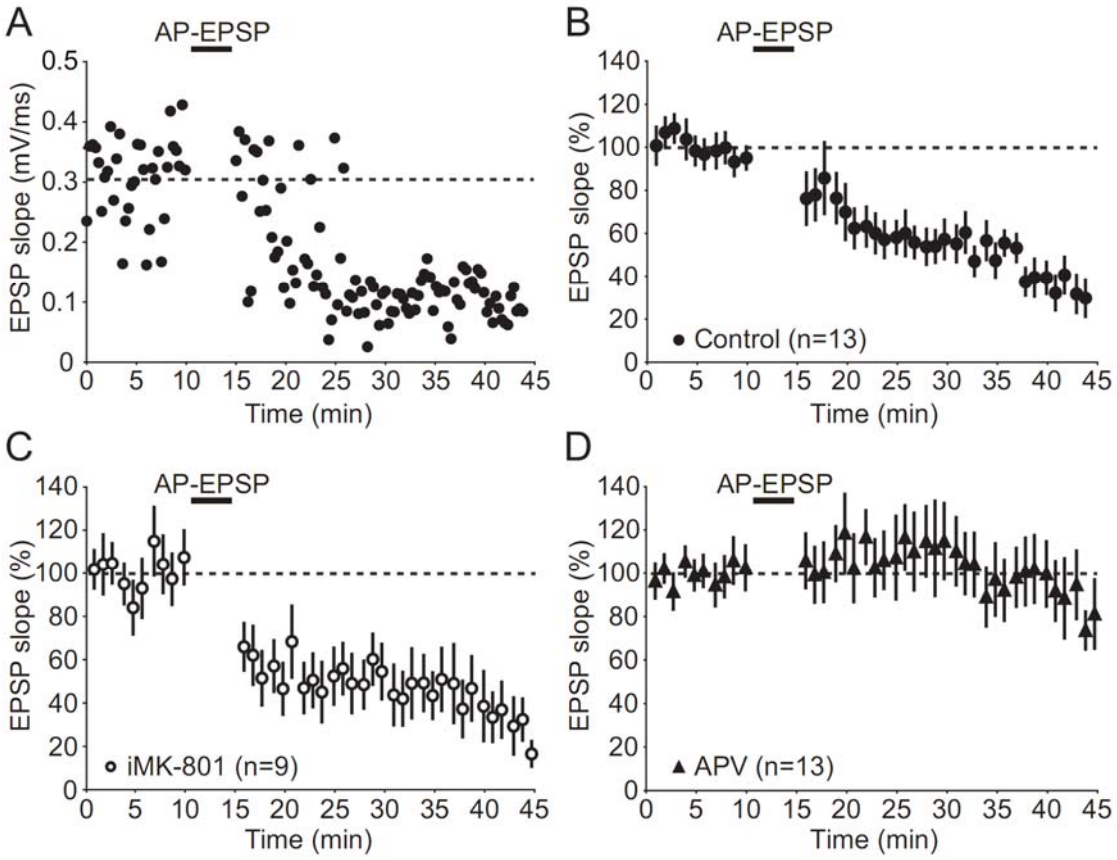
One parsimonious explanation for the developmental loss of preNMDAR function is that fewer synapses contain preNMDARs in older mice. To test this, we used EM to examine the pre- and postsynaptic prevalence of asymmetric (excitatory) synapses in L2/3 containing the NR1 subunit, which is obligatory for NMDAR function (Fig. 2.4). We examined a total of 1073 synapses from two brains each at P16 and P27 (Table 2.2). While the percent of synapses containing NR1 at the postsynaptic density was similar in P16 and P27 brains (77% and 75% at P16, and 84% and 75% at P27), there was a 50% developmental reduction in NR1 at presynaptic sites (Fig. 2.4E). Specifically, a majority (>60%) of presynaptic terminals contained NR1 in each of two brains at P16 (n=240 and 420 synapses examined), while only a minority (<31%) of the presynaptic sites were labeled in two P27 brains (n=254 and 159 synapses examined). The same data revealed approximately a 50% developmental reduction in the ratio of pre- versus postsynaptic localization of NR1 (Table 2.2). Because the prevalence of postsynaptic NR1 was unchanged during development, it is unlikely that the developmental reduction in presynaptic NR1 could be due to differences in either the quality of the perfusions or age-dependent differences of antibody penetration into the tissue. Thus, the anatomical reduction in preNMDARs is consistent with our physiological data demonstrating a dramatic developmental loss of functional preNMDARs in facilitating spontaneous neurotransmitter release over this time course, suggestive of a causal relationship.




**<u>Figure 2.4</u> The presence of presynaptic NR1 is down-regulated with development. (A)** Electron micrograph in L2/3 of a P16 mouse demonstrating an NR1 positive terminal (t+) making a synapse with a spine that is also NR1 positive (s+). An NR1 negative presynaptic terminal (t-) making an asymmetric synapse onto an NR1 negative spine (s-) is present in the same field. Scale bar = 250 nanometers, applies to **(A-D).** Arrows indicate aggregations of DAB in presynaptic terminals. **(B)** In a section from L2/3 of another P16 mouse, a diffusely labeled terminal (t+) is seen forming a synapse onto a spine (s+) that contains NR1 label at the postsynaptic density. **(C)** At P27, most synapses exhibit postsynaptic, but not presynaptic, NR1. An unlabeled terminal (t-) forms a synapse onto a labeled dendrite (d+). **(D)** In a section from another P27 mouse an NR1 positive terminal (t+) makes a synapse onto an NR1 positive spine (s+). **(C)** Scatter plot from 4 mice (two at each age) quantifying the selective loss of presynaptic, but not postsynaptic, NR1 over development. Note that 30% of terminals still contain NR1 at P27.

### 2.3.4 Developmental switch in the role of pre- and postsynaptic NMDARs in tLTD

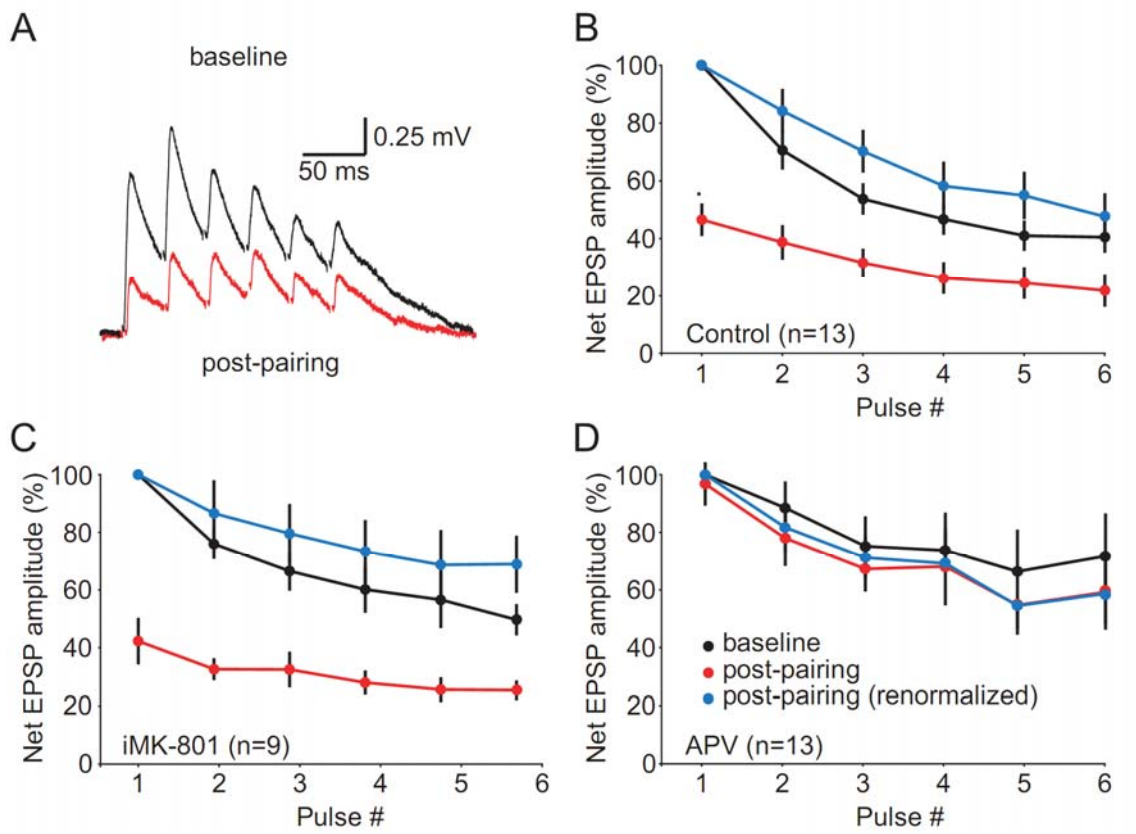
What is the functional significance of this developmental loss of preNMDARs to synaptic plasticity during development? PreNMDARs in young rodents have been implicated in tLTD (Sjöström et al., 2003; Bender et al., 2006b), a form of synaptic plasticity thought to be important for the acquisition of receptive field properties (Dan and Poo, 2006). Thus, we predicted that the developmental loss of preNMDARs in L2/3 would either abolish the ability to induce tLTD or that there would be a different induction mechanism for tLTD later in development. To test these possibilities, we used a standard tLTD induction protocol by repeatedly pairing a postsynaptic action potential (AP) in a L2/3 pyramidal cell closely followed (5-25 ms) by an excitatory postsynaptic potential (EPSP) evoked by L4 stimulation. In young mice (P13-P17), AP-EPSP pairings produced strong tLTD (Fig. 2.5A, B; 43.6 ± 5.6 % baseline slope, n=13, $p<0.007$). Similarly, robust tLTD could be induced when the NMDAR antagonist MK-801 was included in the recording electrode to block postsynaptic NMDARs (Berretta and Jones, 1996; Bender et al., 2006b) (Fig. 2.5C; 36.2 ± 9.1 % baseline, n=9, $p=0.017$). However, bath application of D,L-APV prevented induction of significant tLTD in young mice (Fig. 2.5D; 95.3±9.0 % baseline, n=13, $p=0.80$), suggesting that the synaptic weakening relied on activation of pre- and not postsynaptic NMDARs. Notably, these data also serve as an additional control showing that iMK-801 is not acting by spillover into the extrasynaptic medium, otherwise iMK-801 would have blocked tLTD in a manner similar to D,L-APV.




**<u>Figure 2.5</u> PreNMDARs are required for tLTD at L4-L2/3 synapses in young (<P20) mice. (A)** Example of tLTD induced at L4-L2/3 synapses by AP-EPSP pairings in a P13 mouse. **(B)** Averaged data from control cells demonstrating the strong depression in EPSP slope (43.6 ± 5.6 % baseline slope, n=13, $p<0.007$) induced with AP-EPSP pairings. **(C)** Strong tLTD could be induced with AP-EPSP pairings even when postsynaptic NMDARs were blocked by inclusion of MK-801 in the internal recording solution (iMK-801) (36.2 ± 9.1 % baseline, n=9, $p<0.018$). This suggests that postsynaptic NMDARs are not required for tLTD induction in these young mice. **(D)** Induction of tLTD was prevented by bath application of the NMDAR antagonist D,L-APV (95.3±9.4 % baseline, n=13, $p>0.798$), arguing that preNMDARs are required for the induction of tLTD. Because the inclusion of picrotoxin in the recording ACSF had no effect on the induction of tLTD at this early stage each condition contains experiments with and without picrotoxin.

To determine if the tLTD in young mice was expressed as a presynaptic reduction in neurotransmitter release, we analyzed synaptic depression in a high-frequency stimulus train of 6 pulses at 30Hz before and after tLTD induction. The short-term depression (STD) index was used as a measure of changes in synaptic depression (see Methods). In the STD index, a negative number indicates that a manipulation reduces the rate of short-term depression. Because higher rates of short-term depression are generally associated with a higher initial probability of neurotransmitter release, a negative STD index indicates that a manipulation is likely to have lowered the probability of neurotransmitter release. By analyzing the STD index on the same cells shown in Fig. 2.5, we observed that the rate of synaptic depression during stimulation was reduced after AP-EPSP pairings under control conditions (Fig. 2.6A, B; STD index= -0.30±0.11, n=13) and when the recording electrode contained MK-801 (Fig. 2.6C; STD index= -0.24±0.09, n=9). When AP-EPSP pairings were made during the bath application of D,L-APV to block all NMDARs, there was no change in the STD index (Fig. 2.6D; STD index= -0.005±0.09, n=13). Therefore, in young mice, the induction of tLTD in L2/3 requires the activation of preNMDARs to reduce the probability of neurotransmitter release.

In contrast to the failure of iMK-801 to block tLTD in younger mice, postsynaptic blockade of NMDARs with iMK-801 in older mice (P23-30) prevented the induction of tLTD in L2/3 pyramidal cells (Fig. 2.7B; n=7 p=0.79). Notably, in control conditions, AP-EPSP pairings significantly reduced synaptic strength in the visual cortex of these older mice (Fig. 2.7A; 67.7 ± 7.0 % baseline,




**<u>Figure 2.6</u> Analysis of synaptic depression indicates that tLTD induction in P13-17 mice is expressed as a decrease in release probability. (A)** Sample waveform of 6 pulses evoked by 30 Hz stimulation before and after AP-EPSP pairings. This example recording was made with iMK-801. **(B)** In the control conditions the rate of synaptic depression at L4→L2/3 connections was reduced after tLTD produced by AP-EPSP pairings, (STD index= -0.30±0.11, n=13), suggesting the pairing protocol caused a lasting reduction in neurotransmitter release. The STD index was used here as a measure of synaptic depression (see Methods). **(C)** AP-EPSP pairing induction of tLTD made with iMK-801 also produced a reduction in the rate of synaptic depression (STD index= -0.24±0.09, n=9), indicating that the pairing-induced reduction in neurotransmitter release did not require activation of postsynaptic NMDARs. **(D)** There was no significant change in synaptic depression when tLTD is blocked by bath application of D,L-APV (STD index= -0.005±0.09, n=13). While there was no difference between the STD index for control and iMK-801 experiments p=0.7, the STD index for these experiments combined were different from the STD index of the APV experiments (p <0.03) suggesting a role for presynaptic NMDARs in the tLTD. Data are from the same cells in Figure 2.5.

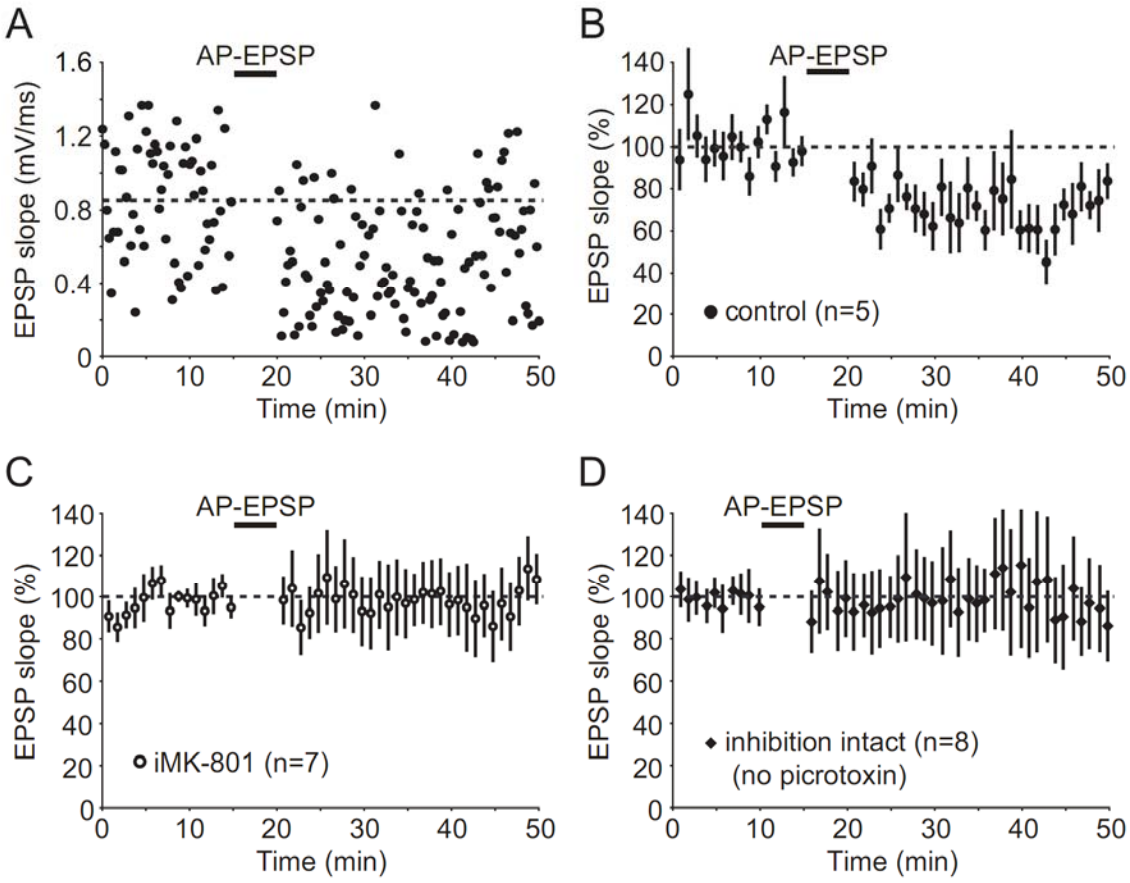
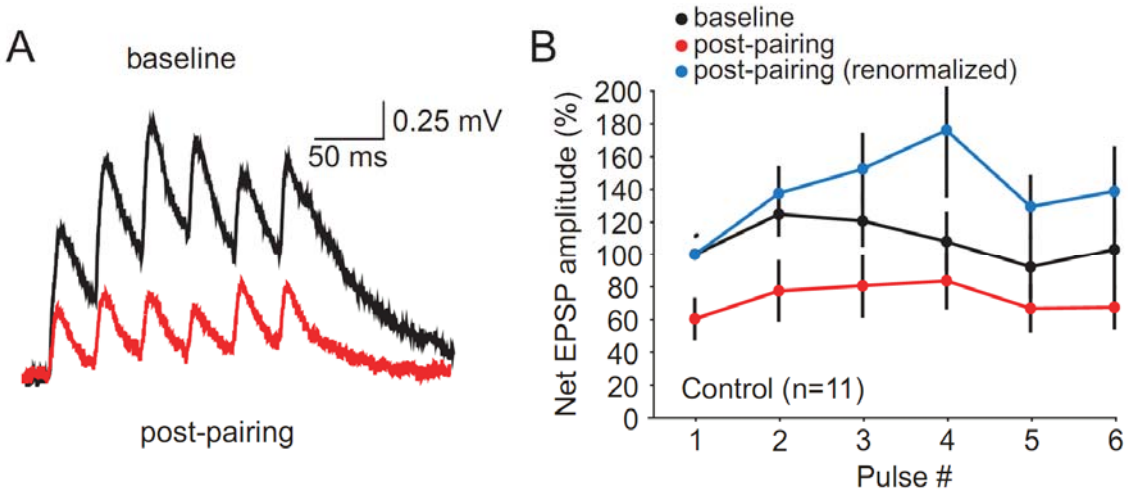



**Figure 2.7 Postsynaptic NMDARs are required for tLTD induction in critical period mice (P23-30). (A)** Example of tLTD induced at L4→L2/3 synapses by AP-EPSP pairings in a P13 mouse. **(B)** In P23-P30 mice, AP-EPSP pairings induced a small degree of tLTD (67.7 ± 7.1 % baseline; n=5, p=0.02). **(C)** No tLTD was induced when postsynaptic NMDARs were blocked by iMK-801 (n=7, p> 0.79), suggesting that the activation of postsynaptic NMDARs is required for the full expression of the depression. **(D)** No tLTD was induced when inhibition (no picrotoxin in the recording ACSF) (95.7 ± 21.0 % baseline; n=8, p=0.30).

n=5, p=0.018). Collectively, these data demonstrate that tLTD can be induced in the L4-L2/3 pathway in the visual cortex of mice aged P23-30, but the induction at this older age requires postsynaptic NMDARs. These results however, do not rule out the possibility that preNMDARs are also required for tLTD at this stage.

Interestingly, we found another developmental shift in the capacity for tLTD induction that depended on the presence of intact inhibition in the slice. We had initially found that, in the P13-20 animals, tLTD induced either in the presence of intact inhibition, or with inhibition blocked in the entire slice by including 50μM picrotoxin in the ACSF. Because the amount and nature of the tLTD did not differ between the two conditions and they were combined. In the older group however, we found that blocking inhibition with picrotoxin was required for tLTD induction as LTD could not be induced in the absence of picrotoxin (Fig. 2.7D; 95.7 ± 21.0 % baseline, n=8, p=0.30).

To determine if the tLTD in older mice was also expressed as a presynaptic reduction in neurotransmitter release, we analyzed the STD index and observed that the rate of synaptic depression during stimulation was decreased after AP-EPSP pairings under control conditions (Fig. 2.6A, B; STD index= -0.72±0.42, n=11). 4 of these experiments showed an increase in STD while 7 showed a decrease. Therefore, in older mice, the induction of tLTD in L2/3 requires the activation of postsynaptic NMDARs and is expressed at least in part by a decrease in neurotransmitter release. Though a change in STD index after tLTD induction suggests a presynaptic tLTD mechanism we can not rule out the possibility that the tLTD is being simultaneously expressed postsynaptically.




**<u>Figure 2.8</u> Analysis of synaptic depression indicates that tLTD induction in P26-28 mice is expressed as a decrease in release probability. (A)** Sample waveform of 6 pulses evoked by 30 Hz stimulation before and after AP-EPSP pairings. **(B)** The rate of synaptic depression at L4→L2/3 connections was reduced after tLTD produced by AP-EPSP pairings, (STD index= -0.72±0.42, n=11). The STD index was used here as a measure of synaptic depression (see Methods).

## 2.4 Discussion

A vast area of neuroscience research is dedicated to the study of NMDAR-mediated synaptic plasticity (Malenka and Bear, 2004). To date, most research has concentrated on the role of postsynaptic NMDARs, while the role and even the very existence of presynaptic NMDARs (preNMDARs) have been controversial or largely ignored. This disregard may be in large part due to the unanswered question "how are preNMDARs tonically activated?". This question has two major parts. The first part relates to the source of glutamate and has been recently explored by several labs. Jourdain et al 2007 showed that preNMDARs in the hippocampus are activated by vesicular release of glutamate from juxtaposed astrocytes. Le Meur et al 2007 recorded tonic NMDA current in hippocampal cells mediated by ambient interstitial glutamate released from glia. The other part of this question relates to the necessity for removal of $Mg^{2+}$ block for NMDAR activation, and has been less explored, but there are two popular possibilities. One possibility is, as suggested by Jourdain et al 2007, that the high input resistance of presynaptic terminals might allow for the voltage dependent removal of $Mg^{2+}$ with very small excitatory currents. The second possibility, which will be explored further in Chapter 3, is that the preNMDARs contain subunits that are less sensitive to the voltage dependent block by $Mg^{2+}$. The NMDAR subunits NR2C, D, and NR3A, all show less voltage sensitivity and are expressed in visual cortex.

Regardless of these unanswered questions, the role of preNMDARs is important to consider, as studies in different cell types, pathways, brain regions,

species, and ages have found varying roles for presynaptic and postsynaptic NMDARs in synaptic plasticity (for example, see (Sjöström et al., 2003; Malenka and Bear, 2004; Froemke et al., 2005; Bender et al., 2006b; Duguid and Sjöström, 2006)). Our study focused on the function of preNMDARs and their putative role in LTD, specifically within the developing mouse visual cortex. Our data make four key observations demonstrating that the role of preNMDARs in synaptic transmission and plasticity is highly age-dependent. (1) PreNMDARs are present and contribute to the spike timing-dependent induction of LTD in the neocortex of young animals. This is consistent with the observations that there appears to be little or no need for postsynaptic NMDARs in the induction of tLTD for pyramidal neurons in L5 (Sjöström et al., 2003) or L2/3 (present study). (2) The tLTD induced in young animals is at least in part expressed as a presynaptic reduction in neurotransmitter release although an additional postsynaptic contribution to expression was not ruled out. (3) The tonic function of preNMDARs is lost in development, at least in the assays used here. (4) The developmental loss of preNMDARs may be compensated for by the emergence of a postsynaptic role for NMDARs in the induction of tLTD although it can't be ruled out that there remains a role for preNMDARs at this stage as well. Our data thus highlight the changing functions of preNMDARs over development and provide the first evidence for a developmental switch in the involvement of NMDARs in timing-dependent plasticity.

The loss of functional preNMDARs has a clear mechanistic importance for the induction of tLTD, but what is the cellular basis of this loss? An obvious

possibility, which we tested, is that the prevalence of preNMDARs decreases with development. Here we show the first anatomical data to indicate both that NMDARs are present presynaptically at the majority of synapses in L2/3 early in development (P16) and that their prevalence is reduced to 50% over the next ten days. The developmental decrease in preNMDAR prevalence correlates temporally with a loss in their contribution to neurotransmitter release, suggesting a causal relationship. However, there may be additional contributing factors to the loss of preNMDAR function, as our anatomical data also indicate that preNMDARs remain at approximately 30% of the synapses even when we no longer observe their effect on mEPSC frequency. At least five possibilities exist for why we observe a complete loss of functional preNMDARs in these older mice despite our anatomical evidence that preNMDARs remain at a small subset of synapses. First, the number of NMDARs remaining at individual boutons may be below a threshold needed to have a measurable impact on neurotransmitter release. Second, preNMDARs may undergo a developmental change in their subunit composition rendering them less effective. Because functional preNMDARs in both the visual cortex (Sjöström et al., 2003) and the entorhinal cortex (Woodhall et al., 2001) are thought to contain NR2B subunits, the developmental increase in the relative expression of NR2A to NR2B NMDAR subunits in the visual cortex (Nase et al., 1999; Quinlan et al., 1999a; Quinlan et al., 1999b) may contribute to the loss of preNMDAR function. Third, the inputs that maintain preNMDARs later in development may not often participate in the spontaneous release of neurotransmitter. Fourth, preNMDARs may become

functionally uncoupled during development from the neurotransmitter release machinery. A final possibility is that the remaining preNMDARs may lose their efficacy due to a developmental reduction in a co-factor that is necessary for them to exert their influences. For example, coincident activation of preNMDARs and endocannabinoid receptors is necessary for the induction of tLTD between L5 cells of young mice (Sjöström et al., 2003), thus a change in endocannabinoid function could alter preNMDAR function. Although we did not observe a functional role for preNMDARs at later stages of development, one may become apparent in assays other than the ones used in this study.

Our data add to recent literature suggesting that preNMDARs are regulated in a developmental and activity-dependent manner (Mameli et al., 2005; Yang et al., 2006). For example, the neurosteroid pregnenolone increases the probability of glutamate release through preNMDARs in the CA3→CA1 hippocampal synapse, and this effect is thought to be lost at P5 due to a decrease in NR2D-containing NMDARs (Mameli et al., 2005). A similar but much more gradual reduction in preNMDAR function occurs in layer 5 of the rat entorhinal cortex, where their function is pronounced at ~5 weeks of age and only modest at ~5 months of age (Yang et al., 2006). Notably, in the entorhinal cortex, preNMDAR function can also be regulated in an activity-dependent manner, as the induction of seizures in adults restores juvenile levels of preNMDAR function (Yang et al., 2006). The combined data demonstrate that preNMDAR function can be bidirectionally regulated to alter neurotransmitter release. The developmental loss of preNMDARs may be a general feature of

neural development, and the time course for this loss could be highly dependent on brain region. We also stress that there may be important differences between species and that this study was performed in mice. Further studies are needed to explore these possibilities.

Why might a mechanistic switch in the induction of synaptic plasticity be important for emerging cortical circuits? We and others (Nosyreva and Huber, 2005) suggest that it may be beneficial to modify synaptic strength by altering the presynaptic probably of release at a time of early circuit formation when postsynaptic sites neither contain many AMPA receptors nor are stabilized. However, when postsynaptic AMPA receptor numbers increase and cortical circuits are being stabilized via the experience-dependent weakening or elimination of synapses, this may be performed more effectively through a postsynaptic mechanism. Consistent with this idea, there is a developmental loss of preNMDARs in the visual cortex coincident with (1) the developmental loss of AMPA receptor "silent synapses" (Rumpel et al., 2004) and (2) the onset of the critical period for deprivation-induced loss of inputs (Gordon and Stryker, 1996). Analogous developmental changes in presynaptic mechanisms occur in other receptor systems, as a presynaptic role for kainate receptors in neurotransmitter release has been observed only in the first postnatal week of rodent hippocampus and cortex development (Kidd and Isaac, 1999; Lauri et al., 2006). Moreover, a presynaptic to postsynaptic switch in the involvement of metabotropic glutamate receptors has been observed to occur over the first few weeks of postnatal development in the hippocampus, and this underlies a

developmental switch in the pre- versus postsynaptic expression of metabotropic glutamate receptor-mediated LTD (Nosyreva and Huber, 2005). Thus, a common theme during brain development may be that plasticity mechanisms are more likely to affect presynaptic release in emerging cortical circuits when there is a high signal to noise ratio, but as synapses become stabilized, it becomes more efficacious to alter synaptic strength via postsynaptic mechanisms. In addition to changing the properties of synaptic plasticity, the developmental loss of the ability of preNMDARs to support neurotransmitter release may also help explain the general observation that synapses switch from depressing to facilitating during development in many brain regions (Pouzat and Hestrin, 1997; Reyes and Sakmann, 1999).

Our findings add to a growing body of literature that the properties of synaptic plasticity must be studied within a developmental context (Yasuda et al., 2003; Frenkel and Bear, 2004; Nosyreva and Huber, 2005; Yashiro et al., 2005; He et al., 2006; Jo et al., 2006). Our data also raise the exciting possibility that preNMDARs might regulate plasticity during a pre-critical period early in development but that postsynaptic NMDARs may be critically involved in experience-dependent synaptic plasticity later in development. Given the observations that preNMDARs exist early in development in the hippocampus (Mameli et al., 2005), entorhinal cortex (Woodhall et al., 2001; Yang et al., 2006), and visual cortex (present study) but not at later stages of maturation, we suggest that the development loss of preNMDAR function may be an important and general property of early circuit formation.

# Chapter 3:

## PreNMDARs contain the novel NMDAR subunit NR3A

**This chapter is a modified version of a manuscript being prepared for submission and is the joint effort of Larsen R. and Corlew R.**

## 3.1 Introduction

The NMDA-type glutamate receptor (NMDAR) is critical for many forms of experience-dependent plasticity (Kirkwood and Bear, 1994; Katz and Shatz, 1996) and the proper development of the brain (Iwasato et al., 2000; Perez-Otano and Ehlers, 2005). In the visual cortex, NMDAR activation is required for neurons to acquire normal ocular-dominance and orientation selectivity (Roberts et al., 1998; Ramoa et al., 2001). A general assumption has been that NMDARs influence the experience-dependent development of these stimulus-selective properties via a postsynaptic mechanism. (Kirkwood and Bear, 1994; Katz and Shatz, 1996). There is now evidence that NMDARs can also powerfully alter the presynaptic release of neurotransmitter through both short-term and long-lasting synaptic modifications (Sjostrom et al., 2003; Bender et al., 2006b; Duguid and Sjostrom, 2006; Corlew et al., 2007; Corlew et al., 2008; Rodriguez-Moreno and Paulsen, 2008; Banerjee et al., 2009). We have recently demonstrated that presynaptically-acting NMDARs (preNMDARs) both enhance the spontaneous release of neurotransmitter and are required to induce timing-dependent long-

term depression of synaptic strength (tLTD) in the visual cortex of young mice (Corlew et al., 2007; Corlew et al., 2008). Despite the role of preNMDARs in these important aspects of synaptic function and plasticity, little is known about how preNMDARs function or why their functional influences diminish with age.

One particularly intriguing feature of preNMDARs is their tonic activity (Berretta and Jones, 1996), suggesting that preNMDARs can influence neuronal communication on a moment-to-moment basis. Because the NMDAR ionophore is typically blocked by magnesium ($Mg^{2+}$) at hyperpolarized potentials, it remains unknown how preNMDARs can be spontaneously active. Several possibilities exist to explain the tonic activity of preNMDARs. First, given the high input resistance of the synaptic terminal, local depolarization of the presynaptic terminal may be sufficient to relieve the magnesium block (Jourdain et al., 2007; Corlew et al., 2008). However, this idea is speculative and is tempered by studies in which presynaptic terminal membrane potentials have been measured in the Calyx of Held, where the resting membrane potential is close to -80mV (Duguid and Smart, 2004; Awatramani et al., 2005). Second, it is conceivable that the binding of glutamate and glycine to preNMDARs can influence neurotransmitter release in the absence of ion flow. Currently, there is little evidence for this idea. Third, preNMDARs may lack block by $Mg^{2+}$ at hyperpolarized potentials, allowing them to be tonically active (Mameli et al., 2005; Rodriguez-Moreno and Paulsen, 2008). This possibility seems promising because NMDARs are composed of the obligatory NR1 subunit in combination with NR2A-D and/or NR3A-B subunits. While most NMDARs in the neocortex

are composed of NR2A and NR2B, which are strongly blocked by $Mg^{2+}$, NMDARs which contain NR2C, NR2D, NR3A, or NR3B subunits exhibit less basal $Mg^{2+}$ block (Burnashev et al., 1992; Cull-Candy et al., 2001; Sasaki et al., 2002).

Here we tested the composition of preNMDARs onto layer (L) 2/3 pyramidal cells in the juvenile mouse primary visual cortex (V1). We demonstrate that the NR3A subunit is required for the ability of preNMDARs to enhance neurotransmitter release. We also show that the requirement for NR3A to affect spontaneous release relies on the $Mg^{2+}$ insensitivity of the receptor, as preNMDAR functions can be restored in low $Mg^{2+}$ conditions. Because the developmental loss of NR3A coincides with the loss of preNMDAR functions, we also tested whether the low $Mg^{2+}$ condition could restore tonic activity of preNMDARs in older mice (Ciabarra et al., 1995; Corlew et al., 2007; Corlew et al., 2008). These data demonstrate an essential role for NR3A-containing NMDARs in supporting a novel form of neurotransmitter release in the developing visual cortex.

## 3.2 Materials and Methods

*Subjects* Mice were obtained and housed as described in Chapter 2, with the exception that both NR2A KO and NR3A KO mice were used, as well as their wild-type controls. NR2A KO mice were generously supplied by S. Nakanishi (Kyoto, Japan, (Kadotani et al., 1996)), but re-derived on a C57BL/6 background

by Charles River Laboratories. NR3A KO mice were generously provided by Stuart Lipton.

*Cortical slice preparation* Cortical slice preparation was identical to methods described in Chapters 2 and 4 (Yashiro et al., 2005; Corlew et al., 2007).

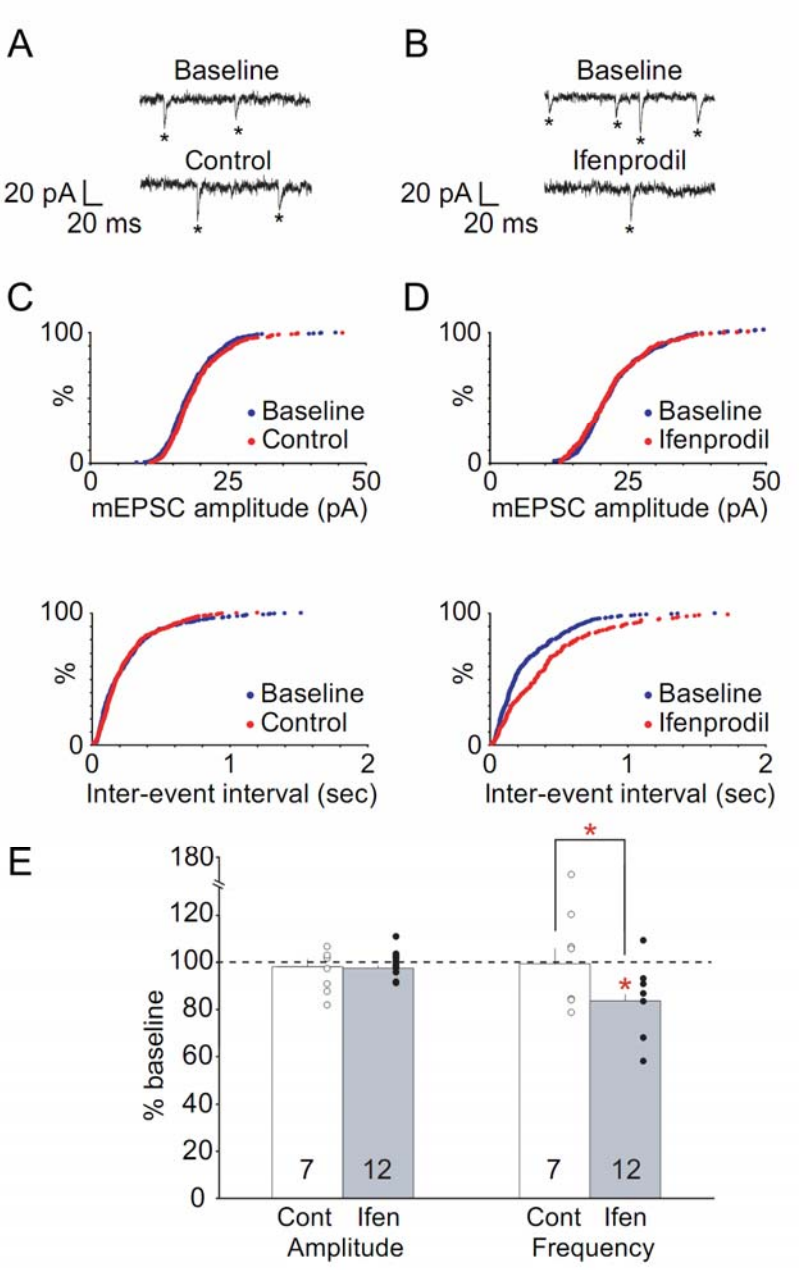
*Voltage-clamp recordings* Voltage-clamp recordings and mEPSC analysis were performed in an identical fashion as in Chapter 2, except that all recordings were performed with MK-801 in the postsynaptic recording pipette to block all postsynaptic NMDARs.

## 3.3 Results

### 3.3.1 NR2B-containing preNMDARs enhance spontaneous neurotransmitter release i

NR2B-containing preNMDARs are required to enhance spontaneous neurotransmitter release in several regions of the brain (Woodhall et al., 2001; Brasier and Feldman, 2008), including the visual cortex (Li et al., 2008). Accordingly, we confirmed this in L2/3 cells of juvenile mouse visual cortex. As described in Chapter 2 (Corlew et al., 2007), we used a common method to measure spontaneous release probability: analyzing the effect of blocking preNMDARs on the frequency of miniature excitatory postsynaptic currents (mEPSCs). By including the NMDAR blocker MK-801 in the postsynaptic recording pipette, we first block all postsynaptic NMDARs in the recorded cell for the entire length of the experiment. Including MK-801 in the postsynaptic recording pipette has been shown to block nearly all of the NMDAR currents in

the postsynaptic cell, but does not leak out to measurably affect any other cells (Bender et al., 2006b; Corlew et al., 2007; Brasier and Feldman, 2008). Action potential-evoked activity is blocked with 200nm tetrodotoxin TTX during the recording, so that only action potential-independent spontaneous release of single vesicles is measured. The $GABA_A$ receptor antagonist picrotoxin (50μm) is also included to block mIPSCs and possible shunting inhibition on mEPSCs. After a steady 10 min baseline is recorded, the NR2B specific antagonist ifenprodil (3μM) is bath-applied to block all NR2B-containing NMDARs in the slice. Because the postsynaptic NMDARs were already blocked, a change in frequency of mEPSCs indicates a change in spontaneous release probability due to blocking of the preNMDARs. Amplitude of the mEPSCs was analyzed to insure that there is no postsynaptic effect of Ifenprodil. Ifenprodil reliably reduced the frequency but not the amplitude of mEPSCs in L2/3 pyramidal cells in young (postnatal day (P) 13-18) mouse V1 (83.8 ± 4.1%, n = 12, $p < 0.02$) (Fig 3.1) (R. Larsen unpublished data). To ensure that there was no decrease in mEPSC frequency over time, recordings with ifenprodil were interleaved with control experiments in which normal ACSF was added to the bath instead of ACSF-containing ifenprodil. Indeed, the decrease in mEPSC frequency was due to the application of ifenprodil, as control recordings showed no decrease in mEPSC frequency (107.4 ± 11.9%, n = 7) and the normalized values of the change with ACSF / ifenprodil application was also significant ($p < 0.04$). This confirms that the NR2B NMDAR subunit is required for the function of preNMDARs to enhance spontaneous neurotransmitter release.




**<u>Figure 3.1</u> NR2B-containing preNMDARs enhance spontaneous neurotransmitter release. (A-B)** Sample voltage-clamp recordings of AMPAR-mediated mEPSCs from L2/3 pyramidal cells during baseline and drug application periods (control **(A),** or the selective NR2B-containing NMDAR antagonist ifenprodil **(B)**). mEPSC events are indicated by "∗". **(C-D)** Amplitude and inter-event interval cumulative probability histograms from the above cells during baseline and control/ifenprodil application. **(E)** Combined data comparing the normalized amplitude and frequency changes during the control (white) and ifenprodil (black) recordings. Bars illustrate the average with S.E.M. while individual experiments are plotted as points within the bars. In this and subsequent figures, sample sizes are given within the bars. * = $p < 0.05$.

### 3.3.2 PreNMDARs do not require the NR2A subunit

Several studies have tested the requirement of the NR2A subunit in the action of preNMDARs on spontaneous release (Li et al., 2008) and evoked release (Chamberlain et al., 2008; Li et al., 2009), as well as the preNMDARs role in tLTD (Banerjee et al., 2009). Here we use a genetic approach to show definitively that preNMDARs do not require the NR2A subunit to enhance spontaneous neurotransmitter release in mouse V1. We used the same methods as described above except that the general NMDAR antagonist APV is used as in Chapter 2 (Corlew et al., 2007) instead of ifenprodil and mice are NR2A knockout (NR2A KO) and NR2A wild type (WT). In WT mice, we show the expected decrease in mEPSC frequency with APV application (80.0 ± 4.9%, $n = 7$, $p < 0.03$) (R. Larsen and R. Corlew unpublished data) (Fig. 3.2A, C). This decrease is similar to that normally seen with APV application at this age (Corlew et al., 2007). In the NR2A KO mice, mEPSC frequency decreases to the same degree as in the wild type (83.5 ± 6.3%, $n = 7$, $p < 0.05$) (Fig. 3.2B, D, E), indicating that the absence of NR2A has no detrimental effect on preNMDARs. Therefore preNMDARs do not require the NR2A subunit.

### 3.3.3 NR3A-containing preNMDARs enhance spontaneous neurotransmitter release

We previously showed that, while the function of preNMDARs is down-regulated with development at around P20, the expression of NMDARs only decreases by 50%, at least for NR1 (Corlew et al., 2007). Therefore,

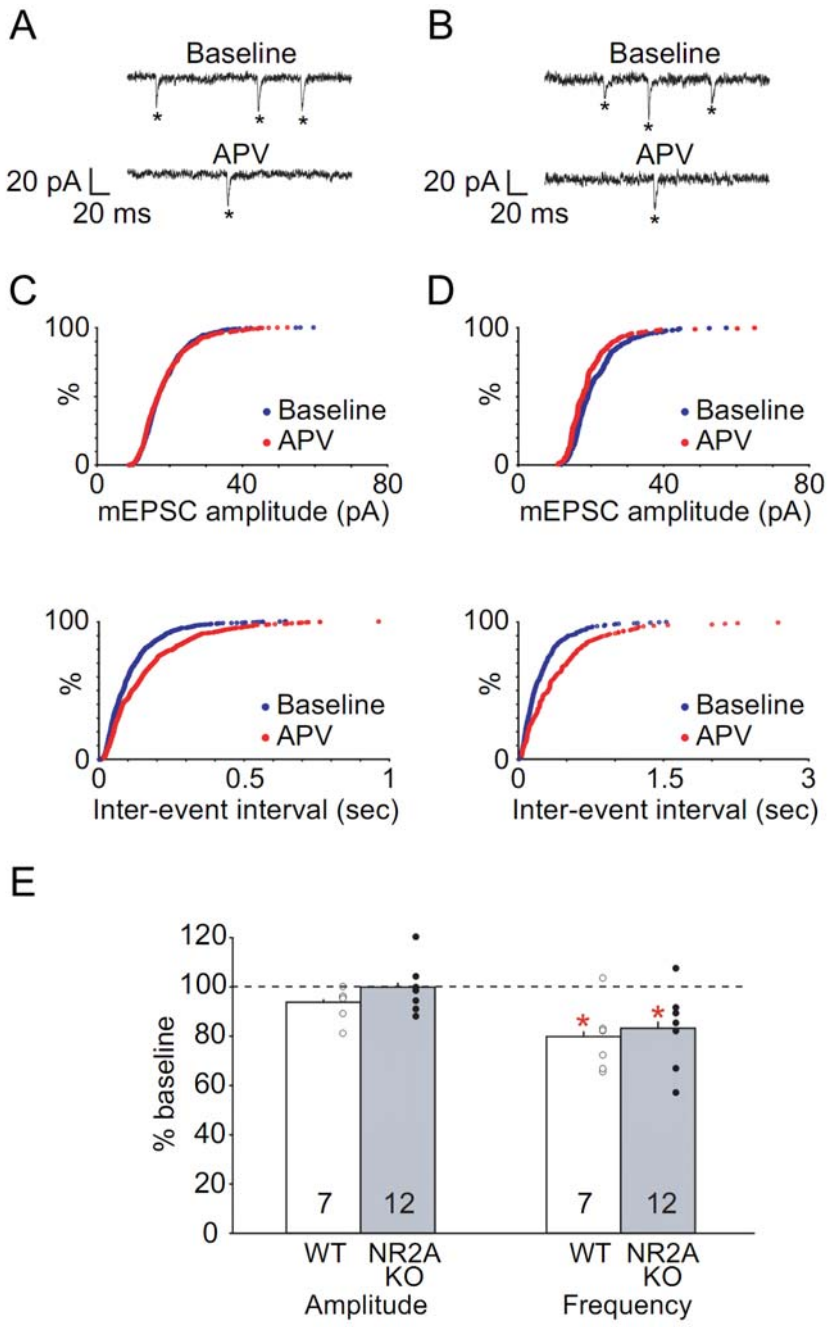
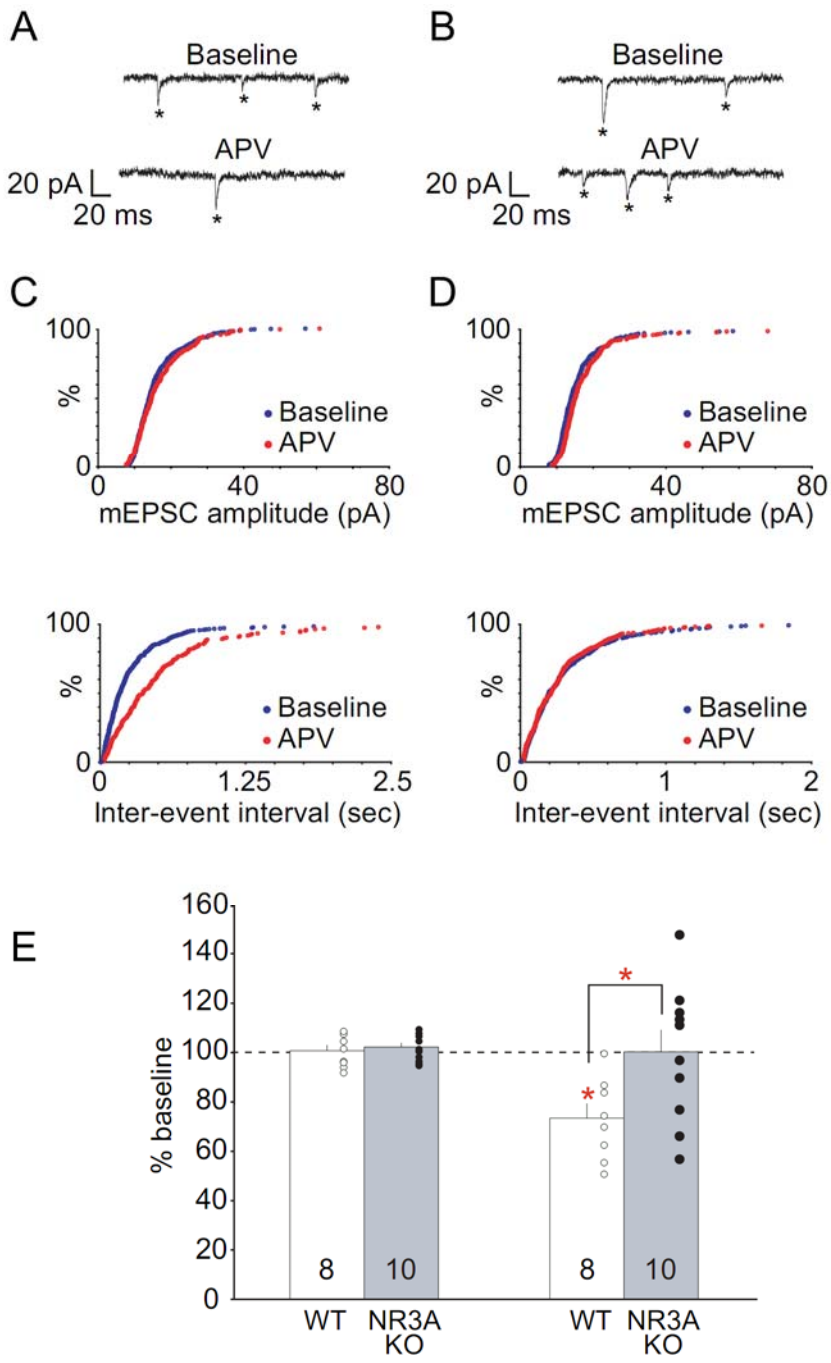



**Figure 3.2 The NR2A subunit is not required for the ability of preNMDARs to promote neurotransmitter release. (A-B)** Sample voltage-clamp recordings from L2/3 pyramidal cells during baseline and APV application periods in WT **(A)** and NR2A KO **(B)** mice. Events are indicated by "∗". **(C-D)** Amplitude and inter-event interval cumulative probability histograms from the above cells during baseline and APV application. **(E)** Combined data comparing the normalized amplitude and frequency changes during the APV recordings in WT (white) and NR2AKO (black) mice. Bars illustrate the average with S.E.M. and individual experiments are plotted as points within the bars. * = $p < 0.05$.

preNMDARs are present later in development (~P26) but unable to enhance neurotransmitter release probability. This decrease in function, but not expression, could be due to the developmental down-regulation of a subunit that is less $Mg^{2+}$ sensitive than either NR2A or NR2B. The developmental expression profile of the $Mg^{2+}$ insensitive receptor should match that of the developmental loss of function that we have previously reported for the preNMDAR (Corlew et al., 2007). NR3A is a novel NMDAR subunit whose expression in the cortex follows this same developmental decrease (Corlew et al., 2007). We have also confirmed this developmental profile in the visual cortex. In wild type C57B6 mice, NR3A protein is high until P16, when it starts to decline and is at extremely low levels by P26 (M. Henson unpublished data). The inclusion of NR3A may allow the preNMDAR to act tonically by removing its $Mg^{2+}$ block. We tested the preNMDARs requirement for the NR3A subunit by using the same mEPSC assay used above to test for the NR2A subunit. We recorded the frequency of mEPSCs in L2/3 pyramidal cells in V1 of NR3A KO and WT mice while blocking postsynaptic NMDARs. When APV was applied, mEPSC frequency, but not amplitude, decreased in the WT animals (73.1 ± 5.9%, n = 8, $p < 0.007$) (Fig. 3.3A, C, E). This decrease did not occur in the NR3A KO animals (99.9 ± 8.8%, n = 10) (Fig. 3.3B, D, E) and the normalized values were different between the WT and NR3A animals ($p < 0.03$) (Fig 3.3E). Therefore, preNMDARs onto L2/3 pyramidal cells in mouse V1 require the NR3A subunit in order to tonically enhance spontaneous neurotransmitter release.



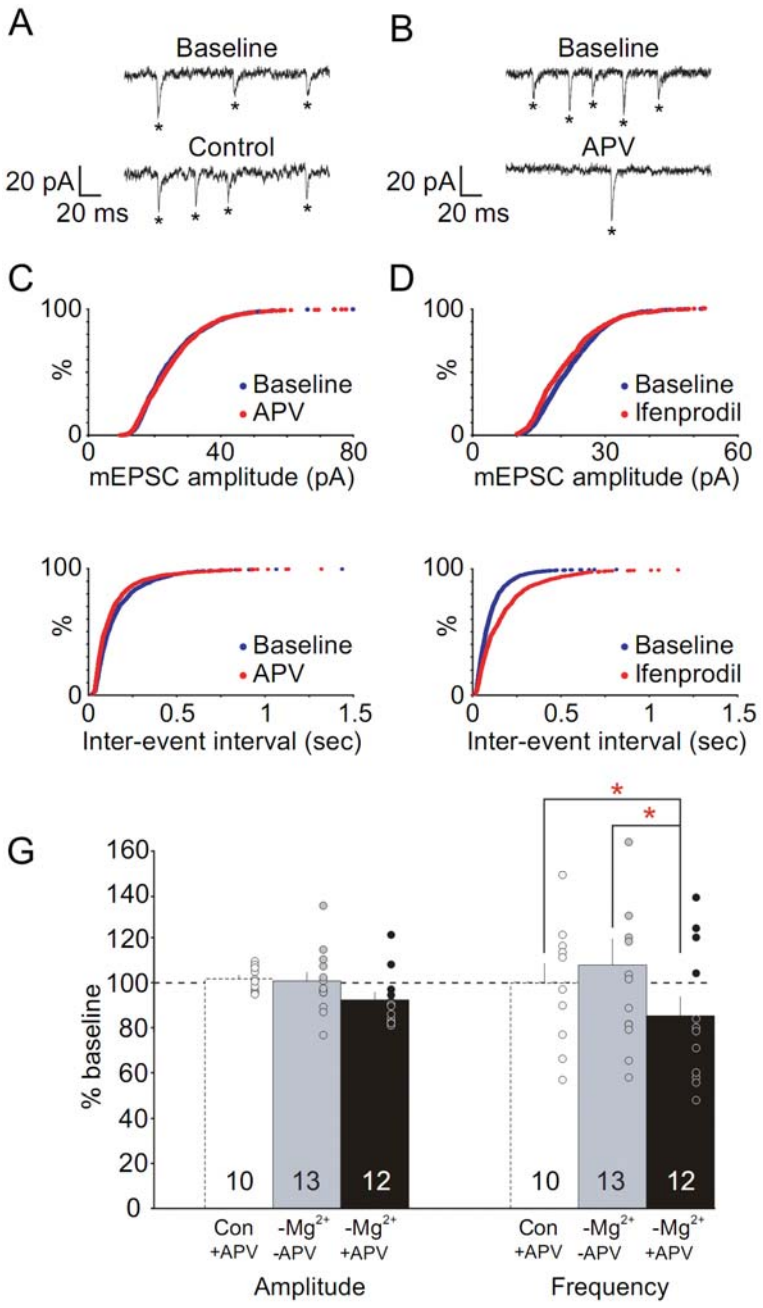

**<u>Figure 3.3</u> The NR3A subunit is required for the ability of preNMDARs to enhance spontaneous neurotransmitter release. (A-B)** Sample voltage-clamp recordings from L2/3 pyramidal cells during baseline and drug application periods in WT **(A)** and NR3A KO **(B)** mice. Events are indicated by "∗". **(C-D)** Amplitude and inter-event interval cumulative probability histograms from the above cells during APV application. **(E)** Combined data comparing the normalized frequency change during the APV recordings in WT (white) and NR3AKO (black) mice. Bars illustrate the average with S.E.M. and individual experiments are plotted as points with in the bars. * = $p < 0.05$.

### 3.3.4 $Mg^{2+}$ insensitivity of the NR3A subunit allows the preNMDAR to be tonically active

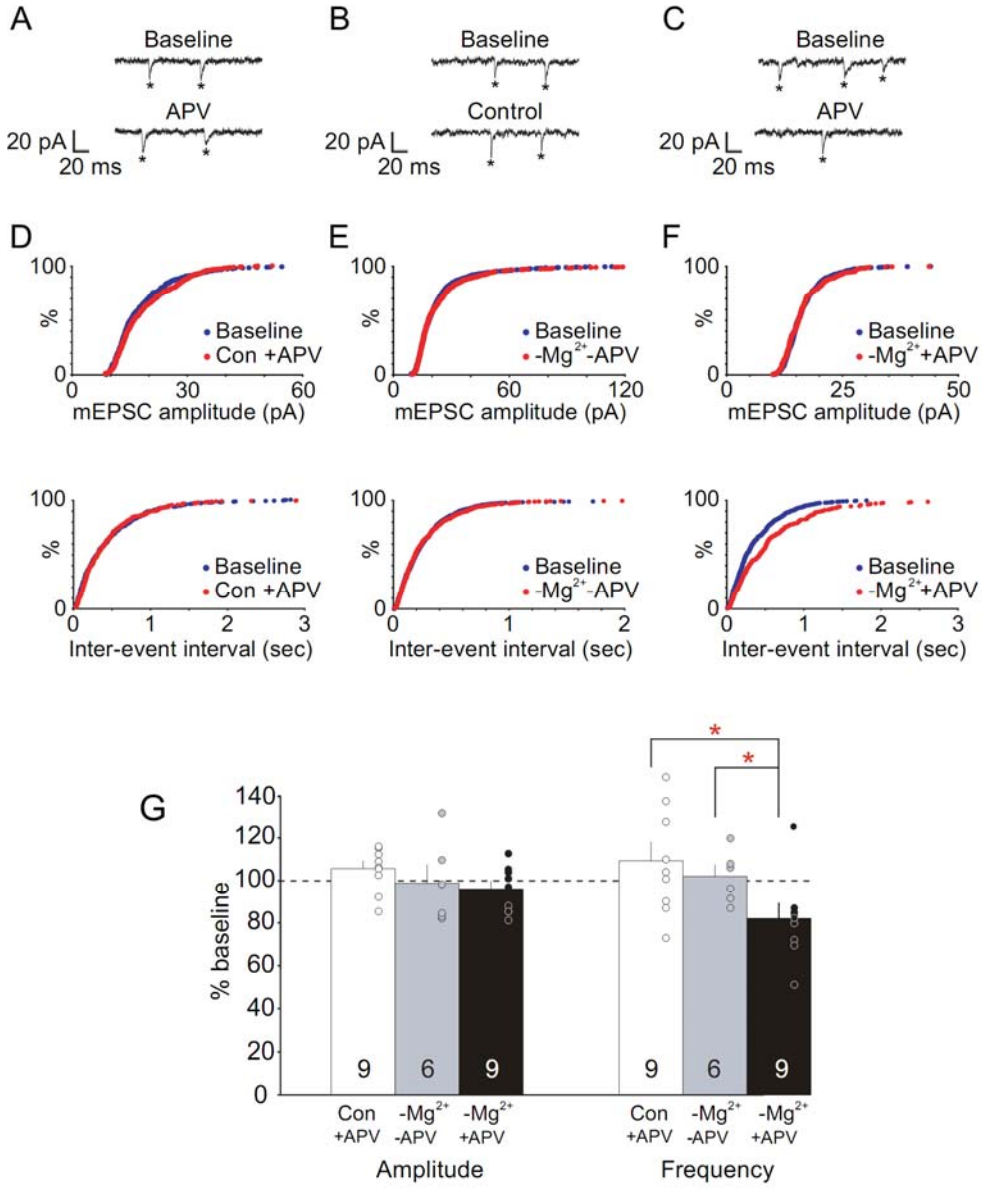
If, later in development, and in the NR3A KO mouse, preNMDARs are in fact present without the NR3A subunit they may still be able to enhance spontaneous neurotransmitter release when the $Mg^{2+}$ is absent from the recording solution. We tested for this $Mg^{2+}$ free enhancement, first in the NR3AKO mouse. We again recorded the effect of blocking preNMDAR with APV in NR3AKO mice, but this time used ACSF free of $Mg^{2+}$. As a control, we measured mEPSC frequency for the same duration without adding APV, thus ensuring that there is no decrease in mEPSC frequency over time in $Mg^{2+}$-free ACSF (107.9 ± 11.7%, n = 13) (Fig. 3.4A, C). APV application, however, did decrease mEPSC frequency in $Mg^{2+}$ free ACSF (85.3 ± 8.6%, n = 12, $p < 0.05$) (Fig. 3.4B, D). For comparison, recordings of NR3A KO mice in regular (1mM) $Mg^{2+}$ +APV are re-plotted in Fig. 3.4E from figure 3.3E (99.9 ± 8.8%, n = 10). Therefore, while NR3A is not required for the ability of the preNMDAR to enhance the probability of neurotransmitter release, it is required for its tonic activation.

### 3.3.5 Developmental loss of the NR3A subunit leaves the preNMDAR functional but $Mg^{2+}$ sensitive

These findings suggest that the previously reported developmental decrease in preNMDARs may not be due to a complete loss of the receptor, but




**<u>Figure 3.4</u> Low $Mg^{2+}$ recovers the ability of preNMDARs to enhance neurotransmitter release in the absence of NR3A. (A-B)** Sample voltage-clamp recordings in $Mg^{2+}$ free ACSF from L2/3 pyramidal cells in NR3A KO mice during baseline and drug application periods (control **(A)**, APV **(B)**). Events are indicated by "∗". **(C-D)** Amplitude and inter-event interval cumulative probability histograms from the above cells during control/APV application. **(E)** Combined data comparing the normalized amplitude and frequency changes during the control **(C)** and APV **(D)** recordings in NR3AKO mice. Bars illustrate the average with S.E.M. and individual experiments are plotted as points with in the bars. * = $p < 0.05$.




**<u>Figure 3.5</u> Low $Mg^{2+}$ recovers the ability of preNMDARs to enhance neurotransmitter release in older mice. (A)** Sample voltage-clamp recording in normal $Mg^{2+}$ (1mM) ACSF from L2/3 pyramidal cells in *P26?* mouse during baseline and APV application periods. **(A-B)** Recordings in $Mg^{2+}$ free ACSF from L2/3 pyramidal cells in *P23-30* mice during baseline and APV application periods (control **(A),** APV **(B)**). Events are indicated by "∗". **(C-D)** Amplitude and inter-event interval cumulative probability histograms from the above cells comparing baseline and control/APV periods. **(E)** Combined data comparing the normalized amplitude and frequency changes during the control + APV, Low $Mg^{2+}$ – APV, and Low $Mg^{2+}$ +APV. Bars illustrate the average with S.E.M. and individual experiments are plotted as points with in the bars * $p < 0.05$.

only a loss of the tonic activation of the preNMDAR. We hypothesized that the loss of this tonic activation is due to the developmental loss of NR3A. To test this possibility, we replicated the measurements that we made previously in older mice (P23-30) but used $Mg^{2+}$ free solution. We first recorded the effect of APV application in L2/3 pyramidal cells in V1 of P23-30 mice in regular (1mM) $Mg^{2+}$. We again show that at this age APV has no effect on either the amplitude or the frequency of mEPSCs (108.7 ± 9.3%, n = 9) (Fig. 3.5A, D, G). We then confirmed that the frequency and amplitude of mEPSCs is stable for the length of a control experiment in $Mg^{2+}$ -free ACSF but no APV (101.5 ± 5.0%, n = 6) (Fig 3.5B, E, G). Finally, we showed that adding APV in $Mg^{2+}$-free ACSF revealed the ability of preNMDARs to enhance spontaneous release at this older age; mEPSC frequency decreased significantly from baseline (81.7 ± 6.6%, n = 9, $p < 0.03$) (Fig. 3.5C, F, G), and normalized values for APV application were significantly different from Low $Mg^{2+}$ –APV ($p < 0.05$) and regular $Mg^{2+}$ +APV ( $p < 0.03$) (Fig.3.5G).

## 3.4 Discussion

Here we have resolved a question that has plagued the study of preNMDARs since their discovery. How are preNMDARs tonically active despite their $Mg^{2+}$ block? After confirming many previous results that show that preNMDARs require the NR2B subunit, we identified another required subunit. We first suspected the NR3A subunit as a likely candidate for inclusion in the preNMDAR because it is dramatically less $Mg^{2+}$ sensitive than the NR2 subunits

and its developmental expression pattern in the neocortex follows the same developmental profile as the preNMDAR. We have previously reported that preNMDARs are tonically active to increase spontaneous neurotransmitter release in the visual cortex during the first 3 weeks—but not after the fourth postnatal week—in a mouse. The NR3A subunit is similarly expressed in the neocortex at high levels only during the first three weeks and then drops off dramatically. The loss of one subunit, and not the whole preNMDAR, reconciles anatomical data that suggest that the preNMDAR is only slightly down-regulated during development while the function of the receptor is completely lost (Corlew et al., 2007).

### 3.4.1 NR2B-containg preNMDARs enhance spontaneous glutamate release

There is both anatomical and functional evidence that preNMDARs are composed of at least one NR2B subunit. Anatomical evidence indicates that NR2B subunits are expressed in the neocortex (Fujisawa and Aoki, 2003), spinal cord (Boyce et al., 1999), and hippocampes (Jourdain et al., 2007). There is also compelling functional evidence that preNMDARs contain the NR2B subunit in the visual and somatosensory cortecies (Sjostrom et al., 2003 ; Bender et al., 2006a; Brasier and Feldman, 2008 ; Li et al., 2009), entorhinal cortex (Yang et al., 2006), and cerebellum (Chamberlain et al., 2008). NR2B containing preNMDARs are required for enhancing spontaneous (Sjostrom et al., 2003; Li et al., 2008) and evoked (Sjostrom et al., 2003; Li et al., 2009) neurotransmitter release, and induction of synaptic plasticity (Banerjee et al., 2009). Here we confirm that

NR2B-containing preNMDARs in L2/3 pyramidal cells in V1 of young mice (P13-18) are tonically activated to enhance spontaneous glutamate release.

Because of the seemingly ubiquitous requirement for NR2B in preNMDARs it is tempting to ascribe the unique properties of the preNMDAR to the inclusion of NR2B. For example, there are suggestions that preNMDARs are insensitive to $Mg^{2+}$ block because NR2B-containing NMDARs are slightly less $Mg^{2+}$ sensitive compared to NR2A (Corlew et al., 2008). The developmental profile of preNMDARs has also been suggested to be caused by the developmental down-regulation of the NR2B subunit (Yang et al., 2006). But NR2B is a less than satisfying explanation because the NR2B subunit is only slightly less $Mg^{2+}$ sensitive than the NR2A subunit (Monyer et al., 1992). Additionally, NR2B's developmental down-regulation has a very shallow decline that does not correlate with the developmental loss of function that has been reported for preNMDARs (Corlew et al., 2007). Nevertheless we do show that preNMDARs require NR2B though it is likely not responsible for their tonic activation.

### 3.4.2 NR2A subunit does not enhance spontaneous glutamate release

Though there is much evidence for a requirement for NR2B in preNMDARs enhancement for evoked and spontaneous release, as well as a role in plasticity, there is very little evidence of a requirement for NR2A. There are a few anatomical reports of NR2A expression in presynaptic terminals of rat neocortex and cerebellum (DeBiasi et al., 1996; Aoki et al., 2003; Duguid and

Smart, 2004). And two studies also suggest that NR2A containing preNMDARs enhance evoked release on Shaffer collateral axons in the hippocampus (Mallon et al., 2004; Suarez and Solis, 2006). However, the drug that was used to test this, NVP-AAM077, has since been shown to be highly non-specific in rodent brain (Neyton and Paoletti, 2006) and it has not been tested for a possible action on NR3A containing NMDARs. In our mEPSC assay, we were unable to show a requirement for the NR2A subunit. This is the first time that this has been confirmed by using a genetic modification to knock out NR2A subunits. Previous studies have shown the lack of NR2A containing preNMDARs using specific NR2A antagonists NVP-AAM077 and $Zn^{2+}$. The two functional studies (Mallon et al., 2004; Suarez and Solis, 2006) that show a role for NR2A in evoked release may reflect a difference between brain regions, or there may be a difference in the roles of preNMDARs depending on their subunit configuration. The reported NR2A requirement in preNMDARs has been suggested to involve preNMDARs located in axons and activated to enhance excitability (Mallon et al., 2004; Suarez and Solis, 2006). Furthermore, there may be differences in timeline of expression as the studies showing NR2B containing preNMDARs are primarily in very young animals (Sjostrom et al., 2003; Yang et al., 2006; Corlew et al., 2007; Corlew et al., 2008) and the evidence for NR2A is shown in older animals (Mallon et al., 2004; Suarez and Solis, 2006). Our results show that in L2/3 pyramidal cells in V1 of young mice the NR2A subunit is not required for enhancement of spontaneous glutamate release.

### 3.4.3 Other possible subunits

NR2D subunit activation has recently been suggested to be required for the induction of preNMDAR-mediated tLTD in the L4 → L2/3 synapse in rodent barrel cortex (Banerjee et al., 2009). We attempted to determine whether NR2D might also be required for the preNMDARs' enhancement of spontaneous release. We used UBP141 (the most specific antagonist for NR2D/2C available) and the one that was used in Banerjee et. al 2009 (Banerjee et al., 2009). Unfortunately, we found that UBP141 not only decreased mEPSC frequency in our recordings, but also significantly decreased the amplitude (R. Larsen unpublished) suggesting a block of AMPAR-mediated currents. We further tested UBP141 on AMPAR-mediated currents and found that, indeed, UBP141 does partially block AMPAR currents (R. Larsen unpublished). It is possible that the decrease in mEPSC frequency is due to a decrease in amplitude. Therefore, we are unable to test for its presence in preNMDARs by this assay. Perhaps future anatomical analysis would be instructive. However, our previous findings that both NR2B and NR3A are required for the activation of preNMDARs make it unlikely that NR2D is also involved. Another possibility is that there are two different populations of preNMDARs with different subunit compositions. One report in barrel cortex suggests that preNMDARs contain the NR2B subunit in the L2/3-L2/3 connection while in the L4-L2/3 connection, preNMDARs contain NR2D (Rodriguez-Moreno and Paulsen, 2008). Our data would suggest that all preNMDARs require NR3A but the other subunit could be NR2B or another subunit. In order to test this, we can record evoked activity in the L4-L2/3

connection and in the L2/3-L2/3 connection and use ifenprodil to selectively block NR2B containing receptors. We can analyze the probability of release, as in Chapter 2, by looking at the synaptic depression before and after ifenprodil is added. If there are two separate populations of preNMDARs, then we would expect to see a decrease in synaptic depression with ifenprodil application in one of the pathways, but not the other. We have already tested the requirement for NR3A in preNMDARs enhancement of evoked activity in the L4-L2/3 synapse in V1 of young mice. (R. Larsen and R. Corlew unpublished). While application of APV increases paired pulse facilitation in WT mice (indicating preNMDARs which contribute to glutamate release probability), it does not change paired pulse facilitation in NR3A KO mice (R. Larsen and R. Corlew unpublished). Therefore, the NR3A subunit is required for preNMDARs' mediated enhancement of spontaneous and evoked glutamate in the young visual cortex at least in the L4-L2/3 synapse.

### 3.4.4 PreNMDARs are NR1/NR3A/NR2B tri-heteromeres

The culmination of the ifenprodil experiments and the NR3A KO experiments lead us to conclude that preNMDARs are tri-heteromeric receptors containing NR2B, NR3A and NR1 subunits. This hypothesis is consistent with findings that NR3A typically co-immunoprecipitates with NR1 and NR2 subunits (Perez-Otano et al., 2001). Importantly, ifenprodil (which blocks preNMDAR functions) antagonizes both NR1/NR2B diheteromers and NR1/NR2B/NR3A triheteromeric receptors (Smothers and Woodward, 2003). While NR3A slightly

reduces the permeability of NMDARs (Perez-Otano et al., 2001), these receptors lack block by $Mg^{2+}$ (Sasaki et al., 2002), which allows them to be activated by glutamate in the absence of strong depolarizations. The developmental profile of NR3A perfectly matches the time-course for the loss of preNMDARs. Furthermore, because NR3A-containing NMDARs have a high glycine affinity (Nilsson et al., 2007), the observation that preNMDARs contain NR3A could explain why presynaptic, but not post-synaptic, NMDARs are saturated by glycine at rest (Li and Han, 2006). One study came to the conclusion that preNMDARs are unlikely to contain NR3A because D-serine enhanced mEPSC frequency (Li and Han, 2006), but this conclusion was based the authors' erroneous belief that D-serine was an *antagonist* of NR3A-containing NMDARs. However, a recent study convincingly demonstrated that D-serine is an *agonist* of NR3A-containing NMDARs (Yao and Mayer, 2006), so the re-interpretation of these studies provides further evidence that preNMDARs may contain NR3A.

### 3.4.5 Maturity quiets the receptor but does not completely remove it

We show that without the NR3A subunit in a NR3A KO, preNMDARs can still affect spontaneous release if the $Mg^{2+}$ is withheld from the recording solution. These data also explain the developmental loss of the functional receptor. While the preNMDAR appears to be completely lost with development, anatomical evidence suggests that the receptor is still present, though to a lesser degree. The presence of the receptor in the older animal was also confirmed. At a time when NR3A subunit is at extremely low levels in the visual cortex, the

preNMDAR appears to be non-functional. However, its activity can be restored with the exclusion of $Mg^{2+}$ from the ACSF. The function of this "quiet" receptor in the adult is unknown. The $Mg^{2+}$ sensitive receptor might function much more like the classic coincidence detector, representing an important, long-term modulator in the presynaptic terminal (Bender et al., 2006b).

# Chapter 4:

# Visual deprivation modifies both presynaptic glutamate release and composition of pre/perisynaptic/extrasynaptic NMDA receptors in adult visual cortex.

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## 4.1 Introduction

Use-dependent modifications of synapses have been well described in the developing visual cortex, but the ability for experience to modify synapses in the adult visual cortex is poorly understood. We have found that 10 days of late-onset visual deprivation (LOVD) modifies both pre- and postsynaptic elements at the layer (L) 4 to 2/3 connection in the visual cortex of adult mice, and these changes differ from those observed in juveniles. While dark-rearing (DR) in juvenile mice modifies the subunit composition and increases the current duration of synaptic NMDA receptors (NMDARs), no such effect is observed at synapses between L4 and L2/3 pyramidal neurons in adult mice. Surprisingly, LOVD in adult mice enhances the temporal summation of NMDAR-mediated currents induced by bursts of high-frequency stimulation. The enhanced temporal summation of NMDAR-mediated currents in deprived cortex could not

be explained by a reduction in the rate of synaptic depression, because our data indicate that LOVD actually increases the rate of synaptic depression. Biochemical and electrophysiological evidence instead suggest that the enhanced temporal summation in adult mice could be accounted for by a change in the molecular composition of NMDARs at peri-/extrasynaptic sites. Our data demonstrate that the experience-dependent modifications observed in the adult visual cortex are different from those observed during development. These differences may help explain the unique consequences of sensory deprivation on plasticity in the developing versus mature cortex.

Sensory experience modifies cortical circuitry by inducing use-dependent changes in synapses (Katz and Shatz, 1996), and these modifications are generally thought to be more dramatic in developing animals than in adults. Monocular deprivation has been a well-studied model for critical period plasticity since the pioneering studies of Wiesel and Hubel (Wiesel and Hubel, 1963). Their finding that cortical neurons lose responsiveness to the deprived eye only if the deprivation begins early in life has led to the assumption that the adult cortex lacks the capacity for experience-dependent modifications.

There is a growing consensus, however, that the adult cortex maintains greater plasticity than originally thought (Buonomano and Merzenich, 1998; Gilbert, 1998; Tagawa et al., 2005). For example, monocular deprivation shifts the ocular dominance of neurons in the primary visual cortex of adult mice, although the manner of the shift differs from that observed in juveniles (Sawtell et al., 2003; Frenkel and Bear, 2004; Lickey et al., 2004). In juvenile mice,

monocular deprivation causes a rapid reduction of the deprived eye response recorded in the contralateral cortex, followed by a potentiation of the responses driven by the non-deprived eye in the ipsilateral cortex. In contrast, monocular deprivation in adult mice fails to cause a loss of the deprived eye response, although a delayed potentiation of the responses driven by the non-deprived eye is still observed. The basis for the different consequences of sensory deprivation in young and mature animals is poorly understood, and we suggest that key differences may lie in the mechanisms that control the properties of synaptic plasticity.

The ocular dominance plasticity observed in both juvenile and adult mice requires activation of NMDARs. NMDARs are required for many forms of synaptic plasticity (Malenka and Bear, 2004), and changes in the receptor's attributes are likely to influence the properties of synaptic plasticity. The NMDAR complex consists of the obligatory NR1 subunit in combination with NR2A-D and NR3A-B subunits that confer distinct receptor properties (Monyer et al., 1992; McBain and Mayer, 1994; Perez-Otano and Ehlers, 2004). NR1, NR2A, and NR2B subunits predominate in the postnatal visual cortex, and during development the ratio of NR2A- to NR2B-containing NMDARs increases (Quinlan et al., 1999a; Quinlan et al., 1999b; Roberts and Ramoa, 1999). Because NR2A-containing NMDARs possess shorter current durations than NR2B-containing receptors, NMDAR-mediated current durations shorten over development (Carmignoto and Vicini, 1992; Hestrin, 1992; Monyer et al., 1992; Priestley et al., 1995; Flint et al., 1997; Vicini et al., 1998). The developmental

increase in NR2A in the visual cortex is experience-dependent, as dark-rearing delays the increase in NR2A in the visual cortex (Nase et al., 1999; Quinlan et al., 1999a). Given the age-dependent differences in the synaptic consequences of sensory deprivation, we examined whether visual deprivation uniquely affects NMDAR composition and function in the visual cortex of juvenile and adult mice. Our results indicate that intracortical synapses of adult mice are highly plastic but undergo use-dependent modifications in a unique manner compared to juveniles.

## 4.2 Materials and Methods

*Animals* C57BL/6 mice (Charles River, MA) of both genders between postnatal (P) day 21-27 or P74-84 were used. These ages represent periods during and after the classically defined critical period for ocular dominance plasticity in mice (Gordon et al., 1996). Normally reared (NR) mice were raised on a 12:12 light:dark cycle, whereas dark-reared (DR) mice were raised in complete darkness from P2. Late-onset visual deprivation (LOVD) was achieved by placing animals into a completely dark room for ~10 days starting at ~P68.

*Slice Preparation* was identical to Chapter 2 (Corlew et al., 2007).

*Voltage-clamp recordings* Electrophysiology was performed as in Chapter 2 (Corlew et al., 2007) except for the following additions. To pharmacologically isolate NMDAR-mediated currents, ACSF modified to contain (in mM): 124 NaCl, 3 KCl, 1.25 $NaH_2PO_4$, 26 $NaHCO_3$, 20 glucose, 4 $MgCl_2$, 4 $CaCl_2$, 0.001 glycine, 0.05 picrotoxin, and 0.02 CNQX or DNQX. CNQX/DNQX was omitted when recording AMPA receptor (AMPAR)-mediated currents. Internal solution

contained (in mM) 102 cesium gluconate, 5 TEA-chloride, 3.7 NaCl, 20 HEPES, 0.3 sodium guanosine triphosphate, 4 magnesium adenosine triphosphate, 0.2 EGTA, 10 BAPTA, and 5 QX-314 chloride (Alomone Labs, Israel), with pH adjusted to 7.2 and osmolarity adjusted to ~300 mmol/kg by addition of sucrose. For voltage-clamp recordings, recorded series resistance averaged 21.8 ± 0.9 MΩ and no series resistance compensation was applied. Input resistances recorded at +40 mV did not differ between deprived and control groups at P21-27 (110.4 ± 6.0 MΩ) or at P74-84 (92.3 ± 5.4 MΩ). Excitatory postsynaptic currents (EPSCs) were evoked from a stimulating electrode (concentric bipolar; 200 μM tip separation) placed in layer 4, and stimulation was given for 200 μs every 15 sec. To describe the deactivation kinetics of NMDAR-mediated currents recorded at +40 mV, 30-60 evoked NMDAR EPSCs were averaged, and the current decays were described using the following formula: $I(t) = I_f \exp(-t/\tau_f) + I_s \exp(-t/\tau_s)$, where I is the current amplitude, t is time, $I_f$ and $I_s$ are the peak amplitudes of the fast and slow components, respectively, and $\tau_f$ and $\tau_s$ are their respective time constants. A nonlinear regression in pCLAMP software was used to fit double exponentials to decay curves. The weighted time constant ($\tau_w$) was used for quantification purposes and was calculated as: $\tau_w = \tau_f * (I_f/(I_f + I_s)) + \tau_s * (I_s/(I_f + I_s))$. To examine functional changes in the short-term depression of AMPAR-mediated currents recorded at -70 mV, 11 pulses at 40 Hz were given every 6 sec. The time constant of AMPAR EPSC depression was obtained by fitting the following single exponential formula: $I_{net}(t) = K\exp(-t/\tau_d) + PL$, where $I_{net}$ is the normalized net current amplitude, $\tau_d$ is the time constant of the synaptic

depression, PL is the normalized steady-state EPSC amplitude, and K + PL = 1. To measure the kinetics of MK-801 blockade, isolated NMDAR EPSCs were first measured at +40 mV and stimulation intensity was adjusted to evoke ~100 pA response. MK-801 (40 μM) was added to the bath and responses were evoked every 15 sec until the NMDAR-mediated response was abolished. The time constant of MK-801 blockade ($\tau_{block}$) was calculated using the following formula: $I(t) = I_1 \exp(-t/\tau_{block})$, where I is the current amplitude, $I_1$ is the amplitude of the first pulse, and t is time.

*Biochemical fractions* Each of the biochemical fractions was prepared using visual or frontal cortices pooled from 3-5 brains with a procedure modified from Cho and colleagues (Aramori and Nakanishi, 1992). Comparisons were made from fractions run in parallel to minimize variability among preparations. Samples were homogenized in HEPES-buffered sucrose (4 mM HEPES, 0.32 M sucrose, pH 7.4) using a motor-driven dounce homogenizer. Post-nuclear supernatant (PNS) fractions were prepared by centrifuging the homogenates twice at 1,000 x *g* for 10 min to eliminate nuclei. The PNS fractions were centrifuged at 10,000 x *g* for 20 min yielding crude synaptic pellets, which were then suspended in HEPES-buffered sucrose and centrifuged. The resulting pellets were lysed in a hypoosmotic buffer (4 mM HEPES, pH 7.4) using the motor-driven dounce homogenizer and mixed constantly for 30 min. The lysates were centrifuged at 25,000 x *g* for 20 min and pellets were suspended in HEPES-buffered sucrose to obtain lysed synaptosomal membrane (LSM) fractions. The LSM fractions were subjected to density centrifugation (150,000 x *g*, 2 hrs) using

a gradient consisting of 0.8 M, 1.0 M, and 1.2 M sucrose in 4 mM HEPES (pH 7.4). Synaptic plasma membrane fractions were collected at the 1.0-1.2 M interface, diluted with 4 mM HEPES, and pelleted (150,000 x *g*, 30 min). These pellets were resuspended in 50 mM HEPES (pH 7.4) containing 0.5% Triton X-100, rotated for 15 min, and centrifuged at 32,000 x *g* for 20 min. The resulting pellets were resuspended in the 0.5% Triton-containing buffer, rotated for 15 min, and centrifuged at 200,000 x *g* for 20 min to obtain postsynaptic density (PSD) fractions, which were suspended in 50 mM HEPES containing 0.2% SDS. Complete protease inhibitor cocktail tablets (Roche, Germany), pepstatin 10 μg/ml, and phosphatase inhibitor cocktail 1 & 2 (Sigma, MO) were added to all buffers. The above procedures were carried out on ice or in a cold room and the fractions were stored at -80$^{o}$C. Protein concentrations were measured using Coomassie Plus reagent (Pierce, IL).

*Immunoblot analysis* PNS, LSM, and PSD fractions (10 μg) were resolved by 7.5% SDS-PAGE (Ready Gels, Bio-Rad, PA) and transferred to nitrocellulose membranes. Both blotting and imaging with the Odyssey imaging system (LI-COR, NE) were carried out following the manufacturer's protocols. Primary antibodies were anti-NR2A rabbit antibody (1:500, sc-9056, Santa Cruz, CA), anti-NR2B goat antibody (1:20,000, sc-1469, Santa Cruz, CA), anti-PSD-95 monoclonal antibody (1:500, MAB1596, Chemicon, CA), and anti-β-tubulin monoclonal antibody (1:3000, MAB3408, Chemicon, CA). The employed secondary antibodies were Alexa Fluor 680-labeled anti-goat IgG antibody (1:5,000, Molecular Probes, OR), Alexa Fluor 680-labeled anti-mouse IgG

antibody (1:5,000, Molecular Probes, OR), and IRDye 800-labeled anti-rabbit IgG antibody (1:3,000, Rockland, PA).

*Statistics* Data are expressed as means ± SEM. ANOVA or *t*-tests were used to test for statistical significance, which was placed at $p < 0.05$.

## 4.3 Results

### 4.3.1 Visual deprivation lengthens the decay kinetics of synaptic NMDAR-mediated currents in developing but not adult mice

We first determined whether visual deprivation affects NMDARs in the visual cortex of juvenile mice in a manner similar to that observed in rats. Previous studies demonstrated that dark-rearing (DR) or 5 days of visual deprivation in juvenile rats decreases the relative expression of NR2A- to NR2B-containing NMDARs, resulting in longer NMDAR-mediated currents (Carmignoto and Vicini, 1992; Flint et al., 1997; Philpot et al., 2001a). We found that a similar process occurs in mice. Pharmacologically isolated NMDAR-mediated currents evoked by stimulating L4 were measured in layer 2/3 pyramidal neurons in primary visual cortical slices. DR until P21-27 significantly increased the duration of NMDAR-mediated currents compared to normally-reared (NR) mice (Fig. 4.1; DR, $\tau_w = 130.7 \pm 6.5$ ms, n = 21 cells; NR, $\tau_w = 82.1 \pm 7.6$ ms, n = 16 cells; $p < 0.00003$). These data suggest that DR in juvenile mice modifies the composition and function of synaptic NMDARs, as has been observed in rats.

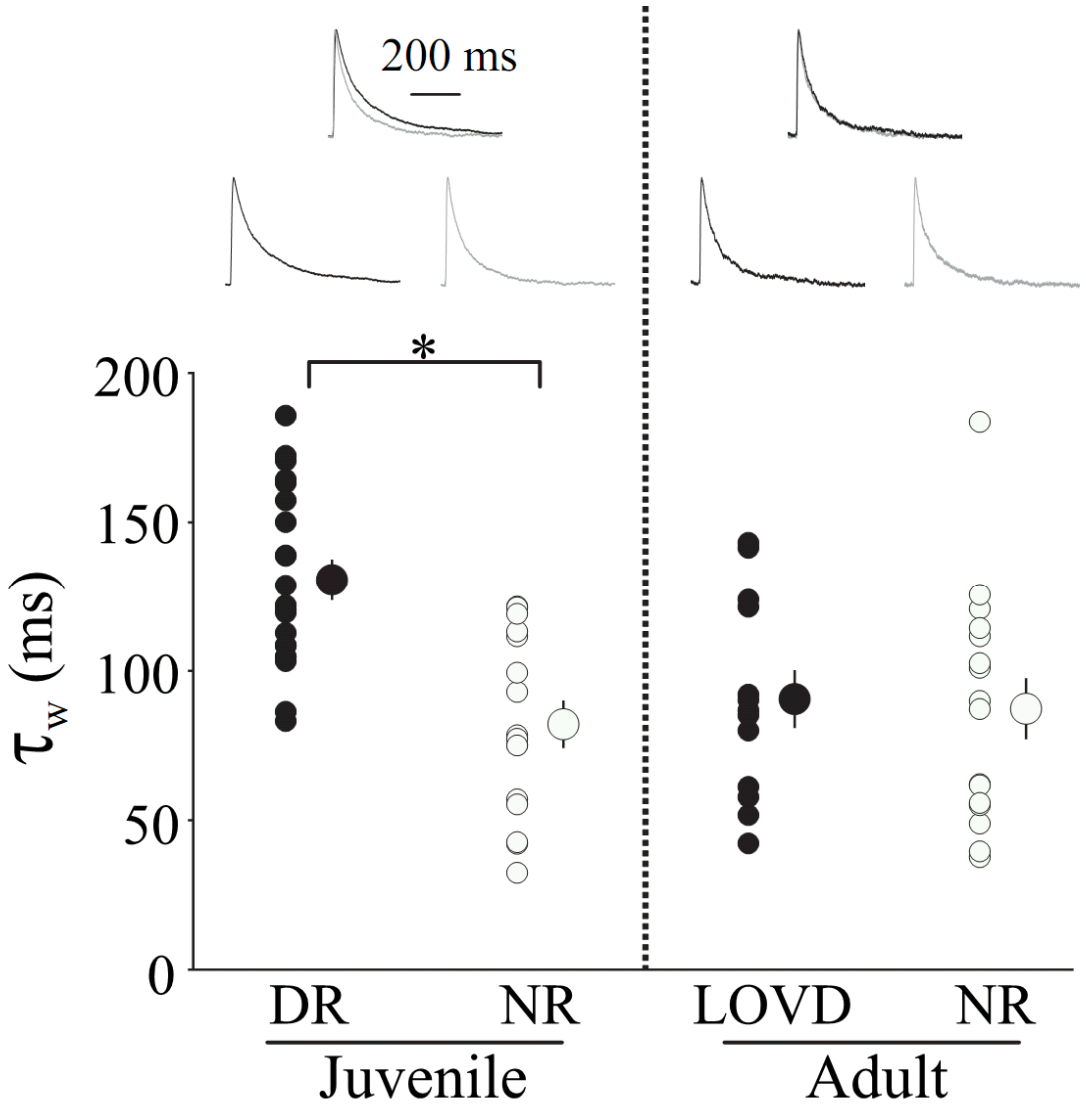
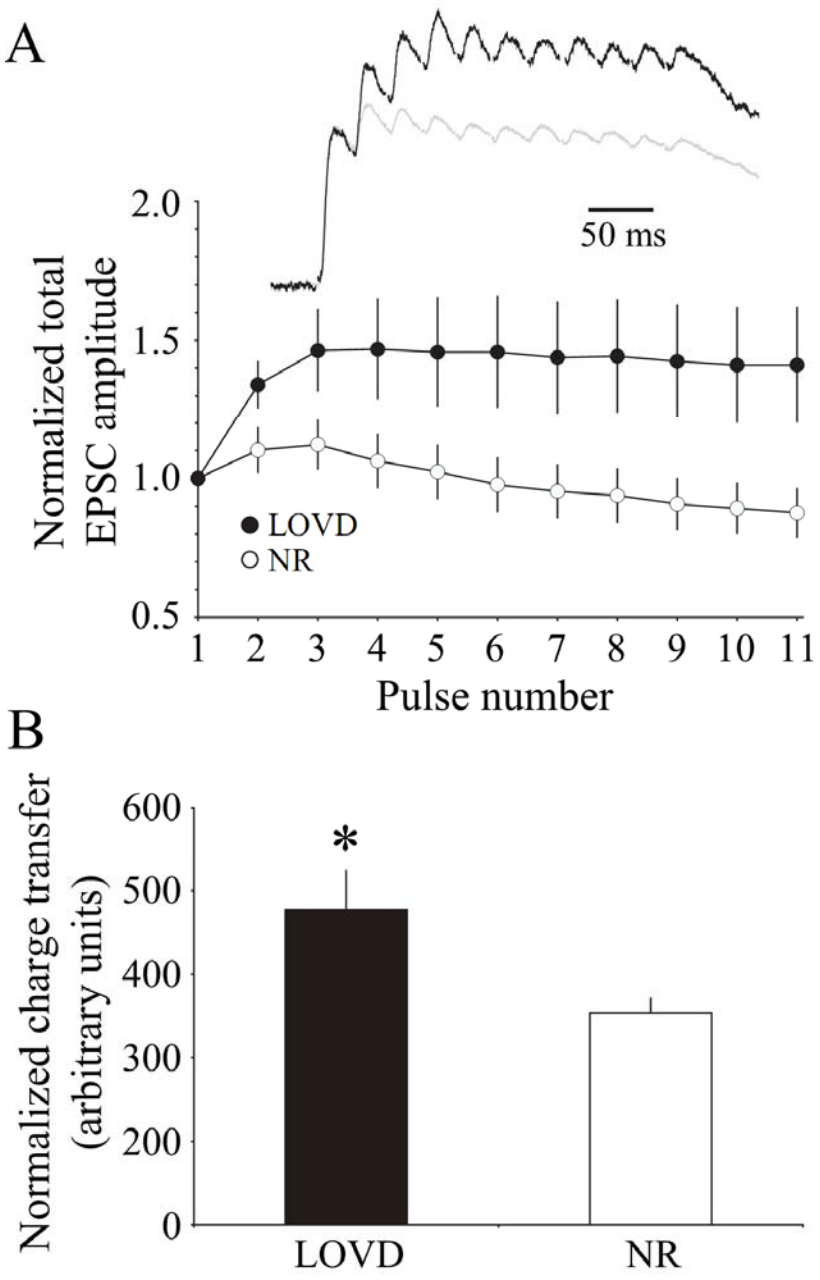



**Figure 4.1 Visual deprivation in adult mice fails to modify NMDAR EPSCs evoked by single pulses.** Scatter plot of the weighted time constants ($\tau_w$) of NMDAR-mediated EPSCs recorded from L2/3 pyramidal cells after stimulating L4 in the visual cortex of DR and NR juvenile mice as well as LOVD and NR adult mice. Small circles represent individual data points and larger circles represent means (±S.E.M.). NMDAR-mediated currents are significantly longer in the visual cortex of DR juvenile mice compared to NR. LOVD in adult mice does not alter NMDAR-mediated current duration. Normalized traces are representative of pharmacologically isolated NMDAR EPSCs recorded at +40 mV, and an overlay of the traces (top) is included as a basis for comparisons. *$p < 0.05$.

To determine whether experience-dependent modifications in synaptic NMDARs could be elicited outside a critical period of development, we examined the consequences of 10 days of late-onset visual deprivation (LOVD) in the visual cortex of adult mice. Unlike juvenile rodents, we failed to observe a change in NMDAR EPSC decay kinetics between LOVD and NR adult mice (Fig. 4.1; LOVD, $\tau_w$ = 90.5 ± 9.3 ms, n = 13 cells; NR, $\tau_w$ = 87.3 ± 9.8 ms, n = 16 cells; p = 0.82). These data indicate that, in adult mice, visual deprivation does not change the function of NMDARs driven by a single synaptic activation of L4-2/3.

### 4.3.2 Visual deprivation enhances the temporal summation of NMDAR EPSCs in adults

We have previously demonstrated in juvenile rodents that the temporal summation of NMDAR-mediated currents is tightly correlated with the duration of individual EPSCs; the longer the NMDAR currents, the greater the magnitude of temporal summation (Philpot et al., 2001a). Because of the similar duration of NMDAR EPSCs in visual cortical pyramidal cells from LOVD and NR adult mice, we expected that the temporal summation of NMDAR-mediated currents would be nearly identical between the groups. To test this possibility, we delivered bursts of 40 Hz stimulation (11 pulses) to L4 and measured the response in L2/3 pyramidal cells in LOVD and NR cortices (LOVD: n = 15 cells; NR: n = 22 cells). We adjusted stimulus intensity to obtain ~100 pA response on the first pulse (LOVD = 113.5 ± 13.1 pA; NR = 103.4 ± 7.6 pA; p = 0.48). Surprisingly, we observed that LOVD greatly enhanced the temporal summation of NMDAR-




**<u>Figure 4.2</u> Visual deprivation in adult mice alters the temporal summation of NMDAR EPSCs evoked by burst stimulation in the visual cortex.** (**A)** Plot of the normalized and averaged amplitudes of NMDAR EPSCs evoked at 40 Hz in the adult visual cortex at a holding potential of +40 mV. Representative traces of pharmacologically isolated NMDAR EPSCs in response to 40 Hz stimulus trains are shown (dark trace = response from pyramidal neuron in LOVD mice; light trace = response from pyramidal neuron in NR mice). Stimulus artifacts were blanked for clarity. (**B)** Same as in A, but recordings were made at a holding potential of -70 mV in ACSF containing nominal magnesium (0.1 mM $MgCl_2$).

mediated currents in the adult visual cortex (Fig. 4.2A: amplitude of the 11$^{th}$ pulse/1$^{st}$ pulse; LOVD = 1.33 ± 0.15; NR = 0.96 ± 0.005; $p < 0.02$). The LOVD-induced enhancement of temporal summation was also significant when quantified by averaging the charge transfer (integral) of the normalized currents (LOVD = 354.7 ± 32.1 arbitrary units = a.u.; NR = 259.4 ± 14.0 a.u.; $p< 0.005$).

To determine whether the experience-dependent differences in temporal summation arose from recording at a depolarized voltage (+40 mV), we repeated the experiment in nominal magnesium (0.1 mM) while clamping cells at -70 mV. The deprivation-induced enhancement of temporal summation was also observed when postsynaptic cells were clamped at the hyperpolarized membrane potential (Fig. 4.2B: amplitude of the 11$^{th}$ pulse/1$^{st}$ pulse: LOVD = 1.41 ± 0.15, n = 16 cells; NR = 0.76 ± 0.08, n = 12 cells; $p < 0.003$; Normalized charge transfer: LOVD = 397.4 ± 36.3 a.u.; NR = 266.5 ± 27.3 a.u.; $p < 0.02$). This observation suggests that the effects on temporal summation are unlikely to be due to an experience-dependent change in an intrinsic membrane current that has voltage-sensitive properties.

Thus, even though visual deprivation did not alter synaptic NMDAR-mediated currents evoked by a single stimulation, visual deprivation nevertheless enhanced the temporal summation of NMDAR-mediated currents. The enhanced temporal summation could be explained by a change in (1) presynaptic neurotransmitter release or (2) a population of peri-/extrasynaptic NMDARs that is activated with bursts of stimulation.

### 4.3.3 Visual deprivation increases the release probability of glutamate in adult mice

The temporal summation of NMDAR-mediated currents is determined by both the postsynaptic summation of currents and the properties of presynaptic neurotransmitter release (e.g. the rate of synaptic depression or facilitation) (Zucker and Regehr, 2002). We initially hypothesized that the enhanced temporal summation of NMDAR-mediated currents could be due to a reduction in the rate of synaptic depression. Because AMPAR-mediated currents are much faster than NMDAR-mediated currents, there is little, if any, temporal summation of AMPAR EPSCs at frequencies $\leq$ 40 Hz. Thus, short-term plasticity of AMPAR-mediated currents is a good measure of changes in presynaptic neurotransmitter release. We examined the short-term plasticity of AMPAR-mediated EPSCs recorded at -70 mV in L2/3 pyramidal cells by giving 11 pulses of 40 Hz stimulation to layer 4. Contrary to our initial hypothesis, our data indicated that the rate of synaptic depression was significantly increased in the visual cortex of the LOVD mice (Fig. 4.3: $\tau_d$ ; LOVD = 64.9 ± 10.3 ms, n = 18 cells; NR = 129.5 ± 18.2 ms, n = 23 cells; $p < 0.007$). The normalized steady-state AMPAR EPSC amplitudes were unchanged by visual experience (LOVD = 0.187 ± 0033; NR = 0.130 ± 0.026; p = 0.17). These data suggest that visual deprivation increases the initial probability of neurotransmitter release and limits the relative amount of neurotransmitter available for subsequent release. Thus, the enhanced temporal summation of NMDAR-mediated currents in the cortex of deprived mice cannot be explained by a reduction in the rate of short-term

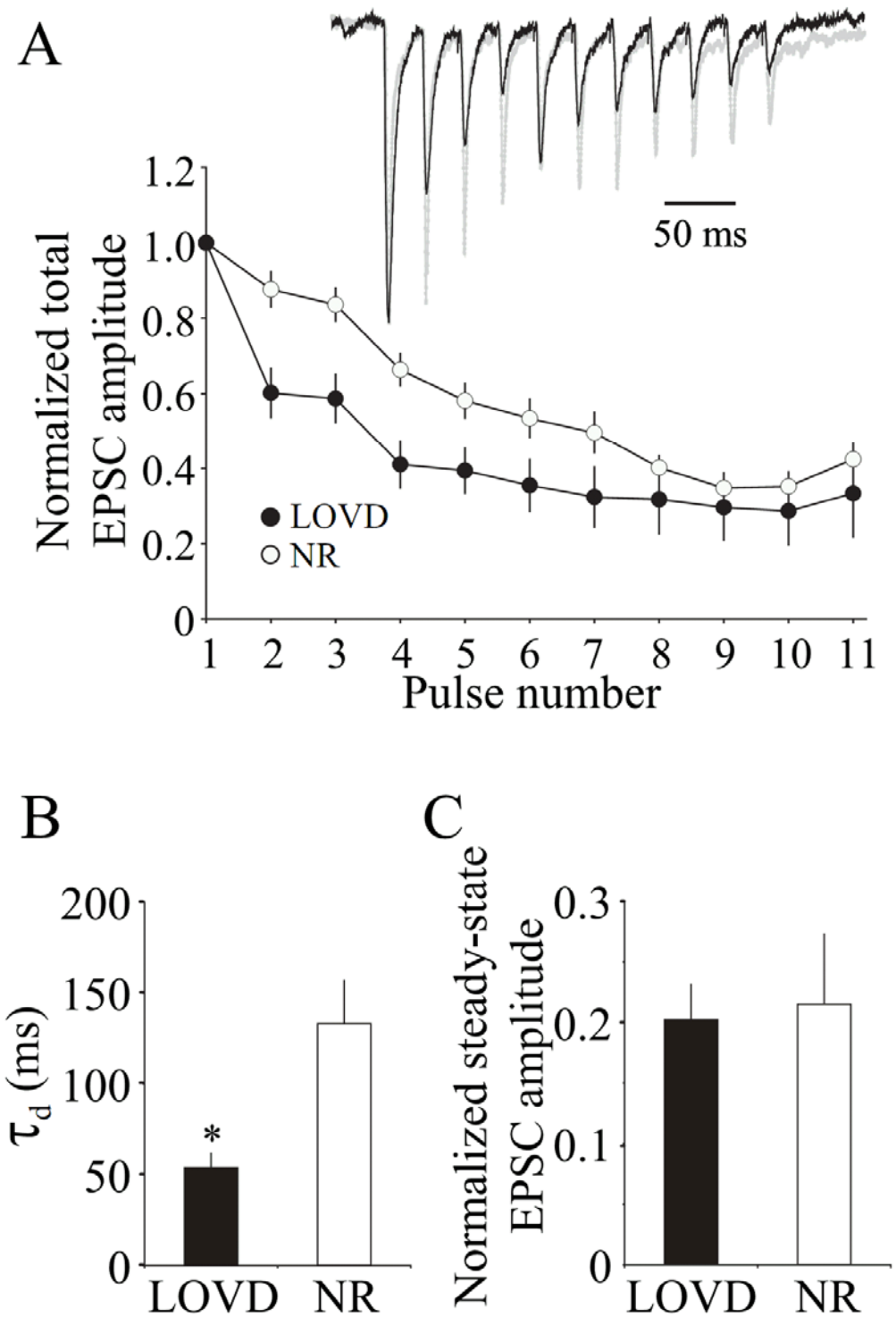



**Figure 4.3 Visual deprivation increases the rate of synaptic depression in adult mice.** Plot of the AMPAR EPSC amplitudes in response to a brief 40 Hz stimulation train. Responses were normalized to the first pulse. Traces are representative AMPAR EPSCs recorded at -70 mV in cells from LOVD (dark trace) and NR (light trace) mice. Stimulus artifacts were blanked for clarity.

synaptic depression. Because of the novel and unexpected observation that LOVD increases the rate of synaptic depression in the visual cortex of adult mice, we wanted to use an independent assay to verify that deprivation increases the probability of neurotransmitter release. We took advantage of the pharmacological properties of MK-801, an irreversible open-channel NMDAR blocker, to examine neurotransmitter release in NR and LOVD adult mice. The rate of block of NMDAR-mediated currents by MK-801 is an indicator of the probability of neurotransmitter release; the higher the probability of release, the faster the rate of block by MK-801 (Hessler et al., 1993). Consistent with the increased rate of synaptic depression of AMPAR-mediated currents, we observed that LOVD significantly accelerated the rate at which MK-801 blocks pharmacologically isolated NMDAR EPSCs (Fig. 4.4: $\tau_{block}$ ; LOVD = 9.30 ± 0.63 ms, n = 4 cells; NR = 20.00 ± 3.16 ms, n = 7 cells; $p < 0.04$). These results confirm that LOVD increases the probability of release in L4-2/3 synapses. Although the enhanced temporal summation of NMDAR EPSCs in adult mice cannot be explained by an increase in the rate of synaptic depression, it is possible that the increased probability of release could facilitate the spillover of glutamate to peri-/extrasynaptic sites (see Discussion).

### 4.3.4 Visual deprivation differentially reduces the NR2A/B ratio in biochemical fractions from the visual cortex of juvenile and adult mice

Our data suggest that the change in the rate of synaptic depression could not account for the enhanced temporal summation of NMDAR-mediated currents

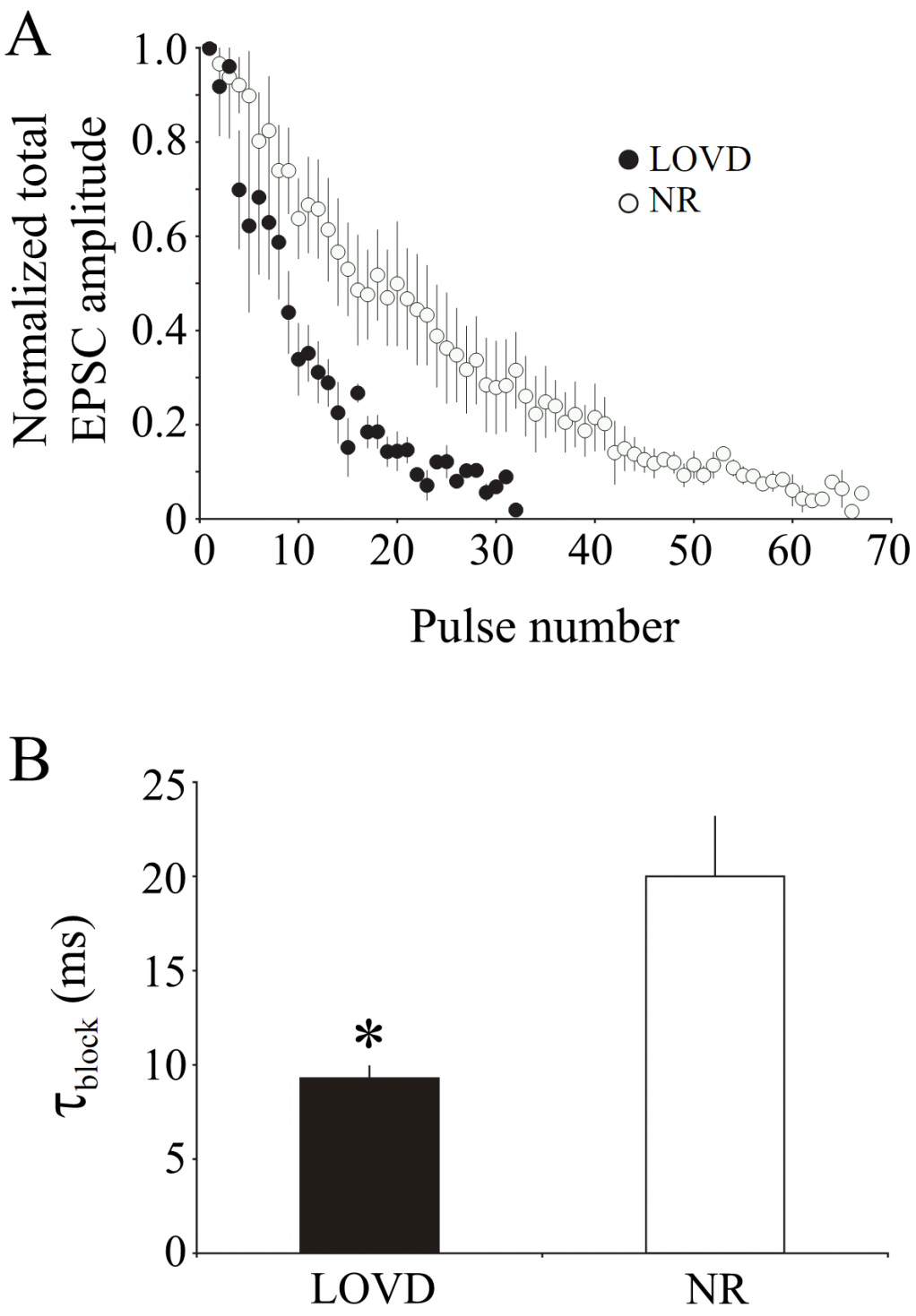
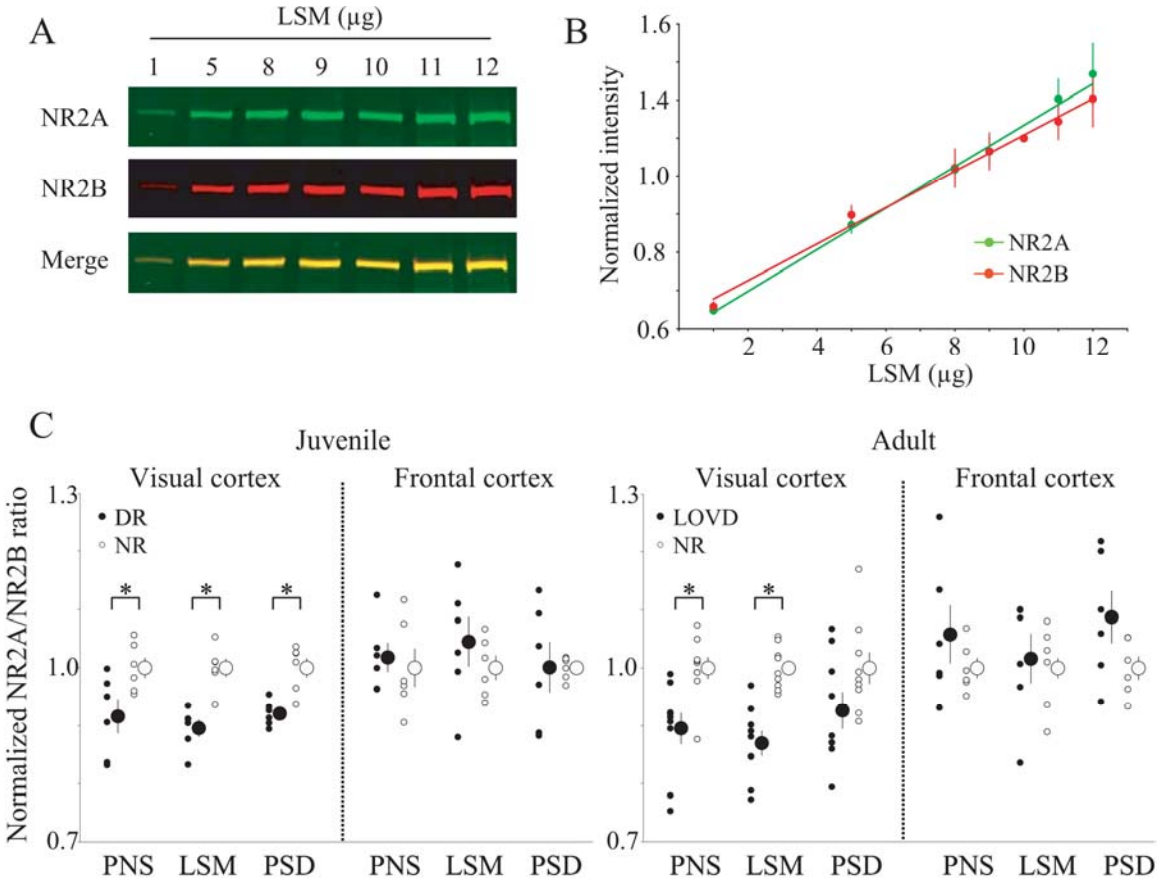



**Figure 4.4 Visual deprivation increases the rate of neurotransmitter release in adult mice.** Plot of the normalized amplitude of NMDAR EPSCs in response to repetitive stimulation in the presence of MK-801. Note that NMDAR EPSC blockade by MK-801 occurs faster in pyramidal cells from LOVD than NR adult mice.

in pyramidal cells of LOVD adult mice. We therefore decided to evaluate possible changes in postsynaptic NMDAR subunit composition. We have previously used the synaptoneurosome preparation to show that there is a correlation between the functional properties of NMDARs observed electrophysiologically and the subunit expression of NMDARs observed biochemically (Quinlan et al., 1999a; Quinlan et al., 1999b; Philpot et al., 2001b). The synaptoneurosome preparation, however, cannot distinguish protein expression in the postsynaptic density (PSD) from expression in other compartments near the synapse. Moreover, it was difficult to detect small changes in NMDAR composition with our previously employed chemiluminescent immunoblot techniques.

To overcome the limitations of our previously techniques, we produced enriched biochemical fractions that allowed us to differentiate proteins in the PSD from other compartments. We then analyzed these fractions using a novel immunoblotting technique using fluorescent secondary antibodies to NR2A and NR2B subunits (see Methods and Fig. 4.5). This method allowed us to achieve a highly quantitative measurement of the NR2A/B ratio due to the elimination of several sources of error. (1) The Odyssey infrared system operates within a very large linear range for quantification, thus errors from working within the small linear range using traditional immunoblots were eliminated. (2) The dual fluorescent labeling of NR2A and NR2B allowed us to compare band intensities within the same gel lane, eliminating errors introduced by variations in sample loading onto SDS-PAGE gels. (3) Membrane stripping was unnecessary, so no




**<u>Figure 4.5</u> Visual deprivation in juvenile mice alters the NR2A/B ratio in the postsynaptic density (PSD), but only in post-nuclear supernatant (PNS) and lysed synaptic membrane (LSM) fractions in adult mice. (A)** Biochemical fractionation progressively enriches NR2A, NR2B, and PSD-95 and eliminates a non-synaptic protein, β-tubulin, in visual cortical samples from LOVD and NR adult mice. 10 µg samples were loaded into each gel lane**. (B)** Quantification of NR2A and NR2B band intensities, which were normalized to the value at 12.5 µg. The inset is a representative NR2A/B immunoblot of a PSD fraction. 1 to 12 µg samples were loaded into each gel lane and results from three blots were averaged. **(C)** NR2A/B ratios were measured in PNS, LSM, and PSD fractions of visual cortices of deprived and control from both juvenile and adult mice. The values (means ± S.E.M.) are normalized to average control values. *$p < 0.05$.

error was introduced by incomplete stripping or overstripping. (4) NR2A and NR2B migrate through the SDS-PAGE gel to almost the same distance due to their similar molecular weights, eliminating errors introduced by differential transfer of the proteins from the gel to the nitrocellulose membrane. Although the technique still has the limitation that it is difficult to determine whether differences in the NR2A/B ratio are due to changes in NR2A, NR2B, or both, the advantages of the technique allowed us to detect modest differences in the ratio of NR2A/B with high precision and little variability.

We first examined NR2A/B expression in the visual cortex from DR and NR juvenile mice (n of each group = 6 pools of 5 mice each). To evaluate changes in NR2A/B expression, we examined three biochemical fractions: 1) the post-nuclear supernatant (PNS) fraction, containing both cytoplasmic and cell membrane contents, 2) the lysed synaptosomal membrane (LSM) fractions, which contained both synaptic and extrasynaptic components of the plasma membrane, and 3) the highly enriched postsynaptic density (PSD). Consistent with previous findings using synaptoneurosome preparations in rats (Quinlan et al., 1999a), we found that the NR2A/B ratios were significantly lower in the PNS, LSM, and PSD visual cortical fractions of DR juvenile mice compared to NR (Fig. 4.5C: PNS, $p < 0.03$; LSM, $p < 0.0006$; PSD, $p < 0.02$). To determine the effects of LOVD in adults, we compared the NR2A/B ratios in NR and LOVD adult mice (n of each group = 12 pools of 3-5 mice each). In contrast to what we observed in the visual cortex of DR juvenile mice, LOVD in adults failed to modify the composition of NMDARs within the highly enriched PSD (Fig. 4.5A,C: $p = 0.22$).

However, LOVD in adults significantly lowered the NR2A/B ratio in the PNS and LSM visual cortical fractions compared to the NR (Fig. 4.5C: PNS, $p < 0.006$; LSM, $p < 0.002$). In both juvenile and adult mice, the change in NMDAR subunit composition in NR and deprived mice did not appear to be the result of a general stress response, because no change in the NR2A/B ratio in the PNS, LSM, and PSD preparations were observed in frontal cortices of deprived mice compared to controls (p-values in all fractions from both juveniles and adults > 0.1, n = 6 pools of tissues for each of the six groups). These data indicate that LOVD in adults might alter the composition of NMDARs located at pre/peri-/extrasynaptic sites but not postsynaptic sites.

### 4.3.5 Visual deprivation does not alter the temporal summation of NMDAR-mediated currents evoked by minimal stimulation

Because high-frequency stimulation can additively facilitate diffusion of glutamate at synapses and induce activation of peri-/extrasynaptic NMDARs that are not activated by a single pulse (Lozovaya et al., 2004; Scimemi et al., 2004), we reasoned that the enhanced temporal summation of NMDAR-mediated currents in LOVD mice could be a consequence of glutamate spillover onto a modified population of peri-/extrasynaptic NMDARs. Previous studies demonstrate that glutamate spillover increases with EPSC size (Scimemi et al., 2004). That is, spillover is more likely to occur with an increase in the number of simultaneously activated synapses.

If synaptic spillover contributes to the deprivation-induced enhancement of NMDAR temporal summation, then we reasoned that we would be less likely to observe the effect when activating a lower density of synapses. To test this possibility, we examined the temporal summation of NMDAR EPSCs elicited by minimal stimulation. The assumption in these studies is that minimal stimulation activates one or a small number of afferents. In this experiment, stimulus intensity was adjusted to elicit a response to the 1$^{st}$ pulse ~50 % of the time. We then delivered 11 pulses at 40 Hz and analyzed only traces where there was a response to the first pulse (1$^{st}$ peak amplitude; LOVD = 14.1 ± 1.0, n = 14 cells; NR = 15.6 ± 1.6, n = 16 cells; p = 0.46). With this minimal stimulation protocol, temporal summation of NMDA EPSCs in LOVD and NR mice were almost identical as measured by the amplitude of the 11$^{th}$ pulse (Fig. 4.6: Amplitude of 11$^{th}$ pulse/1$^{st}$ pulse; LOVD = 1.15 ± 0.14; NR = 1.12 ± 0.12; p = 0.90) or by the normalized charge transfer (LOVD = 283.1 ± 25.7 a.u.; NR = 288.9 ± 30.3 a.u.: p = 0.89). These data indicate that a critical number of synapses must be activated, reflected by EPSC amplitude, to observe the deprivation-induced enhanced temporal summation. The data are consistent with the idea that the coordinated release of glutamate above a certain threshold of activated synapses can produce glutamate spillover sufficient to reach an extrasynaptic population of NMDARs that is modified by visual deprivation.

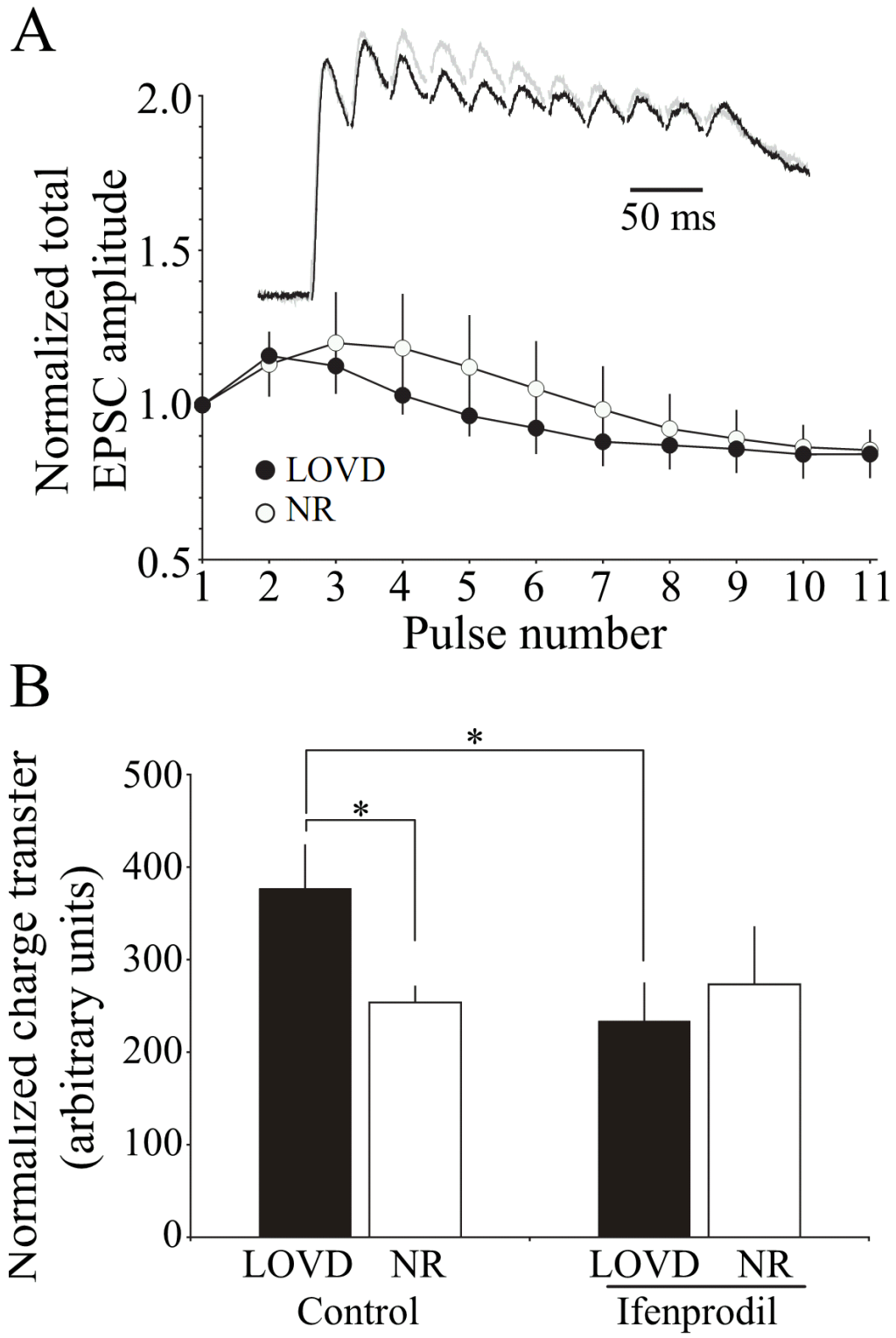



**Figure 4.6 Minimal stimulation fails to reveal deprivation-induced differences in the temporal summation of NMDAR-mediated currents.** Plot of the temporal summation of NMDAR EPSCs in LOVD and NR mice evoked by minimal stimulation at 40 Hz.

### 4.3.6 The NR2B-selective antagonist ifenprodil blocks the enhanced temporal summation of NMDAR EPSCs in deprived visual cortex of adult mice

The above data indicated that neither a change in the rate of synaptic depression nor a change in a voltage-sensitive membrane property could account for the enhanced temporal summation of NMDAR-mediated currents in the cortex of LOVD mice. However, the data also indicated that (1) LOVD in adult mice alters the complement of pre-/peri-/extrasynaptic NMDARs without significantly changing the postsynaptic NMDARs, and (2) a critical threshold of synaptic activation was required to observe the deprivation-induced enhancement of temporal summation. These observations are consistent with LOVD altering a population of peri-/extrasynaptic NMDARs that are activated by glutamate spillover occurring with coordinated bursts of stimulation. Previous studies suggest that NR2B-containing NMDARs can detect glutamate spillover (Scimemi et al., 2004), likely due to their high affinity for glutamate (Priestley et al., 1995). We used the NR2B-specific antagonist ifenprodil to determine whether the deprivation-induced enhancement of NMDAR temporal summation was mediated through activation of NR2B-containing receptors. Ifenprodil blocked the enhanced temporal summation of NMDAR EPSCs in LOVD mice (Fig. 4.7A: amplitude of $11^{th}$ pulse/$1^{st}$ pulse; LOVD = 0.84 ± 0.07, n = 6 cells; NR = 0.85 ± 0.06, n = 8 cells; p = 0.98). While ifenprodil dramatically reduced the temporal summation of NMDAR-mediated currents in the visual cortex of LOVD

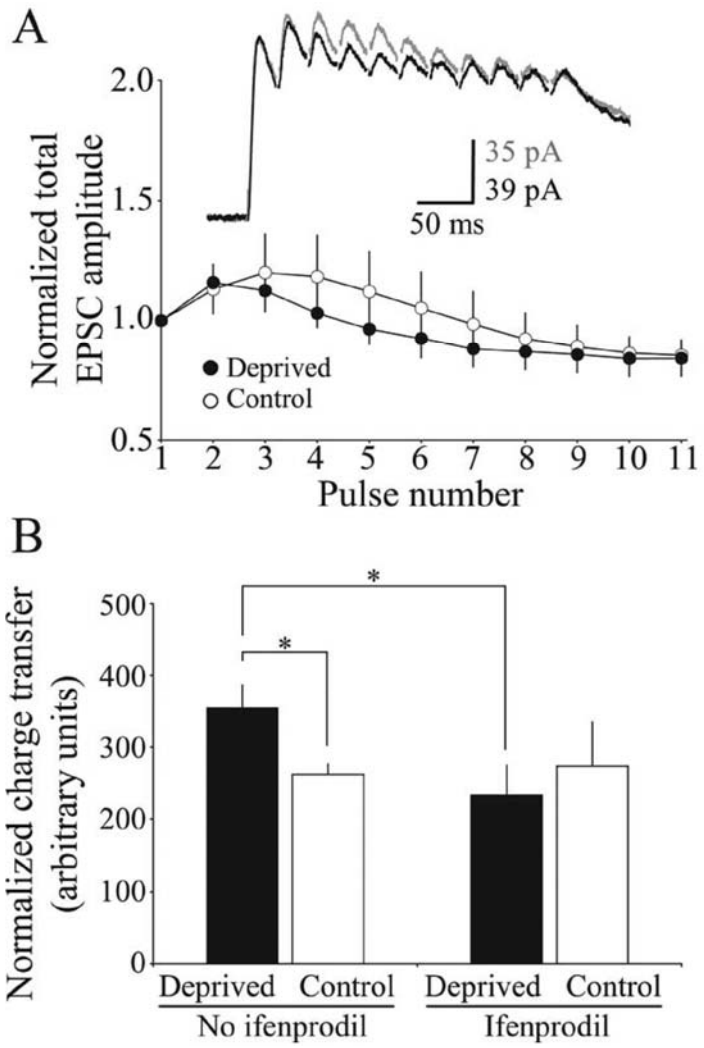



**Figure 4.7 The enhanced temporal summation of NMDAR EPSCs in deprived adult cortex can be blocked by acute administration of ifenprodil, an NR2B specific NMDAR antagonist. (A)** Temporal summation of NMDAR EPSCs in LOVD and NR mice in the presence of ifenprodil. (**B)** The average charge transfer taken from the normalized responses of the eleven pulses evoked at 40 Hz stimulation in the presence or absence of ifenprodil. ANOVA with post hoc analyses: *$p < 0.05$.

mice, the drug had no noticeable consequence in NR mice (Fig. 4.7B). These observations support our hypothesis that LOVD can increase the relative complement of NR2B-containing NMDARs at pre-/peri-/extrasynaptic sites but not synaptic sites. An idea consistent with our data is that these peri-/extrasynaptic NMDARs could be activated by glutamate spillover triggered by consecutive pulses, thus helping to explain why we observed a deprivation-induced enhancement of NMDAR-mediated temporal summation as quickly as the second pulse in a train of stimulation (Fig. 4.2).

## 4.4 Discussion

We demonstrate that a brief period (10 days) of late-onset visual deprivation (LOVD) enhances NMDAR-mediated transmission in the L4 → 2/3 visual cortical synapse by presynaptically increasing the probability of neurotransmitter release and by increasing the relative expression of NR2B-containing NMDARs at pre-/peri-/extrasynaptic sites. These results provide evidence that the history of sensory experience modifies synapses in the mature visual cortex outside of the critical period of receptive field plasticity and that these modifications differ between juvenile and adult animals.

Accumulating evidence indicates that the adult visual cortex is more plastic than previously thought, and our data show that one synaptic basis for adult plasticity is a change in the short-term dynamics of excitatory synaptic responses. The observed deprivation-induced increase in the rate of neurotransmitter release is likely a compensatory mechanism to maintain

synaptic drive in the absence of visually evoked activity. This increase in release is reminiscent of what has been observed in culture systems following manipulations that reduce presynaptic activity (Chavis and Westbrook, 2001) or postsynaptic excitability (Murthy et al., 2001). The deprivation-induced increase in release probability in the adult cortex enhances the likelihood that peri-/extrasynaptic NMDARs may be activated by glutamate spillover (Kullmann et al., 1996), and future studies will need to investigate whether this spillover could be augmented by a decrease in glutamate reuptake (Kim et al., 2005). Nonetheless, our data are consistent with the idea that visual deprivation increases a peri-/extrasynaptic population of ifenprodil-sensitive NMDARs that can be activated by glutamate spillover during bursts of high-frequency stimulation.

In addition to age-dependent differences in the presynaptic consequences of visual deprivation, our data demonstrate that there are also unique postsynaptic consequences to visual deprivation. Deprivation in juvenile animals decreases the NR2A/NR2B ratio at synaptic sites, but LOVD in adults only modifies pre-/peri-/extrasynaptic NMDARs. There is a precedent in the literature that NR2A-containing NMDARs are trafficked to synaptic sites, whereas NR2B-containing NMDARs are preferentially trafficked to peri-/extrasynaptic sites. For example, NMDARs appear to be eliminated from the central portion of the synapse in the superior colliculus of mice lacking the NR2A subunit (Townsend et al., 2003). One possibility is that NR2A is trafficked selectively to the synapse, but NR2B-containing NMDARs might be prevented from remaining in the

synapse once “slot” proteins for anchoring NR2A-containing NMDARs, such as PSD-95, have been delivered to the synapse (Yoshii et al., 2003). In support of this, manipulations of visual experience in developing mice are known to bidirectionally regulate the expression of synaptic NR2A in the visual cortex (Quinlan et al., 1999a). If, on the other hand, visual experience preferentially modifies NR2B but not NR2A levels in the adult cortex, then changes in NR2B-containing NMDARs might be detected only at peri/extrasynaptic sites because NR2A-containing NMDARs are entrenched in the central portion of the synapse. Our data provide evidence that this may indeed be the case, as we observe that the NR2B-selective antagonist ifenprodil eliminates the enhanced temporal summation of NMDAR-mediated currents in the visual cortex of deprived mice. In addition, detailed quantitative measurements of NR2A and NR2B levels suggest that LOVD increases NR2B levels rather than decreasing NR2A (E. Quinlan, personal communications).

We suggest that an increase in peri-/extrasynaptic NR2B-containing NMDARs postsynaptically could account for the deprivation-induced increase in the temporal summation of NMDAR currents. This conclusion is supported by three observations. (1) The deprivation-induced enhancement of temporal summation is only observed in conditions that favor glutamate spillover; the effect is not observed with minimal stimulation but is observed with stronger stimulation intensities. (2) Biochemical data indicate there is an increase in the relative proportion of NR2B-containing NMDARs at pre/extrasynaptic but not postsynaptic sites. (3) The NR2B-containing NMDAR antagonist ifenprodil

blocks the deprivation-induced enhancement of NMDAR EPSC temporal summation.

A possible complication to our interpretation of the data is that presynaptic NR2B-containing NMDARs are known to exist (Aoki et al., 1994) and to enhance neurotransmitter release in the visual cortex (Sjostrom et al., 2003). The deprivation-induced elevation in NR2B proteins within the lysed synaptic membrane fraction (Fig. 4.5) could be explained by an increase in presynaptic NMDARs, contributing to the observed increase in neurotransmitter release following deprivation. However, an increase in presynaptic NR2B-containing NMDARs is unlikely to account for the deprivation-induced enhancement of NMDAR-mediated temporal summation. If an increase in relative NR2B levels were restricted to presynaptic sites, we should have observed a similar trend in the short-term dynamics of AMPAR- and NMDAR-mediated currents following deprivation (Fig. 4.2 and 4.3). Specifically, an increase in release by presynaptic NR2B-containing NMDARs with deprivation would be expected to decrease the temporal summation of NMDAR-mediated currents in deprived mice, which was not what we observed. Hence, the most parsimonious explanation for our data is that visual deprivation in adult mice increases the relative population of NR2B-containing NMDARs at pre-/peri-/extrasynaptic sites. While future research is needed to address the physiological importance of the experience-dependent changes in glutamatergic synaptic transmission in the adult visual cortex, some clues may be provided by the very different consequences of monocular deprivation on ocular dominance in juvenile and adult mice (Sawtell et al., 2003;

Frenkel and Bear, 2004). Because visual cortex responses in rodents are largely driven by the contralateral eye, monocular deprivation essentially eliminates visual activity in the contralateral cortex except for a minor input driven by the ipsilateral eye. In juvenile mice, monocular deprivation leads to a rapid depression of the contralateral deprived-eye inputs, followed by a deprivation-enabled strengthening of the weak ipsilateral inputs from the non-deprived eye. The delayed strengthening of the previously weak inputs might be a consequence of lowering the threshold for synaptic potentiation by increasing the relative expression of NR2B-containing NMDARs in deprived cortex (Quinlan et al., 1999a; Quinlan et al., 1999b; Philpot et al., 2001a). In contrast to what has been observed in juvenile mice, the synapses in the mature cortex are normally stable and relatively resistant to modifications. One possibility is that the limited plasticity in the adult visual cortex is a consequence of low expression levels of NMDARs, especially the NR2B-containing NMDARs. Although monocular deprivation in the mature cortex fails to depress the inputs driven by the deprived eye, this manipulation can cause a delayed strengthening of the weak ipsilateral eye inputs. Perhaps the increase in the relative expression of NR2B subtypes at peri-/extrasynaptic sites, coupled with an increase in neurotransmitter release that can ensure their activation, provides a synaptic milieu that is permissive for the strengthening of normally weak responses.

The dependence of long-term potentiation and depression on the subunit composition of the NMDAR is heavily debated (Liu et al., 2004; Massey et al., 2004), but a number of recent studies indicate that NR2A- and NR2B-containing

NMDARs can both contribute to the induction of long-term depression and potentiation (Hendricson et al., 2002; Berberich et al., 2005; Heinbockel et al., 2005; Toyoda et al., 2005; Weitlauf et al., 2005). An intriguing possibility is that, under certain conditions, an increase in the relative expression of NR2B-containing NMDARs in the adult visual cortex can reinstate some aspects of synaptic plasticity that are normally lost during development. In support of this hypothesis, 10 days of LOVD in adult mice increases NR2B expression and simultaneously reinstates the ability to observe rapid ocular dominance shifts following monocular deprivation (E. Quinlan, personal observations).

In summary, our data provide direct evidence that sensory experience differentially modifies synaptic transmission in the cortex of juvenile and mature animals. These differences may provide a synaptic basis for why sensory deprivation has unique manifestations across development (modeled in Fig. 4.8)

# Chapter 5:

# Experience-dependent expression and function of preNMDARs

## 5.1 Introduction

In the sensory cortices of the immature brain, activity shapes the development of the neuronal circuitry (Fox and Wong, 2005; Tropea et al., 2009). This process determines how the brain will process sensory information for a whole lifetime, making correct sensory experience crucial for proper development. If appropriate sensory input is blocked, there can be devastating consequences on brain function. In humans, incorrect aligning of the eyes (termed strabismus) or childhood cataracts can cause Amblyopia, a condition that can leave afflicted patients cortically blind for the rest of their lives. Lack of proper stimulation can also cause learning disabilities and language disorders (Hall, 1998; Innocenti, 2007). It is commonly thought that there are critical periods in which sensory information must direct normal development, and that after these periods little can be done to correct for deficiencies. However, the neuroscience community has recently been changing its view on critical periods (Morishita and Hensch, 2008). Although there are sensitive periods where sensory information is particularly influential for changing brain function, it is now thought that the brain remains plastic throughout life. While the synaptic plasticity mechanisms may change with development, the ability to change is still

available. Clinical examples of this type of adult experience-dependent plasticity (Birnbaum, 1997; Simmers and Gray, 1999; Fronius et al., 2004) and a growing number of animal model studies (Sawtell et al., 2003; Yashiro et al., 2005; He et al., 2006; Hofer et al., 2006a, b; Sale et al., 2007) show that a remarkable amount of plasticity can be attained in the adult brain using short periods of visual deprivation.

An important affector of experience-dependent synaptic plasticity is the NMDA type glutamate receptor (NMDAR) (Kopp et al., 2007; Philpot et al., 2007; Yashiro and Philpot, 2008). Postsynaptic NMDARs have been thoroughly studied for their involvement in experience-dependent plasticity. However, we have recently found that preNMDARs are also present early in the developing visual cortex, where they enhance neurotransmitter release and support long-term depression (LTD) (Berretta and Jones, 1996; Sjostrom et al., 2003; Bender et al., 2006b; Corlew et al., 2007; Brasier and Feldman, 2008; Corlew et al., 2008). It is possible that, like their postsynaptic counterparts, preNMDARs may be themselves modified by sensory experience and key players in experience-dependent synaptic plasticity.

Here we investigated whether an altered visual environment could alter the function and expression of preNMDAR during development and adulthood. We explored three models for an altered visual environment: 1) We demonstrated that visual deprivation by dark rearing (DR) prevents the normal down-regulation of the preNMDARs' expression and function. 2) However, visual enrichment also produced the same prevention of the normal loss of the

receptor. We found that the effect of enrichment lasted well into adulthood, confirming that visual enrichment not only delays but also completely prevents the loss of this receptor. 3) Finally, we showed that the function of this receptor and its role in plasticity may be plastic well into adulthood. A short treatment of late-onset visual deprivation (LOVD) in adulthood was sufficient to rejuvenate both preNMDARs that had been lost with development and tLTD, a type of plasticity shown to require preNMDARs.

## 5.2 Materials and Methods

Subjects. C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) and used between postnatal day 7 (P7) and P90.
Mice were maintained on a 12h light/dark cycle (except when housed in the dark room) and fed ad libitum. Animals were either raised with a 4-inch length of 3-inch diameter PVC tube in their cage (tube-reared (TR)), or with no tube (normally-reared (NR)). No other enrichment was provided. Dark-reared animals were never raised with tubes. All experiments were performed under the animal care guidelines for the University of North Carolina at Chapel Hill.

Cortical slice preparation Prepared with identical methods as described in Chapters 2 and 4 (Yashiro et al., 2005; Corlew et al., 2007)

Voltage-clamp recordings Voltage clamp recordings and mEPSC analysis was done in an identical fashion as in Chapter 2 except that all recordings were performed with (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-

imine male ate (MK-801) (0.5–1mM) (MK801) in the postsynaptic recording pipette to block all postsynaptic NMDARs.

tLTD induction and short-term plasticity The internal recording solutions used for these experiments consisted of the following (in mM): 100 (K)gluconate, 20 KCl, 4 (Mg)ATP, 10 phosphocreatine, 0.3 GTP, and 10 HEPES, with pH adjusted to 7.25 and osmolarity adjusted to 290–295 mOsm. Extracellular stimulation produced a monophasic and fixed latency response, which we interpreted as predominantly coming from L4 or other vertical inputs. We feel that it is unlikely that we are activating local axon collaterals, because we never evoked antidromic action potentials (APs) at the low stimulation intensities used in these studies. Baseline stimulation was delivered once every 15s. After a 10–15 min stable baseline, AP and EPSP pairings were delivered 100 times (at~0.2Hz) with a postsynaptic action potential produced by brief (< 5ms) depolarization followed 5–25ms later by an EPSP evoked by L4 stimulation. After pairing, stimulation was delivered for 30 min at baseline frequencies.

Electron microscopy Mice were given an overdose of Nembutal and perfused transcardially with 0.9% saline solution for 1 min, followed by a mixture of 2% paraformaldehyde (dissolved in 0.1 M phosphate buffer at pH 7.4 and 2% gluteraldehyde [electron microscope (EM) grade; Electron Microscopy Science Hatfield, PA]) for 15 min. After perfusion, brains were postfixed at 4 ºC for 48 hours in the same fixative. A vibrating microtome was used to cut 200µm sections coronally through the visual cortex. Pieces were then isolated from slices containing L2/3 of V1. Further processing, preparation, and

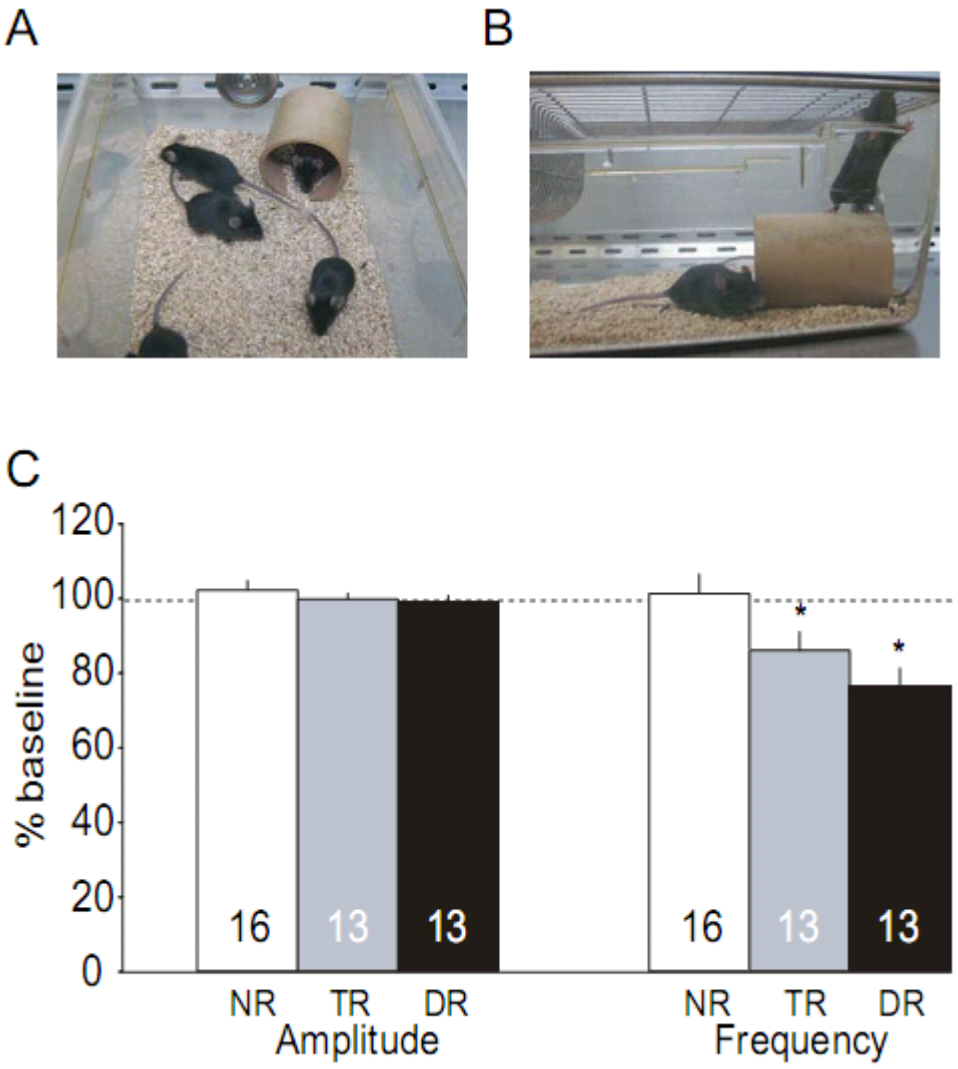
immunohistochemistry, was performed by Kristen Phend in the laboratory of Richard Weinberg. Briefly, these L2/3 V1 pieces were cyroprotected in 30% glycerol, and quick frozen. Freeze substitution was carried out in a Leica Electron Microscopy Automatic Freeze Substitution System and then pieces were embedded in Lowicryl HM-20. Sections were cut at ~100 nm with an ultramicrotome and collected on uncoated nickel grids. Postembedding Immunocytochemistry was performed by Phend K. (Phend et al., 1995; Kharazia and Weinberg, 1999). Briefly, grids were incubated overnight at 37°C in the polyclonal primary antibody NR1 (1:100). This antibody recognizes the most common splice variants of the C-terminal of the obligatory NR1 NMDAR subunit. Grids were rinsed and incubated in anti-rabbit IgG conjugated to 10-nm gold particles (Ted Pella, 1:15) for 1 hour, then dried, counterstained 1% with uranyl acetate and Sato's lead (Sato, 1968) and analyzed. Electron microscopy data collection and quantitative analysis was performed with a Philips Tecnai electron microscope (Hillsboro, OR) at 80 kV with a magnification of 10,000–40,000 and images were acquired with a Gatan 12-bit 1024 x 1024 CCD camera (Pleasanton, CA). Scoring and image acquisition was done in a blind fashion whenever possible. Random grid squares were chosen and scored online or images were taken for post hoc measuring. Synapses were analyzed if they were asymmetric, had well defined membranes, postsynaptic densities and presynaptic terminals with synaptic vesicles. To analyze the developmental decrease in preNMDAR expression and the delay in this decrease by DR, synapses were scored online for presynaptic labeling, postsynaptic labeling, or

no labeling. Three animals were used for each condition, and two grids were used for each animal. 30-48 synapses were recorded from each grid (~250 synapses for each condition). Synapses were counted if they could be identified as excitatory synapses, having clear presynaptic terminals, postsynaptic spines, and an obvious cleft. Synapses were considered labeled if a gold particle lay <20nm from either the pre- or postsynaptic membrane, and only particles that lay within the PSD or active zone were considered for this analysis. For the analysis of the tube-reared (TR) animals, the same requirements were used, but images were taken on a Gatan 12-bit 024 x 1024 CCD camera (Pleasanton, CA) to aid in properly measuring the distance of gold particles to the membranes. Post hoc measurements were made using Image J software. Thus, the two studies can not be combined but only compared.

## 5.3 Results

### 5.3.1 Both DR and TR prevent the loss of functional preNMDARs

We have previously shown a developmental loss of functional preNMDARs at the fourth postnatal week in mouse visual cortex (Corlew et al., 2007). We now show that this decline is not strictly developmental, but instead depends on the visual experience of the animal. Many animal rearing protocols now require the inclusion of some type of enrichment. At the University of North Carolina at Chapel Hill, the enrichment object is a 4-inch length of 3-inch diameter PVC tube (Fig. 5.1). Animals raised with tubes in their cages can climb on top of the tube to access the top of the cage and then invert themselves to




**<u>Figure 5.1</u> Both visual deprivation and environmental enrichment prevent the loss of functional preNMDARs. (A & B)** Example of PVC "enrichment" tube provided to each animal cage in the tube reared (TR) condition. Animals are P30. **(C)** Combined data from voltage-clamp recordings of AMPAR-mediated mEPSCs. Normalized amplitude and frequency changes during APV application. Cells from normally-reared (NR) P23-31 mice show no decrease in mEPSC frequency when APV is applied (101.3 ± 5.2%, n = 16), whereas cells from both TR (86.0 ± 5.1%, n = 13, $p < 0.03$) and dark-reared (DR) animals (76.4 ± 4.9%, n = 13, $p < 0.002$) show a significant decreases in mEPSC frequency and their normalized values are both significantly different from the NR animals (TR $p < 0.05$, DR $p < 0.002$). Bars illustrate the average with S.E.M., sample sizes are given within the bars. * = $p < 0.05$.

climb on the wire lid of the cage. The animals can also use the tube as a shelter that may deprive them of light. We raised animals in three different conditions to determine if visual experience could influence the loss of functional preNMDARs. Normally-reared (NR) animals were reared on a 12:12 light dark cycle and were not given any enrichment. Tube-reared (TR) animals were given a PVC tube in their cage during their entire life beginning prenatally. Finally, dark-reared (DR) animals were placed in a completely dark room shortly after birth with no enrichment (Yashiro et al., 2005). These DR animals were exposed to less than 2 min of light during euthanization and dissection to remove the brain.

To probe for functional preNMDARs, we used an established method (Berretta and Jones, 1996; Sjostrom et al., 2003; Bender et al., 2006b; Yang et al., 2006; Corlew et al., 2007). PreNMDARs have been shown to increase the frequency of miniature excitatory postsynaptic potentials (mEPSCs). The method involves recording the frequency of mEPSCs while blocking postsynaptic NMDARs by including (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801) (0.5–1mM) in the postsynaptic recording pipette and continually hyperpolarizing the cell to maintain $Mg^{2+}$ block. Throughout the recording, 200nM tetrodotoxin (TTX) is included in the bath to block action potential-mediated release, and picrotoxin (50 µM) is included to block $GABA_AR$-mediated miniature inhibitory postsynaptic currents (mIPSCs). After a baseline recording is made, the NMDAR antagonist APV (50µm) is applied to determine its effect on mEPSC frequency.

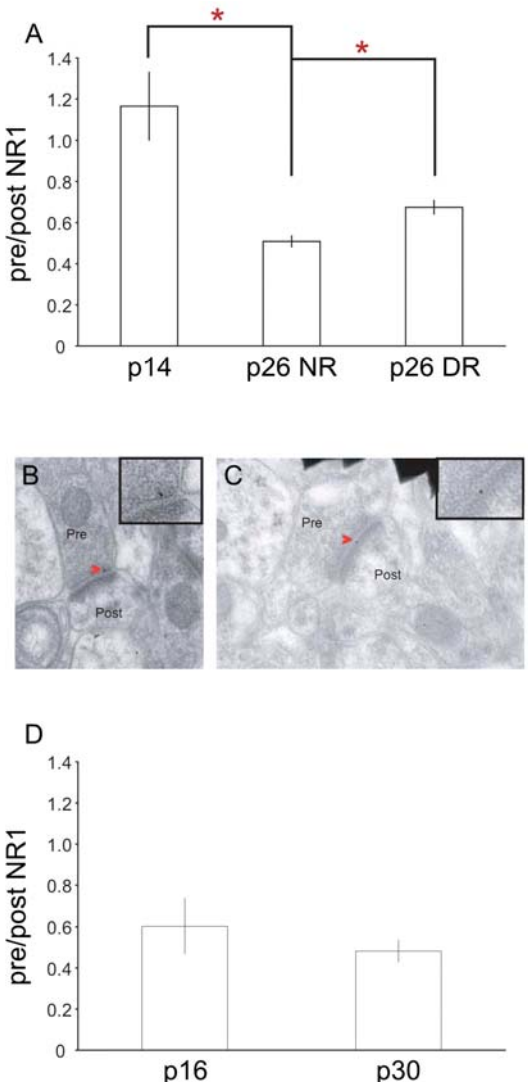
As expected from previous findings (Corlew et al., 2007), NR animals between the ages of postnatal day (P) 23-31 did not show functional preNMDARs by this assay. When APV was applied there was no change in the frequency of mEPSCs (101.3 ± 5.2% n = 16) (Fig. 5.1C). The bar graphs represent the average of many recordings from L2/3 pyramidal cells in primary visual cortex (V1) of P23-31 mice. In TR animals of the same age, however, APV did decrease mEPSC frequency (86.0 ± 5.1%, n = 13). This decrease was significant when comparing baseline frequency to frequency in APV ($p < 0.03$). There was also a significant difference between the normalized change during APV application of NR and TR animals ($p < 0.05$). This prevention of the developmental loss of functional preNMDARs was not unique to TR animals. DR animals also showed a decrease in frequency with APV application (76.4 ± 4.9%, n = 13) (Fig. 5.1C). This decrease was significant when comparing baseline to APV ($p < 0.002$) and when comparing normalized values of NR and DR ($p < 0.002$). There was no change in amplitude of mEPSCs in any of the recordings, confirming that postsynaptic NMDARs are not contributing to our measurements. These data indicate that the loss of preNMDAR function with development is not strictly developmentally controlled, but can be prevented by altering the animals' visual environment with either enrichment or deprivation.

### 5.3.2 Both DR and TR delay the anatomical loss of preNMDARs.

We showed previously, by pre-imbedding immuno-electron microscopy, that a developmental loss of functional preNMDARs coincided with a

developmental decrease in the ratio of synapses that contain preNMDARs and synapses that contain postsynaptic NMDARs. Using an antibody against NR1, the obligatory NMDAR subunit, we showed that while the percentage of postsynaptic spines with NR1 stayed constant, the percentage of presynaptic terminals that contain NR1 decreased by half from P16 to P27 (Corlew et al., 2007). We now show that this decrease, like the loss of functional preNMDARs, depends on visual experience. Here we use a post-imbedding immunogold technique to label NR1. This technique has the advantage of being able to localize the labeled receptor within a small proximity to the gold secondary particle. Thus, we were able to focus our analysis on receptors that are likely to be inserted into the membrane of presynaptic terminals and postsynaptic spines.

In a micrograph of an excitatory synapse in L2/3 in V1 of a P16 mouse, immunogold particles were determined to be “presynaptic” if they were located <20nm from the presynaptic membrane (Fig. 5.2B). For this analysis we concentrated on receptors that were associated with the postsynaptic PSD or the presynaptic active zone. In an excitatory synapse in L2/3 in V1 of a P26 mouse (Fig. 5.2C) a gold particle lies <20nm from the postsynaptic membrane and within the postsynaptic density (PSD). We confirmed our previous developmental findings using this technique. From P14 to P26 there is a >50% decrease in the ratio of synapses with presynaptic NR1 and synapses with postsynaptic NR1 (P14; 1.17± 0.16, n = 3 animals, P26; 0.51± 0.03% n = 3 animals, $p < 0.02$) (Fig. 5.2A). We also showed that visual deprivation prevents this decrease. Although DR animals at P26 show a small and non-significant decrease in the pre/post



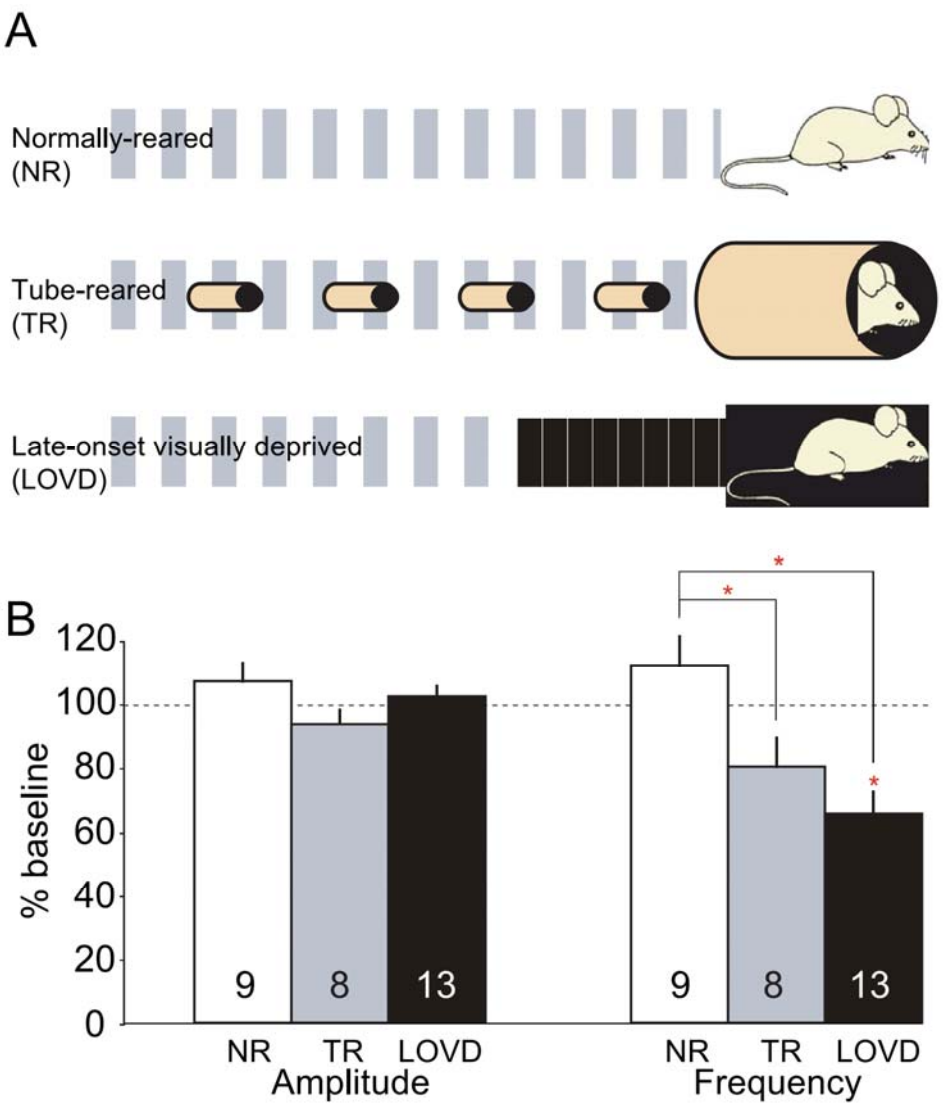

**<u>Figure 5.2</u> Both visual deprivation and environmental enrichment decrease the anatomical down-regulation of preNMDARs. (A)** In normally reared (NR) mice there is a significant decrease in the ratio of synapses with presynaptic, to synapses with postsynaptic immunogold label for NR1 from high at P14 (1.17 ± 0.16, n = 3) to low at P26 (0.51 ± 0.03, n = 3) ($p < 0.017$). This decrease is lessened when animals are dark-reared (DR) (0.67 ± 0.03, n = 3) ($p < 0.05$), and there is a significant difference between NR and DR P26 mice ($p < 0.02$). Bars illustrate the average ratio for three animals in each condition with S.E.M. * = $p < 0.05$. Approximately 85 synapses were analyzed for each animal. **(B & C)** Micrographs of excitatory synapses in L2/3 of primary visual cortex (V1) from a TR P30 mouse. Arrows indicate immunogold labeling of NR1 subunit on the membrane of the **(B)** presynaptic terminal and **(C)** postsynaptic spine. **(D)** Raising animals with environmental enrichment provided by a 4 inch section of 3 inch diameter PVC tube prevents the decrease in pre/postsynaptic labeling. There is no difference in the ratio of pre/postsynaptic labeling from P14 (0.48 ± 0.05, n = 3) to P26 (0.60 ± 0.13, n = 3) ($p < 0.65$).

ratio from the NR P14 animals, the decrease is not as great as in the NR P26 mice. In fact, when comparing the DR and NR P26 animals, the pre/post ratio of DR animals was significantly larger than the pre/post ratio of NR than the pre/post ratio of NR animals (DR 0.67 ± 0.03% n = 3 animals, $p < 0.02$).

A different set of animals were TR to determine if the developmental decline would be affected by an enriched environment. Although the tissue and grids were prepared in the same fashion and the same antibodies were used at the same concentrations, this study was not done simultaneously with the above NR/DR study (Fig. 5.2A). As a result, while the two developmental timelines can be compared, the data could not be combined into the same figure. Here we showed that there is no decrease in the ratio of pre/postsynaptically labeled synapses between P16 and P26 in TR animals (P16 0.6 ± 0.13, n = 3 animals, P26 0.48 ± 0.05, n = 3 animals, $p < 0.65$) (Fig. 5.2D). Therefore, the developmental decrease that we have now shown twice does not appear if the animals are TR. This is consistent with the functional evidence that preNMDARs are maintained with maturity in a visually altered environment.

### 5.3.3 Does TR prevent or simply delay the loss of preNMDARs?

We raised mice to adulthood in three different conditions (Fig. 5.3A). Normally reared (NR) animals were maintained until ~P73-83 with a standard 12:12 light dark cycle and were not provided any enrichment in their cage. Tube reared (TR) animals were also raised with a12:12 light dark cycle until ~P73-83 but were provided a 4-inch length of 3-inch diameter PVC tubing in their cage.




**<u>Figure 5.3</u> A short period of late-onset visual deprivation in adulthood is sufficient to bring back function of the preNMDARs lost during development. (A)** Schematic of the three different rearing conditions. Normally Reared (NR) mice were maintained until P73-83 on a 12:12 light:dark cycle. Late-onset visually deprived (LOVD) animals were reared normally until ~P63-73 and then moved to a darkroom for 10 days. Tube reared (TR) animals were raised on a 12:12 dark cycle but were provided a PVC tube for "enrichment". **(B)** Combined data from voltage-clamp recordings of AMPAR-mediated mEPSCs. Normalized amplitude and frequency changes during APV application. Cells from NR P73-83 mice show no decrease in mEPSC frequency when APV is applied (111.9 ± 3.0%, n = 9), whereas cells from both TR (80.0 ± 9.0%, n = 8), and LOVD (65.6 ± 6.8%, n = 13, p = 0.001) animals show a decrease in mEPSC frequency and their normalized values are both significantly different from the NR animals (TR $p < 0.03$, DR $p < 0.0005$). Bars illustrate the average with S.E.M., sample sizes are given within the bars. * = $p < 0.05$.

This tubing provided both a climbing structure to access the wire top of the cage as well as a shelter to block much of the light from ceiling lights. Finally, late-onset visually deprived (LOVD) animals were maintained as NR animals with no enrichment until ~P63-73 and then placed in a completely darkened room for 10 days. At the end of 10 days animals were exposed to less than 2 min of light during the euthanization and dissection periods in order to minimize possible recovery. To probe for functional preNMDARs, we again used the standard method of recording AMPAR-mediated mEPSCs. First, we recorded a baseline period while blocking postsynaptic NMDARs and then applied APV to block the preNMDARs. Here we have plotted the amplitude and frequency of mEPSCs during the APV application periods, each normalized to the preceding baseline periods. Again, recordings were made from pyramidal cells in L2/3 of V1 in P73-83 animals, TTX and picrotoxin were included in the bath, and MK801 was included in the postsynaptic recording pipette. As expected, NR animals showed no change in mEPSC frequency with APV application (111.9 ± 3.0%, n = 9) (Fig. 5.3B), agreeing with our previous results that preNMDARs are not functional in late adulthood (Corlew et al., 2007). When P73-83 animals were TR, however, APV application decreased mEPSC frequency (80.0 ± 9.0%, n = 8), making it significantly different from NR animals ($p < 0.03$). No conditions showed a change in amplitude with APV application, confirming that the effect is on preNMDARs. This maintenance of the preNMDARs late into adulthood with an altered visual environment suggests one of two possibilities: either the effect of TR during early life has a profound and lasting effect, completely preventing the

loss of functional preNMDARs; or the function of preNMDARs is continually plastic, remaining sensitive to visual experience throughout development and into adulthood.

### 5.3.4 LOVD restores preNMDAR function

If either enrichment, or deprivation throughout life, are capable of preventing the developmental loss of preNMDARs, what effect might a short period of deprivation have on the function of the receptor? Here we used adult mice raised normally until P63-73 and then placed in the dark for 10 days to determine if the effect of DR from birth could be replicated with only a short period of LOVD in adulthood (Fig. 5.3A). LOVD animals showed a decrease in mEPSC frequency with APV (65.6 ± 6.8%, n = 13) similar to both the TR animals and to the adolescent DR animals. The change from baseline was significant ($p = 0.001$), as was the difference between the normalized values of NR and LOVD animals ($p < 0.001$). Thus, the function of the receptor remains plastic and sensitive of experience well into adulthood.

### 5.3.5 LOVD brings back tLTD induction capability

Early in development, induction of timing-dependent long term depression (tLTD) between excitatory L4-L2/3 synapses in the rodent neocortex relies on activation of preNMDARs (Sjostrom et al., 2003; Bender et al., 2006b; Corlew et al., 2007; Corlew et al., 2008; Rodriguez-Moreno and Paulsen, 2008). In V1, this reliance on preNMDARs may be isolated to a short period at the beginning of visual system development <P20 (Corlew et al., 2007; Corlew et al., 2008;

Rodriguez-Moreno and Paulsen, 2008) when preNMDARs are functional. What has not been clear is if this period is strictly developmentally controlled, or if experience can alter preNMDAR function and make this plasticity accessible at more mature ages. There has long been evidence that the expression and function of postsynaptic NMDARs and their role in synaptic plasticity is altered with sensory experience. We have now presented evidence that the function of preNMDARs also changes with altered sensory experience during adolescence and even late into adulthood. In fact, very short periods (10 days) of LOVD are sufficient to restore preNMDAR function (Fig 5.3B). Therefore, we hypothesize that this LOVD may restore preNMDAR mediated tLTD (preNMDAR-tLTD). We used a standard tLTD induction protocol by repeatedly pairing a postsynaptic AP in a L2/3 pyramidal cell closely followed (5–25ms) by an EPSP evoked by L4 stimulation (Corlew et al., 2007). This protocol induces a robust preNMDAR-tLTD in young mice and a modest postsynaptically mediated tLTD in adolescent mice (Corlew et al., 2007). When we used this protocol on NR (P73-83) mice, we could not elicit tLTD even when we doubled the induction protocol from 75 AP-EPSC pairings to 150 pairings (Fig. 5.4A). However, when animals were given 10 days of LOVD, robust tLTD was induced with the same protocol (Fig. 5.4B). This effect is coincident with the reemergence of functional preNMDAR in LOVD animals. Further, this tLTD required preNMDARs in young animals (Corlew et al., 2007) when preNMDARs were also functional.

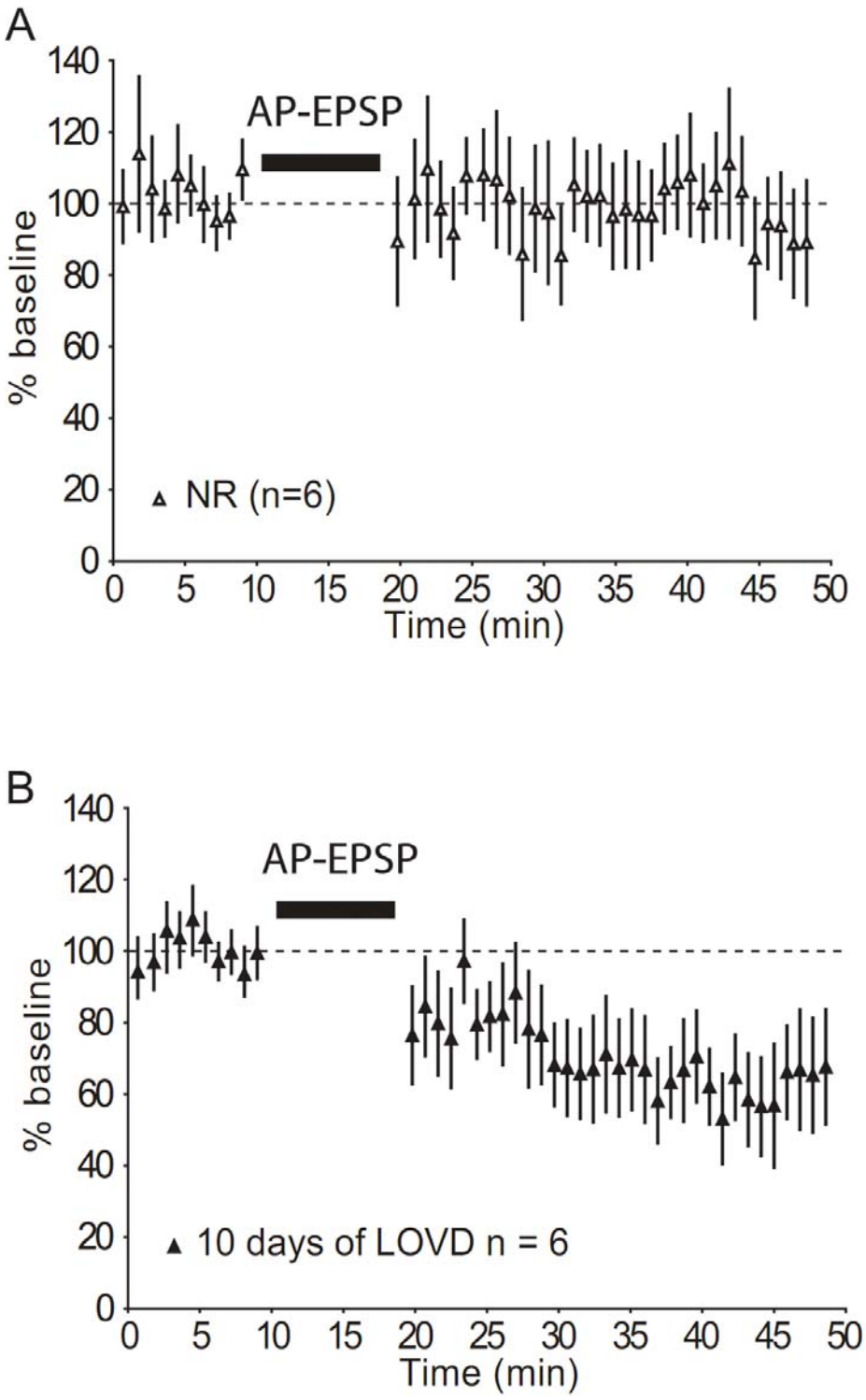



**Figure 5.4 LOVD in adulthood brings back tLTD lost during development.** **(A)** Our standard AP-EPSP pairing protocol fails to induce tLTD in normal adult mice. **(B)** After 10 days of LOVD the same protocol induces tLTD.

## 5.4 Discussion

In a previous study, we showed that LOVD in adult mice could both alter the presynaptic release probability and increase the relative expression of NR2B at presynaptic and/or extrasynaptic sites (Yashiro et al., 2005). Both of these findings suggested that LOVD increased the expression and function of preNMDARs. Here we now provide evidence that further supports the idea that an altered visual environment influences preNMDAR expression and function. We show that two seemingly different alterations in visual environment had the same effect on an important aspect of visual system development. Both visual deprivation and enrichment prevented the down-regulation of preNMDAR function and partially prevented the loss of the preNMDAR anatomically. Additionally, a third manipulation, LOVD in adult mice, had the effect of bringing back both the function of the preNMDAR and a type of tLTD that has been shown to rely on preNMDARs when they are present in the very young animal (Sjostrom et al., 2003; Bender et al., 2006b; Corlew et al., 2007; Rodriguez-Moreno and Paulsen, 2008; Banerjee et al., 2009).

We demonstrated that visual deprivation by dark rearing (DR) prevents both preNMDARs' normal developmental decrease in expression and loss of function. However, it needs to be determined whether the maintenance of preNMDAR function is due simply to continued tonic activity (and $Mg^{2+}$ insensitivity) of the receptor. As suggested in Chapter 3, tonic activity of the preNMDAR may rely on the inclusion of NR3A and it is possible that DR prevents the loss of this subunit. This is an enticing possibility considering the anatomical

evidence that we provide here. We have shown in the past (Corlew et al., 2007) and show again here there is only a 50% reduction in the pre/post synaptic NR1 expression ratio with development, but there is a complete loss of function of the preNMDAR. With DR there is only a small, though significant, increase in the pre/post ratio with DR compared to NR. However, the physiology data in DR animals shows a complete recovery of the preNMDAR function back to juvenile levels. This could be explained by an isolated anatomical loss of NR3A containing receptors with normal development (and selective maintenance with DR). This would explain the seemingly small anatomical change in relation to the large change seen in the functioning of the receptor.

The restoration (or preservation) of preNMDARs with visual deprivation may increase the rate of neurotransmitter release and thereby act as a compensatory mechanism for maintaining synaptic drive in the absence of visually evoked activity. This increased release resembles findings in culture systems after manipulations that reduce presynaptic activity (Chavis and Westbrook, 2001) or postsynaptic excitability (Murthy et al., 2001). PreNMDAR subunit trafficking has also been altered *in vivo* by activation or blockade of NMDARs. There is an increase in the trafficking of NR2B subunits to presynaptic terminals with NMDAR blockade and an increase in the presynaptic trafficking of NR2A with activation of NMDARS (Aoki et al., 2003; Fujisawa and Aoki, 2003).

This interpretation is somewhat complicated by the finding that visual enrichment also produces the same effect--increasing the function and expression of the preNMDAR. The effect of enrichment has normally been

associated with an acceleration of development (Bartoletti et al., 2004; Cancedda et al., 2004; Sale et al., 2004; Guzzetta et al., 2009). Here we possibly have a prevention of normal development. One possibility suggests that the developmental decrease was never "normal" in the first place. Admittedly, rearing conditions in an animal housing facility would never be considered normal compared to the animal's natural habitat. One group suggested that the "non-enriched" or "standard" cages are so deprived that rodents sustain impaired brain development, stereotypies, and an anxious behavior profile. Maternal care is also impaired (Bartoletti et al., 2004; Cancedda et al., 2004; Sale et al., 2004; Wolfer et al., 2004) with less licking behavior and less physical contact. The authors of one study suggest that this lack of maternal care directly inhibits normal development of the visual system. One week after birth, levels of BDNF in the visual cortex were lower in pups raised in a "standard" cage compared to pups raised with enrichment. Visual acuity was also impaired in the standard pups compared to their enriched counterparts, and this impairment lasted for up to a year after birth. Learning abilities measured by behavioral tasks were also higher for the enriched pups (Bartoletti et al., 2004; Cancedda et al., 2004; Sale et al., 2004). Therefore, the environmental enrichment primarily caused a change in maternal behavior affecting the pups' development. This was further supported by study showing that body massage increases and accelerates visual acuity in human infants and rat pups (Guzzetta et al., 2009).

While providing one means to interpret our findings, this explanation cannot account for the fact that LOVD produces the same increase in

preNMDARs as DR and TR. If the enrichment provided by the PVC tubes has the same consequence as deprivation, it would require a revaluation of the current understanding of enrichment.

One possibility is that all three conditions provide varying amounts of visual deprivation. The TR condition may be a mild form of visual deprivation, an explanation that seems likely considering that the effect seen in TR animals is slightly less than that seen in the DR and LOVD animals. The PVC tubes do block some light when animals are inside the tube, and animals provided with them, seem to spend a substantial amount of time clustering in the tubes with other pups, a behavior which would further block light. To determine if the PVC tubes are providing visual enrichment or light deprivation, the same experiments should be performed with clear tubes that do not block any of the light. If the clear tubes cause the same increase in preNMDARs as the PVC tubes, it can be concluded that the effect is not a form of deprivation and must be attributed to maternal care, visual enrichment, lowered stress level, or some other, unknown cause. Whatever the nature of the PVC tube treatment, we found that the effect of TR lasted well into adulthood, confirming that TR not only delays but also completely prevents the loss of this receptor.

Finally, we show that the function of this receptor and its role in plasticity may be modified by experience well into adulthood. A short treatment of LOVD in adulthood was sufficient to rejuvenate both preNMDARs function and tLTD, a type of plasticity shown to require preNMDARs.

Therefore TR, DR and LOVD add to the growing list of environmental manipulations that are able to influence the development of the visual system. When parsed out and understood more thoroughly, they may prove useful in clinical settings. In some cases of cortical blindness in humans (amblyopia), periods of visual deprivation cause recovery of normal vision in adults (Birnbaum, 1997; Simmers and Gray, 1999). Our findings could account for the molecular mechanisms behind these clinical observations. With a better understanding of these mechanisms we will know how to orchestrate plasticity in the adult cortex and reverse conditions such as amblyopia that were once thought to be permanent.

# Chapter 6:

# General discussion

This body of work represents three major contributions to the study of preNMDARs: 1) preNMDARs are down-regulated during development, 2) preNMDARs require the NR3A subunit, and 3) function and expression of preNMDARs is experience-dependent. I will briefly discuss how each finding has, or will make, an impact to the study of preNMDARs, and then suggest how the culmination of this work might impact the understanding and/or treatment of neurological disorders.

## 6.1 PreNMDARs are down-regulated with development.

Since the initial reports indicating that presynaptic NMDARs enhance synaptic transmission and plasticity (Berretta and Jones, 1996; Sjostrom et al., 2003; Bender et al., 2006b), the developmental expression of these receptors has remained unknown. Interestingly, two studies seemed to suggest that these receptors may be developmentally regulated. In the first report**,** preNMDARs in the hippocampus were shown to be modulated by prenenalone sulfate and that this modulation is ineffective after P5 (Mameli et al., 2005) (see Introduction: *Developmental regulation of preNMDARs role in neurotransmission*

*and plasticity*)**.** The second report indicated that preNMDARs in the entorhinal cortex required the NR2B subunit until 5 weeks of age but not after 5 months (Yang et al., 2006). Both of these studies suggested a developmental change in preNMDARs, but neither showed a loss of function or decrease in expression of preNMDARs. Our work, described in Chapter 2, was the first to show a functional and anatomical decrease in preNMDARs with development. Furthermore, we provided evidence that the mechanisms underlying tLTD, switch with development, from depending upon preNMDARs (preNMDAR-tLTD) to depending on postsynaptic NMDARs after the first three weeks postnatal. This developmental change has since been replicated in rodent primary somatosensory cortex (S1). Rodriguez-Moreno et. al. (2008) showed that including the NMDAR antagonist MK801 in the presynaptic recording pipette, significantly disrupted tLTD in young animals (P6-14) (Rodriguez-Moreno and Paulsen, 2008). In another publication by the same group, Banerje e et. al. 2009 were able to induce robust preNMDAR-tLTD in the L4-2/3 connection in rat S1 only until the fourth postnatal week—after which time induction of preNMDAR-tLTD was not possible (Banerjee et al., 2009).

Between these reports, there are clear differences in the in the timeline of developmental down-regulation of the preNMDAR: P5 for hippocampus (Mameli et al., 2005), 5 months for entorhinal cortex (Yang et al., 2006), and~P20 for sensory cortices (Corlew et al., 2007; Rodriguez-Moreno and Paulsen, 2008; Banerjee et al., 2009). These differences have three possible explanations: 1) The differences in developmental profile for preNMDARs may be due to

differences between brain regions. 2) The developmental changes in the hippocampus and entorhinal studies may be (as suggested above) due not to a change in the expression or function of preNMDARs, but to a change in their modulation. Thus the receptor may have the same developmental profile as reported for sensory cortices, but its modulation by pregnenolone and NR2B antagonists may be developmentally regulated. 3) Finally, the differences may also be explained by experience-dependent modifications due to different rearing conditions (discussed further below). The actual nature of the developmental expression in different brain regions can be easily tested (see future directions).

Another interpretation of the developmental differences in preNMDAR function that we observed is a combination of poor space-clamp and expanding neuronal morphology. Space-clamp errors can be a considerable problem when attempting to accurately measure very small currents in distal spines (Simkus and Stricker, 2002). Because patch-clamp electrodes provide only a spatial point source of current they can not uniformly control the voltage at different parts of a branching, twisting cell. The quality of the voltage-clamp in a neuron degrades with distance from the soma where the electrode is placed (Spruston et al., 1993). Therefore the error in measurement of synaptic currents also degrades substantially as a function of distance from the soma. With a rapid decrease in amplitude of already small mEPSCs, events originating even a small distance from the soma, would not be distinguishable above noise in the recording and would thus not be reflected in the frequency measurements. Thus, all synapses in the mEPSC assay must be located very close to the soma. As neurons grow

in size the population of synapses that are subject to this analysis also change as synapses that were originally close enough to the soma now move too distal to be recorded. Therefore, it is conceivable that preNMDARs are present on a subpopulation of synapses close enough to be measured in early development but then grow out of range due to neurons expanding morphology. If so, preNMDARs could be tonically functioning at older ages but on synapses that are too distal to be adequately space-clamped. I have provided several pieces of evidence that argue against this possibility. First, it is unlikely that the short period of visual deprivation LOVD, that reinitiates tonic functioning preNMDARs is dramatic and long enough to cause a major "shrinking" of neuronal morphology back to the infantile state. A more likely explanation is that the receptors on synapses within the recordable distance are being modified. Further evidence is provided by the low $Mg^{2+}$ experiments where tonic functional preNMDARs are detected in adults by withholding $Mg^{2+}$ from the extracellular recording solution. This experiment suggest that functional preNMDARs are present on synapses within the recordable distance in the adult animals but are not tonically active due to $Mg^{2+}$ block in standard recording solution. Therefore, errors introduced by poor space-clamp do not seem to negate the interpretation of the developmental change in preNMDARs.

## 6.2 PreNMDARs require 3A

Despite evidence suggesting a significant role of preNMDARs in synaptic function, the exact mechanisms by which they can remain tonically active, despite $Mg^{2+}$ block, has yet to be resolved (Glitsch and Marty, 1999; Duguid and Sjostrom, 2006; Corlew et al., 2008). It was previously suggested that the presynaptic terminal may be constantly depolarized thereby removing the $Mg^{2+}$ block. (Jourdain et al., 2007). Others have suggested that preNMDARs are insensitive to $Mg^{2+}$ block due to their subunit composition. For example, the NR2B subunit, which is required for preNMDAR activation, has been suggested to confer $Mg^{2+}$ insensitivity. There has been further suggestion that the decline of preNMDARs function is due to the developmental decrease in NR2B expression (Yang et al., 2006). However, as discussed in Chapter 3, the NR2B subunit is only slightly less $Mg^{2+}$ sensitive than the NR2A subunit (Monyer et al., 1992) and it's developmental down-regulation is not coincident with the loss of preNMDARs (Corlew et al., 2007). Despite evidence that NR2B subunits mediate preNMDAR function, the hypothesis that they convey $Mg^{2+}$ insensitivity to the preNMDAR has yet to be tested.

Our work is the first to directly confront the question of how preNMDARs are tonically active to enhance spontaneous neurotransmitter release and why this is lost with development. Here we provide evidence that NR3A, not NR2B, endows preNMDARs with receptor properties which would enable them to remain tonically active and enhance synaptic transmission. In Chapter 3, I explain that the inclusion of the NR3A subunit in the preNMDAR makes these

receptors $Mg^{2+}$ insensitive and, thus, potentially tonically active. I further explain that the precipitous developmental down-regulation of the NR3A subunit in the fourth postnatal week causes a loss of the tonic preNMDAR function. However, the preNMDAR is not totally lost at these older ages and is able to enhance spontaneous transmitter release when $Mg^{2+}$ is removed from the recording solution. Because the pre/post ratio of NMDARs has been shown to decrease only 50%, anatomical data supports the hypothesis that only a subset of preNMDARs (presumably the ones that contain NR3A) are being down-regulated (Corlew et al., 2007). Therefore, it is possible that the 3A subunit, and not the whole preNMDAR, is down-regulated with maturity. When NR3A is down-regulated (or absent as in the NR3AKO), the remaining preNMDARs are likely composed of NR2B and 2A subunits, continuing to increase spontaneous neurotransmitter release in $Mg^{2+}$ free solution. Although they seem not to be tonically active to increase spontaneous neurotransmitter release, it is possible that these “NR3A free” preNMDARs may have some unidentified role in action potential-dependent synaptic transmission or plasticity.

With the discovery that preNMDARs contain this novel subunit, we now must re-evaluate our understanding of the preNMDAR. Almost everything we know about NMDARs has been from the study of what we thought were postsynaptic NMDARs composed of NR2 subunits. But the NR3A subunit differs from NR2 subunits in its permeability, binding partners, subcellular location, as well as its regulation and role in plasticity (Roberts et al. 2009 in press). Thus, the inclusion of NR3A may cause the preNMDAR to be very different from its

postsynaptic counterpart. This difference could be advantageous for the study of preNMDARs, and for the treatment of neurological disorders with which they are associated (discussed further below).

## 6.3 Function and expression of preNMDARs is experience-dependent

### 6.3.1 Experience-dependent plasticity is possible in adults

Our inquiry into the experience-dependence of the preNMDARs was initiated by our more general developmental study of the effect of visual experience on NMDARs. I would like to first discuss these findings before becoming more specific to preNMDARs. In these studies, we were able to induce changes in both neurotransmitter release probability, and NMDAR subunit composition, in V1 of adult visual cortex with a short period of late-onset visual deprivation (LOVD). This finding adds to the growing body of evidence that the adult sensory cortex is more plastic than classically thought.

The idea that previously plastic synaptic connections grow rigid in adulthood began with the work of Hubel and Wiesel. They showed that after a short period of monocular deprivation (MD), responsiveness of the cat visual cortex shifted toward the non-deprived eye (Wiesel and Hubel, 1963) (ocular dominance (OD) shift). Because adult cats showed no OD shift with MD, they hypothesized that this plasticity was only possible during a short period early in visual system development. Rodents were also found to have this “critical period” for OD plasticity (Gordon and Stryker, 1996). However, recent studies make it clear that adult brains can still undergo experience-dependent plasticity.

Although the experience parameters and molecular mechanisms might be slightly different at different ages, adult sensory cortex is still remarkably plastic. Ocular dominance shifts were achieved in adults using a variety of recording and deprivation methods (Sawtell et al., 2003; Tagawa et al., 2005; Hofer et al., 2006a, b).

One proposed mechanism for the experience-dependence of sensory cortices is changes in NMDARs. Visual experience has been shown to alter the composition of NMDARs in the visual cortex; specifically, deprived cortex shows a lower ratio of NR2A- to NR2B-containing NMDARs (Quinlan et al., 1999a; Quinlan et al., 1999b; Roberts and Ramoa, 1999; Kanold et al., 2009). This change in the ratio alters NMRA- mediated current kinetics and presumably shifts the inducibility of plasticity (termed metaplasticity) (Abraham and Bear, 1996; Philpot et al., 2007; Yashiro and Philpot, 2008). We showed that in V1 of LOVD mice the L4-2/3 synapse displayed a higher probability of release than NR mice and the NR2B/2A ratio was higher at presynaptic and extrasynaptic sites than NR mice. This change in NMDARs with deprivation may be the metaplastic event that allows for the reemergence of OD plasticity that has been reported (Sawtell et al., 2003; Tagawa et al., 2005; Hofer et al., 2006a, b).

### 6.3.2 Function and expression of preNMDARs is experience dependent

The findings discussed in Chapter 4 are explained most parsimoniously by a change in preNMDARs. However, it is possible that the increase in probability

of release in the L4-2/3 pathway, and the increase in the NR2B/2A ratio at presynaptic / extrasynaptic sites with LOVD, were independent. Certainly there are many ways that a presynaptic terminal can be modified to increase neurotransmitter release other than preNMDARs. Alterations in release machinery, VSCCs, other ligand gated channels such as presynaptic Kainate or AMPARs, and changes in presynaptic inhibition, are just some of these possible modifications. Additionally, the biochemical fractionization technique that was used is unable to differentiate between presynaptic proteins and extrasynaptic proteins. Thus, the term peri-/extrasynaptic NMDARs was used but it is possible that only extrasynaptic NMDARs were changed with this manipulation. However, we tested the possibility that these two changes were due to a single change, preNMDARs. Indeed, in chapter 5 we found that preNMDAR function, although lost in development, is restored with LOVD.

This finding lead us to ask whether preNMDARs could only be modified with LOVD, or if other more traditional types of visual manipulation could alter the developmental down-regulation of preNMDARs' function and expression. We found that DR until P26 also prevented the normal decrease in preNMDAR function and expression.

More support for the experience model, rather than the developmental model, is provided by the timing of the normal "developmental" decrease. It is interesting that in NR mice, preNMDARs are down-regulated in the visual cortex just a week after eye opening (exposed to light for a week) (Chapter 2). This was previously interpreted as a genetic program to provide a presynaptic type of

plasticity at a time when postsynaptic plasticity would be less effective due to weaker postsynaptic responses (Corlew et al., 2007). This might still be the case, but these findings make it more probable that the system is more dynamic and interactive than a simple developmental program. It is interesting that it also took only 10 days for the receptor to be re-expressed and functional, late in adulthood after it had long since gone quiet (Chapter 4, 5 (Yashiro et al., 2005)). We might suspect that the expression of the receptor is not developmentally regulated at all, but is modified solely by sensory experience. When both the visual environment and synaptic connections in the visual cortex are stable, there is no need for this type of plasticity. As a result, the receptor goes quiet, possibly by removal of NR3A. With an enriched environment, however, the animal is in a position to continually alter its view of the world and can constantly make use of one more type of plasticity. If the animal is raised in the dark, or placed into the dark, the system can also regain plasticity, making it ready to alter its visual processing when it is again brought into the light.

### 6.4 "Experience" is more than just "deprivation"

In our study, three seemingly different alterations in visual environment all had the same effect on the function/expression of the preNMDARs. DR from birth, LOVD in adulthood, and raising the animals with enrichment (TR), all increased the function and expression of preNMDARs or prevented their developmental down-regulation. In the entorhinal cortex, one group found that preNMDARs can be up-regulated in adult rats after induction of epileptic-like

seizures (Yang et al., 2006). It will be interesting to parse the common effect on the brain of seizure, visual deprivation, and visual enrichment. Alternatively, these conditions may have little in common other than a similar outcome for this one assay. However, visual deprivation and enrichment have both been shown to reinstate OD plasticity (He et al., 2006; Sale et al., 2007). Thus, as in our studies, two seemingly opposite manipulations—deprivation and enrichment—produce similar or identical synaptic changes. One possible explanation is that both enrichment and deprivation alter cortical inhibition in the adult, leading to the changes in plasticity as well as changes in preNMDAR expression (He et al., 2004; Sale et al., 2007) (discussed further below)

At the very least, these findings should give caution to investigators that try to extrapolate findings from studies in mice. Subtle environmental differences between mice reared in different animal research facilities could dramatically alter seemingly basic developmental properties such as the composition or localization of NMDARs. Differences observed between studies could be explained by differences in rearing conditions, which we have shown influence preNMDAR function. Thus, discrepancies in the timeline of developmental down-regulation seen in the entorhinal cortex could be due to differences in housing conditions. In the entorhinal cortex, it has been reported that preNMDARs are tonically active as late as 5 weeks of age and are absent by 5 months (Yang et al., 2006). We have now shown that V1 of TR mice maintains active preNMDARs past 5 weeks. We did not test for preNMDARs as late as 5 months. Our “adult” mice were less than 3 months old, but it is possible that with TR,

preNMDARs tonic activity is eventually down-regulated. It may be that NR causes an early decrease in the expression of preNMDARs, and closes the door for preNMDAR-mediated plasticity. Rearing conditions that provide more stimulation may keep preNMDARs tonically active longer and possibly maintain preNMDAR-mediated tLTD. This difference may give us insight into the importance of early experiences and stimulation of human infants for normal neurological development.

### 6.5 PreNMDARs and Disease

NMDARs have a known role in the etiologies of many serious neurological disorders including Huntington's, Parkinson's, stroke, schizophrenia, epilepsy, and neuropathic pain (Liu et al., 1997; Heresco-Levy and Javitt, 1998; Gogas, 2006; Missale et al., 2006; Visser and Schug, 2006; Fan and Raymond, 2007). Until recently NMDARs were assumed to act postsynaptically, so the contribution of preNMDARs to these disorders has not been explored. It is now clear that preNMDARs may have an important role in synaptic transmission and plasticity, especially during very early development when many such neuropathologies are forming. Therefore more research is necessary to investigate a possible role for preNMDARs in these disorders.

Epilepsy is one disorder in which a role for preNMDARs is now being investigated. It is already known that chronic changes in neural activity levels in epileptic patients have been shown to affect NMDAR receptor function and expression (Dalby and Mody, 2001; Avanzini and Franceschetti, 2003; Morimoto

et al., 2004). Though these NMDARs were assumed to be postsynaptic, one report in rodents indicates that epileptic activity could affect the function of preNMDARs. Specifically, preNMDARs were reinstated in the adult entorhinal cortex following 2-4 weeks of lithium-pilocarpine treatment to induce seizures (Yang et al., 2006). Though it is not clear whether this increase was causal to, or a consequence of, increased neural activity, preNMDARs clearly played a role. Gabapentin, a drug prescribed to treat epilepsy, has been shown to decrease neurotransmitter release via preNMDARs, providing further evidence that preNMDARs may be involved in some forms of epilepsy (Suarez et al., 2005). We must now consider that the therapeutic value or deleterious off-target effects of many global NMDAR antagonists being used to treat neurological disorders, may be due to their action on preNMDARs (Suarez et al., 2005). Better pharmacological therapies for disorders involving NMDAR dysfunction may be revealed by selectively targeting pre- versus post-synaptic NMDARs. Therefore, we now need a better understanding of the differences between preNMDARs and postsynaptic NMDARs. It is crucial to understand the differences in when, where, and how each NMDAR affects synaptic communication, plasticity, and neural network function.

### 6.6 Targeting NR3A to treat disease

Although genetic and pharmacological manipulations of NMDARs can alleviate symptoms of neurological disorders, there are also off-target effects due to ubiquitous NMDAR expression. Additionally, the therapeutic benefits are often

outweighed by the harmful side effects. Even targeting specific subunits, until now, has proved non-specific because the targeted subunits, most often NR2B, are also ubiquitously expressed (reviewed in Henson et al., in preparation).

However, accumulating research on NR3A provides exciting hope for treatment of a long list of neuropathologies. There is evidence that the NR3A subunit might be directly involved in schizophrenia, white matter injury, Huntington's, Parkinson's, and Alzheimer's diseases, as well as chronic alcohol exposure, and neuropathic pain (reviewed in Henson et al., in preparation). In addition, targeting the NR3A subunit could provide a possible treatment for acute brain injuries caused by stroke, epilepsy, and trauma (reviewed in Henson et al., in preparation). Thus, the development of specific agonists and antagonists of NR3A may be of therapeutic value. Even in disorders where the direct involvement of NR3A has not yet been shown, manipulation of NR3A could have a therapeutic effect by compensating for genetic abnormalities. Mental retardations such as Fragile X, Rett, and Down Syndromes, are known to change the density of synaptic spines, and genetic manipulations of NR3A also dramatically affect spine density (reviewed in Henson et al., in preparation). In NR3A KO mice spine density is dramatically increased while NR3A OE mice show decreases in spine density (Das et al., 1998). In these developmental disorders, such manipulations of NR3A could be used to compensate for whatever initial abnormality caused changes to the spines.

The novel qualities of NR3A may prove beneficial as a target for treating neurological disorders. Because NR3A has a unique quality—playing an

important role early in development but going away in maturity—NR3A may have very limited action in the adult brain. This limitation offers the possibility that specific actions can be modulated in the adult brain (reviewed in Henson et al., in preparation). However, if the NR3A subunit is largely absent in the normal adult brain, it ceases to be a target. If the NR3A subunit could be reintroduced into the adult brain, it may have limited actions in a brain that has not relied on NR3A since infancy. As a consequence, the problem of excessive action on NMDARs is minimized, an excess that previously made NMDAR manipulation unsuitable for broad treatments. Additionally, if the role of NR3A is specific to presynaptic function, manipulation of this subunit may have fewer consequences than an approach that affects NMDARs throughout the postsynaptic, extrasynaptic, and somatodendritic domains.

### 6.7 Activity dependent regulation of preNMDARs (subunit specific?)

It may be possible to harness the brain's own activity-dependent regulation to reintroduce a receptor, or subunit of a receptor, into specific cortical regions and selectively treat or prevent neurological disorders. Our data suggests that it may be the NR3A subunit, and not the whole preNMDAR, that is highly experience-dependent. The developmental down-regulation of NR3A causes a loss of the tonically active preNMDAR. This developmental decrease in NR3A is correlated with a very small decrease in preNMDAR expression. Conversely, with visual deprivation or enrichment, preNMDARs remain functional, there is an equally small increase in preNMDAR expression (suggesting a small

population of preNMDARs are increased), and tLTD is restored. It is likely then, that the up-regulation of the NR3A subunit in preNMDARs is the change that makes the receptors functional again with visual deprivation or enrichment. If this is the case, then the reemergence of preNMDARs and tLTD that we see with LOVD could all be caused by the "reinsertion" of NR3A into the preNMDARs. This could easily be tested in the NR3A KO and NR3A overexpressing animals and, if true, opens the door to many exciting possibilities (see future directions *Experience dependence of NR3A*).

The instances of reemergence of plasticity in adulthood with short periods of sensory deprivation are growing, and it will be simple to test for a role for NR3A in these instances. It may be that the brain has provided its own method for genetic manipulation and that our awareness of this method will allow us to access it and utilize it for the treatment and prevention of disorders. With evidence that visual deprivation, enrichment, or an epileptically caused increase in activity can all increase the function of preNMDARs, there might be several possible "therapies" that would alter NR3A-containing preNMDARs (see future directions *Reintroducing NR3A in adult brain to treat neurological disorders)*. Exposure to weak magnetic fields is one non- invasive and non-pharmacological treatment that has been shown to alter NR3A mRNA levels (reviewed in Henson et al., in preparation).

### 6.8 Visual experience preNMDARs and inhibition

In Chapter 4 we show both the reemergence of functional preNMDARs, and the restoration of tLTD in adult V1 with LOVD. One explanation for the restoration of the tLTD is that preNMDARs are lost with development, and with deprivation, they reemerge and are able to function both in spontaneous neurotransmitter release and in tLTD mechanisms. This seems likely because of the high experience-dependence of the receptor, and because the receptor's function and role in tLTD are tied at other developmental time points. An alternate explanation however, is that the reemergence of tLTD is related to a change in inhibition in the deprived animal. In Chapter 2 I briefly discuss the relationship of inhibition and tLTD. In young animals (P13-17), the presence or absence of picrotoxin to block GABARs, did not affect our ability to induce preNMDAR-mediated tLTD (Fig. 2.5). It is commonly accepted that inhibition does not affect plasticity mechanisms at this age(Feldman, 2000; Froemke and Dan, 2002), presumably because inhibitory drive and inhibitory connections are less developed. However, in older animals (P23-30), postsynaptic NMDAR-mediated tLTD was prevented when inhibition was intact (Fig. 2.7D). There are two explanations for this age difference. 1) Inhibition is able to block the induction of postsynaptic and presynaptic tLTD, but it is not sufficiently developed in young mice. 2) Inhibition only blocks tLTD that relies on postsynaptic NMDARs.

More experiments are needed to clarify the mechanism of deprivation-restored tLTD in adult animals. All of the tLTD experiments in adult animals were performed with inhibition intact. Therefore, it was not surprising LTD could not be

induced in the NR adult animals (Fig. 5.4A), because LTD was also not induced at P23-30 when inhibition was left intact (Fig. 2.7D). What was surprising though, was that we were able to induce robust tLTD in LOVD animals (Fig. 5.4B). This finding would lead us to two probable explanations. 1) LOVD restores preNMDAR-tLTD. 2) LOVD reduces inhibition to "pre-critical period" levels so that postsynaptic LTD can be induced. Although such a change in inhibition seems extreme, visual deprivation has been shown to induce large changes in both GABAR expression and maturation of inhibitory cells. In a study by Kreczko et al 2009, the number of somatic GAD65-puncta on individual layer 2/3 pyramidal neurons was reduced in DR mice, but increased back to normal levels with light exposure (Kreczko et al., 2009). In another study by Wahle 2009, DR was shown to alter the maturation of potassium channel (Kv 3.1b/3.2) expression in a subset of inhibitory cells, causing deficits in inhibition. Changes in inhibition have been shown to affect experience-dependent plasticity. In a landmark finding Hensch et al. (1998) showed that GAD65KO mice do not show an OD shift with MD during the critical period. Further, local infusion of diazepam, a use-dependent GABA agonist, restored the OD shift with MD at any age in these mice (Hensch et al., 1998). Environmental enrichment also alters OD plasticity in adults by reducing inhibition (Sale et al., 2007).

Because of our evidence that preNMDARs are being restored with LOVD (Chapter 4 & 5), it may be that both inhibition and preNMDARs expression are being altered with visual deprivation and that both contribute to the restored preNMDAR-tLTD. A recent paper suggests that changes in OD plasticity with

dark-rearing could be due to changes in NMDAR composition down-stream of changes in inhibition. The NR2B/NR2A ratio was higher in V1 of GAD65KO mice but this was reversed with benzodiazepine application (Kanold et al., 2009).

## 6.9 Future directions

*More direct studies of preNMDAR function are needed*

Although there is now a large number of studies analyzing preNMDARs there is still skepticism about whether they actually exist. This skepticism is due to the indirect nature of all functional studies to date. Future studies will need to more directly test for the presence of functional preNMDARs. Until recently, no method has existed to selectively activate, block, or eliminate preNMDARs without simultaneously affecting postsynaptic NMDARs in the same way. Today, there should be methods for achieving these aims in order to directly measure preNMDAR functions. One possibility would be to selectively block the expression of NMDARs in a subset of cells using, for example, viral expression of siRNAs or modern genetic approaches (Zong et al., 2005; Miskevich et al., 2006; Muzumdar et al., 2007). As such, synaptic functions at synapses with and without preNMDARs can be compared directly using paired electrophysiological recordings. Global application of NMDAR antagonists would then only be expected to alter presynaptic functions in pathways where preNMDAR functions were not down-regulated. In combination with pharmacological manipulations, we will then be able to compare, in a single slice, the role of preNMDARs in synaptic transmission at the level of contacts between single cells. If we want to

record from neurons in which the endogenous populations of receptors have not been altered, an alternative approach would be to selectively activate preNMDARs by glutamate uncaging. With this method, one could, for example, selectively uncage glutamate and simultaneously observe $Ca^{2+}$ increases in the presynaptic terminal.

*Visual deprivation and inhibition*

We are now carrying out a line of research that will definitively answer whether the restoration of tLTD in LOVD animals is due solely to a change in inhibition or in some part due to preNMDAR re-expression. Experiments to induce tLTD in NR and LOVD animals are being done with, and without, intact inhibition. Recordings are also made with postsynaptic NMDARs blocked with MK801 in the postsynaptic recording pipette or with all of the NMDARs blocked with APV in the bath. These experiments will tell us whether preNMDAR-tLTD is restored in the adult animals with LOVD. If the result of these experiments suggests a change in inhibition, that line of research could also be explored. It is likely, based on previous work discussed above, that visual deprivation has an impact on inhibition.

*TR: visual deprivation or enrichment?*

We showed that rearing mice with PVC tubes in their cages prevents the normal decrease in preNMDAR expression and function. In order to determine whether the effect of the tubes is due to a mild form of visual deprivation, or to environmental enrichment, the opaque tubes need to be replaced with clear tubes that do not block out light. If there is still no developmental decrease in

preNMDARs in TR animals when the tubes are clear, then we can rule out visual deprivation.

*Experience dependence of NR3A*

Our evidence strongly suggests that the developmental decline in the NR3A subunit causes the loss of functional preNMDARs. It is likely that the experience-dependence of the preNMDAR function and expression also depends on the NR3A subunit. In order to test this, we should replicate the EM experiments using an antibody for NR3A. If presynaptic NR3A is highly experience-dependent, then DR animals should maintain NR3A in preNMDARs, while NR mice loose NR3A.

*Are preNMDARs and / or NR3A experience-dependent in other brain regions?*

It will also be instructive to determine whether expression of preNMDARs and NR3A in other brain regions is sensitive to altered experience. Can preNMDARs, or presynaptic NR3A, be modulated in S1 with whisker deprivation? Can socialization affect the expression of preNMDARs or NR3A in the frontal cortex? Can fear conditioning or manipulations that affect hippocampal memory affect preNMDARs in the amygdale or hippocampus?

*Is the role of NR3A evolutionarily conserved?*

If indeed, enrichment is capable of preventing the developmental down-regulation of the receptor, we are left to assume that the maintenance of the receptor is not the pathological state but the healthy state. Therefore, there is presumably a role for these receptors throughout development and into adulthood at least in normal mice. In order to use this to inform us on the human

condition, we need to determine what role NR3A plays in the human brain, or at the very least, determine if these findings are evolutionarily conserved among rodents, carnivores, and non-human primates. One piece of evidence that suggests an evolutionarily conserved mechanism is that preNMDARs have been shown to be present in non-mammalian systems. In the avian midbrain preNMDARs are required for tLTD (Penzo and Pena, 2009). And in the zebrafish, NMJ preNMDARs increase spontaneous acetylcholine release (Todd et al., 2004).

*Reintroducing NR3A in adult brain to treat neurological disorders*

If presynaptic NR3A expression in the adult brain is highly experience dependent, then periods of altered sensory experience may provide therapy to NMDAR associated neurological disorders. This could be easily tested in mice by breading NR3A KO or NR3A OE mice with mouse models for neurological disorders and then providing visual deprivation to test whether an increase in NR3A expression modifies the symptoms of the disorder. Environmental enrichment may also prove effective for reintroducing NR3A into adult brain to treat neurological disorders. Environmental enrichment has already been shown to reduce symptoms and disease progression in models of Huntington's and Alzheimer's, ischemia and traumatic insults (Lazarov et al., 2005; Nithianantharajah and Hannan, 2006).

## References

Abraham WC, Bear MF (1996) Metaplasticity: the plasticity of synaptic plasticity. Trends Neurosci 19:126-130.

Allen CB, Celikel T, Feldman DE (2003) Long-term depression induced by sensory deprivation during cortical map plasticity in vivo. Nat Neurosci 6:291-299.

Aoki C, Venkatesan C, Go CG, Mong JA, Dawson TM (1994) Cellular and subcellular localization of NMDA-R1 subunit immunoreactivity in the visual cortex of adult and neonatal rats. J Neurosci 14:5202-5222.

Aoki C, Fujisawa S, Mahadomrongkul V, Shah PJ, Nader K, Erisir A (2003) NMDA receptor blockade in intact adult cortex increases trafficking of NR2A subunits into spines, postsynaptic densities, and axon terminals. Brain Res 963:139-149.

Aramori I, Nakanishi S (1992) Signal transduction and pharmacological characteristics of a metabotropic glutamate receptor, mGluR1, in transfected CHO cells. Neuron 8:757-765.

Avanzini G, Franceschetti S (2003) Cellular biology of epileptogenesis. Lancet Neurol 2:33-42.

Awatramani GB, Price GD, Trussell LO (2005) Modulation of transmitter release by presynaptic resting potential and background calcium levels. Neuron 48:109-121.

Awobuluyi M, Yang J, Ye Y, Chatterton JE, Godzik A, Lipton SA, Zhang D (2007) Subunit-specific roles of glycine-binding domains in activation of NR1/NR3 N-methyl-D-aspartate receptors. Mol Pharmacol 71:112-122.

Banerjee A, Meredith RM, Rodriguez-Moreno A, Mierau SB, Auberson YP, Paulsen O (2009) Double Dissociation of Spike Timing-Dependent Potentiation and Depression by Subunit-Preferring NMDA Receptor Antagonists in Mouse Barrel Cortex. Cereb Cortex.

Bardoni R, Torsney C, Tong CK, Prandini M, MacDermott AB (2004) Presynaptic NMDA receptors modulate glutamate release from primary sensory neurons in rat spinal cord dorsal horn. J Neurosci 24:2774-2781.

Bartoletti A, Medini P, Berardi N, Maffei L (2004) Environmental enrichment prevents effects of dark-rearing in the rat visual cortex. Nat Neurosci 7:215-216.

Bear MF, Rittenhouse CD (1999) Molecular basis for induction of ocular dominance plasticity. J Neurobiol 41:83-91.

Bender KJ, Allen CB, Bender VA, Feldman DE (2006a) Synaptic basis for whisker deprivation-induced synaptic depression in rat somatosensory cortex. J Neurosci 26:4155-4165.

Bender VA, Bender KJ, Brasier DJ, Feldman DE (2006b) Two coincidence detectors for spike timing-dependent plasticity in somatosensory cortex. J Neurosci 26:4166-4177.

Berardi N, Pizzorusso T, Maffei L (2000) Critical periods during sensory development. Curr Opin Neurobiol 10:138-145.

Berberich S, Punnakkal P, Jensen V, Pawlak V, Seeburg PH, Hvalby O, Kohr G (2005) Lack of NMDA receptor subtype selectivity for hippocampal long-term potentiation. J Neurosci 25:6907-6910.

Berretta N, Jones RS (1996) Tonic facilitation of glutamate release by presynaptic N-methyl-D-aspartate autoreceptors in the entorhinal cortex. Neuroscience 75:339-344.

Bezzi P, Gundersen V, Galbete JL, Seifert G, Steinhauser C, Pilati E, Volterra A (2004) Astrocytes contain a vesicular compartment that is competent for regulated exocytosis of glutamate. Nat Neurosci 7:613-620.

Bezzi P, Carmignoto G, Pasti L, Vesce S, Rossi D, Rizzini BL, Pozzan T, Volterra A (1998) Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. Nature 391:281-285.

Bezzi P, Domercq M, Brambilla L, Galli R, Schols D, De Clercq E, Vescovi A, Bagetta G, Kollias G, Meldolesi J, Volterra A (2001) CXCR4-activated astrocyte glutamate release via TNFalpha: amplification by microglia triggers neurotoxicity. Nat Neurosci 4:702-710.

Birnbaum MH (1997) Full-scope optometry: quality eye and vision care. J Am Optom Assoc 68:73-75.

Bliss TVP, Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361:31-39.

Bolshakov VY, Siegelbaum SA (1995) Regulation of hippocampal transmiter release during development and long-term potentiation. Science 269:1730-1734.

Boyce S, Wyatt A, Webb JK, O'Donnell R, Mason G, Rigby M, Sirinathsinghji D, Hill RG, Rupniak NM (1999) Selective NMDA NR2B antagonists induce

antinociception without motor dysfunction: correlation with restricted localisation of NR2B subunit in dorsal horn. Neuropharmacology 38:611-623.

Brasier DJ, Feldman DE (2008) Synapse-specific expression of functional presynaptic NMDA receptors in rat somatosensory cortex. J Neurosci 28:2199-2211.

Buonomano DV, Merzenich MM (1998) Cortical plasticity: from synapses to maps. Annu Rev Neurosci 21:149-186.

Burkhalter A (1989) Intrinsic connections of rat primary visual cortex: laminar organization of axonal projections. J Comp Neurol 279:171-186.

Burnashev N, Monyer H, Seeburg PH, Sakmann B (1992) Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. Neuron 8:189-198.

Cancedda L, Putignano E, Sale A, Viegi A, Berardi N, Maffei L (2004) Acceleration of visual system development by environmental enrichment. J Neurosci 24:4840-4848.

Carmignoto G, Vicini S (1992) Activity-dependent decrease in NMDA receptor responses during development of the visual cortex. Science 258:1007-1011.

Casado M, Dieudonne S, Ascher P (2000) Presynaptic N-methyl-D-aspartate receptors at the parallel fiber-Purkinje cell synapse. Proc Natl Acad Sci U S A 97:11593-11597.

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

Catterall WA (1998) Structure and function of neuronal Ca2+ channels and their role in neurotransmitter release. Cell Calcium 24:307-323.

Cavelier P, Attwell D (2005) Tonic release of glutamate by a DIDS-sensitive mechanism in rat hippocampal slices. J Physiol 564:397-410.

Celikel T, Szostak VA, Feldman DE (2004) Modulation of spike timing by sensory deprivation during induction of cortical map plasticity. Nat Neurosci 7:534-541.

Chamberlain SE, Yang J, Jones RS (2008) The role of NMDA receptor subtypes in short-term plasticity in the rat entorhinal cortex. Neural Plast 2008:872456.

Charton JP, Herkert M, Becker CM, Schroder H (1999) Cellular and subcellular localization of the 2B-subunit of the NMDA receptor in the adult rat telencephalon. Brain Res 816:609-617.

Chavis P, Westbrook G (2001) Integrins mediate functional pre- and postsynaptic maturation at a hippocampal synapse. Nature 411:317-321.

Chen HS, Lipton SA (2006) The chemical biology of clinically tolerated NMDA receptor antagonists. J Neurochem 97:1611-1626.

Chevaleyre V, Takahashi KA, Castillo PE (2006) Endocannabinoid-mediated synaptic plasticity in the CNS. Annu Rev Neurosci 29:37-76.

Choi S, Lovinger DM (1997) Decreased probability of neurotransmitter release underlies striatal long-term depression and postnatal development of corticostriatal synapses. Proc Natl Acad Sci U S A 94:2665-2670.

Ciabarra AM, Sullivan JM, Gahn LG, Pecht G, Heinemann S, Sevarino KA (1995) Cloning and characterization of chi-1: a developmentally regulated member of a novel class of the ionotropic glutamate receptor family. J Neurosci 15:6498-6508.

Clements JD, Bekkers JM (1997) Detection of spontaneous synaptic events with an optimally scaled template. Biophys J 73:220-229.

Collingridge GL, Bliss TV (1995) Memories of NMDA receptors and LTP. Trends Neurosci 18:54-56.

Contractor QQ, Dubian MK, Boujemla M, Contractor TQ (2001) Endoscopic therapy after laparoscopic cholecystectomy. J Clin Gastroenterol 33:218-221.

Corlew R, Brasier DJ, Feldman DE, Philpot BD (2008) Presynaptic NMDA receptors: newly appreciated roles in cortical synaptic function and plasticity. Neuroscientist 14:609-625.

Corlew R, Wang Y, Ghermazien H, Erisir A, Philpot BD (2007) Developmental switch in the contribution of presynaptic and postsynaptic NMDA receptors to long-term depression. J Neurosci 27:9835-9845.

Crowley JC, Katz LC (1999) Development of ocular dominance columns in the absence of retinal input. Nat Neurosci 2:1125-1130.

Crozier RA, Wang Y, Liu CH, Bear MF (2007) Deprivation-induced synaptic depression by distinct mechanisms in different layers of mouse visual cortex. Proc Natl Acad Sci U S A 104:1383-1388.

Cull-Candy S, Brickley S, Farrant M (2001) NMDA receptor subunits: diversity, development and disease. Curr Opin Neurobiol 11:327-335.

Curtis DR, Watkins JC (1963) Acidic amino acids with strong excitatory actions on mammalian neurones. J Physiol 166:1-14.

Dalby NO, Mody I (2001) The process of epileptogenesis: a pathophysiological approach. Curr Opin Neurol 14:187-192.

Dan Y, Poo MM (2006) Spike timing-dependent plasticity: from synapse to perception. Physiol Rev 86:1033-1048.

Das S, Sasaki YF, Rothe T, Premkumar LS, Takasu M, Crandall JE, Dikkes P, Conner DA, Rayudu PV, Cheung W, Chen HS, Lipton SA, Nakanishi N (1998) Increased NMDA current and spine density in mice lacking the NMDA receptor subunit NR3A. Nature 393:377-381.

DeBiasi S, Minelli A, Melone M, Conti F (1996) Presynaptic NMDA receptors in the neocortex are both auto- and heteroreceptors. Neuroreport 7:2773-2776.

Dekay JG, Chang TC, Mills N, Speed HE, Dobrunz LE (2006) Responses of excitatory hippocampal synapses to natural stimulus patterns reveal a decrease in short-term facilitation and increase in short-term depression during postnatal development. Hippocampus 16:66-79.

Dingledine R, Borges K, Bowie D, Traynelis SF (1999) The glutamate receptor ion channels. Pharmacol Rev 51:7-61.

Duguid I, Sjöström PJ (2006) Novel presynaptic mechanisms for coincidence detection in synaptic plasticity. Curr Opin Neurobiol 16:312-322.

Duguid I, Sjostrom PJ (2006) Novel presynaptic mechanisms for coincidence detection in synaptic plasticity. Curr Opin Neurobiol 16:312-322.

Duguid IC, Smart TG (2004) Retrograde activation of presynaptic NMDA receptors enhances GABA release at cerebellar interneuron-Purkinje cell synapses. Nat Neurosci 7:525-533.

Egger V, Feldmeyer D, Sakmann B (1999) Coincidence detection and changes of synaptic efficacy in spiny stellate neurons in rat barrel cortex. Nat Neurosci 2:1098-1105.

Engelman HS, MacDermott AB (2004) Presynaptic ionotropic receptors and control of transmitter release. Nat Rev Neurosci 5:135-145.

Fan MM, Raymond LA (2007) N-methyl-D-aspartate (NMDA) receptor function and excitotoxicity in Huntington's disease. Prog Neurobiol 81:272-293.

Farb CR, Aoki C, Ledoux JE (1995) Differential localization of NMDA and AMPA receptor subunits in the lateral and basal nuclei of the amygdala: a light and electron microscopic study. J Comp Neurol 362:86-108.

Feldman DE (2000) Timing-based LTP and LTD at vertical inputs to layer II/III pyramidal cells in rat barrel cortex. Neuron 27:45-56.

Feller MB, Scanziani M (2005) A precritical period for plasticity in visual cortex. Curr Opin Neurobiol 15:94-100.

Felmy F, Neher E, Schneggenburger R (2003) The timing of phasic transmitter release is Ca2+-dependent and lacks a direct influence of presynaptic membrane potential. Proc Natl Acad Sci U S A 100:15200-15205.

Fiszman ML, Barberis A, Lu C, Fu Z, Erdelyi F, Szabo G, Vicini S (2005) NMDA receptors increase the size of GABAergic terminals and enhance GABA release. J Neurosci 25:2024-2031.

Flint AC, Maisch US, Weishaupt JH, Kriegstein AR, Monyer H (1997) NR2A subunit expression shortens NMDA receptor synaptic currents in developing neocortex. J Neurosci 17:2469-2476.

Fox K, Zahs K (1994) Critical period control in sensory cortex. Curr Opin Neurobiol 4:112-119.

Fox K, Wong RO (2005) A comparison of experience-dependent plasticity in the visual and somatosensory systems. Neuron 48:465-477.

Frenkel MY, Bear MF (2004) How monocular deprivation shifts ocular dominance in visual cortex of young mice. Neuron 44:917-923.

Froemke RC, Dan Y (2002) Spike-timing-dependent synaptic modification induced by natural spike trains. Nature 416:433-438.

Froemke RC, Poo MM, Dan Y (2005) Spike-timing-dependent synaptic plasticity depends on dendritic location. Nature 434:221-225.

Fronius M, Cirina L, Cordey A, Ohrloff C (2004) Visual improvement during psychophysical training in an adult amblyopic eye following visual loss in the contralateral eye. Graefes Arch Clin Exp Ophthalmol.

Fujisawa S, Aoki C (2003) In vivo blockade of N-methyl-D-aspartate receptors induces rapid trafficking of NR2B subunits away from synapses and out of spines and terminals in adult cortex. Neuroscience 121:51-63.

Gilbert CD (1998) Adult cortical dynamics. Physiol Rev 78:467-485.

Glitsch M, Marty A (1999) Presynaptic effects of NMDA in cerebellar Purkinje cells and interneurons. J Neurosci 19:511-519.

Glitsch MD (2008) Calcium influx through N-methyl-d-aspartate receptors triggers GABA release at interneuron-Purkinje cell synapse in rat cerebellum. Neuroscience 151:403-409.

Gogas KR (2006) Glutamate-based therapeutic approaches: NR2B receptor antagonists. Curr Opin Pharmacol 6:68-74.

Gordon JA, Stryker MP (1996) Experience-dependent plasticity of binocular responses in the primary visual cortex of the mouse. J Neurosci 16:3274-3286.

Gordon JA, Cioffi D, Silva AJ, Stryker MP (1996) Deficient plasticity in the primary visual cortex of a-calcium/calmodulin-dependent protein kinase II mutant mice. Neuron 17:491-499.

Guzzetta A, Baldini S, Bancale A, Baroncelli L, Ciucci F, Ghirri P, Putignano E, Sale A, Viegi A, Berardi N, Boldrini A, Cioni G, Maffei L (2009) Massage accelerates brain development and the maturation of visual function. J Neurosci 29:6042-6051.

Hall FS (1998) Social deprivation of neonatal, adolescent, and adult rats has distinct neurochemical and behavioral consequences. Crit Rev Neurobiol 12:129-162.

Hashimotodani Y, Ohno-Shosaku T, Tsubokawa H, Ogata H, Emoto K, Maejima T, Araishi K, Shin HS, Kano M (2005) Phospholipase Cbeta serves as a coincidence detector through its Ca2+ dependency for triggering retrograde endocannabinoid signal. Neuron 45:257-268.

He HY, Rasmusson DD, Quinlan EM (2004) Progressive elevations in AMPA and GABAA receptor levels in deafferented somatosensory cortex. J Neurochem 90:1186-1193.

He HY, Hodos W, Quinlan EM (2006) Visual deprivation reactivates rapid ocular dominance plasticity in adult visual cortex. J Neurosci 26:2951-2955.

Heinbockel T, Brager DH, Reich CG, Zhao J, Muralidharan S, Alger BE, Kao JP (2005) Endocannabinoid signaling dynamics probed with optical tools. J Neurosci 25:9449-9459.

Hendricson AW, Miao CL, Lippmann MJ, Morrisett RA (2002) Ifenprodil and ethanol enhance NMDA receptor-dependent long-term depression. J Pharmacol Exp Ther 301:938-944.

Hensch TK (2004) Critical period regulation. Annu Rev Neurosci 27:549-579.

Hensch TK, Fagiolini M, Mataga N, Stryker MP, Baekkeskov S, Kash SF (1998) Local GABA circuit control of experience-dependent plasticity in developing visual cortex. Science 282:1504-1508.

Heresco-Levy U, Javitt DC (1998) The role of N-methyl-D-aspartate (NMDA) receptor-mediated neurotransmission in the pathophysiology and therapeutics of psychiatric syndromes. Eur Neuropsychopharmacol 8:141-152.

Herman MA, Jahr CE (2007) Extracellular glutamate concentration in hippocampal slice. J Neurosci 27:9736-9741.

Hessler NA, Shirke AM, Malinow R (1993) The probability of transmitter release at a mammalian central synapse. Nature 366:569-572.

Hestrin S (1992) Developmental regulation of NMDA receptor-mediated synaptic currents at a central synapse. Nature 357:686-689.

Hofer SB, Mrsic-Flogel TD, Bonhoeffer T, Hubener M (2006a) Prior experience enhances plasticity in adult visual cortex. Nat Neurosci 9:127-132.

Hofer SB, Mrsic-Flogel TD, Bonhoeffer T, Hubener M (2006b) Lifelong learning: ocular dominance plasticity in mouse visual cortex. Curr Opin Neurobiol 16:451-459.

Horton JC, Hocking DR (1996) An adult-like pattern of ocular dominance columns in striate cortex of newborn monkeys prior to visual experience. J Neurosci 16:1791-1807.

Humeau Y, Shaban H, Bissiere S, Luthi A (2003) Presynaptic induction of heterosynaptic associative plasticity in the mammalian brain. Nature 426:841-845.

Innocenti GM (2007) Subcortical regulation of cortical development: some effects of early, selective deprivations. Prog Brain Res 164:23-37.

Iwasato T, Datwani A, Wolf AM, Nishiyama H, Taguchi Y, Tonegawa S, Knopfel T, Erzurumlu RS, Itohara S (2000) Cortex-restricted disruption of NMDAR1 impairs neuronal patterns in the barrel cortex. Nature 406:726-731.

Jo J, Ball SM, Seok H, Oh SB, Massey PV, Molnar E, Bashir ZI, Cho K (2006) Experience-dependent modification of mechanisms of long-term depression. Nat Neurosci 9:170-172.

Jourdain P, Bergersen LH, Bhaukaurally K, Bezzi P, Santello M, Domercq M, Matute C, Tonello F, Gundersen V, Volterra A (2007) Glutamate exocytosis from astrocytes controls synaptic strength. Nat Neurosci 10:331-339.

Kadotani H, Hirano T, Masugi M, Nakamura K, Nakao K, Katsuki M, Nakanishi S (1996) Motor discoordination results from combined gene disruption of the NMDA receptor NR2A and NR2C subunits, but not from single disruption of the NR2A or NR2C subunit. J Neurosci 16:7859-7867.

Kanold PO, Kim YA, GrandPre T, Shatz CJ (2009) Co-regulation of ocular dominance plasticity and NMDA receptor subunit expression in glutamic acid decarboxylase-65 knock-out mice. J Physiol 587:2857-2867.

Katz LC, Shatz CJ (1996) Synaptic activity and the construction of cortical circuits. Science 274:1133-1138.

Kew JN, Richards JG, Mutel V, Kemp JA (1998) Developmental changes in NMDA receptor glycine affinity and ifenprodil sensitivity reveal three distinct populations of NMDA receptors in individual rat cortical neurons. J Neurosci 18:1935-1943.

Khakh BS, Henderson G (2000) Modulation of fast synaptic transmission by presynaptic ligand-gated cation channels. J Auton Nerv Syst 81:110-121.

Kharazia VN, Weinberg RJ (1999) Immunogold localization of AMPA and NMDA receptors in somatic sensory cortex of albino rat. J Comp Neurol 412:292-302.

Kidd FL, Isaac JT (1999) Developmental and activity-dependent regulation of kainate receptors at thalamocortical synapses. Nature 400:569-573.

Kim DK, Catterall WA (1997) Ca2+-dependent and -independent interactions of the isoforms of the alpha1A subunit of brain Ca2+ channels with presynaptic SNARE proteins. Proc Natl Acad Sci U S A 94:14782-14786.

Kim MJ, Dunah AW, Wang YT, Sheng M (2005) Differential roles of NR2A- and NR2B-containing NMDA receptors in Ras-ERK signaling and AMPA receptor trafficking. Neuron 46:745-760.

Kirkwood A, Bear MF (1994) Homosynaptic long-term depression in the visual cortex. J Neurosci 14:3404-3412.

Koester HJ, Sakmann B (1998) Calcium dynamics in single spines during coincident pre- and postsynaptic activity depend on relative timing of back-propagating action potentials and subthreshold excitatory postsynaptic potentials. Proc Natl Acad Sci U S A 95:9596-9601.

Kopp C, Longordo F, Luthi A (2007) Experience-dependent changes in NMDA receptor composition at mature central synapses. Neuropharmacology 53:1-9.

Kreczko A, Goel A, Song L, Lee HK (2009) Visual deprivation decreases somatic GAD65 puncta number on layer 2/3 pyramidal neurons in mouse visual cortex. Neural Plast 2009:415135.

Kristiansen LV, Huerta I, Beneyto M, Meador-Woodruff JH (2007) NMDA receptors and schizophrenia. Curr Opin Pharmacol 7:48-55.

Kullmann DM, Erdemli G, Asztely F (1996) LTP of AMPA and NMDA receptor-mediated signals: evidence for presynaptic expression and extrasynaptic glutamate spill-over. Neuron 17:461-474.

Lau CG, Zukin RS (2007) NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders. Nat Rev Neurosci 8:413-426.

Lauri SE, Vesikansa A, Segerstrale M, Collingridge GL, Isaac JT, Taira T (2006) Functional maturation of CA1 synapses involves activity-dependent loss of tonic kainate receptor-mediated inhibition of glutamate release. Neuron 50:415-429.

Laurie DJ, Seeburg PH (1994) Ligand affinities at recombinant N-methyl-D-aspartate receptors depend on subunit composition. Eur J Pharmacol 268:335-345.

Lazarov O, Robinson J, Tang YP, Hairston IS, Korade-Mirnics Z, Lee VM, Hersh LB, Sapolsky RM, Mirnics K, Sisodia SS (2005) Environmental enrichment reduces Abeta levels and amyloid deposition in transgenic mice. Cell 120:701-713.

Le Meur K, Galante M, Angulo MC, Audinat E (2007) Tonic activation of NMDA receptors by ambient glutamate of non-synaptic origin in the rat hippocampus. J Physiol 580:373-383.

Li YH, Han TZ (2006) Glycine binding sites of presynaptic NMDA receptors may tonically regulate glutamate release in the rat visual cortex. J Neurophysiol.

Li YH, Han TZ (2007) Glycine binding sites of presynaptic NMDA receptors may tonically regulate glutamate release in the rat visual cortex. J Neurophysiol 97:817-823.

Li YH, Han TZ, Meng K (2008) Tonic facilitation of glutamate release by glycine binding sites on presynaptic NR2B-containing NMDA autoreceptors in the rat visual cortex. Neurosci Lett 432:212-216.

Li YH, Wang J, Zhang G (2009) Presynaptic NR2B-containing NMDA autoreceptors mediate gluta-matergic synaptic transmission in the rat visual cortex. Curr Neurovasc Res 6:104-109.

Lickey ME, Pham TA, Gordon B (2004) Swept contrast visual evoked potentials and their plasticity following monocular deprivation in mice. Vision Res 44:3381-3387.

Lien CC, Mu Y, Vargas-Caballero M, Poo MM (2006) Visual stimuli-induced LTD of GABAergic synapses mediated by presynaptic NMDA receptors. Nat Neurosci 9:372-380.

Lieske SP, Ramirez JM (2006) Pattern-specific synaptic mechanisms in a multifunctional network. II. Intrinsic modulation by metabotropic glutamate receptors. J Neurophysiol 95:1334-1344.

Liu H, Mantyh PW, Basbaum AI (1997) NMDA-receptor regulation of substance P release from primary afferent nociceptors. Nature 386:721-724.

Liu H, Wang H, Sheng M, Jan LY, Jan YN, Basbaum AI (1994) Evidence for presynaptic N-methyl-D-aspartate autoreceptors in the spinal cord dorsal horn. Proc Natl Acad Sci U S A 91:8383-8387.

Liu L, Wong TP, Pozza MF, Lingenhoehl K, Wang Y, Sheng M, Auberson YP, Wang YT (2004) Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. Science 304:1021-1024.

Lozovaya NA, Grebenyuk SE, Tsintsadze T, Feng B, Monaghan DT, Krishtal OA (2004) Extrasynaptic NR2B and NR2D subunits of NMDA receptors shape 'superslow' afterburst EPSC in rat hippocampus. J Physiol 558:451-463.

Lu CR, Willcockson HH, Phend KD, Lucifora S, Darstein M, Valtschanoff JG, Rustioni A (2005) Ionotropic glutamate receptors are expressed in GABAergic terminals in the rat superficial dorsal horn. J Comp Neurol 486:169-178.

MacDermott AB, Role LW, Siegelbaum SA (1999) Presynaptic ionotropic receptors and the control of transmitter release. Annu Rev Neurosci 22:443-485.

Magee JC, Johnston D (1997) A synaptically controlled, associative signal for Hebbian plasticity in hippocampal neurons. Science 275:209-213.

Malenka RC, Bear MF (2004) LTP and LTD: an embarrassment of riches. Neuron 44:5-21.

Mallon AP, Auberson YP, Stone TW (2004) Selective subunit antagonists suggest an inhibitory relationship between NR2B and NR2A-subunit containing N-methyl-D: -aspartate receptors in hippocampal slices. Exp Brain Res 162:374-383.

Mameli M, Carta M, Partridge LD, Valenzuela CF (2005) Neurosteroid-induced plasticity of immature synapses via retrograde modulation of presynaptic NMDA receptors. J Neurosci 25:2285-2294.

Markram H, Lubke J, Frotscher M, Sakmann B (1997) Regulation of synaptic efficacy by coincidence of postsynaptic APs and EPSPs. Science 275:213-215.

Massey PV, Johnson BE, Moult PR, Auberson YP, Brown MW, Molnar E, Collingridge GL, Bashir ZI (2004) Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. J Neurosci 24:7821-7828.

Mayer MC, Westbrook GL, Guthrie PB (1984) Voltage dependent block by magnesium of NMDA responses in spinal cord neurones. Nature 309:261-267.

McBain CJ, Mayer ML (1994) N-methyl-D-aspartic acid receptor structure and function. Physiol Rev 74:723-760.

Meldrum BS (1994) The role of glutamate in epilepsy and other CNS disorders. Neurology 44:S14-23.

Miskevich F, Doench JG, Townsend MT, Sharp PA, Constantine-Paton M (2006) RNA interference of Xenopus NMDAR NR1 in vitro and in vivo. J Neurosci Methods 152:65-73.

Missale C, Fiorentini C, Busi C, Collo G, Spano PF (2006) The NMDA/D1 receptor complex as a new target in drug development. Curr Top Med Chem 6:801-808.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12:529-540.

Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, Seeburg PH (1992) Heteromeric NMDA receptors: Molecular and functional distinction of subtypes. Science 256:1217-1221.

Morimoto K, Fahnestock M, Racine RJ (2004) Kindling and status epilepticus models of epilepsy: rewiring the brain. Prog Neurobiol 73:1-60.

Morishita H, Hensch TK (2008) Critical period revisited: impact on vision. Curr Opin Neurobiol 18:101-107.

Mower GD, Caplan CJ, Christen WG, Duffy FH (1985) Dark rearing prolongs physiological but not anatomical plasticity of the cat visual cortex. J Comp Neurol 235:448-466.

Murthy VN, Schikorski T, Stevens CF, Zhu Y (2001) Inactivity produces increases in neurotransmitter release and synapse size. Neuron 32:673-682.

Muzumdar MD, Luo L, Zong H (2007) Modeling sporadic loss of heterozygosity in mice by using mosaic analysis with double markers (MADM). Proc Natl Acad Sci U S A 104:4495-4500.

Nase G, Weishaupt J, Stern P, Singer W, Monyer H (1999) Genetic and epigenetic regulation of NMDA receptor expression in the rat visual cortex. Eur J Neurosci 11:4320-4326.

Nevian T, Sakmann B (2006) Spine Ca2+ signaling in spike-timing-dependent plasticity. J Neurosci 26:11001-11013.

Neyton J, Paoletti P (2006) Relating NMDA receptor function to receptor subunit composition: limitations of the pharmacological approach. J Neurosci 26:1331-1333.

Nilsson A, Duan J, Mo-Boquist LL, Benedikz E, Sundstrom E (2007) Characterisation of the human NMDA receptor subunit NR3A glycine binding site. Neuropharmacology 52:1151-1159.

Nithianantharajah J, Hannan AJ (2006) Enriched environments, experience-dependent plasticity and disorders of the nervous system. Nat Rev Neurosci 7:697-709.

Nosyreva ED, Huber KM (2005) Developmental switch in synaptic mechanisms of hippocampal metabotropic glutamate receptor-dependent long-term depression. J Neurosci 25:2992-3001.

Nowak L, Bregostovski P, Ascher P, Herbert A, Prochiantz A (1984) Magnesium gates glutamate-activated channels in mouse central neurones. Nature 307:462-465.

Penzo MA, Pena JL (2009) Endocannabinoid-mediated long-term depression in the avian midbrain expressed presynaptically and postsynaptically. J Neurosci 29:4131-4139.

Perez-Otano I, Ehlers MD (2004) Learning from NMDA Receptor Trafficking: Clues to the Development and Maturation of Glutamatergic Synapses. Neurosignals 13:175-189.

Perez-Otano I, Ehlers MD (2005) Homeostatic plasticity and NMDA receptor trafficking. Trends Neurosci 28:229-238.

Perez-Otano I, Schulteis CT, Contractor A, Lipton SA, Trimmer JS, Sucher NJ, Heinemann SF (2001) Assembly with the NR1 subunit is required for surface expression of NR3A-containing NMDA receptors. J Neurosci 21:1228-1237.

Phend KD, Rustioni A, Weinberg RJ (1995) An osmium-free method of epon embedment that preserves both ultrastructure and antigenicity for post-embedding immunocytochemistry. J Histochem Cytochem 43:283-292.

Philpot BD, Cho KK, Bear MF (2007) Obligatory Role of NR2A for Metaplasticity in Visual Cortex. Neuron 53:495-502.

Philpot BD, Sekhar AK, Shouval HZ, Bear MF (2001a) Visual experience and deprivation bidirectionally modify the composition and function of NMDA receptors in visual cortex. Neuron 29:157-169.

Philpot BD, Weisberg MP, Ramos MS, Sawtell NB, Tang YP, Tsien JZ, Bear MF (2001b) Effect of transgenic overexpression of NR2B on NMDA receptor function and synaptic plasticity in visual cortex. Neuropharmacology 41:762-770.

Pickel VM, Colago EE, Mania I, Molosh AI, Rainnie DG (2006) Dopamine D1 receptors co-distribute with N-methyl-D-aspartic acid type-1 subunits and modulate synaptically-evoked N-methyl-D-aspartic acid currents in rat basolateral amygdala. Neuroscience 142:671-690.

Pinheiro PS, Mulle C (2008) Presynaptic glutamate receptors: physiological functions and mechanisms of action. Nat Rev Neurosci 9:423-436.

Pinheiro PS, Perrais D, Coussen F, Barhanin J, Bettler B, Mann JR, Malva JO, Heinemann SF, Mulle C (2007) GluR7 is an essential subunit of presynaptic kainate autoreceptors at hippocampal mossy fiber synapses. Proc Natl Acad Sci U S A 104:12181-12186.

Pouzat C, Hestrin S (1997) Developmental regulation of basket/stellate cell--
>Purkinje cell synapses in the cerebellum. J Neurosci 17:9104-9112.

Priestley T, Laughton P, Myers J, Le Bourdelles B, Kerby J, Whiting PJ (1995) Pharmacological properties of recombinant human N-methyl-D-aspartate receptors comprising NR1a/NR2A and NR1a/NR2B subunit assemblies expressed in permanently transfected mouse fibroblast cells. Mol Pharmacol 48:841-848.

Quinlan EM, Olstein DH, Bear MF (1999a) Bidirectional, experience-dependent regulation of N-methyl-D-aspartate receptor subunit composition in the rat visual cortex during postnatal development. Proc Natl Acad Sci USA 96:12876-12880.

Quinlan EM, Philpot BD, Huganir RL, Bear MF (1999b) Rapid, experience-dependent expression of synaptic NMDA receptors in visual cortex in vivo. Nat Neurosci 2:352-357.

Rakic P (1977) Prenatal development of the visual system in rhesus monkey. Philos Trans R Soc Lond B Biol Sci 278:245-260.

Ramoa AS, Mower AF, Liao D, Jafri SI (2001) Suppression of cortical NMDA receptor function prevents development of orientation selectivity in the primary visual cortex. J Neurosci 21:4299-4309.

Reyes A, Sakmann B (1999) Developmental switch in the short-term modification of unitary EPSPs evoked in layer 2/3 and layer 5 pyramidal neurons of rat neocortex. J Neurosci 19:3827-3835.

Roberts EB, Ramoa AS (1999) Enhanced NR2A subunit expression and decreased NMDA receptor decay time at the onset of ocular dominance plasticity in the ferret. J Neurophysiol 81:2587-2591.

Roberts EB, Meredith MA, Ramoa AS (1998) Suppression of NMDA receptor function using antisense DNA blocks ocular dominance plasticity while preserving visual responses. J Neurophysiol 80:1021-1032.

Rodriguez-Moreno A, Paulsen O (2008) Spike timing-dependent long-term depression requires presynaptic NMDA receptors. Nat Neurosci 11:744-745.

Rumpel S, Kattenstroth G, Gottmann K (2004) Silent synapses in the immature visual cortex: layer-specific developmental regulation. J Neurophysiol 91:1097-1101.

Sah P, Hestrin S, Nicoll RA (1989) Tonic activation of NMDA receptors by ambient glutamate enhances excitability of neurons. Science 246:815-818.

Sale A, Putignano E, Cancedda L, Landi S, Cirulli F, Berardi N, Maffei L (2004) Enriched environment and acceleration of visual system development. Neuropharmacology 47:649-660.

Sale A, Maya Vetencourt JF, Medini P, Cenni MC, Baroncelli L, De Pasquale R, Maffei L (2007) Environmental enrichment in adulthood promotes amblyopia recovery through a reduction of intracortical inhibition. Nat Neurosci 10:679-681.

Sasaki YF, Rothe T, Premkumar LS, Das S, Cui J, Talantova MV, Wong HK, Gong X, Chan SF, Zhang D, Nakanishi N, Sucher NJ, Lipton SA (2002) Characterization and comparison of the NR3A subunit of the NMDA receptor in recombinant systems and primary cortical neurons. J Neurophysiol 87:2052-2063.

Sawtell NB, Frenkel MY, Philpot BD, Nakazawa K, Tonegawa S, Bear MF (2003) NMDA receptor-dependent ocular dominance plasticity in adult visual cortex. Neuron 38:977-985.

Scanziani M, Gahwiler BH, Charpak S (1998) Target cell-specific modulation of transmitter release at terminals from a single axon. Proc Natl Acad Sci U S A 95:12004-12009.

Scimemi A, Fine A, Kullmann DM, Rusakov DA (2004) NR2B-containing receptors mediate cross talk among hippocampal synapses. J Neurosci 24:4767-4777.

Sengpiel F, Kind PC (2002) The role of activity in development of the visual system. Curr Biol 12:R818-826.

Shin JH, Linden DJ (2005) An NMDA receptor/nitric oxide cascade is involved in cerebellar LTD but is not localized to the parallel fiber terminal. J Neurophysiol 94:4281-4289.

Shouval H, Perrone M (1995) Post-Hebbian Learning Rules. In: The Handbook of Brain Theory and Neural Networks (Arbib M, ed), p 1118. Cambridge, Massachusetts: MIT Press.

Siegel SJ, Brose N, Janssen WG, Gasic GP, Jahn R, Heinemann SF, Morrison JH (1994) Regional, cellular, and ultrastructural distribution of N-methyl-D-aspartate receptor subunit 1 in monkey hippocampus. Proc Natl Acad Sci U S A 91:564-568.

Simkus CR, Stricker C (2002) Properties of mEPSCs recorded in layer II neurones of rat barrel cortex. J Physiol 545:509-520.

Simmers AJ, Gray LS (1999) Improvement of visual function in an adult amblyope. Optom Vis Sci 76:82-87.

Sjostrom PJ, Turrigiano GG, Nelson S (2003) Neocortical LTD via Coincident Activation of Presynaptic NMDA and Cannabinoid Receptors. Neuron 39:641-654.

Sjöström PJ, Turrigiano GG, Nelson SB (2001) Rate, timing, and cooperativity jointly determine cortical synaptic plasticity. Neuron 32:1149-1164.

Sjöström PJ, Turrigiano GG, Nelson SB (2003) Neocortical LTD via coincident activation of presynaptic NMDA and cannabinoid receptors. Neuron 39:641-654.

Smothers CT, Woodward JJ (2003) Effect of the NR3 subunit on ethanol inhibition of recombinant NMDA receptors. Brain Res 987:117-121.

Spruston N, Jaffe DB, Williams SH, Johnston D (1993) Voltage- and space-clamp errors associated with the measurement of electrotonically remote synaptic events. J Neurophysiol 70:781-802.

Strack S, McNeill RB, Colbran RJ (2000) Mechanism and regulation of calcium/calmodulin-dependent protein kinase II targeting to the NR2B subunit of the N-methyl-D-aspartate receptor. J Biol Chem 275:23798-23806.

Stryker MP, Harris WA (1986) Binocular impulse blockade prevents the formation of ocular dominance columns in cat visual cortex. J Neurosci 6:2117-2133.

Suarez LM, Solis JM (2006) Taurine potentiates presynaptic NMDA receptors in hippocampal Schaffer collateral axons. Eur J Neurosci 24:405-418.

Suarez LM, Suarez F, Del Olmo N, Ruiz M, Gonzalez-Escalada JR, Solis JM (2005) Presynaptic NMDA autoreceptors facilitate axon excitability: a new molecular target for the anticonvulsant gabapentin. Eur J Neurosci 21:197-209.

Sun HY, Dobrunz LE (2006) Presynaptic kainate receptor activation is a novel mechanism for target cell-specific short-term facilitation at Schaffer collateral synapses. J Neurosci 26:10796-10807.

Tagawa Y, Kanold PO, Majdan M, Shatz CJ (2005) Multiple periods of functional ocular dominance plasticity in mouse visual cortex. Nat Neurosci 8:380-388.

Taha SA, Stryker MP (2005) Molecular substrates of plasticity in the developing visual cortex. Prog Brain Res 147:103-114.

Thoreson WB, Ulphani JS (1995) Pharmacology of selective and non-selective metabotropic glutamate receptor agonists at L-AP4 receptors in retinal ON bipolar cells. Brain Res 676:93-102.

Todd KJ, Slatter CA, Ali DW (2004) Activation of ionotropic glutamate receptors on peripheral axons of primary motoneurons mediates transmitter release at the zebrafish NMJ. J Neurophysiol 91:828-840.

Townsend M, Yoshii A, Mishina M, Constantine-Paton M (2003) Developmental loss of miniature N-methyl-D-aspartate receptor currents in NR2A knockout mice. Proc Natl Acad Sci U S A 100:1340-1345.

Toyoda H, Zhao MG, Zhuo M (2005) Roles of NMDA receptor NR2A and NR2B subtypes for long-term depression in the anterior cingulate cortex. Eur J Neurosci 22:485-494.

Tropea D, Van Wart A, Sur M (2009) Molecular mechanisms of experience-dependent plasticity in visual cortex. Philos Trans R Soc Lond B Biol Sci 364:341-355.

Trussell LO (2002) Modulation of transmitter release at giant synapses of the auditory system. Curr Opin Neurobiol 12:400-404.

Turecek R, Trussell LO (2001) Presynaptic glycine receptors enhance transmitter release at a mammalian central synapse. Nature 411:587-590.

Valenzuela CF, Partridge LD, Mameli M, Meyer DA (2008) Modulation of glutamatergic transmission by sulfated steroids: role in fetal alcohol spectrum disorder. Brain Res Rev 57:506-519.

Vicini S, Wang JF, Li JH, Zhu WJ, Wang YH, Luo JH, Wolfe BB, Grayson DR (1998) Functional and pharmacological differences between recombinant N-methyl-D-aspartate receptors. J Neurophysiol 79:555-566.

Visser E, Schug SA (2006) The role of ketamine in pain management. Biomed Pharmacother 60:341-348.

Vitten H, Isaacson JS (2001) Synaptic transmission: exciting times for presynaptic receptors. Curr Biol 11:R695-697.

Weitlauf C, Honse Y, Auberson YP, Mishina M, Lovinger DM, Winder DG (2005) Activation of NR2A-containing NMDA receptors is not obligatory for NMDA receptor-dependent long-term potentiation. J Neurosci 25:8386-8390.

Wiesel TN, Hubel DH (1963) Single cell responses in striate cortex of kittens deprived of vision in one eye. J Neurophysiol 26:1003-1017.

Wolfer DP, Litvin O, Morf S, Nitsch RM, Lipp HP, Wurbel H (2004) Laboratory animal welfare: cage enrichment and mouse behaviour. Nature 432:821-822.

Woodhall G, Evans DI, Cunningham MO, Jones RS (2001) NR2B-containing NMDA autoreceptors at synapses on entorhinal cortical neurons. J Neurophysiol 86:1644-1651.

Yang J, Woodhall GL, Jones RS (2006) Tonic facilitation of glutamate release by presynaptic NR2B-containing NMDA receptors is increased in the entorhinal cortex of chronically epileptic rats. J Neurosci 26:406-410.

Yao Y, Mayer ML (2006) Characterization of a soluble ligand binding domain of the NMDA receptor regulatory subunit NR3A. J Neurosci 26:4559-4566.

Yashiro K, Philpot BD (2008) Regulation of NMDA receptor subunit expression and its implications for LTD, LTP, and metaplasticity. Neuropharmacology 55:1081-1094.

Yashiro K, Corlew R, Philpot BD (2005) Visual deprivation modifies both presynaptic glutamate release and the composition of perisynaptic/extrasynaptic NMDA receptors in adult visual cortex. J Neurosci 25:11684-11692.

Yasuda H, Barth AL, Stellwagen D, Malenka RC (2003) A developmental switch in the signaling cascades for LTP induction. Nat Neurosci 6:15-16.

Yoshii A, Sheng MH, Constantine-Paton M (2003) Eye opening induces a rapid dendritic localization of PSD-95 in central visual neurons. Proc Natl Acad Sci U S A 100:1334-1339.

Zong H, Espinosa JS, Su HH, Muzumdar MD, Luo L (2005) Mosaic analysis with double markers in mice. Cell 121:479-492.

Zucker RS, Regehr WG (2002) Short-term synaptic plasticity. Annu Rev Physiol 64:355-405.