Sensory experience-dependent synaptic modifications in the visual cortex

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ABSTRACT

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Under the direction of Benjamin D. Philpot

During early postnatal development, the brain receives tremendous input from developing sensory organs. These sensory inputs shape neuronal networks in the cortex so as to adapt the neuronal circuits to the animal's living environment. Thus, synaptic connections in the cortex mature in a sensory experience-dependent manner. In this thesis, I studied 1) an endogenous mechanism that is crucial for regulating normal, experience-dependent synaptic modification in adult rodents, and 2) how dysregulation of experience-dependent plasticity contributes to a severe mental retardation, Angelman syndrome.

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PREFACE

In any instance of our daily life, we receive constant sensory input through our ears, nose, tongue, skin, and eyes. These sensory inputs are processed by neurons in the brain, and some of these inputs alter neuronal circuits to form memories or to adapt the brain to the environment. It is widely believed that experience modifies neural circuits at specialized contacts between neurons, called synapses, because sensory experiences are known to alter the structures and properties of synapses. Thus, to understand mechanisms of memory formation or sensory adaptation, the question should be asked is, "How does sensory experience modify synapses?" My research seeks to answer this fundamental question and to solve how this process is distorted in a neurodevelopmental disorder. Specifically, I aim to understand 1) how experience modifies synapses in the visual cortex, and 2) how disruption of the experience-dependent modifications results in a severe mental retardation known as Angelman syndrome.

In the first project, I tested if visual deprivation changes properties of synapses in the visual cortex. Using electrophysiological and biochemical means, I showed for the first time that visual experience modifies subunit composition of NMDA receptors in the adult brain. In the second project, I studied synaptic properties of a mutant mouse, which resembles Angelman syndrome. By studying the visual cortex of the mutant mouse, I

found that their synapses are less plastic, explaining learning inabilities in this syndrome. These results have important implications for both mechanisms of sensory information encoding and cognitive disorders.

Chapter 1: Introduction to the roles of NMDA-type glutamate receptors (NMDARs) in synaptic plasticity and Angelman syndrome.

Chapter 2: The results of a study testing sensory experience-dependent modifications of NMDARs. Our results suggest that sensory experience modifies subunit composition of extrasynaptic NMDARs in the visual cortex of adult mice.

Chapter 3: Our initial study of the mouse model of Angelman syndrome is reported. Our results suggest that these mice are incapable of undergoing sensory experience-dependent synaptic development and that the lack of this development results from experience-guided loss of synaptic plasticity.

Chapter 4: Conclusions and future directions. Significance of the findings in the chapter 2 and 3 are discussed in light of existing literatures. Future experiments are proposed.

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LIST OF ABREVIATIONS

ACSF	artificial cerebrospinal fluid
AMPAR	AMPA-type glutamate receptors
APV	(2R)-amino-5-phosphonovaleric acid
AS	Angelman syndrome
BDNF	brain-derived neurotrophic factor
CaMKII	calcium/calmodulin kinase II
CRE	cAMP response element
CSPGs	Chondroitin sulphate proteoglycans
DIV	days *in vitro*
DR	dark-reared
ECM	extracellular matrix
ER	endoplasmic reticulum
fEPSP	field excitatory postsynaptic potential
FMRP	fragile X mental retardation protein
$GABA_ARs$	$GABA_A$ receptors
GABAB3	β subunit of $GABA_A$ receptor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LSM	lysed synaptosomal membrane
LTD	long-term depression
LTP	long-term potentiation
MAGUK	membrane-associated guanylate kinase

MD	monocular deprivation
MeCP2	methyl-CpG-binding protein
mEPSC	miniature excitatory post synaptic currents
mGluR	metabotropic glutamate receptor
MHCI	major histocompatibility complex class I
Mib2	Mind bomb 2
MYCBP2	myc binding protein 2
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
P	postnatal day
PBS	phosphate-buffered saline
PirB	Paired-immunoglobulin-like receptor B
PNS	Post-nuclear supernatant
PSD	postsynaptic density
PSD-95	postsynaptic density protein-95
RasGAP	Ras GTPase activating protein

Ras-GRF1	Ras-guanine nucleotide-releasing factor 1
RPM-1	regulator of presynaptic morphology
RTT	Rett Syndrome
SAP102	synapse-associated protein 102
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
$Ube3A^{m-/p+}$	maternally-deficient Ube3A heterozygous mouse (AS model mouse)
$Ube3A^{m+/p-}$	paternally-deficient Ube3A heterozygous mouse
VEP	visually evoked potential
WT	wild-type

Chapter 1

Introduction

1.1. Regulation of NMDA receptor subunit expression and its implications for LTD, LTP, and metaplasticity

This was written as a review to be submitted for publication.

1.1.1. Abstract

NMDA-type glutamate receptors (NMDARs) mediate many forms of synaptic plasticity. Two main regulatory subunits of NMDARs are NR2A and NR2B. In the neonatal neocortex NR2B-containing NMDARs predominate, and sensory experience facilitates a developmental switch of NMDARs from containing NR2B to predominantly containing NR2A. In this review, I clarify roles of NR2 subunits in synaptic plasticity. While the physiological importance of a shift in the ratio of NR2A and NR2B subunits is multifold, I argue that a primary role of this shift is to control the threshold, rather than determining the direction, for modifying synaptic strength. I also discuss recent studies that illuminate the mechanisms regulating NR2 subunits, and I suggest that the NR2A/NR2B ratio is regulated both locally at individual synapses and globally in a cell-wide manner. Finally, I use the visual cortex as a model system to illustrate how activity-dependent modifications in the NR2A/NR2B ratio may contribute to the development of cortical functions.

1.1.2. Introduction

NMDARs and AMPA-type glutamate receptors (AMPARs) are key mediators of excitatory synaptic transmission in the brain. Both NMDARs and AMPARs are glutamate-gated cation channels that convert a chemical signal (glutamate released from presynaptic terminals) to an electric signal (a membrane voltage change due to cation flow

though the receptors). Most NMDAR subtypes are unique in that their opening requires both presynaptic transmitter release and strong postsynaptic membrane depolarization (Mayer et al., 1984; Nowak et al., 1984). This coincidence detection arises because NMDARs are typically blocked by Mg^{2+} at resting membrane potentials and can only be activated when there is both receptor binding of glutamate and sufficient depolarization to remove the Mg^{2+} block of the receptor (Mayer et al., 1984; Nowak et al., 1984). NMDARs are permeable to Na^{+}, K^{+}, and Ca^{2+} ions, the latter of which acts as a second messenger to modify synapses. These receptor properties insure input specificity of Ca^{2+}-dependent synaptic modifications by NMDARs.

Two paradigmatic examples of changes in synaptic strength are long-term depression (LTD) and long-term potentiation (LTP), which are induced in a variety of brain regions with diverse stimulation protocols (Malenka and Bear, 2004). Many forms of LTD and LTP require NMDAR activation. Stimulations that induce NMDAR activation and subsequent Ca^{2+} influx trigger a cascade of events to express LTD or LTP. Those events include AMPAR removal from, or insertion into, postsynaptic membranes, respectively (Malenka and Bear, 2004) and changes in spine morphology (Matsuzaki et al., 2004; Zhou et al., 2004). The direction of the plasticity (weakening or strengthening) is controlled largely by the kinetics and amount of Ca^{2+} influx through NMDARs. In the case of frequency-dependent forms of synaptic plasticity, the magnitude and time course of Ca^{2+} entry is determined by the frequency of the conditioning stimulation given to axonal fibers. For example, 100 Hz stimulation of axonal fibers for 1-3 seconds induces rapid and robust Ca^{2+} entry through NMDARs resulting in LTP (Bliss and Lomo, 1973). On the other hand,

0.5-5 Hz stimulation lasting for 5-30 minutes allows a smaller magnitude of Ca^{2+} entry through NMDARs over a longer time course leading to LTD (Dudek and Bear, 1992). The level of Ca^{2+} influx through NMDARs is determined in part by the level of postsynaptic membrane depolarization, as this determines the extent that NMDARs are relieved from Mg^{2+} block. Thus, even with low frequency stimulation, LTP can be induced if the postsynaptic cells are held at depolarized membrane potentials (Kelso et al., 1986).

NMDARs are thought to consist of four subunits: two obligatory NR1 subunits and two regulatory subunits that can be NR2A→D, or NR3A or B. The precise combination of NMDAR subunits determines the functional properties of the NMDAR channels (Cull-Candy and Leszkiewicz, 2004). Additional heterogeneity of NMDAR functions can arise through alternative splicing. For example, NR1 and NR2D subunits undergo alternative splicing to yield eight and two splice variants, respectively. Both the NMDAR subunit composition (Chen et al., 2000; Liu et al., 2004b; Nase et al., 1999; Quinlan et al., 1999a; Roberts and Ramoa, 1999) and the alternative splicing of NR1 subunits (Laurie and Seeburg, 1994; Prybylowski and Wolfe, 2000) change during development. NR2A and NR2B subunits, which predominate NMDARs in the forebrain, undergo a particularly well-characterized developmental shift in the cortex. NR2B subunits are abundant in the early postnatal brain, and NR2A levels increase progressively with development (Quinlan et al., 1999a; Roberts and Ramoa, 1999; Sheng et al., 1994). Sensory deprivation retards the NR2B→NR2A shift in NMDAR composition (Liu et al., 2004b; Nase et al., 1999; Quinlan et al., 1999a; Roberts and Ramoa, 1999), suggesting this subunit change is guided in part by sensory experience.

In this review, I will discuss three fundamental questions regarding the NR2A and NR2B subunits. First, what are the molecular bases for the activity-dependent regulation of the NR2A/NR2B ratio? Second, what are the roles of NR2A and NR2B in LTD and LTP? Third, what is the functional consequence of the NR2A/NR2B ratio change in synaptic plasticity *in vitro* and *in vivo*? This review is not meant to be comprehensive, but rather is meant to highlight recent literature, to address controversies in the field, and to put forth one viewpoint about the importance of the developmental changes in NMDAR subunit composition. Readers are directed to other recent reviews for a more in-depth perspective on the functions and regulation of NMDARs (Cull-Candy and Leszkiewicz, 2004; Kopp et al., 2007; Lau and Zukin, 2007).

1.1.3. Characteristics of NR2A and NR2B subunits

Among the six regulatory subunits of NMDARs, NR2A and NR2B have been extensively studied because they are broadly expressed in the brain, predominate in the postnatal cortex, and are believed to play important roles in synaptic plasticity. NR2A and NR2B subtypes of NMDARs are present as either di-heteromers (NR1/NR2A or NR1/NR2B) or tri-heteromers (NR1/NR2A/NR2B). A recent biochemical study involving serial immunoprecipitation from hippocampal lysates in young rats at P42 estimated that 60–70% of NR2A and 70–85% of NR2B subunits were associated in NR1/NR2A or NR1/NR2B di-heteromeric complexes, and one third of NMDARs are NR1/NR2A/NR2B tri-heteromers (Al-Hallaq et al., 2007). It is likely that NMDARs in a single spine contain a heterogeneous combination of NMDARs, because the rate of blockade of NMDAR

currents by NR2B-specific antagonist, ifenprodil, are different among spines (Sobczyk et al., 2005). NR2A and NR2B differ in channel kinetics, synaptic localization, and protein binding partners, all of which are expected to influence the induction of synaptic plasticity (Table 1-1), elaborated as follows:

Channel kinetics

At a macroscopic level, NR1/NR2A di-heteromeric channels exhibit faster rising and decaying currents than NR1/NR2B di-heteromeric channels (Chen et al., 1999; Monyer et al., 1994; Prybylowski et al., 2002; Vicini et al., 1998). NR1/NR2A/NR2B tri-heteromeric channels reveal intermediate decay time courses (Vicini et al., 1998). The difference in the decay kinetics arises from their single channel behaviors. That is, NR1/NR2A channels have higher open probability and faster deactivation than NR1/NR2B channels (Chen et al., 1999; Erreger et al., 2005) (but also see (Prybylowski et al., 2002)). Therefore, in response to glutamate release, NR1/NR2A channels tend to open and close earlier than NR1/NR2B channels, resulting in the faster rise and decay times observed macroscopically for NR2A-containing NMDARs (Chen et al., 1999; Erreger et al., 2005). Although NR1/NR2B channels may have lower peak currents, they carry about two-fold more charge than NR1/NR2A channels (Erreger et al., 2005). This occurs because deactivation of NR1/NR2B receptors is slow enough to compensate for their lower open probability (Erreger et al., 2005). Moreover, Ca^{2+} imaging studies suggest that NR2B-containing NMDARs carry more Ca^{2+} per unit of current than NR2A-containing NMDARs (Sobczyk et al., 2005). Therefore, NR1/NR2B channels may carry a greater Ca^{2+} charge than NR1/NR2A receptors because of their higher charge transfer and Ca^{2+} permeability.

However, I stress that this viewpoint remains highly speculative, as this interpretation awaits studies both that directly measure Ca^{2+} responses in isolated NR2A-only or NR2B-only synapses and that more accurately assess and quantify the open probability statistics of NR1/NR2B and NR1/NR2A receptors in mammalian neurons (see (Prybylowski et al., 2002)).

Synaptic localization

NMDARs are found both at synaptic and extrasynaptic sites including the cell soma and dendritic shaft. The understanding of these studies are, however, complicated by the varied use of terminology to describe different cellular compartments. In this review, I define the compartments of the neuronal surface as follows: the central part of postsynapse = the area activated by spontaneous neurotransmitter release; the postsynapse = area activated by action potential driven (evoked) neurotransmitter release; perisynapse = region of the postsynaptic activated only by glutamate spillover from the synaptic cleft arising from elevated levels of transmitter release driven by trains of action potentials or arising from single action potentials in the presence of glutamate transporter inhibitors; extrasynapse = area activated not by synaptic stimulations but by chemical stimulation such as bath application of NMDA after blockage of synaptic NMDARs.

NR2A-containing NMDARs are thought to be concentrated in the central part of the postsynapse in the adult brain. This view has arisen because, in rat dentate gyrus granule cells, NMDAR-mediated miniature excitatory post synaptic currents (mEPSCs) reveal faster decay kinetics than evoked NMDAR-mediated EPSCs (Dalby and Mody, 2003).

Moreover, spontaneous synaptic events fail to activate NMDAR currents in the midbrain of NR2A knockout mice, while evoked synaptic activity can drive NMDAR currents in the absence of NR2A (Townsend et al., 2003; Zhao and Constantine-Paton, 2007). Thus, miniature synaptic transmission primarily activates NR2A-containing NMDARs, whereas action potential driven synaptic transmission engages both NR2A and NR2B-containing NMDARs.

Subunit complements of NMDARs at extrasynaptic sites are controversial. Whereas some studies suggest that NR2B-containing NMDARs are most prevalent at extrasynaptic sites (Scimemi et al., 2004; Stocca and Vicini, 1998; Tovar and Westbrook, 1999), other studies suggests that both NR2A- and NR2B-containing NMDARs exist extrasynaptically (Mohrmann et al., 2000) and the ratio of the two subtypes is comparable to that of synaptic NMDARs (Thomas et al., 2006). While the subunit composition of NMDARs at extrasynaptic sites remains controversial, it is clear that synaptic and extrasynaptic NMDARs couple to distinct intracellular signaling pathways (Ehlers, 2003; Hardingham et al., 2002; Ivanov et al., 2006). However, because extrasynaptic NMDARs are activated only by non-physiological stimulation such as bath application of NMDA or under pathological conditions, and are unlikely to be activated under basal conditions (Herman and Jahr, 2007), the subunit complement of extrasynaptic NMDARs may be irrelevant to the normal induction of synaptic plasticity (although see (Harris and Pettit, 2008)).

Protein interaction

To induce NMDAR-dependent LTD and LTP, downstream signaling pathways are tightly coupled to NMDARs. Hence, proteins interacting with NMDAR subunits are important determinates for the direction of synaptic plasticity. NR2A and NR2B interact with different proteins intracellularly (reviewed in (Kennedy et al., 2005)). NR2B has many unique or preferential binding partners. For example, NR2B interacts directly with Ras-guanine nucleotide-releasing factor 1 (Ras-GRF1) (Krapivinsky et al., 2003), although whether this interaction is occurring at synapses needs to be shown. NR2B is also indirectly linked to synaptic Ras GTPase activating protein (RasGAP), presumably through synapse-associated protein 102 (SAP102) (Kim et al., 1998). One of the most important NMDAR binding partners is CaMKII, which has a well-documented role in the induction of LTP (reviewed in (Lisman et al., 2002)). CaMKII binds with high affinity to NR2B subunits (Leonard et al., 1999; Strack and Colbran, 1998; Strack et al., 2000), and, to a much lesser extent, with NR2A subunits (Lisman et al., 2002). Ca^{2+} that enters through NMDARs associates with a Ca^{2+} binding protein, calmodulin, and the Ca^{2+}/calmodulin complex interacts with and activates CaMKII. Activated CaMKII binds strongly to NR2B (Strack and Colbran, 1998), allowing CaMKII to remain active even after dissociating from Ca^{2+}/calmodulin (Bayer et al., 2001). It has been shown that CaMKII activation and its association to NR2B are required for LTP induction (Barria and Malinow, 2005).

NR2A also appears to have some unique associations with signaling molecules. A recent study suggests that NR2A co-immunoprecipitates with neuronal nitric oxide synthase more effectively than NR2B (Al-Hallaq et al., 2007). Although this interaction is likely indirect,

the association raises the interesting possibility that NO-mediated presynaptic forms of LTP and LTD (Haghikia et al., 2007; Prast and Philippu, 2001; Zhang et al., 2006) may be preferentially linked to NR2A-mediated signaling pathways.

Both NR2A and NR2B possess PDZ-binding motifs in their c-terminus. Through the PDZ-binding motifs, they interact with membrane-associated guanylate kinase (MAGUK) family of synaptic scaffolding proteins that in turn associate with important synaptic signaling molecules and tether NMDARs to intracellular signaling pathways (Kennedy, 2000). The differential interaction of NR2A and NR2B subunits to MAGUKs is controversial. It was once believed that, whereas NR2A preferentially bound to postsynaptic density protein-95 (PSD-95), NR2B predominantly interacts with SAP102 (Sans et al., 2000; Townsend et al., 2003). Moreover, these interactions were thought to control distinct synaptic localization of NR2A and NR2B (Townsend et al., 2003). However, a recent biochemical study using a serial immunoprecipitation suggests that MAGUK proteins such as PSD-95 and SAP102 interact with di-heteromeric NR1/NR2A and NR1/NR2B receptors at comparable levels (Al-Hallaq et al., 2007). Thus, additional studies are needed to clarify the association of NMDAR subunits with MAGUK family members and what effects these associations may have on receptor localization and on plasticity signaling pathways.

1.1.4. Activity-dependent modulation of NR2A/NR2B ratio

The NR2A/NR2B ratio is not fixed at synapses, rather, it changes with development and sensory experience (Carmignoto and Vicini, 1992; Hestrin, 1992; Liu et al., 2004b; Nase et

al., 1999; Quinlan et al., 1999a; Roberts and Ramoa, 1999) as well as plasticity (Bellone and Nicoll, 2007). This change may help to optimize the threshold for inducing synaptic plasticity at different developmental points and/or under different sensory environments (discussed later). An important question is how sensory experience and neuronal activity regulate the ratio of the two subunits. Recent studies in cultured neurons have revealed differential regulation of NR2A and NR2B subunits at various points in their synthesis, trafficking, and degradation. Here I describe mechanisms that control NR2A and NR2B at each step and how these mechanisms can be regulated by neuronal activity (Fig.1-2).

Transcription and translation

Much of what we understand of the transcriptional and translational regulation of NMDARs comes from studies of cultured neurons, and such studies reveal important differences based on the maturity of neurons in culture. The early developmental increase (days *in vitro* (DIV) 9 to 15) in the NR2A/NR2B ratio is largely due to an increase in NR2A mRNA (Hoffmann et al., 2000), suggesting that the developmental shift is controlled at a transcriptional level (but also see (Follesa and Ticku, 1996)). This increase in NR2A levels can be suppressed by blockers of NMDARs ((2R)-amino-5-phosphonovaleric acid; APV), or voltage-gated calcium channels (Nifedipine). NR2B mRNA levels in immature cultures are insensitive to APV treatment. These data indicate that the early developmental increase in the NR2A/NR2B ratio is caused primarily by an increase in NR2A levels driven by activity-dependent activation of NMDARs. These observations also suggest that NR2A and NR2B transcripts are regulated by distinct Ca^{2+}-dependent mechanisms. The basis for the differential transcriptional response for the NR2

subunits is currently unknown. However, analysis of transcriptional regulatory sequences of NR2A revealed that a sequence between -1253 and -1180 in the up-stream region of NR2A contains a cAMP response element (CRE)-like element and is necessary for the developmental increase in NR2A (Desai et al., 2002a). Although it has not yet been tested if this coding sequence is required for activity-dependent NR2A transcription, the possible regulation of NR2A by CRE gives rise to the interesting possibility that the activity-dependent developmental increases in NR2A may be mediated by the NMDAR/PKA/CREB pathway.

In older cortical cultures (DIV22-30), responses of NR2A and NR2B to APV treatment is quite different to that observed in younger cultures. One day of NMDAR blockade by APV increases NR2B protein without affecting the level of NR2A protein, and this NR2B increase is largely blocked by the translational inhibitors, cycloheximide and anisomycin (Chen and Bear, 2006). Therefore, NMDAR activity in more mature neurons may tonically suppress NR2B translation, and brief (one day) blockade of NMDARs may be sufficient to relieve this suppression. Thus, neuronal activity appears to facilitate transcription of NR2A in immature neurons, while activity and NMDAR activation suppresses translation of NR2B in more mature neurons. Together, both these mechanisms increase the NR2A/NR2B ratio in response to enhanced neural activity.

Forward trafficking

NMDAR subunits are assembled in the endoplasmic reticulum (ER) (Qiu et al., 2005), modified in the ER and Golgi, and then trafficked to the plasma membrane. Both NR2A

and NR2B contain an ER export signal (HLFY) at the base of their c-terminus (Hawkins et al., 2004). Because of the ER export signal on NR2 subunits, overexpression of NR2 subunits enhances the surface delivery of NMDARs by overcoming an ER retention signal located in NR1 subunits (Scott et al., 2001; Standley et al., 2000). In this regard, the increase in NR2B levels by NMDAR blockade seen in more mature neurons (Chen and Bear, 2006) could also facilitate the surface delivery of NMDARs. Consistent with this idea, chronic inactivation of NMDARs has been shown to increase synaptic NR1 (Crump et al., 2001; Rao and Craig, 1997) and synaptic NR2B (Ehlers, 2003).

The activity-dependent mechanisms are distinct for regulating the synaptic delivery of NR2A and NR2B (Barria and Malinow, 2002). Synaptic accumulation of NR2A-containing NMDARs requires glutamate binding to NMDARs, whereas NR2B-containing NMDARs can accumulate at synapses regardless of synaptic activity level or ligand binding (Barria and Malinow, 2002). These results provide further evidence that synaptic activity preferentially drives NR2A-containing NMDARs to the synapse. Thus, neuronal activity not only increases transcription of NR2A (Hoffmann et al., 2000) but also facilitates synaptic delivery of NR2A-containing NMDARs over NR2B-containing NMDARs. This differential regulation of NR2A and NR2B subunits ensures an activity-dependent increase in the NR2A/NR2B ratio. The molecular mechanisms underlying this differential trafficking of NMDAR subunits is an intense area of research.

Surface diffusion

Although it has been traditionally thought that NMDARs are relatively stable and immobile at the cell surface, recent electrophysiological and imaging studies suggest that NMDARs are highly mobile. By taking advantage of the irreversible open channel blocker, MK-801, Tovar and Westbrook have shown in immature cultured hippocampal neurons that NMDAR-mediated EPSC can quickly recover following MK-801 block (Tovar and Westbrook, 2002). This recovery was shown to be due to lateral diffusion, by which 65% of synaptic NMDARs exchange in less than 7 minutes. NMDAR lateral mobility has also been observed by single molecular tracking (Groc et al., 2004, 2006, 2007). These studies reveal that NMDARs are highly mobile both at synaptic and extrasynaptic membranes. Importantly, the surface mobility of NMDARs appears to change with development in a subunit composition specific manner (Groc et al., 2006). For example, NR2A-containing NMDARs are less mobile than NR2B-containing NMDARs, and the synaptic residency time of NR2B-containing NMDARs decreases over development. This decrease in synaptic dwell time of NR2B-containing NMDARs is mediated by Reelin, an extracellular matrix protein (Groc et al., 2007). Interestingly, the lateral mobility of NMDARs is insensitive to acute changes in neuronal activity levels, and this is unlike AMPARs, whose diffusion is bidirectionally controlled by neuronal activity (Groc et al., 2004). It is unknown, however, if chronic activity manipulations for several days affect NMDAR surface mobility and thereby contributes in a homeostatic fashion to differential synaptic accumulations of NR2 subunits. Lastly, these studies were performed primarily in neuronal cultures, where the packing density of cells is lower than in slices or *in vivo*.

Therefore, surface mobility of these receptors need to be examined in more intact preparations.

Endocytosis

The ability of NMDARs to undergo endocytosis decreases with age (Roche et al., 2001). This developmental change is likely a consequence of the fact that NR2B subunits experience more robust endocytosis than NR2A subunits (Lavezzari et al., 2004), and the proportion of NR2B subunits decreases with age. How are the distinct endocytic mechanisms of NR2A and NR2B controlled? Both NR2A and NR2B contain a PDZ binding motif (ESDV) at the c-terminus (Lin et al., 2004; Prybylowski et al., 2005), and this helps to tether the subunits to the postsynaptic density through binding to MAGUK proteins, such as PSD-95 and SAP102. Interestingly, PDZ binding is required for synaptic localization of NR2B, but not NR2A (Lin et al., 2004; Prybylowski et al., 2005). The c-terminus of both NR2A and NR2B contain the endocytic signals LL (Lavezzari et al., 2004) and YEKL (Roche et al., 2001), respectively, which bind the AP2 clathrin adaptor protein to initiate clathrin-dependent endocytosis. Additional regulation of NR2B endocytosis is endowed through phosphorylation of tyrosine 1472 in YEKL by the Src-family kinase Fyn (Prybylowski et al., 2005). This phosphorylation protects YEKL from AP2 binding, and consequently limits NR2B subtypes from clathrin-dependent endocytosis. Thus, localization of NR2B-containing NMDARs to the postsynaptic density is controlled by Fyn and an as yet unidentified phosphatase which removes the phosphate from YEKL. Such phosphorylation-dependent regulation of NR2A endocytosis has not yet been reported.

NR2 subunits can also dictate the fate of endocytosed NMDARs. Both NR2A and NR2B contain proximal motifs that direct NMDARs to the late enodosome/lysosome, where the receptors are degraded. However, NR2B subunits possess an additional proximal motif, which can help drive the receptors along a recycling pathway (Scott et al., 2004). As a consequence, endocytosed NR2B-containing NMDARs are preferentially recycled back to the plasma membrane surface, whereas NR2A di-heteromeric NMDARs are more likely to be degraded when endocytosed.

Whether activity-dependent endocytosis of NMDARs (Morishita et al., 2005) is regulated in a subunit-specific manner has not been examined. Given the recent finding of the rapid increase in the NR2A/NR2B ratio upon LTP induction in hippocampus (Bellone and Nicoll, 2007), one may speculate that LTP-inducing stimulation preferentially induces endocytosis of NR2B-containing NMDARs and/or insertion of NR2A-containing NMDARs. It is possible, however, that the change is mediated by posttranslational modifications.

Degradation

By changing neuronal activity levels, NR2A and NR2B levels can be bidirectionally regulated (Ehlers, 2003). This activity-dependent regulation can be prevented by proteasome inhibitors (Ehlers, 2003), indicating that degradation pathways are important for the activity-dependent regulation of NMDAR subunit levels. A recent study showed that NR2B can be ubiquitinated by an E3 ligase, Mind bomb 2 (Mib2) (Jurd et al., 2007).

Mib2 directly interacts with NR2B and ubiquitinates it when tyrosine 1472 in YEKL is phosphorylated by Fyn. Because neuronal activity facilitates tyrosine phosphorylation of NR2B and Mib2 binding (Jurd et al., 2007), Mib2 may play an important role in the activity-dependent regulation of NR2 subunits. In an apparent paradox, phosphorylation at tyrosine 1472 both increases the proteasomal degradation of NR2B but limits NR2B endocytosis (Prybylowski et al., 2005). Thus, it will be interesting to discern how synaptic localization and Mib2-mediated degradation of tyrosine 1472 phosphorylated-NR2B are balanced.

In conclusion, NR2A and NR2B undergo differential activity-dependent regulation at various points of the subunit turnover. Therefore, multiple layers of regulation contribute to the experience-dependent modifications of the synaptic NR2A/NR2B ratio.

1.1.5. Developmental and experience-dependent modification of the NR2A/NR2B ratio *in vivo*

Developmental regulation

In many parts of the CNS, including the brain stem, hippocampus, and neocortex, the NR2A/NR2B ratio increases during early postnatal development (Barth and Malenka, 2001; Chen et al., 2000; Hestrin, 1992; Liu et al., 2004b; Nase et al., 1999; Quinlan et al., 1999a; Roberts and Ramoa, 1999; Yoshimura et al., 2003). This change can occur both at the mRNA (Liu et al., 2004b; Nase et al., 1999) and protein levels (Chen et al., 2000; Quinlan et al., 1999a; Roberts and Ramoa, 1999). Furthermore, a profound increase in NR2A-

containing NMDARs, rather than a decrease in NR2B subunits, is believed to be the primary factor contributing to the observation that the decay of NMDAR-mediated currents becomes faster with development (Carmignoto and Vicini, 1992; Quinlan et al., 1999a; Yoshimura et al., 2003). However, an increase in the NR2A/NR2B ratio may not be the sole factor that regulates NMDAR decay kinetics. For example, changes in NMDAR phosphorylation and the expression of NR1 splice variants also regulate NMDAR current kinetics (Lieberman and Mody, 1994; Rumbaugh et al., 2000; Tong et al., 1995). In some regions, the largest developmental decline in NMDAR-mediated current duration can actually precede the most profound increase in the NR2A/NR2B ratio (Barth and Malenka, 2001). Moreover, a mild but significant developmental decrease in NMDAR-mediated current decay time is still observed in NR2A knockout mice (Lu et al., 2001). Nonetheless, studies in NR2A knockout mice demonstrate that the upregulation of NR2A underlies the largest developmental changes in NMDAR current duration (Fagiolini et al., 2003).

Experience-dependent regulation

In some parts of the neocortex, including the primary visual cortex, the elevation of the NR2A/NR2B ratio is dependent upon the level of neuronal activity (Liu et al., 2004b; Nase et al., 1999; Quinlan et al., 1999a; Roberts and Ramoa, 1999). Sensory deprivation, such as dark-rearing, reduces the developmental shift in the NR2A/NR2B ratio (Carmignoto and Vicini, 1992; Philpot et al., 2001a; Quinlan et al., 1999a). This visual experience-dependent control in the NR2A/B ratio is not restricted to young animals. I and others have shown that 10 days of visual deprivation in adult rodents can reduce the NR2A/B

ratio in the visual cortex (He et al., 2006; Yashiro et al., 2005). The present evidence suggests, however, that the ratio change may be restricted to perisynaptic sites in adults, unlike the experience-dependent modifications in NR2A/NR2B that can occur at synapses in young rodents (Yashiro et al., 2005). This suggests that NR2A/B protein expression level can be modified throughout development by visual experience, but the ability to control synaptic NMDARs is restricted to young animals.

How is the NR2A/NR2B ratio controlled by visual experience? There are currently conflicting reports on how visual experience regulates NR2A and NR2B at the mRNA level. For example, one comprehensive microarray analysis of mouse visual cortex revealed that both NR2A and NR2B mRNA are elevated in rodents reared in complete darkness until P27 compared to those in age-matched light-reared animals (Tropea et al., 2006). In contrast, single cell RT-PCR analysis of mRNA isolated from neurons in layer 4 of rat visual cortex showed that dark-rearing until P20 significantly retards the developmental increase in NR2A mRNA (Nase et al., 1999). These differences may be a subtle consequence of the ages studied or the techniques employed.

There is general agreement that visual experience increases the NR2A/NR2B ratio at the protein level, although there is some disagreement as to whether this change is due to an increase in NR2A and/or decrease in NR2B. Dark-rearing of rats until 6 weeks of age does not change NR2B protein levels in synatoneurosome fractions, although this manipulation significantly reduces NR2A protein levels starting at 3 weeks of age compared to age-matched normally-reared rats (Quinlan et al., 1999a). Moreover, light exposure rapidly

raises NR2A protein levels within one hour (Quinlan et al., 1999b). Reciprocally, 5 weeks of dark-rearing reduces NR2A protein levels without affecting NR2B protein levels in cats (Chen et al., 2000). Moreover, an immunohistochemical analysis reports that the NR2A protein reductions occur in all layers of the visual cortex in dark-reared rats (Tongiorgi et al., 2003). Collectively, these results indicate that the NR2A is the target of sensory experience-dependent regulation, but NR2B is not. A recent study suggests, however, that visual experience may regulate both NR2A and NR2B levels, although whether visual deprivation enhances NR2B or retards NR2A expression is regulated tightly depending on the age of onset of the visual deprivation (Chen and Bear, 2006). Therefore, both NR2A and NR2B protein levels can be targets of sensory experience-dependent regulation, and the two proteins are inversely regulated by experience.

I suggest that the visual experience-dependent increase in the NR2A/NR2B ratio is controlled by several cellular processes. The developmental NR2A increase is likely driven by activity-dependent facilitation in transcription (Hoffmann et al., 2000). The NR2B level may be chronically attenuated by activity-dependent suppression of its translation (Chen and Bear, 2006). Moreover, neuronal activity may limit NR2B levels by facilitating its degradation by Mib2-mediated ubiquitin proteasome system (Jurd et al., 2007). In the absence of visual experience, NR2A mRNA synthesis is slowed, the suppression of NR2B translation is relieved, and NR2B degradation is attenuated, all processes that result in a net decrease in the NR2A/NR2B ratio. Detailed biochemical studies are required to dissect the visual experience-induced transcriptional and translational controls of NR2 subunits.

1.1.6. Are activity-dependent changes in the NR2A/NR2B ratio input-specific or global?

I have described that the NR2A/NR2B ratio changes in response to neuronal activity both *in vitro* and *in vivo*. An important question is whether the NR2A/NR2B ratio is controlled at the level of individual synapses or in a global, cell-wide manner. This question is important, because it predicts whether the inactivation of a subset of synapses could result in a global reduction in the NR2A/NR2B ratio. In contrast, a global (cell-wide) change in the synaptic levels of NR2A/NR2B would alter the properties of synaptic plasticity throughout a neuron, and such changes obviously have different consequences on dynamic modifications of synapses across a neuronal network.

Most studies that have addressed the regulation of NR2A/NR2B have employed global manipulations of synaptic function, such as chronic treatment with APV or dark-rearing. Because these slow alterations of the NR2A/NR2B ratio are, at least in part, controlled by transcription and translation of NR2 subunits, these slow changes are likely to be achieved by global control of the NR2A/NR2B ratio on the neuronal surface. There is evidence, however, that the NR2A/NR2B ratio may also be controlled in a local (input-specific) manner. For example, the NR2A/B ratio is different between *inter*cortical and *intra*cortical synapses in L5 pyramidal neurons in the cortex (Kumar and Huguenard, 2003). Moreover, the synaptic distribution of NR2B subunits in the adult mouse hippocampus is asymmetrical between the apical and basal dendrites of single neurons (Kawakami et al., 2003). Such observations indicate that the NR2A/NR2B ratio must have a level of regulation at individual synapses. In support of this view, a recent study demonstrated that

the NR2A/NR2B ratio increases immediately after the application of LTP-inducing stimulation in hippocampus in an input-specific manner (Bellone and Nicoll, 2007). Such rapid changes are unlikely to be regulated at the transcriptional or translational levels. Therefore, I hypothesize that the NR2A/NR2B ratio is regulated both at synaptic and cellular levels. Global regulation of the NR2A/NR2B levels might regulate the threshold of synaptic plasticity across the cell to direct the acquisition of stimulus-selective response properties (Philpot et al., 1999, 2007), while a rapid, input-specific regulation of NR2A/NR2B levels might limit runaway potentiation on individual synapses.

1.1.7. Roles of NR2A and NR2B in LTD and LTP

LTP

Given that NR2B-containing NMDARs reveal longer currents, carry more Ca^{2+} per unit of current, and interact preferentially with CaMKII compared to NR2A-containing NMDARs, it is tempting to speculate that NR2B subtypes are more likely to favor the induction of LTP compared to NR2A subtypes (although see (Liu et al., 2004a)). A number of lines of evidence support this contention, some of which are highlighted here. (1) Ifenprodil completely blocks LTP induced by a pairing protocol in immature hippocampal slice cultures, suggesting a critical requirement of NR2B subtypes for the induction of LTP (Barria and Malinow, 2005). (2) Overexpression of NR2A, and a presumptive replacement of NR2B subtypes with NR2A subtypes, attenuates the induction of LTP induced by a pairing protocol (Barria and Malinow, 2005). (3) In the anterior cingulate cortex, an NR2B specific antagonist blocks LTP elicited by either pre- and postsynaptic pairing or

theta-burst stimulation (Zhao et al., 2005). (4) In thalamocortical synapses in the barrel cortex of postnatal day (P) 3-5 mice, ifenprodil blocks LTP induced by a pairing protocol (Lu et al., 2001). (5) Genetic lesion of NR2A fails to abolish hippocampal LTP, suggesting that NR1/NR2B di-heteromic receptors are sufficient to induce hippocampal LTP (Berberich et al., 2005; Weitlauf et al., 2005). (6) Transgenic overexpression of NR2B enhances hippocampal LTP (Tang et al., 1999). (7) Transient overexpression of NR2B c-terminus, which blocks the NR2B and CaMKII interaction, attenuates hippocampal LTP (Zhou et al., 2007). Thus, in various regions of the brain, NR2B-containing NMDARs help to promote the induction of LTP induced by a variety of stimulation protocols.

Contrary to these findings, it has been reported that NR2A-containing, but not NR2B-containing, NMDARs mediate LTP (Liu et al., 2004a). Support for this hypothesis comes from experiments utilizing a new pharmacological tool, NVP-AAM077, which was believed to specifically block NR2A-containing NMDARs. In these experiments, NVP-AAM077 was shown to block hippocampal LTP (Liu et al., 2004a). Similar observations were made using this antagonist in the adult perirhinal cortex (Massey et al., 2004). However, the specificity of NVP-AAM077 has been questioned. While NVP-AAM077 was reported to be 100 times more selective for NR1/NR2A channels than NR1/NR2B channels using a cell line exogenously expressing human NMDARs (Liu et al., 2004a), this antagonist is only 6-12 fold more effective for NR1/NR2A channels than NR1/NR2B channels in rodents (Feng et al., 2004; Neyton and Paoletti, 2006). The low selectivity of NVP-AAM077 in rodents is supported by the finding that NVP-AAM077 blocks more

than 20% of the NMDAR current in hippocampal slices of NR2A-null mice even at 50 nM concentration (Berberich et al., 2005). At this concentration less than 80% of NMDAR-mediated currents are obstructed in a cell line expressing rodent NR1/NR2A channels. These findings suggest that specificity of NVP-AAM077 is not sufficient to determine the role of NR2A in synaptic plasticity.

While I suggest that the induction of LTP is more likely to be favored with NR2B subtypes than NR2A subtypes, I caution that the roles of these receptors must be carefully considered within a developmental and regional context. Moreover, NMDARs are not the sole determinants for inducing plasticity. For example, both signaling molecules and inhibitory inputs are important contributors to the induction of plasticity (Choi et al., 2002; Steele and Mauk, 1999), and these factors clearly change across development and region (Chattopadhyaya et al., 2004; Jiang et al., 2005; Morales et al., 2002 ; Yasuda et al., 2003).

LTD

Which subunits mediate LTD? Unlike the previous demonstration of complete block of hippocampal LTD by ifenprodil (Liu et al., 2004a), studies in three independent laboratories consistently found that hippocampal LTD is insensitive to NR2B blockade by ifenprodil (Morishita et al., 2006). Another study even suggests that ifenprodil enhances the induction of LTD in the hippocampus (Hendricson et al., 2002). These studies demonstrate that the induction of LTD does not require activation of NR2B-containing NMDARs. A caveat in these pharmacological studies are in the complex nature of ifenprodil (Neyton and Paoletti, 2006). Even though ifenprodil blocks NMDARs at high

concentrations of glutamate, it may actually potentiate NMDAR currents at low glutamate concentrations (Kew et al., 1996). Thus, ifenprodil might affect synapses differently depending on the glutamate concentration in the synaptic cleft. Such complexities might partially underlie observations that NR2B can be involved in synaptic weakening in some conditions but not others. For example, in the adult perirhinal cortex, the subunit-dependence of LTD relies on the state of the synapse (Massey et al., 2004); ifenprodil blocks LTD that has been induced at a basal state, but the antagonist fails to block LTD induced after synaptic potentiation (depotentiation).

Studies in mice lacking NR2A have attempted to illuminate a possible role for NR2A in LTD. In the visual cortex of NR2A knockout mice, the standard 1 Hz stimulation protocol (900 pulses), which induces LTD in wildtype mice, gives rise to LTP. On the other hand, 0.5 Hz stimulation (900 pulses) induces LTD in NR2A knockout mice comparable to wildtype mice (Philpot et al., 2007). Therefore, one hypothesis is that the threshold for inducing LTP is lowered in NR2A knockout mice, as activation of NR2B-containing di-heteromeric NMDARs allows greater Ca^{2+} entry than possible through NR2A-containing NMDARs. Such a threshold change by deleting NR2A may not be universal in the brain, because 1 Hz stimulation induces LTD in NR2A knockout mice in the midbrain (Zhao and Constantine-Paton, 2007). Thus, the consequences of deleting NR2A may vary depending on age or brain region. Future studies that take advantage of either conditional NR2A deletion and/or more specific NR2A antagonists are needed to clarify the role of NR2A in LTD.

Taken together, it seems that NR2A-containing NMDARs favor the induction of LTD by limiting Ca^{2+} entry through NMDARs. Future studies examining possible interactions between NR2A and LTD-inducing signaling components such as the PP1/PP2B pathway may strengthen this hypothesis. Also, as mentioned previously, further work will be necessary to determine the differences between NR2A and NR2B subtypes in open probability, as the calcium signaling through these two receptor subtypes hinges critically on how these receptors behave endogenously in mammalian neurons.

NR2A/NR2B ratio controls LTD and LTP

The NR2A/NR2B ratio changes during development (Quinlan et al., 1999a) and likely differs dramatically among synaptic sites on dendritic trees (Kumar and Huguenard, 2003; Sobczyk et al., 2005). How do these differences affect LTD and LTP? Given the contributions of NR2 subunits on LTD and LTP mentioned above, it has been hypothesized that the LTD/LTP induction threshold is determined by the ratio of NR2A/NR2B expressed on dendritic spine surfaces (Fig.1-1). That is, if the ratio of NR2A/NR2B is elevated, stronger stimulation (e.g. a higher stimulus frequency) would be required to induce LTP compared to when the ratio of NR2A/NR2B is low. This hypothesis is based on two observations; a higher NR2A/NR2B ratio limits both Ca^{2+} entry through NMDARs (although see (Erreger et al., 2005)) and the accessibility of CaMKII at the synapse. Therefore, with a high NR2A/NR2B ratio a stronger postsynaptic response is needed to elevate Ca^{2+} and activate CaMKII to a level sufficient to induce LTP, whereas weaker postsynaptic responses might suit activation of calcineurin and activate an LTD pathway (Philpot et al., 1999) (Figure 1B). On the contrary, a low NR2A/NR2B ratio

would lower the LTP induction threshold, making it more likely that a modest response can elevate Ca^{2+} and activate CaMKII to a level sufficient to induce LTP. In light of this hypothesis, it is interesting to consider how experience-dependent changes in the NR2A/NR2B ratio alter the LTD/LTP induction threshold (discussed below).

1.1.8. Interaction between NR2A/NR2B ratio and LTD/LTP in the visual cortex

As I discussed above, the NR2A/NR2B ratio increases in many parts of the brain and this increase is regulated by sensory experience in many regions, including the visual cortex. Like the NR2A/NR2B ratio, properties of LTD and LTP change during development in an experience-dependent manner (Bear, 2003). Here I discuss if the described changes in LTD/LTP are mediated, in part, by the modification of the NR2A/NR2B ratio. I focus my discussion onto two excitatory synaptic connections within the visual cortex, the thalamus to layer 4 (thalamocortical) and the layer 4 to 2/3 (intracortical) connections, because these pathways are well-studied and yet very distinct. Interestingly, although the NR2A/NR2B ratio similarly increases in both of the excitatory connections, those synapses reveal distinctive developmental and experience-dependent regulations of LTD and LTP.

Thalamocortical synapses

In the thalamocortical synapses of somatosensory and visual cortices, the magnitude of both LTD and LTP diminish in the second to fourth postnatal week in rodents (Dudek and Friedlander, 1996; Feldman et al., 1999; Jiang et al., 2007). Since the timing of the developmental increase in the NR2A/NR2B ratio coincides with the loss of LTD and LTP in both the somatosensory (Barth and Malenka, 2001; Lu et al., 2001) and the visual cortex

(Carmignoto and Vicini, 1992; Jiang et al., 2007; Quinlan et al., 1999b), it has been hypothesized that the increase in the NR2A/NR2B ratio may limit the expression of plasticity at thalamocortical synapses. This hypothesis has been challenged by a finding that the developmental reduction in the thalamocortical LTP is shown to be conserved in the somatosensory cortex of the NR2A knock-out mice (Lu et al., 2001). This suggests that the developmental increase in the NR2A/NR2B ratio does not mediate the developmental loss of thalamocortical LTP in the somatosensory cortex. Whether the developmental loss of thalamocortical LTP is also conserved in visual cortex has not yet been tested, but the current evidence indicates that the developmental loss of thalamocortical LTP is not dependent solely on the NR2A/NR2B switch. For example, while dark-rearing delays both the onset of the critical period for ocular dominance plasticity (Mower, 1991b) and the developmental upregulation of the NR2A/NR2B ratio at layer 4 (Carmignoto and Vicini, 1992; Quinlan et al., 1999a), this manipulation does not delay the developmental loss of thalamocortical LTP in the visual cortex (Jiang et al., 2007).

These data argue that the NR2A/NR2B ratio is not the sole determinant for the ability to induce LTD and LTP at thalamocortical plasticity. However, it is still conceivable that the relative levels of NR2A/NR2B might adjust the threshold for inducing plasticity within a developmental time point. For example, it is possible that an increase in the NR2A/NR2B ratio may increase the threshold for inducing LTP at thalamocortical synapses, but whether or not LTP can be induced at all is likely a consequence of other factors including inhibition (Dudek and Friedlander, 1996; Steele and Mauk, 1999), neuromodulators (Seol

et al., 2007), or downstream signaling molecules (Yasuda et al., 2003). Future studies that manipulate NR2A and NR2B levels *in vivo* are needed to test these possibilities.

Intracortical synapses

Although there is general agreement that there is a developmental loss of thalamocortical plasticity, it is less clear whether plasticity persists at the L4-L2/3 synapse, the first intracortical relay. There are reports that both LTD (Kirkwood et al., 1997) and LTP (Yoshimura et al., 2003) at the L4-L2/3 synapses diminish with development, but other studies find that LTD (Jiang et al., 2007) and LTP (Frankland et al., 2001; Jiang et al., 2007; Kirkwood et al., 1997) persist into adulthood at this synapse. These seemingly contradictory findings make it impossible to determine whether observed changes in the NR2A/NR2B ratio (Yoshimura et al., 2003) are associated with the ability to induce LTD and LTP at this intracortical synapse. Because receptive field plasticity remains intact in the superficial layers into adulthood (Daw et al., 1992), suggesting that LTD and LTP also remain intact, it appears as though the developmental increase in NR2A/NR2B does not eliminate the induction of plasticity at the L4-L2/3 synapse.

While the NR2A/NR2B ratio does not gate the absolute ability to induce LTD and/or LTP, changes in NR2A/NR2B appear to affect the *threshold* for the frequency-dependent induction of LTD/LTP. Visual experience/deprivation alters the frequency-response relationship of LTD/LTP in the layer 4-2/3 synapses (Kirkwood et al., 1996; Philpot et al., 2003), such that dark-rearing narrows the window of stimulus frequencies that induce LTD by lowering the threshold for inducing LTP (Kirkwood et al., 1996; Philpot et al., 2003).

Namely, previous sensory experience modifies the properties of synaptic plasticity in the visual cortex, an effect known as "metaplasticity" (Bear, 2003). Because dark-rearing reduces the NR2A/NR2B ratio, there is a striking correlation between the threshold for modifying synaptic strength and the relative ratio of NR2A/NR2B. Thus, it has been hypothesized that sensory experience slides the threshold for inducing LTP/LTD through regulation of the NR2A/NR2B ratio (Philpot et al., 1999), with a low NR2A/NR2B ratio favoring the induction of LTP (by lowering the plasticity threshold).

To test the hypothesis that the NR2A/NR2B ratio regulates the threshold of inducing synaptic plasticity, a process termed metaplasticity, we have taken advantage of mice that lack NR2A. The idea of this study was to lock the NR2A/NR2B in place, as this should prevent experience-dependent modifications in the LTD/LTP threshold if this were normally a consequence of changing the NR2A/NR2B ratio. we first demonstrated that the visual experience-dependent shortening of NMDAR-current decay is absent in NR2A knockout mice (Philpot et al., 2007), indicating that the shortening of NMDAR currents is indeed due to an increase in the NR2A/NR2B ratio. Importantly, dark-rearing, which normally lowers the threshold for inducing LTP in wildtype mice, failed to alter the threshold for frequency-dependent plasticity in mice lacking NR2A. Moreover, the threshold stimulus frequency for inducing LTP is greatly lowered in NR2A knockout mice, such that 1Hz stimulation, which induces LTD in the wildtype mice, is sufficient to give rise to LTP. This is consistent with the idea that a low NR2A/NR2B ratio favors the induction of LTP. Currently the mechanism by which the NR2A/NR2B ratio alters the plasticity threshold is unknown. One possibility is that NR2A limits Ca^{2+} entry through

NMDARs and, in its absence, NR2B lowers the plasticity threshold by promoting a greater degree of Ca^{2+} entry into the postsynaptic neuron. These results clearly suggest that, at least in developing rodents, NR2A is required for metaplasticity in the visual cortex and strongly indicate that visual experience-dependent change in the NR2A/B ratio controls metaplasticity by directly changing biophysical properties of postsynaptic NMDARs.

1.1.9. Roles of NR2A and NR2B in cortical functions *in vivo*

I have described the roles of NR2A and NR2B in synaptic plasticity *in vitro*, but how do changes in these NMDAR types contribute to development of sensory systems? Here I review the roles of the NR2 subunits in the development of two well-studied visual functions: orientation selectivity and ocular dominance plasticity.

Orientation selectivity

Most neurons in the primary visual cortex respond vigorously to light-dark bars or edges presented to animals at a particular range of orientations. Some degree of orientation selectivity is innate in cortical neurons and the selectivity becomes more fine-tuned with development. Visual experience is necessary for the proper development of orientation selectivity, and fewer cells exhibit orientation selectivity in the absence of prior visual experience (Fagiolini et al., 2003; White et al., 2001). Moreover, if the visual environment is largely restricted to one orientation (by rearing in a striped cylinder), animals develop orientation selectivity biased toward the orientation of the stripes (Sengpiel et al., 1999). These results indicate that cortical neurons change their connectivity to respond more to experienced orientations.

What is happening at synapses during the acquisition of enhanced stimulus selectivity? One may hypothesize that patterned visual stimulation potentiates synapses of neurons, which gain preferential responses to repeatedly experienced orientations by an LTP-like mechanism. On the other hand, the same stimulation may depress synapses responding to the non-favored orientation, by an LTD-like mechanism. The acquisition of stimulus selective properties such as orientation selectivity are thought to require experience-dependent modifications in the properties of synaptic plasticity (metaplasticity) (Bienenstock et al., 1982). Thus, orientation selectivity is less likely to occur, and more likely to be broad when it does occur, in the absence of metaplasticity. Given that the NR2A/NR2B ratio controls visual cortex metaplasticity, one would predict that orientation selectivity would be severely retarded if the NR2A/NR2B ratio were fixed (hence preventing metaplasticity). Consistent with this hypothesis, the proportion of orientation selective neurons is severely diminished in the visual cortex of NR2A knockout mice (Fagiolini et al., 2003). It is tempting to speculate that, under normal conditions, a visual experience-dependent increase in the NR2A/NR2B ratio mediates the establishment of orientation selectivity by widening the window for LTD induction and strengthening orientation-specific synaptic connections through an LTP-like mechanism.

Ocular dominance plasticity

One well-studied *in vivo* paradigm of synaptic plasticity is the ocular dominance shift observed in the primary visual cortex. Classical studies demonstrate that closure of one eye by eyelid suturing results in a loss of responsiveness to the closed (deprived) eye and a

gain of responsiveness to the intact (non-deprived) eye (Wiesel and Hubel, 1963). Recent studies, which investigate visually-evoked potential recordings in awake mice, reveal that monocular deprivation induces depression of the closed eye response in the first 2 days after MD followed by potentiation of the open eye response occurring in 5 days (Frenkel and Bear, 2004). Is the initial depression and subsequent potentiation induced by LTD and LTP-like mechanisms? It has been shown that monocular deprivation induces dephosphorylation of AMPARs seen in LTD (Heynen et al., 2003). Moreover, in the visual cortical slices prepared from monocularly deprived rats, LTD was suppressed, suggesting that synaptic depression induced by monocular deprivation occludes LTD (Heynen et al., 2003). Thus, the closed eye depression seems likely to involve a LTD-like mechanism. Because the ocular dominance shift can not be reliably induced in mice carrying a knock-in mutation in CaMKII at threonine 286, autophosphorylation of which is required of LTP induction (Taha et al., 2002), its expression mechanism shares common molecular pathways with LTP. It has not been investigated, however, if open eye potentiation is absent in the CaMKII mutant mice.

Ocular dominance plasticity is most dramatic during a brief period of postnatal life, termed the critical period. In mice, this period lasts roughly from 3 to 5 weeks of age. Interestingly, at the onset of the critical period, NR2A protein levels markedly increase and NMDAR decay kinetics decrease steeply (Chen et al., 2000; Erisir and Harris, 2003; Roberts and Ramoa, 1999). This indicates that, at least in layers 2/3 of cortex, an increase in the NR2A/NR2B ratio does not terminate the critical period and may even help to enable plasticity expressed during this time. Consistent with this view, dark-rearing that

delays the developmental increase in the NR2A/NR2B ratio also delays the initiation of the critical period (Mower, 1991a). Moreover, genetic deletion of NR2A suppresses ocular dominance plasticity without changing the timing or duration of the critical period (Fagiolini et al., 2003). Together, these findings suggest that experience-dependent increases in the NR2A/NR2B ratio may be required to enable certain forms of critical period plasticity. This hypothesis is attractive because it predicts that in order to have full expression of critical period plasticity, the NR2A/NR2B ratio needs to reach a high enough level to help promote weakening (LTD?) of deprived eye inputs.

It was previously thought that the increase in the NR2A/NR2B ratio regulates the end of the critical period (Carmignoto and Vicini, 1992; Fox and Zahs, 1994). But, as mentioned above, studies in NR2A knockout mice revealed that the developmental increase in NR2A is not essential for the end of the critical period either in the somatosensory (Lu et al., 2001) or visual (Fagiolini et al., 2003) cortices. However, it is still premature to conclude that changes in NR2 subunits do not contribute to the termination of the critical period in all pathways. Indeed, a loss of NR2B immunoreactivity at layer 4 tightly coincides with the end of the critical period in both somatosensory (Liu et al., 2004b) and visual (Erisir and Harris, 2003) cortices. Therefore, although the end of the critical period is not regulated by changes in NR2A, it may instead be controlled by the decrease in NR2B at layer 4 synapses (independent of changes in NR2A). Because NR2B knockout mice die shortly after birth (Kutsuwada et al., 1995), future studies taking advantage of conditional NR2B deletion are needed to test the hypothesis that a sharp reduction in NR2B at thalamocortical synapses may terminate critical period plasticity.

Interestingly, recent findings suggest that the change in the NR2A/NR2B ratio could also be involved in the dynamic regulation of the ocular dominance shift. Although monocular deprivation initially causes deprivation of the deprived eye, the subsequent potentiation of the open eye inputs at 5 days after monocular deprivation may be a result of a reduction in the NR2A/NR2B ratio (Chen and Bear, 2006). This reduction in NR2A/NR2B may lower the LTP induction threshold and allow weak ipsilateral inputs to induce LTP. This hypothesis would explain why the potentiation of open eye responses is slow to emerge (Frenkel and Bear, 2004). Moreover, the above observations are consistent with a global regulation of NR2A/NR2B levels, as the reduced response at one set of inputs following deprivation eventually leads to a change in the LTP threshold at a second set of synapses corresponding to the open eye.

In conclusion, the experience-dependent increase in the NR2A/NR2B ratio is likely to regulate the threshold for inducing plasticity. As such, the NR2A/NR2B switch is important both for the acquisition of stimulus-selective properties such as orientation selectivity and for the full expression of ocular dominance plasticity. Moreover, a change in the NR2A/NR2B ratio might underlie the naturally occurring metaplasticity observed in visual cortex, with an initial deprivation-induced depression and a delayed potentiation of the open eye response. Clever uses of genetic manipulation of NR2 subunits combined with chronic *in vivo* measurements will clarify the roles of NR2 subunits in *in vivo* synaptic plasticity.

1.1.10. Conclusion

Here I proposed that the ratio of NR2A/NR2B in synaptic NMDARs controls the direction and extent of synaptic modifications by controlling Ca^{2+} entry and intracellular signaling cascades. In my model, NR2A-dominated synapses are more likely to induce LTD than NR2B-dominated synapses, while NR2B-dominated synapses have a greater disposition to being potentiated. This idea is largely consistent with the available data. However, LTD and LTP are complicated cellular processes involving many signaling proteins and expressed by different mechanisms among brain regions and developmental stages (Malenka and Bear, 2004). Thus, although regulation of the NR2A/NR2B ratio is clearly a major determinant of the properties of synaptic plasticity, it is certainly only one of several important factors that affect the developmental and experience-dependent properties of synaptic plasticity.

The activity-dependent control of the NR2A/NR2B ratio provides another layer for regulating synapses in addition to activity-dependent modifications of synaptic strength. Since the NR2A/NR2B ratio affects induction thresholds for LTD and LTP, the synapse modification thresholds are controlled by sensory experience. Therefore, the NR2A/NR2B ratio changes occurring after synaptic stimulation or sensory experience alter how synapses change in response to subsequent synaptic stimulations or sensory experience. Thus, both the current state and the future destiny (predisposition) of synapses are modified by ongoing sensory experience. This process endows neuronal networks not only with a feedback mechanism to adjust to an ever-changing environment, but also with a further competency in processes of their rearrangement by sensory experience.

Table 1-1, Comparison of NR2A and NR2B-containing NMDARs

	NR2A	NR2B	Reference
Open probability	High	Low	Chen et al., 1999 Erreger et al., 2005 (but also see Prybylowski et al., 2002)
Deactivation	Fast	Slow	Erreger et al., 2005
Peak current	High	Low	Erreger et al., 2005
Rise time	Fast	Slow	Chen et al., 1999 Monyer et al., 1994 Prybylowski et al., 2002 Vicini et al., 1998
Decay time	Fast	Slow	
Charge transfer	Low	High	Erreger et al., 2005
Ca^{2+}/EPSC	Low	High	Sobczyk et al., 2005
Location	Central synapse	Peri-synapse	Dalby and Mody, 2003 Townsend et al., 2003 Zhao and Constantine-Paton, 2007
CaMKII binding	Weak	Strong	Mayadevi et al., 2002 Strack and Colbran, 1998
Plasticity	LTD/LTP	LTD/LTP	Barria and Malinow, 2005 Berberich et al., 2005 Morishita et al., 2006 Philpot et al., 2007 Tang et al., 1999 Weitlauf et al., 2005 Zhao et al., 2005 (but see Massey et al. 2004, Liu et al. 2004)

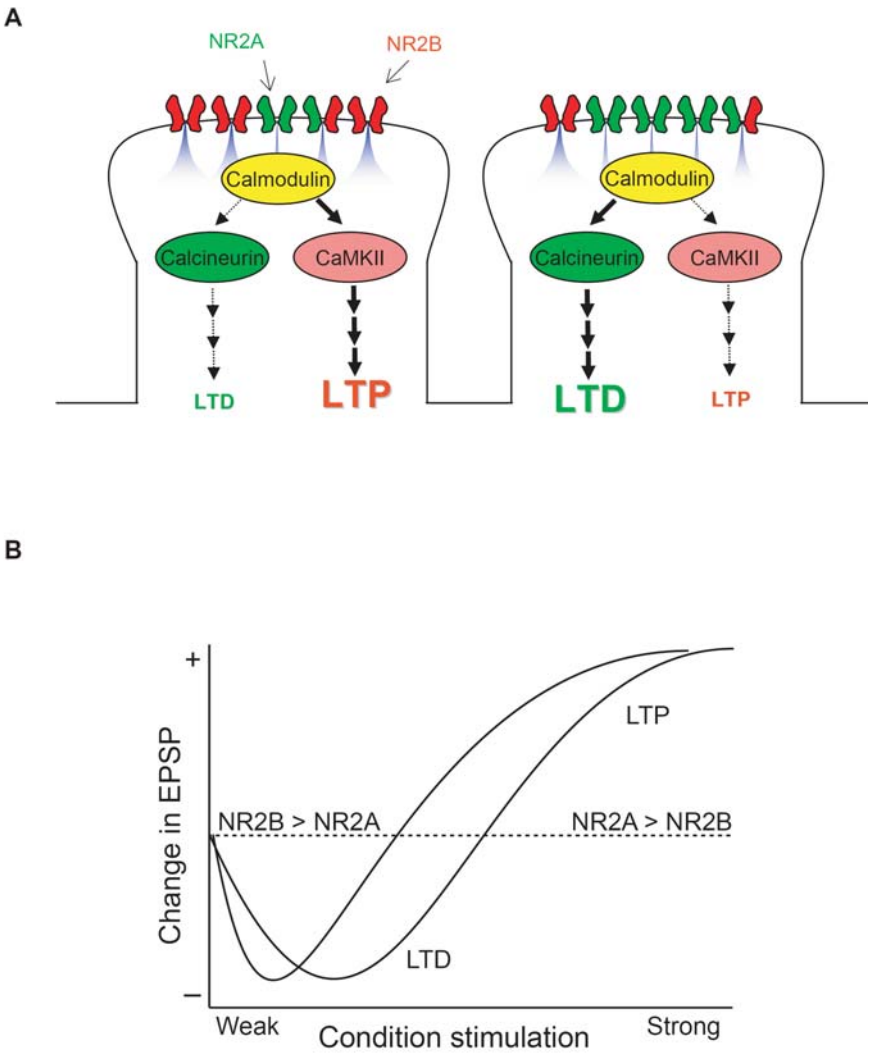

Figure 1-1. Hypothetical model of synaptic plasticity regulation by NMDAR subunits.

A) When NR2B-containing NMDARs dominate the postsynaptic membrane, synaptic stimulations induce high Ca^{2+} entry through NMDARs into spines. In this high Ca^{2+} concentration, calmodulin favors activating CaMKII resulting in activation of LTP pathways. On the other hand, when NR2A-containing NMDARs dominate, Ca^{2+} entry through NMDARs is limited. In the low Ca^{2+} milieu, calmodulin tends to activate calcineurin, stimulating LTD pathways.

B) How NMDAR subunit regulates the properties of synaptic modification. The x-axis represents the level of the integrated postsynaptic response, while the y-axis represents the lasting change in synaptic strength. The curves are schematized from the data of (Kirkwood et al., 1996; Philpot et al., 2003, 2007). When the synaptic NR2A/B ratio is high, the LTD-LTP crossover point (θ_m) shifts to the right decreasing the likelihood that LTP will occur. Conversely, when the synaptic NR2A/B ratio is low, θ_m slides to the left, favoring LTP over LTD.

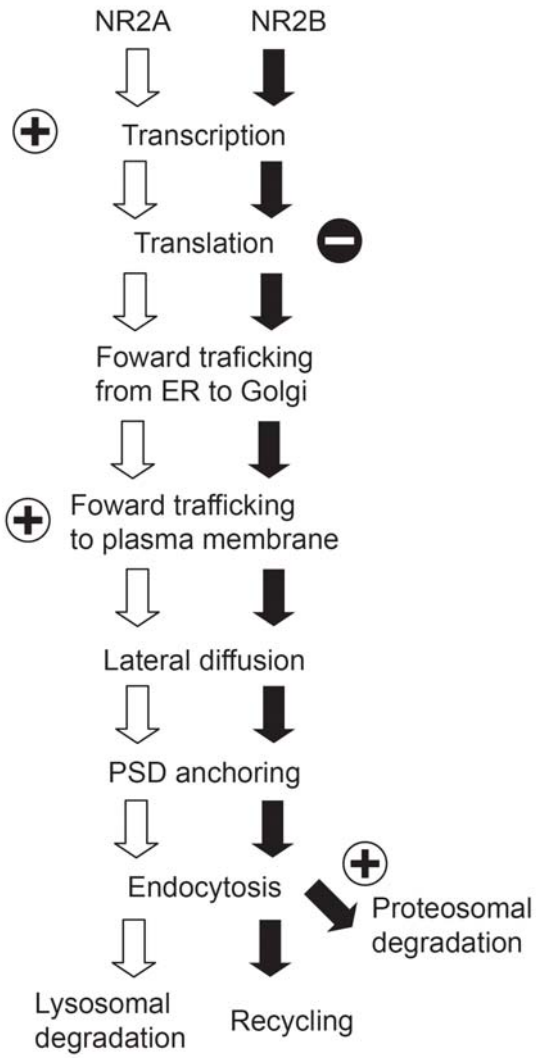

Figure 1-2. Activity-dependent regulation of NR2A and NR2B

NR2A and NR2B contain distinct signals that control synaptic presentation of NMDARs. Whereas neuronal activity facilitates (+) transcription of NR2A subunit, it attenuates translation of NR2B (-). Neuronal activity also facilitates surface delivery of NR2A and proteasomal degradation of NR2B.

1.2. Angelman syndrome

Above I have described a mechanism that adjusts the ability to strengthen and weaken synapses. However, what happens when the properties of synaptic plasticity are poorly regulated or expressed? During early postnatal development, neuronal circuits normally become functional through an iterative process of synaptic modifications and reorganization. This sculpting of cortical synapses and networks is driven in part by ongoing sensory processing, which in turn is required for normal sensory perceptions and cognitive abilities (Katz and Shatz, 1996), which are distorted in neurodevelopmental disorders such as autism and mental retardation. Therefore, it has been hypothesized that, in those disorders, sensory experiences fail to shape the maturation of neuronal circuits (Zoghbi, 2003). There has been, however, no experimental evidence to substantiate this hypothesis to date.

Angelman syndrome (AS) is a neurodevelopmental disorder characterized by happy demeanors, ataxia, a high incidence of seizures, and severe mental retardation (Clayton-Smith and Laan, 2003). Despite the profound cognitive and behavioral defects that become apparent in childhood, AS patients often have no apparent abnormalities at birth. Ataxia becomes evident in AS patients during early postnatal life (Laan et al., 1999), and these patients exhibit profound cognitive deficits represented by their lack of language acquisition. The time course of AS suggests that motor and cognitive development diminishes early in postnatal life of these patients (Philippart, 2001). Many AS patients show behaviors, such as showing a strong fascination with water and reflective surfaces, that seem to reflect unusual sensory processing. In addition, AS patients are commonly

hypo-sensitive to tactile and vestibular inputs (Walz and Baranek, 2006). These observations are consistent with the idea that AS patients fail to develop normal neuronal circuits for sensory processing and, therefore, they are severely deficient in acquiring learned skills.

A loss of Ube3A is sufficient to cause AS. Because of maternal genetic imprinting of Ube3A in many regions of the brain, mutation of the *UBE3A* gene on the maternal chromosome can cause AS (Clayton-Smith and Laan, 2003). Ube3A is an E3 ubiquitin ligase, which is a critical component of the ubiquitin proteasome system that targets proteins for degradation. A number of recent studies demonstrate involvement of the ubiquitin proteasome system in synaptic development and plasticity (Yi and Ehlers, 2005; Yi and Ehlers, 2007), raising the possibility that Ube3A-mediated protein degradation is involved in these processes. Although several substrates for Ube3A have been identified, none to date has been implicated in the pathophysiology of AS. AS has been modeled in Ube3A-deficient mice, and these mice exhibit phenotypes consistent with AS including , poor learning, uncoordinated movement, and inducible seizures (Jiang et al., 1998; van Woerden et al., 2007). An extensive biochemical analyses of the mouse model of AS revealed that the loss of Ube3A results in inactivation of calcium/calmodulin kinase II (CaMKII) in the hippocampus due to the elevated inhibitory phosphorylation of the kinase (Weeber et al., 2003). In support of the hypothesis that dysfunction of hippocampal CaMKII signaling is in part responsible for the AS phenotype, introduction of a point mutation at the inhibitory phosphorylation site of CaMKII rescues many abnormalities in

Ube3A-defecient mice, including the deficits in hippocampal synaptic plasticity (long-term potentiation; LTP) and memory.

There is no current knowledge of the role of Ube3A in the neocortex, a region of the brain heavily sculpted through experience-dependent development and thought to be a repository for memories. One of the brain areas where sensory experience-dependent brain development is most studied is the visual cortex (Hensch, 2005). Immature neuronal circuits in the visual cortex are modified and refined by sensory experiences during early life. Notably, visual experiences sculpt many fundamental receptive field properties, such as receptive field size, visual acuity, orientation tuning, direction selectivity, and ocular dominance plasticity (Fagiolini et al., 2003; Gianfranceschi et al., 2003; Li et al., 2006). These sensory experiences modify neural circuits at the level of the synapse. For example, visual experience reduces the size of dendritic spines on pyramidal neurons (Wallace and Bear, 2004). Moreover, visual experience modifies NMDAR subunit composition to alters the induction threshold for LTP and long-term depression (LTD) (Kirkwood et al., 1995; Philpot et al., 2007).

Several lines of evidence indicate the visual system in AS patients is abnormal both functionally and anatomically. Visual acuity is low in AS patients, and these visual deficits are unique in AS patients among patients with a wide range of other neurodevelopmental disorders (Van Splunder et al., 2003). Consistent with abnormal visual cortex maturation, measurements of visually-evoked potentials in an AS patient revealed a left-right asymmetry in visual cortex responses (Thompson et al., 1999).

Finally, one of the only anatomical studies to date in a postmortem brain of an AS patient revealed that dendritic arborizations and spine densities are reduced in pyramidal neurons of the visual cortex (Jay et al., 1991). These observations indicate that visual deficits in AS patients may arise because their visual cortex fails to undergo normal experience-dependent refinements, and encouraged us to examine the role of sensory experience in visual cortex development in Ube3A-deficient mice.

By taking advantage of the well documented role of experience in visual cortical development, I was able to examine the role of sensory experiences in synaptic maturation using the mouse model of AS. My results demonstrate an unexpected role for Ube3A in maintaining synaptic plasticity in the face of sensory processing and provide a basis for the learning deficits associated with Angelman syndrome.

Chapter 2

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

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2.1. Abstract

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 modifies both pre- and postsynaptic elements at the layer 4 to 2/3 connection in the visual cortex of adult mice, and these changes differ from those observed in juveniles. While visual deprivation 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 layer 4 and layer 2/3 pyramidal neurons in adult mice. Surprisingly, visual deprivation 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 late-onset visual deprivation 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.

2.2. Introduction

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; Kaas, 1991; 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 (Frenkel and Bear, 2004; Lickey et al., 2004; Pham et al., 2004; Sawtell et al., 2003). 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 NMDA-type glutamate receptors (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 (McBain and Mayer, 1994; Monyer et al., 1992; 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; Flint et al., 1997; Hestrin, 1992; Monyer et al., 1992; Priestley et al., 1995; 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.

2.3. 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). Control mice were raised on a 12:12 light:dark cycle, whereas deprived, dark-reared mice were raised in complete darkness from P2. Late-onset visual deprivation was achieved by placing animals into a completely dark room for ~10 days starting at ~P68.

Slice Preparation

Mice were anesthetized with an overdose of pentobarbital barbiturate and decapitated following the disappearance of corneal reflexes, in compliance with the U.S. Department of Health and Human Services and the University of North Carolina guidelines. Brains were rapidly dissected and the visual cortices cut in 400 μm coronal slices as previously described (Kirkwood et al., 1993; Philpot et al., 2001a), with the exception that the dissection buffer contained (in mM): 75 sucrose, 87 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 7 $MgCl_2$, 0.5 $CaCl_2$, and 1.3 ascorbic acid. We focused our study on the layer 4 to 2/3 pathway, as this is thought to be the initial site for receptive field plasticity (Trachtenberg et al., 2000).

Voltage-clamp recordings

Slices were allowed to recover for 20 min at 35°C in a submersion chamber containing oxygenated artificial cerebrospinal fluid (ACSF) and then moved to room temperature for

at least 40 min before use as described (Philpot et al., 2001a). For pharmacologically isolating NMDAR-mediated currents, slices were placed in a submersion chamber, maintained at 30°C, and perfused at 2 ml per min with oxygenated 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 for recording AMPA receptor (AMPAR)-mediated currents. Cells were visualized using a Nikon E600FN microscope equipped with infrared differential interference contrast optics. Patch pipettes were pulled from thick-walled borosilicate glass. Open tip resistances were 3-8 MΩ when pipettes were filled with the internal solution 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, Israel), with pH adjusted to 7.2 and osmolarity adjusted to ~300 mmol/kg by addition of sucrose. Voltage-clamp recordings were performed in the whole-cell configuration using a patch-clamp amplifier (Multiclamp 700A, Axon Instruments), and data were acquired and analyzed using pCLAMP 9.2 software (Axon Instruments, CA). Pipette seal resistances were >1 GΩ, and pipette capacitive transients were minimized prior to breakthrough. Input and series resistance were determined throughout the experiment by measuring the response to small, intermittent test pulses. The 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 τ_f and τ_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 (τ_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, τ_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 (τ_{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 (Cho et al., 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 $p < 0.05$.

2.4. Results

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 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 layer 4 were measured in layer 2/3 pyramidal neurons in primary visual cortical slices. Dark-rearing until P21-27 significantly increased the duration of NMDAR-mediated currents compared to controls (Fig. 2-1; Deprived, τ_w = 130.7 ± 6.5 ms, n = 21 cells; Control, τ_w = 82.1 ± 7.6 ms, n = 16 cells; $p < 0.00003$). These data suggest that visual deprivation in juvenile mice modifies the composition and function of synaptic NMDARs, as has been observed in rats.

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 in the visual cortex of adult mice. Unlike juvenile rodents, we failed to observe a change in NMDAR EPSC decay kinetics between deprived and control adult mice (Fig. 2-1; Deprived, τ_w = 90.5 ± 9.3 ms, n = 13 cells; Control, τ_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 the layer 4 to 2/3 pathway.

Visual deprivation enhances the temporal summation of NMDAR EPSCs in adult mice

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 deprived and control 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 layer 4 and measured the response in layer 2/3 pyramidal cells in deprived and control cortices (Deprived: n = 15 cells; Control: n = 22 cells). We adjusted stimulus intensity to obtain ~100 pA response on the first pulse (Deprived = 113.5 ± 13.1 pA; Control = 103.4 ± 7.6 pA; $p = 0.48$). Surprisingly, we observed that visual deprivation greatly enhanced the temporal summation of NMDAR-mediated currents in the adult visual cortex (Fig. 2-2A: amplitude of the 11^{th} pulse/1^{st} pulse; Deprived = 1.33 ± 0.15; Control = 0.96 ± 0.005; $p < 0.02$). The deprivation-induced enhancement of temporal summation was also significant when quantified by averaging the charge transfer (integral) of the normalized currents (Deprived = 354.7 ± 32.1 arbitrary units = a.u.; Control = 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. 2-2B: amplitude of the 11^{th} pulse/1^{st} pulse: Deprived = 1.41 ± 0.15, n = 16 cells; Control = 0.76 ± 0.08, n = 12 cells; $p < 0.003$; Normalized charge transfer: Deprived = 397.4 ± 36.3 a.u.; Control = 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.

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 ≤ 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 layer 2/3 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 deprived mice (Fig. 2-3: τ_d ; Deprived = 64.9 ± 10.3 ms, n = 18 cells; Control = 129.5 ± 18.2 ms, n = 23 cells; $p < 0.007$). The normalized steady-state AMPAR EPSC amplitudes were unchanged by visual experience (Deprived = 0.187 ± 0033; Control = 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 synaptic depression.

Because of the novel and unexpected observation that visual deprivation 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 control and deprived 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 visual deprivation significantly accelerated the rate at which MK-801 blocks pharmacologically isolated NMDAR EPSCs (Fig.2-4: τ_{block} ; Deprived = 9.30 ± 0.63 ms, n = 4 cells; Control = 20.00 ± 3.16 ms, n = 7 cells; $p < 0.04$). These results confirm that visual deprivation increases the probability of release in layer 4 to 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).

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 in pyramidal cells of deprived 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 (Philpot et al., 2001b; Quinlan et al., 1999a; Quinlan et al., 1999b). 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. 2-5). 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 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 deprived and control 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 deprived juvenile mice compared to controls (Fig. 2-5C: PNS, $p < 0.03$; LSM, $p < 0.0006$; PSD, $p < 0.02$). To determine the effects of visual deprivation in adults, we compared the NR2A/B ratios in control and deprived adult mice (n of each group = 12 pools of 3-5 mice each). In contrast to what we observed in the visual cortex of deprived juvenile mice, late-onset visual deprivation in adults failed to modify the composition of NMDARs within the highly enriched PSD (Fig. 2-5A,C: $p = 0.22$). However, late-onset visual deprivation in adults significantly lowered the NR2A/B ratio in the PNS and LSM visual cortical fractions compared to the controls (Fig. 2-5C: PNS, $p < 0.006$; LSM, $p < 0.002$). These data indicate that visual deprivation in adults might alter the composition of NMDARs located at peri-/extrasynaptic sites but not synaptic sites. In both juvenile and adult mice, the change in NMDAR subunit composition in control and deprived mice did not appear to be the result of a general stress response, because we failed to observe a change in the NR2A/B ratio in the PNS, LSM, and PSD preparations taken from 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 suggest that late-onset visual deprivation alters the composition of peri-/extrasynaptic NMDARs but not the complement of postsynaptic NMDARs.

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 deprived 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; Deprived = 14.1 ± 1.0, n = 14 cells; Control = 15.6 ± 1.6, n = 16 cells; p = 0.46). With this minimal stimulation protocol, temporal summation of NMDA EPSCs in deprived and control mice were almost identical as measured by the amplitude of the 11^{th} pulse (Fig. 2-6: Amplitude of 11^{th} pulse/1^{st} pulse; Deprived = 1.15 ± 0.14; Control = 1.12 ± 0.12; p = 0.90) or by the normalized charge

transfer (Deprived = 283.1 ± 25.7 a.u.; Control = 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.

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 deprived mice. However, the data also indicated that (1) visual deprivation in adult mice alters the complement of peri-/extrasynaptic NMDARs without significantly changing the synaptic 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 visual deprivation 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 deprived mice (Fig. 2-7A: amplitude of 11^{th} pulse/1^{st} pulse; Deprived = 0.84 ± 0.07, n = 6 cells; Control = 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 deprived mice, the drug had no noticeable consequence in control mice (Fig. 2-7B). These observations support our hypothesis that late-onset visual deprivation can increase the relative complement of NR2B-containing NMDARs at 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. 2-2).

2.5. Discussion

We demonstrate that a brief period (10 days) of visual deprivation enhances NMDAR-mediated transmission in the layer 4 to 2/3 visual cortical synapse by presynaptically increasing the probability of neurotransmitter release and by increasing the relative expression of NR2B-containing NMDARs at peri-/extrasynaptic sites. Both the presynaptic and postsynaptic changes are unique to the mature cortex, because visual deprivation in juvenile rodents does not alter the probability of release but does decrease the expression of NR2A-containing NMDARs at the postsynaptic density (Philpot et al., 2001a; Quinlan et al., 1999a; Quinlan et al., 1999b); present study). 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 enhanced neurotransmitter release at vertical intracortical connections following global sensory deprivation appears to be unique to adult sensory cortices, as global reductions in sensory activity in the developing somatosensory or visual cortices fail to modify neurotransmitter release (Finnerty and Connors, 2000; Philpot et al., 2001a). 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 (Shen and Linden, 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 late-onset visual deprivation in adults only modifies 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 late-onset visual deprivation 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 peri-/extrasynaptic but not synaptic 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. 2-5) 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. 2-2 and 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 peri-/extrasynaptic sites, although our data does not rule out a possibility that the increased rate in neurotransmitter release is due to an increase in presynaptic NR2B-containing NMDARs.

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 (Frenkel and Bear, 2004; Sawtell et al., 2003). 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 (Philpot et al., 2001a; Quinlan et al., 1999a; Quinlan et al., 1999b) present study). 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., 2004a; 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 (Berberich et al., 2005; Hendricson et al., 2002; Toyoda et al., 2005; Weitlauf et al., 2005; Zhao 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 visual deprivation in adult mice increases NR2B expression and simultaneously reinstates the ability to observe rapid ocular dominance shifts following monocular deprivation (He et al., 2006).

A number of clinical studies suggest that some recovery from amblyopia is possible well past the classically defined critical period in humans, which is generally considered to last up to about nine years of age (Birnbaum et al., 1977; Fronius et al., 2004; Simmers and Gray, 1999). These studies indicate that long periods of depriving the dominant eye in

amblyopes are needed for recovery of vision in the weak eye. We speculate that patching of the non-amblyopic eye initially mimics visual deprivation, because vision is blurred through the amblyopic eye, and these long periods of patching might be needed to drive an increase in NR2B-containing NMDARs. Our findings could account for the molecular mechanisms behind the clinical observations that visual deprivation in mature humans can facilitate the strengthening of weakened connections from the amblyopic eye.

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 Supplemental Fig. 2-1).

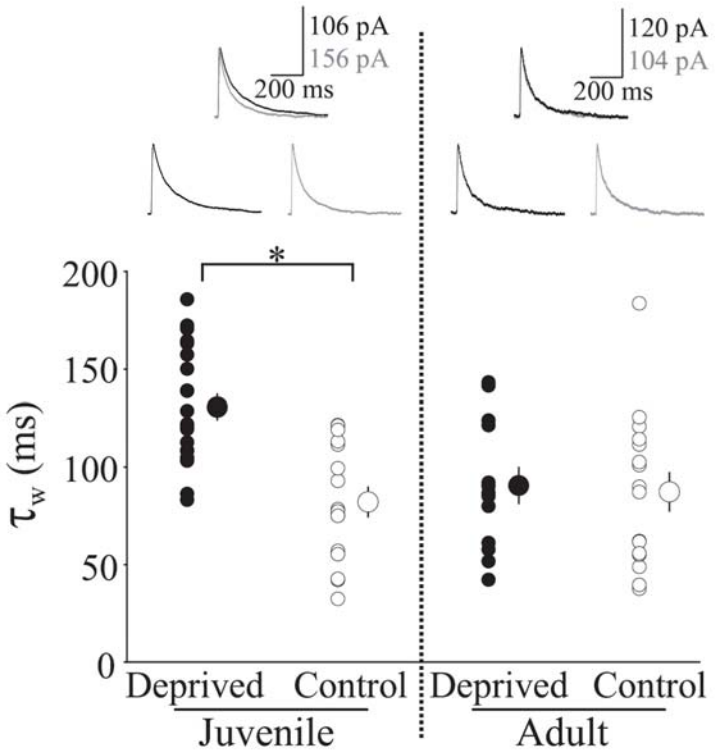


Figure 2-1: Visual deprivation in adult mice fails to modify NMDAR EPSCs evoked by single pulses. Scatter plot of the weighted time constants (τ_w) of NMDAR-mediated EPSCs recorded from layer 2/3 pyramidal cells after stimulating layer 4 in the visual cortex of visually deprived and control juvenile mice as well as deprived and control adult mice. Small circles represent individual data points and larger circles represent means (±SEM). NMDAR-mediated currents are significantly longer in the visual cortex of deprived juvenile mice compared to controls. Visual deprivation 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. *$p < 0.05$.

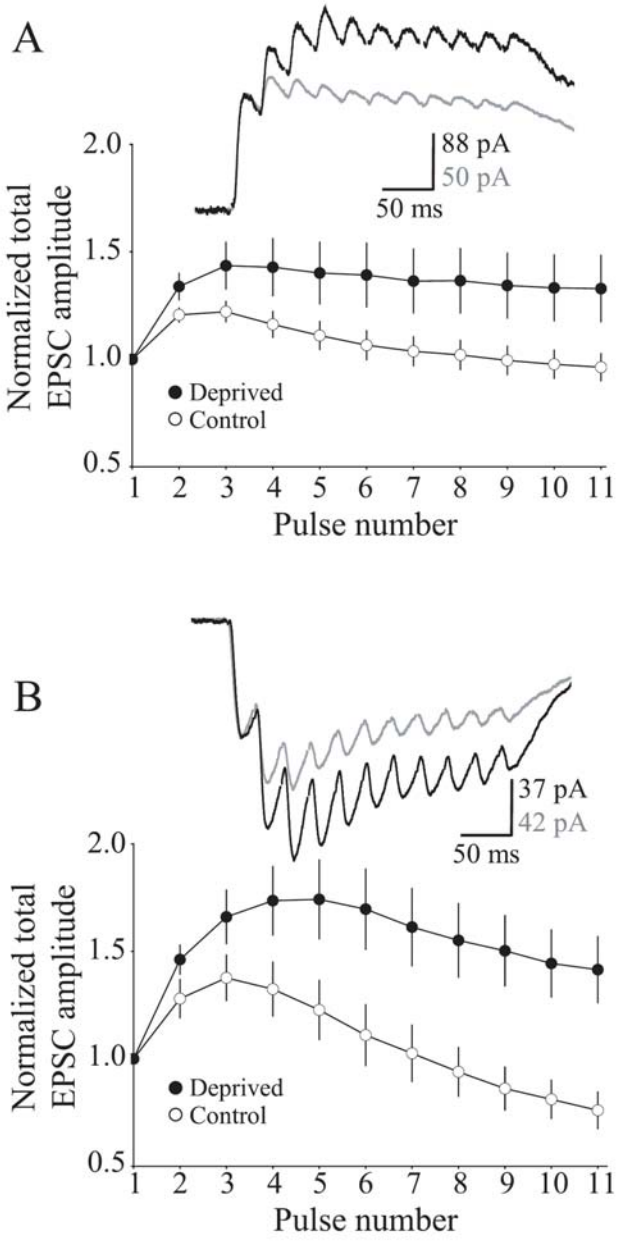


Figure 2-2: Visual deprivation in adult mice alters the temporal summation of NMDAR EPSCs evoked by burst stimulation in the visual cortex. A. Plot of the normalized and averaged amplitudes of NMDAR EPSCs evoked at 40 Hz in the adult visual cortex at a holding potential of +40 mV. Representative traces of pharmacologically isolated NMDAR EPSCs in response to 40 Hz stimulus trains are shown (dark trace = response from pyramidal neuron in deprived mice; light trace = response from pyramidal neuron in control 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 $MgCl_2$).

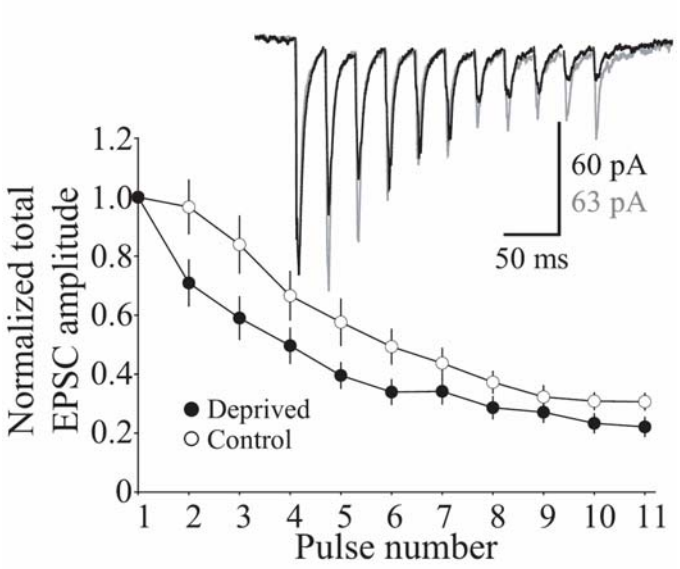


Figure 2-3: Visual deprivation increases the rate of synaptic depression in adult mice. Plot of the AMPAR EPSC amplitudes in response to a brief 40 Hz stimulation train. Responses were normalized to the first pulse. Traces are representative AMPAR EPSCs recorded at -70 mV in cells from deprived (dark trace) and control (light trace) mice. Stimulus artifacts were blanked for clarity.

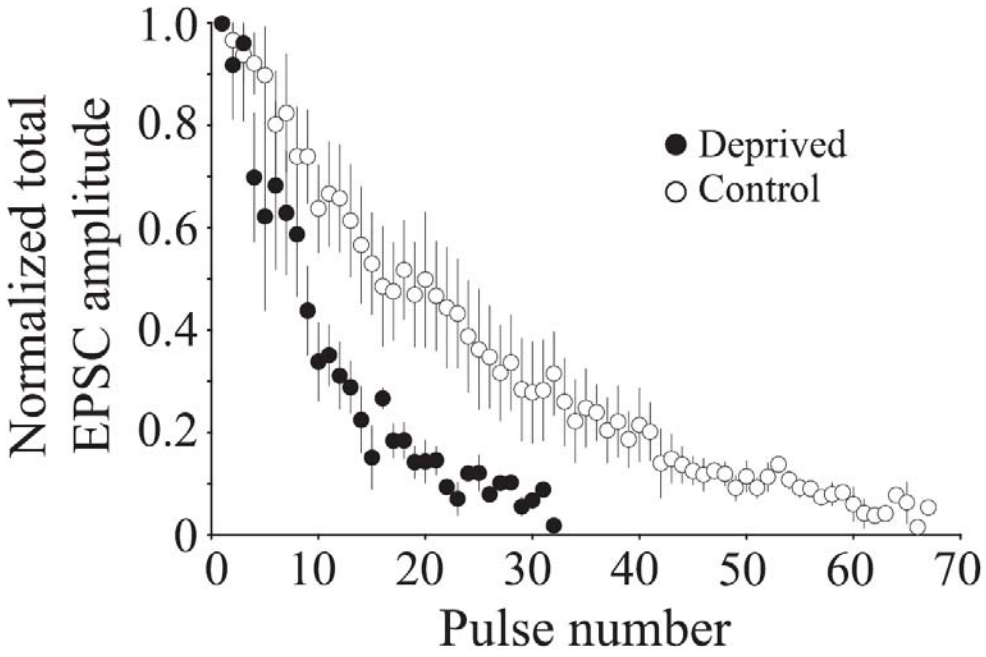


Figure 2-4: Visual deprivation increases the rate of neurotransmitter release in adult mice. Plot of the normalized amplitude of NMDAR EPSCs in response to repetitive stimulation in the presence of MK-801. Note that NMDAR EPSC blockade by MK-801 occurs faster in pyramidal cells from deprived adult mice than controls.

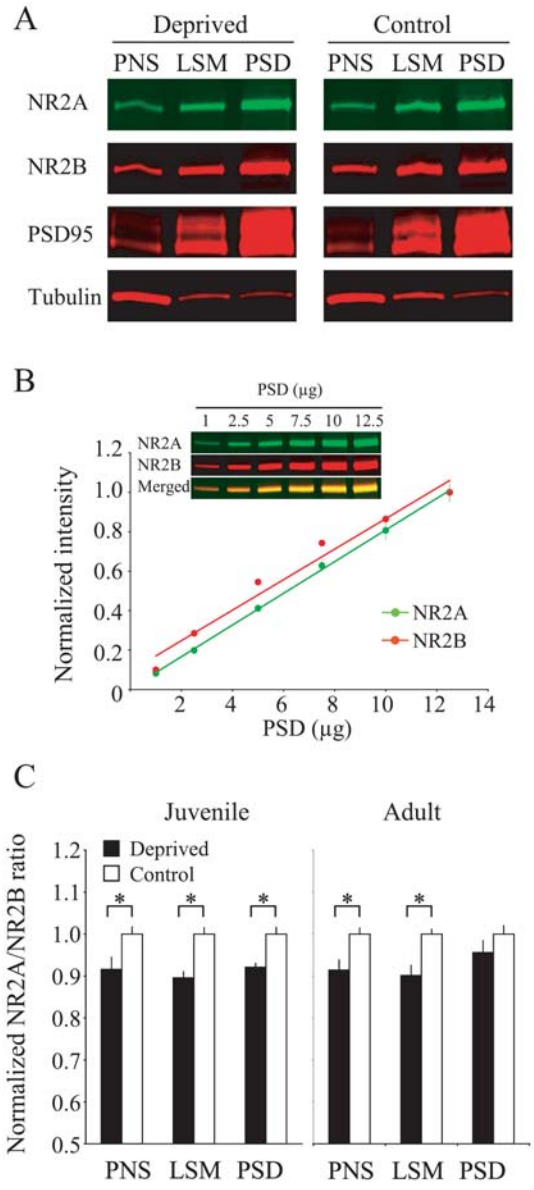


Figure 2-5: Visual deprivation in juvenile mice alters the NR2A/B ratio in the postsynaptic density (PSD), but only in post-nuclear supernatant (PNS) and lysed synaptic membrane (LSM) fractions in adult mice. A. Biochemical fractionation progressively enriches NR2A, NR2B, and PSD-95 and eliminates a non-synaptic protein, β-tubulin, in visual cortical samples from deprived and control 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. *$p < 0.05$.

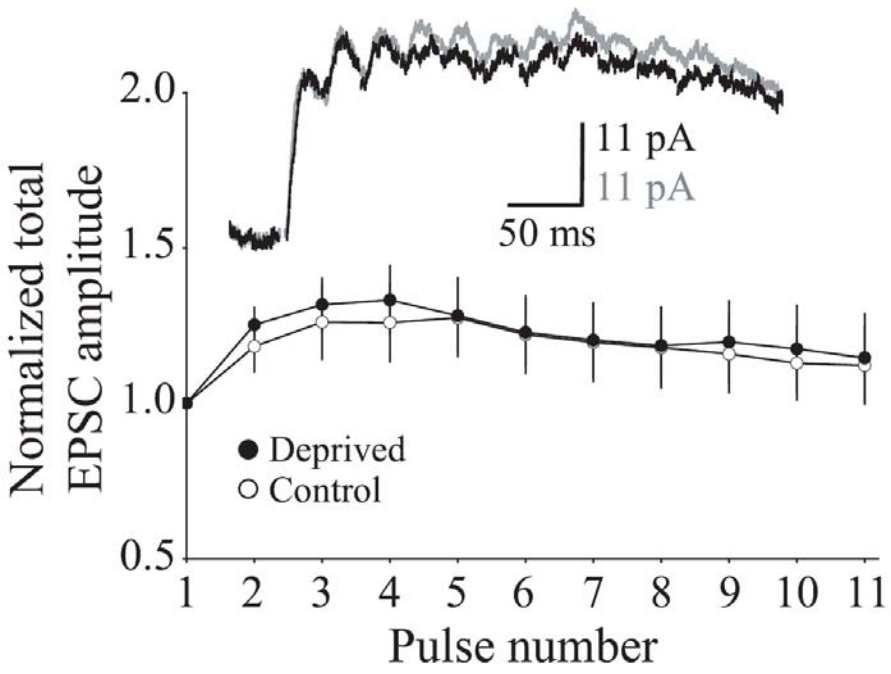


Figure 2-6: Minimal stimulation fails to reveal deprivation-induced differences in the temporal summation of NMDAR-mediated currents. Plot of the temporal summation of NMDAR EPSCs in deprived and control mice evoked by minimal stimulation at 40 Hz.

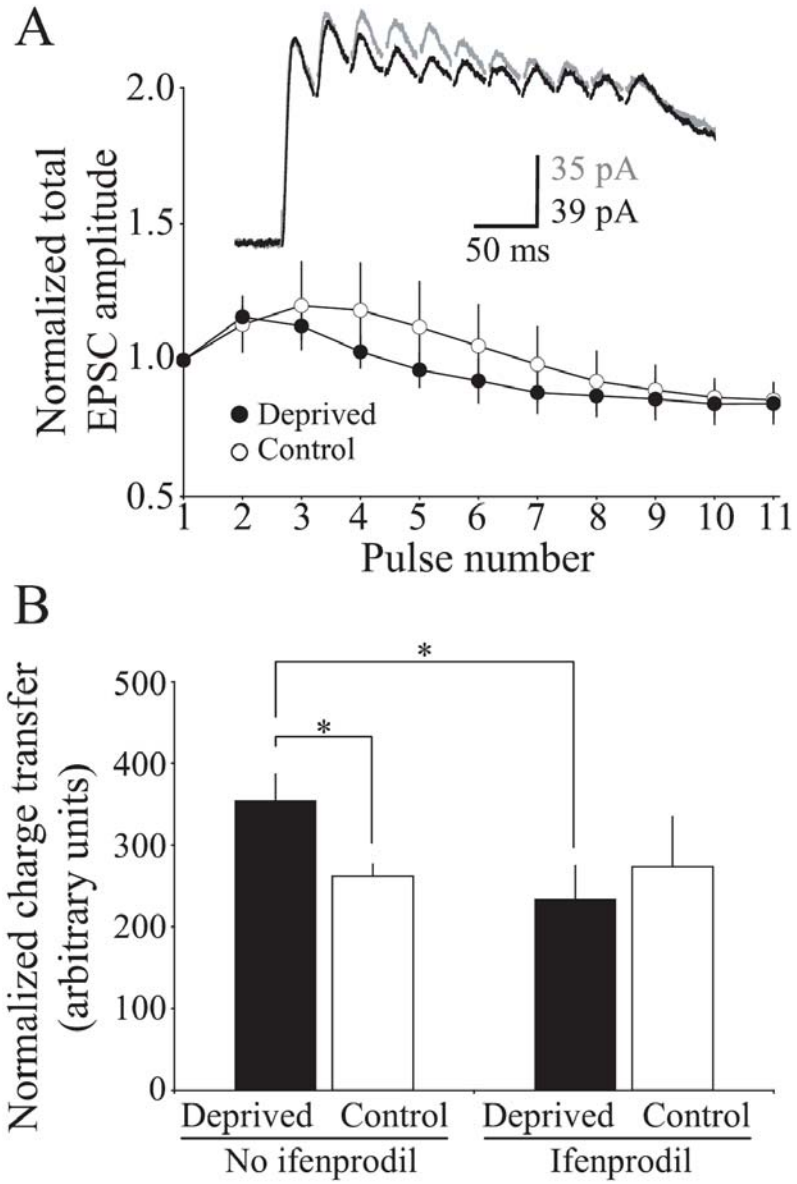
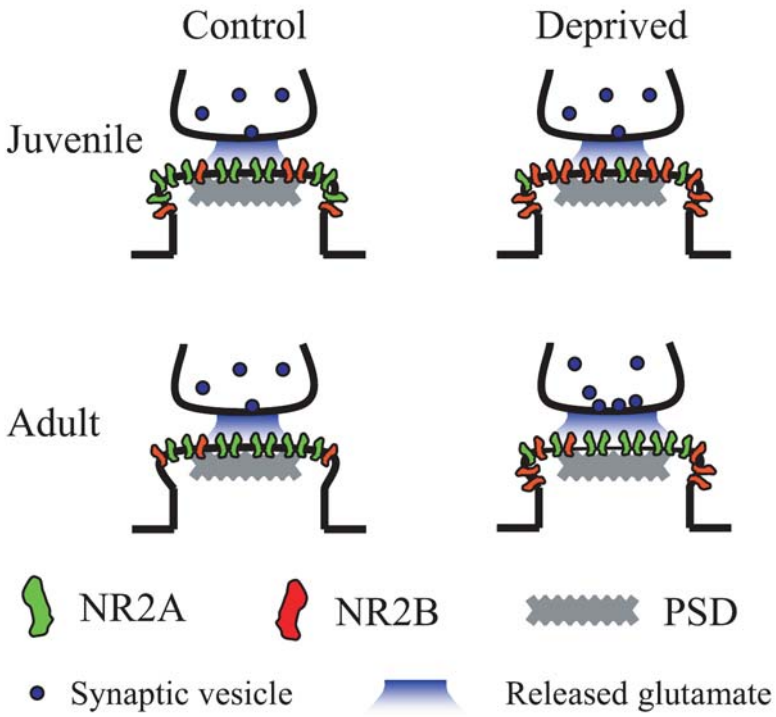


Figure 2-7: The enhanced temporal summation of NMDAR EPSCs in deprived adult cortex can be blocked by acute administration of ifenprodil, an NR2B specific NMDAR antagonist. A. Temporal summation of NMDAR EPSCs in deprived and control mice in the presence of ifenprodil. **B.** The average charge transfer 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: *$p < 0.05$.



Supplemental figure 2-1: Proposed model for visual deprivation-induced synaptic changes in juvenile and adult animals. In the visual cortex of juvenile animals, both NR2A- and NR2B-containing NMDARS are present at the synapse. Visual deprivation in juveniles does not alter release probability but causes a loss of NR2A subtypes at the PSD and at peri-/extrasytnaptic sites. In the visual cortex of adult animals, NR2A-containing NMDARs predominate at synaptic sites. Visual deprivation in adults increases both neurotransmitter release and the expression of NR2B subtypes restricted to peri-/extrasynaptic sites, which may be activated by glutamate spillover.

Chapter 3

3. Sensory deprivation restores neocortical plasticity in a mouse model of Angelman syndrome

A modified version of this chapter has been submitted for publication together with Kathryn H. Condon, Dr. Adam C. Roberts, Dr. Michael D. Ehlers, and Dr. Benjamin D. Philpot

3.1. Abstract

Sensory experience guides development of neocortical neuronal circuits. This activity-dependent circuit maturation is required for normal sensory (Putzar et al., 2007) and cognitive abilities (Hong et al., 2005), which are distorted in neurodevelopmental disorders such as autism and mental retardation. Here we have tested whether experience-dependent cortical modifications require Ube3A, an E3 ubiquitin ligase whose dysregulation has been implicated in both autism (Schroer et al., 1998) and Angelman syndrome (AS) (Rougeulle et al., 1997). Using the visual cortex as a model system of neocortical development, we demonstrate that experience-dependent maturation of excitatory cortical circuits is severely impaired in AS model mice deficient in Ube3A. This developmental defect likely arises from profound impairments in cortical plasticity, as both long-term depression (LTD) and long-term potentiation (LTP) of synaptic strength in layer 2/3 are nearly absent. Remarkably, experience-dependent synaptic plasticity in Ube3A-deficient mice is restored under conditions of sensory deprivation. Conversely, brief sensory experience rapidly reinstates a plasticity deficiency. These results show that Ube3A is necessary for experience-dependent synaptic plasticity during neocortical development, and suggest that loss of cortical plasticity contributes to deficits associated with AS. Further, our findings demonstrate the potential for non-invasive interventions to restore plasticity at the level of the synapse in AS and perhaps other cognitive disorders.

3.2. Main text

Angelman syndrome (AS) is a severe hereditary mental retardation characterized by outwardly normal development during the first year of life and normal life span, but a profound absence of subsequent cognitive milestones such as speech, and an absence of neurodegeneration (Clayton-Smith and Laan, 2003; Jiang et al., 1998). AS is caused by loss-of-function mutations or deletions in the maternally inherited allele of *Ube3A* (Rougeulle et al., 1997). *Ube3A* encodes a HECT domain ubiquitin ligase which, in the brain, is expressed primarily from the maternal allele due to tissue-specific imprinting. The apparent lack of neurodegeneration (Jiang et al., 1998) and the sharp postnatal onset has suggested a developmental defect in synaptic circuits (Zoghbi, 2003), the origin of which is unknown. Interestingly, mice deficient in maternal Ube3A exhibit genetically-reversible impairments both in learning and hippocampal LTP (Jiang et al., 1998; van Woerden et al., 2007; Weeber et al., 2003), pointing to deficits in synaptic plasticity. Clinically, AS patients present behaviors consistent with altered sensory processing, such as reduced sensitivity to sensory inputs (Walz and Baranek, 2006) and a strong fascination with water and reflective surfaces (Williams et al., 2006). We thus hypothesized that Ube3A regulates experience-dependent refinement of neuronal circuits dependent upon sensory input.

Despite a documented role of Ube3A in learning, there is no knowledge of how its loss affects the neocortex, a brain region that is heavily sculpted through experience-dependent development and whose disruption could explain most AS deficits. We used the visual cortex as a model system (Fagiolini et al., 2003; Gianfranceschi et al., 2003; Hensch, 2005;

Kirkwood et al., 1996; Li et al., 2006; Philpot et al., 2001a; Wallace and Bear, 2004) to study the role of Ube3A in experience-dependent plasticity of the neocortex in a maternally-deficient AS mouse model ($Ube3A^{m-/p+}$). Although $Ube3A^{m-/p+}$ mice exhibit extensive loss of Ube3A due to maternal imprinting in many areas of the brain including the cerebellum, hippocampus, and parts of the neocortex (Albrecht et al., 1997; Jiang et al., 1998; Jordan and Francke, 2006), it is unknown whether this also occurs within the visual cortex. Immunoblot analysis revealed that paternal deficiency in Ube3A ($Ube3A^{m+/p-}$) does not alter Ube3A protein levels in the hippocampus, cerebellum, and visual cortex (Fig. 3-1a). In contrast, Ube3A expression was drastically reduced in $Ube3A^{m-/p+}$ mice compared to wild-type (WT) mice in all three regions of the brain. Consistent with previous observations (Rougeulle et al., 1997; Vu and Hoffman, 1997), this attenuation was brain-specific, because Ube3A was highly expressed in the liver of both $Ube3A^{m+/p-}$ and $Ube3A^{m-/p+}$ mice (Fig.3-1a). We verified maternal imprinting of Ube3A protein using immunohistochemistry. Whereas Ube3A was detected in the visual cortex of WT mice, it was absent in that of $Ube3A^{-/-}$ and $Ube3A^{m-/p+}$ mice (Fig. 3-1b and Supplemental Fig. 3-1). In WT mice, intense Ube3A immunoreactivity was present on cells positive for the neuronal marker NeuN (Mullen et al., 1992) (Fig. 3-1b).

To determine the physiological consequence of Ube3A loss on neocortical development, we examined the developmental acquisition of spontaneous excitatory synaptic transmission by recording miniature excitatory postsynaptic currents (mEPSCs) in layer 2/3 pyramidal neurons of visual cortex (see Supplemental Table for intrinsic membrane properties of recorded neurons). We first compared developmental changes in mEPSC

amplitude and frequency in WT and Ube3A$^{m-/p+}$ mice raised with normal visual experience. We took measurements in brain slices from mouse pups before eye opening (~postnatal day (P)10: WT, n = 9 cells; Ube3A$^{m-/p+}$, n = 7 cells), from young animals during the peak of the critical period for ocular dominance plasticity (~P25: WT, n = 11 cells; Ube3A$^{m-/p+}$, n = 12 cells), and from adults (~P100: WT, n = 12 cells, Ube3A$^{m-/p+}$, n = 12 cells). Consistent with previous findings (Desai et al., 2002b; Goel and Lee, 2007), mEPSC amplitudes in WT mice decreased steeply during early postnatal life and were then maintained into adulthood (Fig. 3-1c, d). The same postnatal decrease in mEPSC amplitude was seen in Ube3A$^{m-/p+}$ mice (Fig. 3-1c, d: P10, WT 15.4 ± 1.2 pA, Ube3A$^{m-/p+}$ 17.6 ± 1.5 pA, p= 0.2; P25, WT 11.8 ± 0.3 pA, Ube3A$^{m-/p+}$ 11.0 ± 0.4 pA, p = 0.2; P100, WT 10.1 ± 0.3 pA, Ube3A$^{m-/p+}$ 11.1 ± 0.5 pA, p = 0.1). In WT mice, mEPSC frequency was also strongly regulated over early development, increasing sharply from P10 to P25 and remaining stable into adulthood, consistent with previous results (Desai et al., 2002b; Goel and Lee, 2007). In Ube3A$^{m-/p+}$ mice, mEPSC frequency was comparable to WT mice at infancy (P10: WT, 0.9 ± 0.2 Hz; Ube3A$^{m-/p+}$, 0.6 ± 0.1 Hz; p = 0.2). However, the subsequent developmental increase in mEPSC frequency at P25 was severely diminished in Ube3A$^{m-/p+}$ animals compared to WT (Fig. 3-1c, e: WT, 10.7 ± 0.8 Hz; Ube3A$^{m-/p+}$, 6.8 ± 0.9 Hz; p < 0.003), and this reduction persisted into adulthood (Fig. 3-1c, e: WT, 10.9 ± 1.3 Hz; Ube3A$^{m-/p+}$, 7.5 ± 0.7 Hz; p < 0.03). Thus, although normal in infancy, spontaneous excitatory synaptic transmission in layer 2/3 fails to mature in Ube3A$^{m-/p+}$ mice, consistent with a reduction in spine density (Dindot et al., 2007) and functional synapses caused by the absence of Ube3A.

Because mEPSC deficits began to appear after the onset of eye opening and patterned vision (P11-12 in the mouse), we investigated the role of visual experience in regulating the development of spontaneous synaptic activity in Ube3A$^{m-/p+}$ mice. Toward this end, we dark-reared (DR) animals from P10 until P25 to deprive them of visual experience after eye-opening (P11-12), and compared mEPSC frequency and amplitude in layer 2/3 pyramidal neurons to that of normally-reared (NR) mice (Fig. 3-2a: DR WT, n = 12 cells; DR Ube3A$^{m-/p+}$, n = 14 cells). Although dark-rearing from P10 had no measurable effect on mEPSC amplitude in WT mice recorded at P25 (Fig. 3-2b, c: NR, 11.8 ± 0.3 pA; DR, 11.5 ± 0.5 pA; p = 0.7), sensory deprivation strongly attenuated the developmental increase in mEPSC frequency in WT mice (Fig. 3-2b, d: NR, 10.7 ± 0.8; DR, 7.0 ± 0.5; p < 0.0005). In contrast, dark-rearing had no effect on either mEPSC amplitude (Fig. 3-2b, c: NR, 11.0 ± 0.4 pA; DR 11.7 ± 0.3 pA; p = 0.16) or frequency (Fig. 3-2b, d: NR, 6.8 ± 0.9 Hz; DR, 6.0 ± 0.6 Hz; p = 0.46) in Ube3A$^{m-/p+}$ mice. Moreover, the mEPSC frequency in NR Ube3A$^{m-/p+}$ mice was not significantly different from that of DR WT mice (p = 0.8). These findings demonstrate that, although Ube3A is not necessary for the initial activity-independent establishment of synaptic connectivity, it is selectively required for experience-dependent maturation of excitatory cortical synapses.

One possible explanation for the lack of experience-dependent synaptic development in Ube3A$^{m-/p+}$ mice is the absence of activity-dependent plasticity at neocortical synapses. We therefore compared properties of neocortical LTD and LTP at layer 2/3 synapses in visual cortex of WT and Ube3A$^{m-/p+}$ mice, at both young (~P25) and adult (~P100) ages. Because layer 2/3 pyramidal neurons receive major inputs from layer 4 pyramidal neurons,

layer 2/3 field potentials were evoked by layer 4 stimulation. We began by measuring LTD in young mice using a standard stimulation protocol (1 Hz for 15 min). Whereas LTD was reliably induced in young WT mice, it was completely absent in young Ube3A$^{m-/p+}$ mice (Fig. 3-3b; % field excitatory postsynaptic potential (fEPSP): WT, 84.3 ± 3.5, n = 13; Ube3A$^{m-/p+}$, 97.9 ± 4.9, n = 7; $p < 0.04$). We also observed deficits in LTP induction. While a relatively weak induction protocol (three 1 second trains of 40 Hz stimulation) elicited LTP in young WT mice, this protocol failed to induce LTP in young Ube3A$^{m-/p+}$ mice (Fig. 3-3c; %fEPSP: WT, 114.8 ± 3.3, n = 12; Ube3A$^{m-/p+}$, 104.2 ± 2.0, n = 10 slices; $p < 0.02$). To test whether the neocortex of young Ube3A$^{m-/p+}$ mice was capable of expressing LTP, we also applied a strong LTP stimulation protocol (two 1 second trains of 100 Hz stimulation). This protocol reliably induced similar LTP in both young Ube3A$^{m-/p+}$ and WT mice (Fig. 3-3d; %fEPSP: WT, 110.4 ± 3.3, n = 12; Ube3A$^{m-/p+}$, 112.7 ± 2.2, n = 10 slices; $p = 0.6$). Thus, as with deficits in hippocampus (Weeber et al., 2003), LTP induction machinery is suppressed in the visual cortex of Ube3A$^{m-/p+}$ mice and this deficit in LTP can be overcome with stronger stimulation (Fig. 3-3e). Moreover, and unexpectedly, the impairment in plasticity was bidirectional, as Ube3A is also required for the normal expression of LTD (Fig. 3-3b, e). The impairment of both LTP and LTD produced a flattened stimulus-response curve indicating profound synaptic rigidity (Fig. 3-3e).

We tested whether the plasticity deficits in AS mice persisted into adulthood. In adult WT mice, LTD induced by 1 Hz stimulation was absent as expected(Kirkwood et al., 1997), and LTP could be induced with strong stimulation (two trains of 100Hz stimulations). In

adult Ube3A$^{m-/p+}$ mice, however, none of these protocols was effective at modifying synaptic strength (Supplemental Fig. 3-2b, c, d). These results indicate that WT animals lose neocortical plasticity as they mature and this loss of plasticity is even more severe in the absence of Ube3A (compare Fig. 3-3e and Supplemental Fig. 3-2e). Further, these data show that plasticity defects in AS mice persist into adulthood.

Visual experience affects the ability to induce LTD and LTP (Kirkwood et al., 1996; Philpot et al., 2007). Moreover, visual deprivation has been shown to recover juvenile-like cortical plasticity in adult rodents (He et al., 2006; He et al., 2007). Therefore, we speculated that the loss of LTD and LTP in Ube3A$^{m-/p+}$ mice could result from downregulation of synaptic plasticity induced by visual experience. In this case, one would expect that reducing sensory experience would recuperate synaptic plasticity. We investigated this possibility by measuring LTD and LTP in young WT and Ube3A$^{m-/p+}$ mice reared in the dark (Fig. 3-4a, b). Indeed, LTP induced by the 40 Hz protocol was similar for both WT and Ube3A$^{m-/p+}$ mice following dark rearing (Fig. 3-4c: WT, 113.2 ± 2.4 % and n = 18 slices, Ube3A$^{m-/p+}$, 113.6 ± 2.4 % and n = 18 slices; p = 0.9). Similarly, visual deprivation restored the normal induction and expression of LTD in Ube3A$^{m-/p+}$ mice (Fig. 3-4d, g: WT, 86.5 ± 4.3 % and n = 16 slices; Ube3A$^{m-/p+}$, 89.7 ± 2.9 % and n = 17 slices; p = 0.7). Thus, sensory deprivation restores bidirectional synaptic plasticity in Ube3A$^{m-/p+}$ mice. These data indicate that, although Ube3A$^{m-/p+}$ mice are born with normal LTD and LTP at neocortical synapses, ongoing sensory experience induces a profound loss of synaptic plasticity in the absence of Ube3A that can be restored by sensory deprivation.

Finally, to confirm that visual experience, rather than an intrinsic developmental program, caused the loss of plasticity in Ube3A$^{m-/p+}$ mice, we investigated the effect of reinstating sensory experience following dark rearing. To this end, we dark-reared mice until ~P26, and then provided them with four days of a normal visual environment (Fig. 3-4e). Under this condition, we found that, although LTD was normally induced in the layer 4 to 2/3 pathway of visual cortex in WT mice, it was now once again absent in Ube3A$^{m-/p+}$ mice (Fig. 3-4f, g: WT, 86.1 ± 4.8 % and n = 9 slices; Ube3A$^{m-/p+}$, 107.0 ± 3.4 % and n = 6 slices; $p < 0.01$), indicating that as little as four days of visual experience was sufficient to cause a loss of plasticity in AS animals. Together, these findings demonstrate that Ube3A is required to maintain synaptic plasticity in the face of sensory activity at both early and late developmental stages (Fig. 3-4g).

In this report, we have studied the role of Ube3A in experience-dependent cortical development. We found that visual experience fails to strengthen functional connectivity of excitatory neurons in the visual cortex of Ube3A$^{m-/p+}$ mice. Moreover, we demonstrated that neocortical synaptic plasticity is bidirectionally blunted in an experience-dependent manner, presumably limiting subsequent development of excitatory circuits in the neocortex. Thus, Ube3A is necessary to maintain synaptic plasticity during ongoing activity-dependent remodeling. Given the known function of Ube3A in ubiquitin-dependent protein degradation[5], the absence of Ube3A may facilitate accumulation of Ube3A-substrate proteins that impair synaptic plasticity.

Functional and anatomical abnormalities in the visual system of human AS patients are consistent with the observed aberrant development of visual cortical circuits in Ube3A$^{m-/p+}$ mice. Visual acuity is reported to be low in AS patients, and this visual deficit is unique to AS among a wide range of neurodevelopmental disorders (Van Splunder et al., 2003). Moreover, recordings of visual responses in an AS patient revealed possible dysfunction of visual circuitry (Thompson et al., 1999). Finally, the only anatomical study to date from postmortem tissue from an AS patient revealed reduced dendritic arborization and spine density in pyramidal neurons of visual cortex (Jay et al., 1991). These observations imply that visual deficits in AS patients may arise due to a lack of normal experience-dependent refinement. If the deficits in experience-dependent encoding in the visual cortex are generalizable to other areas of the brain, then these same changes in synaptic physiology may help explain the observed deficiencies in learning and cognition.

Our observations raise the possibility that sensory experience-dependent synaptic development may be abnormal in human AS patients, and further show that a non-invasive manipulation can restore plasticity in a model of mental retardation. The demonstration that the physiological substrates of synaptic plasticity remain intact thus raises the possibility that behavioral or pharmacological manipulations could improve brain function in patients with AS or other neurodevelopmental disorders.

3.3. Methods

Animals

Mice deficient in Ube3A originally developed by Jiang et. al.(Jiang et al., 1998) were obtained through the Jackson Laboratory (Bar Harbor, ME). Mice were bred in a 129S7 background. To obtain heterozygous mice lacking Ube3A gene paternally (m+/p-) or maternally (m-/p+), a heterozygous male or female mouse was crossed with a wild-type female or male mouse, respectively. WT and maternal heterozygous mice were used for electrophysiological recordings at three age groups: infant (P8-11; ~P10); young (P21-28; ~P25); and adult (P94-121; ~P100). Control mice were raised on a 12 h light/dark cycle, whereas DR mice were raised in complete darkness starting from P9-P11 (before eye opening) until ~P25. Light exposure was achieved by transferring DR mice into the control condition at P24-P28 and providing 4 days in the normal light/dark cycle. Electrophysiological recordings were obtained from at least three mice for each condition. All animal procedures were performed in compliance with the U. S. Department of Health and Human Services and the animal care guidelines at the University of North Carolina and Duke University.

Immunoblot analysis

Tissue lysates were prepared in lysis buffer containing (in mM): 50 Tris, 150 NaCl, 50 NaF, 2 EDTA, 2 EGTA, 1% (v/v) Triton, pH 7.4, and a cocktail of protease and phosphatase inhibitors. Protein concentrations were determined using DC Protein Assay (BioRad). 20 μg of each lysate were resolved on a 7.5% Tris-HCl gel (BioRad), and

transferred to a PVDF membrane. The membrane was probed with the primary antibody anti-Ube3A (1:1000, Bethyl Labs) and subsequently with horseradish peroxidase-conjugated anti-rabbit polyclonal antibody (1:10,000, Cell Signaling). Signals were visualized with ECL Plus reagent (Amersham) and a LAS-3000 Intelligent Dark Box (FujiFilm).

Immunohistochemistry

Mice were anesthetized with pentobarbital and perfused intracardially with cold phosphate-buffered saline (PBS) followed by freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were postfixed in the same solution overnight at 4°C. Brains were then cut into 50 μm coronal sections in a vibratome (Leica). For immunohistochemistry experiments, sections from wild-type, Ube3a$^{m-/p+}$, and Ube3a knock-out mice were always processed simultaneously. Sections were incubated in 10 mM sodium citrate buffer (pH 8.65) at 80^{o}C for 15 min prior to the immunoreactions. Sections were reacted with anti-Ube3A rabbit polyclonal antibody (1:500, Bethyl Labs) and anti-NeuN monoclonal mouse antibody (1:3,000, Chemicon) overnight at 4^{o}C, and were then incubated with Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG secondary antibodies (1:500, Invitrogen). Images were captured with a Zeiss LSM 510 microscope.

Visual cortical slice preparation

Mice were anesthetized with an overdose of pentobarbital barbiturate and decapitated after the disappearance of corneal reflexes. Brains were dissected rapidly and the visual cortex

cut coronally at 300 μm (voltage-clamp recordings) or 400 μm (field potential recordings) in dissection buffer containing the following (in mM): 75 sucrose, 87 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 7 $MgCl_2$, 0.5 $CaCl_2$, and 1.3 ascorbic acid. Prior to recordings, slices were allowed to recover for one hour in a submersion chamber containing oxygenated artificial cerebrospinal fluid (ACSF) consisting of the following (in mM): 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 20 glucose, 2 $MgCl_2$, and 1 $CaCl_2$.

Voltage-clamp recordings

Slices were placed in a submersion chamber maintained at 30°C and perfused at 2 ml/min with oxygenated ACSF supplemented with 200 nM Tetrodotoxin, 100 μM APV, and 50 μM picrotoxin. Neurons were visualized with a Carl Zeiss Axioskop (Germany) equipped with infrared differential interference contrast optics. Patch pipettes were pulled from thick-walled borosilicate glass. Open tip resistances were 3–5 MΩ when pipettes were filled with the internal solution containing (in mM): 20 KCl, 100 (K)Gluconate, 10 (K)HEPES, 4 (Mg)ATP, 0.3 (Na)GTP, 10 (Na)Phosphocreatine, and 0.01% w/v Alexa 488, adjusted with KOH to pH 7.4, and with sucrose to 290–300 mOsm. Voltage-clamp recordings were performed in the whole-cell configuration with a patch-clamp amplifier (Multiclamp 700A, Molecular Devices, Sunnyvale, CA), and data were acquired with pClamp 9.2 software (Molecular Devices). Input and series resistances were determined throughout the experiment by measuring the response to small intermittent test pulses. Recordings were discarded if the series resistance grew larger than 25 MΩ, or the resting potential was more positive than -60 mV (for the recordings in infant mice, the resting potential was not used as a criterion, as many of neurons typically exhibit resting potentials

higher than -60 mV). Analysis was restricted to neurons that were anatomically verified by intracellular fills to be pyramidal neurons. MiniAnalysis (Synaptosoft) was used to detect and measure mEPSCs. The threshold to detect mEPSC was set at 5 pA, which is greater than 2.5 times the root mean square of the noise, and mEPSCs with 10−90% rise times more than 3 ms were excluded from the analysis. Analysis of mEPSCs was performed blind to age, genotype, and rearing condition.

Field potential recordings

Slices were maintained at 30°C and perfused with ACSF at a rate of 2.0 ml/min. A concentric bipolar tungsten stimulation electrode was positioned in layer 4, and a glass recording electrode (1-3 MΩ) filled with ACSF was positioned in layers 2/3. The magnitude of responses evoked by a 200 μs pulse was monitored by the amplitude of the field potential. Stimulation intensity was adjusted to elicit half the maximal response, and stable baseline responses were elicited every 30 s. The resulting signals were filtered between 0.1 Hz and 3 kHz, amplified 1000 times, and captured at 10 kHz on an IBM-compatible computer using pCLAMP 9.2 software (Molecular Devices). After achieving a stable baseline (<5% drift) for 15 min, slices were stimulated with one of the following three protocols: 100 Hz stimulation for 1 s, repeated two times with a 15 s interval; 40 Hz stimulation for 1 s, repeated three times with a 10 s interval; or 900 pulses at 1 Hz. Field excitatory postsynaptic potential (fEPSP) amplitudes were recorded every 30 s for 45 min following the cessation of the stimulation protocol. Control and experimental subjects were run in an interleaved fashion. The data were normalized, averaged, and reported as means ± SEM. Changes in synaptic strength were measured by comparing the average

response amplitude 30-45 min after conditioning stimulation to the pre-conditioning baseline response.

Statistics

Data are expressed as the means ± SEM. Two-tailed Student's t tests were used to test for statistical significance, which was placed at $p < 0.05$.

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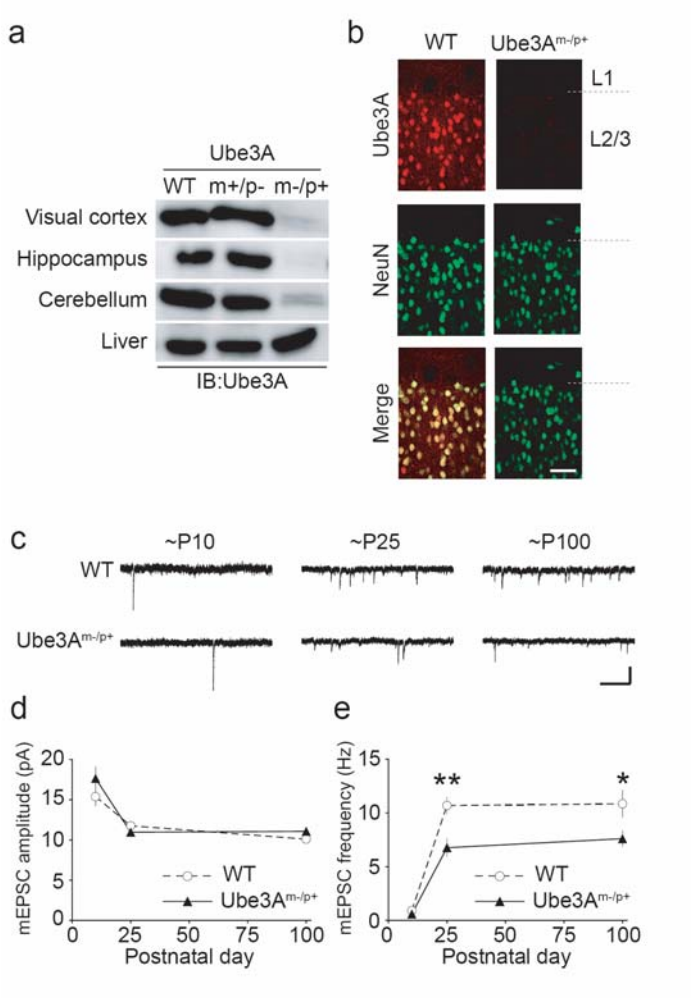


Figure 3-1: Reduced functional maturation of neocortical synapses in AS mice. a, Immunoblot analysis of Ube3A in tissue from young (P26) WT, Ube3a$^{m+/p-}$, and Ube3a$^{m-/p+}$ mice. **b**, Immunohistochemical analysis of Ube3A expression in the visual cortex from young (P24) WT, and Ube3a$^{m-/p+}$ mice. Strong Ube3A immunoreactivity was observed in layer 2/3 (L2/3) neurons of WT mice. NeuN antibody stains cell bodies of neurons. Scale bars: 50 μm. **C**, Representative traces of mEPSCs recorded in layer 2/3 pyramidal neurons from WT (upper) or Ube3a$^{m-/p+}$ (lower) mice at ~P10, ~P25, and ~P100. Scale bars: 0.2 sec, 20 pA. **d and e,** Average mEPSC amplitude (**d**) and frequency (**e**) as a function of postnatal age in WT and Ube3a$^{m-/p+}$ mice. * $P < 0.05$, ** $P < 0.005$.

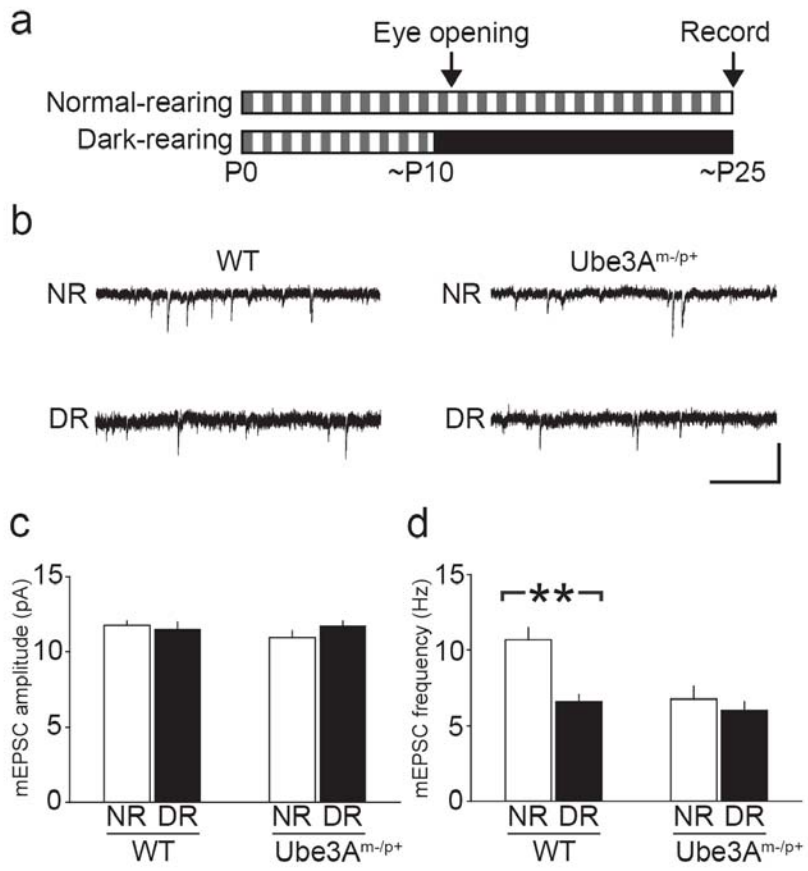


Figure 3-2: Sensory experience augments excitatory synaptic transmission in the neocortex of WT mice, but not AS mice. **a**, Schematic for the rearing conditions. Normally-reared animals were maintained in a 12 hour consecutive dark:light cycle, and DR animals were kept in complete darkness from ~P10. **b**, Representative traces of mEPSCs recorded in layer 2/3 pyramidal neurons in WT (left) or Ube3A$^{m-/p+}$ (right) mice reared normally (NR, upper) or in complete darkness (DR, lower). Scale bar: 0.2 sec, 20 pA. **c**, Dark-rearing does not affect mEPSC amplitude in WT or Ube3A$^{m-/p+}$ mice. **d**, Dark-rearing significantly reduces mEPSC frequency in WT mice, but does not affect mEPSC frequency in Ube3A$^{m-/p+}$ mice.

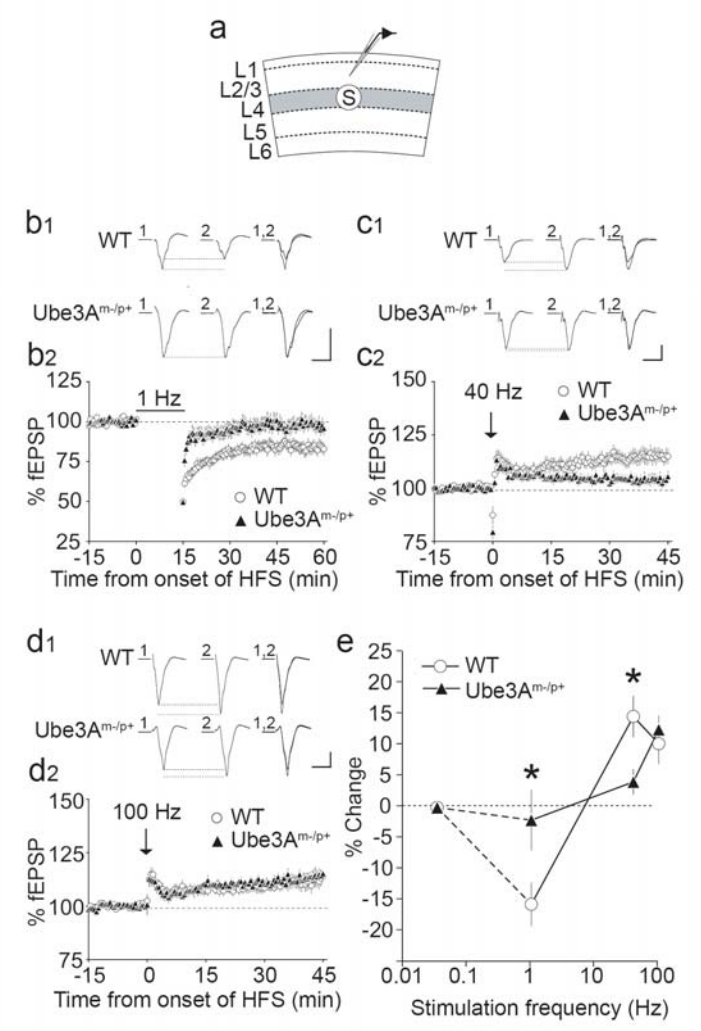


Figure 3-3: Synaptic plasticity is impaired bidirectionally in the neocortex of AS mice. **a**, Schematic diagram of stimulating (S) and recording configuration. **b**, Baseline synaptic responses of WT (open circle) and Ube3A$^{m-/p+}$ (closed triangle) mice were measured before and after application of conditioning stimuli to the layer 4 to layer 2/3 pathway of the visual cortex. LTD was induced with 1 Hz stimulation for 15 min in visual cortical slices from young (P25) mice. b1, top traces are representative averaged traces of 15 min baseline (1), 30-45 min period after LTD inductions (2), and their overlays (1, 2). Scale bars: 5 ms, 1 mV. b2, average change in field EPSP (% fEPSP) upon delivery of a 1 Hz stimulus (indicated by the bar). **c**, Same as b, except that LTP-inducing stimulation consisted of three 40 Hz trains (indicated by an arrow in c2). **d**, Same as b, except that the LTP-inducing stimulation consisted of two 100 Hz trains (indicated by an arrow in d2). **e**, Frequency-response functions derived from visual cortex of WT and Ube3A$^{m-/p+}$ mice. Data points represent percent changes in fEPSP 30-45 min after the delivery of conditioning stimuli. The data points for 0.033 Hz are inferred from baseline stimulation delivered once every 30 sec which does not induce synaptic modification.

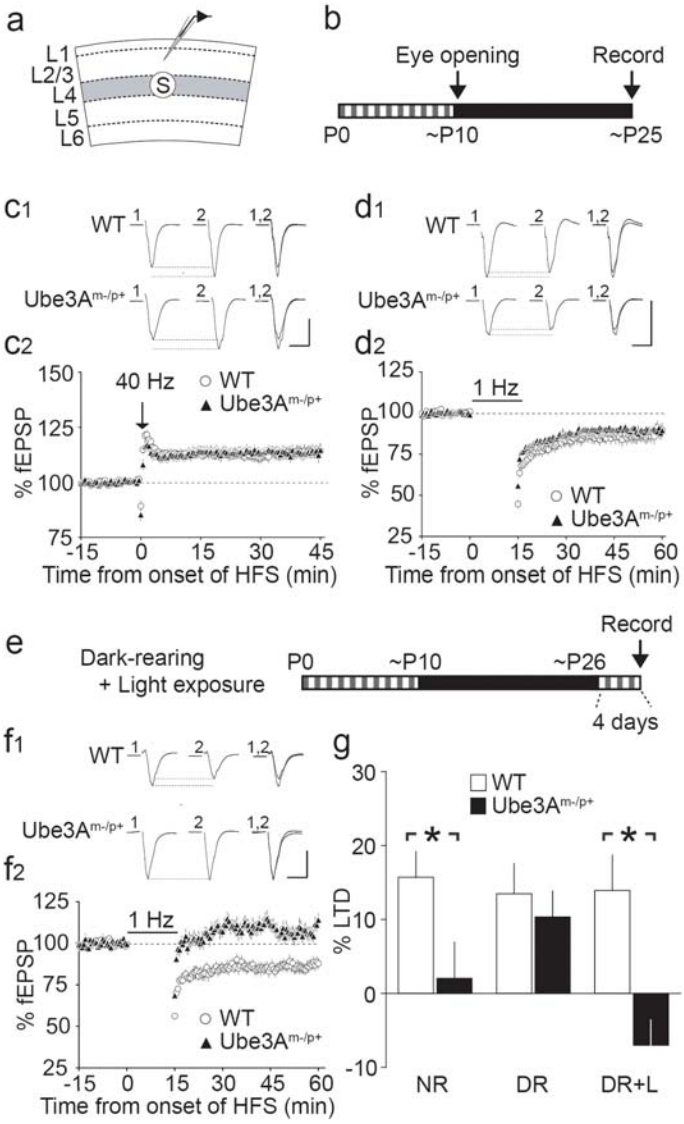
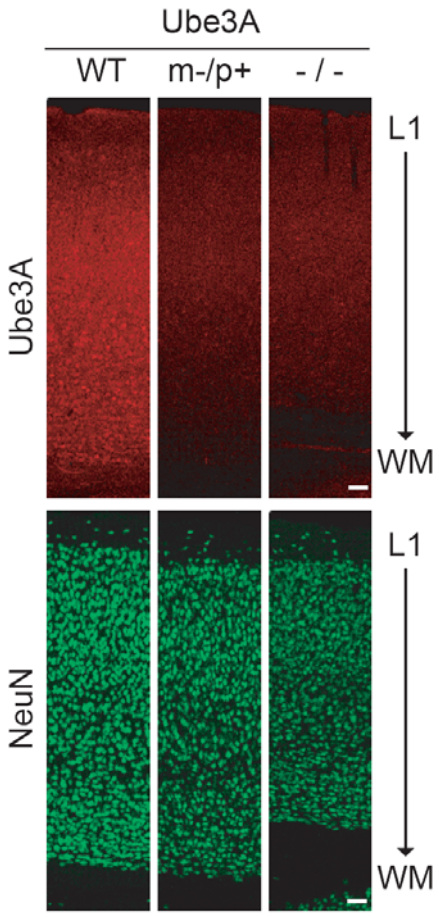
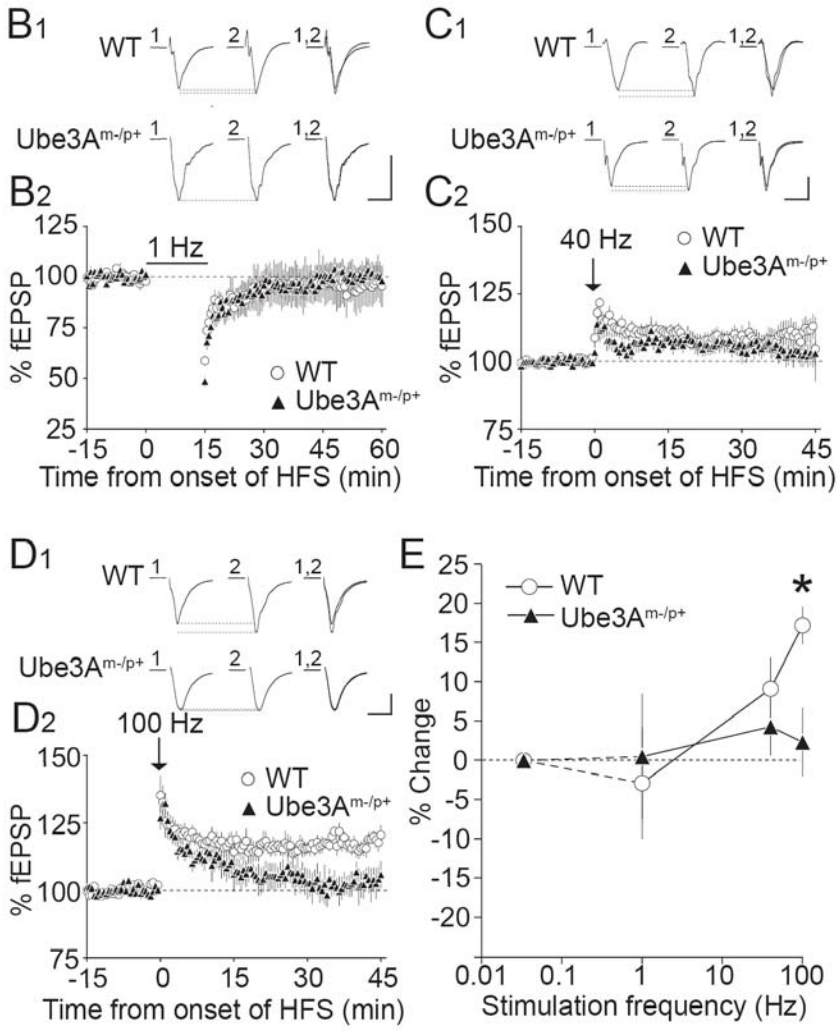


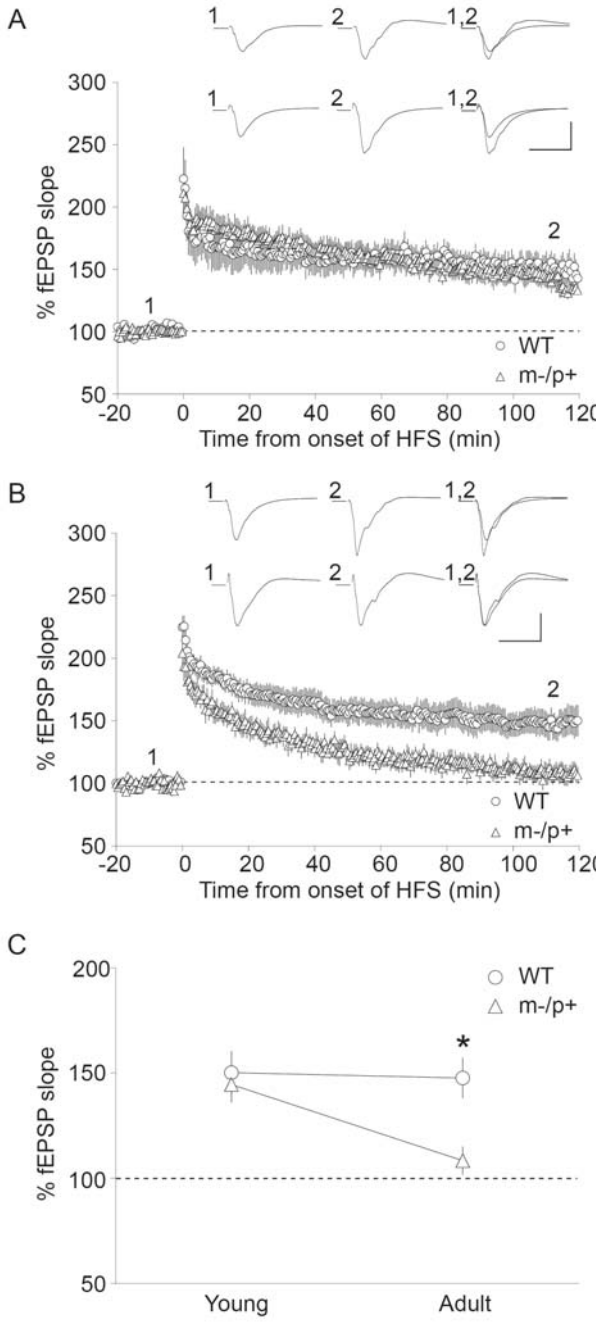
Figure 3-4: Sensory deprivation restores neocortical plasticity in AS mice. **a**, Schematic of the recording configuration. **b**, Schematic for the dark-rearing condition. **c**, Representative waveforms (1) and averaged data (2) demonstrating that the level of LTP induced with 40 Hz stimulation is comparable between WT and Ube3A$^{m-/p+}$ mice reared in complete darkness. Scale bars: 5 ms, 0.5 mV. **d**, the level of LTD is also comparable between WT and Ube3A$^{m-/p+}$ dark-reared mice. **e**, Schematic showing the schedule for exposing dark-reared mice to light. **f**, 4 days of normal-rearing following dark-rearing attenuates LTD in visual cortical slices from Ube3A$^{m-/p+}$ mice. **g**, Visual experience dampens LTD in the visual cortex of Ube3A$^{m-/p+}$ mice. Data represent means ± SEM of the percent reduction in fEPSP 30-45 min after the delivery of conditioning stimuli measured in normally-reared (NR), dark-reared (DR), and dark-then-light exposed (DR-L) mice.



Supplemental figure 3-1. Immunohistochemical analysis of Ube3A expression in the visual cortex from young (P24) WT, Ube3a$^{m-/p+}$, and Ube3a KO mice. Whereas strong Ube3A immunoreactivity was observed in all cortical layers except for layer 1 (L1) and white matter (WM) in WT mice, it was absent in both Ube3a$^{m-/p+}$ and KO mice. NeuN antibody stains neurons. Scale bars: 100 μm.



Supplemental figure 3-2. The absence of Ube3A causes severe deficits in bidirectional synaptic plasticity in the visual cortex of <u>adult</u> mice. **a**. Schematic of the recording configuration. **b**. Baseline synaptic responses of WT (open circle) and AS (closed triangle) mice were measured before and after application of a conditioning stimulation to the layer 4 to 2/3 pathway of the visual cortex. LTD was induced with 1 Hz stimulation for 15 minutes in visual critical slices from adult mice. b1, Top traces are representative averaged traces of 15 min baseline (1), 30-45 min period after LTD inductions, (2) and their overlays (1, 2). Scale bars: 5 ms, 1 mV. b2, average change in field EPSP (% fEPSP) upon delivery of a 1 Hz stimulus (indicated by the bar) **c**. Same as b, except that LTP-inducing stimulation consisted of three 40 Hz trains (indicated by an arrow). **d**. Same as a, except that the LTP-inducing stimulation consisted of two 100 Hz trains (indicated by an arrow). **e**, Frequency-response functions derived from visual cortex of WT and Ube3a$^{m-/p+}$ mice. Data points represent percent changes in fEPSP 30-45 minutes after the delivery of conditioning stimulations. The data points for 0.033 Hz are inferred as baseline stimulation once every 30 seconds does not induce synaptic modifications in the visual cortex.



Supplemental figure 3-3. Developmental loss of hippocampal LTP in AS mice.
a, Baseline synaptic responses of WT (open circle) and AS (open triangle) mice were measured before and after application of LTP-inducing HFS (indicated by arrows) to area CA1 of hippocampus. LTP was induced with two trains of 100 Hz stimulation for 1 second separated by 20 seconds. Top traces are representative averaged traces of 15 min baseline (1), 102-120 min period after LTP inductions (2) and their overlays (1+2) **b**, Same as a, except that hippocampal slices are from adult mice.LTP. * $P < 0.05$. Scale bars: 10 ms, 1 mV.

Supplemental table 3-1. Passive membrane properties of layer 2/3 pyramidal neurons in which mEPSC's are recorded

	Infant (P8 – 11)			Young (P21 – 28)			Adult (P98 – 101)		
	WT	Ube3A$^{m-/p+}$	P-value	WT	Ube3A$^{m-/p+}$	P-value	WT	Ube3A$^{m-/p+}$	P-value
Neuron number	9	7		13	12		12	12	
V_m (mV)	-59.9 ± 2.4	-54.8 ± 4.8	0.33	-73.0 ± 1.5	-70.0 ± 1.9	0.24	-75.0 ± 1.4	-72.6 ± 1.7	0.29
Ri (MΩ)	568 ± 42.4	560.1 ± 50.3	0.91	114.3 ± 10.1	163.4 ± 13.6	0.01	122.0 ± 1.4	140.7 ± 13.5	0.30
C_m (pF)	65.6 ± 4.5	66.6 ± 3.7	0.87	147.8 ± 5.5	115.2 ± 5.5	0.0004	151.7 ± 8.7	121.6 ± 9.6	0.03
Tau (ms)	1.1 ± 0.1	1.2 ± 0.2	0.43	2.8 ± 0.2	2.3 ± 0.1	0.06	3.1 ± 0.1	2.7 ± 0.2	0.10

	Young WT (P21 – 28)			Young Ube3A$^{m-/p+}$ (P21 – 28)			DR WT vs m-/p+
	NR	DR	P-value	NR	DR	P-value	P-value
Neuron number	13	12		12	14		
V_m (mV)	-73.0 ± 1.5	-71.9 ± 1.9	0.48	-70.0 ± 1.9	-73.6 ± 1.4	0.14	0.49
Ri (MΩ)	114.3 ± 10.1	144.7 ± 11.7	0.06	163.4 ± 13.6	156.7 ± 13.3	0.73	0.51
C_m (pF)	147.8 ± 5.5	129.6 ± 8.0	0.08	115.2 ± 5.5	117.1 ± 5.4	0.81	0.26
Tau (ms)	2.8 ± 0.2	2.4 ± 0.2	0.20	2.3 ± 0.1	2.1 ± 0.1	0.43	0.26

Presting membrane potential (V_m), input resistance (Ri), Membrane capacitance (Cm), and Membrane time constant (Tau) were measured using Mutliclamp (Axon instruments) while cells are voltage-clamped at -70 mV. Significant differences were found in membrane capacitance between the two genotypes of normally reared young and adult mice, and in input resistance of normally reared young mice. These differences may indicate smaller cell size of Ube3A$^{m-/p+}$ mice.

Chapter 4

Implications and Future Directions

In the studies presented in this thesis, using the visual cortex as a model system, I have revealed important attributes of how sensory experience affects the brain. In the first study, I have shown that 10 days of visual deprivation increases extrasynaptic NR2B-containing NMDA receptors (NMDARs) in the visual cortex. The significance of this finding is two fold. First, this study shows a high degree of experience-dependent plasticity in adults at a molecular level for the first time. Second, this study may provide a molecular mechanism for visual deprivation-enabled synaptic plasticity in adults (He et al., 2006; He et al., 2007). These two points will be discussed in light of recent findings of experience-dependent plasticity and its recovery in adult animals.

In the second study, I showed that visual experience acts to attenuate synaptic plasticity in the visual cortex and, therefore, fails to modify synapses in the mouse model of Angelman syndrome (AS). In this section, first, I will have an extended discussion of our findings. Second, I will discuss hypothetical molecular mechanisms by which Ube3A controls experience-dependent development of excitatory synapses. Third, I will speculate on a possible role of Ube3A in controlling inhibitory synaptic development. Fourth, I will propose experiments to elucidate roles of Ube3A in sensory system development *in vivo*. Finally, I will argue for potential applications of my experimental approach involving sensory deprivation for elucidating the pathophysiology of other neurodevelopmental disorders such as Rett syndrome, Fragile X syndrome, and autism.

4.1. Experience-dependent synaptic modifications in adults

4.1.1. Plasticity of adult brains

Traditionally, it was thought that synaptic connections in the cortex of adult animals are much more stable than those of young animals. Pioneering efforts studying the visual cortex of cats have shown that monocular deprivation (MD) induces ocular dominance shift only in the restricted period of juvenile life called the critical period (Hubel and Wiesel, 1970). The presence of the critical period is evolutionally conserved, because it was also demonstrated in rodents. For example, the initial study using single unit recordings has shown that 4 days of MD induces appreciable ocular dominance shift in mice only at the fourth postnatal week (Gordon and Stryker, 1996). A later study using the same recording technique confirmed the existence of a clear critical period and showed the lack of ocular dominance shift even after 15 days of MD in adult mice (Fagiolini and Hensch, 2000). Moreover, a recent study, using endogenous flavoprotein fluorescence as a marker for cellular metabolic activity, also show a clear loss of ocular dominance shift in adult mice (Tohmi et al., 2006). These studies have led to the idea that, whereas synaptic connections are modifiable in young animals, they are largely rigid in adult animals.

Contrary to the traditional view, however, many recent studies find that cortical synapses of adult mice possess a high degree of plasticity (Hooks and Chen, 2007). Using recordings of visually evoked potentials (VEPs), it has been shown that 5 days of MD induces a clear ocular dominance shift in adult mice (Sawtell et al., 2003). Another independent study using imaging of intrinsic activity also found a large ocular dominance

shift after 5 days of MD in adult rodents (Hofer et al., 2006). Moreover, a recent study using the single unit recording technique has detected surprisingly rapid changes after one day of MD in adult mice (Fischer et al., 2007). Furthermore, Tagawa et al. reported similar degrees of ocular dominance shift before, during, and after the classically defined critical period (Tagawa et al., 2005). In this study, mice were monocularly deprived for 4 days by enucleating one eye. Then, the mice were provided with 30 minutes of monocular vision followed by 24 hours dark-adaptation and ocular dominance was measured by expression of Arc, an immediate early gene, in the visual cortex. It is difficult to find out the causes of the discrepancy in the degree of ocular dominance shift in adult rodents between earlier and recent studies, but new imaging and recording techniques seem to reveal a higher degree of plasticity than previously recognized in adult rodents.

The ocular dominance shift is not the only form of visual experience-induced synaptic modifications observed in adults. A recent study by Goel and Lee have shown that synaptic scaling occurs in adult visual cortex (2007). Synaptic scaling describes a slow and global change in synaptic strength in response to neuronal activity and often is expressed as a global increase in miniature excitatory postsynaptic current (mEPSC) amplitude. Earlier studies found that, in the visual cortex of young rodents, binocular deprivation achieved by dark-rearing or monocular inactivation can increase the average mEPSC amplitude in a multiplicative manner (Desai et al., 2002a; Goel et al., 2006). Dark-rearing induced similar degree of synaptic scaling in adults, but the multiplicative nature of the scaling is lost (Goel and Lee, 2007). Therefore, in adults, only a subset of

synaptic population may undergo synaptic scaling, presumably due to the reduced number of motile spines in adults (Grutzendler et al., 2002).

My study adds one more dimension to the visual experience-driven synaptic modifications. I showed that 10 days of visual experience increases extrasynaptic, but not synaptic, NR2B-containing NMDARs (Yashiro et al., 2005). This is the first evidence for sensory experience-dependent synaptic modifications in adults at the molecular level. Later, this finding was partly reproduced by another group, which showed that visual deprivation increases the NR2B/NR2A ratio in a crude synaptic fraction (He et al., 2006). This study further showed that visual deprivation changes subunit composition of $GABA_A$ receptors ($GABA_ARs$), suggesting that visual deprivation may induce a large change in the molecular organization of synaptic proteins in adults akin to that observed in young rodents (Tropea et al., 2006).

All together, recent studies provide evidence for a high degree of plasticity in adult rodents both at physiological and molecular levels. However, it is unknown if these findings can be extended to other animals such as cats and primates. Previous studies using cats report a severe loss of ocular dominance plasticity after the critical period (Hubel and Wiesel, 1970; Wiesel and Hubel, 1963). Classical studies in primates find that reversal of MD (e.g. reverse lid suture) does not recover the MD-induced loss of vision in adults (Hubel et al., 1977), suggesting the more rigid nature of visual circuits in adult primates. However, unlike what was suggested in the primate visual cortex, several studies find a high degree of synaptic plasticity in the somatosensory cortex of adult animals. For example, in the

somatosensory cortex of primates, silencing the principal input to a region causes a potentiation of the initially weak inputs from other regions of the body surface (Diamond et al., 1993; Fox, 2002; Merzenich et al., 1984). In addition, single-digit amputation in adult raccoons leads to transiently increase AMPARs and $GABA_A$Rs at the deafferented somatosensory cortex (He et al., 2004). Future studies should re-examine experience-dependent synaptic modifications in the visual cortex of adult cats and primates to detect extant plasticity in these adult model systems.

4.1.2. Reinstating synaptic plasticity in adult brains

Although a number of studies find a high degree of synaptic plasticity in adult animals, it is true that plasticity in adults is less robust than that observed in young animals. Recent studies, however, show that it is possible to restore plasticity in adult animals to the level of young animals. One such procedure is dark-rearing, which I have examined in this thesis. Other procedures include genetic, pharmacological/enzymatic, and environmental manipulations. These manipulations have high therapeutic potentials for neurological disorders, such as amblyopia, that require rearrangements of neuronal networks in the adult brains. I describe such manipulations in this section.

Genetic manipulations to enhance ocular dominance plasticity in adults

There are two genetic manipulations shown to enhance ocular dominance plasticity. One is the gene deletion of Nogo receptor that is involved in myelination of axonal fibers (McGee et al., 2005). Maturation of intracortical myelination correlates with the end of the critical period. Suppressing myelination of cortical axonal fibers by a gene deletion of

Nogo receptor prevents critical period closure and maintains high ocular dominance plasticity into adulthood. The second example is a gene deletion of Paired-immunoglobulin-like receptor B (PirB), a major histocompatibility complex class I (MHCI) receptor (Syken et al., 2006). These mutant mice reveal higher ocular dominance plasticity throughout life than wildtype controls suggesting that PirB-MHCI interaction stabilizes synapses by limiting their plasticity.

Pharmacological/enzymological manipulations to enhance ocular dominance plasticity in adults

Pharmacological/enzymatic manipulations targeting diverse cellular processes are also effective in reinstating plasticity. Chondroitin sulphate proteoglycans (CSPGs), which are a major component of the extracellular matrix (ECM), prevent axonal sprouting. CSPGs establish perineuronal nets around soma and dendrites in a visual experience-dependent manner, and the timing of the establishment of these nets roughly coincides with the end of the critical period (Pizzorusso et al., 2002). Chondroitinase-ABC, which disrupts CSPGs, restores plasticity in adult rodents (Pizzorusso et al., 2002). Therefore, by giving some flexibility to synapses by "loosening" the ECM, critical period plasticity can be restored.

Interestingly, the critical period plasticity can also be reinstated by generally altering gene expression. Closure of the critical period is shown to be associated with a decrease in visual experience-driven changes in histone phosphorylation and acetylation that control gene transcription. Trichostatin, which blocks histone deacetylation, promotes ocular dominance plasticity in the adult visual cortex (Putignano et al., 2007).

Environmental manipulations to enhance ocular dominance plasticity in adults

Two environmental manipulations are shown to enhance ocular dominance plasticity in adults. First, as described above, 10 days of dark rearing is shown to promote ocular dominance plasticity in adult rodents (He et al., 2006). Interestingly, the same group recently reported that dark-rearing also promotes visual acuity in an amblyopic eye in adults (He et al., 2007). That is, 10 days of dark-rearing facilitated recovery of visual acuity in chronically-deprived eye (P13-100) in adults enabling strengthening of the previously weakened inputs. Intriguingly, a seemingly opposite manipulation to deprivation also promotes plasticity in adults. Sale and colleagues showed that environmental enrichment in adult amblyopic rats reinstated normal visual acuity and ocular dominance (Sale et al., 2007). Those two distinct manipulations may promote cortical plasticity by a similar mechanism in adults, because they are shown to reduce cortical inhibition (He et al., 2006; Sale et al., 2007).

What do these findings suggest collectively? First, it seems that diverse mechanisms including myelination, formation of extracellular matrix, and suppression of gene expression, are required for the closure of the critical period. Second, it appears that recovery of one of the mechanisms is sufficient to reinstate the critical period plasticity, although it cannot be ruled out that removal of one inhibitory constraint on plasticity leads to changes in the other inhibitory constraints. Therefore, these studies, including the one presented in this thesis, suggest that synaptic plasticity in adults can be reinstated with relatively straightforward manipulations, raising the exciting possibility that genetic,

pharmacological, and environmental manipulations can be developed in the near future to regain plasticity in adult humans.

4.2. Lack of sensory experience-dependent synaptic maturations in Angelman syndrome

4.2.1. Roles of Ube3A in the experience-dependent synaptic development

In the current study, we revealed an essential role for Ube3A in sensory experience-dependent synaptic development in the neocortex. The primary findings in this study are four fold: 1) Ube3A expression is maternally imprinted in the visual cortex; 2) Visual experience fails to mature neuronal circuits in the visual cortex of $Ube3A^{m-/p+}$ mice; 3) Both LTD and LTP are attenuated in the visual cortex of $Ube3A^{m-/p+}$ mice; and 4) Early sensory experiences drive the loss of bidirectional synaptic plasticity in $Ube3A^{m-/p+}$ mice. From these observations, we speculate that initial visual experience suppresses synaptic plasticity in the visual cortex of $Ube3A^{m-/p+}$ mice and, because of this suppression, visual experience is unable to properly sculpt excitatory synaptic connections in the visual cortex (Fig. 4-1).

We demonstrated that a developmental increase in spontaneous synaptic activity, normally occurring in visual cortical pyramidal neurons in WT mice (present study; (Desai et al., 2002b; Goel and Lee, 2007), fails to occur in the absence of Ube3A. We hypothesized that Ube3A is required for the experience-dependent maturation of synapses. Consistent with this hypothesis, mEPSC frequency was similar in WT and Ube3A-deficient mice reared in

complete darkness, yet Ube3A$^{m-/p+}$ mice failed to achieve the increase in mEPSC seen in normally-reared wildtype mice. Thus, Ube3A-deficient mice appear to lack the sensory experience-dependent component of synaptic maturation. The reduction in mEPSC frequency in the absence of Ube3A could be a consequence of either a reduced probability of glutamate release or a reduced number of functional synapses. We favor the idea that synapse number is reduced in Ube3A-deficient mice, as this is consistent with the observations that spine density in basal dendrites of layer 2/3 pyramidal neurons is reduced in the absence of experience-dependent maturation (Valverde, 1967; Wallace and Bear, 2004) and, in the only study to date, that dendritic spine density appears to be reduced in human visual cortex of an Angelman syndrome patient (Jay et al., 1991). In contrast to other studies, we failed to observe a deprivation-induced synaptic scaling of mEPSC amplitude in WT mice (Desai et al., 2002b; Goel and Lee, 2007), and this apparent discrepancy may be attributed to differences in the time of onset or the duration of visual deprivation or to differences between species/strains.

We found that spontaneous glutamatergic activity in Ube3A-deficient mice aged ~P25 or ~P100 was about 60-70 % of that in WT mice. While this observation might appear at odds with the fact that both AS patients and Ube3A$^{m-/p+}$ mice are prone to seizures, such an observation is not without precedent. For example, in Rett syndrome, which is a neurodevelopmental disorder characterized by mental retardation with Autistic features (Moretti and Zoghbi, 2006), seizure is common, although its mouse model also exhibits reduced glutamatergic drive (Dani et al., 2005). Moreover, we cannot preclude the possibilities that the absence of Ube3A could greatly diminish inhibition, enhance evoked

recurrent excitation, cause circuit miswiring, or alter another factor that would increase seizure susceptibility despite reductions in spontaneous glutamate activity.

Our finding that the absence of Ube3A prevents experience-dependent maturation of visual cortex circuitry does not imply that visual abilities are lost. Ube3A-deficient mice are clearly able to recognize visual cues, given that they can learn the Morris water maze task which relies on visual recognition (van Woerden et al., 2007). Because a large component of visual wiring does not require visual experience (Crowley and Katz, 2000), a loss of experience-dependent refinement of visual cortex would not be expected to cause complete blindness but could account for the assorted visual problems reported in AS (Thompson et al., 1999; Van Splunder et al., 2003). A possible basis for the lack of experience-dependent synaptic development is that visual cortical synapses lack an ability to undergo activity-dependent modifications. This hypothesis is strengthened by the previous observations showing that LTP is attenuated in the hippocampus of $Ube3A^{m-/p+}$ mice and strong learning deficits are observed in both AS patients (Laan et al., 1999) and $Ube3A^{m-/p+}$ mice (Jiang et al., 1998; van Woerden et al., 2007). Given our observations that bidirectional synaptic plasticity, both LTP and LTD, are impaired in $Ube3A^{m-/p+}$ mice, we predict that the absence of Ube3A would attenuate the dramatic experience-dependent ocular dominance shifts normally observed after monocular deprivation.

The loss of bidirectional synaptic plasticity in the absence of Ube3A is expected to have severe consequences. Cortical synapses undergo use-dependent modifications, which are believed to be required for forming long-term memories (Frankland et al., 2001), adjusting

synaptic networks to changes in the environment, and establishing functional neuronal circuits during development (Sur and Rubenstein, 2005). Normal sensory abilities frequently fail to develop in animal models that lack normal synaptic plasticity. For example, mice lacking NR2A, one of the regulatory subunits of NMDA-type glutamate NMDA receptors, exhibit a low and fixed threshold for LTP induction (Philpot et al., 2007), and these mice fail to develop orientation selectivity (Fagiolini et al., 2003). Moreover, mice over-expressing brain-derived neurotrophic factor have an accelerated loss of thalamocortical LTP, and these mice have a premature onset and termination of their critical period for ocular dominance plasticity compared to WT mice (Huang et al., 1999). These examples suggest that normal LTP and LTD are associated with the proper development of visual cortical circuits.

While a deficit of LTP was observed early in development in Ube3A$^{m-/p+}$ mice, the severity of the LTP deficit appeared to increase with age (compare Fig. 3-3 and Supplemental figure 3-2). That is, weak stimulation (40 Hz) failed to induce LTP in either young or adult mice lacking Ube3A, and a stronger (100 Hz) induction protocol could rescue LTP induction only in younger mice. Thus, the absence of Ube3A increases the threshold for inducing LTP in the visual cortex, consistent with previous observations in the hippocampus (Weeber et al., 2003). Our novel finding that there is a progressive loss of LTP in the visual cortex of Ube3A$^{m-/p+}$ mice may be a feature generalizable to other areas of the brain, as we also observed for the first time that the hippocampus exhibits an age-dependent loss of LTP in the absence of Ube3A(Supplemental figure 3-3).

What might account for the loss of cortical LTP and the progressive nature of the LTP deficit in Ube3A$^{m-/p+}$ mice? One proposed mechanism for the hippocampal LTP attenuation is reduced activity of calcium/calmodulin-dependent protein kinase II (CaMKII) (Weeber et al., 2003), which activates with LTP induction and stimulates downstream proteins to induce LTP (Xia and Storm, 2005). CaMKII activity in the hippocampus is reduced because its inhibitory phosphorylation is elevated (Weeber et al., 2003). Consistent with an important role for CaMKII in LTP induction, genetic deletion of CaMKII attenuate LTP both in the hippocampus (Silva et al., 1992) and in the visual cortex (Frankland et al., 2001; Kirkwood et al., 1997). It is possible that cortical LTP is suppressed because of the reduced CaMKII activity in the visual cortex. Intriguingly, CaMKII knockout mice show a developmental decline in LTP (Kirkwood et al., 1997). Thus a parsimonious explanation for the developmental decline in LTP induction in Ube3A-deficient mice is that this arises due to an increasing requirement of CaMKII for LTP induction (Yasuda et al., 2003).

We are the first to demonstrate that LTD is severely attenuated in Ube3A-deficient mice. This may suggest that cortically-based deficits in experience-dependent learning might equally arise from a deficit in synaptic weakening as from a deficit in synaptic strengthening. What is the mechanism for this loss of LTD? Activation of calcineurin is required for LTD induction in the visual cortex (Torii et al., 1995), and function of protein phosphatases such as PP1 is attenuated in the hippocampus of Ube3A$^{m-/p+}$ mice (Weeber et al., 2003). Since protein phosphatase 1 (PP1) forms a cascade to induce LTD with calcineurin (Mulkey et al., 1994) to induce LTD, the loss of cortical LTD in Ube3A$^{m-/p+}$

mice could be due to the attenuated PP1 activity in the visual cortex. An alternative explanation for the loss of bidirectional synaptic plasticity in Ube3A-deficient mice is that Ube3A has a direct role in the induction of both LTD and LTP. Given that the proteasome system is necessary for both LTP (Fonseca et al., 2006) and LTD (Colledge et al., 2003), it is tempting to speculate that ubiquitination of proteins by Ube3A may occur upon synaptic stimulation and be required for LTP and LTD induction.

We were surprised to find that bidirectional synaptic plasticity can be recovered in the visual cortex of Ube3A-deficient mice by raising mice in complete darkness. To our knowledge, this is the first study reporting that sensory experiences can be a cue to inappropriately drive down synaptic plasticity in genetically-modified mice. Our findings are consistent with recent observations showing that visual deprivation can reinstate forms of plasticity that are normally lost with development (He et al., 2006; He et al., 2007). What is the mechanism behind the unique observation that sensory deprivation can restore synaptic plasticity in Ube3A-deficient mice? Extensive GeneChip analyses of the murine visual cortex have revealed that visual experience affects the expression of many genes (Tropea et al., 2006). If one of these visual experience-induced proteins is a substrate for Ube3A, this protein would be enriched in $Ube3A^{m-/p+}$ mice given visual experience. Furthermore, if the protein has an ability to suppress synaptic plasticity, experience-dependent accumulation of such a protein would attenuate LTD and LTP in the absence of Ube3A (modeled in Fig. 4-1).

In conclusion, by using a traditional experimental paradigm of cortical development, we provide strong evidence for the absence of sensory experience-dependent brain development in the mouse model of AS. These observations suggest that sensory experience-dependent synaptic development may similarly fail to occur in human AS patients. Given our findings that Ube3A is necessary to maintain synaptic plasticity in the face of ongoing sensory experience-driven activity, early sensory experiences in AS patients may provide the signal to drive down cortical plasticity and limit further experience-dependent brain development (Fig. 4-1). Because it has been speculated that sensory experience-dependent brain development is abnormal in other neurodevelopmental disorders such as Rett syndrome, Fragile X syndrome, and Autism (Zoghbi, 2003) in addition to Angelman syndrome, our experimental approach may help to elucidate roles of experience in these disorders.

4.2.2. Potential substrates for Ube3A involved in Angelman syndrome

Involvement of Ube3A in AS was demonstrated more than 10 years ago (Sutcliffe et al., 1997). However, Ube3A substrates involved in the disease expression are unknown. Ube3A, also called as E6-AP, was initially identified as an E3 ubiquitin ligase that leads to the ubiquitin-dependent degradation of p53, when it is bound by a human papillomavirus E6 protein (Huibregtse et al., 1991; Scheffner et al., 1993; Scheffner et al., 1990). Later, several proteins, such as Bak, c-Myc, Mcm7, and Scrib, were identified to be substrates of the Ube3A-E6 complex (Mantovani and Banks, 2001; Scheffner and Whitaker, 2003).

Ube3A can function as an E3 ubiquitin ligase even without E6 binding, suggesting that Ube3A is involved in normal cellular processes (Scheffner and Whitaker, 2003). Several E6-independent substrates of E6-AP have been reported, including the human homologs of *Saccharomyces cerevisiae* RAD23, HHR23A, and HHR23B (Kumar et al., 1999), a member of the Src-family tyrosine kinases, Blk (Oda et al., 1999), a human proapoptotic protein, Bak (Thomas and Banks, 1998), and Mcm7, which is involved in DNA replication (Kuhne and Banks, 1998). None of these proteins, however, is shown to date to be involved in the brain development and function. Moreover, whether they are involved in AS has not yet been examined.

There are some proteins reported to form a complex with Ube3A. Those proteins were discovered by pull-down assays. Although whether they can be ubiquitinated by Ube3A has not been tested, some of these Ube3A-associating proteins have been implicated in synaptic development and plasticity. For example, Src is shown to interact with Ube3A in GST-pull-down experiments (Oda et al., 1999). Src regulates the conductance of NMDARs altering the properties of synaptic plasticity (Yu et al., 1997). Therefore, a loss of regulation of Src by Ube3A may have adverse effects on activity-dependent synaptic development.

Another interesting Ube3A interacting molecule is a ubiquitin-like protein, Plic-1. Plic-1 associates with Ube3A and the proteasome, and prevents degradation of p53, a well-known Ube3A substrate (Kleijnen et al., 2003). Plic-1 also binds many $GABA_AR$ subunits and prevents proteasome-dependent degradation of the subunits (Bedford et al., 2001).

Therefore, the findings from two independent groups provide a molecular link between Ube3A to $GABA_A$Rs (Dan and Boyd, 2003). Thus, it can be speculated that $GABA_A$R subunits are Ube3A substrates and Plic-1 regulates proteasome-dependent degradation of $GABA_A$R subunits. Hence, loss of Ube3A may disrupt brain function by taking out one layer of regulation of $GABA_A$R. In fact, a number of studies suggest that inhibition is altered in AS, as discussed in the next section.

The other intriguing Ube3A interacting molecule is myc binding protein 2 (MYCBP2), which is identified in a protein complex immunoprecipitated from a HeLa cell culture with an Ube3A antibody (Jung et al., 2005). MYCBP2 is a mammalian homologue of the *Drosophila* protein highwire and *C.elegans* protein called regulator of presynaptic morphology (RPM-1). Highwire/RPM-1 is a RING-domain containing ubiquitin E3 ligase. Highwire/RPM-1 localizes at presynapses and negatively regulates synaptic formation. In a *Drosophila* highwire mutant, neuromuscular junction synapses grow exuberantly and are greatly expanded in both the number of boutons and the extent and length of branches (Wan et al., 2000). Moreover, highwire is shown to control structural plasticity of the synapses by regulating gene expression through a MAP kinase signaling pathway (Collins et al., 2006). Therefore, if MYCBP2 is regulated by Ube3A, the loss of Ube3A can result in dysregulation of MYCBP2-mediated synaptic modifications.

As I discussed, there are number of probable Ube3A substrate proteins that may be involved in AS. Future molecular biological studies will reveal the molecular pathway by

which the loss of Ube3A induces brain dysfunction. Such studies will help to identify targets for drug development and treatment of AS.

4.2.3. Development of inhibitory connections in Ube3A$^{m-/p+}$ mice

The current study focused on sensory experience-dependent development in excitatory connections in AS. However, there are clinical indications that inhibitory neurotransmission might also be altered in AS (Dan and Boyd, 2003). In fact, one of the clinical diagnostic criteria of AS is rhythmic EEG patterns (Williams et al., 1995) that depend on activation of thalamic $GABA_A$Rs (Blumenfeld and McCormick, 2000; Staak and Pape, 2001). In addition, lamotrigine, which blocks voltage-gated sodium channels but also is known to increase *GABAB3* (β subunit of $GABA_A$R) gene expression *in vivo* (Gibbs et al., 2002), has been used as an anticonvulsant for AS (Wang et al., 2002). These clinical observations suggest that some AS symptoms are caused by altered inhibition, and thus Ube3A may regulate $GABA_A$Rs. There is, however, a major caveat in the human AS studies. Because the 15q11-q13 region contains genes not only for Ube3A but also for α5, β3, and γ3 subunits of $GABA_A$R, AS patients often lack one copy of the genes encoding $GABA_A$R subunits. Although expression of these subunits are not imprinted, the thalamocortical defect might result from a haploinsufficiency of any of the subunits (Handforth et al., 2005). This interpretation, however, seems unlikely, because, whereas Ube3A$^{m-/p+}$ mice exhibit rhythmic EEG patterns (Jiang et al., 1998), GABAB3 knockout (-/-) mice do not (DeLorey et al., 1998). Therefore, Ube3A might have direct roles in the development of not only excitatory circuits but also inhibitory circuits.

It is well known that sensory experience contributes to the development of inhibitory connections (Hensch, 2005; Jiang et al., 2005). During early postnatal development, miniature inhibitory postsynaptic current frequency measured in layer 2/3 pyramidal neurons increases in a visual experience-dependent manner (Morales et al., 2002). Moreover, there is good histological evidence suggesting that visual experience facilitates perisomatic innervations of inhibitory synapses onto pyramidal cells (Chattopadhyaya et al., 2004). Because Ube3A is expressed in inhibitory neurons (K.Y. unpublished data), it is likely that Ube3A is involved in activity-dependent development of not only excitatory synapses but also inhibitory synapses. Whether Ube3A is involved in activity-dependent inhibitory synaptic maturation can be elucidated by comparing mISPCs in normally- or dark-reared WT and Ube3A$^{m-/p+}$ mice.

4.2.4. *In vivo* visual cortical plasticity

We have shown that sensory experience fails to mature excitatory synaptic connections in Ube3A$^{m-/p+}$ mice, because both LTD and LTP are attenuated in Ube3A$^{m-/p+}$ mice in a sensory experience-dependent manner. These findings raise the question of whether sensory experience also fails to shape visual cortex functions and whether synaptic plasticity is attenuated *in vivo*. The former question can be asked by measuring visual acuity that develops with visual experience. On the other hand, the latter can be answered by measuring ocular dominance plasticity.

Visual acuity

Visual acuity of rodents is traditionally measured by recording visually-evoked responses in the primary visual cortex in response to visual stimulations with different spatial

frequencies (Gordon and Stryker, 1996). Visual acuity develops as animals grow. For example, 4-week-old mice exhibit less than a half the visual acuity of 5-week-old mice (Huang et al., 1999). The development of visual acuity depends on visual experience (Gianfranceschi et al., 2003). Because Ube3A$^{m-/p+}$ mice fail to develop excitatory synaptic connections in a visual experience-dependent manner, it is intriguing to see if they fail to develop visual acuity. This hypothesis is supported by a human study finding that visual acuity of AS patients is low (Van Splunder et al., 2003).

Ocular dominance plasticity

One well-studied *in vivo* paradigm of synaptic plasticity is the ocular dominance shift observed in the primary visual cortex. Classical studies suggested that closure of one eye by eyelid suturing results in the loss of responsiveness to the closed eye and an increase of responsiveness from the intact eye (Frenkel and Bear, 2004; Wiesel and Hubel, 1963). This ocular dominance plasticity is highest only in a particular period of life termed the critical period. It is hypothesized that ocular dominance plasticity shares common mechanisms with LTD and LTP (Bear, 2003; Heynen et al., 2003; Taha et al., 2002) (but also see (Hensch, 2005)). Therefore, I hypothesize that Ube3A$^{m-/p+}$ mice, which have severe loss in both LTD and LTP, may show reduced ocular dominance plasticity. Moreover, ocular dominance plasticity may recover with dark-rearing like LTD and LTP in Ube3A$^{m-/p+}$ mice. These experiments would demonstrate the extent to which Ube3A contributes to synaptic plasticity *in vivo*.

4.2.4. Elucidating mechanisms of neurodevelopmental disorders in the primary sensory cortex

By using the visual cortex as a model, we have found that sensory experience acts to diminish synaptic plasticity in the mouse model of AS. This study has proved the usefulness of the sensory deprivation paradigm to elucidate disease expression mechanisms of a neurodevelopmental disorder. Here, I will discuss possible applications of this visual deprivation paradigm for three other well-investigated neurodevelopmental disorders: Fragile X syndrome, Rett syndrome, and autism.

Fragile X syndrome

Fragile X syndrome is the most frequent form of a heritable mental retardation and the leading recognized cause of autism (Penagarikano et al., 2007). Fragile X syndrome is caused by transcriptional silencing of the FMR1 gene, which encodes the fragile X mental retardation protein (FMRP). This silencing results from the expansion of a CGG repeat sequence in the 5'-untranslated region of the FMR1 gene. FMRP can function as a repressor of mRNA translation at synapses (Brown et al., 2001). Therefore, in the absence of FMRP, protein synthesis of certain gene targets is likely increased.

Roles of FMRP in synaptic development have been well-studied in the somatosensory and visual cortices. A number of reports suggest that FMRP is required for synaptic elimination that normally occurs followed by a sharp increase in synapse numbers after birth (Grutzendler et al., 2002; Rakic et al., 1986; Zuo et al., 2005a). For example, in the somatosensory cortex of FMRP knockout mice, dendritic spine numbers do not decrease

from P25 to P76 and maintain a high rate of filopodia-like immature spines (Galvez and Greenough, 2005). Moreover, in the visual cortex of FMRP knockout mice at P25-30, the number of spines is reported to be higher than that in age-matched wildtype mice (Dolen et al., 2007; Irwin et al., 2002). These observations suggest that FMRP is involved in spine elimination that occurs in an experience-dependent manner (Zuo et al., 2005b). Because sensory stimulation is shown to facilitate FMRP expression (Todd and Mack, 2000), it is tempting to speculate that the spine elimination is mediated, in part, by experience-induced expression of FMRP. Such question can be easily addressed by comparing the number of synapses between normally- and dark-reared FMRP knockout mice, as I did so for the mouse model of AS.

The other well-known synaptic abnormality of FMRP knockout mice is enhanced metabotropic glutamate receptor (mGluR)-dependent LTD observed in hippocampus (Bear et al., 2004; Huber et al., 2002). Although mGluR-LTD has not been examined in the cortex of FMRP knockout mice, this enhancement is believed to contribute to the unusually rapid ocular dominance shift observed in FMRP knockout mice (Dolen et al., 2007). The enhancement of mGluR-LTD in FMRP knockout mice might occur only with visual experience, because FMRP expression increases with sensory experience (Todd and Mack, 2000) and, therefore, FMRP may limit mGluR-LTD in a sensory experience-dependent manner. This hypothesis can be tested by measuring mGluR-LTD in FMRP knockout and wildtype mice reared normally or in complete darkness.

Rett syndrome

Rett Syndrome (RTT) is an X-linked neurological disorder occurring almost exclusively in females (Rett, 1966). It is characterized by arrested development between 6 and 18 months of age, regression of acquired skills, loss of speech, stereotypical movements, microcephaly, seizures, and mental retardation (Chahrour and Zoghbi, 2007). The symptoms of RTT become apparent during early childhood, when sensory experience modifies neuronal circuits to establish functionally mature brain. Therefore, it is hypothesized that RTT phenotypes are caused by inappropriate synaptic connectivity resulting from abnormal experience-dependent synaptic development (Zoghbi, 2003). This hypothesis is further supported by the molecular function of the causal gene of RTT, methyl-CpG-binding protein, MeCP2 (Amir et al., 1999; Moretti and Zoghbi, 2006). MeCP2 binds to methylated DNA presumably functioning as a transcriptional repressor (Lewis et al., 1992). The DNA binding of MeCP2 is prevented by phosphorylation at serine 421 that is induced by synaptic activity and requires CaMKII activity (Zhou et al., 2006). The removal of MeCP2 from DNA allows expression of activity-regulated genes such as brain-derived neurotrophic factor (BDNF) that facilitates dendritic and synaptic development. Therefore, MeCP2 is thought to mediate activity-dependent neuronal development and RTT symptoms are likely to result from a lack of experience-dependent synaptic modifications.

Consistent with this view, abnormality in neuronal circuits, which may arise from distorted activity-dependent development, has been reported in MeCP2 knockout mice. In the somatosensory cortex of MeCP2 knockout mice, while inhibition is strengthened,

excitation is weakened compared to littermate wildtypes (Dani et al., 2005). Moreover, in hippocampal cultures established from MeCP2 knockout or duplicated mice, the number of functional glutamatergic synapses is reduced to half or doubled, respectively (Chao et al., 2007). Therefore, it can be speculated that MeCP2 is required for the normal activity-dependent establishment of excitatory circuits. Once again, such hypotheses can be readily tested by comparing visual experience-induced synaptic development between MeCP2 knockout and wildtype mice.

Autism

Autism is characterized by impairments in social interactions, including verbal communication and social play, and can be accompanied by stereotyped patterns of behaviors. Because the ability for social interactions is only acquired with experience, autism is thought to result from a failure in experience-dependent development of neuronal circuits governing social activity (Zoghbi, 2003). The social interaction is complicated process that is likely to involve many brain regions including primary sensory areas of the cortex. Therefore, it is reasonable to investigate sensory experience-dependent synaptic modifications in mouse models of autism to find out disease expression mechanisms.

Recently, Tabuchi et. al. developed a new autism model mouse, which carries a knock-in mutation in neuroligin-3 gene (Tabuchi et al., 2007). This mutation is an $Arg^{451} \rightarrow Cys^{451}$ (R451C) substitution that is found in a subset of autism spectrum disorder patients (Jamain et al., 2003). Neuroligins are postsynaptic cell-adhesion molecules that function as ligands for the neurexin family of cell surface receptors. They control the formation and

functional balance of excitatory and inhibitory synapses (Chih et al., 2005). In the somatosensory cortex of neuroligin-3 R451C mutant mice, although excitatory synaptic connections are not altered, inhibitory synaptic connections are strengthened compared to littermate wildtypes (Tabuchi et al., 2007). Because the strengthening of inhibitory synapses was observed at P13-16 when experience-dependent circuit maturation has already occurred (Fox and Wong, 2005), it is tempting to speculate that sensory experience may guide to form unusually strong inhibitory synaptic connections because of the mutation in neuroligin 3. Such hypotheses can be tested by examining the effects of visual experience on inhibitory synaptic connections in the mutant mice.

Collectively, these neurodevelopmental disorders may share an inability to encode experience-dependent synaptic development. Therefore, genes causing these disorders may normally mediate activity-dependent synaptic development and may normally interact with one another. In fact, although controversial (Jordan and Francke, 2006), it has been shown that Ube3A expression is reduced in MeCP2-deficient brain of both human and mouse (Samaco et al., 2005). This reduction is thought to be due to elevated Ube3A antisense expression caused by an epigenetic aberration at AS imprinting center (Makedonski et al., 2005). Moreover, it has been demonstrated that Ube3A expression is altered in autistic patients (Jiang et al., 2004). Furthermore, gene duplication of the maternal chromosome 15q11-13, which includes the *UBE3A* gene, is found in autism (Schroer et al., 1998). Finally, FMRP is shown to be a target of proteasomal degradation (Hou et al., 2006), raising a possibility that FMRP may be a substrate for Ube3A. In conclusion, it is very tempting to speculate that these genes are all in an interconnected

pathway that regulates experience-dependent synaptic development. Future studies are needed to elucidate this possibility.

4.3. Closing remark

One common theme spanning through my two thesis projects is recovering synaptic plasticity by sensory deprivation. In the first project, I showed in adult mice that 10 days of sensory deprivation increases extrasynaptic NR2B-containg NMDARs, which is likely a major contributing factor to reinstating rapid ocular dominance shift (He et al., 2006). In other words, I found that sensory experience suppresses extrasynaptic NR2B-containing NMDARs to restrain synaptic plasticity. In the second project, I have demonstrated that sensory deprivation recovers both LTD and LTP in the model mouse of AS. Thus, Ube3A may act to maintain synaptic plasticity in the face of experience-dependent synaptic remodeling by bringing plasticity-suppressing molecules to a proteasomal degradation. Therefore, these two studies reveal a new role and mechanisms of sensory experience to suppress synaptic plasticity and hence stabilize neuronal networks.

The effect of sensory experience to stabilize neuronal circuits is counterintuitive, because sensory experience is also known to modify neuronal circuits. However, if you think of the delayed closure of the critical period for ocular dominance plasticity in dark-reared animals (Mower, 1991a), it would be clear to you that sensory experience has an active role in eventually stabilizing neuronal circuits as well. It seems that neurons can sense the history of their experience-driven activity and suppress their plasticity after undergoing a

critical period experience-dependent refinements. I have revealed such mechanisms by reinstating synaptic plasticity in adult and diseased brain.

In the first project, I have shown that 10 days of dark-rearing increases extrasynaptic NR2B-containing NMDARs in the visual cortex of adult mice. Conversely, I have revealed that ongoing visual experience has an effect to suppress the level of extrasynaptic NR2B-containing NMDARs. Because extrasynaptic NR2B-containing NMDARs are shown to be important for LTD induction in adults (Massey et al., 2004), sensory experience may stabilize synaptic connections by suppressing LTD mediated by extrasynaptic NR2B-containing NMDARs. Moreover, it is tempting to speculate that the recovery of rapid ocular dominance shift involving both the depression of the closed eye response and the open eye potentiation is enabled by visual deprivation-induced extrasynaptic NR2B-containing NMDARs (He et al., 2006). Altogether, it can be hypothesized that one of the mechanisms for sensory experience to stabilize neuronal networks is to suppress extrasynaptic NR2B-containing NMDARs, thus reducing the ability to induce LTD. In this regard, it is tempting to speculate that experience-dependent reduction in extrasynaptic NR2B-containing NMDARs contributes to the termination of the critical period for ocular dominance plasticity. Such a possibility can be tested by carefully measuring extrasynaptic NR2B around the time when the critical period closes.

In the second project, I have shown that, whereas both LTD and LTP are severely attenuated in the visual cortex of the mouse model of AS, they are normally induced if these mutant mice are reared in complete darkness. Therefore, in the absence of Ube3A,

sensory experience drives down synaptic plasticity and Ube3A plays a role in maintaining synaptic plasticity during activity-dependent synaptic modifications. Given the function of Ube3A as an E3 ubiquitin ligase, it is tempting to speculate that one or more Ube3A substrate(s) has an activity to limit synaptic plasticity and, in the absence of Ube3A, it accumulates to abolish synaptic plasticity. Although none of the known Ube3A substrates is shown to limit synaptic plasticity, there are precedents for such a plasticity-limiting protein. For example, Paired-immunoglobulin-like receptor B (PirB), a major histocompatibility complex class I (MHCI) receptor is reported to limit ocular dominance plasticity throughout life in rodents (Syken et al., 2006). Therefore, the role of Ube3A may be to degrade a yet unidentified plasticity-limiting protein, such as PirB, whose expression is induced with sensory experience. Such a protein would normally serve to limit synaptic plasticity and stabilize neuronal networks activity-dependent manner. Therefore, Ube3A-dependent degradation of the protein may be a system, by which neurons maintain synaptic plasticity at a certain level in the face of sensory experience-induced synaptic modifications.

In conclusion, through the two independent projects, I have revealed fundamental mechanisms for sensory experience to maintain the integrity of neuronal circuits by suppressing extrasynaptic NR2B or by degrading a to-be-identified Ube3A substrate. A role of neuronal activity in synaptic stabilization is just beginning to be elucidated. For example, a recent study reports that mEPSC stabilizes synaptic function via tonic suppression of local dendritic protein synthesis (Sutton et al., 2006). A whole picture of

dynamic regulation of neuronal network by activity that both modifies and stabilizes is just beginning to emerge.

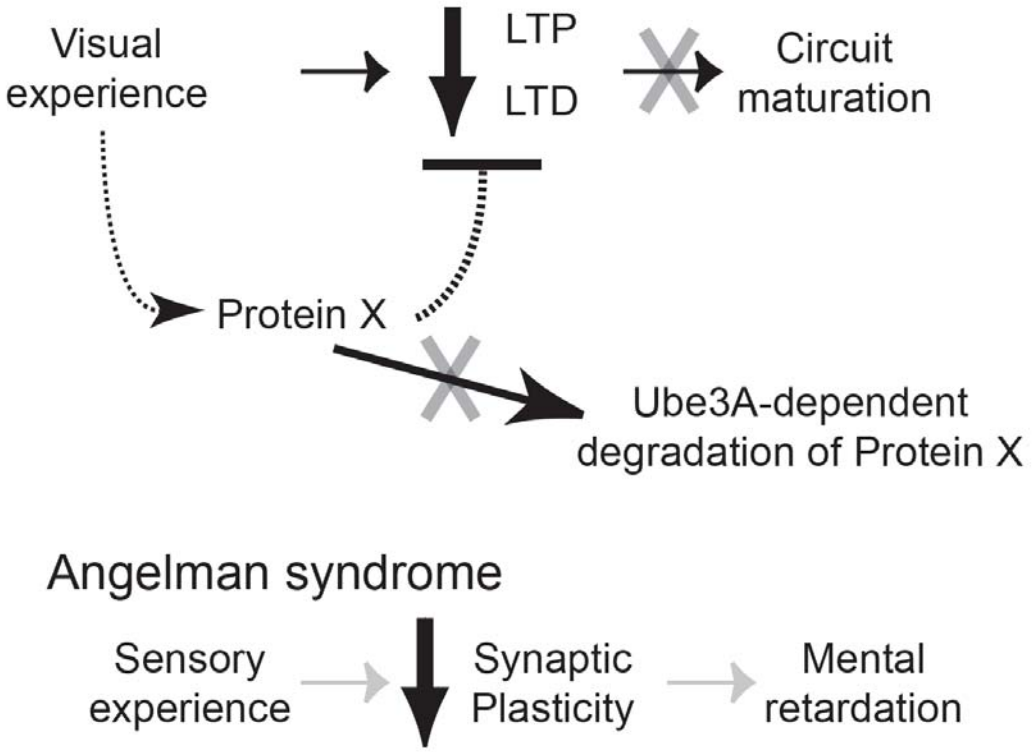


Figure 4-1. Hypothetical model for the abnormal neuronal circuit development in AS mice. **A**. In the visual cortices of AS mice, early visual experience acts to attenuate LTP and LTD in the visual cortex. Since synaptic plasticity is lost, neuronal circuits fail to develop normally. The loss of plasticity may be due to accumulation of Ube3A substrates, which are synthesized in an activity-dependent manner and act as suppressors of synaptic plasticity. **B**. In the brains of AS patients, early sensory experience similarly may suppress synaptic plasticity and therefore sensory-dependent neuronal circuit development, resulting in severe mental retardation.

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