Mechanisms of Ocular Dominance Plasticity in the Juvenile and Adult Mouse Visual Cortex

by

Lena A. Khibnik

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Signature of Author: _____
Department of Brain and Cognitive Sciences
January, 2011

Certified by: _____
Dr. Mark F. Bear
Picower Professor of Neuroscience
Thesis Supervisor

Accepted by: _____
Dr. Earl K. Miller
Picower Professor of Neuroscience
Director, BCS Graduate Program

"I know that I know nothing"

-Socrates

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Abstract

Ocular dominance (OD) plasticity is a classic example of bidirectional experience-dependent plasticity in the primary visual cortex. This form of plasticity is most robust during early postnatal development (termed the "critical period"), when monocular deprivation (MD) leads to a rapid weakening of responses evoked through the deprived eye followed by a delayed strengthening of non-deprived eye inputs. It has been proposed that these bidirectional changes occur as a three-stage process: first, degradation of patterned visual input weakens deprived-eye responses via homosynaptic long-term depression (LTD); this is accompanied by a shift in the plasticity modification threshold (θ_m) that determines the direction of synaptic plasticity, such that synaptic strengthening is favored over synaptic weakening; finally, weak open-eye responses are strengthened via the mechanisms of homosynaptic long-term potentiation (LTP). Despite the growing evidence supporting this model of experience-dependent synaptic modification, the exact molecular and synaptic mechanisms that are responsible for these processes remain controversial. In my thesis work, I address three questions. First, I attempt to parse the relative contribution of excitatory and inhibitory processes to expression of the OD shift in order to understand how deprived-eye depression is expressed in the cortex. To address this, I first induce a shift in OD with 3 days of MD and then use several pharmacological methods to shut off cortical inhibitory synaptic transmission. I demonstrate that rapid deprived-eye depression is strongly expressed at excitatory thalamocortical synapses without any influences of polysynaptic intracortical inhibition. In the second part of my work, I try to resolve the nature/identity of the molecular mechanism that underlies the regulation of θ_m. Using a transgenic mouse model, I find that a reduction in the NR2A/B subunit ratio of the N-methyl-d-aspartate (NMDA) receptor during MD alters the qualities of OD plasticity by impairing weakening of deprived-eye inputs and enhancing strengthening of open-eye inputs. These findings suggest that NMDAR subunit composition may specify the value and the rate of adjustment of synaptic θ_m, which in turn determines the bidirectional cortical response to MD. The final portion of my thesis addresses the factors that limit OD plasticity beyond the critical period. I test the hypothesis that the developmental increase in intracortical GABAergic inhibitory synaptic transmission is a fundamental restricting factor for adult cortical plasticity and demonstrate that parvalbumin-expressing fast-spiking basket cells are specifically implicated in the absence of juvenile-like deprived-eye depression in adult mice.

Thesis Supervisor: Mark F. Bear, Ph. D.
Title: Picower Professor of Neuroscience

Lena A. Khibnik

khibnikl@mit.edu

Education:

Massachusetts Institute of Technology **Doctor of Philosophy – June 2010**
Cambridge MA
Ph. D. in Neuroscience, Department Brain and Cognitive Sciences

Brandeis University **Bachelor of Science – May 2003**
Waltham, MA
B.S. in Neuroscience and Biology with High Honors, *Magna cum laude*

Research Experience:

September 2003 - Present: Graduate Studies, Dr. Mark Bear, MIT
Use in vivo electrophysiology and genetic manipulations to study experience-dependent developmental plasticity in the mouse visual cortex.

February 2002 – June 2003: Honors Thesis student, Dr. John Lisman, Brandeis University
Used slice electrophysiology in rodent hippocampus to establish a reliable protocol for induction of chemical long-term potentiation.

June 2001 – August 2001: Summer student, Dr. Roddy Williamson, Marine Biological Association of the UK, Plymouth, UK
Combined voltage-sensitive dyes and theoretical modeling to study visual processing in cephalopods; performed behavioral studies to study saccadic eye movements in cephalopods.

September 2000 – February 2002: Research Assistant, Dr. Jeff Hall, Brandeis University
Performed genetic and behavioral studies to dissect the neural circuit underlying courtship and reproductive behaviors in the fruit fly.

Teaching Experience:

Fall 2008: Museum of Science Initiative: Designed and implemented a hands-on neuroscience laboratory activity for high school students, Museum of Science, Boston, MA.

Fall 2006: Introduction to Neuroscience – Head Teaching Assistant, MIT.

Fall 2005: Introduction to Neuroscience – Teaching Assistant, MIT.

Fall 2004: Experimental Molecular Neuroscience – Teaching Assistant, Cellular Neurobiology (Lecture) MIT.

Awards:

Society for Neuroscience/Women in Neuroscience Graduate Student Travel Award, 2007
Singleton Fellowship, Department of Brain and Cognitive Sciences, MIT, 2005-2006
Walle Nauta Award for Continuing Dedication in Teaching, MIT, 2005
BCS Team Award for Outstanding Teaching, MIT 2004
Dean's List, Brandeis University, 2000-2004
Louis, Frances and Jeffery Sachar Travel Award, Brandeis University, 2001

Publications:

Khibnik, L.A., K.K.A. Cho and M.F. Bear. Relative contribution of feed-forward excitatory connections to expression of ocular dominance plasticity in layer 4 of visual cortex. **Neuron**., 66:493-500, May 27, 2010.

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* Indicates co-first authors.

Invited Talks:

EMBO Conference Series: The Assembly and Function of Neuronal Circuits. "Persistence of the Ocular Dominance Shift in the Absence of Cortical Inhibition." Ascona, Switzerland, 2009.

Picower Institute departmental seminar series. "Persistence of Ocular Dominance Shift in the Absence of Cortical Inhibition." MIT, Cambridge, MA, 2007.

Professional Associations:

BCS Brain Lunch Student Seminar Series, Coordinator, 2006-2007
BCS Women's Group, Member, 2003-present
Society for Neuroscience, Member, 2004-present

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Chapter 1

Mechanisms of bidirectional ocular dominance plasticity in the primary visual cortex

1.1 Introduction

Synaptic modifications are thought to form a substrate for experience-dependent plasticity in the brain, and elucidation of the mechanisms by which these modifications occur has been a very important problem in neuroscience. A preeminent model system for studying experience-dependent changes is the visual cortex because of the vast history of anatomical and physiological studies performed in this system. A classic model of experience-dependent plasticity in the primary visual cortex is ocular dominance (OD) plasticity. The relative response of a cortical neuron to stimulation of either eye is expressed as "ocular dominance" and the convergence of the retinotopically matched inputs onto common postsynaptic neurons in the primary visual cortex underlies binocular vision.

These binocular connections are capable of considerable functional and structural reorganization during normal development and following perturbations of an animal's visual experience. For example, degrading vision in one eye shifts the response properties of cortical neurons such that they cease to respond to stimulation of the deprived eye. Using acute extracellular (single-unit) recordings of individual cortical neurons in kittens, Hubel and Wiesel demonstrated that in animals with normal visual experience, approximately 80% of cortical neurons are binocular (Hubel and Wiesel, 1962). If one of the eyes is deprived of patterned vision by suturing the eyelid closed for a period of 2-3 months, a manipulation termed monocular deprivation (MD), responses to the deprived eye are dramatically reduced and the majority of neurons shift their responsiveness to the non-deprived eye (Wiesel and Hubel, 1963).

The effects of unbalanced visual experience are not limited to a functional loss of

responsiveness, as prolonged MD is accompanied by profound anatomical consequences, the most striking of which is the plasticity of ocular dominance (OD) columns observed in cats and macaque monkeys. Neuroanatomical tracing techniques that allow the labeling of afferent projections reveal in normally reared animals that eye-specific inputs are organized into alternating bands, such that both contralateral and the ipsilateral eyes occupy roughly equal cortical territory (Hubel and Wiesel, 1969, 1972; Wiesel et al., 1974). Prolonged MD initiated early in life leads to shrinkage in the size of columns receiving input from the deprived eye, which is accompanied by an expansion of columns subserving the open non-deprived eye (Hubel et al., 1977).

Importantly, the weakening of visual responses following MD is not merely a consequence of degraded visual input; rather a decorrelation of activity from the two eyes appears necessary to induce a shift in ocular dominance. Binocular deprivation, which reduces inputs to both eyes equally, fails to induce an OD shift (Wiesel and Hubel, 1965; Gordon and Stryker, 1996; Frenkel and Bear, 2004). In contrast, induction of strabismus by artificially misaligning the eyes produces a strong OD shift, with a loss of binocularly driven neurons (Hubel and Wiesel, 1965). Coupled with the results of binocular deprivation, this finding clearly highlights the importance of a mismatch between two inputs in driving OD plasticity. A recent result further demonstrates that it is the degraded quality of visual experience following MD and not simply the reduction in light intensity reaching the retina that is required for OD plasticity. Blurring visual images to one eye using an over-correcting contact lens produces an equivalent OD shift to monocular lid suture (Rittenhouse et al., 2006).

How soon following deprivation is binocularity lost? In the initial experiments

Hubel and Wiesel used rather lengthy periods of deprivation to induce plasticity; while the experiments that followed discovered that a functional OD shift could be induced following as few as 6-8 hours of deprivation (Movshon and Dursteler, 1977; Freeman and Olson, 1982; Mioche and Singer, 1989). This rapid onset of plasticity in the cortex was intriguing and the search for the molecular and synaptic mechanisms responsible for this form of plasticity became central to the field for several important reasons. First of all, unbalanced vision can lead to lasting visual impairments in humans: strabismus, uncorrected refractive errors and cataracts can each lead to amblyopia, a debilitating condition that affects 1-4 % of people in the United States (Doshi and Rodriguez, 2007). Amblyopia is a form of cortical blindness because the deficit arises not due to the loss of functionality of the visual pathway, but because of a functional disconnection between the early visual pathway and the visual cortex. Thus, even upon correcting a problem surgically, the visual impairment remains. Therefore, understanding the molecular mechanisms and consequences underlying such loss in visual responsiveness can lead to novel forms of treatment for amblyopia (Mitchell and Sengpiel, 2009). Secondly, the processes revealed by ocular dominance plasticity are likely to share common features with those that are responsible for refinement of other primary sensory cortical circuits during development (Fox and Wong, 2005; Hooks and Chen, 2007). Finally, visual cortical plasticity is an example of how neuronal receptive fields are modified by experience. These changes in receptive fields reflect synaptic modifications that distributed over a neuronal network can store information (Bear, 2003). Thus, understanding the underlying features of OD plasticity may shed the light on the fundamental principles of cortical learning and memory storage.

A large body of classic work in the cat and monkey visual cortex has provided a detailed description of the physiology underlying the shift in ocular dominance. However, in recent decades the mouse visual cortex has emerged as a preferred preparation for the mechanistic dissection of OD plasticity. The most important advantage of mice over other species is their genetic homogeneity and relative abundance, allowing for rapid progress and more controlled experimental design, where biochemical and electrophysiological studies can be coordinated both *in vivo* and *in vitro*. The mouse also offers a great advantage as a platform for transgenic technology, which makes possible the investigation of the involvement of specific gene products and entire cortical microcircuits in the development, plasticity, and function of the visual cortex. Of course, the ability to use the mouse visual system as a model system requires that mice display robust OD plasticity in response to visual deprivation, which has been demonstrated to be very similar in magnitude, kinetics, as well as a behavioral outcome, compared to other species (Smith et al., 2009). The visual system of a mouse shares a number of similarities with that of other animals, but is overall simpler, in that 1) binocularity is established by the convergence of thalamic inputs onto layer 4 neurons, the earliest stage in cortical processing, and 2) the columnar organization of the visual cortex is absent. The integration of the inputs from the two eyes so early in cortical processing and the fact that the mouse visual cortex is relatively undifferentiated has a potential for simplifying the analysis of the underlying synaptic changes brought about by changes in the visual experience.

1.2 Features and the organization of the mouse visual system

Mice are nocturnal animals and therefore rely more on tactile and sensory, rather

than visual cues for survival, rendering their vision rather poor. This may appear as a confound in using these animals as an experimental model for visual plasticity, as compared to cats, which are natural predators and greatly rely on patterned vision. However, in addition to great advantages of rodents described above, the mouse visual system shares many fundamental features with those found in higher mammals.

Representations of binocular visual space are projected to the primary visual cortex contralateral to the visual hemifield, consisting of overlapping inputs from both eyes. Visual information is transmitted from the retina to the dorsal lateral geniculate nucleus of the thalamus (dLGN), where eye-specific inputs remain segregated and neurons are anatomically organized into regions receiving either contralateral or ipsilateral eye inputs (Figure 1.1). In the canonical cortical circuit, the dLGN projects to layer 4 of the visual cortex, which in turn projects to layer 2/3, followed by layer 5 (Douglas and Martin, 2004).

The location of first convergence of retinotopically matched eye-specific inputs varies across species: in macaque monkeys and cats, where much of the pioneering research in ocular dominance was performed, monocularly driven layer 4 neurons converge to produce binocular neurons in layer 2/3 (Hubel and Wiesel, 1968, 1969; Shatz and Stryker, 1978). In these species, layer 4 neurons are anatomically organized similarly to the dLGN, with alternating regions of cortex receiving projections from only the contra- or ipsi- lateral eye (Hubel and Wiesel, 1968, 1969; Shatz and Stryker, 1978). In contrast, in mice the convergence of monocular inputs occurs at the thalamocortical synapse in layer 4 where the majority of the neurons receive inputs from the two eyes (Drager, 1974) (Figure 1.1).

Electrophysiological characterization of mouse visual cortex revealed the presence of all major receptive field types previously characterized in cats and monkeys (Drager, 1975; Mangini and Pearlman, 1980; Metin et al., 1988). Many neurons are highly tuned for orientation and direction of the visual stimulus. Systematic mapping of the mouse primary visual cortex with single-unit recordings, as well as optical imaging of intrinsic signals, revealed a retinotopic organization, where each hemisphere contains a continuous representation of the contralateral visual field (Drager, 1975; Schuett et al., 2002). The arrangement of the retinotopic map is similar to that found in most mammals and is highly reproducible between mice (Hubener, 2003).

There are also several major differences between the mouse visual cortex and that of cats and macaques that can be regarded as both advantageous due to the more simple organization of the system, but should also be viewed as cautionary when interpreting the data obtained from a rodent model. First, receptive field size in the mouse cortex is much larger compared to other species, alluding to the fact that visual acuity of a mouse is far worse than that of higher mammals (Metin et al., 1988). Second, a hallmark of functional architecture of the visual cortex of many higher mammals is the columnar organization of response properties such as orientation selectivity and ocular dominance (Hubel et al., 1977). This type or organization has not been revealed in the mouse with the available techniques, such as transneuronal labeling with tracer injections, optical imaging or imaging using calcium indicators (Drager, 1974; Antonini et al., 1999; Schuett et al., 2002; Ohki and Reid, 2007). Whether the organization exists but is more subtle than that in cat and monkey remains to be determined. Finally, due to the lateral position of the eyes, the binocular field of vision in mice is relatively small (Drager, 1978).

Representations of binocular visual space are projected to the primary visual cortex contralateral to the visual hemifield, consisting of overlapping inputs from both eyes. The binocular segment occupies about a third of the area of the mouse primary visual cortex, where the majority of the neurons can be stimulated by either eye, in sharp comparison to the monocular region, where the neurons responds exclusively to the stimulation of the contralateral input (Figure 1.1). Overall, however, there is a strong contralateral bias in the binocular region, with the response to the contralateral eye being about double that of the ipsilateral eye (Hubener, 2003).

1.3 Bidirectional features of OD plasticity in response to brief periods of MD

Despite the relative simplicity of the organization of the mouse visual cortex compared to that of cats and macaques, it is able to undergo dramatic plastic changes. A robust shift in ocular dominance has been documented using a variety of techniques including single-unit recordings, optical imaging of intrinsic signals, visually evoked potentials (VEPs), immediate early gene expression, and two-photon imaging using calcium sensors (Mioche and Singer, 1989; Mower, 1991; Gordon and Stryker, 1996; Antonini et al., 1999; Frenkel and Bear, 2004; Tagawa et al., 2005; Hofer et al., 2006a; Mrsic-Flogel et al., 2007). Importantly, all of these methods have recapitulated the bidirectional consequences of monocular deprivation: first, a weakening of the deprived-eye response, followed by an increase in the responsiveness of the non-deprived eye, which has also previously been described in kittens (Mioche and Singer, 1989).

The method of measuring VEPs has been particularly useful in addressing the bidirectional changes evoked by MD. Unlike all other methods mentioned above, VEPs offer an advantage in that the signals can be recorded with chronically implanted

electrodes from awake and alert animals. This means that the pre- and post-MD signals can be recorded from the same mouse, affording within-animal experimental control. Varying periods of deprivation revealed the exact time course of the kinetics of the OD shift. First, there is a rapid depression of the deprived-eye responses, which becomes significant within 24 hours of MD and maximal following 3 days of MD (Frenkel and Bear, 2004; Liu et al., 2008). Within 7 days of MD, this is followed by a delayed potentiation of the non-deprived eye response (Frenkel and Bear, 2004).

Measurements of visual acuity following MD, revealed that both stages of the OD shift bear functional consequences. In a visually guided task, animals previously subjected to a period of MD showed a dramatic loss of acuity in the deprived eye (Prusky et al., 2000; Iny et al., 2006), while the performance through the non-deprived eye was enhanced (Iny et al., 2006). These experiments suggest that changes in ocular dominance measured physiologically reflect behaviorally meaningful changes in sensory processing.

The molecular and synaptic changes involved in deprived-eye depression and open-eye potentiation have been the subject of much investigation and controversy in recent decades. One model that has been proposed recently provides a useful framework for the data that has been collected from both the cat and rodent visual cortex. Namely, it proposes a three step process for the functional changes brought about by MD. First, weak activation of the deprived-eye functionally decouples the afferents from postsynaptic cortical neurons through the mechanisms of homosynaptic long-term depression (LTD). This is followed by an overall reduction in cortical activity, which lowers the plasticity threshold, favoring strengthening of weak inputs. This, in turn, allows the initially weaker inputs from the non-deprived eye to potentiate through

homosynaptic long-term potentiation (LTP) (Bear, 2003). This model is by no means comprehensive and alternative theories exist for each of the three stages of the OD shift. The evidence for both will be discussed in further sections of this chapter.

1.4 The role of excitatory and inhibitory networks in OD plasticity: a historical debate

The most clear and undisputable consequence of MD is the loss of responsiveness of cortical neurons to stimulation of the formerly deprived eye. What is less clear is what synaptic changes are responsible for this loss of visual responsiveness. A large body of classic work in kittens has supported the idea that excitatory drive into the cortex from the deprived eye becomes weakened, thereby decreasing cortical responsiveness to the deprived input. For instance, it was found that following MD in young kittens, there is a significant rearrangement of thalamocortical afferents: the area of cortex subserving the deprived eye shrinks, while the cortical area subserving the open eye expands (Shatz and Stryker, 1978). Furthermore, electrophysiological recordings provided strong evidence for considerable weakening of the deprived-eye inputs following MD (Singer, 1977; Tsumoto and Suda, 1978; Mitzdorf and Singer, 1980). However, an alternative hypothesis was proposed suggesting that intracortical inhibition of deprived-eye inputs could increase following MD, thereby suppressing or "masking" visual responses evoked by the deprived eye. This idea first gained ground over 30 years ago based on an observation that following a period of MD, enucleation of the non-deprived eye led to a substantial recovery of cortical responses driven by the deprived eye (Kratz and Spear, 1976). This idea was further investigated by Duffy and colleagues when they intravenously infused a $GABA_A$ receptor antagonist, bicuculline methiodide (BMI) into deprived kittens and observed that the drug partially reversed the effect of MD and

restores responsiveness to as many as 50% of cortical neurons (Duffy et al., 1976). The finding was further strengthened by demonstrating that iontophoretic administration of BMI also reverses the effect of MD (Burchfiel and Duffy, 1981). Unfortunately, the authors did not repeat the experiments in kittens that did not undergo MD, and therefore it is unclear if the findings are entirely due to the effect of deprivation. For example, other investigators demonstrated that in cats with normal vision, visually unresponsive cells become responsive following application of intracortical BMI (Sillito, 1975b). Finally, in another study, Sillito investigated the role of GABAergic inhibition in the OD shift by iontophoretic delivery of BMI and found that the application of the drug results in a major reduction of inhibition-dependent receptive field properties such as direction and orientation selectivity, which together with an increased magnitude of the visually evoked response, suggested a general loss of inhibitory influences (Sillito et al., 1981). Sillito also reported that about 70% of cells still failed to respond to deprived eye stimulation after application of the drug, suggesting that intracortical GABA-mediated inhibition is not making a significant contribution to domination by the non-deprived eye. This study concluded that there is a proportion of cortical cells that are dominated by the non-deprived eye that actually also receive inputs from the deprived-eye which are normally subliminal, as a result of local inhibitory circuits, but it is not clear whether plastic changes in the GABAergic processes are contributing to the expression of the OD shift seen during MD. Together with other findings which failed to detect sprouting of inhibitory neurons or detect any changes in strength of inhibitory synaptic transmission following MD, while clearly observing a reduction in the excitatory transmission, it was postulated that the intracortical inhibition has a passive, rather than active role in the OD

shift resulting from MD (Singer, 1977; Sillito et al., 1981; Bear et al., 1985).

1.5 Homosynaptic LTD as a mechanism for deprived-eye depression

If MD results in weakening of excitatory synaptic transmission, what is the molecular mechanism that can account for this change? A synaptic mechanism for persistent weakening of synaptic transmission – LTD – was characterized extensively in the hippocampus (Dudek and Bear, 1992; Mulkey and Malenka, 1992) and was later found to exist in visual cortex (Kirkwood et al., 1993). Multiple forms of homosynaptic LTD (referring to the synaptic modifications being driven by the affected inputs) depend on weak activation of the *N*-methyl-D-aspartate (NMDA) receptor and subsequent dephosphorylation and internalization of α-animo-3-hydroxy-5-methyl-4-isoxazoleproprionate (AMPA) receptors through clathrin-dependent receptor endocytosis (Malenka and Bear, 2004). Since the induction of an OD shift has also been demonstrated to be NMDAR-dependent (Bear et al., 1990), it was reasonable to investigate whether LTD-like changes could be detected in the visual cortex following MD. Subsequent work in rodents demonstrated that changes precipitated by MD share many common molecular pathways with LTD and vice versa (Bear, 2003; Smith et al., 2009).

The LTD mechanism first characterized in the CA1 region of the hippocampus and later discovered also in the primary visual cortex was found to have a 'biochemical fingerprint': weak activation of NMDA receptors trigger a postsynaptic protein phosphatase cascade that mediates dephosphorylation of the AMPA receptors at the protein kinase A (PKA) site (ser-845) of GluR1 (Malenka and Bear, 2004). The altered state of phosphorylation was shown to be involved in the subsequent internalization of

AMPA receptors, a mechanism considered to be central to the expression of LTD (Malenka and Bear, 2004). Later studies demonstrated that the same molecular signature of LTD was detected on surface AMPA receptors in the visual cortex following a brief period of monocular deprivation (Heynen et al., 2003). An elegant recent study conclusively demonstrated that NMDAR-dependent AMPAR endocytosis is essential for ocular dominance plasticity in the visual cortex. In this study, the authors disrupted AMPA receptor endocytosis exclusively in excitatory neurons with a virally introduced peptide and showed that while this did not alter basal synaptic transmission, both the induction of visual cortical LTD *in vitro* and the induction of OD plasticity *in vivo* was completely blocked (Yoon et al., 2009). Other molecular manipulations that also disrupt AMPAR internalization, such as deletion of the *Arc* gene also disrupts OD plasticity (McCurry et al.). Additional evidence of LTD mechanisms being responsible for deprived-eye depression are electrophysiological recordings in visual cortical slices where changes induced by MD occlude subsequent expression of homosynaptic LTD *ex vivo* (Heynen et al., 2003). A complementary finding demonstrated that low-frequency stimulation of thalamic afferents (in dLGN) produces an NMDAR-dependent LTD in the visual cortex that was found to be associated with both dephosphorylation of ser-845 on the GluR1 subunit of the AMPA receptor, as well as a decrease in the visual evoked responses, comparable to those caused by monocular deprivation (Heynen et al., 2003). Together, these findings demonstrate that monocular deprivation and LTD share molecular mechanisms and induction of LTD is necessary for induction of deprived-eye depression produced by a brief period of monocular deprivation.

More detailed investigations of LTD mechanisms in visual cortex have painted a

much more complicated picture of the exact relationship between LTD and visual cortical plasticity evoked by visual deprivation. One of the complicating factors was the discovery that the mechanisms of LTD appear to vary according to cortical layer (Daw et al., 2004). A detailed study of LTD induction in layers 3 and 4 of mouse visual cortex has been recently carried out by Crozier and colleagues who found that while LTD of similar magnitude could be induced in either layer, LTD induced in layer 4 was mediated via endocytosis of AMPA receptors, while LTD in layer 3 was sensitive to the blockade of cannabinoid receptors. Nevertheless, a prior period of MD occluded further LTD induction *ex vivo* in both layers (Crozier et al., 2007). This is in line with the work *in vivo* that demonstrated that in mice, brief MD produces an OD shift simultaneously in layers 3 and 4: the shift in layer 4 neurons is dependent on AMPA receptor internalization, while the shift in layer 3 neurons requires transmission through the cannabinoid receptor, but is insensitive to blockade of AMPAR endocytosis (Liu et al., 2008; Yoon et al., 2009). Given the existence of different kinds of synaptic plasticity, as well as independent forms of OD plasticity in various layers of the visual cortex, the relationship between the OD shift and LTD is still valid, but appears to be more complicated than the first studies of LTD in the hippocampus have revealed (Smith et al., 2009).

The picture becomes even less simple with the characterization of several mutant mice that attempted to produce a dissociation between mechanisms of LTD and those of OD plasticity. A mouse lacking glutamic acid decarboxylase 65 (GAD65), which has impaired activity-dependent inhibition was shown to be deficient in OD plasticity, with seemingly normal layer 3 LTD (Hensch et al., 1998b). However, a later study

definitively proved that cortical LTD was clearly impaired in this mutant (Choi et al., 2002). On the other hand, another mutant where one of the PKA subunits (RIβ) was knocked out, exhibited impaired layer 3 LTD, but normal OD plasticity (Hensch et al., 1998a). The inconsistency found in these studies can probably be explained by the fact that the mechanisms of OD plasticity vary by cortical layer and the disruption of layer-specific plasticity mechanisms are likely to affect OD plasticity in a complex fashion. Furthermore, many studies using single-unit recordings pool neurons recorded across all layers, preventing the analysis of layer-specific deficits of plasticity.

1.6 Plasticity of inhibitory networks following brief periods of MD

Fueled by the inconsistencies observed between LTD and OD plasticity, several laboratories have revisited the consequence of MD on intracortical inhibition and asked whether plasticity of inhibitory connections may be responsible for deprived-eye depression. Recent work by Maffei and colleagues demonstrated that cortical inhibitory connections can also be strongly affected by MD (Maffei et al., 2006). By studying pairs of neurons in visual cortex *ex vivo*, the authors found that following deprivation there is an increase in the inhibitory tone via strengthening of excitatory cell connections onto inhibitory interneurons and vice versa, but no change in the strength of excitatory connections onto other excitatory cells. The strengthening of inhibitory transmission occurs through a novel form of potentiation (LTPi), which was occluded by prior MD. One major caveat of this study is that the recordings were performed in the monocular zone of visual cortex that may have different plasticity rules, since it lacks the input from the non-deprived eye and, therefore, does not have ocular dominance plasticity.

In a very recent report, Yazaki-Sugiyama and colleagues took a direct approach to

test MD-evoked plasticity of inhibitory neurons by performing intracellular *in vivo* recordings from layer 2/3 of anesthetized mice to assay the OD score of individual neurons following MD (Yazaki-Sugiyama et al., 2009). This approach enabled a direct comparison of the consequences of MD on both pyramidal and fast-spiking (FS) inhibitory neurons. The authors found that in the baseline state, the inhibitory neurons are binocular and display no contralateral bias found in excitatory cells. This dramatically reverses following a short period of deprivation, and the FS cells become sharply tuned to activation of the deprived eye. The findings are, however, not in agreement with several recent studies that used two-photon calcium imaging and found that the inhibitory neurons show a significant contralateral bias in the non-deprived state (Gandhi et al., 2008; Kameyama et al., 2010). These studies also report that the ocular dominance of inhibitory neurons undergoes an OD shift, but this shift lags the shift in excitatory neurons.

There is considerable evidence suggesting that the inhibitory networks are modified by visual experience (Smith and Bear, 2010). However, the exact contribution of the inhibitory neurons to OD plasticity remains unclear. It is worth mentioning at this point that there are two phases through which deprived-eye depression occurs: induction and expression. Inhibitory tone and activity of the inhibitory networks have been largely implicated in the induction of the OD shift and may well influence whether OD plasticity can occur or alters the qualities of the OD shift (Hensch, 2005; Hensch and Fagiolini, 2005; Jiang et al., 2005). The second phase of OD plasticity has to do with the expression of the OD shift which, assuming that the changes promoting the shift have already taken place, considers the molecular and synaptic correlates that allow the shift to

be *maintained.* The relative significance of inhibitory plasticity in maintaining the OD shift following MD remains to be determined, especially in light of the fact that a direct reduction of excitatory synaptic transmission following deprivation has never been demonstrated. This question has been one focus of my dissertation and is addressed in detail in Chapter 2.

1.7 Metaplasticity: a mechanism for bidirectional plasticity in the visual cortex

Following the rapid onset of deprived-eye depression, which becomes maximal after 3 days of MD, there is a delayed gradual increase in cortical responsiveness to the non-deprived eye (Frenkel and Bear, 2004). It seems reasonable to assume that following a functional decoupling of the deprived-eye from the cortex that cortical activity must adapt in a way that would allow the non-deprived inputs to gain enhanced function and strength. The idea came forth to explain how the cortex could accommodate such bidirectional synaptic plasticity following visual deprivation from the group of Leon Cooper and was popularized as the BCM theory (Bienenstock et al., 1982). The theory postulates that active synapses can be bidirectionally modified as a function of postsynaptic activation. Secondly, there is an activity threshold which dictates whether a given activity pattern will elicit LTP or LTD at the synapse. The third and probably the key assumption of the BCM theory is that the plasticity threshold, θ_M, is not fixed, but can vary with the history of cortical activity and previously induced plasticity at a given synapse (Figure 1.2). This third assumption of the BCM model gave rise to the notion of "metaplasticity": plasticity of synaptic plasticity (Abraham and Bear, 1996; Bear, 2003). This model was tested in visual cortex and proved to be correct: synapses were bidirectionally modifiable as a function of stimulation frequency and θ_M could be shifted

following just a few days of dark rearing such that LTP could be induced by lower stimulation frequencies (Kirkwood et al., 1996; Philpot et al., 2003; Philpot et al., 2007). These experimental results further encouraged the application of the BCM theory to receptive field plasticity. It was hypothesized that perhaps following MD there is a reduction of overall cortical activity caused by the degradation of patterned vision in one eye, which decreases the value of the sliding modification threshold, θ_M, thereby facilitating potentiation of correlated open-eye inputs (Bear, 2003; Smith et al., 2009).

What is the molecular mechanism that could support such forms of metaplasticity and allow for the regulation of the sliding modification threshold? Although there is a number of ways to regulate bidirectional synaptic plasticity, the mechanism that received most attention based on a wealth of experimental evidence involves the experience-dependent regulation of the NMDA receptors. The NMDA receptor consists of an obligatory NR1 subunit and non-obligatory NR2 subunits. In the brain, the most widely expressed NR2 subunits are NR2A and NR2B and they have been studied extensively, as they are believed to be important for synaptic plasticity (Yashiro and Philpot, 2008). The relative number of NR2A- and NR2B-containing NMDA receptors varies with cortical development and with sensory experience. In many parts of the brain, the NR2A/NR2B ratio increases during early postnatal development at both mRNA and protein levels (Nase et al., 1999; Quinlan et al., 1999a; Roberts and Ramoa, 1999; Barth and Malenka, 2001; Yoshii et al., 2003). There is a developmentally regulated acceleration of NMDAR-mediated decay currents, attributed to a postnatal gradual increase in NR2A-containing receptors (Carmignoto and Vicini, 1992; Quinlan et al., 1999a). Furthermore, the NR2A/2B ratio can be regulated with varied visual experience. Rats that are dark

reared or dark exposed show reduction in the ratio of NR2A/2B protein levels, which is rapidly reversible upon exposure to light (Quinlan et al., 1999a; Quinlan et al., 1999b). In line with what has been observed in normal visual development, dark rearing increases the decay times of synaptic NMDA currents (Carmignoto and Vicini, 1992). In addition, dark rearing also increases sensitivity to NR2B selective antagonists, suggesting that dark rearing reverses the increase in the NR2A subunit in favor of the NR2B-containing receptors (Philpot et al., 2001a). As NR2B subunits confer longer current decay kinetics on the NMDA receptors, the presence of these subunits has been proposed to facilitate the summation of inputs and thereby promote more coincidence detection, possibly facilitating LTP (Monyer et al., 1994; Flint et al., 1997; Yashiro and Philpot, 2008). Conversely, an elevation in NR2A/2B ratio would require stronger stimulation to induce LTP and favor induction of LTD, due to shorter current decay times of the NR2A subunit. The rationale for this idea comes from the observation that NR2A/2B ratio limits the accessibility of the Ca^{2+}/Calmodulin Kinase II (CaMKII), as well as the amount of Ca^{2+} that can enter through the NMDARs with a given level of synaptic activation. Therefore, a high ratio of NR2A/2B subunits would require a stronger postsynaptic response to elevate Ca^{2+} and activate CaMKII to a level sufficient to induce LTP. On the other hand, weaker postsynaptic activation and smaller Ca^{2+} influx could activate the calcineurin signaling cascade and tip the synapse toward induction of LTD. If plasticity threshold were then decreased by lowering the NR2A/2B ratio, a modest postsynaptic response might be sufficient to adequately elevate calcium, activate CaMKII and induce LTP (Yashiro and Philpot, 2008).

The critical question is whether a shift in NR2A/2B ratio could account the

modification of the sliding threshold following MD and be permissive for potentiation of the open-eye inputs (Figure 1.2). To determine this, Philpot et al. took advantage of a genetically modified mouse which had a deletion of the NR2A subunit, rendering a fixed ratio of the NMDAR subunits (Philpot et al., 2007). The examination of the NMDAR currents in this mouse showed that the absence of the NR2A subunit both mimicked and occluded the effects of dark rearing on the amplitude, decay kinetics and temporal summation of synaptic currents. 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 wildtype mice, is sufficient to induce LTP. These observations are consistent with the idea that a low NR2A/2B ratio favors the induction of LTP, although this may vary according to brain region (Zhao and Constantine-Paton, 2007).

Assuming the sliding modification threshold for plasticity is altered by a changing NR2A/2B ratio and this change is permissive for open-eye potentiation, then it would be reasonable to hypothesize that changes in the subunit ratio should slightly precede the potentiation of non-deprived eye inputs. Indeed, when the ratio of the NR2A and NR2B protein levels were examined in mouse visual cortex following MD, a significant decrease in the ratio was found as early as after 5 days of MD, whereas open-eye potentiation becomes significant after 5-7 days of deprivation (Frenkel and Bear, 2004; Chen and Bear, 2007). The obvious question that follows from these studies is whether ocular dominance would be affected in the NR2A knockout mouse. Since the NR2A/2B ratio is fixed in this mouse, with induction of LTP favored at low frequencies that usually

induce LTD, it would be reasonable to predict that open-eye potentiation would be favored over deprived-eye depression. This question is another focus of my dissertation and will be addressed in detail in Chapter 3.

Although the regulation of NR2A/2B ratio is an attractive mechanism for regulating the sliding modification threshold and bidirectional synaptic plasticity, a host of studies provide evidence that this model may be insufficient to explain all features of plasticity and metaplasticity in the brain (Abraham, 2008; Yashiro and Philpot, 2008). Besides the regulation of the relative amounts of NMDAR subunits, factors that could also be responsible for regulating plasticity could include phosphorylation states of the NMDA receptor, downstream signaling cascades, other glutamate receptor subtypes, such as metabotropic glutamate receptors (mGluRs), voltage-gated calcium channels, changes in neuronal excitability, neurotrophins, and changes in inhibitory tone to name a few (Cohen et al., 1998; Huber et al., 1998; Huang et al., 1999; Abraham et al., 2001; Choi et al., 2002; Huber et al., 2002; Ohmura et al., 2003; Yoshimura et al., 2003; Chattopadhyaya et al., 2004; Abraham, 2008; Yoshimura et al., 2008). Furthermore, LTP and LTD are umbrella terms for complicated cellular processes that involve many signaling cascades and the rules for induction and expression of plasticity varies among brain regions and developmental stages. For example, a recent study showed an important role of cholinergic and adrenergic neuromodulation in governing the polarity of plasticity (Seol et al., 2007). The authors showed that activation of adenylate cyclase (AC) and phospholipase C (PLC) signaling cascades results in specific changes of glutamate receptor phosphorylation states, making a synapse more or less susceptible to LTP vs. LTD. Consequently, the polarity of synaptic plasticity maybe be, as least in part,

gated by the relative activation of neuromodulatory receptors.

1.8 Mechanisms underlying open-eye potentiation following brief periods of MD

Much effort has been devoted to uncovering molecular and synaptic processes supporting deprived-eye depression following MD. In contrast, the second phase of the OD shift, potentiation of the non-deprived inputs has received relatively little attention. According to the prediction of the BCM model, the depression of deprived-eye inputs accompanying the leftward shift of the sliding modification threshold, which would enable the open-eye inputs to potentiate via a homosynaptic mechanism, such as NMDAR-dependent LTP. Experimental findings have demonstrated that LTP can indeed be induced at cortical synapses. For example, homosynaptic NMDAR-dependent LTP has been shown at layer 3 synapses in the rat visual cortex (Kirkwood and Bear, 1994). NMDAR-dependent LTP has also been demonstrated *in vivo* in layers 4 and 2/3 following a tetanic stimulation of the LGN (Heynen and Bear, 2001). As this form of LTP was sufficient to increase the magnitude of visually evoked responses, it is reasonable to suggest that homosynaptic LTP and open-eye potentiation could share the same molecular pathways. Whether this potentiation occurs at thalamocortical or intracortical synapses remains to be determined.

Most of what we can infer about open-eye potentiation in the context of OD plasticity comes from studies using various methods of disruption of homosynaptic LTP (Daw et al., 2004; Hensch, 2005; Hooks and Chen, 2007; Smith et al., 2009), although care must be taken not to over-interpret the results, as the mechanisms of LTP have been shown to vary by cortical layer, similar to LTD (Wang and Daw, 2003). One example that seems to suggest a strong role for LTP in OD plasticity comes from a finding that

disrupting αCaMKII, an isoform of CaMKII necessary for NMDAR-dependent LTP, abolishes both the induction of LTP and blocks the OD shift in mice (Gordon et al., 1996; Taha et al., 2002). One large technical difficulty in studying open-eye potentiation is that there is currently no method for isolating responses of the open-eye afferent from those of the deprived-eye afferents, so when studying the effect of MD *ex vivo* in slices, it is impossible to isolate changes in the deprived-eye pathway from that serving the open-eye. This can present a big challenge when trying to understand the relationship between disruption in the LTD/LTP pathways and the effect of this disruption on OD plasticity. For instance, in the studies described above, is it that LTP is necessary for open-eye potentiation? Or is αCaMKII required for deprived-eye depression and this is why the OD shift is blocked?

Further studies have yielded more evidence suggesting a pivotal role for LTP in strengthening of open-eye inputs. One finding is that in adult mice with a deletion of the obligatory NR1 subunit from layers 2-4 or pharmacological blockade of NMDA receptors, open-eye potentiation is absent, suggesting that NMDAR-mediated plasticity is a requirement in this process (Sawtell et al., 2003; Sato and Stryker, 2008). The interpretation of these results are clouded by the fact that it was assumed that deprived-eye depression is absent in adult mice, even with a long period of deprivation, which was later shown not to be the case (Frenkel et al., 2006), so absence of open-eye potentiation could be a result of the requirement of NMDAR-mediated plasticity for deprived-eye depression and sliding of θ_M. Even though NMDAR-dependence of open eye potentiation has been established in the adult cortex, this has not been investigated in juveniles. I address this question experimentally in Chapter 3.

1.9 Alternative mechanisms for open-eye potentiation

The BCM sliding threshold model describes a means to achieve homeostasis of postsynaptic activation in the face of decreased synaptic drive. The modification threshold will adopt a position optimal for maintaining the appropriate firing rate. The homeostatic adjustment of the firing rate can be achieved by changing synaptic weights via LTP or LTD. According to this scenario, a prolonged MD leads to a leftward shift in θ_M, which facilitates LTP of the open-eye inputs, thereby increasing synaptic drive and restoring the postsynaptic level of activity to its original value. An alternative mechanism of homeostatic regulation is synaptic scaling. This phenomenon was first described by Gina Turrigiano in rat cortical cultures, where pharmacological blockade of activity with TTX resulted in a global multiplicative scaling up of synaptic weights, in an attempt to maintain the basal firing levels of neurons (Turrigiano et al., 1998). Several recent studies have proposed that synaptic scaling could account for the strengthening of open-eye responses following MD (Turrigiano and Nelson, 2004; Smith et al., 2009). Visual deprivation, either via monocular retinal inactivation with TTX or dark rearing has been shown to increase mEPSC amplitude in layers 2/3 of juvenile rat visual cortex (Desai et al., 2002; Goel et al., 2006). A recent study of OD plasticity using *in vivo* two-photon calcium imaging suggested that a similar scaling mechanism might manifest following MD (Mrsic-Flogel et al., 2007). The authors observed that in addition to the expected shift in open-eye dominance, responses of isolated cells in the binocular segment that are driven exclusively by the deprived eye were also larger following MD. Unfortunately, since the responses of individual cells could not be compared pre- and post-MD, it is unclear whether the response of the previously monocular cells scales up following deprivation or whether cells that were originally binocular have become

monocular following MD.

The two models proposed for open-eye potentiation, whether homosynaptic LTP or synaptic scaling, are both forms of homeostatic regulation and the biggest distinction between the two is whether homeostasis occurs globally at all synapses in a network (a hallmark of scaling) or whether it is input-specific (i.e. homosynaptic). One way to dissociate homosynaptic plasticity from heterosynaptic scaling is to turn to the outcome of binocular deprivation (BD). Upon closure of both eyelids, cortical responses must adjust to the diminished visual drive. A synaptic scaling model predicts that BD would lead to potentiation of responses to stimulation of both eyes, whereas a BCM-type homosynaptic model would argue for no change in responsiveness (Blais et al., 1999; Blais et al., 2008)(Blais et al., 1999; Blais et al., 2008). Several studies measuring single-unit responses and chronically-recorded VEPs have reported no change in visual responsiveness following BD (Gordon and Stryker, 1996; Frenkel and Bear, 2004; Blais et al., 2008). However, Mrsic-Flogel and colleagues did observe potentiation of responses following BD when measuring calcium signals in layer 2/3 (Mrsic-Flogel et al., 2007).

Another difference between the two potentiation mechanisms is requirement for NMDA receptor activation. Both open-eye potentiation and LTP have been demonstrated to depend on activation of NMDAR currents (Sawtell et al., 2003; Sato and Stryker, 2008), while the expression mechanism for synaptic scaling does not have the same requirement (Turrigiano et al., 1998; Turrigiano and Nelson, 2004).

A further study attempted to distinguish the two models of plasticity using genetically modified mice that were deficient in synaptic scaling (Kaneko et al., 2008).

This knockout mouse lacked tumor necrosis factor-α (TNFα) and did not exhibit scaling up of synaptic currents in response to decreased activity *in vitro*. The authors further found that the TNFα mice did not have open-eye potentiation, as assessed with chronic optical imaging of intrinsic signals; however, LTP in layer 2/3 was normal. This finding would suggest a strong role for scaling in open-eye potentiation, apart from the one caveat: the deprived-eye responses did not increase proportionally to open-eye responses during the later stages of MD (Aizenman and Pratt, 2008; Kaneko et al., 2008). This point is crucial because a key feature of synaptic scaling is that all synapses are scaled equally so as to preserve the relative strength of distinct inputs (Turrigiano et al., 1998). Therefore, the disproportionate effect on open-eye responses during the later stages of MD would argue against synaptic scaling as the sole mechanism of open-eye potentiation (Smith et al., 2009).

1.10 Critical periods for ocular dominance plasticity

During a brief period in postnatal life the growing brain is most malleable and shows heightened responsiveness to external sensory influences. Such critical periods seem to exist for human language acquisition, bird song learning and binocular vision to name a few. The question of what mechanisms underlie the activation and regulation of critical periods in the brain is of great interest, because manipulation of such mechanisms may provide a way to allow expression of plasticity in a fully mature individual and would be invaluable in the context of recovery from brain trauma.

The concept of a critical period was introduced by Hubel and Wiesel when they first characterized the OD shift in response to MD (Wiesel and Hubel, 1963). They found that deprivation was effective at shifting eye dominance in young cortex, but the

adult cortex was unresponsive to even a prolonged deprivation of visual experience (Hubel and Wiesel, 1970). It has now been well documented that windows for experience-dependent plasticity exist in various brain regions and occur at different times (Hensch, 2005), although capacity for plasticity beyond traditionally defined critical periods still exists (as will be discussed later in more detail) (Hofer et al., 2006b; Hooks and Chen, 2007). From a clinical point of view, this capacity becomes extremely relevant in the treatment of amblyopia. In children a variety of conditions causing asymmetric vision, such as a cataract in one eye, can result in functional blindness in the abnormal eye, leading to loss of stereoscopic vision and amblyopia. Restoration of binocular vision and recovery of function has been very successful in children between ages of several months and approximately seven years (Mitchell and Sengpiel, 2009). In fact, until recently, it was believed that if a child was diagnosed after the age of eight, treatment was completely ineffective. However, in a recent clinical trial for treatment of amblyopic patients it was found that in about one fourth of cases, treatment was effective up until seventeen years of age (Scheiman et al., 2005). It is clear, however, that the young brain can exhibit very dramatic plasticity and understanding the mechanisms that permit this plasticity is essential to tap into the plastic potential of the adult brain.

1.11 Mechanisms controlling the critical period onset

There is no firm consensus in the field of what defines a critical period for a specific neural circuit (Hooks and Chen, 2007). The general agreement seems to define at least three stages of a critical period: 1) a pre-critical period window during which the neuronal circuits develop mostly via internally set cues and are not very sensitive to changes in the external sensory world, 2) a critical period of robust plasticity during

which an initially formed circuit can be modified by experience, and 3) a closure of the critical period, a period of diminished sensitivity and relative stability of a neuronal circuit, during which the same stimulus can no longer elicit the same degree of plasticity.

The classically defined critical period for ocular dominance plasticity in rodents begins during the third postnatal week, about a week after eye-opening (Gordon and Stryker, 1996). This period is preceded by weeks during which the visual circuitry matures. There is much debate over the influence of vision over the formation of the neuronal circuits (Hooks and Chen, 2007). There is no doubt that the brain is very plastic during this time, but the prevalent current belief is that the initial formation of the OD structure does not depend on vision, but rather on spontaneous activity originating within the visual pathway, such as retinal waves (Feller and Scanziani, 2005; Hooks and Chen, 2007).

Once the initial circuits are formed, the animals have a window of time during which ocular dominance can be modified in response to visual experience (Wiesel and Hubel, 1963; Hubel and Wiesel, 1970; Fagiolini et al., 1994; Gordon and Stryker, 1996). It has been suggested that a critical period cannot begin until the brain circuitry has developed reliability and precision (Knudsen, 2004). It appears that mechanisms underlying a critical period onset are not simply driven by activity, as part of the precritical period occurs after eye opening. Instead it seems that something during precritical period development sets the molecular stage for the activation of the critical period (Feller and Scanziani, 2005; Hooks and Chen, 2007). A number of studies showed that the onset and the closure of the critical period can be manipulated, suggesting these events do not occur at fixed points in the life cycle of the animal, but

that critical period timing can be regulated by physiological and molecular processes. For instance, it has been shown that dark rearing can delay the onset of plasticity (Fagiolini et al., 1994). One of the explanations that could support this data is the role of BDNF in maturation of the visual cortex. Expression of BDNF in the visual cortex increases following light stimulation (Castren et al., 1992; Bozzi et al., 1995; Schoups et al., 1995; Cabelli et al., 1996). In line with these data, overexpression of BDNF in excitatory neurons of the mouse visual cortex resulted in premature onset and closure of the critical period (Hanover et al., 1999; Huang et al., 1999). Consistent with these data, increasing cortical BDNF levels in dark-reared mice normalized the onset and closure of OD plasticity (Gianfranceschi et al., 2003; Bartoletti et al., 2004).

Interestingly, BDNF was also found to play a role in the development of inhibitory circuits in the visual cortex (Huang et al., 1999). This finding spurred interest in the inhibitory networks as a gating mechanism for critical period onset and closure (Jiang et al., 2005). Another clue came from a study that used a mouse with deficient inhibitory transmission (GAD65 knockout) to demonstrate a complete absence of OD plasticity (Hensch et al., 1998b). This deficit could be rescued by a potentiation of inhibitory transmission via an infusion of a benzodiazepine compound, diazepam. The authors showed that the onset of plasticity could be induced at any point in an animal's lifetime: before or after a traditionally defined critical period, although, once plasticity was induced, diazepam was no longer effective in producing additional plasticity later in life. This finding is consistent with the BDNF overexpression result, suggesting that a certain level of inhibitory tone must be reached in order for cortical circuits to respond effectively to visual experience (Hensch, 2005). These findings were followed up with

additional studies to dissect out specific GABAergic circuits that are responsible for triggering the critical period plasticity. Genetically altered mice in which various $GABA_A$ receptor alpha subunits have been mutated to render them insensitive to benzodiazepines show that inhibitory circuits containing the $GABA_AR$ alpha1 subunit are required for induction of plasticity by diazepam infusion (Fagiolini et al., 2004; Katagiri et al., 2007). This implicates a specific class of inhibitory interneurons, fast-spiking basket cells, in triggering OD plasticity.

In addition to playing a role in critical period onset, inhibition may also influence the closure of the critical period. Experiments in GAD65 KO mice where the critical period could be activated once, but not subsequently, by infusion of diazepam have suggested that an increase in GABAergic inhibition once triggered remains potent and contributes to closure of OD plasticity, an idea that will be discussed in more detail in the subsequent sections of this chapter.

1.12 Potential for plasticity in the adult brain

In contrast to the classical notion of a critical period for experience-dependent plasticity, an increasing number of studies are reporting that OD shifts, along with various other forms of plasticity can be induced in adulthood (Hofer et al., 2006b; Hooks and Chen, 2007). For instance, studies of the tectum in the barn owl, an area of the central nervous system where the visual and the auditory spatial maps are aligned, have shown that large shifts in visual maps in juvenile birds are eventually followed by corresponding realignment of the auditory map (Knudsen and Brainard, 1991; Brainard and Knudsen, 1998). In contrast, adult owls show very little plasticity when subjected to similar experimental conditions; however, the authors discovered that map realignment

could be induced in the owl as long as the shifts in the visual map were smaller and more incremental (Linkenhoker and Knudsen, 2002).

In rodents, evidence for adult plasticity is becoming increasingly abundant. Several studies found that and OD shift can be induced in adult mice, just with a longer period of deprivation, suggesting that plasticity is not absent, but requires a more potent or longer-lasting stimulus to induce it (Sawtell et al., 2003; Frenkel et al., 2006; Sato and Stryker, 2008). Findings from other labs support this result, finding that only five days are required to induce an OD shift in adults (Hofer et al., 2006b).

Given these results, the concept of an abrupt closure of the critical period seems to not be entirely true, although it seems that the degree of plasticity in the adult animals is decreased and longer periods of deprivation seem necessary to induce OD plasticity (Sawtell et al., 2003; Lickey et al., 2004; Pham et al., 2004; Frenkel et al., 2006; Hofer et al., 2006a; Fischer et al., 2007b; Sato and Stryker, 2008; Hofer et al., 2009).

Interestingly, the potential for adult plasticity can be increased by previously experienced juvenile plasticity. This phenomenon, first observed in the barn owl (Linkenhoker et al., 2005), was recently observed in the mouse (Hofer et al., 2006a). In this study juvenile mice were subjected to a short period of MD and allowed complete recovery of binocular vision. The same mice were subjected to a subsequent period of MD in adulthood and, strikingly, as few as three days of deprivation were enough to induce robust OD plasticity.

Another form of adult plasticity is revealed by studies of recovery from MD (Mitchell and Sengpiel, 2009). OD shifts and visual acuity has been shown to recover in cats and rats and mice following prolonged visual deprivation (Mitchell, 1991; Iny et al.,

2006; Fischer et al., 2007a; Mitchell and Sengpiel, 2009). In most cases the recovery was enhanced by occluding the previously open eye as well as correlated binocular vision (Blakemore and Van Sluyters, 1974; Kind et al., 2002; He et al., 2006; Fischer et al., 2007a; He et al., 2007). In rats, the recovery from visual deprivation has been shown to be readily reversible by a subsequent MD (Iny et al., 2006), suggesting that traces of previous plasticity may facilitate further plasticity in adult brain.

1.13 Critical periods termination and reactivation of juvenile plasticity in the adult brain

The exact molecular and synaptic mechanisms that constrain synaptic modifications to a short critical period remain obscure (Berardi et al., 2000; Hensch, 2005; Hooks and Chen, 2007). Recently, however, GABAergic inhibitory circuits have become increasingly implicated in determining the timing of the critical period for synaptic modification (Jiang et al., 2005). The studies of molecular and physiological maturation of inhibitory circuits have unambiguously shown that synaptic inhibition matures largely postnatally, lagging behind the formation of excitatory cortical circuits (Blue and Parnavelas, 1983; Luhmann and Prince, 1991; Benevento et al., 1992; Guo et al., 1997; Gao et al., 2000; Chattopadhyaya et al., 2004; Chattopadhyaya et al., 2007).

Perinatal GABAergic responses are slow and depolarizing (Luhmann and Prince, 1991; Agmon et al., 1996). The shift in reversal potential, final changes in the subunit composition underlying the kinetics of the GABAergic responses, and precise targeting of interneurons to specific subcellular compartments are largely completed by eye-opening, suggesting that there features of circuit development rely on internal cues, rather than visual experience (Jiang et al., 2005). On the other hand, synaptic maturation and proliferation of inhibitory contacts onto pyramidal neurons continues throughout the

critical period and is most likely to affect experience-dependent plasticity (Jiang et al., 2005; Huang et al., 2007). In rodents, the total number of GABAergic synapses and the magnitude of inhibitory synaptic responses undergo a sharp three-fold increase between eye-opening (~2^{nd} postnatal week) and the end of critical period (~5^{th} postnatal week) (Huang et al., 1999; Morales et al., 2002). Similar to the timing of the critical period, proliferation of inhibitory synapses can be manipulated by sensory experience. Dark rearing postpones the increase in inhibitory responses and the effect of dark rearing can be reversed rapidly by transient light exposure (Morales et al., 2002; Chattopadhyaya et al., 2004; Di Cristo, 2007). Recent studies using transgenic mice that express green fluorescent protein (GFP) in a subset of fast spiking interneurons provided evidence that individual axons increase the number of postsynaptic boutons contacting a target pyramidal cell (Chattopadhyaya et al., 2004). The functional maturation of inhibitory synaptic transmission mediated by an endocannabinoid-dependent LTD of inhibitory inputs is completed by approximately fifth postnatal week (Jiang et al., 2007; Jiang et al., 2010). During this time the inhibitory synapses transition from an immature state with high release probability to a mature state with low release probability and increased fidelity of transmission.

Besides the overall increase in efficacy of synaptic transmission, another feature that marks the maturation of the GABAergic circuit is the development of perineuronal nets (PNNs), a matrix of extracellular proteins that envelops the fast-spiking basket cells (Hartig et al., 1992; Hartig et al., 1999), reaching asymptotic levels around the closure of the critical period. In line with data mentioned above, dark rearing prolongs the formation of PNNs (Hockfield et al., 1990), which presumably contributes to stalling of

maturation of inhibitory responses.

By controlling excitation, GABAergic circuits are ideally poised to control (and limit) the activity-dependent modifications of excitatory synaptic transmission and the delay in the maturation of inhibition may define a window of opportunity where experience-dependent plasticity is allowed to occur (Jiang et al., 2005; Sale et al., 2010).

The idea that GABAergic inhibition might play a role in terminating the critical period was first suggested by an *in vitro* analysis of developmentally regulated forms of NMDAR-dependent plasticity (Artola and Singer, 1987; Kirkwood and Bear, 1994). In cortical brain slices, LTP can be readily elicited in the upper cortical layers (layers 2/3) by white matter stimulation in young animals, but in adults only stimulation of cortical layer 4 can evoke LTP at the same synapses, while stimulation of white matter is no longer effective at eliciting plasticity. However, reducing the level of inhibition pharmacologically with GABAergic antagonists enables LTP to be elicited by white matter activation even in adults. This has led to the notion that inhibitory circuits in layer 4 act as a "gate" for synaptic modifications during the critical period: early on, inhibition is weak and the gate is open, allowing for synaptic modifications to occur; subsequently, the development of inhibitory circuits in layer 4 closes the gate, limiting any further plasticity in supragranular layers (Kirkwood and Bear, 1994). Consistent with this idea, rearing the animals in the dark, which attenuates the maturation of inhibition also postpones the termination of the critical period for induction of white matter to layer 3 LTP (Rozas et al., 2001). Conversely, accelerating maturation of inhibition with BDNF lead to a precociously terminated critical period expressed either through lack of responsiveness to MD or through the attenuation of white matter-layer 3 LTP (Huang et

al., 1999).

The studies that could manipulate the timing of the onset and closure of the critical period (Hensch et al., 1998b; Huang et al., 1999; Jiang et al., 2005; Sale et al., 2010) have led to a popular view in the field that synaptic modifications require the strength of GABAergic inhibition to be within a range permissive for plasticity. That is to say that in the process of maturation inhibition crosses a minimal threshold for enabling excitatory synaptic transmission to undergo changes; inhibitory circuits continue maturing and gaining strength throughout the critical period until an upper limit is reached beyond which plasticity of excitatory transmission cannot occur (at least as easily). This would suggest that decreasing the level or the efficacy of inhibition in an adult brain could potentially revert the brain back to the plasticity mode. In fact, a number of recent papers have proposed several strategies that could reactivate plasticity in adult animals (Spolidoro et al., 2009). Curiously, all of the manipulations directly or indirectly lowered cortical inhibition, reinforcing the role of this circuit in gating adult plasticity.

The first demonstration of reactivation of plasticity in the adult rodent cortex came for the laboratory of Lamberto Maffei. His group made the connection between the importance of the PNNs for functionality of the inhibitory circuit and the possibility that inhibition may restrict post-critical period plasticity. Enzymatic degradation of the proteins that make up PNNs (chondroitin-sulfate glycosaminoglycans or CSPGs) uncovered rapid OD plasticity in the rat primary visual cortex in response to a short period of MD (Pizzorusso et al., 2002). Other components of the extracellular matrix (ECM) or signaling from the ECM proteins, such as myelin proteins on Nogo receptors

might contribute to limiting plasticity in the adult cortex, as it has been demonstrated that a deletion of the Nogo protein facilitates adult plasticity (McGee et al., 2005).

An alternative paradigm for enhancement of OD plasticity in adult rats has been proposed recently where a ten-day period of complete visual deprivation prior to an MD resulted in activation of a juvenile-like level of OD plasticity (He et al., 2006). The plasticity was expressed in a manner similar to that in young animals with deprived-eye depression preceding open-eye potentiation (Frenkel and Bear, 2004). Moreover, this experimental paradigm also allowed for significant functional recovery of visual acuity in adult subjects that have previously undergone long-term MD (He et al., 2007). Interestingly, the transient dark exposure led to reduced expression of $GABA_A$ receptors relative to AMPA receptors in the visual cortex, suggesting that an altered balance between excitation and inhibition might be permissive for plasticity (He et al., 2006; He et al., 2007).

Another study employed a different strategy to reactivate plasticity in adults: environmental enrichment (Sale et al., 2007; Baroncelli et al., 2010). The authors demonstrated that raising animals in an enriched environment promotes complete recovery of visual acuity and ocular dominance in adult animals that have been rendered amblyopic with a prolonged period of MD. Recovery of plasticity was associated with a marked reduction of GABA release in the visual cortex. Additionally, LTP of layer 3 field potentials evoked by stimulation of white matter, which is not normally present in the adult cortex was completely restored. Restoration of plasticity was also accompanied by a reduction in the PNN density in the visual cortex. The involvement of inhibitory processes in gating inhibition was further confirmed by a finding that diazepam infusion

during enrichment completely blocked all restoration of plasticity. Of course, it is important to note that environmental enrichment has profound effects on the brain, such an marked improvements in learning and memory, enhanced neural plasticity and even improvement of certain cognitive impairments (van Praag et al., 2000; Spolidoro et al., 2009).

It is known that environmental enrichment has a great influence over various neuromodulatory systems in the brain (Spolidoro et al., 2009; Baroncelli et al., 2010). This has led the same group to explore a possibility that directly manipulating levels of neuromodulators, such as serotonin, in the brain could have similar effects to environmental enrichment. To test this hypothesis, the authors chronically treated rats with fluoxetine, a selective serotonin reuptake inhibitor, and discovered that such treatment successfully restored plasticity in the adult visual system (Maya Vetencourt et al., 2008). Plasticity was manifest both as a juvenile-like OD shift following a brief period of MD and a recovery of visual function in adult amblyopic rats. Similar to the effects of enrichment, fluoxetine treatment was also associated with a marked reduction in GABAergic inhibition and cortical administration of diazepam completely blocked fluoxetine-induced plasticity.

Finally, an acute blockade of inhibitory function in the visual cortex has most recently been shown to restore adult plasticity in rats (Harauzov et al., 2010). The authors employed pharmacological methods to block inhibition for several days via infusion of MPA (a GABA synthesis inhibitor) or picrotoxin (a $GABA_AR$ antagonist) and demonstrated that an OD shift could be induced with as few as three days of MD.

An intriguing finding was recently reported by Southwell and colleagues: the

authors were able to induce plasticity after the end of the critical period by transplanting immature inhibitory neurons into young adult mice (Southwell et al., 2010). They found that transplanted interneurons integrated into the circuit and formed connections with the excitatory neurons. Interestingly, however, plasticity was only permitted when the transplanted interneurons reached the developmental stage equivalent to that of endogenous inhibitory neurons during the normal critical period.

Developmental increase in inhibitory control of excitatory synaptic transmission is an attractive mechanism for regulating the timing of critical period plasticity. It is still unknown, however, which subset of a diverse family of inhibitory cortical interneurons might play the pivotal role in this regulation. Fast-spiking parvalbumin-positive basket cells are ideal candidates, due to their exquisite ability to control both the input and the output of pyramidal cells (Huang et al., 2007; Kuhlman et al., 2010). The involvement of this neuronal population in adult visual cortical plasticity has been another focus of my dissertation and I address this issue experimentally in Chapter 4.

1.14 Conclusion

Recent advances in recording techniques together with the development of molecular tools and transgenic mice have led to the identification of many molecular pathways that may be involved in OD plasticity (Hooks and Chen, 2007; Smith et al., 2009). Based on the current evidence, a model emerges of how plasticity is achieved in juvenile visual cortex: 1) first deprived-eye responses weaken through a homosynaptic LTD and the deprived-eye afferents become functionally decoupled from cortical neurons, leading to loss of visual function, 2) an overall decrease in visual drive leads to a drop in the threshold for induction of plasticity, possibly through a modification of the

ratio of NR2 subunits of the NMDA receptor, enabling potentiation of the weak inputs, and 3) correlated activity through the open eye allows strengthening of these inputs via a homosynaptic potentiation, possibly LTP or synaptic scaling. While this model is overall plausible, based on available experimental evidence, its specific predictions remain to be tested.

Although a large number of studies agree that visual cortical plasticity is most robust in juvenile cortex, the idea of a strictly defined critical period has been challenged by the demonstration of a great capacity for plasticity in the adult brain, which may be in part gated by an increased level of GABAergic inhibition. Whether critical periods have a stricter onset and termination in some species, but not in others and whether the molecular rules of adult plasticity are the same as the rules governing juvenile plasticity remains to be resolved.

Advancement in our understanding of mechanisms underlying these forms of plasticity will ultimately lead to improved treatments of neurological disorders involving disruptions in neuronal circuitry, such as amblyopia. Moreover, the discovery of residual plasticity in the adult brain has been especially encouraging, and further work to discover the mechanisms responsible for this plasticity will offer the potential for new therapeutic strategies for reorganizing neuronal circuits in the mature nervous system.

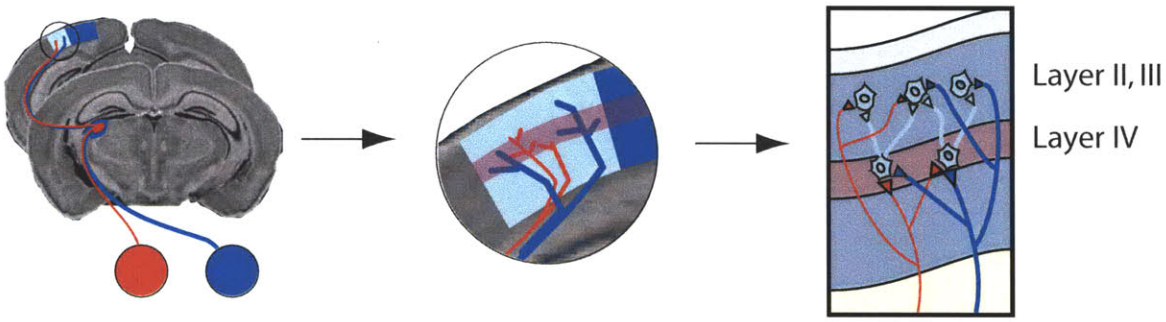

Figure 1.1 – Organization of the visual pathway in a mouse

Schematized organization of the visual pathway in a mouse visual system is shown. Visual information is transmitted from both retinas (ipsilateral – red; contralateral – blue) to the dorsal lateral geniculate nucleus (dLGN) of the thalamus. Eye-specific thalamic afferents make synaptic connections in the primary thalamorecipient layer 4 of the primary visual cortex. Note that there is also thalamic input into layers 2/3. Part of the primary visual cortex (V1) that receives both contralateral- and ipsilateral-eye projections is the binocular segment, occupying roughly one-third of the total V1 area. Convergence of monocular inputs occurs in layer 4, in contrast to higher mammals, like cats and primates, where eye-specific thalamic inputs into layer 4 remain segregated and first converge only in layers 2/3. Parts of the figure are adapted from Coleman *et al.*, 2010.

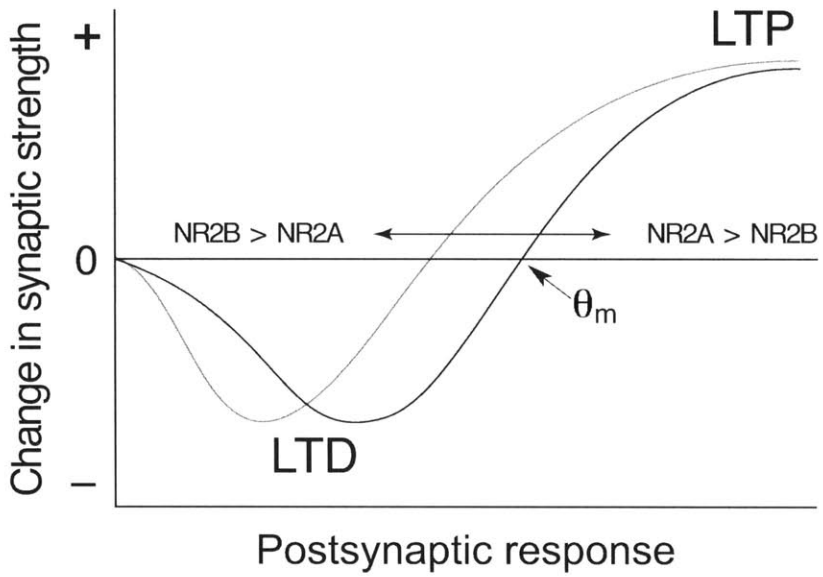

Figure 1.2 – BCM model for bidirectional synaptic plasticity

Model for the relationship between the strength of postsynaptic activation and long-lasting changes in synaptic strength. Low levels of conditioning stimulation evoke low levels of postsynaptic activation and lead to synaptic weakening (LTD), while high levels of stimulation favor synaptic strengthening (LTP). The LTD-LTP crossover point is the sliding modification threshold (θ_m). The value of θ_m is bidirectionally modifiable. Perturbations in visual experience, such as dark rearing, monocular or binocular deprivation can lower the value of θ_m, sliding the BCM curve to the left, thus increasing the likelihood that synaptic strengthening will occur. Conversely, light exposure following a period of dark rearing slides the curve to the right, favoring induction of LTD over LTP. The model also proposes the NMDAR subunit regulation underlies the modification of θ_m. Decreases in the ratio of NR2A to NR2B NMDAR subunits is depicted as a leftward shift in the modification curve (gray line). This leftward shift facilitates synaptic potentiation at intermediate levels of postsynaptic activation. Figure is based on data from Kirkwood *et al.*, 1996 and Philpot *et al.*, 2001, 2003, and 2007.

Chapter 2

Relative contribution of feed-forward excitatory connections to expression of ocular dominance plasticity in layer 4 of mouse visual cortex

Portions of this chapter were published together with Dr. Kathleen K. A. Cho, and Dr. Mark F. Bear in Neuron (2010) Vol.66, pp. 493-500.

2.1 Abstract

Brief monocular deprivation (MD) shifts ocular dominance (OD) in primary visual cortex by causing depression of responses to the deprived eye. Here we address the extent to which the shift is expressed by a modification of excitatory synaptic transmission. An OD shift was first induced with 3 days of MD, and then the influences of intracortical polysynaptic inhibitory and excitatory synapses were pharmacologically removed, leaving only "feed-forward" thalamocortical synaptic currents. The results show that the rapid OD shift following MD is strongly expressed at the level of thalamocortical synaptic transmission.

2.2 Introduction

A robust example of experience-dependent brain plasticity first described by Hubel and Wiesel is the ocular dominance (OD) shift in the primary visual cortex following monocular deprivation (MD). Temporary closure of one eyelid results in a selective and persistent decrease in cortical responsiveness to stimulation of the deprived eye (Wiesel and Hubel, 1963; Mioche and Singer, 1989). The OD shift following brief MD has now been documented in a number of species with various methods, including single unit recordings, intrinsic signal imaging, visually evoked potentials (VEPs), two-photon calcium imaging and immediate-early gene induction (Mioche and Singer, 1989; Mower, 1991; Gordon and Stryker, 1996; Antonini et al., 1999; Frenkel and Bear, 2004; Tagawa et al., 2005; Hofer et al., 2006a; Mrsic-Flogel et al., 2007). With long periods of MD, there are clear and well-documented structural changes in the excitatory thalamocortical inputs to cortex (Shatz and Stryker, 1978; LeVay et al., 1980). However, the OD shift measured electrophysiologically saturates with just a few days of MD, well before structural plasticity has reached an asymptote. Thus, rapid changes in synaptic transmission are among the first consequences of deprivation, but the precise nature and locus of these early changes remain unclear and have been vigorously debated for many years.

The simplest explanation for the selective loss of responsiveness to deprived-eye stimulation is the depression of excitatory synaptic strength by afferents conveying information from this eye (reviewed by (Smith et al., 2009)). However, an alternative hypothesis—first proposed over 30 years ago (Duffy et al., 1976; Burchfiel and Duffy, 1981)— is that adjustments in the strength of cortical inhibition are induced by MD and

these lead to selective suppression of responses evoked by deprived eye stimulation. The notion of inhibitory plasticity has recently been supported by several studies in rodent visual cortex. By recording from synaptically coupled pairs of neurons in cortical layer 4, it was shown that brief visual deprivation increases the strength of inhibitory, $GABA_A$ receptor-mediated synaptic responses (Maffei et al., 2006). In addition, by measuring the early consequences of MD in different classes of cortical neurons *in vivo*, it has been shown that responses of inhibitory cells are also modified by deprivation (Gandhi et al., 2008; Yazaki-Sugiyama et al., 2009; Kameyama et al.).

The precise contribution of inhibitory plasticity to the OD shift following MD is unclear, however. One view is that adjustments in inhibition following MD are ancillary, serving mainly to facilitate Hebbian plasticity of excitatory connections (Gandhi et al., 2008). According to this model, the OD shift is *expressed* by changes in the strength of excitatory synaptic connections, but at a rate that is determined in part by inhibitory tone. The alternate view is that the early OD shift is actually a consequence of inhibitory suppression of deprived-eye responses in visual cortex (Duffy et al., 1976; Burchfiel and Duffy, 1981; Maffei et al., 2006; Yazaki-Sugiyama et al., 2009). The latter model predicts that acute relief from intracortical inhibition would preferentially restore responsiveness to the deprived eye and at least partially reverse the OD shift.

In this study, we attempt to parse the relative contribution of excitation and inhibition to *expression* of the OD shift in mice. Our approach was to first induce an OD shift with 3 days of MD, and then acutely probe for its persistence under conditions of either reduced cortical inhibition, produced by the $GABA_A$ receptor antagonist bicuculline methiodide (BMI), or silenced polysynaptic intracortical transmission,

produced by the $GABA_A$ receptor agonist muscimol (Liu et al., 2007). We found that the OD shift induced by brief MD persists in the absence of cortical inhibition and is supported fully by the decrease in excitatory synaptic transmission at the thalamocortical synapse.

2.3 Results

2.3.1 Effect of reduced cortical inhibition on expression of the ocular dominance shift following monocular deprivation

In rodents, it has been shown that brief MD causes changes in the strength of inhibitory synaptic transmission in cortical layer 4 (Maffei et al., 2006). Our objective was to test the hypothesis that these changes contribute to expression of the OD shift. To assess the strength of responses evoked by contralateral (C) and ipsilateral (I) eye stimulation, we monitored visually evoked potentials (VEPs). Because VEPs can be recorded through chronically implanted electrodes, it is possible to obtain measurements of absolute and relative visual responsiveness to each eye before and after MD in the same animals, without use of anesthesia (Sawtell et al., 2003; Frenkel and Bear, 2004). Changes in VEPs following MD agree both qualitatively and quantitatively with other measurements of visual responsiveness. Before MD, the C/I VEP ratio is approximately 2; when the contralateral eyelid is closed for 3 days, the ratio shifts to ~1 due to a selective loss of cortical responsiveness to the stimulation of the contralateral eye (Frenkel and Bear, 2004).

We initially used BMI to remove inhibition following MD, a traditional approach first introduced in the cat by Duffy, *et al.* (1976). If selective inhibitory suppression of excitatory responses occurs after MD in the mouse, we should observe a recovery of deprived eye responses relative to non-deprived eye responses during $GABA_A$ receptor

blockade—*i.e.*, the C/I ratio should increase towards the pre-MD value when inhibition is removed. A limitation of this approach is that it is impossible to completely eliminate inhibition without causing epileptiform activity in the cortex. This limitation is not specific to BMI; it applies to any approach that attempts to remove all inhibition while leaving polysynaptic excitatory transmission intact. Thus, the best outcome that could be achieved using this approach is a partial reversal of the OD shift caused by a partial reduction in inhibition.

With these caveats in mind, we set out to produce a maximal reduction in cortical inhibition. The experimental design appears in Figure 2.1. A guide cannula was chronically implanted in close proximity to the tip of the VEP recording electrode (separated by 640 ± 40 μm, n = 29). Drug infusion (0.5 μl of 10-500 μM BMI over 5 min) invariably increased the magnitude of the VEPs (Figure 2.2A), which ultimately recovered to baseline levels. In control (non-deprived) mice, we investigated the effects of various doses of BMI on visual responses and the C/I ratio (Figure 2.2B, Figure 2.3A, B). These experiments revealed that the maximum tolerated dose of BMI was 95 μM. Above that concentration we saw the abrupt emergence of epileptiform activity in visual cortex that obscured visual responses (see Figure 2.2B). Infusion of 95 μM BMI still produced significant disinhibition as evidenced by an approximately 3-fold increase in VEP amplitude (Figure 2.3A; baseline = 207.7 ± 9.22 μV, peak = 639 ± 73.8 μV, washout = 201 ± 19.4 μV, n = 4, post hoc Fisher test, p = 0.001). Although the amplitude changes were substantial, BMI infusion in control mice did not alter the C/I ratio (Figure 2.3B; baseline = 2.22 ± 0.25, peak = 2.21 ± 0.21, washout = 2.44 ± 0.34, n = 4).

We then proceeded to infuse 95 μM BMI into visual cortex of mice that had been

monocularly deprived for 3 days to probe the persistence of the OD shift with reduced cortical inhibition. Three-day MD was chosen specifically because this is when the OD shift is asymptotic and is caused solely by loss of responsiveness to the deprived eye (Frenkel and Bear, 2004; Liu et al., 2008; Mrsic-Flogel et al., 2007; Sawtell et al., 2003).

As expected, before BMI treatment we observed depression of the contralateral deprived-eye VEP (246 ± 29.1 μV pre-MD vs. 120 ± 13.8 μV post-MD baseline, n = 7 post hoc Fisher test, p = 0.04) and a shift in the C/I ratio following MD (Figure 2.3C, D). BMI infusion caused an increase in the amplitude of the contralateral deprived-eye VEP (to 392 ± 47.4 μV) and the ipsilateral non-deprived eye VEP (to 361 ± 43.5 μV); and both responses recovered following washout (to 155 ± 19.8 μV and 161 ± 21.5 μV, respectively). Again, however, the C/I ratio appeared to remain stable before, during, and after BMI (pre-MD C/I = 2.3 ± 0.23; post MD = 0.77 ± 0.08; peak BMI = 1.1 ± 0.1; washout = 0.98 ± 0.06; n = 7, pre-MD value vs. all other values, p = 0.02, but post-MD baseline not significantly different from the BMI peak or the washout; Wilcoxon Signed Rank test).

2.3.2 Pharmacological isolation of a monosynaptic field potential in visual cortex in vitro

The possibility remained that residual inhibition in the presence of BMI might be sufficient to support selective suppression of the deprived eye response relative to the non-deprived eye response. We therefore decided to take a different approach: we asked if the full OD shift was already manifest by changes in "feed-forward" excitatory thalamocortical (TC) synaptic transmission after 3 days of MD.

To address this question, we adopted a clever method introduced recently by Liu *et al.* (2007) in mouse auditory cortex. Because sensory evoked potentials reflect

excitatory synaptic currents, blockade of spiking in cortical neurons should leave a residual field response that reflects the sum of TC synaptic input to cortex. Liu et al. (2007) showed that infusion *in vivo* of a cocktail of muscimol and SCH50911 (we term muscimol+) eliminates postsynaptic cortical spiking, but leaves intact the isolated TC synaptic response. Muscimol, a $GABA_A$ receptor agonist, silences spiking of all cortical neurons by strongly inhibiting them. SCH50911, a $GABA_B$ receptor antagonist, preserves TC synaptic transmission by preventing the non-specific activation by muscimol of presynaptic $GABA_B$ receptors.

To ensure that polysynaptic responses could be blocked while leaving intact monosynaptic responses, we examined the action of the muscimol+ cocktail in a cortical slice preparation. We performed field potential (FP) and whole-cell recordings in layer 4 in response to white matter stimulation, with bath application of SCH50911 (70 μM), followed by muscimol (50 μM), and finally by CNQX (10 μM), an AMPA receptor antagonist, to eliminate all synaptic activity. In current clamp recordings, stimulation of the white matter evoked a rapid EPSP followed by a disynaptic IPSP (Figure 2.4A,B). As expected, application of SCH50911 eliminated the slow $GABA_B$ receptor-mediated IPSP, but did not affect the EPSP or the early $GABA_A$ receptor mediated IPSP. Addition of muscimol to the bath eliminated all polysynaptic responses as evidenced by the complete loss of the disynaptic IPSP (peak IPSP in muscimol + SCH50911 = 1.65 ± 1.37 % of baseline, $n = 11$, $p < 0.0001$). However, a smooth EPSP remained, albeit at a moderately reduced amplitude (EPSP in muscimol + SCH50911: 71.42 ± 8.88 % of baseline, $n = 11$, $p = 0.002$). The remaining EPSP was virtually eliminated by addition of CNQX (EPSP in CNQX = 10.9 ± 1.84 % of baseline, $n = 11$, $p < 0.0001$). These

experiments indicate that the EPSP in the muscimol+ cocktail is monosynaptic—*i.e.*, it is evoked by direct electrical stimulation of axons making glutamatergic synapses on the recorded layer 4 neurons.

Stimulation of the white matter also evoked a negative-going FP in layer 4, believed to reflect summed excitatory synaptic currents. Bath application of SCH50911 alone did not alter FP responses (Figure 2.4D, E; 100.22 ± 3.4 % of baseline, n = 9) and, although reduced in amplitude, a clear FP response also remained following addition of muscimol to the bath (Figure 3E; 47.1 ± 5.7 % of baseline, n = 7, $p < 0.0001$). Bath application of CNQX eliminated this remaining FP, showing that it is indeed synaptic (Figure 2.4E; 11.5 ± 11.5 % of baseline, n = 5, $p < 0.0001$). These experiments strongly support the claim by Liu *et al.* (2007) that it is possible to isolate a monosynaptic excitatory FP by suppressing spiking of cortical neurons with a muscimol+ cocktail.

It is interesting to note that the latency to the peak negative FP did not change after isolating the monosynaptic component (Figure 2.4F; baseline: 5.48 ± 0.29 ms, n = 9; SCH50911: 5.30 ± 0.25 ms, n = 9, p = 0.67; muscimol + SCH50911: 5.31 ± 0.36 ms, n = 7). This observation suggests that even under control conditions, the peak negative FP reflects monosynaptic activation. Much of the reduction in FP amplitude during muscimol+ can be explained by shunting of depolarizing currents caused by tonic activation of $GABA_A$ receptors.

2.3.3 Contribution of modified thalamocortical input to the ocular dominance shift following 3 days of monocular deprivation

Next, we tested whether the application of the muscimol+ cocktail *in vivo* also eliminates intracortical activity, while preserving a measurable TC response. The cocktail was infused into the binocular segment of primary visual cortex according to the

exact protocol established by Liu et al. (2007), and we recorded single-unit activity at multiple cortical depths using a multichannel array ("stagger dagger") electrode (Liu et al., 2008). The approximate distance between the infusion cannula and the electrode was 500 μm, and the electrode sampled units in all layers. Treatment eliminated all spiking activity in the large majority of units (Figure 2.5A, B). On rare occasions a unit continued to fire post drug applicaton. Analysis of the spike waveforms revealed that these non-responding units had extremely narrow spike widths (Figure 2.5B, inset) and were largely monophasic; histological reconstruction showed they were located in layer 4. These findings indicate that these are afferent axons (Hubel and Wiesel, 1968; Chapman et al., 1991) which, indeed, should not be affected by the drug cocktail.

We also took advantage of the cellular activity reporter protein c-fos to determine the spatial extent of the cortical activity blockade following muscimol+ infusion. Animals were placed in the dark for 24 hours to reduce c-fos expression. Visual cortex of one hemisphere was then infused with the muscimol+ cocktail (the other hemisphere serving as control) and the animals were exposed to light to induce c-fos in active neurons. Following one hour of light exposure, animals were euthanasized and perfused transcardially with fixative (Figure 2.6A). After histological processing, c-fos expression in both hemispheres was examined by immunofluorescence. As shown in Figure 2.6B, we found that muscimol+ treatment blocked c-fos induction in all cortical layers over a wide spatial extent (essentially all of V1). Therefore any synaptic response remaining in visual cortex following mucimol+ treatment is unlikely to derive from intracortical connections.

Infusion of the muscimol+ cocktail reduced the trough-to-peak amplitude of the

VEP but did not change the latency to the peak negativity, similar to what was observed in the slice preparation (Figure 2.4D, E). The negative-going VEP had a simple morphology in the presence of the drug, similar to what was reported by Liu et al. (2007) and consistent with the interpretation that this is a monosynaptic field EPSP evoked by TC inputs (Figure 2.7A,B). The residual VEP in muscimol+ was completely eliminated by subsequent infusion of CNQX (Figure 2.8A; baseline: 217 ± 22.4 μV; muscimol+: 74.0 ± 8.53 μV; CNQX: 16.4 ± 1.30 μV; n = 5; post-CNQX value was significantly reduced from both baseline ($p < 0.001$) and muscimol+ ($p = 0.003$), post hoc Fisher test), confirming that this response reflects excitatory postsynaptic currents. Furthermore, the residual VEP in muscimol+ was also eliminated by silencing the ipsilateral lateral geniculate nucleus (LGN) with a stereotaxic injection of muscimol (Figure 2.8B; baseline: 207 ± 13.2 μV; cortical muscimol+: 77.0 ± 3.5 μV; LGN muscimol: 16.0 ± 4.9 μV; n = 3; post-LGN injection value was significantly reduced from both baseline ($p = 0.006$) and muscimol+ ($p = 0.01$), post hoc Fisher test). This finding supports the interpretation that the VEPs are of thalamocortical origin and rules out any contribution of interhemispheric input via the corpus callosum (Restani et al., 2009). As an additional confirmation that the residual VEP reflects direct TC input, we performed an analysis of response latency before and after muscimol+ infusion. If muscimol+ treatment were to unmask long-tract, cortico-cortical inputs, we should observe a shift to longer latencies due to the requirement for polysynaptic transmission and impulse conduction over greater distances. However, there was no shift to longer latencies after muscimol+ (Figure 2.7C, D), supporting the interpretation that the VEPs in muscimol+ cocktail are solely of thalamocortical origin.

Although the VEP amplitude is reduced by muscimol+, in control (non-deprived) mice there was no significant effect on the C/I ratio (Figure 2.9A; baseline C/I = 2.2 ± 0.13; muscimol+ = 2.0 ± 0.21 (peak), n = 7, Wilcoxon Signed Rank test). This finding is consistent with the conclusion that the baseline C/I ratio in mouse visual cortex reflects mainly the differential innervation by thalamic (lateral geniculate nucleus) axons serving the two eyes (Coleman et al., 2009).

To test whether the expression of the full OD shift is apparent in the TC component of the VEP, we induced the OD shift with 3 days of MD and then infused the muscimol+ cocktail to probe for the persistence of the shift in the absence of all polysynaptic cortical activity (Figure 2.9B). Before drug infusion, we observed deprived-eye depression of the VEP (232 ± 13.3 μV pre-MD vs. 160 ± 12.7 μV post-MD baseline, n = 5 post hoc Fisher test, $p < 0.001$) and a shifted C/I ratio. Following muscimol+ application, the deprived eye response decreased to 58.6 ± 2.42 μV; however, the shifted C/I ratio was not significantly altered by muscimol+ infusion (C/I = 2.8 ± 0.24 pre-MD vs. 1.1 ± 0.14 post-MD baseline, and 1.4 ± 0.14 during muscimol+, n = 5, pre-MD value was significantly different from all other values, $p = 0.04$, but post-MD baseline was not significantly different from the muscimol+ value, Wilcoxon Signed Rank test). Thus, the effect of MD on OD appears to manifest fully at the level of thalamocortical excitatory transmission.

2.4 Discussion

To our knowledge, the current work represents the first demonstration that the rapid OD shift caused by brief MD is expressed by a modification of TC synaptic transmission. Elimination of all polysynaptic, local circuit influences—both excitatory

and inhibitory—had no significant effect on the magnitude of the OD shift as reflected by the C/I ratio of the VEP. These data argue strongly that a major locus of ocular dominance plasticity is the excitatory synapses formed by lateral geniculate axons onto cortical neurons. Understanding how these synapses modify likely holds the key to understanding how deprivation leads to permanent visual disability.

When considering the meaning of the current findings, it is appropriate to draw a distinction between induction and expression of the OD shift. A host of mechanisms have been implicated in induction (*e.g.*, NMDA receptors), which may determine whether OD plasticity can occur and may influence the qualities of the OD shift (Smith et al., 2009). Expression mechanisms, on the other hand, are the molecular and synaptic changes that allow the shift to be measured and maintained. The mechanisms mediating the expression of the rapid OD shift have been debated for decades.

Early studies in young kittens that were monocularly deprived for weeks found a significant rearrangement of thalamocortical afferents where the area of cortex serving the deprived eye shrinks, while the cortical area serving the open eye expands (Shatz and Stryker, 1978). Furthermore, electrophysiological recordings provided strong evidence for considerable weakening of the deprived-eye inputs following MD (Tsumoto and Suda, 1978; Mitzdorf and Singer, 1980). However, an alternative hypothesis of interocular inhibition was put forth based on an observation that following a period of MD, enucleation of the non-deprived eye leads to a substantial recovery of cortical responses driven by the deprived eye (Kratz and Spear, 1976). This idea was further investigated by Duffy et al who intravenously infused BMI into visually deprived kittens and observed that the drug restored deprived-eye responsiveness to as many as 50% of

cortical neurons (Duffy et al., 1976). The finding was additionally strengthened by demonstrating that local iontophoretic administration of BMI in the cortex also restored deprived-eye responsiveness (Burchfiel and Duffy, 1981).

It was not clear, however, if these observations reflect plasticity of inhibitory connections. For example, even in cats with normal vision, visually unresponsive cells become responsive following application of intracortical BMI (Sillito, 1975a). Indeed, a follow-up study in monocularly deprived kittens by Sillito et al. (1981) concluded that although subliminal excitatory influences could be revealed by reducing inhibition, this effect was not specific to the deprived eye. Considered together with contemporaneous studies which failed to detect sprouting of inhibitory neurons or to detect any changes in strength of inhibitory synaptic transmission following MD, scientific consensus grew that the intracortical inhibition has a passive, rather than active role in the OD shift resulting from MD (Singer, 1977; Sillito et al., 1981; Bear et al., 1985).

With the advent of rodent models, much more sophisticated mechanistic studies of OD plasticity became feasible. One such study revisited the hypothesis that intracortical inhibition may be responsible for maintaining the OD shift by focusing on changes in intracortical inhibition in layer 4 following MD (Maffei et al., 2006). Maffei et al studied synaptically coupled pairs of neurons in visual cortex *ex vivo* and found that following deprivation there is an increase in the inhibitory tone via strengthening of excitatory cell connections onto inhibitory interneurons and vice versa, but no change in the strength of excitatory connections onto other excitatory cells. The strengthening of inhibitory transmission occurs through a novel form of potentiation (LTPi), which was occluded by prior MD.

While the Maffei et al (2006) study provides important validation for the concept of experience-dependent plasticity of intracortical inhibition, the relevance to OD plasticity remains to be determined. The experiments were performed in the monocular segment of visual cortex, receiving input only from the contralateral eye. It is not known if monocular and binocular segments of cortex modify according to the same rules and mechanisms. Moreover, even if they do, the observations by Maffei et al may have more relevance to the effect of total visual deprivation than to the OD shift caused in binocular cortex by MD.

Several very recent reports have addressed the question of how inhibitory interneurons respond to MD in binocular visual cortex of the mouse *in vivo*. Although there is consensus that the visual responsiveness of interneurons is modified by deprivation, these studies disagree on both the qualities and the rate of the OD shift in these cells (Gandhi et al., 2008; Yazaki-Sugiyama et al., 2009; Kameyama et al.). Of greatest relevance to the current findings, Yazaki-Sugiyama et al. (2009) examined the effect of intracellular infusion of picrotoxin on the OD shift expressed by cortical neurons after 3 days of MD. This treatment was designed to block inhibition by suppressing membrane chloride conductance. Although the picrotoxin often changed the eye dominance of responses in individual neurons, on a population level there was no effect on the OD shift.

Thus, it continues to be an open question of whether modifications of inhibitory synaptic transmission play a role in the selective depression of deprived-eye responses. Our findings certainly do not rule out the possibility of inhibitory plasticity in binocular cortex. However, it seems unlikely that inhibitory plasticity plays a major role in

expression of the OD shift, which appears to be fully manifest by a change in TC excitatory synaptic transmission, at least after 3 days of MD.

Our findings do not contradict abundant evidence for a role for inhibition in modulating the induction of ocular dominance plasticity. For instance, it was previously demonstrated that a level of inhibitory tone is required for the ocular dominance shift to occur (Ramoa et al., 1988; Hensch, 2005). Perhaps strengthening of inhibition precedes and is necessary for the subsequent modification of excitatory synapses that express the OD shift. Results of a recent study by Gandhi et al (2008) are consistent with this possibility. These investigators report that brief MD first produces a shift in the responses of excitatory cells well before a shift in the responses of inhibitory cells. Although contradictory reports have appeared recently {Yazaki-Sugiyama, 2009 #173; Kameyama, 2010 #201}, the findings of Ghandi et al. agree nicely with the early observation that disynaptic inhibition in the cortex is less affected by MD than monosynaptic excitation (Singer, 1977). Gandhi et al argue that the slow modification of inhibition could actually accelerate Hebbian long-term depression (LTD) of excitatory synapses by accentuating the decorrelation of pre- and postsynaptic responses to deprived-eye input activity.

The current findings support the conclusion that LTD at TC synapses is a primary cause of the OD shift after brief MD. It is noteworthy that the magnitude of the C/I ratio shift observed in the TC field EPSPs is as great as that observed in various measures of cortical neuronal responses, including single units and calcium spikes (Gordon and Stryker, 1996; Hofer et al., 2006a; Mrsic-Flogel et al., 2007). Thus, although modifications of intracortical connections clearly occur following deprivation, our data

suggest that the OD shift is already expressed fully by modification of feed-forward TC inputs.

To conclude, our study attempts to identify the mechanism which supports deprived-eye depression following a brief MD. We first induce an OD shift in the mouse visual cortex and then acutely block cortical inhibition or eliminate cortical activity altogether. We find that both of these manipulations do not alter the OD shift and do not restore visual responsiveness through the formerly deprived eye. Our data suggest that the enhancement of cortical inhibition is not the primary mechanism by which the deprived-eye depression is maintained. Furthermore, we directly demonstrate a change in the strength of thalamocortical synaptic transmission following MD and show that this change can fully account for the magnitude of the observed OD shift.

2.5 Methods

2.5.1 Animal preparation

Male C57Bl/6 mice were used for all experiments. Animals were group housed and kept on a 12hr light/dark cycle. All animals were treated in accordance with National Institutes of Health and Massachusetts Institute of Technology guidelines. Mice were anesthetized with 50 mg/kg ketamine and 10 mg/kg xylazine i.p. A local anesthetic, 0.1% lidocaine, was injected under the scalp. A head post was attached just anterior to bregma with cyanoacrylate glue (Small parts Inc., Miami Lakes, FL). Reference electrodes were placed bilaterally in prefrontal cortex. A small craniotomy (~1 mm) was made over binocular visual cortex (3 mm lateral to lambda), and tungsten microelectrodes (FHC, Bowdoinham, ME) were inserted 450 μm below the cortical surface. A second small craniotomy was made 0.5 mm lateral and 0.5 mm posterior to the electrode placement. A guide cannula (Plastics One, Roanoke, VA) was inserted 150 μm below the cortical surface at ~45° angle to the plane of electrode placement, thereby minimizing the distance between the tip of the electrode and the tip of the cannula. Electrodes and guide cannulae were secured in place by cyanoacrylate glue and dental cement (Lang Dental Inc., Nashua, NH). Animals were monitored postoperatively and were allowed at least 24 hr recovery period before habituation to the restraint apparatus. For single unit recordings, a custom-made multichannel linear array ("stagger dagger") consisting of eight tungsten microwires (Calilfornia Fine Wire, Grover Beach, CA) attached by instant adhesive were implanted and fixed in place with dental acrylic. Details of the stagger dagger electrode construction are described in Liu et al (2008).

2.5.2 Monocular deprivation

Mice were anesthetized by inhalation of isoflurane (IsoFlo 2%-3%) and placed under a surgical microscope. Lid margins were trimmed and antibiotic ophthalmic ointment (Vetropolycin, Pharmaderm) was applied to the eye. Eyelids were sutured with mattress stitches opposing the full extent of the trimmed lids using 6-0 vicryl. Mice were recovered by breathing room air and were monitored daily to ensure that the sutured eye remained shut and uninfected. Animals whose eyelids were not fully shut for the entirety of the deprivation period were excluded from the study. At the end of the deprivation period, mice were reanesthetized, stitches were removed, and lid margins were separated. Eyes were then flushed with sterile saline and checked for clarity under a microscope. Mice with corneal opacities or signs of infection were excluded from the study.

2.5.3 Visual stimuli

Stimuli consisted of full-field sine-wave gratings of 100% contrast, square-reversing at 1Hz, and presented at 0.05 cycles/degree. Stimuli were generated by a VSG2/2 card (Cambridge Research System, Cheshire, UK) and presented on a CRT computer monitor. VEPs were elicited by either horizontal or vertically oriented gratings. Orientation of stimuli during the first recording was randomized, but the stimuli presented to the same animal before and after deprivation were always orthogonal to each other to avoid the confound of stimulus-selective response potentiation(Frenkel et al., 2006). The display was positioned 20 cm in front of the mouse and centered at the vertical meridian, occupying 92° x 66° of the visual field. Mean luminance was 27 cd/m^2.

2.5.4 In vivo electrophysiology

All recordings were conducted in awake mice. The animals were alert and head-

restrained during recording. Following postoperative recovery, the animals were habituated to the restraint apparatus for 1-2 hrs. For recording sessions visual stimuli were presented to left and right eyes randomly. A total of 100 to 200 stimuli were presented per each condition. VEP amplitude was routinely quantified by measuring peak negativity to peak positivity of the response amplitude. For single-unit analysis, FPs and spike activity were evoked by sinusoidal gratings and collected simultaneously from all channels of the implanted multichannel array (Liu et al., 2008).

2.5.5 Infusion

On the day of the infusion, the dummy cannula was removed and replaced with a 33 GA infusion cannula, attached with tubing to a 100 μL Hamilton syringe (VWR, West Chester, PA). 0.5 μL of bicuculline methiodide (BMI; 10-500 μM; Sigma, St. Louis, MO), or a cocktail of muscimol (4 mM; Sigma, St. Louis, MO) and SCH50911 (6 mM; Sigma, St. Louis, MO), or cyano-7-nitroquinoxaline-2,3-dione disodium salt hydrate (CNQX, 3 mM; Sigma, St. Louis, MO)was infused with an infusion pump (VWR, West Chester, PA) over a 5 min period at a rate of 6 μl/hr. VEPs were recorded throughout the infusion and for at least an additional hour or, in the case of BMI infusions, until the drug washout was observed.

2.5.6 Cortical slice preparation

Following an overdose of barbiturates (i.p.), mice were decapitated upon disappearance of corneal reflexes in compliance with the U.S. Department of Health and Human Services. The brain was rapidly removed and immersed in ice-cold dissection buffer (composition: 87 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 75 mM sucrose, 10 mM dextrose, 1.3 mM ascorbic acid, 7 mM $MgCl_2$, 0.5 mM and

$CaCl_2$) bubbled with 95% O_2 and 5% CO_2. The visual cortex was rapidly removed and 350 μm coronal slices were cut using a vibrating microtome (Leica VT100S). Slices recovered for 15 min in a submersion chamber at 32 °C filled with warmed artificial cerebral spinal fluid (ACSF; 124 mM NaCl, 5 mM KCl, 1.25 mM Na_2PO_4, 26 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM dextrose, saturated with 95% O_2 and 5% CO_2) and then cooled gradually to room temperature until use.

2.5.7 Extracellular in vitro electrophysiology

Slices were transferred to an interface recording chamber maintained at 30 °C and perfused with ACSF at a rate of 2.5 ml/min. A stimulation electrode (concentric bipolar tungsten) was positioned in white matter, and a glass recording electrode (~1 MΩ) filled with ACSF was positioned in layer IV. The magnitude of responses evoked by a 200 μsec pulse was monitored by the amplitude of the FP. Stimulation intensity was adjusted to elicit half the maximal response, and stable baseline responses were elicited every 30 sec. Objective criteria (baseline drifts no greater than 5% and proper waveform alignment) were applied as inclusion criteria for further analysis. The data were normalized, averaged, and reported as means ± SEM. For the drug cocktail experiments, responses were recorded every 20 sec. 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).

2.5.8 Current-clamp recordings

The internal solution consisted of: 130 mM K-gluconate, 4 mM KCl, 2 mM NaCl, 10 mM HEPES, 0.2 mM EGTA, 4 mM Mg–ATP, 0.3 mM Na-GTP, 14 mM phosphocreatine, 0.2% biocytin, with pH adjusted to 7.26, and osmolarity adjusted to 296

mOsm using ddH_2O. Pipette resistances were ≈6 MΩ when filled with internal solution. For current-clamp recordings, stimuli (0.2 msec) were delivered at 0.05 Hz. Recordings were considered acceptable if membrane potentials were maintained between -55 and -70 mV. To clearly resolve EPSPs and IPSPs, depolarizing current was passed through the pipette to maintain the membrane potential at –40 mV. At least 5 stable responses were collected before and after infusion of SCH50911, muscimol, and CNQX (Sigma, St. Louis, MO). EPSPs and IPSPs were acquired and analyzed via pClamp and Clampfit software. The reversal potential of chloride of these solutions is –82 mV. All neurons studied fell within the borders of layer 4, as identified by trans-illumination. In addition, neurons were routinely filled with biocytin and confirmed to be in layer 4 and to have spiny dendrites.

2.5.9 Data analysis

The peak BMI-evoked response was the maximum observed response (a summed mean response over 100 stimulus presentations) after infusion of the drug and usually occurred within a window of 20-30 min after the start of infusion. In the muscimol+SCH50911 experiments, all responses over an hour of recording were summed and the mean was reported. All statistical analyses were performed using StatView 5.0.1 (Abacus Concepts, Berkeley, CA). A Student's paired t-test or a global ANOVA was always performed where appropriate, and relevant post hoc comparisons were made using Fisher's protected least square difference analysis. In all cases, significance was set at $p < 0.05$. For single-unit analysis, spikes were sorted using offline discrimination of single unit activity that was based on waveform shape, while multiunit activity was excluded. Firing rate was calculated as the (total number of spikes)/(total

recording time). Single-unit firing rates and C/I ratios were not normally distributed (Lillie test for normality of distribution), therefore statistics were performed using Wilcoxon Signed Rank test, Kruskal-Wallis test and Mann-Whitney U test.

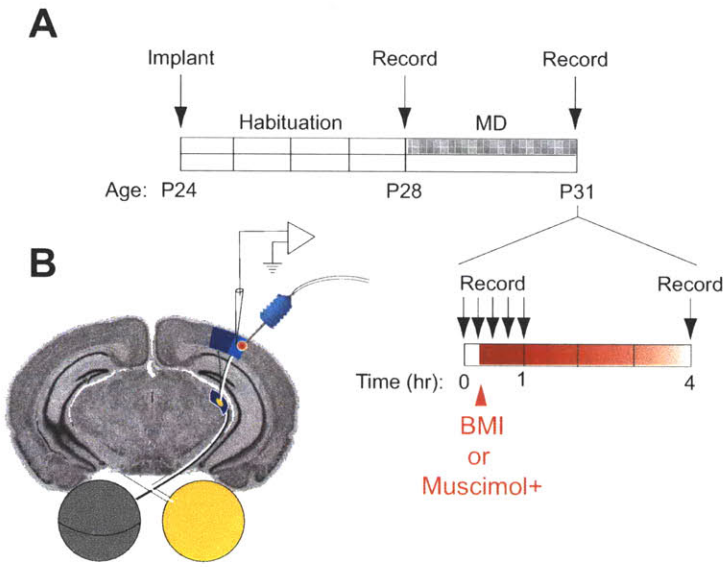

Figure 2.1 – Experimental design

A. Mice were chronically implanted with an electrode and a cannula in binocular visual cortex at P24. Following habituation to the recording apparatus (P25-27), baseline VEPs were recorded at P28 and mice were monocularly deprived for 3 days. At P31, the eye was reopened and VEPs were recorded. After baseline recordings, 0.5 μL of BMI or muscimol+SCH50911 (hereafter referred to as muscimol+) was infused at a rate of 6 μL/hr for 5 min and VEPs were continuously recorded for 1 hour. Final recordings (for BMI only) were made approximately 4 hrs post drug infusion in order to allow for a complete washout of the drug. B. Diagram of recording electrode and cannula placement in the visual cortex. The recording electrode was implanted at a depth of 450 μm and the cannula was implanted at a depth of 150 μm at a 45° angle to the electrode.

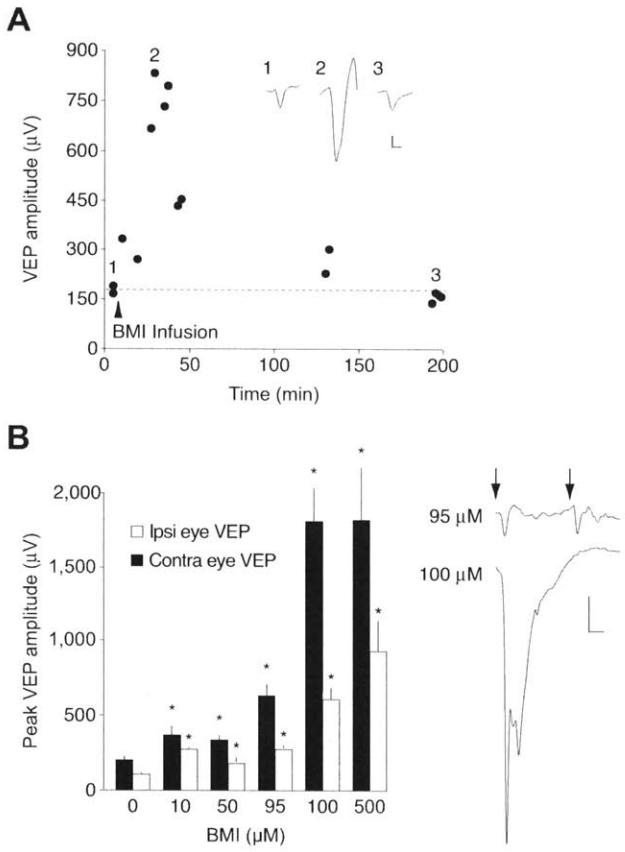

Figure 2.2 – Changes in VEP amplitude in response to BMI application

A. A representative example of the change in VEP amplitude in response to visual stimulation of the contralateral eye after infusion of 95 μM BMI. Traces represent baseline contralateral VEP prior to BMI application (1), peak VEP following BMI (2) and VEP after drug washout (3). Scale bar: 0.1 mV, 25 ms. B. Peak VEP amplitudes before and after infusion of increasing concentrations of BMI. "0 μM" represents the average pre-infusion VEP amplitude for all BMI concentrations. The increase in the contralateral and ipsilateral eye VEP amplitudes is significant across all drug concentrations ($n = 4$-8 for all groups, $p < 0.05$, one-way ANOVA, stars indicate a significant difference of the contralateral and the ipsilateral VEP from baseline respectively). Right panel shows representative VEP traces evoked by 1Hz visual stimulation (arrows indicate time of stimulus reversal). 95 μM BMI produces an increased, but faithful VEP response to each stimulus reversal, whereas 100 μM BMI produces an epileptiform discharge. Scale: 0.5 mV, 100 ms.

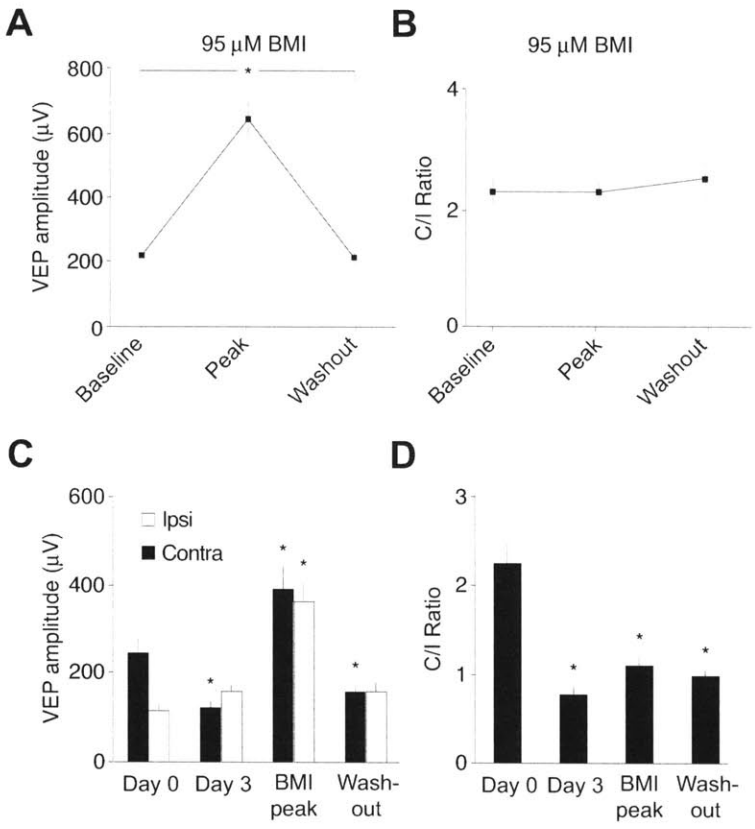

Figure 2.3 – Reducing cortical inhibition does not alter the OD shift that accompanies brief MD

A. Application of BMI in non-deprived mice increases VEP amplitude, which return to baseline levels following washout of the drug. Peak amplitude of VEPs during BMI application was significantly different from both baseline and washout ($n = 4$, post hoc Fisher test, $p < 0.05$). B. Application of BMI in non-deprived mice has no effect on the C/I ratio ($n = 4$, repeated measures ANOVA). C. MD produces a depression of contralateral-eye responses (black bars), but no change in ipsilateral-eye responses (white bars; day 0 vs. day 3, $n = 7$). BMI infusion caused an increase in both contralateral- and ipsilateral-eye responses, which recovered following washout. * indicates a level of significance of $p < 0.05$ as compared to day 0 (post hoc Fisher test). D. There is a decrease in the C/I ratio after MD that remains constant throughout BMI infusion and after washout (day 3 baseline is not significantly different from the BMI peak or washout values and BMI peak is not significantly different from washout; all comparisons were made using post hoc Fisher test).

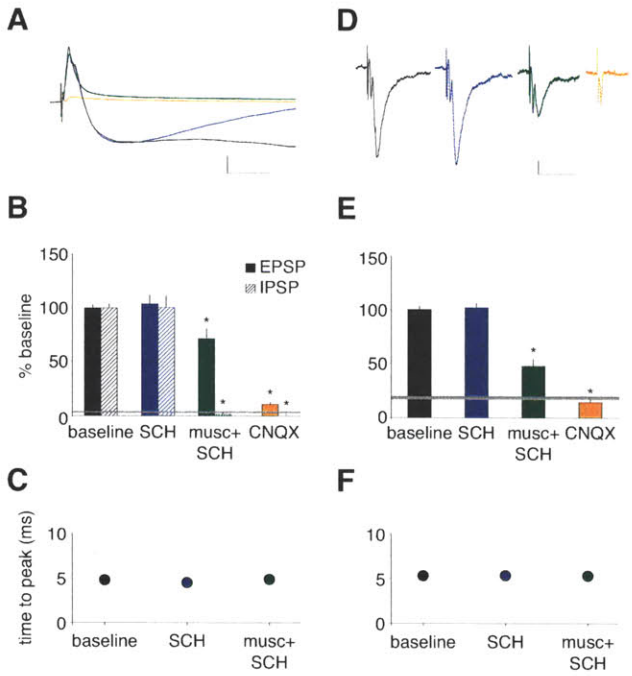

Figure 2.4 – Application of muscimol+ cocktail blocks polysynaptic transmission but preserves monosynaptic excitation in visual cortex *in vitro*

A-C. Current-clamp recordings of postsynaptic potentials evoked by WM stimulation. Neurons were maintained at –40 mV, just below spiking threshold, to clearly resolve EPSPs and IPSPs. A. Overlay of averaged PSP waveforms before (black) and after bath application of SCH50911 (blue), muscimol+SCH cocktail (green), and CNQX (orange). B. Normalized PSP amplitudes as a percentage of baseline (± SEM) before and after drug cocktail application. EPSP and IPSP amplitudes are not changed by SCH50911 application; EPSP amplitude is significantly reduced in the presence of muscimol+SCH ($n = 11$, $p = 0.002$), while IPSPs are completely eliminated ($n = 11$, $p < 0.0001$). Further application of CNQX abolished the remaining EPSP ($n = 11$, $p < 0.0001$). C. Averaged time to peak (± SEM) latencies of the EPSPs remain unchanged by the application of SCH50911 alone or muscimol+SCH ($n = 11$). D. Averaged FP waveforms before (black) and after bath application of SCH50911 (70 μM) (blue), muscimol (50 μM) +SCH50911 (green), and CNQX (10 μM) (orange). Scale bar: 25 μV, 25 ms. E. Normalized FP amplitude as a percentage of baseline (± SEM) before and after drug cocktail application. FP amplitude is not changed by SCH50911 application, but is significantly reduced in the presence of muscimol+SCH ($n = 9$, $p < 0.0001$). CNQX completely abolishes the remaining synaptic FP ($n = 5$, $p < 0.0001$). The short latency FP (< 3 msec) observed in CNQX is not synaptic, as it persists in 0 Ca^{2+} and under total pharmacological blockade of excitatory synaptic transmission (Bear et al., 1992; Kimura et al., 1989). This response is excluded from analysis. F. Averaged time to peak (± SEM) latencies of the synaptic FP remain unchanged by the application of SCH50911 alone or muscimol+SCH. (B,E) The grey line, indicating the limit of detection in our measurements, is the mean ± SEM fluctuation in voltage measured 5 ms prior to electrical stimulation (3.47% ± 0.62

for B and 18.5% ± 1.67 for E) taken across 5 ms prior to electrical stimulation. (This experiment was performed by Kathleen Cho.)

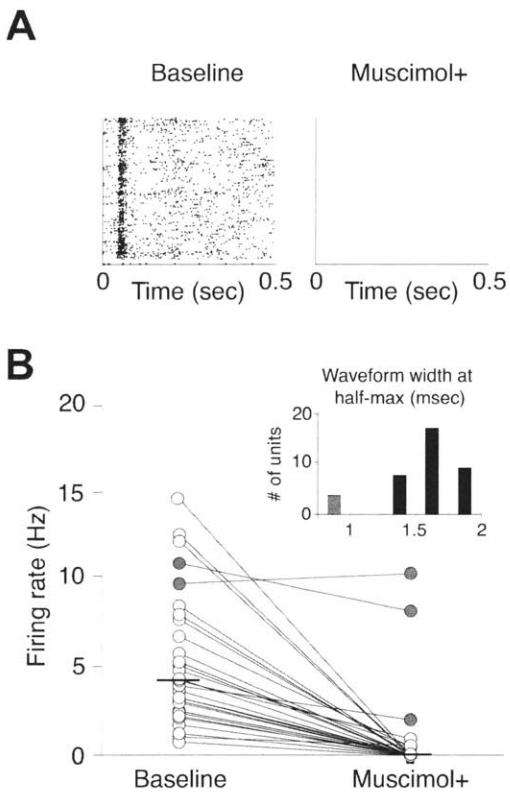

Figure 2.5 – Muscimol+ cocktail eliminates cortical spiking *in vivo*

A. Raster plot of a representative unit recorded before and after muscimol+ infusion demonstrating that cortical spiking was completely eliminated. B. Single unit firing rate is dramatically reduced following muscimol+ infusion (n = 29, black bars represent medians (4.4 and 0 for baseline and drug respectively); $p < 0.0001$, Mann-Whitney U test). Inset shows waveform width analysis of the units: filled gray circles correspond to the gray bar and the narrowest spike width. Data are plotted in 0.25 ms bins. Units were sampled in all layers: 11 in superficial layers, 8 in layer 4, and 9 in deep layers. Units unresponsive to muscimol+ were axons in layer 4.

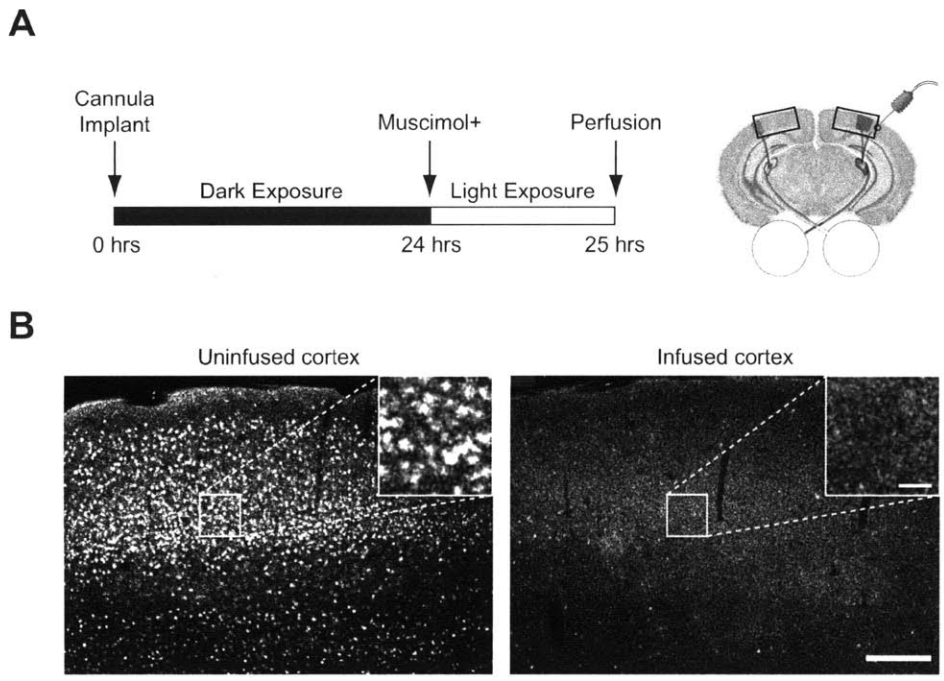

Figure 2.6 – Muscimol+ eliminates cortical activity over a wide spatial extent

A. Experimental design for induction of c-fos expression after muscimol+ infusion. 24 hour dark exposure of mice with cannulated mice was followed by infusion of 0.5 µl of muscimol+ and an hour of subsequent light exposure. Mice were then perfused and stained for c-fos. Black boxes on the diagram represent the regions that were analyzed for c-fos expression. B. c-fos expression is normal in the uninfused hemisphere (left) and completely abolished in the infused hemisphere (right). Scale: 100 µm. Insets represent enlarged areas from the control and infused cortices to clearly illustrate the absence of stained nuclei in the hemisphere treated with muscimol+. Scale: 15 µM.

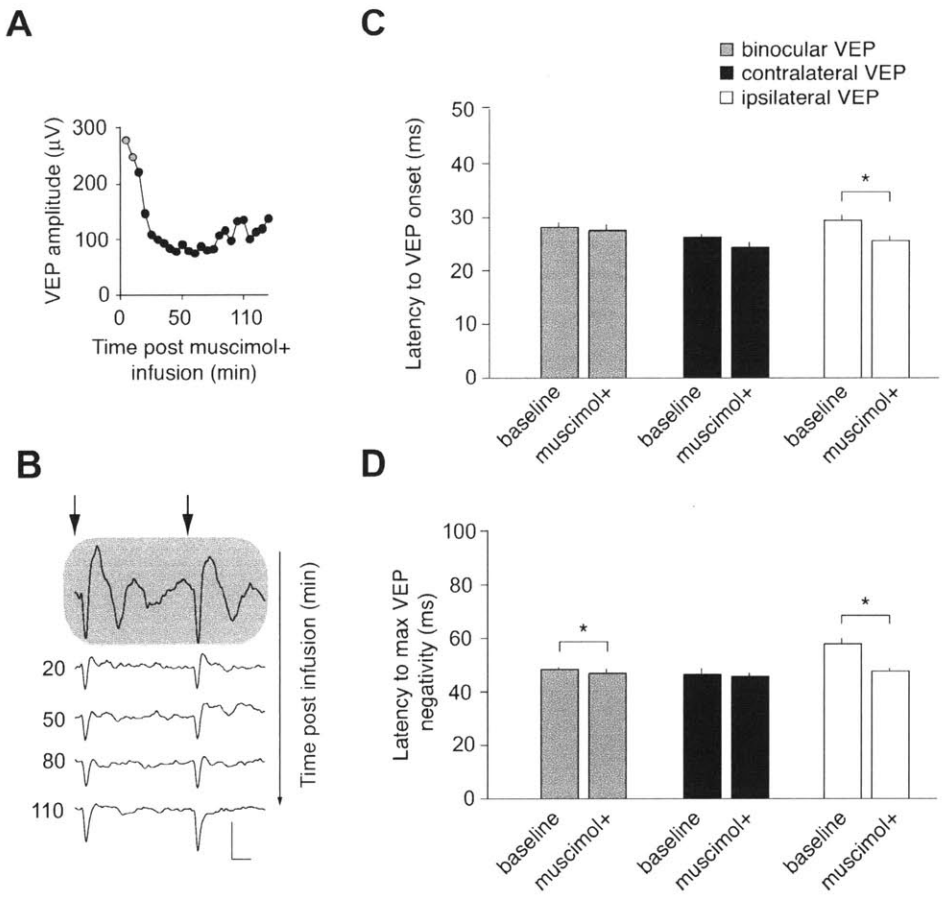

Figure 2.7 – Changes in VEP morphology and latency following muscimol+ infusion

A. A representative example of the change in VEP amplitude to binocular stimulation before and after infusion of muscimol+. Grey circles represent baseline VEP amplitude and black circles represent VEP amplitude following muscimol+ infusion. B. Representative VEP traces from (A) evoked by 1 Hz visual stimulation (arrows indicate time of stimulus reversal). Grey rectangle highlights the baseline VEP trace. Scale bar: 0.1 mV, 0.1 s. C. Measurements of latency to VEP onset before and after muscimol+ infusion (baseline 28 ± 0.8 ms (binocular – grey), 26 ± 0.8 ms (contralateral – black), and 30 ± 1 ms (ipsilateral – white); post-muscimol+: 28 ± 0.9 ms (binocular), 25 ± 1ms (contralateral), and 26 ± 1 ms (ipsilateral). Latency to binocular or contralateral VEP onset did not change significantly post muscimol+ infusion (Student's paired t test). Latency to ipsilateral VEP onset decreased ($p = 0.0001$, Student's paired t test). D. Measurements of latency to maximal VEP negativity before and after muscimol+ infusion (baseline: 50 ± 0.9 ms (binocular – grey), 48 ± 1 ms (contralateral – black), and 58 ± 2 ms (ipsilateral – white); post-muscimol+: 47 ± 1 ms (binocular), 46 ± 1 ms (contralateral), and 48 ± 1 ms (ipsilateral). Latency to maximal negativity of the contralateral VEP did not change post muscimol+ infusion, and decreased for both binocular and ipsilateral VEPs ($p = 0.01$ and $p = 0.001$ respectively, Student's paired t test). Error bars represent ± SEM.

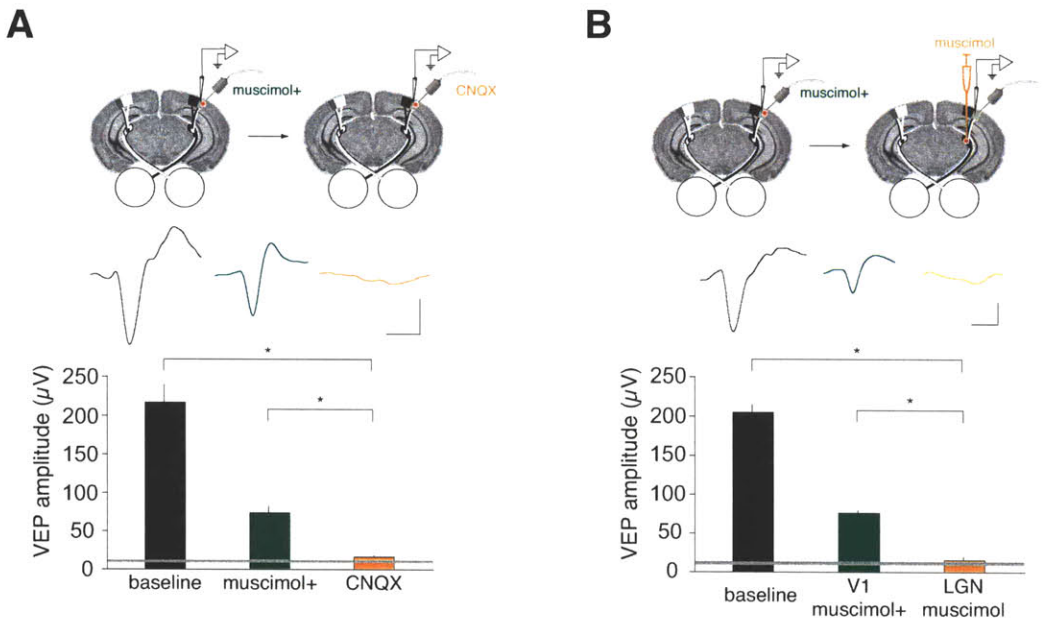

Figure 2.8 – Residual VEPs in muscimol+ reflect excitatory postsynaptic currents that originate in LGN

A. Traces indicate representative FPs evoked by contrast reversing grating stimuli during baseline (left), after infusion of muscimol+ (middle), and after infusion of CNQX (right). Scale: 50 μV, 50 ms. The baseline VEP is significantly reduced by infusion of muscimol+ and is completely eliminated by the infusion of CNQX. The grey line represents the limit of detection (see Figure 3). B. Traces indicate representative FPs evoked by contrast reversing grating stimuli during baseline (left), after infusion of muscimol+ into the cortex (middle), and after injection of muscimol into the ipsilateral LGN (right). Scale: 50 μV, 50ms. The baseline VEP is significantly reduced by infusion of muscimol+ and is completely eliminated by the injection of muscimol into LGN. The grey line represents the limit of detection.

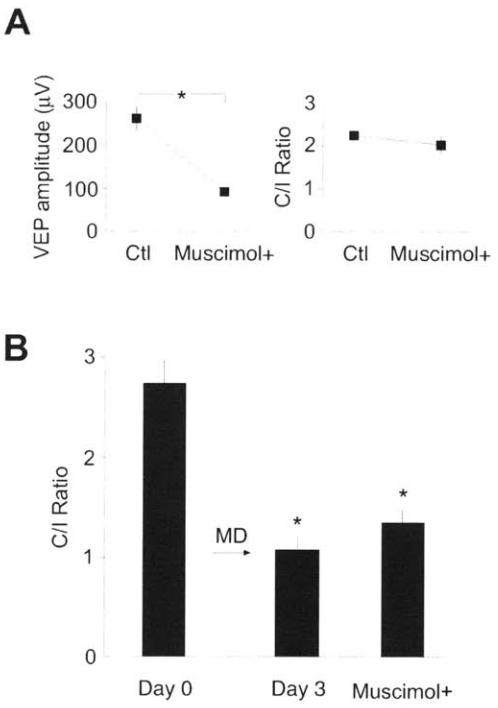

Figure 2.9 – The OD shift following brief MD is expressed in the isolated thalamocortical component of the VEP

A. Application of muscimol+ significantly reduces VEP amplitude (n = 7, paired t-test, p = 0.0004), but has no significant effect on the C/I ratio. B. The decrease in the C/I ratio following MD persists following acute muscimol+ infusion (day 3 baseline is not significantly different from the muscimol+ values (Wilcoxon Signed Rank test). * indicates a level of significance of $p < 0.05$ as compared to day 0.

Chapter 3

The ratio of NR2A/B NMDA receptor subunits determines the qualities of ocular dominance plasticity in visual cortex

*Portions of this chapter were published together with Dr. K.K.A. Cho, Dr. Benjamin D. Philpot, and Dr. Mark F. Bear in Proceedings of the National Academy of Sciences of the United States of America (2009) Vol. **106**, pp. 5377-5382.*

3.1 Abstract

Bidirectional synaptic plasticity during development ensures that appropriate synapses in the brain are strengthened and maintained, while inappropriate connections are weakened and eliminated. This plasticity is well-illustrated in mouse visual cortex, where monocular deprivation during early postnatal development leads to a rapid depression of inputs from the deprived eye and a delayed strengthening of inputs from the non-deprived eye. The mechanisms that control these bidirectional synaptic modifications remain controversial. Here we demonstrate, both *in vitro* and *in vivo*, that genetic deletion or reduction of the NR2A NMDA receptor subunit impairs activity-dependent weakening of synapses and enhances the strengthening of synapses. While brief monocular deprivation in juvenile wild-type mice normally causes a profound depression of the deprived eye response without a change in the non-deprived eye response, NR2A knockout mice fail to exhibit deprivation-induced depression and instead exhibit precocious potentiation of the non-deprived eye inputs. These data support the hypothesis that a reduction in the NR2A/B ratio during monocular deprivation is permissive for the compensatory potentiation of non-deprived inputs.

3.2 Introduction

The circuitry of primary visual cortex is susceptible to changes in sensory experience during early postnatal development, as evidenced by the well-studied paradigm of monocular deprivation (MD) (Hubel and Wiesel, 1970). MD and reverse occlusion studies demonstrate that the strength of synapses is bidirectionally modifiable (Blakemore and Van Sluyters, 1974; Movshon, 1976; Mioche and Singer, 1989). A detailed time course of the synaptic events following MD in mice shows that the initial consequence is a rapid depression of the deprived eye inputs followed by a delayed strengthening of the non-deprived eye inputs (Frenkel and Bear, 2004). However, little is known about the molecular mechanisms that regulate the susceptibility of synapses to bidirectional modifications in their strength.

Bidirectional synaptic plasticity has been studied in slice recordings of visual cortex in the form of long-term potentiation (LTP) and long-term depression (LTD), whereby synapses strengthen and weaken in response to stimulation (Kirkwood et al., 1993). These activity-dependent modifications can be modeled by a learning rule where high levels of postsynaptic activation (evoked electrically by high-frequency stimulation) induce LTP and smaller levels of postsynaptic activation (evoked electrically by lower frequency stimulation) induce LTD (Bienenstock et al., 1982). The crossover point from synaptic weakening to strengthening is called the modification threshold (θ_m). An important feature of this model is that the value of θ_m is not fixed; rather, its value can "slide" as a function of the history of postsynaptic activation. According to the BCM theory, closing the dominant contralateral eye first leads to depression of the deprived synapses, followed by a leftward shift in θ_m due to the reduction in average cortical

activity. This shift in θ_m is permissive for the subsequent increase in the responses to the non-deprived, ipsilateral eye (Frenkel and Bear, 2004).

A wealth of data now indicates that deprivation and experience during early postnatal development can indeed modify the plasticity threshold. For example, a period of complete darkness lowers the plasticity threshold such that LTP is enhanced and LTD is attenuated across a range of stimulation frequencies (Kirkwood et al., 1996; Philpot et al., 2003; Philpot et al., 2007). These observations demonstrate that the susceptibility of synapses to plastic changes in visual cortex modifies in relation to their history of experience-driven activity.

Data suggest that the shift in the modification threshold is caused by a change in *N*-methyl-d-aspartate (NMDA) receptor function (Philpot et al., 2003), and regulation of the molecular composition of the NMDA receptor provides a powerful means to achieve this change. The NMDA receptor is a heteromer that contains the obligatory NR1 subunit and a mixture of NR2A-D subunits that alter receptor properties (McBain and Mayer, 1994; Monyer et al., 1994). At birth, most cortical NMDA receptors contain the NR2B subunit (Monyer et al., 1994). NR2A subunit levels gradually increase with development and reach a maximal expression between the peak and end of juvenile plasticity (Flint et al., 1997; Roberts and Ramoa, 1999). This switch from predominantly NR2B to NR2A subtypes is experience-dependent and reflects the recent history of visual experience (Quinlan et al., 1999a; Quinlan et al., 1999b; Philpot et al., 2001a). During MD, after the initial depression of deprived eye responses, there is a transient reduction in the NR2A/B ratio that slightly precedes open-eye response potentiation (Chen and Bear, 2007). Because lowering the NR2A/B ratio reduces the threshold for inducing LTP in

mouse visual cortex (Philpot et al., 2007), it has been proposed that activity-dependent regulation of NR2A and/or NR2B receptor expression is the molecular basis for the sliding modification threshold.

In the current study we examined the connection between NMDA receptor subunit composition and the qualities of bidirectional synaptic plasticity in the visual cortex of NR2A knockout (KO), heterozygote (Het), and wild-type (WT) mice. We confirm in layer IV that reducing NR2A expression shifts to lower frequencies both the LTP threshold and the optimal stimulation for LTD. In response to MD, VEPs evoked *in vivo* through the deprived eye fail to depress normally in NR2A mutants. Instead, an ocular dominance shift occurs by precocious potentiation of responses through the non-deprived eye. These data support the hypothesis that experience-dependent modifications in the NR2A/B ratio at synapses provides a powerful *in vivo* mechanism for regulating subsequent induction of plasticity.

3.3 Results

3.3.1 Effect of NR2A gene dosage on the synaptic modification threshold in layer IV of mouse visual cortex

The goal of this study was to determine how decreasing the NR2A/B ratio alters the LTD-LTP modification threshold *in vitro* and compare this with changes in the properties of naturally occurring plasticity in the visual cortex *in vivo* as a consequence of MD. We examined this question using mice with targeted disruption of one or both alleles of the NR2A gene (Kadotani et al., 1996). Because NR2A mutant mice do not display compensatory alterations in NR1 or NR2B subunit expression in visual cortex at the ages of interest, reducing NR2A expression effectively changes the NR2A/B ratio (Philpot et al., 2007).

The bidirectional changes in visual responsiveness that occur after MD were established using visually evoked potentials (VEPs) recorded in layer IV of visual cortex (Frenkel and Bear, 2004). Current source density (CSD) analysis *in vivo* has confirmed that changes in the amplitude of layer IV VEPs reflect changes in synaptic current sinks in this layer (Sawtell et al., 2003; Liu et al., 2008). However, previous studies of LTP and LTD in NR2A mutant mice were performed in layer III (Philpot et al., 2007), and it is now understood that there are significant laminar differences in the mechanisms of visual cortical plasticity in mice (Crozier et al., 2007; Liu et al., 2008). Therefore, our study began with an analysis of the effect of NR2A gene dosage on synaptic plasticity in layer IV of slices of mouse visual cortex. A CSD analysis confirmed that the negative extracellular field potential (FP) evoked in layer IV of mouse visual cortex by white matter (WM) stimulation *in vitro* reflects a current sink qualitatively similar to the VEP *in vivo* (Figure 3.1). Therefore, we attempted to replicate in layer IV FPs the effects previously described in layer III of reducing NR2A on the LTD-LTP threshold (Philpot et al., 2007).

To test whether the plasticity threshold was altered by reducing NR2A gene dosage, we examined the consequences of a stimulation protocol (1 Hz for 15 min) in KO and Het mutants that typically results in LTD in normally reared WT mice (aged between postnatal days (P) 21-28). Following collection of a baseline, 1 Hz stimulation produced reliable depression in WT mice (Fig. $3.2A_1$; $83.03 \pm 2.56\%$ of baseline, $n = 8$ slices from 7 mice). However, as previously shown in layer III of the KO mouse, we discovered that 1 Hz stimulation causes LTP of layer IV FP amplitudes in mice lacking NR2A (Fig. $3.2A_1$; $111.39 \pm 2.33\%$ of baseline, $n = 9$ slices from 7 mice). Moreover, in the NR2A

Het mice, 1 Hz stimulation resulted in a modest depression of synapses (Fig. 3.2A_1; 93.77 ± 5.33% of baseline, n = 9 slices from 7 mice) that was intermediate between the WT (p = 0.034) and NR2A KO (p = 0.001) values. Importantly, basal synaptic transmission was comparable between genotypes (Fig, 3.2A_2) and there was no correlation between baseline FP amplitude and the percent change in synaptic transmission following 1 Hz stimulation (Fig. 3.2A_3). There was also no effect of genotype on AMPA receptor-mediated miniature EPSCs in layer IV neurons (NR2A KO: 18.58 pA ± 1.76, n = 10 mice; WT: 18.90 pA ± 1.30, n = 13 mice). Together, these results support previous conclusions that that the LTD-LTP threshold is proportional to the level of NR2A expression in mouse visual cortex (Philpot et al., 2007).

To confirm that the plasticity observed in the NR2A mutants was still NMDA receptor-dependent, we repeated the experiment in the presence of the competitive NMDA receptor antagonist APV (D-2-amino-5-phosphonopentanoic acid; 50 μM). In addition to the expected effect of APV on LTD in WT mice (n = 5 slices from 3 mice) (Crozier et al., 2007), we found that blocking NMDA receptors prevented both the residual LTD in the Het mice (n = 5 slices from 3 mice) as well as the LTP induced by 1 Hz stimulation in the KO animals (n = 5 slices from 3 mice; Fig. 3.2B).

Finally, to confirm that reducing NR2A caused a change in the induction requirements for LTD/P rather than a dose-dependent loss of LTD, we repeated the experiment using 0.5 Hz stimulation—a frequency that was shown previously to be optimal for LTD induction in visually deprived animals (Philpot et al., 2003). We found that 900 pulses at 0.5 Hz elicited reliable and statistically significant depression in mice of all genotypes, with the greatest effect in the NR2A KO (Fig. 3.2C; NR2A KO: 70.56 ±

6.41% of baseline, n = 10 slices from 5 mice; Het: 79.05 ± 6.86%, n = 6 slices from 3 mice; WT: 84.43 ± 5.64%, n = 6 slices from 3 mice). Taken together, these data lead us to conclude that lowering the NR2A/B ratio shifts the stimulation-response curve to the left, and the degree of this shift is proportional to the amount of NR2A present in visual cortex.

3.3.2 Effect of NR2A gene dosage on the ocular dominance shift following MD in layer IV of mouse visual cortex

We next examined the impact of altered NR2A and synaptic plasticity on ocular dominance plasticity in layer IV of mouse visual cortex. Electrodes were chronically implanted in layer IV of the binocular zone in primary visual cortex. Baseline VEPs were measured at P27-P29, and the eyelid of the eye contralateral to the experimental hemisphere was sutured closed. After three days of MD, the sutured eye was opened, the animal was allowed to recover from anesthesia, and VEPs were again recorded. We assessed ocular dominance plasticity by determining the ratio of contralateral to ipsilateral eye responses (C/I ratio), which is normally about 2:1 at baseline and decreases after MD to around 1:1. Our results show that NR2A KO and Het mice, as well as their WT littermates, exhibit a normal shift in the C/I ratio (Fig. 3.3A; day 0: 1.67 ± 0.21, day 3: 0.89 ± 0.13 in KO, n = 8, $p = 0.01$; day 0: 1.75 ± 0.09, day 3: 1.05 ± 0.14 in Het, n = 9, $p < 0.01$; day 0: 1.95 ± 0.22, day 3: 0.90 ± 0.13 in WT, n = 10, $p < 0.001$), similar to what has been reported previously (Fagiolini et al., 2003). The degree of the shift is indistinguishable among the three genotypes (Kruskal-Wallis test, n = 27; $p = 0.81$).

However, upon closer examination of the deprived and non-deprived eye responses, we discovered profound differences in the qualities of the ocular dominance shift between the genotypes (Fig. 3.3B). As previously reported (Frenkel and Bear,

2004), we found that deprived eye responses in WT mice were significantly depressed (Fig. 3.3B; day 0: 211.2 ± 17.1 μV; day 3: 127.6 ± 30.5 μV, n = 10, p = 0.002), and non-deprived eye responses remained at baseline levels (day 0: 114.5 ± 13.2 μV, day 3: 151.9 ± 26.4 μV, n = 10, p = 0.07). In stark contrast to WT mice, the deprived eye responses in NR2A KO mice were unchanged (Fig. 3.3B; day 0: 188.2 ± 19.7 μV, day 3: 202.6 ± 21.4 μV, n = 8, p = 0.55), whereas the non-deprived eye responses dramatically potentiated (day 0: 118.5 ± 11.6 μV; day 3: 247.9 ± 34.3 μV, n = 11, p = 0.005). Results in the Het mice were intermediate: there was still a significant depression of the deprived eye (Fig. 3.3B; day 0: 221.3 ± 14.7 μV, day 3: 180.0 ± 26.5 μV, n = 9, p = 0.04) and a slight but statistically significant potentiation of the non-deprived eye responses (day 0: 129.1 ± 10.4 μV; day 3: 175.2 ± 14.9 μV, n = 9, p = 0.03).

These findings are consistent with the idea that reducing the NR2A/B ratio promotes the deprivation-induced adjustment of the BCM modification threshold, and thereby enhances open-eye response potentiation and reduces deprived-eye response depression *in vivo*, similar to what we observed in the slice experiments. However, an alternative explanation is that the shift occurs normally, but is superimposed on an exaggerated global upward scaling of responses caused by visual deprivation. To investigate the possibility of enhanced synaptic scaling in response to deprivation, we recorded VEPs before and after 3 days of binocular lid suture in NR2A KO and WT littermates. An increased homeostatic scaling response should lead to substantially increased visual responses after binocular deprivation (BD).

Our results show that the C/I ratios of both NR2A KO and WT do not change following this visual manipulation (Fig. 3.4A; day 0: 2.16 ± .19, day 3: 1.92 ± 0.20 in

KO, n = 7; day 0: 2.34 ± 0.15; day 3: 2.57 ± 0.43 in WT, n = 7; p = 0.31). More importantly, BD did not affect the VEP amplitudes of contralaterally-projecting eyes (Fig. 3.4B; day 0: 228.4 ± 16.2 μV, day 3: 232.0 ± 35.5 μV in KO, n = 7; day 0: 236.5 ± 35.7 μV, day 3: 220.8 ± 35.1 μV in WT, n = 7; p = 0.72) nor the VEP amplitudes of ipsilaterally-projecting eyes (Fig. 3.4B; day 0: 111.9 ± 11.5 μV, day 3: 121.6 ± 12.0 μV in KO, n = 7; day 0: 99.2 ± 12.2 μV, day 3: 87.0 ± 6.8 μV in WT, n = 7; p = 0.26). These data indicate that reduction of NR2A does not promote synaptic scaling in response to 3 days of visual deprivation.

3.3.3 Open-eye potentiation in WT mice requires NMDAR activation

In WT mice, MD for > 5 days causes potentiation of visual responses that we hypothesize is enabled by a deprivation-induced decrease in the NR2A/B ratio (Chen and Bear, 2007). This hypothesis rests on the assumption that response potentiation is an NMDAR-dependent form of "Hebbian" synaptic plasticity (Bear, 2003; Smith et al., 2009). The alternative hypothesis is that responses increase by global upward scaling (Mrsic-Flogel et al., 2007), a process that has been shown to be independent of NMDAR activation (Turrigiano and Nelson, 2004). To distinguish among these hypotheses, we designed experiments in which NMDARs were blocked pharmacologically during the time span when response potentiation occurs (Fig. 3.5A).

Following 3 days of MD, which allowed for deprived eye depression, either CPP ((R, S)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid; 10 mg/kg) or saline were injected *i.p.* every 6 hours over the course of 4 additional days of MD. The saline controls showed a normal response to 7 day MD. First, deprived eye responses were significantly depressed relative to baseline (Fig. 3.5B; day 0: 198.2 ± 16.6 μV; day 7:

155.4 ± 17.7 μV, n = 7, p = 0.007), but as described in previous studies (Frenkel and Bear, 2004), this depression was less than that observed after 3 days MD (*cf.* Fig 3.3B, WT). Second, open-eye responses were significantly potentiated (Fig. 3.5B; day 0: 104.7 ± 13.0 μV; day 7: 163.0 ± 21.0 μV, n = 7, p = 0.035). In contrast, the mice that received CPP injections exhibited deprived eye depression similar to what is obtained after 3 days of MD (Fig. 3.5B; day 0: 200.4 ± 22.8 μV; day 7: 115.7 ± 31.8 μV, n = 7, p = 0.004), and the ipsilateral eye responses remained unchanged (Fig. 3.5B; day 0: 94.8 ± 6.1 μV; day 7: 106.3 ± 24.9 μV, n = 7, p = 0.62). The blockade of response potentiation with an NMDAR antagonist is not consistent with the scaling hypothesis.

3.4 Discussion

Our data show that even a graded reduction of the NR2A subunit can dramatically alter the qualities of NMDAR-dependent bidirectional synaptic plasticity in layer IV of visual cortex. Reduced NR2A expression shifts the LTD-LTP threshold to the left; consequently, some stimulation frequencies that would normally lead to LTD cause LTP instead. *In vivo*, the patterns of synaptic activity that normally cause depression of responses from the deprived eye no longer have that effect, and the patterns of synaptic activity through the open eye that normally have no effect, cause precocious potentiation of responses instead. Our results are consistent with the hypothesis that the NR2A/B ratio specifies the value of the synaptic modification threshold that choreographs the bidirectional cortical response to monocular deprivation.

A considerable body of work in the visual cortex has shown how the subunit composition of NMDARs varies during the course of early postnatal development and after periods of visual deprivation. As the cortex matures, the NR2A/B ratio

progressively increases, reaching an asymptote around the time of adolescence. This developmental profile is at least partially experience-dependent, as even brief episodes of visual deprivation can reversibly lower the NR2A/B ratio. Changes in NR2A and NR2B expression also occur during the course of MD. In the hemisphere contralateral to the deprived eye, the NR2A/B ratio is significantly reduced after 5 days of MD (Chen and Bear, 2007).

Three primary theories have been advanced in the literature regarding the possible significance of NMDAR subunit composition changes: (1) the subunit switch might bring the classically-defined critical period for ocular dominance plasticity to a close (Nase et al., 1999), (2) an increase in NR2A might favor the induction of LTP versus LTD (Liu et al., 2004; Massey et al., 2004), or (3) the activity-dependent increase in the NR2A/B ratio adjusts the threshold for synaptic plasticity and facilitates the refinement of receptive field properties in juveniles (Quinlan et al., 1999a; Quinlan et al., 1999b; Philpot et al., 2001a; Philpot et al., 2001b).

The idea that the NMDAR subunit switch might bring the critical period to a close was attractive because the timing of the NMDAR subunit switch seemed to coincide with a reduction in NMDAR function and the end of the critical period. However, closer examination demonstrated that NR2A levels in layer IV are maximal during the period of maximal plasticity, not at the end, suggesting that the increase in NR2A is not the ultimate signal for terminating juvenile ocular dominance plasticity ((Roberts and Ramoa, 1999) although see (Erisir and Harris, 2003)). Moreover, NR2A KO mice continue to exhibit an age-dependent decline in ocular dominance plasticity (Fagiolini et al., 2003), corroborating findings in the somatosensory cortex (Lu et al., 2001).

The second putative role for NMDA receptor subunits was that NR2A-containing receptors were a requirement for the induction of LTP, while NR2B receptors were a requirement for the induction of LTD (Liu et al., 2004; Massey et al., 2004). This possibility was attractive because it provided a simple mechanism to describe the developmental loss of NMDAR-dependent LTD observed in many regions of the brain. However, the validity of these findings is now being questioned because these studies were conducted using non-specific concentrations of NR2A-selective antagonists (Neyton and Paoletti, 2006). Moreover, recent data contradict the initial findings that NR2A and NR2B play distinct roles in regulating the polarity of synaptic plasticity (Berberich et al., 2005; Weitlauf et al., 2005; Zhao et al., 2005; Morishita et al., 2007; de Marchena et al., 2008). Finally, accumulating evidence (Sakimura et al., 1995; Lu et al., 2001; Weitlauf et al., 2005; Philpot et al., 2007), including findings from the present study, demonstrate that LTP can be induced in NR2A KO mice, suggesting that a synaptic requirement of NR2A for LTP is overly simplistic.

The current findings fit best the theory that NMDA receptor subunit composition regulates a sliding threshold for bidirectional synaptic plasticity (Bienenstock et al., 1982; Philpot et al., 2001b). As previously demonstrated in layer III (Philpot et al., 2007), we find in layer IV that reducing NR2A expression shifts the optimal LTD stimulation frequency leftward and enables LTP at low stimulation frequencies. It has been suggested previously that the drop in NR2A/B protein that normally occurs between 3 and 5 days of MD enables the potentiation of the non-deprived eye by shifting the modification threshold to the left (Chen and Bear, 2007). Our finding of reduced deprived eye depression and precocious open-eye potentiation after 3 days of MD in the

Het and KO animals are consistent with this theory. However, rather than setting the threshold *per se*, reducing NR2A appears to remove a constraint on how fast it can adjust, so that 3 days of contralateral-eye MD is sufficient to cause potentiation of the ipsilateral eye responses. Additional mechanisms for adjusting the threshold independently of NR2A could include regulation of NR2B (Chen and Bear, 2007) and/or the total number of NMDARs at the synapse (Philpot et al., 2007), among other possibilities (Abraham, 2008; Yoshimura et al., 2008).

The current data are relevant to the recent debate over whether the compensatory potentiation of the non-deprived eye after MD reflects a process analogous to input-specific LTP enabled by metaplastic adjustment of the modification threshold (Sawtell et al., 2003; Frenkel and Bear, 2004), or a cell-wide process of homeostatic synaptic scaling (Mrsic-Flogel et al., 2007; Kaneko et al., 2008). Scaling is a phenomenon that does not require NMDAR activation (Turrigiano and Nelson, 2004), so the OD plasticity phenotype in the NR2A mutant mice is unlikely to result from altered scaling. Moreover, consistent with findings in adult mice (Sawtell et al., 2003; Sato and Stryker, 2008), we find that the response potentiation caused by 7 day MD in juvenile mice requires NMDAR activation. Therefore, the current findings implicate metaplasticity rather than scaling as the mechanism for deprivation-induced response potentiation, at least in layer IV.

In conclusion, our data support the hypothesis that the experience-dependent regulation of the NR2A/B ratio is critical for adjusting the threshold for synaptic modifications, both *in vitro* and *in vivo*. These data suggest that lowering the NR2A/B ratio might provide a permissive milieu for strengthening weak cortical inputs. An

exciting possibility is that manipulation of this ratio, either experientially or pharmacologically, could be exploited therapeutically to promote synaptic rewiring after brain injury or disease.

3.5 Methods

3.5.1 Subjects

Mice deficient in NR2A were generously supplied by S. Nakanishi. The mice were developed by replacing the region spanning the M2 transmembrane segment of NR2A subunits with the neomycin resistance gene as previously described (Kadotani et al., 1996). A pathogen-free line was rederived on a C57BL/6 background by Charles River Laboratories. WT (+/+), heterozygote (+/-), and NR2A-KO (-/-) mice were used between postnatal days (P) 21-28 for *in vitro* experiments and P24-P36 for *in vivo* experiments. Subjects were fed *ad libitum* and reared in normal lighting conditions (12/12 light/dark cycle). There was no significant difference in AMPAR-mediated responses across genotypes, as evidenced by the facts that (1) the baseline VEP amplitudes were not different (Figs 2B, 3B), (2) the baseline FPs evoked in layer IV by white matter stimulation were not different (Figs $1A_2$ and $1A_3$), and (3) the stimulation intensities required to evoke a half-maximal FPs was not different. Whole-cell recordings of AMPA/NMDA receptor ratios in layer IV cells revealed no difference between KO and WT, suggesting a normal level of NMDA receptor expression at these ages (data not shown). As described previously, changes in NR2A gene dosage systematically alter NR2A protein and the properties of NMDA receptor-mediated synaptic currents in visual cortex (Philpot et al., 2007).

3.5.2 Cortical slice preparation

Following an overdose of barbiturates (i.p.), mice were decapitated upon disappearance of corneal reflexes in compliance with the U.S. Department of Health and Human Services. The brain was rapidly removed and immersed in ice-cold dissection

buffer (composition in mM: NaCl, 87; KCl, 2.5; NaH_2PO_4, 1.25; $NaHCO_3$, 25; sucrose, 75; dextrose, 10; ascorbic acid, 1.3; $MgCl_2$, 7; and $CaCl_2$, 0.5) bubbled with 95% O_2 and 5% CO_2. The visual cortex was rapidly removed and 350 μm coronal slices were cut using a vibrating microtome (Leica VT100S). Slices recovered for 15 min in a submersion chamber at 32° C filled with warmed artificial cerebral spinal fluid (ACSF; 124 mM NaCl, 5 mM KCl, 1.25 mM Na2PO_4, 26 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM dextrose, saturated with 95% O_2, 5% CO_2) and then cooled gradually to room temperature until use.

3.5.3 Extracellular electrophysiology

Slices were transferred to an interface recording chamber maintained at 30°C and perfused with ACSF at a rate of 2.5 mL/min. A stimulation electrode (concentric bipolar tungsten) was positioned in layer 6/white matter, and a glass recording electrode (~ 1 MΩ) filled with ACSF was positioned in layer IV. 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 sec. 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 minutes, slices were stimulated with 900 pulses at 1 Hz or with 900 pulses at 0.5 Hz. Field excitatory postsynaptic potential (FP) amplitudes were recorded every 30 seconds for 45 minutes following the cessation of the stimulation protocol. The concentration used for bath application of D-APV was 50 μM. Control and experimental subjects were run in an interleaved fashion. Objective criteria (baseline drifts no greater

than 5% and proper waveform alignment) were applied as inclusion criteria for further analysis. The data were normalized, averaged, and reported as means ± SEM. Changes in synaptic strength were measured by comparing the average response amplitude 35-45 minutes after conditioning stimulation to the pre-conditioning baseline response.

3.5.4 Current source-density analysis

CSD analysis was performed to determine the spatiotemporal pattern of current sinks and sources evoked in layer IV by biphasic stimulation at the layer VI/white matter boundary of primary visual cortex. The glass recording electrode (~ 1 MΩ) filled with ACSF was tracked down through the layers in 100 μm steps. At each recording depth, ten 200 μsec pulses were delivered by biphasic stimulation (A-M Systems Isolated Pulse Stimulator Model 2100) and the responses were averaged. At the completion of the recording session, the recording electrode was lifted along the z-plane and its tip immersed in FluoSpheres polystyrene microspheres and returned to its recording site to verify layer IV localization. The section was then mounted on gelatin-coated slides and fluorescently stained for Nissl substance (Neurotrace, Molecular Probes).

From the FPs collected, the corresponding one-dimensional (depth) CSD profile was constructed according to the method described by Mitzdorf (Mitzdorf, 1985), using a spatial differentiation grid of 200 μm. A full account of the theoretical basis of CSD analysis has previously been presented (Freeman and Nicholson, 1975; Mitzdorf, 1985).

3.5.5 Miniature EPSC recordings

Slices were maintained in ACSF containing (in mM) 124 NaCl, 3 KCl, 1.25 Na_2PO_4, 26 $NaHCO_3$, 1 $MgCl_2$, 2 $CaCl_2$, and 20 D-glucose, saturated with 95% O_2 and 5% CO_2 (315 mOsm and pH 7.25). Recording electrodes were filled with internal

containing (in mM) 20 KCl, 100 (K)gluconate, 10 HEPES, 4 (Mg)ATP, 0.3 (Na)GTP, and 10 (Na) phosphocreatine with pH adjusted to 7.25 and osmolarity adjusted to 300 mOsm. AMPA receptor-mediated miniature EPSCs (mEPSCs) were recorded in the presence of blockers for voltage-gated sodium channels (tetrodotoxin; 200 nM), $GABA_A$ receptors (picrotoxin; 50 μM), and NMDA receptors (D,L-APV; 100 μM). To further block NMDA receptor currents the internal recording solution contained 1 μM MK801 and mEPSCs were recorded at negative holding potentials (–80 mV). Events were first identified using an automatic template detection program (pCLAMP; Molecular Devices) and then manually verified so that only events with a monotonic rise time and exponential decay were included in the analysis. Over 100 events were analyzed for each data point for each cell.

3.5.6 In vivo electrophysiology

VEP recordings were conducted in awake mice as described previously (Frenkel and Bear, 2004). Mice were anesthetized with 50 mg/kg ketamine and 10 mg/kg xylazine *i.p.* Tungsten microelectrodes (FHC, Bowdoinham, ME) were chronically implanted into binocular visual cortex at P24. Reference electrodes were placed bilaterally into prefrontal cortex. All electrodes were secured in place with cyanoacrylate and the entire exposure was covered with dental cement.

For MD and BD, P27-29 mice were anesthetized by inhalation of isoflurane (IsoFlo 2-3%). Lids were sutured using 6-0 vicryl. Animals were monitored daily to ensure a full seal. Mice whose eyelids did not remain fully shut for the entire duration of MD were excluded from the study. For CPP experiments, CPP (Tocris Bioscience) or saline was delivered intraperitoneally every 6 hours at 10 mg/kg (Heynen et al., 2003;

Frenkel et al., 2006).

Visual stimuli consisted of full-field sine-wave gratings of 0% and 100% contrast, square reversing at 1 Hz, and presented at 0.05 cycles/degree. VEPs were evoked by either horizontal or vertical stimuli. As described previously, stimuli of orthogonal orientations were presented pre- and post- MD in order to avoid the phenomenon of stimulus-selective response potentiation (SRP) (Frenkel and Bear, 2004; Frenkel et al., 2006). Visual display occupied 92° x 66° of the animal's visual field. Visual stimuli were presented to left and right eyes randomly. A total of 100-200 stimuli were presented per each condition. VEP amplitude was quantified by measuring trough-to-peak response amplitude, as described previously (Sawtell et al., 2003).

3.5.7 Statistics

Global ANOVA's with a repeated measures factor were run with post hoc analyses (Fisher's PLSD) to test for statistical significance between multiple groups. Data expressed as means ± SEM, and significance was placed at $p < 0.05$.

3.5.8 Drugs

Unless otherwise noted, drugs were purchased from Sigma.

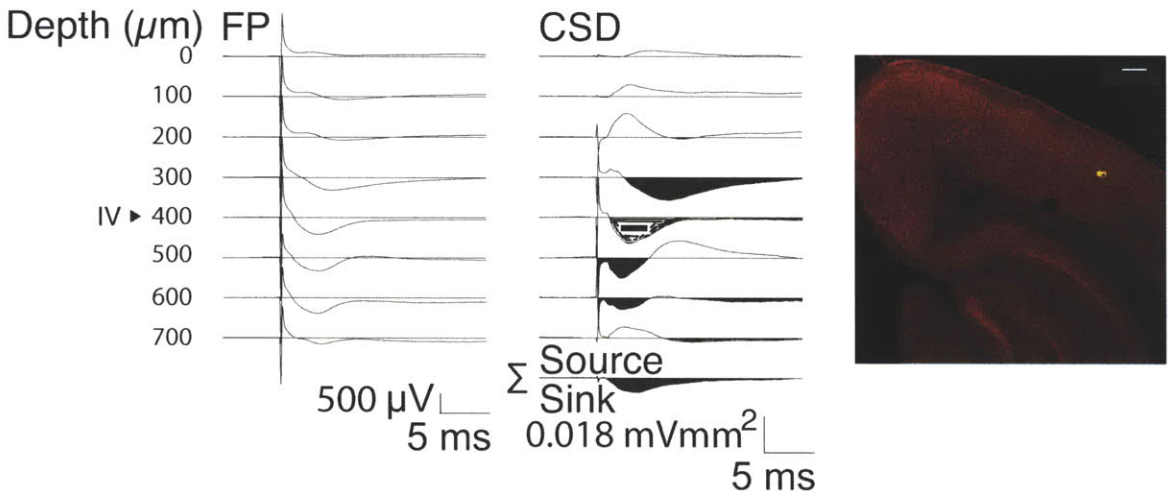

Figure 3.1 – *In vitro* CSD analysis in primary visual cortex in mouse

Left, Fps recorded at different cortical depths in response to biphasic stimulation in layer VI/white matter. (Scale bar: 5 ms, 500 μV.) Cortical layers are indicated. *Middle,* CSD profiles obtained from the Fps using a spatial differentiation grid of 200 μm. (Scale bar: 5 ms, 0.018 $mVmm^2$.) Current sinks are downward and shaded, and current sources are upward. The bottom trace (Σ) is summation of all CSD traces across depth. *Right,* Nissl-stained coronal section showing fluorescent yellow beads in layer IV, indicating position of recording electrode tip at layer IV. (Scale bar: 200 μm.) (This experiment was performed by Kathleen Cho.)

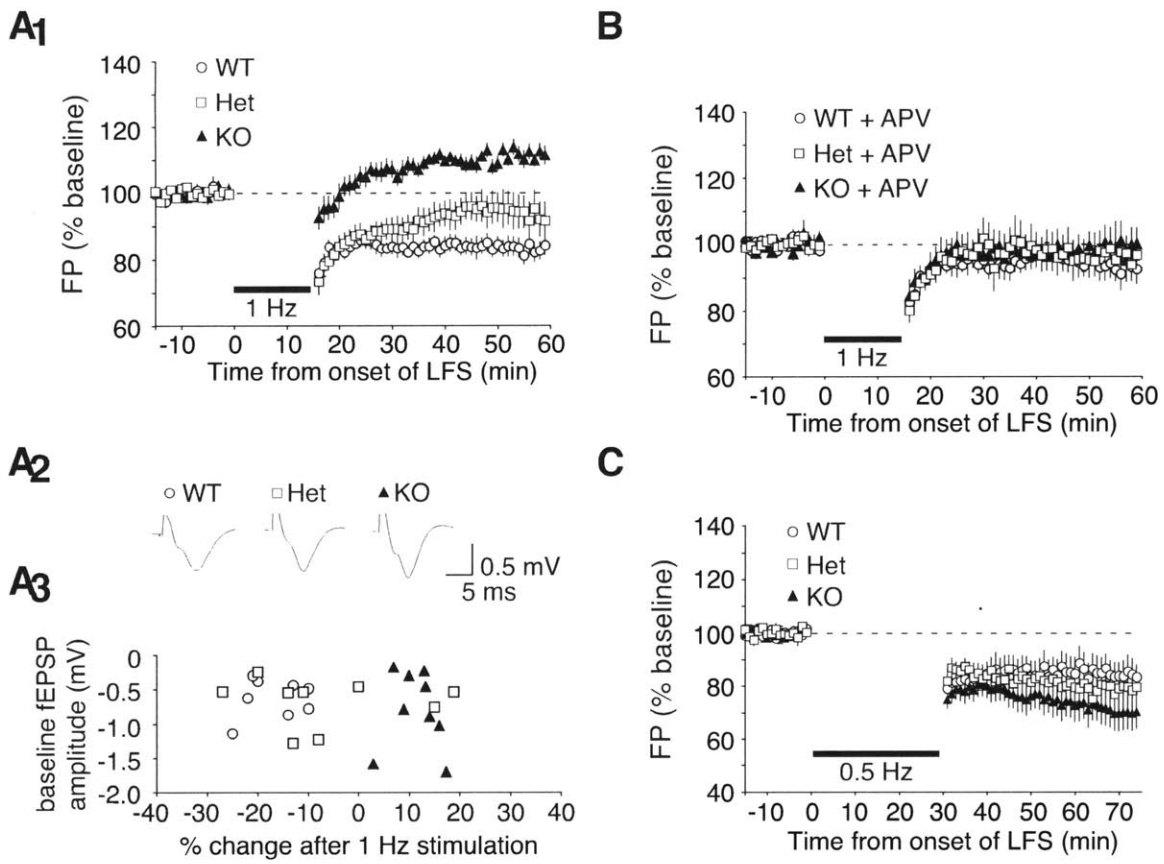

Figure 3.2 – Loss of NR2A lowers the LTP threshold and the optimal LTD stimulation frequency

A_1. Averaged data (± SEM) demonstrating that 1 Hz stimulation (900 pulses) induces LTD in WT and Het mice and LTP in NR2A KO mice. A_2. Baseline waveforms averaged across all the individual experiments that are summarized in A_1 (WT, n = 8; Het, n = 9; KO, n = 7). Note that averaged waveforms are of comparable size and shape in all three genotypes. A_3. Scatter plot of individual baseline FP amplitudes of each genotype and percent change of synaptic transmission following 1 Hz stimulation. Note that the effect of 1 Hz stimulation is not correlated with initial response amplitude. B. Averaged data demonstrating that bath-applied APV prevents the effects of 1 Hz stimulation in all genotypes. C. Averaged data demonstrating that stimulation at 0.5 Hz (900 pulses) yields LTD in all genotypes, with maximal effect in NR2A KO mice. (This experiment was performed by Kathleen Cho.)

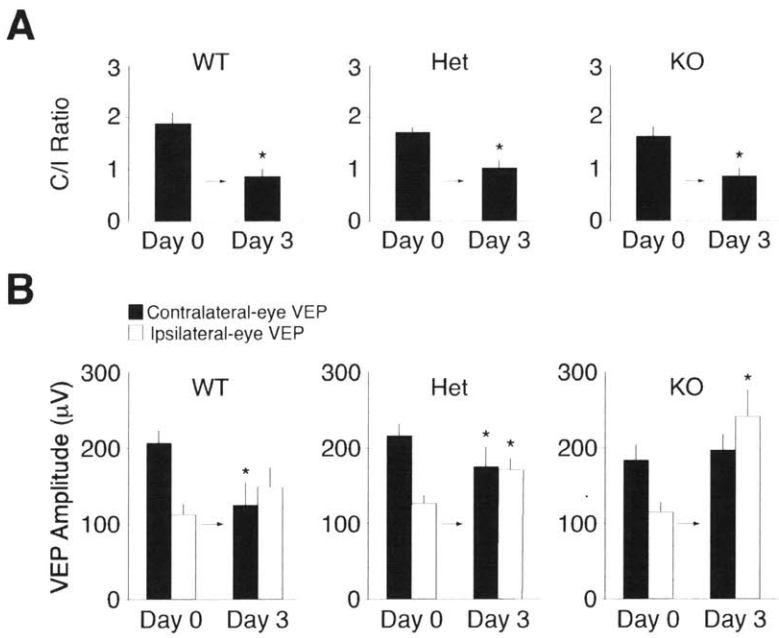

Figure 3.3 – The ocular dominance shift following three days of MD is qualitatively different in NR2A KO, Het, and WT mice

A. There is a significant decrease in C/I ratios of WT (n = 10), Het (n = 9), KO (n = 8) mice following 3 days of MD. Average values of C/I ratios (± SEM) are plotted. Asterisks indicate $p < 0.01$. B. Day 0 and day 3 waveforms, averaged across all individual experiments (scale: 100 ms, 100 μV). Average trough-to-peak amplitude (±SEM, n = 10) of VEPs in WT mice in response to deprived eye (filled bars) and non-deprived eye (open bars) stimulation during baseline (day 0) and after 3 days of MD. There is a significant decrease in the deprived eye VEP amplitude and no change in the non-deprived eye VEP amplitude (left panel). Average amplitude (±SEM, n = 9) of VEPs in Het mice in response to deprived eye (filled bars) and non-deprived eye (open bars) stimulation during baseline (day 0) and after 3 days of MD. There is a significant decrease in the deprived eye VEP amplitude and significant increase in the non-deprived eye VEP amplitude (middle panel). Average amplitude (±SEM, n=8) of VEPs in KO mice in response to deprived eye and non-deprived eye stimulation during baseline (day 0) and after 3 days of MD. No change in deprived eye VEP amplitude is observed, but the non-deprived eye VEP amplitude is significantly increased (right). Average amplitude (±SEM) of VEPs in WT mice (left), Het (middle), and KO mice (right panel) in response to deprived eye and non-deprived eye stimulation after three days of normal visual experience are comparable to baseline values. Asterisks indicate $p < 0.05$.

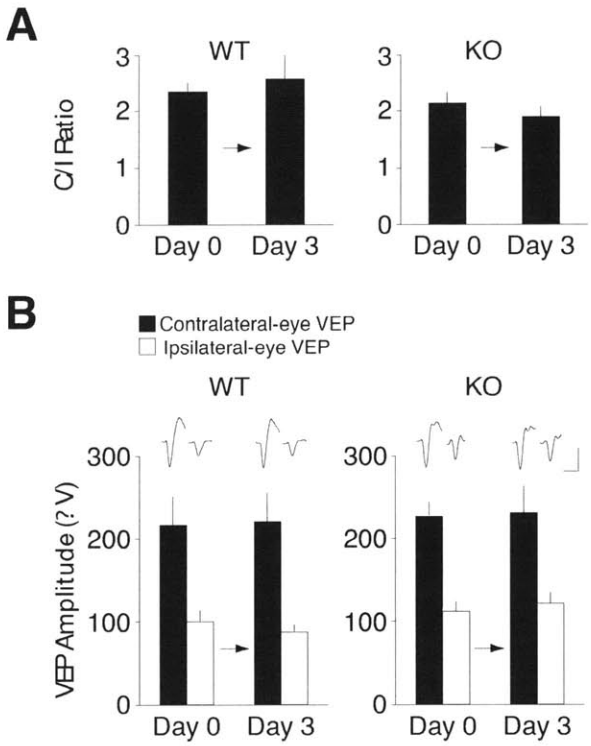

Figure 3.4 – No evidence of synaptic scaling following three days of binocular deprivation in NR2A KO and WT mice

A. Three days of binocular deprivation fail to modify the C/I ratio of VEPs in either WT or NR2A KO mice. B. Neither the ipsilateral nor contralateral eye VEP responses are modified by three days of binocular deprivation in WT and NR2A KO mice. The scale for the averaged waveforms is 100 ms, 100 μV.

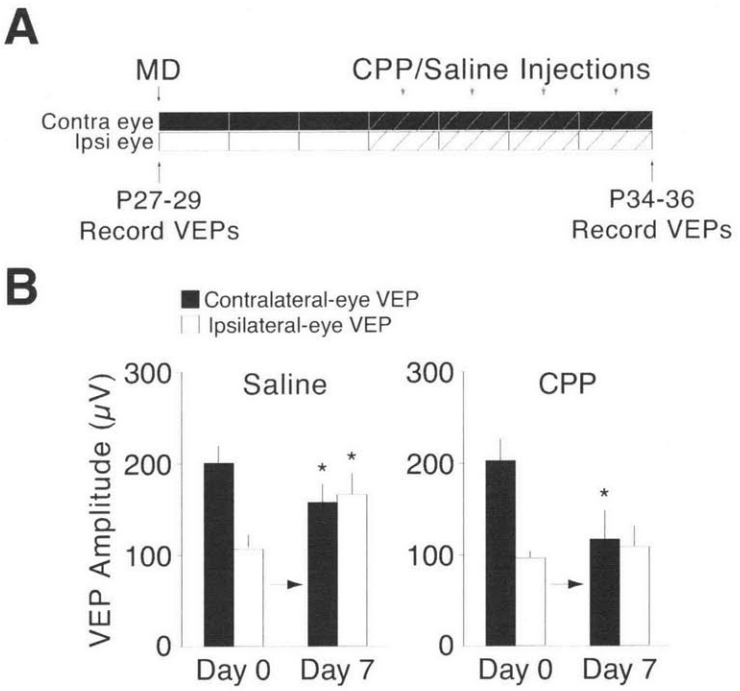

Figure 3.5 – Ipsilateral-eye response potentiation following seven days of monocular deprivation is NMDAR-dependent in juvenile wild-type mice

A. Juvenile mice were treated with saline or CPP for the last four days of a seven day MD. B. Ipsilateral eye potentiation was blocked in mice treated with CPP.

Chapter 4

Parvalbumin-expressing interneurons gate ocular dominance plasticity in the adult mouse visual cortex

4.1 Abstract

During a brief postnatal critical period, synaptic connections that make up the visual cortical circuit are highly malleable in response to alterations in visual experience, an ability that declines in older animals. Recent studies in the rodent primary visual cortex have implicated the maturation of cortical GABAergic inhibition in the timing of the critical period and in subsequent gating of plasticity in the adult cortex. Among the many subtypes of GABAergic interneurons, a subtype that has been highly implicated in limiting plasticity in adults consists of parvalbumin (Pv)-expressing fast-spiking basket cells. Here we used a transgenic strategy to ablate this class of interneuron from the primary visual cortical circuit in the mouse and test the consequences of a brief period of monocular deprivation on ocular dominance (OD) plasticity. We found that lowering the cortical inhibitory tone by specifically removing the Pv^+ basket cells reinstated juvenile-like qualities of OD plasticity in adult mice.

4.2 Introduction

Maturation of the visual cortical circuit takes place over a protracted period during postnatal development. During this period, the developing circuit is highly malleable and susceptible to plastic changes in response to changes in the visual environment. During this time, often referred to as the critical period, changes in visual experience can drive both functional and structural changes in the circuit, a capacity that diminishes with age. As a result of this decline in plasticity, the ability of a cortical network to recover from the effects of abnormal sensory development (such as in the case of long-term amblyopia) is dramatically reduced or even lost after the end of the critical period. Recent studies, however, have demonstrated a great capacity for plasticity in the adult visual cortex (Spolidoro et al., 2009; Sale et al.). Much effort has been put forth to understand the underlying mechanisms that both limit plasticity in the adult brain and are responsible for the reactivation of plasticity in the adult visual cortical circuit.

Intracortical GABAergic inhibition has been suggested to be the fundamental factor in controlling both developmental and adult cortical plasticity. Postnatal maturation of the inhibitory circuit is believed to set the pace for the timing of the critical period for synaptic plasticity in the visual cortex (Jiang et al., 2005; Huang et al., 2007; Sale et al.). Inhibition matures both structurally (by increasing the number of synaptic contacts onto pyramidal neurons) and functionally (by increasing the efficacy of inhibitory synaptic transmission), both of which play a central role in the development of visual cortical receptive fields and visual acuity (Huang et al., 1999; Morales et al., 2002; Chattopadhyaya et al., 2004; Jiang et al.). The development of the inhibitory circuit has been demonstrated to be highly susceptible to changes in visual experience. For instance,

dark rearing slows down developmental increases in intracortical inhibition (Benevento et al., 1992; Morales et al., 2002; Gianfranceschi et al., 2003; Chattopadhyaya et al., 2004; Di Cristo, 2007; Jiang et al.). Conversely, manipulations that accelerate development of visual acuity, such as overexpression of brain derived neurotrophic factor (BDNF), have also been shown to speed up the maturation of the inhibitory circuit (Hanover et al., 1999; Huang et al., 1999). These results suggest that GABAergic inhibition might set the stage for plasticity during the critical period.

A reliable measure of receptive field plasticity in the visual cortex is ocular dominance (OD) plasticity, where manipulations of visual experience, such as monocular deprivation (MD) shift cortical responsiveness of the binocular neurons away from the deprived eye in favor of the open eye (Wiesel and Hubel, 1963). OD plasticity is maximal during the critical period and gradually declines, as the animal ages (Hubel and Wiesel, 1970; Freeman and Olson, 1982; Gordon and Stryker, 1996). Using OD plasticity as a model for assaying developmentally regulated synaptic plasticity, several laboratories have demonstrated that the strength of GABAergic inhibition must cross a threshold in order to be permissive for OD plasticity at the onset of the critical period. One such study showed that a mouse lacking glutamic acid decarboxylase 65 (GAD65), and thus, deficient in GABA release, lacks OD plasticity throughout life, which can be rescued by an injection of benzodiazepines that transiently increase inhibitory tone in the cortex (Hensch et al., 1998b). Conversely, a mouse overexpressing BDNF has a precocious onset of OD plasticity (Huang et al., 1999).

The idea that maturation of GABAergic inhibition also regulates the termination of the critical period came from a finding that the ability to induce long-term potentiation

(LTP) in the visual cortical slice declines with age, but can be restored by application of $GABA_A$ receptor antagonists (Kirkwood and Bear, 1994). This 'inhibitory gate' hypothesis was confirmed both *in vitro* and *in vivo* in the BDNF overexpressing mice, which showed a precocious decline in the cortical LTP, as well as early termination of OD plasticity (Huang et al., 1999). Together, these findings suggest that as inhibitory cortical circuits mature throughout development, the levels of GABAergic inhibition crosses two thresholds: the first one is crossed at the beginning of the critical period and allows OD plasticity to be expressed. Then, as inhibition develops further, a second threshold is crossed, after which the inhibitory tone dramatically limits plasticity, signifying the end of the critical period.

In agreement with this hypothesis it was recently demonstrated that a direct pharmacological reduction of cortical inhibition reactivates OD plasticity in response to a brief MD in adult rats (Harauzov et al.). A number of other studies corroborated this finding by implementing a variety of methods that either promoted reactivation of plasticity in adult cortex or a recovery from long term MD, while, at the same time lowering the inhibitory tone in the cortex or putting the cortex into a juvenile-like state (Pizzorusso et al., 2002; He et al., 2007; Sale et al., 2007; Maya Vetencourt et al., 2008; Huang et al.).

Most of the studies described so far have been carried out in a rat or a cat visual cortex. However, in the recent decade, the mouse has become a widely used model animal for studying experience-dependent plasticity(Smith et al., 2009). A growing body of literature, however, demonstrates that mice might not have a very tightly regulated closure of the critical period, as plasticity in these animals extends well into adulthood

(Sawtell et al., 2003; Lickey et al., 2004; Tagawa et al., 2005; Frenkel et al., 2006; Hofer et al., 2006a; Fischer et al., 2007b; Lehmann and Lowel, 2008; Sato and Stryker, 2008). Nevertheless, it is clear that the capacity for plasticity in the adult mouse visual cortex is diminished compared to juveniles and the qualities of the plasticity are different. While in the juvenile cortex, the consequences of brief MD are largely expressed as a depression of the deprived-eye response; in the adult cortex, the predominant feature of plasticity is the potentiation of the non-deprived eye input. It has also been demonstrated that although recovery from long-term MD is possible in the mouse, the process is slow and the final recovery is only partial (Fischer et al., 2007a). It is reasonable to assume that, much like, in the rat, developmental increase in cortical inhibitory tone is responsible for restricting juvenile-like plasticity in the adult mouse cortex, but direct demonstration of this has been lacking.

Cortical inhibitory circuits are made up of multiple subtypes of interneurons, which target distinct regions on the excitatory pyramidal cells, each providing a different aspect of inhibitory control (Markram et al., 2004; Xu and Callaway, 2009). A specific subtype of cortical interneuron has been implicated in regulating the timing of the critical period: the parvalbumin (Pv)-expressing fast-spiking basket cells (Fagiolini and Hensch, 2000; Pizzorusso et al., 2002; Di Cristo, 2007; Sugiyama et al., 2008). These cells are ideally situated to control the output of excitatory cells, making numerous synaptic connections onto the cell body and the axon initial segment. Studies which demonstrated restoration of juvenile-like plasticity in adults have only provided indirect evidence that the activity of Pv^{+} basket cells was specifically compromised by their manipulations (Pizzorusso et al., 2002; Sale et al., 2007; Maya Vetencourt et al., 2008). The direct

demonstration that these interneurons control the amount and the quality of plasticity in the adult cortex has not yet been performed.

In this study we attempt to answer two questions: 1) does reducing inhibitory tone restore juvenile-like plasticity in the adult mouse visual cortex, much like what has been observed in adult rats and 2) are Pv^+ fast-spiking basket cells directly responsible for gating plasticity in the adult brain? We develop a strategy to reduce the inhibitory cortical tone by specifically ablating Pv^+ interneurons from a fully mature cortical circuit and find that this manipulation prevents the expression of adult forms of plasticity in favor of reactivating juvenile-like OD plasticity.

4.3 Results

4.3.1 Expression of ocular dominance plasticity in the adult mouse primary visual cortex

There is a general agreement in the literature that the qualities of OD plasticity in the adult rodent visual cortex are different than those in juvenile animals in that the magnitude of the OD shift is smaller and plasticity in response to a brief period of MD is expressed mostly as a potentiation of the non-deprived input. However, the available data is highly variable, most likely due to the method of assaying ocular dominance, whether the animal is awake or anesthetized, and the influence of a genetic background and housing conditions. Our method of measuring ocular dominance plasticity using visually evoked potentials (VEPs) offers a unique advantage in that 1) the electrodes are implanted chronically, allowing pre- and post-MD within-animal comparisons, 2) recordings are made in awake and alert animals, eliminating the confounding effects of anesthesia. Therefore, we first decided to reassess the qualities of the adult OD shift in response to a brief 3 day period of MD using our methodologies. We implanted mature

adult mice (postnatal day (P) 90-120) with field recording electrodes in the thalamorecipient layer 4 of the binocular segment of the primary visual cortex. Following recovery and habituation to the restraint and recording apparatus, baseline VEP recordings were obtained. Mice were then unilaterally eye-lid sutured for 3 days and upon eye opening VEPs were measured again. We observed a significant decrease in the ratio of the deprived (contralateral) to the non-deprived eye (ipsilateral) responses, C/I ratio (Figure 4.1A; 2.25 ± 0.14 pre-MD versus 1.45 ± 0.13 post-MD, n = 10, p = 0.009, Wilcoxon Signed Rank test). This was in agreement with most previous studies, although the period of MD was shorter (3 days) than what was used previously (5-7 days). When we examined the cortical response to visual stimulation of the inputs from the two eyes independently, we found that the deprived-eye response was unaltered (138.8 μV ± 9.6 pre-MD versus 129.6 μV ± 14.6 post-MD, n = 10, p = 0.58, Student's paired t test) and that the OD shift was expressed entirely through potentiation of the open-eye response (Figure 4.1B; 62.3 μV ± 2.67 pre-MD versus 92.2 μV ± 10.5 post-MD, n = 10, p = 0.03, Student's paired t test). This result confirmed that while OD plasticity can be induced in adult mice, the qualities of the OD shift are fundamentally different from those in juvenile animals. The nature of the molecular mechanisms responsible for the difference between the two types of plasticity and the elimination of juvenile-like plasticity in adults remains unclear. We next tested the role of a specific inhibitory cortical circuit in gating juvenile plasticity in the adult visual cortex.

4.3.2 Targeted ablation of Pv^+ interneurons in the mouse primary visual cortex

The goal of this study was to determine the role that Pv^+ interneurons play in gating ocular dominance plasticity in the adult cortex. We examined this question by

first developing a strategy to selectively ablate this class of inhibitory neurons from the primary visual cortical circuit in the adult mouse. To accomplish this we took advantage of two transgenic mouse lines: parvalbumin-Cre (Pv-Cre) and a "floxed" diphtheria toxin receptor (DTR). The Pv-Cre line was generated by an insertion of an *IRES-Cre-pA* cassette under a Pv promoter, resulting in the expression of Cre recombinase in all cells that contain parvalbumin (Hippenmeyer et al., 2005). Since parvalbumin is a calcium binding protein that is highly expressed in fast spiking basket cells, it is a good marker for targeting this cell population. The DTR line was generated by placing a transgene (the simian form of DTR or heparin-binding epidermal growth factor (HB-EGF)) under control of a ubiquitously expressed ROSA26 promoter along with insertion of a *loxP*-flanked STOP cassette into the open reading frame of the DTR transgene (Buch et al., 2005). When the two lines are crossed, Cre in Pv^{+} cells can excise the STOP cassette and allow expression of the diphtheria toxin receptor (Figure 4.2). Thus, mouse cells, which are naturally insensitive to diphtheria toxin (DT), now express the simian form of the DTR, rendering them DT-sensitive. The expression of the DTR on the cell surface allows binding of DT. Upon entry into the cell, DT disrupts protein synthesis, resulting in apoptotic death of the target cell. A single molecule of DT is sufficient to kill a cell, making DT-mediated ablation very sensitive and efficient.

The Pv-Cre mice were originally maintained on the SVJae129 background. We backcrossed this line to C57BL/6 for 10 generations before crossing them to the DTR line, as we know that some qualities of visual plasticity differ between the two strains (Muhammad et al., unpublished observations). To assess the efficiency of Cre recombination, we crossed the Pv-Cre mice to a GFP reporter line (ROSA-GFP).

Qualitatively, the vast majority of the Pv-expressing cells also express GFP, suggesting that most of the Pv^+ cells will also be expressing the DTR (Figure 4.3).

To avoid the confound of ablating Pv^+ inteneurons in the entire central and peripheral nervous system, we restricted the ablation to the binocular primary visual cortex by a local injection of DT. We found that delivery of just 15 pg of DT was enough to kill most of the Pv^+ cells in a defined region without affecting other cell populations in the area, as assessed by a fluorescent Nissl counterstain (Figure 4.4).

4.3.3 Effect of Pv^+ interneuron ablation on visual responses

We next examined the effect of Pv^+ interneuron ablation on the magnitude and stability of the VEPs and C/I ratios in the non-deprived cortex. Adult mice (P180-360) were injected with DT and chronically implanted with electrodes in the visual cortex of the left hemisphere on the same day. Following recovery, VEPs were monitored for 12 days, with recordings performed during 6 sessions: on days 2, 4, 6, 8, 10 and 12 post DT injections (DPI). Visual stimuli presented during each recording were of different orientation, separated by at least 30° to avoid the confound of stimulus-selective response potentiation (Frenkel et al., 2006). During the first recording session (2 DPI), VEP amplitudes were comparable to those recorded in baseline conditions in all of our previous studies (Figure 4.5A; contra = 182.3 μV ± 25.6; ipsi = 85.4 μV ± 14.6, n = 8). During the subsequent recording sessions (4 and 6 DPI), the VEP amplitude evoked by stimulation of both contralateral and ipsilateral pathways increased dramatically (Figure 4.5A; ANOVA = 0.004 (contra) and < 0.0001 (ipsi); 4 DPI: contra = 308.9 μV ± 37.8, p = 0.017; ipsi = 166 μV ± 22.2, p < 0.0008; n = 8; 6 DPI: contra = 373 μV ± 70.4, p = 0.0006; ipsi = 214.9 μV ± 38.8, p < 0.0001; n = 8;). Strikingly, by 8 DPI, VEP amplitude

of both ipsilateral and contralateral responses returned to the original baseline value and remained stable during the subsequent recording sessions (Figure 4.5A; 8 DPI: contra = 261.5 μV ± 78.1, p = 0.12; ipsi = 91.7 μV ± 13.8, p = 0.8; n = 8; 10 DPI: contra = 214.9 μV ± 28.1, p = 0.52; ipsi = 88 μV ± 11.1, p = 0.91; n = 8; 12 DPI: contra = 198.2 μV ± 28.3, p = 0.75; ipsi = 72.9 μV ± 10.6, p = 0.57; n = 8;). Importantly, even though the VEP amplitude was changing in the animals with compromised inhibition, the C/I ratio was maintained at 2-3:1 throughout the entire period (Figure 4.5B; 2.28 ± 0.3 at 2 DPI; 1.91 ± 0.16 at 4 DPI; 1.85 ± 0.19 at 6 DPI; 2.72 ± 0.5 at 8 DPI; 2.54 ± 0.26 at 10 DPI, and 2.79 ± 0.26 at 12 DPI; n = 8; Kruskal-Wallis test = 0.3), suggesting that the ratio of amplitudes is not maintained via fast-spiking basket cells. VEP amplitudes and the C/I ratio recorded from littermate controls injected with DT were stable over the entire 12 days (Figure 4.5C,D; 2 DPI: contra = 191.6 μV ± 22.2; ipsi = 131.1 μV ± 17.9; C/I ratio = 1.77 ± 0.48; n = 4; 4 DPI: contra = 221.1 μV ± 33.5; ipsi = 119.9 μV ± 29.1; C/I ratio = 2.11 ± 0.42; n = 4; 6 DPI: contra = 173.2 μV ± 29; ipsi = 108.1 μV ± 28; C/I ratio = 1.88 ± 0.39; n = 4; 8 DPI: contra = 154 μV ± 28.4; ipsi = 95.4 μV ± 25.1; C/I ratio = 1.77 ± 0.2; n = 4; 10 DPI: contra = 152.5 μV ± 34.9; ipsi = 101.5 μV ± 32; C/I ratio = 1.72 ± 0.28; n = 4; 12 DPI: contra = 159.8 μV ± 32.7; ipsi = 96.5 μV ± 28.7; C/I ratio = 1.79 ± 0.16; n = 4; ANOVA = 0.07 (contra) and 0.3 (ipsi); Kruskal-Wallis test (C/I ratio) = 0.78).

Our observation of VEP amplitude dynamics following Pv^+ cell ablation suggests that there is a decrease in inhibitory tone over the first week that causes the increase in the amplitude of the VEP responses; however, after the first week VEP responses return to their baseline values. Since our ablation was spatially restricted, some Pv-expressing

cells lateral to the DT injection site were spared. Therefore, one possibility for the decrease in VEP amplitude over time could be due to an increase in the inhibitory tone due to the spared basket cells extending axons into the area of ablation. We tested this hypothesis by injecting DT into the Pv-Cre/DTR mice along with an injection of an adeno-associated virus (AAV) expressing a "flexed" tomato red fluorescent protein lateral to the DT injection site. Upon Cre recombination, tomato was expressed in Pv^+ cells that were spared by the injection of DT. We observed no invasion by the laterally located Pv^+ interneurons into the area of ablation (Figure 4.6), ruling out this possibility. Alternative explanations could include a compensatory increase in inhibitory responses supported by non-Pv-expressing interneurons or a scaling down of excitatory responses. Regardless of the mechanism by which the cortex achieves this homeostasis, we were sure that following the stabilization of the visual responses, Pv^+ interneurons were ablated in the area of recording. Since our objective was to test the role of this specific subset of interneurons in gating plasticity in adults, and lateral invasion of axons from other Pv^+ cells seemed an unlikely candidate for mediating the decrease in VEP amplitude, we performed our MD studies starting at 8 DPI when VEP amplitudes were found to be stable.

4.3.4 Effect of Pv^+ interneuron ablation on the OD shift in adult mice

Using the technology for Pv^+ interneuron ablation we proceeded to perform monocular deprivation studies in adult mice. As described previously, mice were injected with DT and immediately implanted with recording electrodes in V1. Following recovery, baseline VEPs were collected at 8 DPI. The eye contralateral to the hemisphere of interest was then sutured closed for 3 days. Upon eye opening, VEPs were recorded

again.

Before doing Pv^+ cell ablation studies, we first wanted to replicate the adult OD shift that we have observed in C57BL/6 wild-type (WT) mice in the littermate control animals from the Pv-Cre/DTR line. We discovered that much like WT animals, the mice from this transgenic background exhibit a strong OD shift in the C/I ratio following 3 days of MD at P90-120 (Figure 4.7A; 2.18 ± 0.28 pre-MD versus 1.18 ± 0.15 post-MD; n = 7; p = 0.028, Wilcoxon Signed Rank test). As expected, we also found that the shift was expressed as potentiation of the open-eye response, while the deprived-eye response was unchanged (Figure 4.7B; contra: 195.2 μV ± 25.1 pre-MD versus 157.7 μV ± 18.7 post-MD; ipsi: 94.6 μV ± 14 pre-MD versus 134.5 μV ± 14.6 post-MD; n = 7; p = 0.09 and 0.048 for contra and ipsi respectively, Student's paired t test). However, we did observe a trend toward deprived-eye depression in this set of animals and we wondered whether at this age the mice are still not completely mature. Therefore, decided to test even older mice (P240-360) to see how they respond to 3 days of MD. The results were very similar to our observations in both WT mice and Pv-Cre/DTR-background mice at P90-120: a robust shift in the C/I ratio (Figure 4.7C; 2.4 ± 0.39 pre-MD versus 1.5 ± 0.2 post-MD; n = 6; p = 0.046, Wilcoxon Signed Rank test) and potentiation of the non-deprived eye responses without any change (or downward trend) in the deprived-eye VEPs (Figure 4.7D; contra: 211.1 μV ± 25.9 pre-MD versus 199.9 μV ± 30.3 post-MD; ipsi: 94.9 μV ± 14.7 pre-MD versus 134.6 μV ± 16.9 post-MD; n = 6; p = 0.4 and 0.01 for contra and ipsi respectively, Student's paired t test).

We, then, repeated this experiment in the littermate control mice (P240-360) that were injected with DT to test whether the toxin has any non-specific effects on OD

plasticity. We found that plasticity in these mice was not distinguishable from plasticity in control mice that were not injected with DT. Following 3 days of MD, there was still a robust OD shift, expressed as a decrease in C/I ratio (Figure 4.8A; 1.69 ± 0.12 pre-MD versus 1.15 ± 0.9 post-MD; n = 6; p = 0.028, Wilcoxon Signed Rank test), which was accounted for by an increase in the VEPs driven by the open eye and no change in the VEPs evoked by the deprived eye (Figure 4.8B; contra: 239.7 μV ± 33.3 pre-MD versus 217.7 μV ± 30 post-MD; ipsi: 142.3 μV ± 15.9 pre-MD versus 195.7 μV ± 29.5 post-MD; n = 6; p = 0.18 and 0.04 for contra and ipsi respectively, Student's paired t test). In Pv-Cre/DTR mice where the injection of DT resulted in ablation of Pv^+ cells, the consequences of a 3 d MD were dramatically different. There was a trend towards a decrease in the C/I ratio, although it did not achieve statistical significance (Figure 4.8C; 2.15 ± 0.18 pre-MD versus 1.80 ± 0.21 post-MD; n = 11; p = 0.09, Wilcoxon Signed Rank test). Strikingly, the prominent potentiation of open-eye responses, observed in all groups of control adult mice was completely absent, lending way to a significant depression of the deprived-eye responses (Figure 4.8D; contra: 207.8 μV ± 0.18 pre-MD versus 163.2 μV ± 20.7 post-MD; ipsi: 98.5 μV ± 8.2 pre-MD versus 91.7 μV ± 7.67 post-MD; n = 11; p = 0.02 and 0.46 for contra and ipsi respectively, Student's paired t test). This result was intriguing because removing Pv^+ interneurons from the visual cortical circuit that was subject to a 3d MD seemed to eliminate the hallmark quality of the adult OD shift – non-deprived response potentiation; and reinstate the qualities inherent to the juvenile OD shift – deprived-eye depression without open-eye potentiation.

4.3.5 Inhibition level is critical for induction of juvenile-like plasticity in adult mice

One shortcoming of our finding is that even though we restored the qualities of juvenile OD plasticity following Pv^+ cell ablation, the magnitude of the shift in ocular dominance seemed to be much smaller than that observed in juvenile mice (Frenkel and Bear, 2004). Since it has been demonstrated that a critical level of inhibition is required to promote an OD shift (Hensch et al., 1998b; Huang et al., 1999), we wondered whether our method of ablation of Pv^+ interneurons was too efficient in reducing the inhibitory tone to a level not permissive for full OD plasticity.

One clue to this hypothesis came from a post-hoc histological examination of the brains that were subject to MD. We looked at several representative sections stained for Pv and correlated the number of surviving Pv^+ neurons in the area of ablation with the magnitude of deprived-eye depression observed following 3 days of MD. Qualitatively, we found that in two animals where the level of deprived-eye depression was similar in magnitude to what is normally seen in juveniles (Figure 4.9E,F; example 1: 180 μV pre-MD versus 79.0 μV post MD; example 2: 206.8 μV pre-MD versus 150.6 μV post-MD), there was a number of spared Pv^+ cells in the vicinity of the recoding site (Figure 4.9A,B). In contrast, another representative animal that had nearly a complete ablation of Pv^+ cells in the vicinity of the recording site (Figure 4.9C) displayed no deprived-eye depression following MD (Figure 4.9G; mouse 1: 234.4 μV pre-MD versus 271 μV post MD; mouse 2: 209.6 pre-MD versus 150.6 post-MD).

In order to test the hypothesis that partial reduction of inhibitory processes leads to juvenile-like plasticity in the adult animals, we decided to use another transgenic line (fNR1) which relatively mildly affects the activity of the inhibitory network via the deletion of the NMDA receptor in the Pv^+ fast-spiking basket cells. This line was

generated by inserting *loxP* sequences to flank the gene encoding the NR1 subunit of the NMDA receptor (Tsien et al., 1996). Since NR1 is an obligatory subunit, excision of this gene from a cell will result in the complete absence of NMDA receptors in that cell. Therefore, by crossing this line to the Pv-Cre line, the F1 generation will have NMDA receptors missing in the Pv^+ cells. It is known that NMDARs dominate subthreshold dynamics in interneurons and it has been shown that deletion or blockade of the NMDAR on the Pv^+ cells results in a decreased expression of glutamic acid decarboxylase 67 (GAD67), leading to functional disinhibition of the postsynaptic cells (Goldberg et al., 2003; Zhang et al., 2008; Belforte et al., 2010). It was also demonstrated that Pv-Cre/fNR1 mice exhibit less spatially and temporally precise firing in the pyramidal neurons, which may resemble the juvenile cortical state when GABAergic inhibition is still immature (Korotkova et al., 2010). We used adult animals from this cross to test the effect of a 3 day MD. The results of this study, although preliminary, were in partial agreement with what we have observed in Pv-Cre/DTR mice. As expected, the littermate control animals exhibited a strong OD shift of the C/I ratio (Figure 4.10A; 2.32 ± 0.34 pre-MD versus 1.20 ± 0.2 post-MD; n = 4; p = 0.016, Wilcoxon Signed Rank test), that appeared to be primarily due to the increase in the open-eye response, although statistical significance was not achieved, probably due to the low number of animals tested (Figure 4.10B; contra: 180.6 μV ± 14.5 pre-MD versus 145.4 μV ± 36.0 post-MD; ipsi: 83.2 μV ± 14.5 pre-MD versus 122.5 μV ± 25.5 post-MD; n = 4; p = 0.31 and 0.07 for contra and ipsi respectively, Student's paired t-test). A robust OD shift of the C/I ratio was also observed in the transgenic mice, Pv-Cre/fNR1 (Figure 4.10C; 2.87 μV ± 0.37 pre-MD versus 1.22 μV ± 0.15 post-MD; n = 8; p = 0.012, Wilcoxon Signed Rank test), although,

as we have seen in the case of Pv^+ interneuron ablation and in striking contrast to the controls, the shift was mostly due to a marked depression of the deprived-eye response (Figure 4.10D; contra: 220.1 μV ± 28.0 pre-MD versus 156.4 μV ± 27.2 post-MD; n = 8; p = 0.003, Student's paired t-test). Unexpectedly, however, open-eye potentiation was also significant in these mice (Figure 4.10D; ipsi: 79.3 μV ± 8.7 pre-MD versus 138.5 μV ± 27.0 post-MD; n = 8; p = 0.04, Student's paired t-test). These results indicate that even a mild disruption of Pv^+ interneuron activity is sufficient to restore a hallmark signature of juvenile OD plasticity – deprived-eye depression. Additionally, the magnitude of the depression was very similar to what is observed in the juvenile. However, the presence of open-eye potentiation in these animals complicates the interpretation of these results, as juvenile mice usually don't exhibit this response with only 3 days of MD (Frenkel and Bear, 2004). The fact that potentiation was observed in the Pv-Cre/fNR1 animals, but was blocked in the Pv-Cre/DTR animals injected with DT could indicate that some features of adult OD plasticity (namely non-deprived eye potentiation) are impervious to mild perturbations in activity of the inhibitory circuit.

4.4 Discussion

Our data show that modulating the inhibitory tone in the adult mouse visual cortex by manipulating fast-spiking basket cell activity can have profound effects on the qualities of ocular dominance plasticity. Deprived-eye depression in response to a brief MD is a definitive characteristic of the juvenile visual cortex, a feature that is lost as the animal matures. Here we tested the hypothesis that inhibitory interneurons, specifically Pv^+ fast-spiking basket cells, are responsible for gating juvenile-like plasticity in the adult mouse visual cortex. We found, in accordance with many previous studies, that the adult

cortex is capable of significant plasticity mediated primarily by strengthening of the open-eye input in response to a brief MD. However, elimination of Pv^+ interneurons from the circuit blocked this potentiation and instead allowed for some deprived-eye depression to be expressed, much like what is seen in juveniles during the critical period for developmental plasticity. Additionally, we found that the magnitude of the deprived-eye depression is even greater when the inhibitory tone is only slightly lowered by deleting the NMDA receptor from Pv^+ cells.

Previous studies have reported that non-specific decrease in the cortical inhibitory tone reactivates OD plasticity in adult rats (Harauzov et al.). Other manipulations, including rearing animals in an enriched environment or chronically administering fluoxetine, also led to reactivation of juvenile-like plasticity in adult animals and seemed to specifically affect Pv^+ interneurons, as assayed by the decreased density of perineuronal nets (components of the extracellular matrix that form a tight mesh around the basket cells) (Sale et al., 2007; Maya Vetencourt et al., 2008). Additional studies examining the recovery from long-term deprivation in adult rats found that dark exposure promotes recovery by decreasing the number of the $GABA_A$ receptors (He et al., 2006; He et al., 2007). However, our study is the first to directly demonstrate that removing Pv^+ fast-spiking basket cells from the adult visual cortical circuit is vital for reinstating juvenile-like OD plasticity.

The importance of the identification of the defined class of interneurons that can be manipulated to reactivate juvenile-like plasticity in the mature brain cannot be overstated, because it provides a specific target for clinical treatments of conditions such as amblyopia or recovery from other forms of neurological trauma or developmental

abnormalities in cortical circuits. Our results lead to a hypothesis that perhaps to achieve the greatest potential for circuit plasticity, the activity of Pv^+ cells should be mildly modulated, instead of being completely abolished. A clue for this came from the observation that a relatively light disturbance in activity of basket cells via elimination of NMDARs led to a much more dramatic deprived-eye depression compared to what we found when we ablated the interneurons. To further test this hypothesis, one could determine if ablating only part of the basket cell population (by titrating the amount of DT delivered to the visual cortex) can restore the degree of deprived-eye depression normally observed in juveniles during the critical period. If mild reduction of the inhibitory tone proves to be optimal for restoring juvenile-like plasticity, we can next test whether this strategy would be effective in recovering visual acuity in adult animals following a long-term monocular deprivation. As mentioned before, temporary dark exposure, a paradigm that is used for recovery from long-term MD, is associated with reduced levels of inhibition (He et al., 2007). Therefore, a direct mild reduction in cortical inhibitory tone via manipulation of the Pv^+ basket cells might prove successful in restoring visual function in adult amblyopic mice.

It is important to note that a direct demonstration of the involvement of a Pv-expressing class of interneurons in gating plasticity in adults does not eliminate the possibility that other subtypes of interneurons are involved. Pv^+ interneurons are the most abundant inhibitory cells in the visual cortex (Xu et al.) and are also ideally suited for controlling activity of the pyramidal neurons because they form most of their synaptic contacts on the cell body and the axon initial segment. However, there are other large classes of inhibitory cells that express somatostatin (SOM), VIP and other markers that

also could contribute to gating adult plasticity. It would be of great interest to repeat our study with a mouse line that has Cre recombinase expressed under a promoter that defines a separate class of interneurons, such as the SOM-Cre line.

One finding in our study appears curious. Since 2003, a flurry of reports have demonstrated that adult mice can have OD shift under normal conditions (Sawtell et al., 2003; Lickey et al., 2004; Tagawa et al., 2005; Frenkel et al., 2006; Hofer et al., 2006a; Fischer et al., 2007b; Lehmann and Lowel, 2008; Sato and Stryker, 2008). There is a general agreement in the literature that the maximal OD shift in juvenile animals is induced with a brief 3-4 day period of MD (Gordon and Stryker, 1996; Frenkel and Bear, 2004), while adult animals are susceptible to plasticity, but only with longer periods of deprivation (5-7 day at the minimum) (Frenkel et al., 2006; Hofer et al., 2006a; Sato and Stryker, 2008). Only one report found that a robust OD shift in adult mice could be detected with just 1 day of MD (Fischer et al., 2007b). We found that in our experiments, a very robust OD shift could be induced with 3 days of MD, much like what is seen in a juvenile mouse. We were puzzled by this result, which prompted us to repeat this experiment at different ages and in a variety of strains of WT mice, as there can be great variability in OD plasticity among different background strains (Heimel et al., 2007) but the result held up in every group of mice we tested. There are two factors that could account for the discrepancy between our result and those reported previously: anesthesia and method of measuring OD plasticity. Almost all of the previous studies which measured plasticity in the adult cortex did so in anesthetized animals. Several kinds of anesthesia that are routinely used for these studies can increase the inhibitory tone in the cortex, which would likely disturb plasticity. Secondly, OD plasticity is often measured

with recordings of single units, across all layers of the cortex or with optical imaging of intrinsic signals, which restricts measurements to the superficial layers of the cortex. It is known, however, that mechanisms and timing of OD plasticity vary widely by layer, and therefore could contribute to the observed differences (Smith et al., 2009). In our study, we record from awake chronically-implanted animals and our recording are restricted to layer 4. This method has been previously employed to measure OD plasticity induced by a 7 day period of MD (Frenkel et al., 2006), but here we provide the first demonstration that 3 days are sufficient to induce OD plasticity in the adult mouse.

There is also general agreement in the literature that unlike the juvenile OD shift, where the first consequence of visual deprivation is the depression of the deprived-eye response, the shift in the adults is expressed mainly as potentiation of the non-deprived input, with only very mild deprived-eye depression (Lickey et al., 2004; Frenkel et al., 2006; Hofer et al., 2006a; Sato and Stryker, 2008). We confirmed this result in our studies, observing robust open-eye potentiation with no alterations in the deprived-eye response. In juvenile mice, the OD shift is thought to be expressed as a three-stage process. First, weak activation of the deprived-eye functionally decouples the afferents from postsynaptic cortical neurons through the mechanisms of homosynaptic long-term depression (LTD). This is followed by an overall reduction in cortical activity, which lowers the plasticity threshold, favoring strengthening of weak inputs. This, in turn, allows the initially weaker inputs from the non-deprived eye to potentiate through homosynaptic long-term potentiation (LTP). Whether induction of open-eye potentiation requires previous deprived-eye depression or whether the two processes can occur independently has remained an open question. Our data provide a nice dissociation of the

two events, with open-eye potentiation occurring even in the absence of deprived-eye depression. It is likely that metaplasticity mechanisms responsible for strengthening of open-eye inputs require an average reduction in cortical activity, which is normally facilitated by MD. An alternative explanation is that the increase in the open eye is supported by mechanisms of synaptic scaling, whereby cortical neurons are trying to preserve the baseline level of firing in light of an overall diminished cortical activity (Turrigiano and Nelson, 2004).

In conclusion, we have demonstrated that even though the adult visual cortex of a mouse is susceptible to experience-dependent plastic changes, adult plasticity is limited and its qualities are dramatically different from those observed during early development: in adults a brief 3 day MD drives a potentiation of the non-deprived input in the adult cortex, while in juveniles the shift in OD is expressed as depression of the deprived-eye response. We also showed that much like what has been reported in the adult rats, reduction of the inhibitory tone in the cortex of a mouse is responsible for reactivation of juvenile-like plasticity. Furthermore, we ascribed the 'inhibitory gate' function specifically to a subclass of cortical interneurons: the Pv-expressing fast-spiking basket cells. We observed that removing this population from the visual cortical circuit of the adult mice blocked fast open-eye potentiation and reinstated a juvenile-like depression of deprived-eye responses.

4.5 Methods

4.5.1 Subjects

All animals were handled according to the rules and guidelines set by the MIT Animal Care Committee and the National Institutes of Health. Mice were fed *ad libidum* and reared in normal lighting conditions (12 hours light/12 hours dark cycle). C57BL/6 mice (postnatal day (P) 60) were obtained from Charles River Laboratories and aged at MIT until P90-120. Parvlbumin-Cre mice were generously supplied by S. Arber (Hippenmeyer et al., 2005). The line was backcrossed to the C57BL/6 background for 7-10 generations. Diphtheria toxin receptor (DTR) mice were generously supplied by A. Waisman (Buch et al., 2005). Pv-Cre and DTR lines were crossed and F1 generation animals (P90-120 or P240-360) were used for experiments, including Pv-Cre/DTR, Pv-Cre/WT (wild-type), WT/DTR, and WT/WT. The generation of the "floxed" NMDA receptor subunit-1 (fNR1) mouse line is described in (Tsien et al., 1996). Pv-Cre and fNR1 lines were crossed and F1 generation animals were used for experiments, including Pv-Cre/fNR1 and WT/fNR1 (P90-150). Rosa26-GFP reporter line is described in (Soriano, 1999). Pv-Cre and Rosa-GFP lines were crossed and F1 progeny was used to assess the efficiency of Cre/loxP recombination. All transgenic mice used in electrophysiology experiments were maintained on the C57Bl/6 background.

4.5.2 Animal preparation

Mice were anesthetized by inhalation of isoflurane (IsoFlo 2%-3%). A local anesthetic, 0.1% lidocaine, was injected under the scalp. A head post was attached just anterior to bregma with cyanoacrylate glue (Small parts Inc., Miami Lakes, FL). Reference electrodes were placed bilaterally in prefrontal cortex. A small craniotomy (~1

mm) was made over binocular visual cortex (3.2 or 3.3 mm lateral to lambda for P90-120 and P>120 animals respectively), taking great care to leave dura intact, and tungsten microelectrodes (FHC, Bowdoinham, ME) were inserted ~470 μm from dural surface. Electrodes were secured in place by cyanoacrylate glue and dental cement (Lang Dental Inc., Nashua, NH). Animals were monitored postoperatively for 48 hours and were allowed at least 24 hr recovery period before habituation to the restraint apparatus.

4.5.3 Microinjections

Mice were anesthetized by inhalation of isoflurane (IsoFlo 2%-3%). A local anesthetic, 0.1% lidocaine, was injected under the scalp. A head post was attached just anterior to bregma with cyanoacrylate glue (Small parts Inc., Miami Lakes, FL). A small craniotomy (~1 mm) was made over binocular visual cortex (3.2 or 3.3 mm lateral to lambda for P90-120 and P>120 animals respectively), taking great care to leave dura intact. Diphtheria toxin (Sigma, St. Louis, MO) or ACSF was injected using a Nanoject II injection system (Drummond Scientific, Broomall, PA). The toxin (100 ng/ml) was injected with a glass micropipette with a tip diameter of approximately 20 μm. 18 injections of 9.2 nL were made at three cortical depths (600 μm, 450 μm, and 300 μm from dural surface), 6 injections/depth. The pipette was left in place for 5 min following the last injection. Following removal of the pipette, the exposure was either covered with 1% agar solution or an electrode was implanted and secured with cyanoacrylate glue. The exposed skull was covered with dental cement and the animals were monitored post-operatively for 48 hours.

For AAV2.8-CMV-Flexed-tomato virus injections, a second craniotomy was made at 3.7 mm lateral to lambda; the virus was loaded into a glass micropipette and

injected with a Nanoject II injection system at three cortical depths (600, 450, and 300 μm below dural surface, total of ~165 nL) and allowed to express for ~30 days before the animal was sacrificed for histological analysis (plasmid received from Sternson lab (Atasoy et al., 2008) and packaged by the MIT Viral Core Facility).

4.5.4 Tissue preparation, immunohistochemistry and imaging

Animals were euthanized by an overdose of pentobarbital and transcardially perfused with ~50 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and postfixed for 1 day at room temperature. 50 μm slices were cut in the coronal plane using a vibratome. Slices were blocked in 20% fetal bovine serum (FBS) and 0.1% Triton X-100. Slices were then incubated overnight at 4°C in 20% FBS, 0.1% Triton X-100 and either a monoclonal antibody to Pv (1:1000; Millipore, Bellerica, MA), a rabbit antibody to GFP (1:3000; Abcam, Cambridge, MA). Sections were then incubated for 1 hour at room temperature with the appropriate Alexa488- or Alexa555-conjugated goat IgG (1:400; Millipore, Bellerica, MA). Sections were additionally stained with a fluorescent Nissl stain (1:300, Molecular Probes. Stained sections were mounted onto glass slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA), allowed to adhere and were covered with an aqueous-based mounting medium (Biomeda GelMount, Foster City, CA) and coverslipped, allowed to cure at room temperature for 12-24 h and then sealed with clear nail polish to prevent drying. Stained sections were imaged using a laser-scanning confocal microscope (Olympus).

4.5.5 Monocular deprivation

Mice were anesthetized by inhalation of isoflurane (IsoFlo 2%-3%) and placed under a surgical microscope. Lid margins were trimmed and antibiotic ophthalmic

ointment (Vetropolycin, Pharmaderm) was applied to the eye. Eyelids were sutured with mattress stitches opposing the full extent of the trimmed lids using 6-0 vicryl. Mice were recovered by breathing room air and were monitored daily to ensure that the sutured eye remained shut and uninfected. Animals whose eyelids were not fully shut for the entirety of the deprivation period were excluded from the study. At the end of the deprivation period, mice were reanesthetized, stitches were removed, and lid margins were separated. Eyes were then flushed with sterile saline and checked for clarity under a microscope. Mice with corneal opacities or signs of infection were excluded from the study.

4.5.6 In vivo electrophysiology

All recordings were conducted in awake mice. The animals were alert and head-restrained during recording. Following postoperative recovery, the animals were habituated to the restraint apparatus for 1-2 hrs. For recording sessions visual stimuli were presented to left and right eyes randomly. A total of 100 to 200 stimuli were presented per each condition. VEP amplitude was routinely quantified by measuring trough-to-peak response amplitude, as described previously (Sawtell et al., 2003).

4.5.7 Visual stimuli

Stimuli consisted of full-field sine-wave gratings of 100% contrast, square-reversing at 1Hz, and presented at 0.05 cycles/degree. Stimuli were generated by a VSG2/2 card (Cambridge Research System, Cheshire, UK) and presented on a CRT computer monitor. VEPs were elicited by either horizontal or vertically oriented gratings. In MD experiments, orientation of stimuli during the first recording was randomized, but the stimuli presented to the same animal before and after deprivation were always orthogonal to each other to avoid the confound of stimulus-selective

response potentiation (Frenkel et al., 2006). In non-MD experiments, visual stimuli differed by 30 or more degrees in each recording session. The display was positioned 20 cm in front of the mouse and centered at the vertical meridian, occupying 92° x 66° of the visual field. Mean luminance was 27 cd/m^2.

4.5.8 Statistics

All statistical analyses were performed using StatView 5.0.1 (Abacus Concepts, Berkeley, CA). A Student's paired t-test or a global ANOVA was always performed where appropriate, and relevant post hoc comparisons were made using Fisher's protected least square difference analysis. For comparison of C/I ratios, which were not normally distributed, statistics were performed using Wilcoxon Signed Rank test or Ksuskal-Wallis test. Data expressed as means ± SEM, and significance was placed at $p < 0.05$.

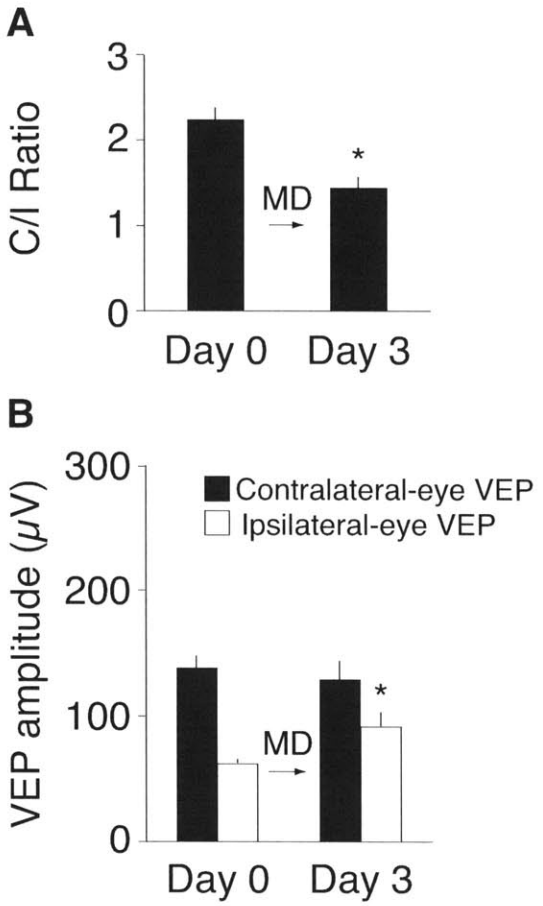

Figure 4.1 – The ocular dominance shift following 3 days of MD in adult mice (P90-120) is expressed as potentiation of non-deprived eye responses

A. There is a significant decrease in C/I ratios following 3 days of MD (n = 10). Average values of C/I ratios (±SEM) are plotted. Asterisk indicates p = 0.009. B. Average trough-to-peak amplitude (±SEM, n = 10) of VEPs in response to deprived eye (filled bars) and non-deprived eye (open bars) stimulation during baseline (day 0) and after 3 days of MD. There is a significant increase in the non-deprived eye VEP amplitude and no change in the deprived-eye VEP amplitude. Asterisk indicates p = 0.03.

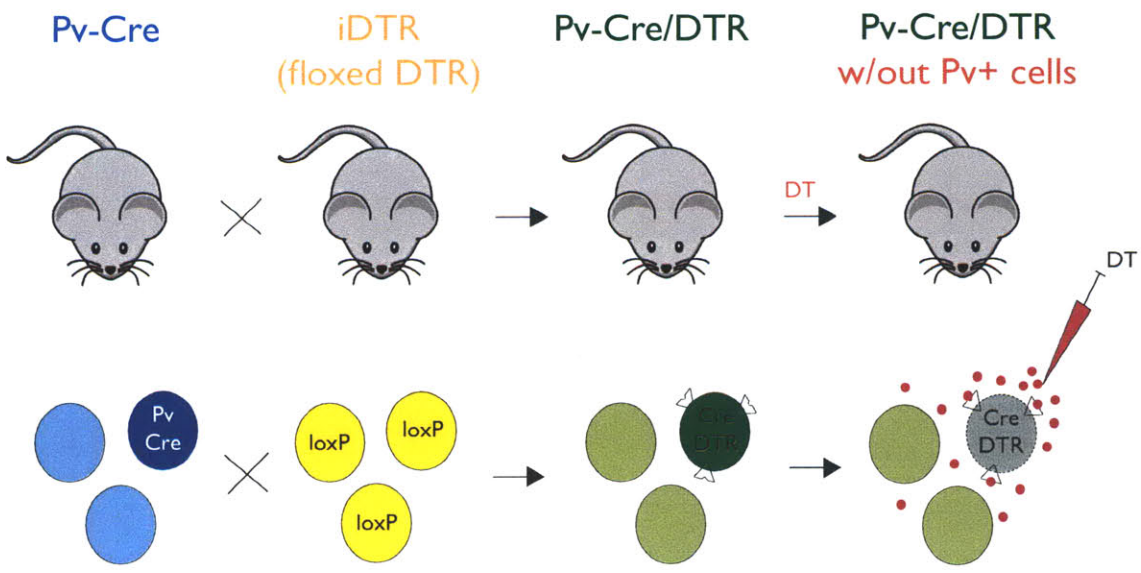

Figure 4.2 – Experimental strategy for selective ablation of Pv^+ cortical interneurons

Mice fom a Pv-Cre transgenic line, expressing Cre under the parvablumin promoter, are crossed to mice from the iDTR transgenic line, containing a floxed simian form of the diphtheria toxin receptor. Mice that result from the cross express DTR in all cells that express parvalbumin. A local injection of diphtheria toxin (DT) into the visual cortex selectively targets and ablates Pv^+ interneurons within the visual cortical circuit.

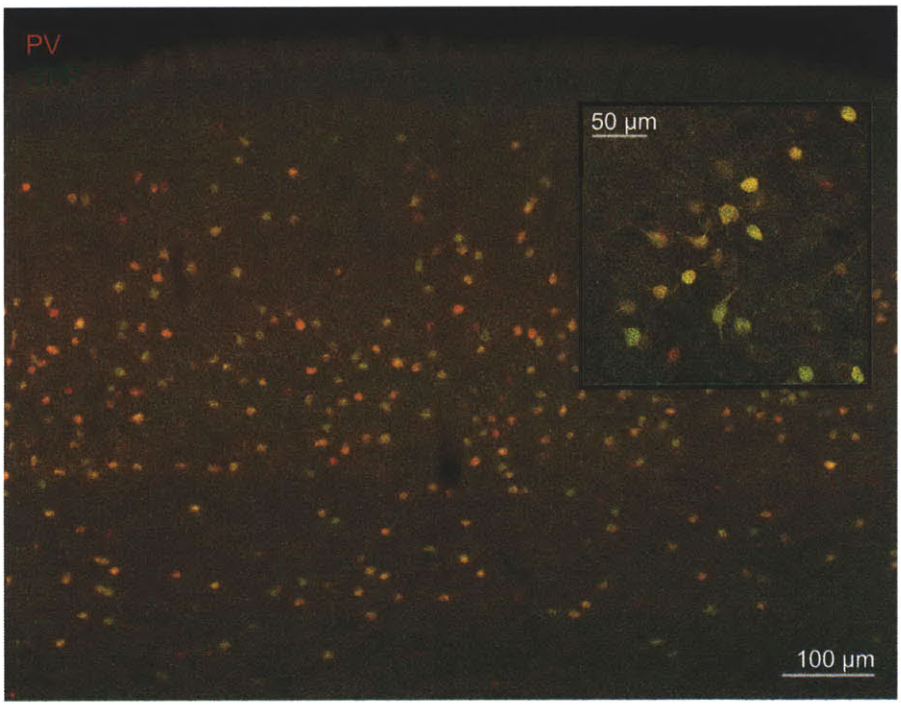

Figure 4.3 – Cre-mediated recombination in the Pv-Cre line is highly efficient

Pv-Cre mice were crossed to a GFP reporter line (ROSA-GFP) to assess the efficiency of Cre-mediated recombination. Mice resulting from this cross express GFP in all cells where Cre recombination occurred. The visual cortical section shown above has been co-immunostained for GFP and Pv. The overlay of the two images demonstrates that most of the GFP^+ (green) and Pv^+ (red) cells co-localize with each other, indicating that recombination occurred in most Pv-expressing cells. The inset shows that among stained cells very few cells are positive for Pv and negative for GFP, while the majority of cells are yellow (i.e. positive for Pv and GFP).

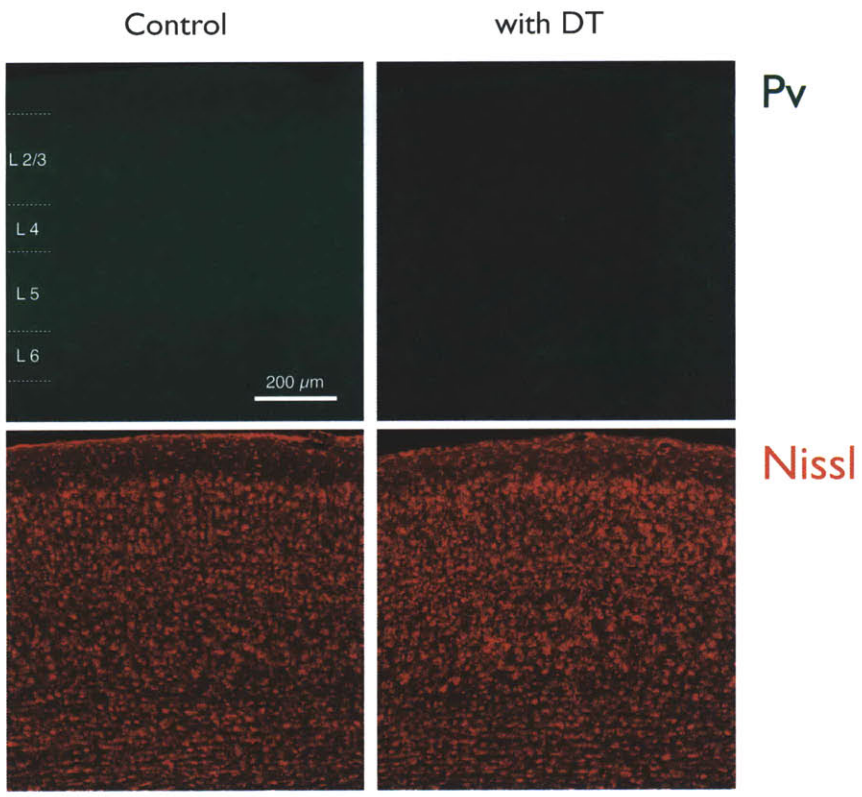

Figure 4.4 – Near complete ablation of Pv^+ interneurons with an injection of DT into the Pv-Cre/DTR mice

(*Left column*) Immunostaining of the visual cortical section of a brain that has not been injected with diphtheria toxin (DT) demonstrates a normal distribution of the parvalbumin (Pv)-positive interneurons (*top*) and an overall normal gross appearance and laminar structure as revealed by the fluorescent Nissl staining (*bottom*). (*Right column*) Immunostaining of the visual cortical section of a brain injected with DT demonstrates a nearly complete ablation of Pv^+ interneurons throughout all cortical layers (*top*). The overall gross appearance and laminar structure of the cortex, however, is not affected by the injection of DT (*bottom*).

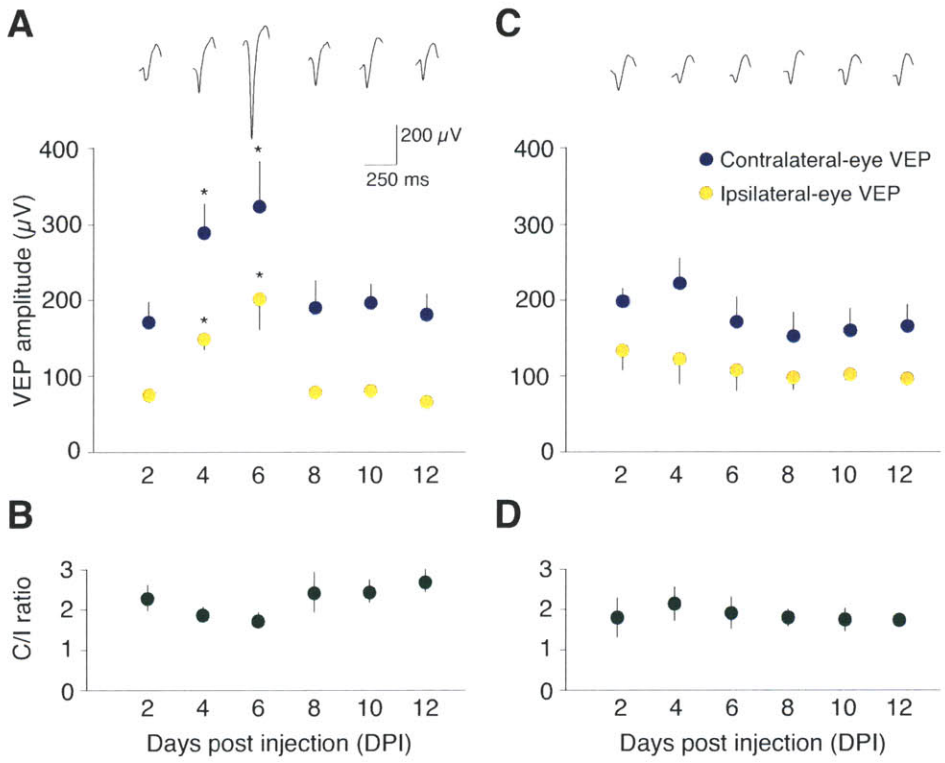

Figure 4.5 – Effect of DT on VEP amplitude and C/I ratios in the Pv-Cre/DTR mice

A,C. Average trough-to-peak VEP amplitudes (±SEM) driven through the contralateral (blue) and the ipsilateral (yellow) eyes were monitored for 12 days post injection (DPI) of diphtheria toxin (DT). In the Pv-Cre/DTR mice injection of DT elicited a gradual increase in VEP amplitude over the first week post injection of the toxin, but the amplitudes returned to baseline at 8 DPI (A, n = 8). In littermate controls, injection of DT did not have a significant effect on the amplitu

de of the responses (C, n = 4). Asterisks indicate $p < 0.05$ compared to baseline values (at 2 DPI). B,D. The C/I ratios were unaffected by the DT injection in both Pv-Cre/DTR mice and littermate controls. Average values of C/I ratios (±SEM) are plotted.

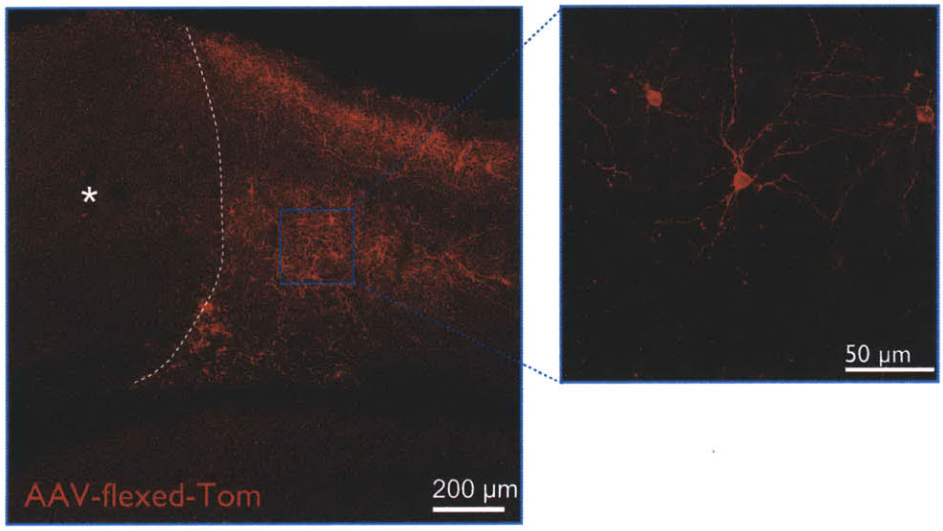

Figure 4.6 – Spared laterally-located Pv^+ basket cells do not extend axons into the DT-induced area of Pv^+ interneuron ablation

Representative visual cortical section from a Pv-Cre/DTR brain injected with diphtheria toxin (DT) and AAV-flexed-tomato virus lateral to the DT injection (white asterisk indicates the location of the DT-injection micropipette). Red neurons expressing tomato fluorescent protein were confirmed to be large basket cells by morphological characteristics (example shown in inset). Tomato fluorescent protein highlights dense arborization of the basket cells lateral to the site of ablation (white dashed line roughly delineates ablation area) with no discernable axons extending into the area of ablation.

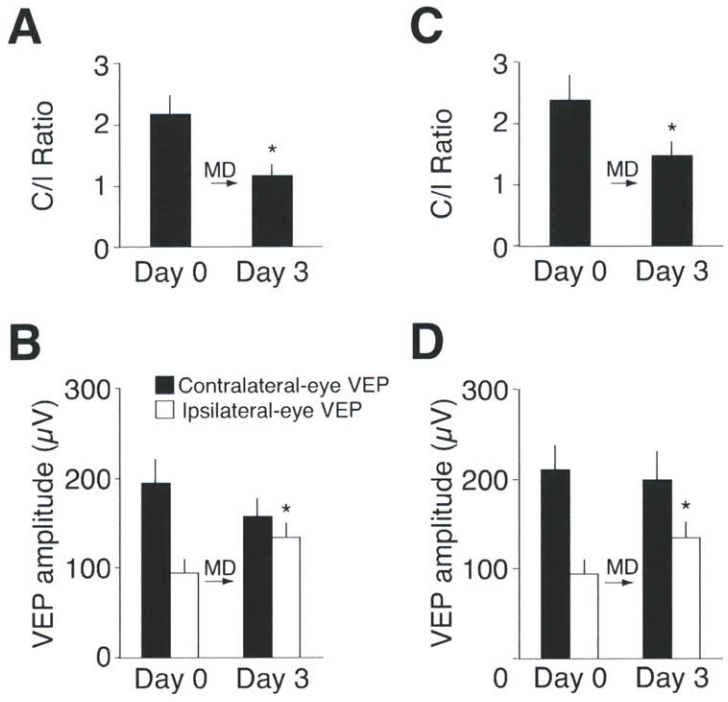

Figure 4.7 – The ocular dominance shift following 3 days of MD in adult Pv-Cre/DTR mice is expressed as potentiation of non-deprived eye responses

A,C. There is a significant decrease in C/I ratios following 3 days of MD in Pv-Cre/DTR mice at both P90-120 (n = 7) (A) and P240-260 (n = 6) (C). Average values of C/I ratios (±SEM) are plotted. Asterisks indicate $p < 0.05$. B,D. Average trough-to-peak amplitude of VEPs in response to deprived eye (filled bars) and non-deprived eye (open bars) stimulation during baseline (day 0) and after 3 days of MD. There is a significant increase in the non-deprived eye VEP amplitude and no change in the deprived-eye VEP amplitude at both ages. Asterisks indicates $p < 0.05$.

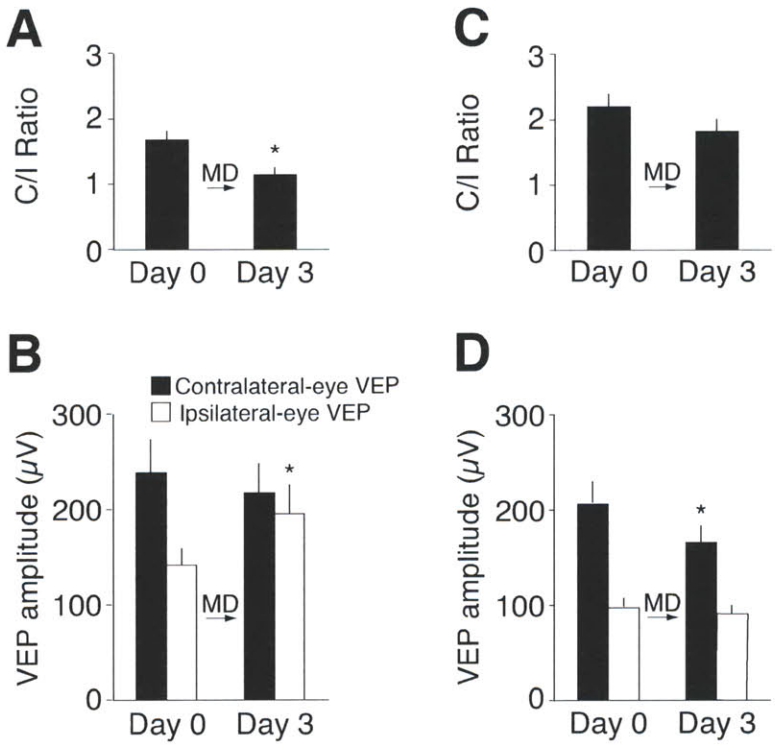

Figure 4.8 – The effect of DT on the qualities of the ocular dominance shift following 3 days of MD in adult Pv-Cre/DTR mice

A. There is a significant decrease in C/I ratios (n = 6) following 3 days of MD in Pv-Cre/DTR littermate control (WT) mice injected with DT. Average values of C/I ratios (±SEM) are plotted. Asterisk indicates p = 0.03. B. Average trough-to-peak amplitude (±SEM, n = 6) of VEPs in response to deprived eye (filled bars) and non-deprived eye (open bars) stimulation during baseline (day 0) and after 3 days of MD. There is a significant increase in the non-deprived eye VEP amplitude and no change in the deprived-eye VEP amplitude. Asterisk indicates p = 0.04. C. There is no significant decrease in C/I ratios (n = 11) following 3 days of MD in Pv-Cre/DTR injected with DT. Average values of C/I ratios (±SEM) are plotted. D. Average trough-to-peak amplitude (±SEM, n = 11) of VEPs in response to deprived eye (filled bars) and non-deprived eye (open bars) stimulation during baseline (day 0) and after 3 days of MD. There is a significant decrease in the deprived-eye VEP amplitude and no change in the non-deprived eye VEP amplitude. Asterisk indicates p = 0.02.

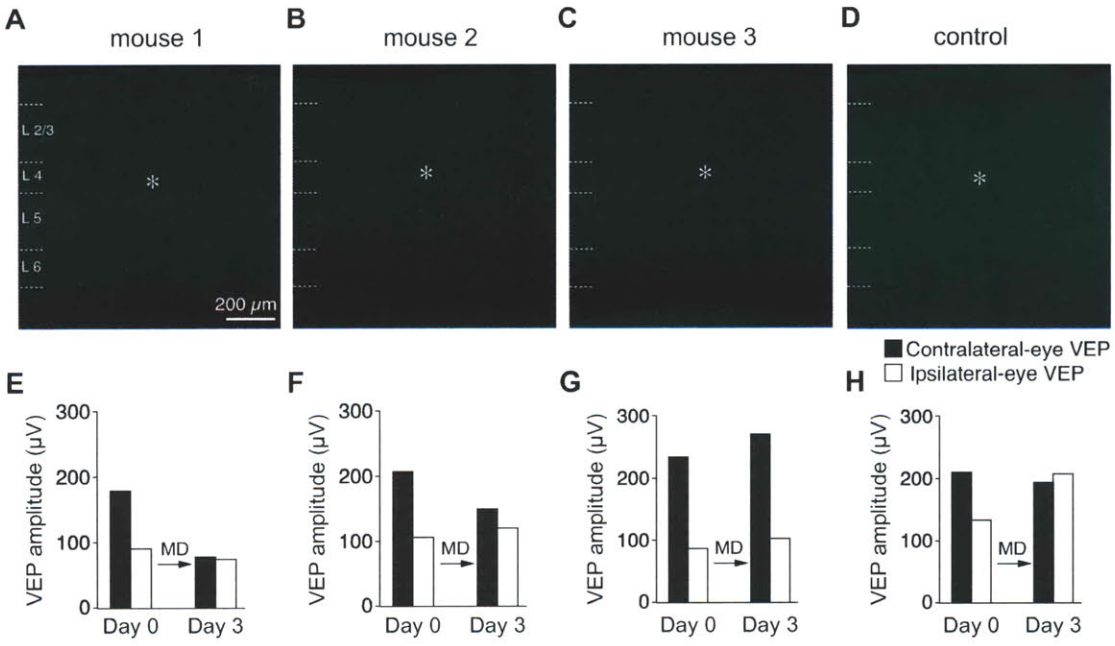

Figure 4.9 – The magnitude of deprived-eye depression in Pv-Cre/DTR mice correlates with the degree of ablation of Pv^+ interneurons

A-D. Representative examples of visual cortical sections from four mouse brains that were injected with diphtheria toxin (DT) and later immunostained for parvalbumin (Pv). Mouse 1-3 were Pv-Cre/DTR, while the "control" mouse was a littermate control mouse. Mouse 1 (A) and mouse 2 (B) have a number of spared Pv^+ interneurons, while ablation is nearly complete in the example section from mouse 3. Control mouse (D) demonstrates a normal distribution of Pv^+ interneurons. White asterisks indicate the approximate position of the recording electrode tip. Dashed lines roughly indicate laminar boundaries. E-H. Representative ocular dominance shifts recorded from the four mice following 3 days of MD. Average trough-to-peak amplitude of VEPs in response to deprived eye (filled bars) and non-deprived eye (open bars) stimulation during baseline (day 0) and after 3 days of MD are plotted. There is a robust decrease in the deprived-eye VEP amplitude and no change in the non-deprived eye VEP amplitude in mouse 1 (E) and mouse 2 (F). In mouse 3 (G) there is no decrease in the deprived-eye VEP amplitude no increase in the non-deprived eye VEP amplitude. In the control mouse (H) there is no decrease in the deprived-eye VEP amplitude and a robust increase in the non-deprived VEP amplitude.

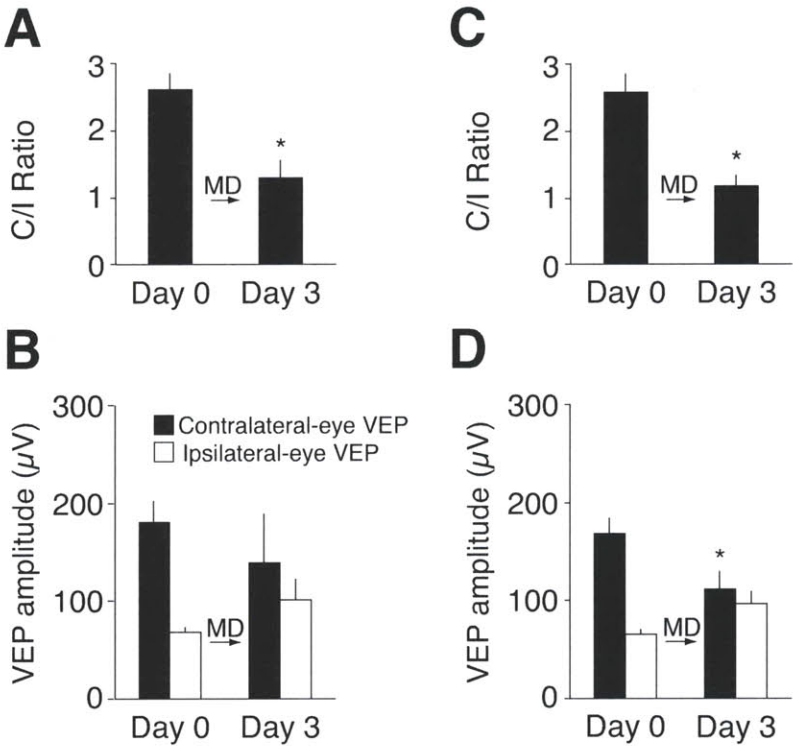

Figure 4.10 – The qualities of the ocular dominance shift following 3 days of MD in adult Pv-Cre/fNR1 mice

A. There is a significant decrease in C/I ratios (n = 4) following 3 days of MD in Pv-Cre/fNR1 littermate control mice. Average values of C/I ratios (±SEM) are plotted. Asterisk indicates p = 0.016. B. Average trough-to-peak amplitude (±SEM, n = 4) of VEPs in response to deprived eye (filled bars) and non-deprived eye (open bars) stimulation during baseline (day 0) and after 3 days of MD. There is no significant increase in the non-deprived eye VEP amplitude and no significant change in the deprived-eye VEP amplitude. C. There is a significant decrease in C/I ratios (n = 8) following 3 days of MD in Pv-Cre/fNR1 mice. Average values of C/I ratios (±SEM) are plotted. Asterisk indicates p = 0.012. D. Average trough-to-peak amplitude (±SEM, n = 8) of VEPs in response to deprived eye (filled bars) and non-deprived eye (open bars) stimulation during baseline (day 0) and after 3 days of MD. There is a significant decrease in the deprived-eye VEP amplitude and a significant increase in the non-deprived eye VEP amplitude. Asterisk indicates p = 0.003 for contralateral VEP amplitudes and p = 0.04 for ipsilateral VEP amplitudes.

Chapter 5

Implications and future directions

5.1 Introduction

Amblyopia is a devastating visual impairment that affects 1-2% of people worldwide (Doshi and Rodriguez, 2007). This condition arises from the failure of cortical visual circuit to mature properly during early development because of a functional mismatch between the two eyes due to a variety of perturbations, such as cataracts, astigmatism or strabismus. It has been demonstrated that a recovery from amblyopia is possible, but it restricted to a narrow window (the critical period) in childhood, such that in the majority of patients recovery is successful only before 7 years of age (Mitchell and Sengpiel, 2009). After this age, even though a cataract removal reveals a nearly perfect condition of the eye itself, the connection between the eye and the visual cortex can no longer be restored through normal binocular vision. It has been a great challenge to understand how the cerebral cortex is modified by the quality of sensory experience in order to elucidate potential treatments for amblyopia and other developmental disorders that result in improper cortical circuit maturation. Furthermore, understanding the mechanisms that govern the decline in cortical plasticity in an adult brain is crucial not only for developing treatments for amblyopia that has failed to be corrected in childhood, but could also provide invaluable insight into recovery from neurological trauma.

To understand how the visual cortical circuit is shaped and modified by the quality of visual experience, investigators have addressed three broad questions: 1) what are the consequences of visual deprivation at the molecular, synaptic and structural levels, 2) what are the factors responsible for a developmental decline in cortical

plasticity and what mechanisms gate plasticity in the adult, and 3) what interventions can promote functional recovery of vision in the neocortex.

Ocular dominance plasticity has served as a sensitive assay for determining the consequences of visual deprivation on the synapses in the visual cortex. One of the models that helps explain the observed changes in OD following monocular deprivation (MD) is the BCM model of bidirectional plasticity (Bienenstock et al., 1982; Blais et al., 1999; Smith et al., 2009). According to this model, decreased visual drive through the deprived eye weakens the connection between this input and visual cortical neurons via homosynaptic long-term depression (LTD). This leads to the overall decrease in cortical activity, and shifts the synaptic threshold for bidirectional plasticity in favor of synaptic strengthening over synaptic weakening. This, in turn, enables the weaker, open-eye inputs to potentiate via the mechanisms of heterosynaptic long-term potentiation (LTP). This model can explain a great deal of the data that has been collected thus far in the field, although certain studies shed light on other outstanding issues that remain unanswered.

5.2 Mechanisms of deprived-eye depression in juvenile mice

Many studies have attempted to establish a causal link between depression of excitatory synaptic transmission (via mechanisms of LTD) and deprived-eye depression that occurs following MD by correlating the effect of pharmacological or genetic manipulations on both processes (Smith et al., 2009). Several recent studies from our laboratory showed that monocular deprivation occludes further induction of LTD *ex vivo* and inhibition of LTD induction blocks deprived-eye depression *in vivo* (Heynen et al., 2003; Yoon et al., 2009). However, it has also recently been demonstrated that both LTD and shifts in

ocular dominance can occur in parallel in different layers of the cortex via different mechanisms (Daw et al., 2004; Crozier et al., 2007; Liu et al., 2008; Yoon et al., 2009). Since input from the lateral geniculate nucleus of the thalamus (LGN) sends most of its afferents into layer 4 of the primary visual cortex, one of the important aims was to demonstrate that depression of excitatory synaptic transmission at the thalamocortical (TC) synapses occurs as a consequence of MD. In Chapter 2, I addressed this question and demonstrated that following 3 days of MD, when the magnitude of deprived-eye depression is maximal, the strength of the deprived-eye input at the thalamocortical synapse is dramatically reduced, while the strength of the non-deprived eye input is unchanged. Additional corroborating anatomical evidence for the loss in strength of the afferent TC inputs carrying information from the deprived eye came from a recent study which discovered a striking decrease in the TC synaptic density following a brief 3 day period of MD (Coleman et al., 2010). These studies were the first to localize deprived-eye depression to a specific set of synapses. However, given that functional shifts can also be detected in layer 2/3 following only 1 or 2 days of MD (Trachtenberg et al., 2000; Liu et al., 2008), it remains unclear whether a functional shift at the TC synapses is a direct consequence of MD or whether the shift first occurs in layer 2/3 and is then transferred to layer 4. One of the arguments against this model is the finding that blocking OD plasticity in layers 2/3 does not affect plasticity in layer 4 (Yoon et al., 2009). However, a better experiment would be to chronically monitor activity in both layers 2/3 and in layer 4 before and after MD. Unfortunately, there are technical difficulties in carrying out this study. Routine methods for assaying OD plasticity include single-unit recording, optical imaging of intrinsic signals, two-photon calcium

imaging and visually evoked potentials (VEPs). Among these, only VEPs can be recorded chronically, but this method limits the study to layer 4 because VEPs largely reflect a current sink created by the TC afferents in this layer. Conversely, imaging techniques are largely limited to the superficial layers. Single-units, on the other hand, can be sampled from all layers, but historically analysis of this data has not be done by layer, which is inadequate for assaying laminar-specific OD shifts. However, recent technological development of better resolution microscopy and genetically-encoded calcium sensors (Knopfel et al., 2006) will enable chronic imaging of both layer 2/3 and layer 4 neuronal activity. This technology combined with the use of various transgenic lines of mice will allow not only for the elucidation of the timing of the OD shifts in each layer, but also to determine the effect and the contribution of various neuronal subtypes to the OD plasticity following brief MD.

It is well established that with longer periods of visual deprivation, functional OD shifts are accompanied by structural changes, which are likely to be the cause of lasting visual disability (Antonini and Stryker, 1996; Antonini et al., 1999). TC afferents serving the deprived eye retract, while the axons from the non-deprived input expand their area of cortical innervation. However, it remains unclear whether the rapid molecular changes precede or are necessary for the structural modifications of the circuit. New *in vivo* imaging technology such as two-photon (2-P) laser excitation microscopy have allowed longitudinal studies of both pre- and postsynaptic structures, revealing remarkable degrees of rapid structural plasticity in the neocortex (Majewska and Sur, 2003; De Paola et al., 2006; Lee et al., 2006; Holtmaat and Svoboda, 2009). The use of 2-P *in vivo* microscopy in combination with new genetic and viral labeling methods allow a direct

examination of axonal branch stability over days and in response to MD. This method would enable testing of the hypothesis that structural changes in the visual cortical circuit (specifically remodeling of the TC inputs) occur on the same time scale as the changes in visual responses and, at least in part, underlie the rapid decrease in visual acuity.

Of particular interest would be to independently track axons serving the deprived and the non-deprived eyes. For instance, data from our laboratory showed that the loss of TC synapses is maximal following 3 days of MD, but returns back to normal levels by 7 days of MD (Coleman et al., 2010). This result maps perfectly onto the timing of the OD shift measured physiologically (Frenkel and Bear, 2004). We speculate that the decrease in synaptic density contributes to deprived-eye depression and the apparent gain of TC synapses after 7 days of MD can be attributed to potentiation of the non-deprived eye responses. However, there are no techniques currently available that would allow for discrimination between the two eye-specific inputs. Methods for input-specific labeling are currently in development in several laboratories and take advantage of the AAV-based labeling systems that allow for transneuronal labeling of synaptically-connected neurons (Gradinaru et al., 2010).

5.3 Mechanisms of open-eye potentiation in juvenile mice

In mice MD produces a biphasic response in the visual cortex. Initial changes in OD occur through the weakening of deprived-eye responses, whereas longer periods of deprivation also drive open-eye potentiation. As discussed in Chapter 1, based on the BCM theory, we favor LTP as a molecular mechanism for the delayed open-eye potentiation. As the length of MD increases beyond 3 days, the sliding modification threshold determining the boundary between depressing and potentiating stimuli would

shift to favor response potentiation. Although there is general agreement among laboratories that this process occurs following visual deprivation, several important questions remain unanswered: 1) what is the molecular mechanism underlying strengthening of open-eye inputs, 2) what is the cortical locus of synaptic and structural changes underlying the increase in open-eye responses, and 3) does open-eye potentiation require deprived-eye depression to precede it or are the two processes independent.

In Chapter 3, I present evidence that potentiation of the non-deprived eye inputs is dependent on the activation of the NMDA receptor and a decrease in the NR2A/2B ratio of NMDAR subunits favors induction of the synaptic potentiation following MD. Since the visual input through the open eye is unaltered during MD, the change in the modification threshold brought about by a decrease in the NR2A/2B ratio is likely to be permissive for the induction of LTP-like processes at cortical synapses receiving open-eye afferents.

An alternative mechanism for the increase in the open-eye responses is synaptic scaling, which relies on the global homeostatic elevation of output activity in response to reduced levels of cortical activity (Turrigiano et al., 1998). In the context of OD plasticity in rodents, the closure of the dominant eye would result in an overall decrease of cortical output, eliciting an upward scaling of all synapses, including the weak inputs coming from the non-deprived eye (Turrigiano and Nelson, 2004).

A third possibility was recently suggested by a study showing a significant role of interehemispheric callosal inputs in OD plasticity (Restani et al., 2009). One of the findings in this study is that in non-deprived animals, blockade of activity in the hemisphere opposite to the recorded one shows a marked decrease in ipsilateral ("open-

eye") driven responses, while following a 7 day period of MD, the same paradigm does not affect the strength of this input. This alludes to the possibility that potentiation of open-eye responses may involve the strengthening of callosal projections subserving the open eye. Support for this idea comes from the finding that MD can affect OD not only in the cortex contralateral to the deprived eye, but also in the hemisphere ipsilateral to the deprived eye (He et al., 2007; Mrsic-Flogel et al., 2007).

The three mechanisms proposed to account for open-eye potentiation – homosynaptic LTP of thalamocortical afferents subserving the non-deprived eye, heterosynaptic scaling of responses, or potentiation of trans-callosal drive dominated by the ipsilateral-eye – are not mutually exclusive and all three processes may, in varying degrees, be recruited following prolonged MD. However, it is important to be able to dissociate these processes from one another, as well as the relative contribution of each to the strengthening of the open-eye responses.

One of the distinctions between LTP and synaptic scaling is the differential requirement for the synaptic insertion of GluR1 containing AMPA receptors. It has been shown that intracellular introduction of a region of the GluR1 C-terminal tail (GluR1CT) into neurons is sufficient to both prevent the delivery of GluR1 to synapses and block LTP (Shi et al., 2001). Other studies additionally report that GluR1 delivery is required for certain experience-dependent forms of plasticity *in vivo* (Rumpel et al., 2005; Frenkel et al., 2006). Viral delivery of GluR1CT peptide into the visual cortex could be used to determine the contribution of LTP mechanisms in open-eye potentiation. If expression of GluR1CT blocks open-eye potentiation, this would strongly argue for the role of LTP in this process. Synaptic scaling, on the other hand, depends on delivery of GluR2-

containing AMPARs and is not blocked by GluR1CT (Gainey et al., 2009). Therefore, the consequence of MD in a GluR1CT-expressing visual cortex with respect to open-eye potentiation will likely distinguish between LTP and synaptic scaling as necessary components underlying this phenomenon.

Additionally, it would be useful to perform studies investigating whether induction of LTP in the visual cortex and open-eye potentiation can occlude each other, much like what has been done with LTD and deprived-eye depression (Heynen et al., 2003; Crozier et al., 2007). In such an experiment, non-deprived eye potentiation would be induced with 7 days of MD and then the ability to further induce LTP in slices *ex vivo* would be tested. Inability to elicit LTP would suggest that same molecular pathways are engaged in the two processes. Conversely, LTP could be induced *in vivo* by stimulation of LGN as has already been demonstrated in our laboratory (Heynen and Bear, 2001) while the animal is undergoing MD (for instance after 3 days of MD, when deprived-eye depression has already occurred) and then, following 4 more days of MD, VEPs could be assayed. If LTP induction and open-eye potentiation do not share the same mechanism, then a greater open-eye potentiation should be detected in the LTP-induced animals compared to controls.

It is unclear whether contribution of callosal inputs to open-eye potentiation is significant, since the callosal fibers provide input to a very small part of the binocular visual cortex (Restani et al., 2009). Nevertheless, a chronic blockade of activity in the cortex or LGN ipsilateral to the deprived eye during the last 4 days of a 7-day MD would reveal whether callosal projections play a vital role in induction of open-eye potentitation.

It is clear that like LTD, the mechanisms of LTP vary across cortical layers (Wang and Daw, 2003). With regards to deprived-eye depression, these mechanistic differences lead to independent responses to MD in layers 4 and 2/3 (Liu et al., 2008). What remains to be determined is whether open-eye potentiation can occur in a similar layer-specific manner. Moreover, it would be important to find out whether the strengthening occurs at the thalamocortical synapses (similar to what I have shown for deprived-eye depression in Chapter 2) or whether most of the potentiation occurs intracortically. One way to assay this would be to perform an experiment similar to the one I have described in Chapter 2. If the cortex is silenced acutely after 7 days of MD, the strength of TC input through the open eye can be measured and compared to that in non-deprived controls. If the magnitude of VEPs is the same as in non-deprived animals, the locus of open-eye potentiation is likely to be intracortical. If, on the other hand, the magnitude is greater than in controls, it would suggest that TC synapses have been strengthened.

Another way to address the laminar specificity of open-eye potentiation would be to combine eye-specific input labeling with the use of genetically-encoded calcium sensors (Knopfel et al., 2006; Gradinaru et al., 2010). This would allow a chronic monitoring of both activity and structure of the open-eye axons in different cortical layers. This is a rather difficult experiment, but the improvements in technology are likely to make these studies possible in the near future.

Given the differential timecourses of deprived-eye depression and open-eye potentiation following MD (Frenkel and Bear, 2004), it is possible that the prior weakening of deprived-eye inputs is required to allow subsequent potentiation to occur.

Alternatively, input activity to the cortex may be sufficiently decreased following MD to allow changes in the modification threshold in the absence of any decreases in synaptic strength. To address this question, it is possible to utilize a peptide that our lab previously used to block deprived-eye depression in layer 4 (G2CT) and test for changes in open-eye responses following 7 days of MD. Given that G2CT is ineffective in blocking LTD in layer 2/3 (Crozier *et al.*, 2007), a potential finding of open-eye potentiation measured through layer 4 VEPs in G2CT expressing animals could result from processes dependent on LTD in supragranular layers. This possibility could be addressed by pairing G2CT expression with the endocannabinoid receptor antagonist AM251 to block LTD and deprived-eye depression in both layer 4 and layer 2/3 (Crozier *et al.*, 2007; Liu *et al.*, 2008).

One interesting observation from the data I present in Chapter 3 that speaks to the dissociation between deprived-eye depression and open-eye potentiation is that in the NR2A KO mouse 3 days of MD leads to open-eye potentiation in the absence of any observable deprived-eye depression. One interpretation of this result is that a decrease in the NR2A/2B ratio in this mouse underlies the reduction in the plasticity modification threshold that would preferentially allow induction of LTP-like processes. Another interpretation of this data is that the decrease in the NR2A/2B ratio accelerates the lowering of the plasticity modification threshold, and thereby accelerates the OD shift. If the first hypothesis is true, then the data would suggest that open-eye potentiation can occur independently of deprived-eye depression, provided there are other means of lowering the plasticity threshold. Alternatively, if open-eye potentiation requires induction of deprived-eye depression, then a shorter (1 day) period of MD should reveal

precocious deprived-eye depression.

5.4 Mechanisms of OD plasticity in adult mice

The recent discovery that mice exhibit a degree of cortical plasticity in adulthood is highly exciting because it will allow us to decipher what mechanisms underlie plasticity in a mature cortical circuit and how these mechanisms could be employed to enable repair and recovery of adult cortex from the effects of long-term sensory deprivation and other forms of neurological trauma.

It has been demonstrated that OD shifts can be induced in adult mice. Interestingly, many of these studies report that the main consequence of monocular deprivation in adult cortex is potentiation of the open-eye responses (Sawtell et al., 2003; Hofer et al., 2006a; Fischer et al., 2007b; Sato and Stryker, 2008). Given that in juvenile mice open-eye potentiation is generally preceded by deprived-eye depression (Frenkel and Bear, 2004), a question that remains to be resolved is whether plasticity observed in adults follows the rules of metaplasticity that are used to describe the consequences of MD in juvenile animals. In young mice, it is thought that deprived-eye depression, resulting from the lack of patterned visual input, brings down the overall level of cortical activity that leads to a modification in the threshold for plasticity induction to favor potentiation of the weak (open-eye) inputs (Smith et al., 2009). If in adult mice deprived-eye depression is not a consequence of MD, then how do open-eye inputs strengthen? What are the mechanisms that allow these inputs to potentiate? Curiously, the data that I report in Chapter 4 shows potentiation of non-deprived eye responses after as few as 3 days of MD without any change in deprived-eye responses. It is unclear both how this potentiation occurs and whether it results in a functional enhancement (e.g. acuity) of the

responses driven through that eye. Several simple experiments, such as measuring the dependence of this potentiation on the activation of the NMDA receptor (by using an NMDAR antagonist) and assessing visual acuity by measuring VEPs at a variety of spatial frequencies could answer these questions.

On the other hand, several studies have shown that it is possible to elicit deprived-eye depression in adult mice with longer periods of deprivation (Lickey et al., 2004; Frenkel et al., 2006). Furthermore, several other reports have provided strategies to reinstate deprived-eye depression in adult rats following short periods of MD (Sale et al., 2007; Maya Vetencourt et al., 2008; Harauzov et al., 2010). Both of these avenues of study provide evidence that induction of juvenile-like plasticity (i.e. deprived-eye depression in response to a short period of MD) is possible in the adult cortex. Since plasticity in juvenile animals is heightened, it would be of great interest to show that adult cortex can be brought back into the juvenile state, as that would dramatically increase the chances of promoting recovery from insult. Therefore, it is imperative to show that juvenile-like plasticity in adults and developmental plasticity share induction mechanisms. In particular, it would be useful to show that deprived-eye depression in adults is supported by induction of LTD. One way to test this would be to deliver the G2CT peptide into the adult visual cortex and test whether deprived-eye depression is blocked with either 7 days of MD (Frenkel et al., 2006) or with "rejuvenating" paradigms, such as environmental enrichment or administration of fluoxetine (Sale et al., 2007; Maya Vetencourt et al., 2008).

A factor that has been most implicated in restricting plasticity in the adult cortex is maturation of the GABAergic cortical circuits (Jiang et al., 2005; Sale et al., 2010).

Several recent studies provide evidence that decreasing the level of inhibition through direct pharmacological means or indirect means, such as rearing animals in enriched environments, administration of antidepressants or dark exposure can render the adult cortex plastic (He et al., 2007; Sale et al., 2007; Maya Vetencourt et al., 2008; Harauzov et al., 2010). In Chapter 4 I demonstrate that a specific subpopulation of the cortical inhibitory interneurons (the parvalbumin-expressing fast-spiking basket cells) is largely responsible for limiting plasticity in the adult mouse visual cortex. Therefore, this population represents a potential therapeutic target for recovery of amblyopic mice from the effects of long-term MD. One way to test this would be to employ the same strategy described in Chapter 4 to compromise the activity of the Pv^+ cells (via ablation or knocking out the NMDARs) and test whether recovery of normal visual acuity can be promoted in mature mice that have been visually deprived for months.

Another possibility is that promoting a decrease in the plasticity modification threshold, such as via a reduction in the NR2A/2B ratio, could also induce a recovery of visual acuity following a long-term MD. It would be useful to test NR2A KO mice that were used in Chapter 3 to see whether these animals are more amenable to rapid recovery from deprived-eye depression in adulthood. Alternatively, a shift in the plasticity modification threshold could potentially be induced pharmacologically with a mild NMDAR antagonist memantine (Rogawski and Wenk, 2003). One curious finding is that Pv^+ interneurons express five-times more NR2A protein than pyramidal cells (Kinney et al., 2006). Thus, it is possible that lowering the NR2A/2B ratio would also lower the activity of the Pv^+ cells, thereby removing the brake from plasticity in the adult brain.

5.5 Concluding remarks

Amblyopia in humans arises from a functional mismatch between the two eyes early in life, leading to a re-wiring of the visual cortex to favor processing of inputs subserving the dominant (non-amblyopic) eye. This human condition has been successfully modeled in mice by temporarily closing the eyelid of one eye early in development and observing functional shifts in ocular dominance in the primary visual cortex. In recent years, much progress has been made in understanding the mechanisms that underlie ocular dominance plasticity and the inappropriate circuit re-wiring in response to perturbations of visual experience. Furthermore, a great potential for experience-dependent plasticity has also been discovered in adult mice, while it was previously thought that this type of plasticity was restricted to a narrow developmental window. Since there is a strong phylogenetic conservation of mechanisms across mammalian primary visual cortices, findings in the mouse model of amblyopia may lead to the development of therapeutic interventions for adult human patients suffering from this devastating form of blindness.

Abbreviations

αCAMKII – calcium calmodulin kinase II, α isoform
AAV – adeno-associated virus
ACSF – artificial cerebrospinal fluid
AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR – AMPA receptor
BD – binocular deprivation
BDNF – brain derived neurotrophic factor
CSD – current source density
dLGN – dorsal LGN
DT – diphtheria toxin
DTR – diphtheria toxin receptor
ECM – extracellular matrix
EPSC – excitatory postsynaptic current
fEPSP – field excitatory postsynaptic potential
FS – fast spiking
GABA – γ-aminobutyric acid
GAD65 – glutamic acid decarboxylase 65
GAD67 – glutamic acid decarbosylase 67
GFP – green fluorescent protein
KO - knockout
LGN – lateral geniculate nucleus
LTD – long term depression
LTP – long term potentiation
MD – monocular deprivation
NMDA – N-methyl-d-aspartate
NMDAR – NMDA receptor
NR2A – NMDA receptor subunit 2A
NR2B – NMDA receptor subunit 2B
OD – ocular dominance
P – postnatal day
PKA – protein kinase A
Pv^{+} – parvalbumin-positive
SOM - somatostatin
TNFα – tumor necrosis factor-α
V1 – primary visual cortex
VEP – visually evoked potential
WM – white matter
WT – wild type

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