

## ABSTRACT

Title of Document: **MOLECULAR MECHANISMS OF  
SYNAPTIC PLASTICITY IN ADULT  
MAMMALIAN SENSORY CORTEX**

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Experience-dependent changes in synaptic composition and function (synaptic plasticity) underlie many brain functions including learning and memory, formation of sensory maps, as well as the capability to recover from injury. Most of these functions decline with age, supporting the observation that synaptic plasticity is greater in juveniles than in adults. However, it has been known for some time that peripheral deafferentation in adult animals can induce large-scale reorganization of sensory cortex, which suggests that the adult cortex retains a level of synaptic plasticity that is typically masked by normal activity inputs. Here I present a series of experiments in the adult mammalian sensory cortex that examine the mechanisms and methods to enhance experience-dependent synaptic plasticity. The results showed: 1. Peripheral deafferentation of a single digit in the adult raccoon induces progressive modulation of excitation and inhibition in the deafferented somatosensory cortex that

might be needed for the reorganization of receptive fields; 2. Visual deprivation in adult rats reverses three molecular mechanisms that have been correlated with the decrease in synaptic plasticity in adult cortex. These include the developmental increase in the level of inhibition relative to excitation, the development switch in NMDA receptor subunit composition from NR2b to NR2a, and the developmental decrease in tPA activity in visual cortex; 3. Visual deprivation rejuvenates ocular dominance plasticity in the adult visual cortex, and promotes the functional recovery of an eye deprived of vision from birth in adulthood. This work provides further understanding of the molecular mechanisms underlying experience-dependent cortical plasticity. In addition, it demonstrates that ocular dominance plasticity can be regulated throughout life, and proposes a non-invasive method to enhance synaptic plasticity in adult cortex.

MOLECULAR MECHANISMS OF SYNAPTIC PLASTICITY  
IN ADULT MAMMALIAN SENSORY CORTEX

By

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## Preface

All experiments presented in this dissertation were conducted by Hai-yan He. Work in this dissertation has resulted in three publications, which are listed as following:

1. He HY, Rasmusson DD, Quinlan EM.

Progressive elevations in AMPA and GABA<sub>A</sub> receptor levels in deafferented somatosensory cortex. *J Neurochem.* 2004 Sep;90(5):1186-93.

Restricted receptive fields (RFs), a hallmark of mature sensory systems, are actively maintained by a balance between ascending excitation and local inhibition in the cortex. Deafferentation disrupts this balance, allowing a sequence of changes in neuronal response properties that culminate in the reorganization of cortical RFs. To explore the molecular basis of deafferentation-induced RF reorganization, we tracked changes in AMPA receptor (AMPA), NMDA receptor (NMDAR) and GABA<sub>A</sub> receptor (GABA<sub>A</sub>R) levels in the deafferented somatosensory cortex of adult raccoons following single-digit amputation. In synaptoneurosome preparations from deafferented cortex, we observe a significant increase in AMPARs, but no change in NMDARs, 1-9 days post-deafferentation, coincident with the appearance of new excitatory inputs and enlarged RFs. We observe a significant increase in GABA<sub>A</sub>Rs 2-7 weeks post-deafferentation, coincident with a return of inhibitory input and shrinking RFs. These experience-dependent changes in the levels of the major cortical ionotropic receptors were transient, returning to pre-experimental baseline by > or = 17 weeks post-deafferentation, when RFs return to original size, but are remapped to different loci. This suggests that deafferentation-induced cortical reorganization may be generated by activity-dependent potentiation of weak excitatory synapses, followed by an increase in the strength of inhibitory synapses, resulting in finely tuned, remapped cortical RFs.

Author Contributions:

H.Y.H. conducted the biochemical experiments, analyzed the data, and participated in writing the manuscript.

D.D.R. contributed to devising the project, conducted animal preparation and dissection of brain tissue, and provided helpful discussion on the manuscript.

E.M.Q. designed and supervised the project, conducted some of the biochemical experiments, wrote the manuscript.

2. He HY, Hodos W, Quinlan EM.

Visual deprivation reactivates rapid ocular dominance plasticity in adult visual cortex. *J Neurosci.* 2006 Mar 15;26(11):2951-5.

Brief monocular deprivation (< or =3 d) induces a rapid shift in the ocular dominance of binocular neurons in the juvenile rodent visual cortex but is ineffective in adults. Here, we report that persistent, rapid, juvenile-like ocular dominance plasticity can be

reactivated in adult rodent visual cortex when monocular deprivation is preceded by visual deprivation. Ocular dominance shifts in visually deprived adults are caused by a rapid depression of the response to stimulation of the deprived eye, previously only reported in juveniles, and a simultaneous potentiation of the response to stimulation of the non-deprived eye. The enhanced ocular dominance plasticity induced by visual deprivation persists for days, even if binocular vision precedes monocular deprivation. Visual deprivation also induces a significant decrease in the level of GABA<sub>A</sub> receptors relative to AMPA receptors and a return to the juvenile form of NMDA receptors in the visual cortex, two molecular changes that we propose enable the persistent reactivation of rapid ocular dominance plasticity.

Author Contributions:

H.Y.H. conducted the experiments, analyzed the data, and participated in writing the manuscript.

W.H. contributed to building the electrophysiological recording rig, and provided helpful discussion on the manuscript.

E.M.Q. designed and supervised the project, conducted some of the experiments, wrote the manuscript.

3. He HY, Ray B, Dennis K, Quinlan EM.

Experience-dependent recovery of vision following lifelong deprivation amblyopia. (submitted)

In the mammalian visual system, deprivation of patterned vision to one eye in juveniles, either experimentally via lid suture, or naturally via a cataract, induces a shift in the ocular dominance of binocular neurons away from the deprived eye. If the occlusion persists until late in postnatal life, the probability of recovering function in the deprived eye is extremely low. However, here we demonstrate that ocular dominance plasticity can be reversibly regulated by visual experience throughout lifetime. Visual deprivation, experienced in adulthood, reinstates rapid ocular dominance plasticity in the adult visual cortex. In addition, visual deprivation experienced in adulthood allows for a recovery of function in an eye deprived of vision from birth. This is the first demonstration of experience-dependent restoration of ocular dominance plasticity in mammals, a method that holds therapeutic promise, as the potential to reverse the visual deficits in human amblyopia decline significantly with age.

Author Contributions:

H.Y.H. contributed to designing the experiments, conducted the electrophysiological experiments and most of the animal surgeries, analyzed the VEP data, and participated in writing the manuscript.

B.R. conducted the behavioral experiments and some of the animal surgeries, analyzed the behavioral data, and participated in writing the manuscript.

K.D. conducted the behavioral experiments.

E.M.Q. designed and supervised the project, conducted some of the experiments, wrote the manuscript.

*I dedicate this dissertation with all my love and gratitude to my mother*

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It's not a tradition for Chinese people to speak out appreciation for the other half, but here you are, always stand by my side, supporting me, correcting me, sharing my ecstasies and frustrations...

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## List of Abbreviations

ANOVA	analysis of variance
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BDNF	brain-derived neurotrophic factor
BV	binocular vision
CCtx	control cortex
CNS	central nervous system
DCtx	deafferented cortex
EEG	electroencephalography
GABA	gamma-aminobutyric acid
GAD	glutamate decarboxylase
HFS-LTP	high frequency stimulus-induced long-term potentiation
IPSC	inhibitory postsynaptic synaptic current
LGN	lateral geniculate nucleus
LTD	long-term depression
LTP	long-term potentiation
MD	monocular deprivation
mEPSC	mini excitatory postsynaptic current
mGluR	metabotropic glutamate receptors
N-CAM	neural cell adhesion molecule
NMDA	N-methyl-D-aspartate
NR	normal reared (reared in normal lighted environment)
OD	ocular dominance
RF	receptive field
RO	reverse occlusion
tPA	tissue plasminogen activator
TTX	tetrodotoxin
VCtx	visual cortex
VD	visual deprivation
VEP	visually evoked potential

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# **CHAPTER 1: GENERAL INTRODUCTION AND BACKGROUND STUDIES**

The ability of synaptic strength to be regulated by activity (synaptic plasticity) underlies many brain functions including learning and memory, perception, motor control, decision-making, and recovery from injury. Understanding synaptic plasticity will have important implications for understanding normal brain functions, as well as give insight into potential therapies of central nervous system disorders. Many types of synaptic plasticity decline with age. For example, it is well known that it is easier to learn a new language at younger age (Hurford, 1991). Other types of synaptic plasticity persist into adults, such as the cortical reorganization induced by single digit deafferentation (Merzenich et al., 1984). My dissertation examines experience-dependent synaptic plasticity involved in receptive field reorganization in adult mammalian cortex.

## **1.1 Theories of synaptic plasticity**

### **1.1.1 Hebb's rule, LTP and LTD**

It seems impossible to write about synaptic plasticity without starting with Hebb's postulate. In 1949, Donald Hebb proposed a theory for the formation of associative and distributed memory -- if the activation of a postsynaptic cell is

correlated with the firing of a presynaptic cell, the connections (synapses) between the two cells will be strengthened. In other words, cells that fire together, wire together (Hebb, 1949).

Later, the discovery of long-term potentiation (LTP) provided experimental evidence for Hebb's hypothesis. In 1973, Bliss and Lomo showed that high frequency stimulation of the hippocampal perforant pathway of an anesthetized rabbit could reliably induce a long-lasting potentiation of excitatory synaptic transmission in the dentate gyrus (Bliss and Lomo, 1973). Further investigations of LTP demonstrated that simultaneous activation of the pre- and postsynaptic neuron is necessary for the induction of LTP, a characteristic predicted by Hebb's postulate (Bliss and Collingridge, 1993). LTP appears to be a general characteristic of synapses. It has been found in multiple species, from *Aplysia* to human. LTP or LTP-like activity has been shown to be involved in many brain functions, including the formation of receptive fields, learning and memory. In fact, the ability to induce LTP has become a standard method to measure the level of synaptic plasticity in neural circuits. From then on, LTP has been one of the hottest topics in neuroscience (Cooke and Bliss, 2006).

However, the organization of the central nervous system and experience-dependent synaptic modifications cannot be explained solely by activity-dependent strengthening of synapses, since cortical circuits would rapidly saturate with irreversible LTP. There has to be a mechanism account for the opposite effect, by which synaptic strength can be depressed and even silenced. In 1992, Dudek and Bear published the first experimental demonstration of synaptic long-term depression

(LTD) (Dudek and Bear, 1992). In hippocampal slices, by applying prolonged low frequency stimuli (in contrast to the brief trains of high frequency stimuli that are used to induce LTP), they were able to reliably induce a depression in the strength of excitatory synapses. Just like LTP, LTD has been demonstrated in many cortical areas and has been shown to be involved in all sorts of plasticity-related paradigms (Malenka and Bear, 2004).

A pronounced characteristic of LTP and LTD is that they are activity-dependent and synapse-specific, which fits nicely with the input specificity that has been observed in the receptive field properties of cortical neurons. This makes LTP and LTD the most popular cellular correlates for learning and memory (Malenka and Bear, 2004).

### **1.1.2 Metaplasticity determines the characteristics of synaptic plasticity and maintains the stability of the nervous system**

Hebbian learning explains many brain functions, but it also bears the risk of bringing great instability to the system. According to Hebb's rule, when correlated activity occurs between pre- and postsynaptic neurons, the connection will be strengthened, which will further strengthen the correlated activity. Without counteracting mechanisms, the positive feedback eventually would lead to seizure-like firings in the associated neurons. Clearly this is not the case in a normal brain.

In fact, the level of available synaptic plasticity is also regulated, by the level of synaptic activity (Abraham and Bear, 1996). The plasticity of synaptic plasticity, or metaplasticity, changes the way synaptic efficacy will be regulated by subsequent activity. The synaptic response (potentiation or depression) to a given frequency of presynaptic stimulation depends on the history of activity at that synapse. Activation of postsynaptic NMDA receptors (ionotropic glutamate receptors) by high frequency stimulation not only induces LTP, but also increases the threshold for further LTP and lowers the threshold to induce LTD (Wagner and Alger, 1995). Metaplasticity has been documented in behavioral paradigms. Motor skill learning induces strengthening of the horizontal synapses in rat motor cortex through LTP-like mechanisms. At the same time motor skill learning occludes the further induction of LTP, and facilitates the induction of LTD (Rioult-Pedotti et al., 2000). The absence of activity also regulates synaptic plasticity. Giving animal complete visual deprivation by dark rearing from birth shifts the LTP threshold (the minimum frequency needed to induce LTP) to lower frequencies in the rat visual cortex (Kirkwood et al., 1996). Stimulation frequencies that formerly induced no change in synaptic strength now induce synaptic potentiation. Bringing dark reared animals into light quickly shifts the threshold back to higher frequencies.

One theory of metaplasticity is the sliding synaptic modification threshold proposed by Bienenstock, Cooper and Munro (BCM theory, Bienenstock et al., 1982). BCM theory suggests that a 'slowly varying time-averaged value of the postsynaptic activity' exerts an instructive effect on how 'instantaneous pre- and postsynaptic activity' modify synaptic efficacy. According to BCM theory, there is a

putative frequency-response curve (the LTP/LTD curve) at all synapses. The change in synaptic strength (LTP or LTD) is determined by the frequency of presynaptic stimulation. There is a crossover point in the curve, called the null-stimulation frequency ( $\theta_m$ ), which is the presynaptic stimulation frequency that induces no net change in synaptic strength. Stimuli of frequencies higher than  $\theta_m$  produce an increase in synaptic strength (LTP) and stimuli of frequencies lower than  $\theta_m$  produce a decrease in synaptic strength (LTD). The position of this curve along the horizontal axis (frequency) is determined by the activation history of the synapse. BCM theory can explain the emergence of orientation selectivity and binocularity in visual cortex (Bienenstock et al., 1982), and has been supported by a wealth of experimental evidence (Kirkwood et al., 1996; Quinlan et al., 1999; Philpot et al., 2001; Kind et al., 2002). For example, over the course of normal visual cortex development, the LTP/LTD curve is shifted to the right (higher frequency). This results in reduced susceptibility to LTP (Kirkwood et al., 1995). However, depriving the animal of visual input by dark rearing from birth, slides the threshold to the left (lower frequency) resulting in enhanced LTP and reduced LTD over a range of stimulation frequencies (Kirkwood, et al., 1996).

Another theory, synaptic scaling, has also been proposed to counteract the runaway potentiation in Hebbian learning (Turrigiano et al., 1998; Turrigiano and Nelson, 2000). Synaptic scaling works by scaling up or down all of the synaptic weights on a given neuron. Suppressing the electrical activity in cell cultures by application of tetrodotoxin (TTX) results in an increase in the strength of excitatory synapses as shown by increased mEPSC (mini excitatory postsynaptic current)

amplitudes. In contrast, increasing the overall synaptic activity with bicuculline (inhibitory GABA<sub>A</sub> receptor antagonist) results in a decrease in mEPSC amplitude (Turrigiano et al., 1998). *In vivo* evidence for synaptic scaling comes from visual cortical slices prepared from neonatal rats that had received two days monocular deprivation. The decrease in the total activity in the deprived monocular region (contralateral to the deprived eye) induces a significant increase in the EPSC amplitude and the spontaneous activity level (Maffei et al., 2004). Sensory deprivation by dark rearing has also been reported to scale up the excitability in the deprived visual cortex with a concomitant compensatory scaling down of excitability in the non-deprived barrel (somatosensory) cortex (Goel et al., 2006). In synaptic scaling, the change in the efficacy of each synapse is proportional to the previous level, so that the relative distribution of the synaptic strength across a neuron would remain unchanged.

The molecular mechanism of synaptic scaling remains illusive. There is evidence suggesting that scaling is accompanied by changes in the number and properties of postsynaptic AMPA receptors (Wierenga et al., 2004; Goel et al., 2006) and morphological properties of dendritic spines (Wallace and Bear, 2004). A neurotrophic factor, pro-inflammatory cytokine tumor-necrosis factor- $\alpha$  (TNF- $\alpha$ ), has been reported to mediate AMPA receptor insertion into the postsynaptic site during low level of activity in cell cultures, suggesting that neurotrophins and glia might also be involved in mechanisms of synaptic scaling (Stewaggen and Malenka, 2006).



## **1.2 The level of synaptic plasticity is high in juveniles and is constrained in adults**

### **1.2.1 Critical period for sensory and cognitive development**

A well-documented characteristic of experience-dependent synaptic plasticity is that it decreases with age. The existence of critical periods at early postnatal ages highlights the difference in synaptic plasticity in juveniles and adults. A critical period is a time in early postnatal development with the highest level of synaptic plasticity for a certain neuronal function. After the critical period, the neural circuits tend to be rather stable and aplastic.

Many cognitive and sensory functions have critical periods during early development. For example, in the auditory system of many vertebrates, depriving cochlear nucleus neurons of auditory nerve input during a critical period in early postnatal life results in dramatic neural death, while the same manipulation does not cause significant cell loss after the critical period (Harris and Rubel., 2006). In songbirds, multiple critical (sensitive) periods have been identified that are critical for different aspects of learning species-specific song (White, 2001). In humans, there is a critical period for first language acquisition (Hurford, 1991). There might also be a critical period for the learning of basic aspects for musical development (Trainor, 2005).

In rat somatosensory cortex, there is a large area dedicated to representations for the whiskers that is arranged in topographic way corresponded to the location of each

whisker on the snout. The cortical neurons that represent each whisker naturally segregate into barrel-shape columns, which earn them the name barrel cortex. Depriving barrel cortex input by trimming some of the whiskers will result in shrinkage of the deprived whisker barrels and expansion of the remaining whisker barrels (Fox 1992). The ability of this manipulation to affect thalamocortical synapses peaks at around postnatal day 4 and decreases very quickly with age.

### **1.2.2 Ocular dominance plasticity: a popular model for studying critical period phenomenon**

In the mammalian visual system, retinal ganglion cell axons cross at the optic chiasm, and project onto the eye specific lamina of lateral geniculate nucleus (LGN). However, in different species, a different percentage of axons cross over to the contralateral LGN. In cat, monkey and human, approximately 50-60% of retinal ganglion cell axons cross over and project to the contralateral LGN (Butler and Hodos, 2005). Neurons from LGN then synapse onto neurons in layer IV, which are segregated into ocular dominance columns, containing neurons receiving inputs from either the ipsilateral eye or the contralateral eye (Shatz and Stryker, 1978). In cats and monkeys, most of the neurons in the primary visual cortex are binocular, which means that they receive inputs from both eyes. The ocular dominance of binocular neurons in the visual cortex can be modified by visual experience, which was first demonstrated by Hubel and Wiesel (1964; 1970). During a critical period in early postnatal development, monocularly depriving one eye (by enucleation or eye-lid suturing) induces a shift in the ocular dominance of binocular neurons toward the non-deprived eye. Following a prolonged period of monocular deprivation (MD), the

majority of neurons in the primary visual cortex loses responsiveness to stimulation of the deprived eye and preferentially responds to stimulation of the non-deprived eye. The phenomenon of ocular dominance plasticity is one of the best-studied critical period phenomena, and has been observed in many mammals. Table 1 lists the critical periods for ocular dominance plasticity in several different species.

In rodents, the percentage of retinal ganglion cell axons that cross over to the contralateral side is much higher (about 90%, Gordon and Stryker, 1996). Only 10% of retinal ganglion axons project to the ipsilateral LGN (Grafstein, 1971; Dräger and Olsen, 1980). There is no column like structure that alternatively clusters visual cortical neurons receiving input from ipsilateral or contralateral eye in layer IV. Nonetheless, the rodent primary visual cortex contains a clearly defined binocular region, which receives input from both eyes. Due to the high cross over percentage of retinal ganglion cell axons, neurons in binocular region of the rodent primary visual cortex respond more strongly to stimulation of the contralateral eye than stimulation of the ipsilateral eye. This contralateral bias is shifted by monocular deprivation, similar to the ocular dominance shift observed in other mammals such as monkeys and ferrets (Fagiolini et al., 1994; Gordon and Stryker, 1996). For rodents, the critical period for ocular dominance plasticity is from P19 to P35 (Gordon and Stryker, 1996).

Recently, a higher level of plasticity has been reported in adult mouse (but not adult rat) visual cortex than had been previously appreciated. With prolonged period of monocular deprivation (>5 days), the ocular dominance in adult mouse visual cortex can be shifted toward the non-deprived eye (Guire et al., 1999; Sawtell et al., 2003; Pham et al., 2004; Tagawa et al., 2005; Hofer et al., 2006; Frenkel et al., 2006).

Nonetheless, the sensitivity to brief (1-3 days) monocular deprivation decreases in adult mouse visual cortex, suggesting the validity of a critical period for ocular dominance plasticity in mice. In all species, the response of primary visual cortex to monocular deprivation is a very useful index for detection of the level of available synaptic plasticity in the visual cortex (Sengpiel and Kind, 2002; Maffei, 2002; Daw et al., 1999).

The ability to recover function in a deprived eye is also limited to a critical period. If monocular deprivation due to congenital cataract is diagnosed and treated early in life,  $\leq 4$  months of age, the patient would be expected to acquire normal vision in both eyes later in life (Mitchell and MacKinnon, 2002). However, the success rate for the treatment decreases abruptly  $> 4$  months of age. If the patient is not treated until  $> 3$  years old, in more than half of the cases studied, patients recovered only rudimentary form vision in the deprived eye.

**Table 1: Critical period for ocular dominance plasticity in different species**

<b>Species</b>	<b>Critical Period (postnatal days)</b>	<b>Deprivation duration</b>	<b>Reference</b>
Rat	P19 – P35	< 4day	Fagiolini et al., 1994; Sawtell et al., 2003
Cat	P6wk – P16wk	2day	Mower, 1991
Ferret	P42 – P100	2day, 7day	Issa et al., 1999
Monkey	P1wk – P12wk  < 2 years	6-8 months	Horton and Hocking, 1997  Daw NW. Visual development. New York: Plenum Press. 1995
Human	< 12 years	N/A*	Vaegan and Taylor, 1979

**\* Data from clinical literature. 12 years is the oldest age that visual cortical function could be disrupted by unilateral cataract.**

### **1.2.3 Induction of activity-dependent synaptic modification decreases with age**

Susceptibility of cortical circuits to the induction of LTP also is greatest during a postnatal critical period (Kirkwood et al., 1995). In slices prepared from juvenile rat visual cortex, LTP could be induced in layer II/III neurons by stimulating white matter with high frequency stimulation. The same protocol fails to induce LTP in adult visual cortex, which might be partially responsible for the decrease in ocular dominance plasticity seen in adults.

Similarly, there is an age-dependence in the induction of LTD in rat hippocampal slices (Wagner and Alger, 1995). LTD can be induced in the CA1 region by low frequency stimulation applied to the Schaffer collateral pathway in brain slices taken from young animals. No LTD is expressed if the slice is taken from rats older than 5 weeks. This age-dependent decline in LTD induction has also been observed in thalamocortical synapses of rat barrel cortex, which coincides with the critical period for modification of thalamocortical synapses with whisker trimming (Feldman et al., 1998).

There is also a report of age-dependence for experience-dependent synaptic scaling in rat visual cortex (Desai et al., 2002). Two days of monocular deprivation induces a scaling up of excitatory synaptic strength in the layer IV of the deprived monocular visual cortex, which is not observed in rats older than P18. In layer II/III, the scaling effect of two-day monocular deprivation on excitatory synaptic transmission has a delayed critical period, starting at P21.

## **1.3 Age-independent effects of sensory deprivation on sensory cortex**

### **1.3.1 Somatosensory cortex**

The somatosensory system is organized in an orderly fashion such that cortical neurons that receive input from adjacent peripheral areas are located next to each other. The receptive field of a neuron in the somatosensory cortex is the area of skin in which stimulation induces a neuronal response. The somatotopic map therefore is the reflection of the cortical representations of receptive fields. Synchronized patterns of spontaneous activity during development guide the formation of such functional somatotopic maps. Also, the more an animal makes use of the sensory input from certain body surface, the larger cortical area is dedicated to the representation of that area (Buonomano and Merzenich, 1998; Butler and Hodos, 2005). However, the size of receptive fields is inversely correlated with the number of cortical neurons represent that part of skin area. Thus for those areas of body surface that have large cortical representations (such as the finger tips), the receptive field size for the neurons representing the area is very small. In fact, restricted receptive fields are hallmarks of adult sensory systems. In adult mammals, these maps are quite stable and are maintained by a use-dependent process. By using peripheral deafferentation, it is possible to induce large-scale reorganization of these maps. This phenomenon has been reported in monkeys, flying foxes, rats, raccoons and other animals (Merzenich et al., 1984; Calford and Tweedale, 1990; Rasmusson, 1982). After deafferentation of one of the forepaw digits either by amputation or by nerve cutting, neurons representing the deafferented digit in the somatosensory cortex will become

silent (Merzenich et al., 1984; Rasmusson, 1982) After a period, these neurons begin to respond to stimulation of a larger area of the adjacent skin (adjacent digits and palm). Eventually a new somatotopic map without representation of the deafferented digit will form in the somatosensory cortex. The new receptive fields resemble those seen before the deafferentation in size and can be very restricted. This remapping can be very dramatic, for example, amputating one digit in adult monkey resulted in a shift of cortical representational areas as much as 1 mm (Pons et. al., 1991).

### **1.3.2 Visual cortex**

Although ocular dominance plasticity decreases over development, retinal lesion continues to induce reorganization of the retinotopic map in the primary visual cortex in adult cats (Dreher et al., 2001). Receptive field reorganization induced by circumscribed, topographically matched retinal lesions are similar to the response of the adult somatosensory cortex to peripheral deafferentation, and is likely due to changes in intracortical connections between neurons in supra-granular and infra-granular layers.

Completely depriving an animal of vision from birth by dark rearing also regulates synapses in the visual cortex. On one hand, it maintains the visual system in an immature state, characterized by low stimulus selectivity and large receptive fields (Fagiolini et al., 1994). On the other hand, dark rearing attenuates the development decline in ocular dominance plasticity (Cynader, 1983; Mower et al., 1991), extending the time period during which the visual cortex will respond to brief



monocular deprivation. The molecular mechanisms that might be involved in the developmental decline in ocular dominance plasticity are also affected by dark rearing from birth, which I will discuss in more detail in section 4.

## **1.4 Molecular correlates underlying the developmental decrease in activity-dependent synaptic plasticity in the mammalian cortex**

The pattern of expression of many different molecules has been demonstrated to be correlated with the developmental decrease in activity-dependent synaptic plasticity in the mammalian cortex. Here I will focus on three key factors that will be re-examined in my experiments.

### **1.4.1 Balance between inhibition/excitation**

The level of cortical activity is determined by the balance between the level of excitation and the level of inhibition. In cortex, the major excitatory neurotransmitter is glutamate and the major inhibitory neurotransmitter is  $\gamma$ -aminobutyric acid (GABA). Levels of postsynaptic neurotransmitter receptors are a primary determinant of synaptic strength. There are three major types of glutamate receptors in the mammalian brain, AMPA ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, NMDA (N-methyl-D-aspartate) receptors, and mGluR receptors (Borchert and Rossier, 1993). Among them, AMPARs are ionotropic and are responsible for fast excitatory synaptic transmission. NMDARs are ligand and voltage-gated ion channels with relatively slow current dynamics. Similarly, three types of GABA

receptors have also been found in the cortex, GABA<sub>A</sub>, GABA<sub>B</sub> and GABA<sub>C</sub> receptors (Chebib and Johnston, 1999). Among them, GABA<sub>A</sub>Rs are the major ionotropic receptor and responsible for fast inhibitory synaptic transmission. GABA<sub>B</sub>Rs are G-protein coupled metabotropic receptors and will activate intracellular signaling cascades upon activation. GABA<sub>C</sub> is also ionotropic, but the function of it is still not well known.

The level of excitation and inhibition in the cortex is determined by activity at glutamatergic and GABAergic synapses. Regulation of synaptic strength can occur at each step of synaptic transmission, including the synthesis of the neurotransmitter in the presynaptic neurons, the release probability at presynaptic terminals, the removal and reuptake of released neurotransmitter at the synaptic cleft, the number of receptors at the postsynaptic membrane, and the electrical properties of pre- and postsynaptic neurons (Bi and Poo, 1998; Shen and Kim, 2002; Zucker and Regher, 2002). For example, experience or activity-dependent modification of synaptic strength is often accompanied by a change in postsynaptic receptor number. The level of synaptic AMPARs has been shown to be highly correlated with the synaptic strength at excitatory synapses in the hippocampus (Heynen et al., 2000). AMPARs are driven into active synapses by experience in the young rodent barrel cortex, which is believed to be part of the mechanism underlying the maintenance of barrel fields (Takahashi et al., 2003). The strength of GABAergic synapses can also be regulated by the number of postsynaptic receptors. An increase in the number of GABA<sub>A</sub>Rs is observed following potentiation of inhibitory synapses (Nusser et al., 1998).

Over development, there is an increase in the level of the inhibition relative to excitation in the visual cortex (Huang et al., 1999; Fagiolini and Hensch, 2000; Rozas et al., 2001; Desai et al., 2002). A direct consequence of this might be the restriction of the receptive field size of cortical neurons, which is characteristic of mature sensory neurons. Decreasing the level of inhibition with GABA antagonist or glutamate agonist results in enlargement of receptive fields (Dykes et al., 1984; Alloway and Burton, 1991). Recent studies in the visual cortex suggest that the developmental increase in the ratio of GABAergic inhibition to glutamatergic excitation may also play an important role in regulating the level of experience-dependent synaptic plasticity available to cortical circuits.

The developmental increase in inhibition relative to excitation observed in the developing visual cortex coincides with the decline of ocular dominance plasticity (Huang et al., 1999; Fagiolini and Hensch, 2000; Rozas et al., 2001; Desai et al., 2002). Dark rearing from birth, which maintains a high level of ocular dominance plasticity in visual cortex, also delays the developmental increase in the level of GABAergic inhibition over glutamatergic excitation (Cotrufò et al., 2003; Morales et al., 2002; Desai et al., 2002). In rodents, there is no ocular dominance plasticity in visual cortex before the third postnatal week (Gordon and Stryker, 1996), which coincides with a low level of GABAergic inhibition and high level of glutamatergic excitation. In addition, experimental evidence from genetically modified animals and pharmacological studies suggest that a basal level of inhibition may be necessary for the initiation of synaptic plasticity. In other words, an optimal range for the inhibition/ excitation might be necessary to allow expression of synaptic plasticity.

The neurotransmitter GABA is synthesized in the presynaptic neuron by two isoforms of the enzyme glutamic acid decarboxylase (GAD65 and GAD67). GAD67 is located in cell bodies and is responsible for maintaining the constitutive level of GABA. GAD65 is found at the presynaptic site, and is involved in replenishing synaptic GABA, especially during repeated presynaptic activation. Genetically knocking out GAD65 results in prolonged excitatory discharge in response to stimulation at all ages (Hensch et al., 1998). As a consequence of this sub-threshold level of inhibition, GAD65 knock out mice never demonstrate ocular dominance plasticity. The lack of ocular dominance plasticity could be rescued by application of the GABA agonist diazepam, at any time during the life, suggesting a certain threshold level of inhibition over excitation is needed for the initiation of ocular dominance plasticity. On the other hand, once the balance of inhibition over excitation goes above certain level, as seen over development (Rozas et al., 2001), or by pharmacological application of a GABA agonist (Fagiolini and Hensch, 2000), the level of ocular dominance plasticity declines. A similar phenomenon has also been observed in mice over expressing brain derived neurotrophin factor (BDNF), which elevates cortical inhibition and results in a precocious critical period (with early onset and early closure) for ocular dominance in visual cortex (Huang et al., 1999).

The role of GABAergic inhibition on the regulation of cortical plasticity has also been explored in other sensory systems. GABA receptor agonists can inhibit the adaptive adjustment of the owl's auditory space map induced by experimental uncoupling of the visual and auditory inputs (Zheng and Knudsen, 2001). Removal of inhibition has also been observed preceding large-scale synaptic reorganization in

response to deafferentation. A decrease in GABA agonist binding to GABA<sub>A</sub> receptors in layer IV neurons of the deafferented cortex has been observed in the primate somatosensory cortex within 5 hours of peripheral nerve transection (Wellman et al., 2002) and within one week of deafferentation in the mouse barrel cortex (Skangiel-Kramska et al., 1994).

#### **1.4.2 NMDA receptor subunit composition is regulated during development**

NMDA receptors are ionotropic glutamate receptors with special requirements for activation. First, NMDA receptors must bind glutamate released from the presynaptic terminal. Second, at resting membrane potential, the ion channel of NMDA receptor is blocked by an Mg<sup>2+</sup> ion. When the postsynaptic neuronal membrane is depolarized above the reversal potential for Mg<sup>2+</sup>, the Mg<sup>2+</sup> ion is repelled and the channel is open to allow ion flux across the membrane. Thus the activation of NMDA receptors requires both glutamate release from the presynaptic neuron and depolarization of the postsynaptic neuron. Therefore, NMDA receptors have been proposed to act as a coincidence detector for synaptic modification as described in Hebbian learning (Bliss and Collingridge, 1993). Indeed, many forms of activity-dependent synaptic plasticity have been shown to be NMDA receptor dependent, including LTP, LTD, and ocular dominance plasticity (Malenka and Bear, 2004).

Each NMDA receptor is composed of two obligatory NR1 subunits and two NR2 subunits. There are four subtypes of NR2 subunit (named a-d), each conveying

different electrophysiological features to the NMDA receptor channel. In neonatal cortex, the majority of NMDA receptors is NR2b-containing, and conducts a longer duration of current with a slower decay time. Over the course of development, NR2b-containing NMDA receptors are switched to NR2a-containing NMDA receptors, which mediate shorter and faster currents (Carmignoto and Vicini 1992; Quinlan et al., 1999). Subunit switching from NR2b to NR2a during development results in a decreased current duration mediated by NMDA receptors and allows a smaller time window for coincidence detection.

It has been previously proposed that the ratio of synaptic NR2b- to NR2a-containing NMDARs determines the threshold for NMDAR-dependent long-term potentiation (Abraham and Bear, 1996). A theoretic model showed that switching of synaptic NMDA receptor subunit composition from NR2b to NR2a would shift the LTP/LTD curve (the sliding threshold for BCM theory) to the right on the frequency axis (Castellani et al., 2001). In another words, a higher frequency will be needed to produce LTP in these synapses. Switch back from NR2a-dominating to NR2b-dominating would lower the threshold for LTP induction. This is supported by the experimental observation that in NR2b over expressing mice, LTP is more readily induced than in wild type controls (Tang et al., 1999). Also, dark rearing is known to maintain the high level of NR2b/NR2a in the visual cortex (Quinlan et al., 1999), which is consistent with the observation that after dark rearing the threshold for LTP is lowered (Kirkwood et al., 1996).

### **1.4.3 Maturation of extracellular matrix may form physical barriers to constrain synaptic plasticity**

Another molecular factor that is thought to constrain the level of activity-dependent synaptic plasticity in adult cortex is the maturation of extracellular matrix. The extracellular matrix is a glycoprotein complex surrounding neuronal and glia cells in the brain. Camillo Golgi first described the extracellular matrix in 1893 as 'perineuronal nets', which appear only after birth and mature over development (Celio and Blumcke, 1994). Perineuronal nets are thought to be connected to the extracellular domain of cell adhesion molecules and form an extracellular protein scaffold to support cells and hold them in place. It is not surprising that perineuronal nets may exert an inhibitory effect on morphological changes of axons given its physical presence as physical barriers intimately surrounding the neurons. In addition, component proteins of perineuronal nets such as tenascin and chondroitin sulfate proteoglycans act as repellents for neurons and their processes, thus preventing neurons from making new synapses (Grumet et al., 1993). In visual cortex, the maturation of perineuronal nets coincides with the end of critical period for ocular dominance plasticity, and can be delayed by dark rearing from birth (Sur et al., 1988). This also supports the view that the perineuronal net plays an inhibitory role in the experience-dependent remodeling of neural circuits. Moreover, a primary component of the mammalian extracellular matrix is chondroitin sulfate proteoglycans (Viapiano and Matthews, 2006). Degrading chondroitin sulfate proteoglycans by applying

exogenous chondroitinase-ABC to adult rat visual cortex degrades perineuronal net and increases the sensitivity to monocular deprivation (Pizzorusso et al., 2002).

The extracellular matrix can also be degraded by endogenous proteolytic enzymes. One candidate for such an endogenous enzyme is tissue plasminogen activator (tPA), which is a serine protease that cleaves plasminogen into the active form plasmin. Plasmin then cleaves the proteins that compose extracellular matrix (Muller and Griesinger, 1998). Extracellular substrates for tPA-plasmin system include neural cell-adhesion molecule (N-CAM) and laminin (Endo et al., 1999; Chen and Strickland, 1997). tPA activity levels in the visual cortex decreases during development (Mataga et al., 2002). Application of tPA to visual cortex increases spine motility and mimics the effect of monocular deprivation in juvenile animals (Oray et al., 2004).

## **1.5 Efforts to enhance ocular dominance plasticity in adults**

Manipulations of molecules that have been hypothesized to constrain synaptic plasticity over development have been used to restore/enhance the level of synaptic plasticity in adult cortex. Here I review the successful examples in enhancing ocular dominance plasticity in adult visual cortex.

### **1.5.1 Degrading extracellular matrix with exogenous proteolytic enzymes**

As mentioned above, Pizzorusso et al. injected the proteolytic enzyme chondroitinase-ABC into adult visual cortex to degrade the chondroitin sulfate



proteoglycan proteins of the extracellular matrix. This manipulation successfully increased the sensitivity to monocular deprivation in adult visual cortex (Pizzorusso et al., 2002). Injection of chondroitinase-ABC alone did not change ocular dominance or the response properties of neurons in the visual cortex, suggesting that degrading extracellular matrix produces a more permissive environment to allow experience-dependent synaptic modifications. The same group also demonstrated that injection of chondroitinase-ABC into visual cortex could facilitate the structural and functional recovery from early monocular deprivation in adults (Pizzorusso et al., 2006).

### **1.5.2 Knocking out Nogo**

Another successful effort in enhancing synaptic plasticity in adult cortex targeted Nogo-A, an inhibitor of axonal growth found on oligodendrocytes (Liu et al., 2006). Nogo-A is known to inhibit axon regeneration in adult central nervous system, and is thought to decrease functional regeneration following injury in the CNS. Transgenic mice that are missing the ligand Nogo, or mutating the NogoR, retain the juvenile level of ocular dominance plasticity in adult visual cortex (MacGee et al., 2005).

### **1.5.3 Prior experience facilitates response to later monocular deprivation.**

Hofer and colleagues recently reported a form of enhanced ocular dominance plasticity in adulthood based on prior visual experience (Hofer et al., 2006). They

showed that the cortical response to monocular deprivation in adult mouse visual cortex is greatly enhanced if the animal has been subjected to monocular deprivation at an earlier time point. The enhanced synaptic plasticity is not the result of an elongated monocular deprivation, since the subjects were allowed to fully recover from the first monocular deprivation. The effect of the previous monocular deprivation is also restricted to the cortical circuits innervated by the deprived eye. The mechanism underlying such kind of plasticity remains unknown. A series of similar experiments in the auditory system of owls provide some very intriguing insights (DeBello et al., 2001; Linkenhoker et al., 2005). In barn owls, the ability of midbrain neurons representing auditory space to adapt to visual inputs skewed by prism-wearing is extended into adulthood if the animal has had similar experience as a juvenile. The early prism-wearing experience is thought to result in cortical reorganization to support to the skewed visual inputs. The remaining anatomical traces from the previous manipulation facilitate the subsequent synaptic modification in response to the same experience in adulthood. This suggests that the anatomical changes in response to the first manipulation are functionally suppressed by normal visual inputs following the removal of prism, however, they are not completely eliminated.

# **CHAPTER 2 DEAFFERENTATION INDUCES PROGRESSIVE CHANGES IN THE LEVEL OF EXCITATION AND INHIBITION IN ADULT RACCOON SOMATOSENSORY CORTEX**

## **2.1 Introduction**

### **2.1.1 Deafferentation induced large-scale reorganization in adult raccoon somatosensory cortex**

A major principle for the anatomical construction of the central nervous system is topographic organization. For example, the primary somatosensory cortex in mammals is organized into somatotopic maps. Neurons that receive input from adjacent areas of body surface are located next to each other in the somatosensory cortex. Synchronized patterns of activity in adjacent peripheral inputs during development play an essential role in the formation of the functional topographic maps in sensory cortices (Buonomano and Merzenich, 1998). Hebbian rule originally predicted the benefits of such activity-dependent organization of cortical maps. In adult mammals, these maps are quite stable and are maintained by a use-dependent process. The more the animal makes use of the sensory input from a certain body surface area, the larger cortical area is dedicated to the representation of that surface area (Buonomano and Merzenich, 1998). By using peripheral deafferentation, it is possible to induce large-scale reorganization of these maps, which allows elucidation of important information on cortical architecture and processing. This phenomenon has been reported in monkeys, flying foxes, rats, raccoons and other animals, and has

become a great animal model for the studying of experience-dependent synaptic plasticity in the cortex (Merzenich et al., 1984; Calford and Tweedale, 1990; Rasmusson, 1982).

In the adult somatosensory cortex, neurons respond to stimulation of a very small area of the paw skin. Such restricted receptive fields are characteristic of adult sensory systems. Following deafferentation of one of the forepaw digits by amputating the digit or by severing the nerve that connects the digit to the central nervous system, neurons representing the deafferented digit in the somatosensory cortex will lose their receptive field and become silent. After a period of time, many of the silenced neurons will begin to respond to stimulations of the adjacent digits and palm area. At this stage, the newly formed receptive fields are usually quite expanded. Eventually those silenced neurons will regain receptive fields which are refined and restricted, similar to receptive fields seen in normal animals, but remapped onto a different part of the skin area. Consequently, a new somatotopic map without representation of the deafferented digit will form in the somatosensory cortex. This reorganization effect can be very dramatic, especially when severe deafferentation is given and long recovery time is allowed after deafferentation for reorganization. For example, sectioning of the dorsal root (C2-T4) in adult monkey resulted in deafferentation of neurons over  $1\text{cm}^2$  of cortical area. Several years after deafferentation, electrophysiological recordings in the deprived cortical area showed that all of the neurons had regained receptive fields in neighboring body surfaces and developed normal response to peripheral stimulations (Pons et al., 1991).

To look at the molecular basis for cortical reorganization in adult somatosensory cortex, we collaborated with Dr. Douglas Rasmusson in Dalhousie University (Halifax, Nova Scotia), who had been working on receptive field plasticity in adult raccoons for more than 20 years. The Rasmusson lab has established the electrophysiological characteristics of large-scale cortical reorganization that could be induced in adult raccoon somatosensory cortex by deafferentation of a single forepaw digit. One of the advantages of using raccoon somatosensory cortex as model for receptive field reorganization is that the cortical representation for each of the ten forepaw digits is exceptionally large due to the high dexterity of raccoons. The large representation of each digit in the raccoon somatosensory cortex has been mapped electrophysiologically (Figure 1A). In adult raccoon somatosensory cortex, there is a well-defined somatotopic map of the forepaw. More importantly, for biochemical studies, these areas are readily identifiable by anatomical landmarks such as cortical sulci, thus the cortical areas representing each digit can be confidently dissected without the necessity of individual electrophysiological receptive field mapping (Welker and Seidenstein, 1959). This makes raccoon a very good animal model for the study of deafferentation-induced cortical reorganization.

After amputation of one of the digits, cortical neurons that had receptive fields on the deafferented digit start to respond to stimulation of glabrous skin areas adjacent to the digit. Over time the deafferented neurons form new receptive fields. A series of electrophysiological studies have outlined the time course of physiological changes following digit amputation in raccoon somatosensory cortex (Rasmusson, 1982; Turnbull and Rasmusson, 1990 and 1991; Figure 1B and C). A schematic depicts the

typical process of deafferentation-induced receptive field reorganization for a neuron with a small, excitatory receptive field. The neuron receives strong excitation from stimulation of a very restricted skin area (receptive field) located on the right digit 4 (normal restricted receptive field, Figure 1C1). Immediately after deafferentation of the primary digit (right digit 4), the neuron loses its receptive field and no longer responds to peripheral stimulation (no receptive field, Fig. 1C2). Over the next one to two weeks, new excitatory responses emerge, and the neuron starts to respond to stimulation of a large skin area (usually much larger than its original receptive field) on the neighboring digits and palm area (mixed receptive field; Figure 1C3). Several months after deafferentation, the receptive field is refined and appears to be small and similar in size to the pre-deafferentation receptive field, but has been remapped to another digit (restricted receptive field; Fig. 1C4). As shown in figure 1B, 1-2 weeks after deafferentation, more than half of the neurons in the deafferented cortex are excited by stimulation of an enlarged skin area (mixed receptive fields). The rest of neurons still show no response to peripheral stimulations (no receptive field). At this time point, almost no neurons have the normal type of restricted receptive field characteristic of adult somatosensory cortex. As time goes on, the number of neurons with no receptive field goes down, and the number of neurons that have regained restricted receptive fields increase. By 18-24 weeks post-deafferentation, the majority of neurons in the deafferented cortex have remapped restricted receptive fields typically seen the adult somatosensory cortex, and only very few neurons remain silent (with no receptive field).

### **2.1.2 Molecular mechanisms underlying cortical reorganization**

Removing the peripheral inputs of a cortical area induces a series of changes in the neuronal response properties in the deafferented cortex that eventually lead to remapping of cortical receptive fields. However, it remains to be established how changes in synaptic activity patterns induced by deafferentation are translated into changes in synaptic composition and function.

Strengthening of excitatory synapses between simultaneously active neurons is thought to play an important role in the initiation and maintenance of topographic maps during the development of the sensory cortices (Buonomano and Merzenich, 1998). A wealth of data suggests that levels of the AMPA ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) subtype of glutamate receptor is a primary determinant of the strength of excitatory synapses, and that activity-dependent insertion or removal of AMPARs results in synaptic potentiation or depression (Malinow and Malenka, 2002). Experience-dependent synaptic strengthening has recently been shown to drive AMPARs into synapses during the formation of S1 circuits in the rodent barrel cortex (Isaac et al., 1997; Takahashi et al., 2003; Ehrlich and Malinow, 2004; Clem and Barth, 2006), while sensory deprivation can induce AMPAR endocytosis and synaptic depression in rodent barrel cortex and visual cortex (Allen et al., 2003; Heynen et al., 2003). Transgenic manipulations that interrupt AMPAR trafficking also disrupt the proper maturation of topography in the barrel cortex (Lu et al., 2003).

A developmental increase in synaptic strength also occurs at inhibitory GABAergic ( $\gamma$ -aminobutyric acid) synapses, but is delayed relative to strengthening

of excitatory synapses (Fagiolini and Hensch, 2000; Rozas et al., 2001; Morales et al., 2002). Recent studies in the visual cortex suggest that a developmental increase in the ratio of GABAergic inhibition to glutamatergic excitation may be necessary to initiate synaptic plasticity (Fagiolini and Hensch, 2000), while further strengthening of cortical inhibition constrains plasticity (Rozas et al., 2001).

An active balance between ascending excitation and local inhibition may serve to maintain the restricted receptive fields, which are characteristic of mature sensory systems. The optimal balance between excitation and inhibition naturally declines with aging, resulting in compromised stimulus selectivity (Leventhal, 2003). Acute, local antagonism of GABA<sub>A</sub> receptors produces an expansion of receptive fields in somatosensory cortex (Dykes et al., 1984; Alloway and Burton, 1991; Tremere et al., 2001a). This suggests that neurons in the somatosensory cortex receive excitatory inputs from a larger spatial range than is indicated by their receptive fields, and that weaker secondary inputs are masked by GABAergic inhibition. GABAergic inhibition may also inhibit the initiation of cortical plasticity in the adult animal. GABA receptor agonists can inhibit the adaptive adjustment of the owl auditory space map induced by experimental uncoupling of the visual and auditory inputs (Zheng and Knudsen, 2001). Removal of inhibition may be required to permit large-scale synaptic reorganization. A decrease in binding to GABA<sub>A</sub> receptors in layer IV neurons of the deafferented cortex has been observed in the primate somatosensory cortex within 5 hours of peripheral nerve transection (Wellman et al., 2002) and within one week of deafferentation in the mouse barrel cortex (Skangiel-Kramska et al., 1994).



Together these data suggest that the release from GABAergic inhibition may be required to allow cortical map plasticity, but how are new receptive fields formed following a disruption in normal afferent activity? The pharmacological expansion of receptive fields in somatosensory cortex via antagonism of GABA<sub>A</sub> receptors is typically limited to adjacent surfaces of the body, such as a larger part of the same digit (Dykes et al., 1984; Tremere et al., 2001a). However following deafferentation, denervated cortical neurons acquire receptive fields on adjacent digits. Thus, deafferentation-induced receptive field plasticity cannot be explained solely by removal of inhibitory cortical connections.

Here we test the hypothesis that progressive changes occur in both excitatory and inhibitory synaptic function during the reorganization of cortical receptive fields, by tracking changes in the levels of ionotropic receptors in the somatosensory cortex of adult raccoons following amputation of a single forepaw digit.

## 2.2 Material and Methods

Animal care and surgery, receptive field mapping in the adult raccoon somatosensory cortex performed in the Rasmusson lab in Dalhousie University.

Adult male and female raccoons (4.5 to 13.4 kg) underwent surgical amputation of the right fourth digit 1 to 9 days (short-term, n=7), 2 to 7 weeks (mid-term, n=7), or 17 to 28 weeks (long-term, n=6) prior to sacrifice. Sham-operated animals (n=3), in which the nerves to the 4th digit were exposed but not transected one day before sacrifice, served as controls. All experimental procedures conformed to guidelines of the Canadian Council on Animal Care and the Dalhousie University Committee on Laboratory Animals. Using sterile surgical conditions, the digit was amputated at the metacarpal-phalangeal joint under isoflurane anesthesia as previously described (Rasmusson 1996). The four digital nerves were ligated, cut and folded proximally. The dorsal hairy skin was sutured to the glabrous skin of the palm. Immediately after surgery, cesazolin sodium (Keszol; Lilly; 15 mg/kg i.m.) was injected to prevent infection and buprenorphin (Buprenex, Reckitt-Coleman; 0.3 mg/kg i.m.) was injected to reduce postoperative pain. All wounds healed within 3 days.

On the day of sacrifice subjects were anesthetized with ketamine HCl (MTC Pharmaceutical, 10-20 mg/kg, i.m.) followed by isoflurane during insertion of a catheter into a forelimb vein for maintenance on  $\alpha$ -chloralose (Sigma, i.v., 5% in propylene glycol). Bilateral craniotomies were performed over somatosensory cortex, the dura mater opened and the brain photographed. In some animals single-unit mapping was performed to confirm the location of digit representational areas, as

previously described (Chowdhury and Rasmusson, 2002) but in most animals these areas were determined from the location of the tri-radiate and surrounding sulci (Welker & Seidenstein, 1959). Small blocks of tissue (approximately 3 mm<sup>3</sup>) were dissected from the representational areas of the deafferented 4<sup>th</sup> digit (deafferented cortex; DCtx), the adjacent 5<sup>th</sup> digit (D5) from the left hemisphere and the control 5<sup>th</sup> digit (control cortex; CCtx) from the right hemisphere. We used the representational area of contralateral digit 5 as the within-animal control because it has been subjected to the same history of use as the other digits when the animals were in the wild, but receives no direct excitatory connections from the cortical area that represents contralateral digit 4. The tissue was rinsed in dissection buffer (Quinlan et al., 1999), rapidly frozen in liquid nitrogen, and shipped on dry ice to the Quinlan lab at the University of Maryland.

#### Biochemical analysis of receptor protein levels performed in the Quinlan lab

##### Synaptoneurosome Preparation

Synaptoneurosomes were prepared by using a procedure adapted from Hollingsworth et al. (1985), as described in Quinlan et al., 1999. The frozen tissue of raccoon somatosensory cortex was homogenized in ice-cold homogenization buffer (10mM Hepes, 1.0mM EDTA, 2.0mM EGTA, 0.5mM DTT, 0.1 mMPMSF, 10mg/liter leupeptin, 50mg/liter soybean trypsin inhibitor, 100nM microcystin). Tissue was homogenized in a Dounce glass/glass tissue homogenizer (Kontes, pestle B), and the homogenate was passed sequentially through two 100- $\mu$ m-pore nylon mesh filters, followed by a 5- $\mu$ m-pore filter, and centrifuged at 1000g for 10min. The

pellet that is the product of this centrifugation contains the synaptoneuroosomes, which are re-suspended in boiling 1% SDS to denature the proteins and stored at -80°C.

### Quantitative Immunoblots

Equal concentrations of synaptoneurosome or homogenate protein (10 µg/sample), determined using the BCA assay (Pierce), were resolved on polyacrylamide gels, transferred to nitrocellulose and probed with anti-GluR2 (mouse monoclonal, 1:500, clone 6C4, Zymed), anti-GABA<sub>A</sub> β2/3 (mouse monoclonal, 1:500, clone 62-3G1, Upstate Biotechnology), anti-actin (mouse monoclonal, 1:500, clone JLA20, Oncogene), anti-NR2A (rabbit polyclonal, 1:500, Upstate Biotechnology), anti-NR2B (rabbit polyclonal, 1:500, Upstate Biotechnology) or anti-NR1 (mouse monoclonal, 1:500, clone 54.1, Zymed) antibodies in Tris buffered saline, pH7.4 containing 1% non fat powdered milk and 0.1% Triton X-100, followed by incubation in the appropriate secondary IgG coupled to horseradish peroxidase (1:3500, Amersham). Visualization of immunoreactive bands was induced by enhanced chemiluminescence (Amersham ECL), and captured on autoradiography film (Amersham, Hyper film ECL). Each immunoblot is exposed to autoradiography film multiple times (3-5) to ensure that we are operating within the linear range of the film. Digital images, produced by densitometric scans of autoradiographs on a ScanJet 7400C (Hewlett Packard), were quantified using NIH Image 1.60 software. The optical density (O.D.) of each immunoreactive band was quantified relative to a baseline immediately above and below the band, and normalized to the O.D. of an immunoblot for actin, which serves as a gel loading control, produced by stripping and reprobing the same blot. This method has been shown to allow quantification of

small changes in protein levels, and that the relationship between O.D. and protein concentration is linear along the range used in this study (Heynen et al., 2000). All immunoblots and densitometry were performed with the experimenter blind to the surgical history of the animal. Group data are represented as the mean  $\pm$  SEM of the normalized O.D. in the deafferented cortex (DCtx) relative to the within-animal control cortex (DCtx/CCtx). Statistical significance was assessed by one-way ANOVA, followed by Tukey-Kramer post-hoc comparisons. Data are presented as a percentage of sham-operated control.

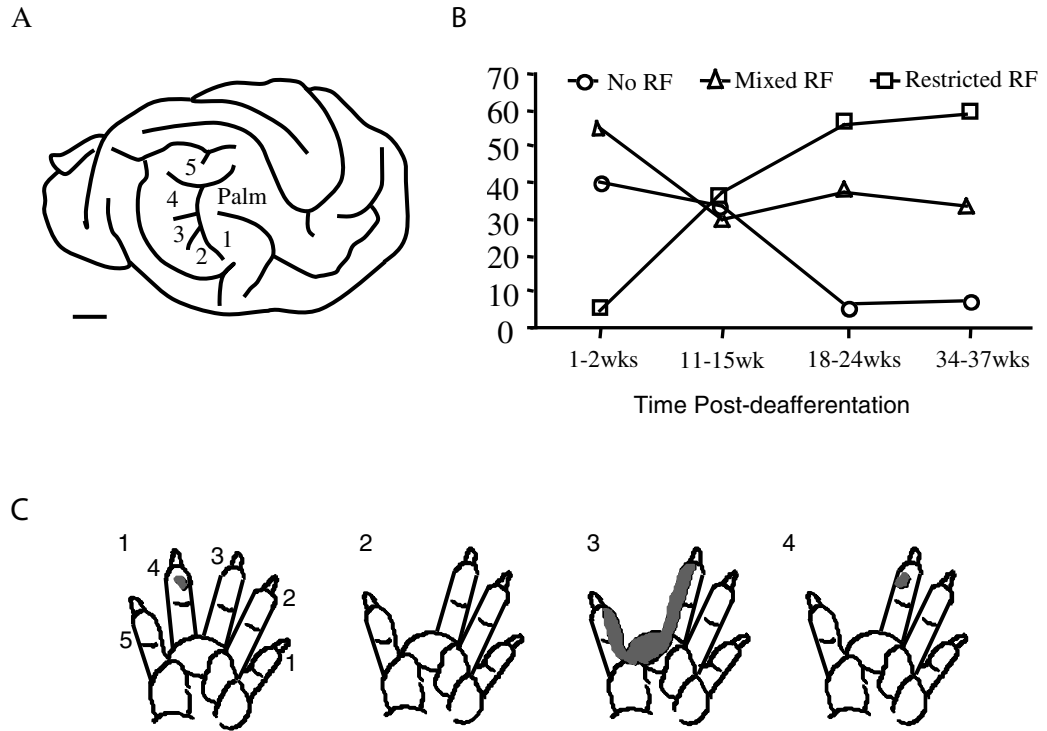
## **2.3 Results**

As the primary mediators of fast synaptic transmission, changes in the number and/or subtype of ionotropic glutamate or GABA receptors will have a significant and long-lasting impact on synaptic strength. Therefore, we tracked the levels of 1) AMPARs, the ionotropic glutamate receptor that mediates fast depolarization at the majority of excitatory synapses, 2) NMDARs, the ligand and voltage-gated ionotropic glutamate receptor which is linked to the induction of many forms of activity-dependent synaptic plasticity, and 3) GABA<sub>A</sub> receptors, the ionotropic receptor that mediates fast hyperpolarization at the majority of inhibitory synapses. We examined receptor protein levels in deafferented somatosensory cortex (DCtx) versus control somatosensory cortex (CCtx) at three intervals after deafferentation: short-term (the first 9 days) when new excitatory inputs and enlarged receptive fields first appear, mid-term (2 to 7 weeks), when enlarged receptive fields begin to constrict, and long-term (> 17 weeks), when final stabilization of remapped receptive fields are observed.

### **2.3.1 AMPA receptor**

To examine the balance between glutamatergic excitation and GABAergic inhibition at different stages of reorganization of the somatosensory cortex following deafferentation, we tracked the levels of ionotropic receptor proteins in the representational areas of the deafferented right 4<sup>th</sup> digit (deafferented cortex; DCtx) and the control left 5<sup>th</sup> digit (control cortex; CCtx). In adult mammalian cortex, the majority of fast excitation is mediated by calcium-impermeable AMPARs at

**Figure 1. Reorganization of neuronal receptive fields in the deafferented somatosensory cortex.** (A) Schematic of the left hemisphere of the adult raccoon brain, illustrating the area of somatosensory cortex representing each digit. Small blocks of tissue (approximately 3 mm<sup>3</sup>) were dissected from the representational area of the deafferented right digit 4 (deafferented cortex; DCtx) and control left digit 5 (control cortex; CCtx) for biochemical study. (B) Changes in receptive field properties of neurons in DCtx versus time post-deafferentation. 1-2 weeks after deafferentation, > 90% of neurons in the DCtx have either no receptive field or poorly defined/enlarged mixed receptive fields. Few neurons at this time point have the restricted receptive field which is characteristic of the adult raccoon somatosensory cortex. Over time, the percentage of neurons with a restricted receptive field increases while the percentage of neurons with no or mixed receptive fields declines. Adapted from (Rasmusson, 1982; Rasmusson and Turnbull, 1983; Turnbull and Rasmusson, 1990; Turnbull and Rasmusson, 1991; Rasmusson et al., 1992) (C) Schematic showing the typical receptive field of a neuron in DCtx at progressive stages of reorganization. Originally, the receptive field is restricted to a small area on the ventral surface of digit 4 (control). Shortly after deafferentation of digit 4, the neuron either has no obvious receptive field (C2) or an enlarged/poorly defined receptive field. Enlarged receptive fields often encompass areas of the adjacent digits (3 and 5) and/or part of the palm (C3). Over time, the receptive field decreases in size, and by 4 months post-deafferentation, it resembles the original receptive field in size, but is now restricted to a small area on an adjacent digit (C4).



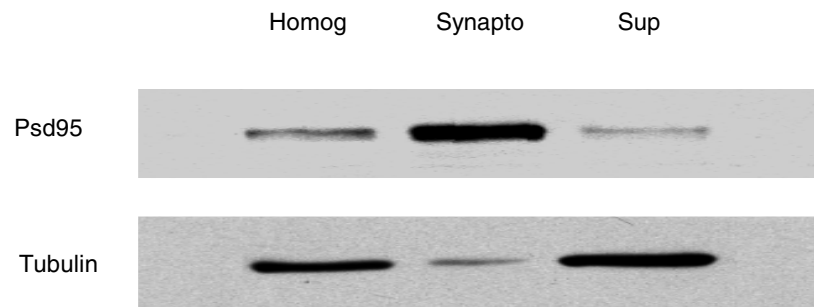
**Figure 1**



glutamatergic synapses. The impermeability of calcium is due to the presence of the GluR2 subunit protein (Hollmann et al., 1991; Wenthold, et al., 1992), suggesting that the majority of AMPARs in the adult cortex contain the GluR2 subunit. Therefore an antibody against GluR2 was used to track changes in AMPAR levels. Quantitative immunoblotting for GluR2 was performed in synaptoneurosomes, a subcellular fraction that specifically enriches for glutamatergic synaptic profiles (Figure 2, Hollingsworth et al., 1985; Quinlan et al., 1999), prepared from DCtx and CCtx somatosensory cortex. It has been shown that the levels of GluR2 in synaptoneurosomes are highly correlated with synaptic strength (Heynen et al., 2000). To control for individual differences, levels of GluR2 in the DCtx were normalized to levels of GluR2 in CCtx from the same animal and run on the same gel. Following deafferentation, levels of GluR2 in DCtx/CCtx were significantly increased between 1 and 9 days post-deafferentation (short-term) relative to control ( $160 \pm 20\%$  of control, Fig. 3). The increase was transient, as GluR2 levels returned to control levels by 2 weeks post-deafferentation, mid-term and long-term. In contrast, no significant difference was observed in the level of GluR2 in the adjacent non-denervated digit 5 representation region at any time point post-deafferentation (One-way ANOVA,  $F_{(3, 10)} = 0.302$ ,  $p=0.823$ , data not shown).

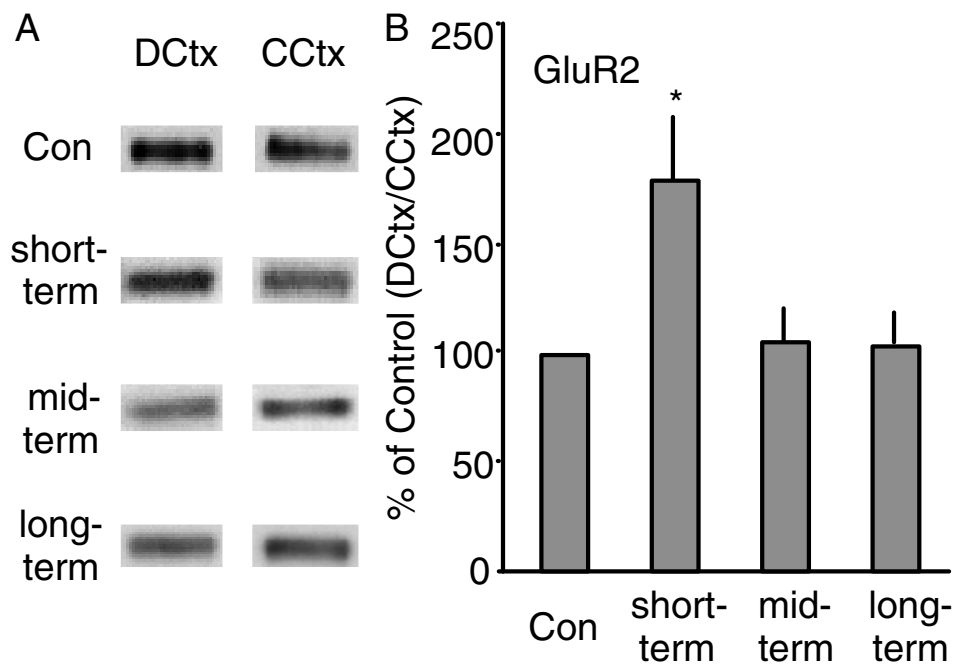
**Figure 2. Protein distributions in different subcellular fractions demonstrate that synaptoneurosome specifically enriches for proteins at excitatory synapses.**

The amount of two proteins, psd95 and tubulin, is visualized by immunoblotting in three preparations: homog (cortical tissue homogenate), synpto (synaptoneurosome prepared from cortical tissue homogenate), and sup (supernatant, the non-synaptoneurosome component of the cortical homogenate). Psd95 is a scaffold protein that is exclusively located at excitatory synapses. As shown by the immunoblots, Psd95 is present in homogenate, greatly enriched in synaptoneurosome. In contrast, tubulin, a cytoskeleton protein that is not present in the synapse, is present in homogenate but excluded from synaptoneurosome.



**Figure 2**

**Figure 3. Deafferentation regulates the level of AMPA receptor subunits in deafferented somatosensory cortex (DCtx)** (A) Representative immunoblots for the GluR2 subunit of the AMPAR in synaptoneurosomes prepared from DCtx and CCtx at each experimental time point. (B) Summary data from all experiments. The optical density of GluR2 from DCtx was normalized to CCtx, allowing a within-animal control. GluR2 protein in DCtx is increased significantly shortly (1 – 9 days) after deafferentation, but returns to baseline levels after longer time periods (mid-term = 2 – 7 weeks; long-term = 17 – 28 weeks). \* : One-way ANOVA ( $F_{(3, 10)} = 4.855$ ,  $p < 0.05$ , with Tukey-Kramer post-hoc comparison. Summarized data are presented as % of averaged sham-operated control (mean  $\pm$  SEM).

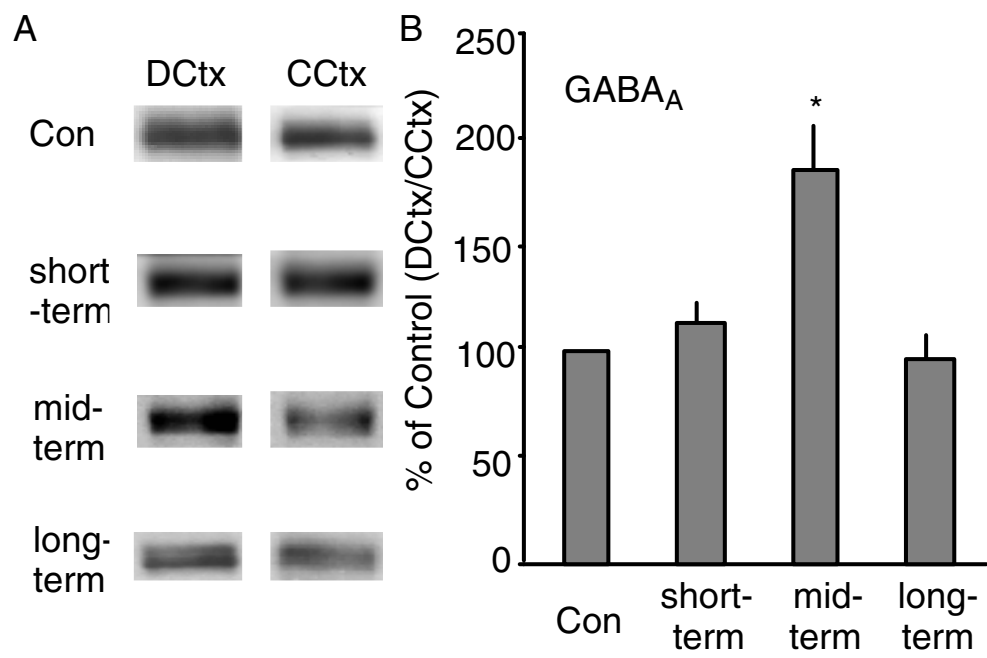


**Figure 3**

### 2.3.2 GABA<sub>A</sub> receptor

The fast inhibition at GABAergic synapses is mediated by the ionotropic GABA<sub>A</sub> receptor. The majority of cortical GABA<sub>A</sub> receptors contain either the  $\beta$ 2 or the  $\beta$ 3 subunit (Li and de Blas, 1997). Therefore, an antibody that recognizes both the  $\beta$ 2 and  $\beta$ 3 subunits was used to track changes in GABA<sub>A</sub> receptors. In this case, quantitative immunoblotting was performed in homogenates prepared from DCtx and CCtx, because synaptoneuroosomes and other subcellular fractions do not enrich for synaptic GABA<sub>A</sub> receptors. Levels of GABA<sub>A</sub> receptor in DCtx/CCtx were significantly increased between 2 and 7 weeks post-deafferentation (mid-term) ( $165 \pm 16\%$  of control) versus control, short-term and long-term (Figure 4). In contrast, no significant differences were observed in the levels of GABA<sub>A</sub> receptors in the representational area of right digit 5 at any time point post-deafferentation (One-way ANOVA ( $F_{(3, 10)} = 1.346$ ,  $p=0.31$ , data not shown).

**Figure 4. Deafferentation regulates the level of GABA<sub>A</sub> receptor subunits in deafferented somatosensory cortex (DCtx)** (A) Representative immunoblots for  $\beta 2/3$  subunits of GABA<sub>A</sub> receptors in homogenates prepared from DCtx and CCtx at each experimental time point. (B) Summary data from all experiments. The optical density of GABA<sub>A</sub> $\beta 2/3$  in DCtx was normalized to CCtx, allowing a within-animal control. GABA<sub>A</sub>  $\beta 2/3$  protein in DCtx is significantly increased at intermediate time points (2 – 7 weeks) after deafferentation, but returns to baseline levels after longer time periods (long-term = 17 – 28 weeks). \* One-way ANOVA ( $F_{(3, 10)} = 6.512$ ,  $p < 0.01$ ; with Tukey-Kramer post-hoc comparison. Summarized data are presented as % of averaged sham-operated control (mean  $\pm$  SEM).



**Figure 4**



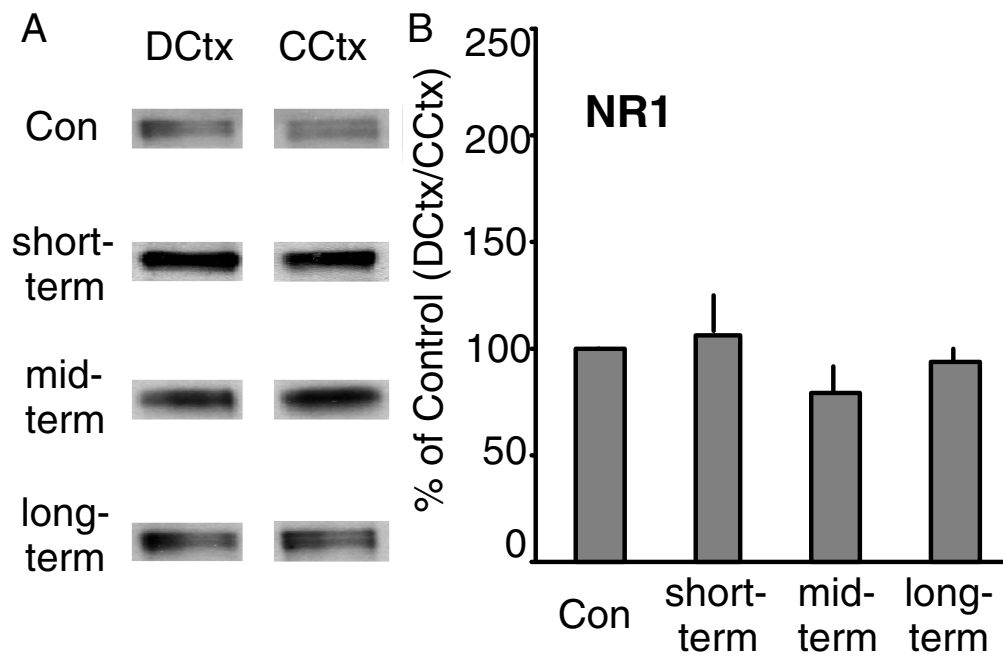
### 2.3.3 NMDA receptors

Many forms of activity-dependent synaptic plasticity have been shown to be dependent on activation of the NMDA subtype of glutamate receptor (Malinow and Malenka, 2002). NMDARs are heteromeric protein complexes, comprised of multiple subunits with developmental and regional heterogeneity. In the adult mammalian cortex, NMDARs contain obligatory NR1 subunits and variable NR2 (gene products NR2a - NR2d) subunit proteins. We used an antibody against the NR1 subunit to track changes in total NMDAR levels in synaptoneurosomes prepared from DCtx relative to CCtx. No significant difference was observed in the level of the NR1 subunit of the NMDAR across experimental conditions (One-way ANOVA ( $F_{(3, 10)} = 0.794$ ,  $p=0.52$ ; Fig 5). In addition, we saw no significant difference in the levels of NR2a (One-way ANOVA ( $F_{(3, 10)} = 3.110$ ,  $p=0.08$ ), NR2b (One-way ANOVA ( $F_{(3, 9)} = 0.821$ ,  $p=0.51$ ) or the ratio of NR2a/NR2b (One-way ANOVA ( $F_{(3, 9)} = 0.270$ ,  $p=0.85$ ) at any time point post-deafferentation. Together these data suggest that deafferentation does not induce a net change in NMDAR number in the deafferented region of somatosensory cortex and the reorganization following deafferentation does not involve a change in the NMDAR subunit compositions.

Comparison of the levels of GluR2, NR1 and GABA<sub>A</sub> receptors across animals demonstrates the progressive changes in excitatory and inhibitory receptors in deafferented cortex during cortical reorganization (Figure 6A-C). Best-fit curves were derived by smoothing the raw data of GluR2, NR1, and GABA<sub>A</sub> receptor levels using a least square fit polynomial ratio with 6 free parameters (coefficients), with both the numerator and denominator as 4<sup>th</sup> order polynomials. The purpose for using the

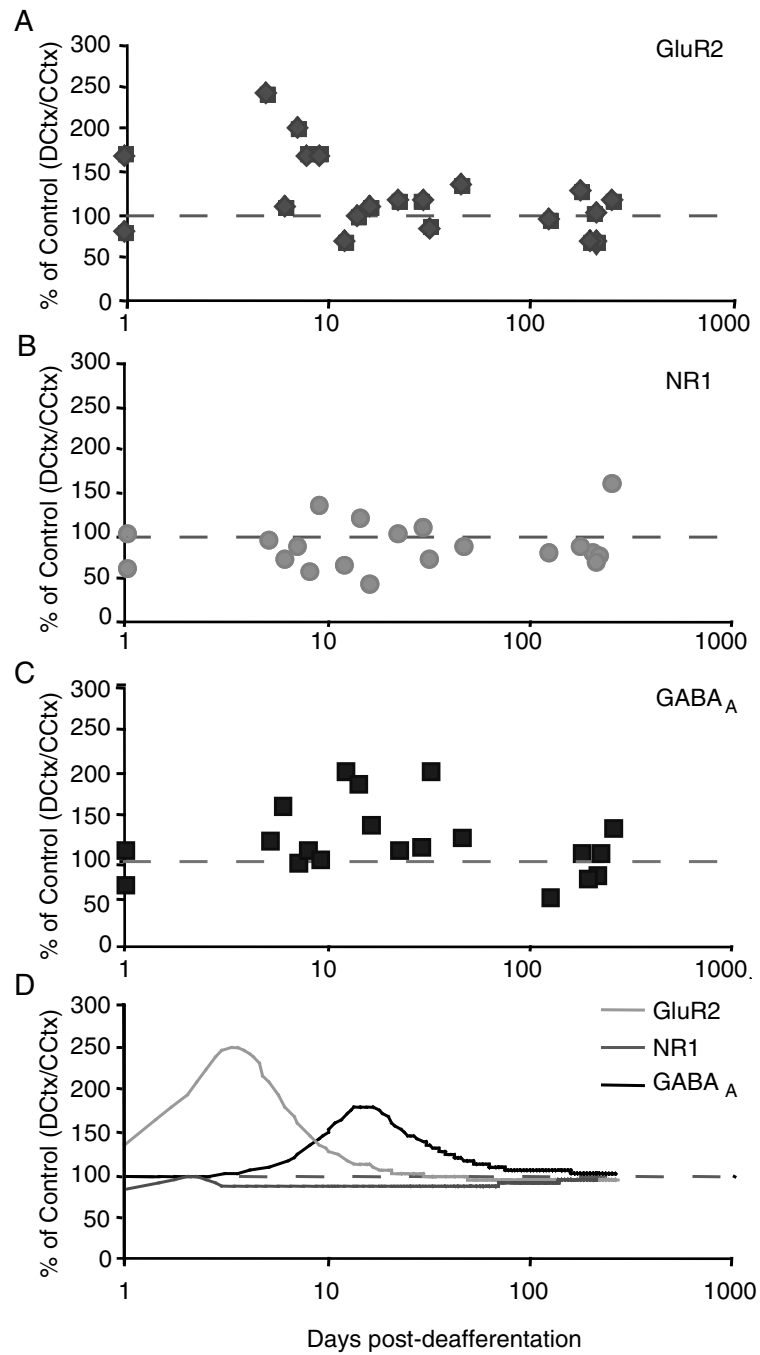
polynomial ratio is to remove the irrelevant high frequency information (the noise) in the raw data, so that the most interesting low frequency information of changes in the receptor levels over a long time scale is retained for further analysis. Comparison of the best-fit curves for GluR2, NR1 and GABA<sub>A</sub> on the same timeline demonstrates that the increase in GluR2 peaks between 3–5 days post deafferentation (Figure 6D), while receptive fields are expanding. The increase in GABA<sub>A</sub> receptor levels peaks between 14-16 days post-deafferentation, just as GluR2 levels are beginning to decline, and receptive fields are shrinking.

**Figure 5. NMDA receptor subunit levels are unchanged in deafferented somatosensory cortex (DCtx)** (A) Representative immunoblots for the NR1 subunit of the NMDAR in synaptoneurosomes prepared from DCtx and CCtx at each experimental time point. (B) Summary data from all experiments. The optical density of NR1 in DCtx was normalized to CCtx, allowing a within-animal control. Level of NR1 protein in DCtx remains unchanged over post-deafferentation time. Summarized data are presented as % of averaged sham-operated control (mean  $\pm$  SEM).



**Figure 5**

**Figure 6. Progressive changes in AMPA and GABA<sub>A</sub> receptor proteins in DCtx during remapping of cortical receptive fields in response to deafferentation.** (A) Values for levels of GluR2 in DCtx/CCtx for each experimental animal versus time post-deafferentation. Dashed line indicates average control level. (B) Values for levels of NR1 in DCtx/CCtx for each experimental animal versus time post-deafferentation. Dashed line indicates average control level. (C) Values for levels of GABA<sub>A</sub> in DCtx/CCtx for each experimental animal versus time post-deafferentation. Dashed line indicates average control level. (D) Best-fit curves for GluR2, NR1 and GABA<sub>A</sub> receptor levels derived by fitting individual data points with a polynomial ratio. The increase in GluR2 peaks between 3-5 days post deafferentation and returns to pre-deafferentation baseline by two weeks. The increase in GABA<sub>A</sub> receptor levels peaks between 14 - 16 days post-deafferentation, at the time when AMPAR levels are declining, and returns to pre-deafferentation baseline by 119 days post-deafferentation.



**Figure 6**

## 2.4 Discussion

Although normally somatotopic maps in adult cortex are highly stable, plasticity of topographic maps can be revealed in the adult somatosensory cortex by removing primary inputs. Here we report that progressive changes in the levels of two major cortical ionotropic receptors are correlated with different stages of deafferentation-induced receptive field reorganization. Deafferentation induces an early, transient elevation in the level of synaptic AMPARs in the deafferented cortex, which coincides with the expansion of receptive fields. This is followed by an increase in GABA<sub>A</sub> receptor levels, which coincides with the contraction of recently expanded receptive fields following deafferentation. Both AMPAR and GABA<sub>A</sub> receptor levels return to pre-deafferentation baseline levels by 17 weeks post-deafferentation, at which time the receptive fields of neurons in the deafferented cortex have remapped to a different part of the body surface. Interestingly, the size of the remapped receptive field is comparable to the normal receptive fields observed in adult somatosensory cortex.

### **2.4.1 The transient increase in AMPAR might be required for the emergence of the enlarged new receptive field shortly after deafferentation**

Several mechanisms have been proposed to underlie deafferentation-induced cortical reorganization, such as strengthening of previously existing weak excitatory synapses from the body surface surrounding the original primary receptive field, or sprouting of new connections from inputs of the surrounding areas. Nonetheless, both

strengthening of pre-existing weak synapses and formation of newly sprouted synapses may require activity-dependent delivery of AMPARs to the synapses. Previously reported electrophysiology recordings (Tremere et al., 2001b; Chowdhury and Rasmusson, 2002) and our biochemical results suggest that there is also a later increase in the strength of inhibitory synapses, which may result in finely tuned, remapped cortical RFs. The progressive elevation in the levels of cortical excitation and inhibition during the receptive fields remapping recapitulates the changes in the balance between excitation and inhibition that accompanies the formation and maturation of receptive fields in developing cortical neurons.

One immediate consequence of peripheral deafferentation may be a decrease in the strength of lateral inhibition due to the removal of the primary excitatory drive. The reduced inhibition may unmask weaker excitatory inputs carrying information from outside of the primary receptive field (Dykes et al., 1984; Tremere et al., 2001a). An initial decrease in GABA<sub>A</sub> receptor binding in layer IV of the deafferented cortex has been observed following peripheral denervation in the mouse barrel cortex (Skangiel-Kramska et al., 1994) and following peripheral nerve transection in the primate somatosensory cortex (Wellman et al., 2002), suggesting that GABA<sub>A</sub> receptors are rapidly endocytosed following deafferentation. Surprisingly, we did not see a decrease in GABA<sub>A</sub> receptor levels immediately following deafferentation. This might be because we had to use cortical homogenate to study the level of GABA<sub>A</sub> receptors. Synaptoneurosomes specifically enrich for the glutamatergic excitatory synapses based on the unique sedimentary coefficient of the postsynaptic density. Because the GABAergic inhibitory synapses do not have a



postsynaptic density, they are not enriched by synaptoneurosomes. The cortical tissue homogenate could not distinguish synaptic protein and non-synaptic proteins, thus is unable to detect a decrease in synaptic GABA<sub>A</sub> receptors if the receptors are endocytosed instead of degraded. In addition, our analysis combines synapses from all layers of S1. Hence, the possibility that receptor expression is decreasing in some layers while increasing in others may result in the absence of change in total GABA<sub>A</sub> receptor levels immediately following deafferentation.

Nonetheless, deafferentation-induced receptive field plasticity cannot be explained solely by the removal of inhibitory cortical connections. The expansion of receptive fields induced pharmacologically by antagonism of GABA<sub>A</sub> receptors is typically limited to adjacent surfaces of the body, such as larger part on the same digit (Dykes et al., 1984; Tremere et al., 2001a). In contrast, following deafferentation, the deafferented cortical neurons could acquire new receptive fields on adjacent digits. The significant, transient increase in the levels of synaptic AMPAR proteins we see at brief period (1-9 days) post-deafferentation is consistent with the hypothesis that the excitatory synapses between weaker secondary inputs in the deafferented cortex are undergoing activity-dependent synaptic strengthening during this period. It is well-documented that the number of synaptic AMPARs determines the strength of excitatory synapses, and that activity-dependent insertion or removal of AMPARs results in synaptic potentiation or depression (Malinow and Malenka, 2002). Sensory experience induces insertion of AMPAR into synaptic membrane and potentiation of synaptic strength (Isaac et al., 1997; Takahashi et al., 2003), while sensory deprivation induces AMPAR endocytosis and depression of synaptic strength (Allen

et al., 2003; Heynen et al., 2003). Cortical remapping is also dependent on peripheral activity (Wallace et al., 2001) and NMDAR activation (Kano et al., 1991; Rema et al., 1998; Myers et al., 2000; Kleinschmidt et al., 1987), suggesting that activity-dependent delivery of AMPARs is involved in the experience-dependent formation of the new receptive fields.

The increase in AMPAR levels could result either from strengthening of weak, pre-existing excitatory synapses or the formation of new excitatory synapses (Jones, 2000). Newly formed excitatory synapses are typically silent at resting membrane potential, due to the presence of voltage-dependent NMDARs and the absence of AMPARs (Liao et al., 1995; Isaac et al., 1995; Isaac, 2003). It is unknown if delivery of NMDARs precedes delivery of AMPARs during synaptogenesis in the adult, however, there have been no descriptions (physiological or biochemical) of AMPA-only synapses. We see an increase in AMPARs in the absence of a change in the levels of NMDAR proteins, suggesting that cortical reorganization may begin with the potentiation of pre-existing excitatory synaptic connections rather than the formation of new synapses. Interestingly, a transient increase in the number of excitatory synapses followed by more persistent increase in inhibitory synapse number has recently been demonstrated in rodent barrel cortex following repetitive single whisker stimulation (Knott et al., 2002).

Regulation of the strength of horizontal excitatory connections between layer 2/3 neurons has recently been shown to be a primary site of experience-induced map plasticity in the adult visual and somatosensory cortices (Fox, 2002; Stern et al., 2001; Trachtenberg et al., 2000; Heynen et al., 2003; Calford et al., 2003; Hickmott

and Merzenich, 2002; Finnerty et al., 1999). Because our analysis was performed on a combination of synapses from all layers of S1, we are unable to tell if deafferentation-induced changes in AMPAR levels we observe are layer-specific.

#### **2.4.2 The delayed increase in GABAergic inhibition might be required for the refinement of the newly formed receptive fields**

An equally important component of long-term cortical reorganization following deafferentation is the reduction of expanded receptive field size and re-establishment of restricted receptive fields (Tremere et al., 2001b; Chowdhury and Rasmuson, 2002). In developing sensory systems, an increase in the ratio of GABAergic inhibition to glutamatergic excitation may constrain synaptic plasticity and serve to maintain restricted receptive field size (Hensch et al., 1998; Huang et al., 1999; Rozas et al., 2001). The significant increase in the levels of GABA<sub>A</sub> proteins that we observed 2-7 weeks post-deafferentation would serve to constrain synaptic strengthening during receptive field reorganization of the deafferented cortex. The deafferentation-induced increase in GABA<sub>A</sub> receptors is likely due to the synthesis of new receptor proteins, or an increase in the half-life of pre-existing receptors, because the movement of GABA<sub>A</sub> receptors from one cellular compartment to another (i.e. non-synaptic to synaptic) would go undetected by our methods.

Accumulating evidence suggests that there may be some difference between the mechanisms by which map topography are regulated in adults and juveniles. For example, in neonates, the reorganization of barrels observed following whisker trimming and the ocular dominance shift following monocular deprivation is due to

regulation of thalamocortical synapses in layer IV. In contrast, in post-neonatal animals, deprivation induces a long-term depression of layer IV to layer II/III synapses, suggesting an intracortical locus of plasticity (Allen et al., 2003; Heynen et al., 2003).

The mechanisms underlying deafferentation-induced reorganization may also differ between different cortical systems. In adult somatosensory cortex, deafferentation-induced cortical reorganization does not correlate with changes in mRNA levels in the deafferented cortex for many markers of synaptic efficacy including AMPA and NMDA receptor subunits (Jones et al., 2002; but also see Gierdalski et al., 1999). This is in contrast to the robust changes in the levels of synaptic mRNAs observed in primary visual cortex following brief monocular deprivation in adults (Hendry et al., 1990; Benson et al., 1994). Together with changes in AMPAR and GABA<sub>A</sub> receptor proteins reported here, this raises the intriguing possibility that deafferentation-induced regulation of synaptic composition in the adult cortex may be post-transcriptional. Nonetheless, the fact that peripheral deafferentation can induce large-scale reorganization of the cortical circuits in adult cortex shows that there is certain level of synaptic plasticity that is retained in the adult cortex.

# **CHAPTER 3: VISUAL DEPRIVATION REACTIVATES JUVENILE-LIKE OCULAR DOMINANCE PLASTICITY IN ADULT VISUAL CORTEX**

## **3.1 Introduction**

### **3.1.1 Rodent visual system and ocular dominance plasticity**

In the rodent visual system, approximately 90% of retinal ganglion cell axons cross over at the optic chiasm and project onto the contralateral lateral geniculate nucleus (LGN). Axons from LGN then project to layer IV of the ipsilateral primary visual cortex. The relatively high percentage of contralateral projections has been hypothesized to be a result of the lateral placement of the eyes, which also results in panoramic vision (Butler and Hodos, 2005). In rodent V1, approximately two-thirds is monocular, and receives input only from the contralateral eye; the other one third receives inputs from both eyes and is therefore binocular. Within the binocular region of the V1, neurons respond more strongly to stimulation of the contralateral eye than the ipsilateral eye. Thus in rodent binocular visual cortex, the ocular dominance can also be described as a contralateral bias. The contralateral bias in rodent visual cortex has been detected by many methods, including single unit recording (Fagiolini et al., 1994; Gordon and Stryker, 1996), visually evoked potentials (VEPs) (Porciatti et al., 1997; Pizzarusso et al., 1999; Heynen et al., 2003; Sawtell et al., 2003; Pham et al., 2004), optical imaging (Cang et al., 2005; Hofer et al., 2006), and functional mapping by immediate early gene expression (Pham et al., 2004; Tagawa et al., 2005). All of these methods show that in the binocular region of rodent V1, the neuronal response

to stimulation of the contralateral eye is greater than the response to stimulation of the ipsilateral eye.

The ocular dominance of binocular neurons in the visual cortex is actively maintained by competition between synapses serving the two eyes, which has been shown in many species including cats, monkeys, ferrets, rats, mice, and humans. Early in development (*in utero*), the spontaneous activity generated in the retinal ganglion cells shapes the formation of the highly ordered retinogeniculate projection (Penn and Shatz, 1999). Precise ocular dominance is maintained by competition between the geniculocortical projections receiving input from the two eyes (Katz and Shatz, 1996). Disrupting this competition by depriving one eye of vision in juvenile animals induces a shift in ocular dominance in favor of the non-deprived eye (Hubel and Wiesel, 1970). In juvenile rodents, the ocular dominance shift induced by monocular deprivation results in a loss of contralateral bias. This is also characterized by a rapid depression of the response to stimulation of the deprived eye, followed by a slower potentiation of the response to stimulation of the non-deprived eye (Frenkel and Bear, 2004). The level of ocular dominance plasticity decreases with age. For example, in rodents, brief monocular deprivation (< 3 days) induces an ocular dominance shift in juveniles. However, the same duration of the monocular deprivation does not induce an ocular dominance shift in adults (Gordon and Stryker, 1996; Sawtell et al., 2003). On the other hand, increasing the duration for monocular deprivation to  $\geq 5$  days effectively shifts the ocular dominance in adult mice. An important difference in the characteristics of ocular dominance plasticity between juveniles and adults needs to be pointed out here. As mentioned above, in juveniles,

the ocular dominance shift induced by monocular deprivation (especially with brief durations) in the visual cortex contralateral to the deprived eye mainly results from a depression in the response to stimulation of the contralateral deprived eye (Sawtell et al., 2003; Frenkel and Bear, 2004; Hofer et al., 2006). However, in adult mice, the ocular dominance shift induced by prolonged monocular deprivation in the visual cortex contralateral to the deprived eye is preferentially due to a potentiation of the response to stimulation of the ipsilateral non-deprived eye (Sawtell et al., 2003; Hofer et al., 2006). This subtle difference might reflect the different mechanisms for synaptic plasticity employed by juvenile and adult cortex due to different levels of available synaptic plasticity.

### **3.1.2 Molecular mechanisms underlying OD plasticity**

The effect of monocular deprivation in juvenile kittens is blocked by antagonists of the NMDA subtype of glutamate receptor (Bear et al., 1990). In addition, monocular deprivation occludes long-term depression of layer IV synapses induced by low frequency stimulation of LGN in juvenile rats (Heynen et al., 2003). In adults, the potentiation of the response to stimulation of the non-deprived eye induced by monocular deprivation shares many features with the long-term potentiation of synaptic strength induced by high frequency stimulation (HFS-LTP), including dependence on NMDAR activation (Heynen and Bear, 2001; Sawtell et al., 2003). These data suggest that both LTD and LTP are involved in ocular dominance plasticity.

A primary determinant of the level of synaptic plasticity available within the visual cortex is hypothesized to be the balance between cortical inhibition and excitation (Hensch, 2004; Rozas et al., 2001). Over the course of postnatal development, there is an increase in the strength of GABAergic inhibition relative to glutamatergic excitation, and a concomitant decrease in the level of ocular dominance plasticity (Desai et al., 2002; Huang et al., 1999; Rozas et al., 2001). The level of inhibition relative to excitation is highly correlated with the level of ocular dominance plasticity, and both are regulated by experience. Dark rearing from birth delays the developmental increase in inhibition over excitation as well as the closure of the critical period for ocular dominance plasticity (Morales et al., 2002; Mower, 1991), maintaining the visual cortex in a highly plastic immature state. Over expressing the neurotrophin factor BDNF causes a precocious increase in the inhibition over excitation level in the visual cortex, together with an early onset and an early closure of the critical period (Huang et al., 1999).

A second molecular determinant for the level of synaptic plasticity is the subunit composition of NMDA receptor. There is developmental regulation of NMDA receptor composition and function (Carmignoto and Vicini, 1992; Sheng et al., 1994; Quinlan et al., 1999). Each NMDA receptor is composed of two obligatory NR1 subunits and two NR2 subunits. There are four subtypes of NR2 subunit (a-d), each conveying different features of the NMDA receptor channel. In neonatal cortex, the majority of NMDA receptors are dominated by NR2b subunit, which conducts a long duration of current with a slower decay time (Carmignoto and Vicini 1992; Sheng et al., 1994). Over the course of the development, NR2b-containing NMDA receptors



are switched to NR2a-containing NMDA receptors, which mediate a shorter and faster current (Carmignoto and Vicini 1992; Sheng et al., 1994). Because the activation of NMDA receptors depends on both binding of the glutamate released from pre-synaptic neuron and depolarization of the post-synaptic neuron (Seeburg et al., 1995), it has been proposed to be a molecular coincidence-detector that is needed for Hebbian learning. The subunit switch from NR2b to NR2a during development results in a decreased current duration mediated by NMDA receptors and allows a smaller time window for coincidence detection, hence changing the properties of NMDAR-dependent synaptic plasticity (Dumas, 2005).

Other molecular mechanisms thought to be involved in the developmental decline in ocular dominance plasticity include the maturation of the extracellular matrix, also known as perineuronal nets, that forms a protein scaffold around the neurons and glia (Celio and Blumcke, 1994). The extracellular matrix is composed of proteins such as chondroitin sulfate proteoglycans. The extracellular matrix forms a physical barrier surrounding the neurons and limits the experience-dependent remodeling of neural circuits by increasing the stability of already existing synapses and blocking synaptogenesis (Grumet et al., 1993). Degrading the extracellular matrix by application of a chondroitinase increases ocular dominance plasticity in adult rats (Pizzorusso et al., 2002), suggesting that removal of the inhibitory effects of the extracellular matrix might be a requirement for inducing synaptic modifications in adult cortex. There are endogenous enzymes that could also digest extracellular matrix such as tissue plasminogen activator (tPA), which is a serine protease that cleaves plasminogen into the active form plasmin. Plasmin then cleaves the proteins

that compose the extracellular matrix (Muller and Griesinger, 1998). Extracellular substrates for tPA-plasmin system include neural cell-adhesion molecule (N-CAM) and laminin, both of which are primary constituents of the extracellular matrix (Endo et al., 1999; Chen and Strickland, 1997). The tPA activity level in the visual cortex decreases during development (Mataga et al., 2001). Application of tPA to the visual cortex increases the spine motility and mimics the effect of monocular deprivation in juvenile animals (Oray et al., 2004).

### **3.1.3 Dark rearing delays the developmental decrease of ocular dominance plasticity in juveniles**

Depriving an animal of vision from birth maintains the visual system in an immature state, characterized by low stimulus selectivity and large receptive fields (Fagiolini et al., 1994). Dark-rearing also attenuates the developmental decline in ocular dominance plasticity (Cynader, 1983; Mower et al., 1991), extending the time period during which the visual cortex will respond to brief monocular deprivation. Logically, the molecular changes hypothesized to be involved in the developmental decline in ocular dominance plasticity would also be inhibited by dark rearing from birth. The developmental increase in inhibition relative to excitation (Morales et al., 2002; Desai et al., 2002), the maturation of NMDA receptor subunit composition (Carminigno and Vicini, 1992; Quinlan et al., 1999), and the maturation of the extracellular matrix are all delayed by dark rearing from birth (Pizzorusso et al., 2002), suggesting that dark rearing may maintain the high level of synaptic plasticity in the visual cortex through these mechanisms.

On the other hand, the effects of visual deprivation in normally reared adults are less well understood. Previous studies of the effect of visual deprivation after normal patterned visual experience in juveniles and young adults are controversial. Giving dark reared kittens a brief period (6hrs) of light exposure has been reported to decrease the sensitivity to monocular deprivation comparing to those without light exposure. And the effect seemed to be irreversible even if subjects were returned to darkness after the light exposure (Mower et al., 1983). However, allowing kittens to have two months of normal vision after birth and then putting them into darkness for a longer period (months) of visual deprivation seemed to be able to restore at least part of the ocular dominance plasticity as demonstrated by higher sensitivity to monocular deprivation comparing to age-matched controls that had been reared in normal lighted environment for the whole time (Cynader, 1983; Mower, 1991). In addition, as I have discussed in Chapter 2, strong peripheral deafferentation can induce reorganization of the cortical circuits in adult somatosensory cortex, which shows that adult cortex still retains a certain level of synaptic plasticity that could be revealed by deprivation. It remains to be seen if visual deprivation in adulthood can enhance the level of ocular dominance plasticity, and if an enhancement is due to a reversal of the developmental maturation of the visual cortex.

## 3.2 Material and Methods

### Animal care

Adult (P70-100) male and female pigmented Long-Evans rats were raised with 12 hours light and 12 hours dark per day, with food and water available *ad libitum*. Subjects were moved into a light-tight dark room at indicated ages for a period of visual deprivation. All procedures conform to the guidelines of the U.S. Department of Health and Human Services and the University of Maryland Institutional Animal Care and Use Committee.

### Monocular deprivation

Subjects were anesthetized with ketamine/xylazine (50mg/10mg/kg, i.p.). The proper depth of anesthesia was confirmed with slow deep breathing, loss of corneal reflexes and loss of response to pain (paw pinch). The margins of the upper and lower lids of one eye were trimmed. All procedures were performed under aseptic conditions, including sterile surgical site and surgical instruments (autoclaved before and after each surgery day, sterile bead bath in between animals on the same day). A drop of triple antibiotic ointment (Phoenix, St Joseph, MO) was placed on the eyeball to prevent dryness and infection and the lids were sewn together with about five stitches with chromic gut suture (5-0, Ethicon, Somerville, NJ). This surgical procedure would result in the fusing of the two eyelids over the course of several days. It remains the most reliable and least behaviorally disruptive method of monocular deprivation. Subjects were kept in their home cages (with 12 hours light: 12 hours dark/day) for the period of monocular occlusion as indicated. The wound was

inspected daily. Subjects with signs of suture opening or infection were excluded from the experiments.

### VEP recordings

Animals were anesthetized with 20% urethane (1.6g/kg i.p). Body temperature and dominant EEG frequency (in response to blank screen) were used to monitor anesthetic depth. The target frequency of bursting activity of local field potentials recorded in the visual cortex was 0.5-2Hz (Fox and Armstrong-James, 1986). A bilateral craniotomy was performed over binocular visual cortex (centered at 7.0mm posterior to Bregma and 4mm lateral to midline, diameter ~3mm), keeping dura intact. VEPs were recorded from the binocular visual cortex with blunt tip tungsten microelectrodes (0.5M $\Omega$ ; MPI, Gaithersburg, MD). A reference electrode was placed in the dorsal neck muscle. A ground screw was mounted on the frontal bone of the skull. Assessment of the VEP in response to stimulation of ipsilateral eye confirmed electrode placement in binocular visual cortex.

The animal was placed 25 cm from the monitor. The visual stimulus was a 1 Hz full screen vertical square wave grating [subtending 74.8 (horizontal) by 49.5 (vertical) degrees of the visual angle, 0.04cycles/deg], with 96.28% maximal contrast and a mean luminance of 94 cd/m<sup>2</sup>. VEPs were amplified (1000X), filtered (0.5Hz and 300Hz), and averaged (100 repetitions) in synchrony with the stimulus using an IBM compatible computer with ENFANT software (Enfant 4010, NeuroScientific Corp., Farmingdale, NY; courtesy of Dr. William Hodos). The data were then digitally filtered with a 60Hz low-pass filter prior to analysis. The amplitude of the primary positive component was used to assess the cortical response to visual

stimulation. The latency of the primary positive peak vary from 110–180 msec. VEPs in response to a stimulus of 0% contrast was recorded every 10–20 minutes during the experiment to serve as an estimate of stimulus-independent cortical activity (noise).

### Quantitative Immunoblots

On the day of sacrifice subjects were anesthetized with sodium pentobarbitol (75mg/kg i.p.). Binocular visual cortex was dissected bilaterally in ice cold dissection buffer (2.6mM KCl, 1.23mM NaH<sub>2</sub>PO<sub>4</sub>, 26mM NaCO<sub>3</sub>, 212.7mM sucrose, 10mM dextrose, 0.5mM CaCl<sub>2</sub>, 1mM MgCl<sub>2</sub>, 100μM Kynurenic acid, pH 7.2, oxygenated on ice for 15 min before use). Pieces of left and right frontal cortex were also taken to serve as controls. Each piece of cortical tissue was then processed for synaptoneurosomes immediately after dissection. Synaptoneurosome preparation and quantitative immunoblotting were performed as described (see Chapter 2, Methods). Primary antibodies used included anti-GluR2 (clone 6C4, Zymed), anti-GABA<sub>A</sub>β2/3 (clone 62-3G1, Upstate Biotechnology), anti-actin (clone JLA20, Oncogene), anti-NR2A (rabbit polyclonal, Upstate Biotechnology) and anti-NR2b (rabbit polyclonal, Upstate Biotechnology). Data were expressed as a ratio of optical density from sequential probes of the same immunoblot, circumventing the need to normalize to a gel-loading control. The relationship between optical density and protein concentration is linear along the range used in this study (Heynen et al., 2000). All immunoblots were performed with the experimenter blind to experimental conditions.

### tPA activity assay

The protocol for tPA activity assay was modified from Xanthopoulos et al., 2005. Binocular visual cortices and frontal cortices were dissected in dissecting buffer (10mM HEPES, 0.32M sucrose, pH 7.2), and snap-frozen in a dry ice/methanol slurry. Cortex samples were stored at -80°C. On the day of assay, cortical samples were homogenized in 1ml of 0.1M PBS buffer with Na deoxycholate (0.5% w/v) and Igepal CA-630 (0.5% v/v) with a polytron homogenizer (Wheaton, PA). Protein concentration was assayed with Quick Start protein assay kit (BioRad, Hercules, CA). All samples were diluted to the same total protein level (~1mg/ml) with homogenization buffer. The tPA activity assay was performed in a 96-well microplate. To each well, 30µl of distilled H<sub>2</sub>O; 130µl of 0.1M Tris-HCl (pH 8.0, with 0.1% v/v Tween 80); 10µl of 8.4 uM plasminogen; 20µl of 3mM substrate D-Ile-Pro-Arg-p-nitroanilide dihydrochloride; 10µl of 5mg/ml fibrin I; and 10µl of brain homogenate (or tPA standard sample) were added, and the solutions were incubated in a 37 °C incubator for 3 hours. In the presence of exogenous fibrin, which acts as a catalyst, endogenous tPA in the homogenate prepared from visual cortex will convert plasminogen to plasmin, which in turn cleaves the chromogenic substrate into a yellow colored reaction product. Absorbance readings at 405nm were taken every 10 to 20 minutes. A standard curve for each incubation period was made by plotting the tPA activity for the standards on the x-axis against their absorbance at 405nm. tPA activity of the cortical sample was determined from the standard curve with the best linear regression (usually 120-160min after the reaction started). tPA activity was normalized to the average of LR control samples run on the same plate to allow comparison of data from different activity assay runs.

### 3.3 RESULTS

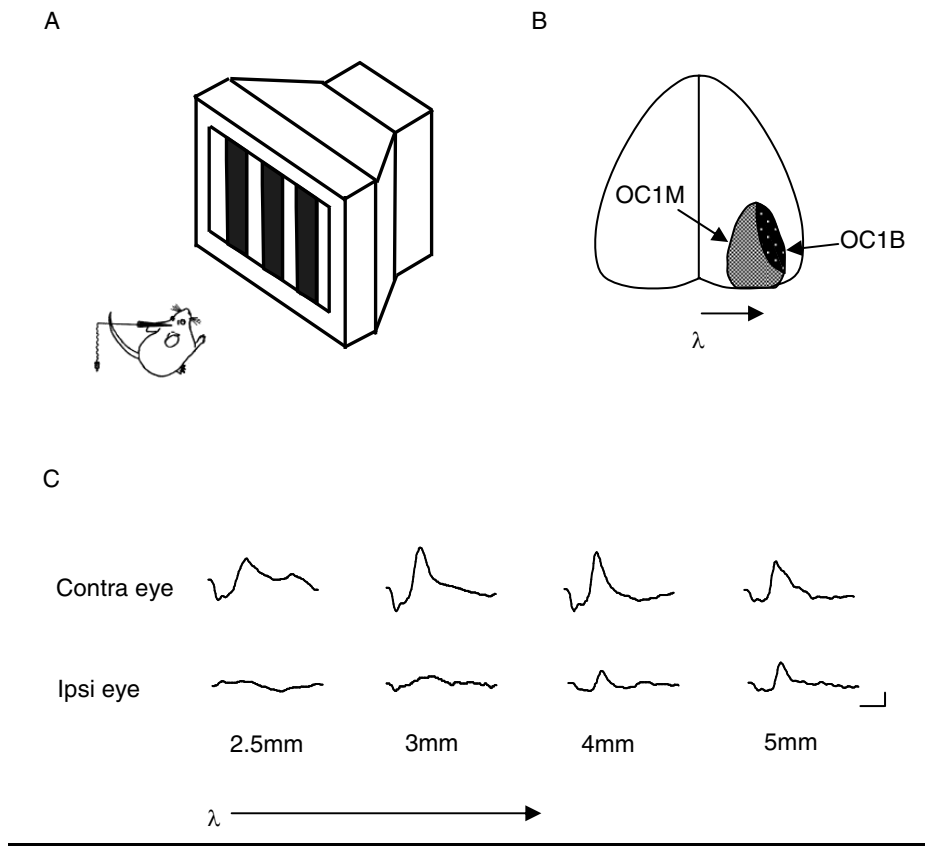
#### 3.3.1 The contralateral bias in rats visual cortex assessed by VEPs

Visually evoked potentials (VEPs) were used to measure ocular dominance in the binocular visual cortex of adult Long-Evans rats. The visual stimulus was composed of vertical square wave gratings, presented on a CRT monitor placed 25cm in front of the subject (Figure 7a). In rodents, approximately 90% of retinal ganglion cell axons decussate at the optic chiasm, leaving only 10% of ganglion cell axons to project onto the ipsilateral visual cortex. The primary visual cortex is naturally divided into two parts in rodents: the monocular visual cortex, which receives inputs from the contralateral eye only and occupies the medial two thirds of the primary visual cortex; and the binocular visual cortex, which receives inputs from both the contralateral and ipsilateral eyes and occupies the lateral one third part of the primary visual cortex (Figure 7b). Even within the binocular visual cortex, there is an innate contralateral bias, evident by a higher responsiveness of neurons to stimulation of the contralateral than to stimulation of the ipsilateral eye. The recorded VEP amplitudes are sensitive to the ocular dominance. With the electrode placed above the binocular visual cortex, the VEPs in response to stimulation of contralateral eye are approximately twice as the VEPs in response to stimulation of the ipsilateral eye, reflecting the innate contralateral bias. Moving the electrode closer to the midline ( $\lambda$ ), VEPs in response to contralateral eye stimulation become larger while VEPs in response to the ipsilateral eye stimulation diminish until indistinguishable from noise (Figure 7c). Within binocular visual cortex, VEPs yields reliable evaluation of the contralateral bias that is consistent with results of other methods used such as single unit recordings and



optical imaging (Porciatti et al., 1999; Sawtell et al., 2003; Pham et al., 2004; Cang et al., 2005; Fagiolini et al., 1994). To be consistent, all of the VEP recordings in the rest of the experiments were recorded within  $5\pm 0.5$ mm lateral to  $\lambda$ .

**Figure 7. VEPs recorded at different sites demonstrate primary features of the organization of the primary visual cortex of the adult rat.** A. Sketch of the VEP recording set up. An anesthetized subject is placed 25cm in front of a CRT monitor presenting the visual stimulus (1Hz full screen vertical square wave gratings). A tungsten electrode is placed on the dura surface overlaying the primary visual cortex to record the cortical response to the visual stimulus. B. Sketch of the dorsal surface of the rat cortex. The shaded area is primary visual cortex. The medial two thirds is monocular visual cortex (OC1m) receiving inputs from the contralateral eye only. The lateral one third is binocular visual cortex (OC1b), which receives inputs from both eyes. C. Representative VEP waveforms recorded at 2.5mm, 3mm, 4mm and 5mm from  $\lambda$  in response to stimulation of contralateral eye and ipsilateral eye. The binocular region of V1 centers around 5mm from  $\lambda$ . Scale bar: 100 $\mu$ V, 100msec.

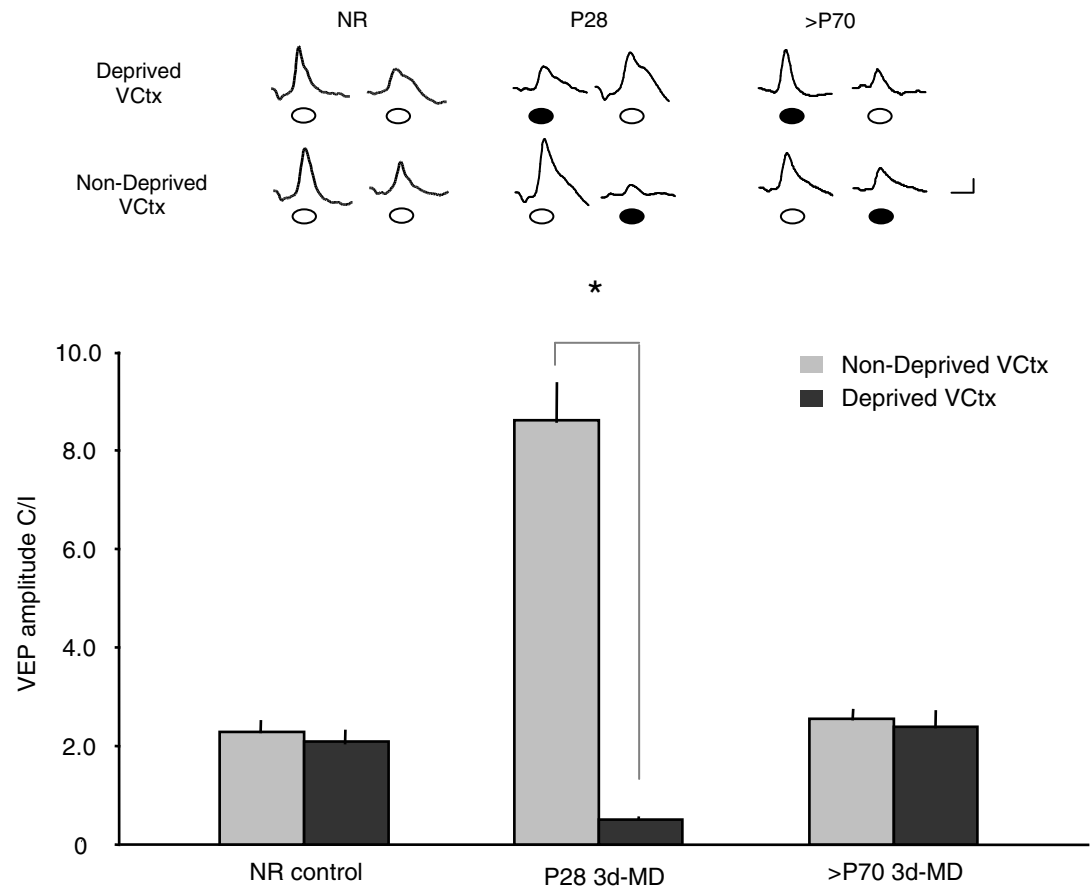


**Figure 7**

### 3.3.2 Sensitivity to brief monocular deprivation decreases with age

The response of binocular neurons to brief (3 days) monocular deprivation is a sensitive method to detect the level of available synaptic plasticity in primary visual cortex. In juvenile rats, the visual cortex is highly plastic, and 3 days of monocular deprivation induces a significant decrease in the natural contralateral bias of the binocular visual cortex. The contralateral bias is the ratio of the amplitude of VEPs in response to stimulation of the contralateral eye over the amplitude of VEPs in response to stimulation of the ipsilateral eye (VEP C/I). The contralateral bias of normally reared rats is approximately 2, in both the left and right binocular visual cortex (VEP amplitude C/I: Left =  $2.28 \pm 0.27$ ; Right =  $2.09 \pm 0.29$ ;  $n=4$ , figure 8). Three days of monocular deprivation within the classical critical period (P25-28) induces an ocular dominance shift shown as a significant difference in the VEP amplitude C/I between the visual cortex contralateral to the deprived eye (the deprived VCtx) and the visual cortex ipsilateral to the deprived eye (the non-deprived VCtx) (VEP amplitude C/I: Deprived =  $0.51 \pm 0.06$ ; Non-deprived =  $8.62 \pm 0.81$ ;  $n=3$ ,  $p < 0.01$ , paired student's t-test). In contrast, if 3 days monocular deprivation starts at a later age ( $>P70$ ), it does not affect the contralateral bias of VEPs in either cortical hemisphere (VEP C/I: deprived VCtx =  $2.42 \pm 0.33$ ; non-deprived VCtx =  $2.48 \pm 0.24$ ;  $n=6$ ,  $p > 0.05$ , paired t-test), confirming the absence of rapid ocular dominance plasticity in adult rats.

**Figure 8. Sensitivity to brief MD is constrained in the adult VCtx.** VEP amplitude C/I in binocular region of primary visual cortex is approximately 2 in normal adult rats. At P28, 3 days of MD induces an ocular dominance shift resulting in a significant difference in the contralateral bias between the non-deprived (the visual cortex ipsilateral to the deprived eye) and deprived visual cortex (the visual cortex contralateral to the deprived eye). When 3 days of MD is applied after P70, no ocular dominance shift is induced, and the VEP amplitude C/I of both deprived and non-deprived visual cortex remain similar to the normal reared age-matched controls. Insets: representative VEP waveforms recorded from the deprived visual cortex and non-deprived visual cortex in response to stimulation of the deprived (filled oval) and non-deprived eye (open oval). Scale bar: 100 $\mu$ V, 100msec.



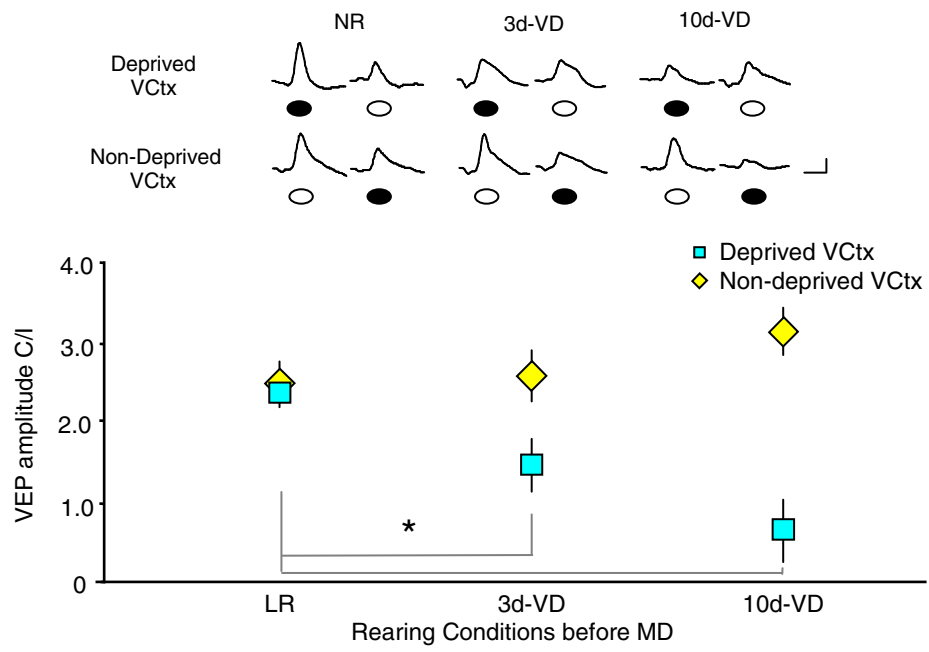
**Figure 8**

### 3.3.3 Visual deprivation enhances sensitivity to brief monocular deprivation in adults

Deprivation of primary input enhances plasticity in adult somatosensory cortex. To explore the effect of visual deprivation on plasticity in the visual cortex, pigmented Long Evans rats were raised in a normally lighted environment (12 hours light and 12 hours dark/day) until adulthood (>P60), then placed into a light-tight dark room for a period of visual deprivation. Visual deprivation for as short as 3 days resulted in enhanced sensitivity to brief (3-day) monocular deprivation in adult visual cortex, demonstrated by a significant reduction in the contralateral bias in the visual cortical dominated by input from the deprived eye (deprived visual cortex) which is contralateral to the occluded eye (VEP C/I: deprived VCtx=1.44±0.27 vs. non-deprived VCtx=2.55±0.32; n=5, Figure 9). The response was significantly different from the response of the normal-reared controls to brief MD. A longer duration of visual deprivation (10 days) also resulted in an enhancement in the response to brief MD (VEP C/I: deprived VCtx=0.82±0.04 vs. non-deprived VCtx=3.13±0.17, n=5; one-way ANOVA,  $F_{(2, 13)}=9.53$ ,  $p<0.01$  versus normal controls with Fisher's PLSD post hoc). This demonstrates that visual deprivation in adulthood can reactivate rapid ocular dominance plasticity, allowing a rapid reorganization of response properties in response to changes in visual input.

**Figure 9. Visual deprivation reactivates rapid ocular dominance plasticity in the adult visual cortex.** The normal contralateral bias of binocular region of the primary visual cortex of the normal-reared adult rat (NR) is unchanged by brief (3 days) of monocular deprivation. A brief period of visual deprivation (3 days VD) enhances the sensitivity of the adult rat visual cortex to brief MD, resulting in a significant shift in ocular dominance. A longer duration of visual deprivation (10 days VD) also induces an enhanced sensitivity to brief MD in the adult rat visual cortex, shown as significant difference in contralateral bias between the deprived and non-deprived visual cortex (One-way ANOVA ( $F_{(2, 13)} = 9.530$ ,  $p < 0.01$ , \*:  $p < 0.05$ , with Fisher's PLSD post hoc test). Inset: representative VEP waveforms recorded from the visual cortex contralateral to the deprived eye (deprived visual cortex) and the visual cortex ipsilateral to the deprived eye (non-deprived visual cortex) in response to stimulation of the deprived (filled oval) and non-deprived (open oval) eye. Scale bar: 100 $\mu$ V, 100msec.



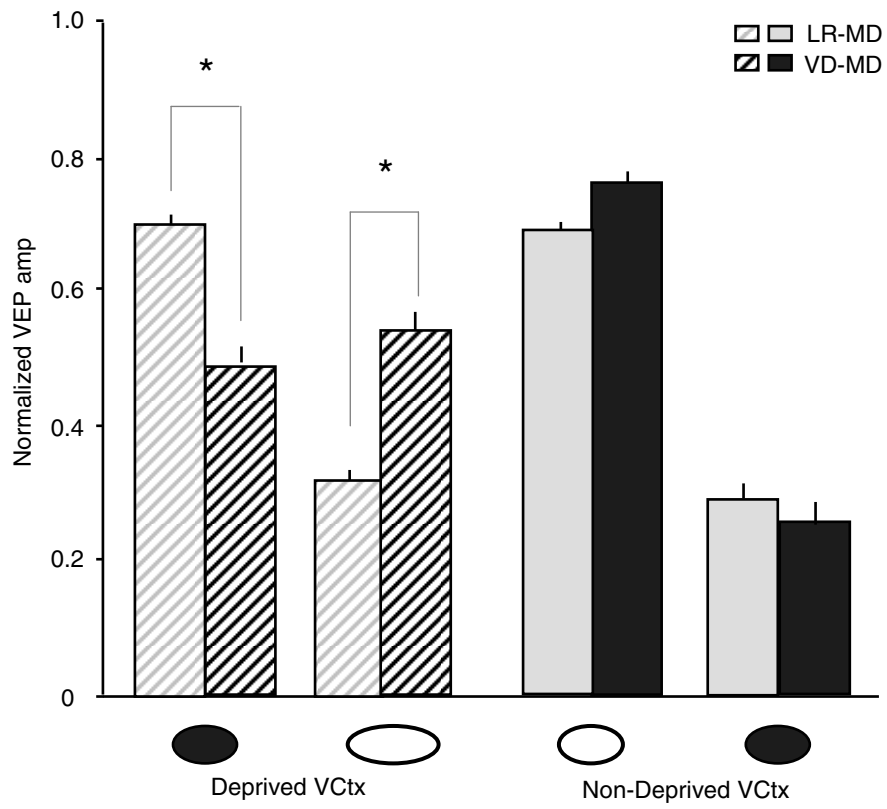


**Figure 9**

### **3.3.4 The enhanced ocular dominance plasticity in adult visual cortex induced by visual deprivation is juvenile-like**

The rapid ocular dominance shift that we observe in the deprived visual cortex of visually-deprived adults may be due to a rapid depression of the response to stimulation of the deprived eye, typically seen only in juveniles, or a potentiation of the response to stimulation of the non-deprived eye, which emerges slowly in juveniles and adults. To distinguish between these two possibilities, we compared VEP amplitudes in response to stimulation of each eye following brief monocular deprivation. To facilitate comparison across animals, VEP amplitudes ( $\mu\text{V}$ ) at each recording site were normalized to the sum of the response to stimulation of the contralateral and ipsilateral eye (C+I), which did not differ across hemispheres ( $p>0.05$ , paired t-test). VEPs recorded in the visual cortex contralateral to the occlusion revealed two responses to brief monocular deprivation in visually-deprived adults: a decrease in the response to stimulation of the contralateral (occluded) eye (average  $\pm\text{SEM}$  (norm to C+I): LR=0.67 $\pm$ 0.02, n=5; VD=0.46 $\pm$ 0.02, n=5;  $p<0.01$ , unpaired t-test, Figure 10) and an increase in the response to stimulation of the ipsilateral (open) eye (LR=0.33 $\pm$ 0.02; VD=0.54 $\pm$ 0.02;  $p<0.01$ , unpaired t-test). We observed no effect in the hemisphere ipsilateral to the occlusion in response to stimulation of the contralateral (open) eye (LR=0.71 $\pm$ 0.01; VD=0.74 $\pm$ 0.01;  $p>0.05$ , unpaired t-test) or ipsilateral (occluded) eye (LR=0.29 $\pm$ 0.01; VD=0.26 $\pm$ 0.01;  $p>0.05$ , unpaired t-test, Figure 10).

**Figure 10. Visual deprivation reactivates juvenile-like ocular dominance plasticity in the adult visual cortex.** In visually-deprived adults, brief MD induces a significant depression in the response to stimulation of the deprived eye (filled oval) and a significant potentiation in the response to stimulation of open eye (open oval) in the visual cortex contralateral to the occlusion (\*\* $p < 0.01$  vs. light-reared controls, unpaired t-test). No difference in the response to stimulation of either eye was observed in the visual cortex ipsilateral to the occlusion ( $p > 0.05$ , unpaired t-test).



**Figure 10**

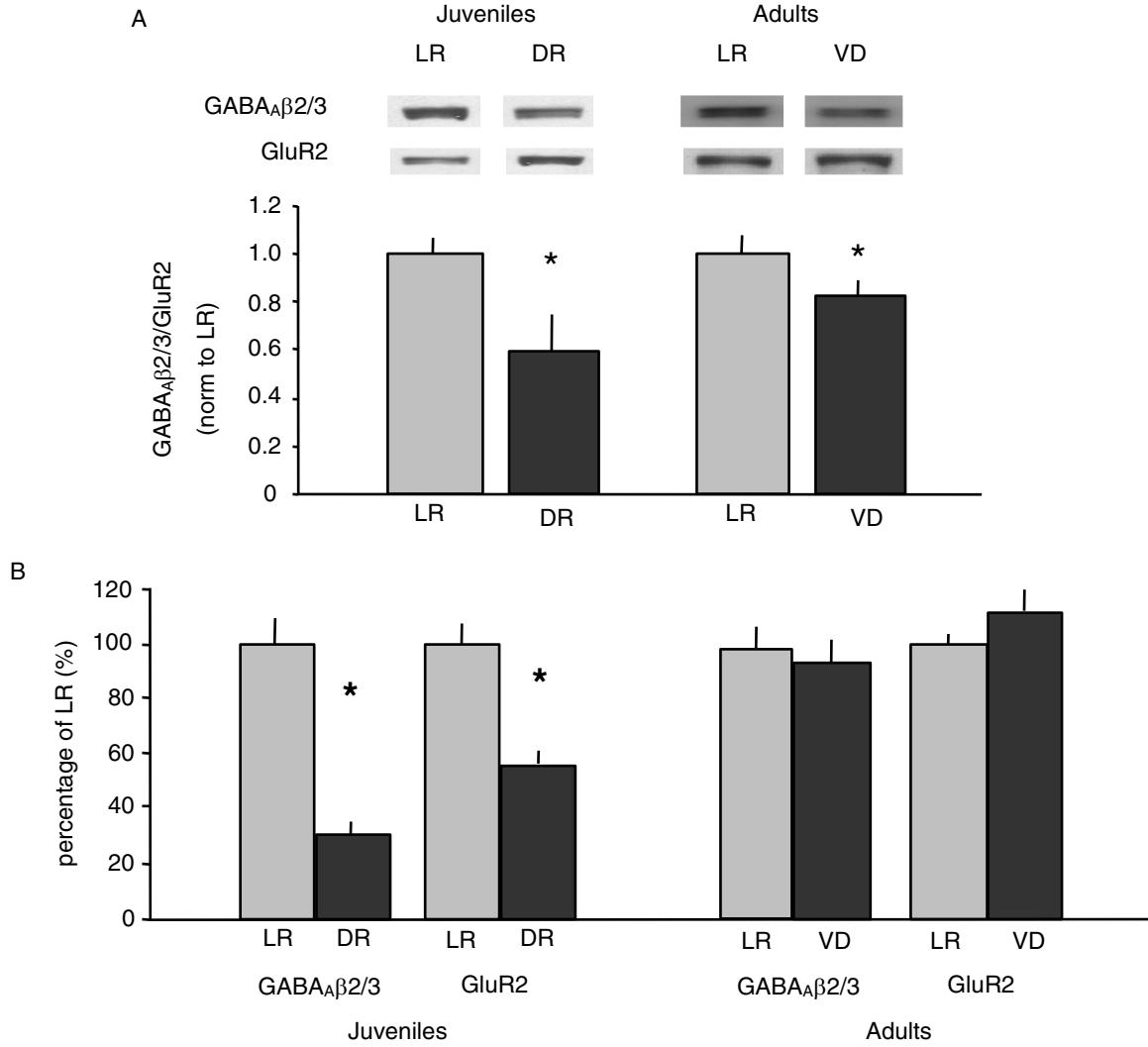
### **3.3.5 Visual deprivation reverses the developmental increase in inhibition over excitation in adult visual cortex**

To explore the mechanisms by which visual deprivation enhances ocular dominance plasticity, we examined known molecular correlates of cortical maturation in visually-deprived juveniles and adults. One of the well-established correlates of cortical maturation is an experience-dependent increase in the level of inhibition relative to excitation. To track changes in the level of cortical inhibition, we examined the levels of postsynaptic GABA<sub>A</sub>Rs, as the number of postsynaptic receptors is a primary determinant of synaptic strength at inhibitory synapses. An antibody that recognizes the  $\beta 2$  and the  $\beta 3$  subunits of the GABA<sub>A</sub>R was used to track changes in inhibition, because the majority of fast cortical inhibition is mediated by GABA<sub>A</sub>Rs containing these subunits (Li and De Blas, 1997). Similarly, we used an antibody that recognizes the GluR2 subunit of the AMPAR to track changes in excitation, because the majority of fast cortical excitation is mediated by AMPARs containing this subunit (Wenthold et al., 1992).

Dark rearing from birth is known to maintain a lower level of inhibition relative to excitation in juvenile visual cortex (Morales et al., 2002). Therefore we decided to use dark rearing from birth as a control to test the validity of our biochemical method to detect the level of inhibition relative to excitation in the cortex. Quantitative immunoblotting revealed a significant decrease in GABA<sub>A</sub> $\beta 2/3$  relative to GluR2 in visual cortex in juveniles (P28) dark-reared from birth, compared to age-matched light-reared controls (LR=1.15±0.08, n = 7; DR=0.67±0.21, n=7; p<0.05, unpaired t-test; Figure 11A), demonstrating that the level of AMPA receptors and GABA<sub>A</sub>

receptors detected in the homogenate of cortical tissue is a reliable index for changes in the level of inhibition and excitation documented physiologically. The biochemical method is sensitive enough to catch the experience-dependent change in cortical level of inhibition relative to excitation. To examine the effect of visual deprivation on the adult visual cortex, quantitative immunoblotting was performed on the visual cortex of light-reared adults (P70), or age-matched littermates visually-deprived for 10 days beginning at P60 (VD). We see a significant decrease in the level of GABA<sub>A</sub>β2/3 relative to GluR2 in visually-deprived versus light-reared adults (LR=1.36±0.13, n=8; VD=1.07±0.08, n=8; p<0.05, unpaired t-test, Figure 11), demonstrating that 10 days of visual deprivation induces a significant decrease in the level of inhibition relative to excitation. No difference in GABA<sub>A</sub>β2/3 relative to GluR2 was observed in the frontal cortex of these subjects (average±SEM: LR=1.41±0.12, n=8; VD=1.48±0.14, n=8; p>0.05, unpaired t-test), demonstrating that the effect of visual deprivation on inhibition over excitation level is specific to the visual cortex.

**Figure 11. Visual deprivation regulates the ratio of inhibition and excitation in binocular visual cortex in both juveniles and adults.** A. Summary data demonstrate that visual deprivation induces a significant decrease in GABA<sub>A</sub>β2/3 relative to GluR2 in juveniles and adults (\*p<0.05, unpaired t-test). Inset: representative immunoblots for GABA<sub>A</sub>β2/3 and GluR2 from juvenile and adult visual cortical homogenate. B. Dark rearing in juveniles results in significant decrease in the level of the both GABA<sub>A</sub>β2/3 and GluR2 receptors (\*: p<0.05, unpaired t-test). The effect of visual deprivation in adults is much less robust, resulting in a trend towards a decrease in GABA<sub>A</sub>β2/3 and a trend towards an increase in GluR2. Data normalized to LR controls for display.



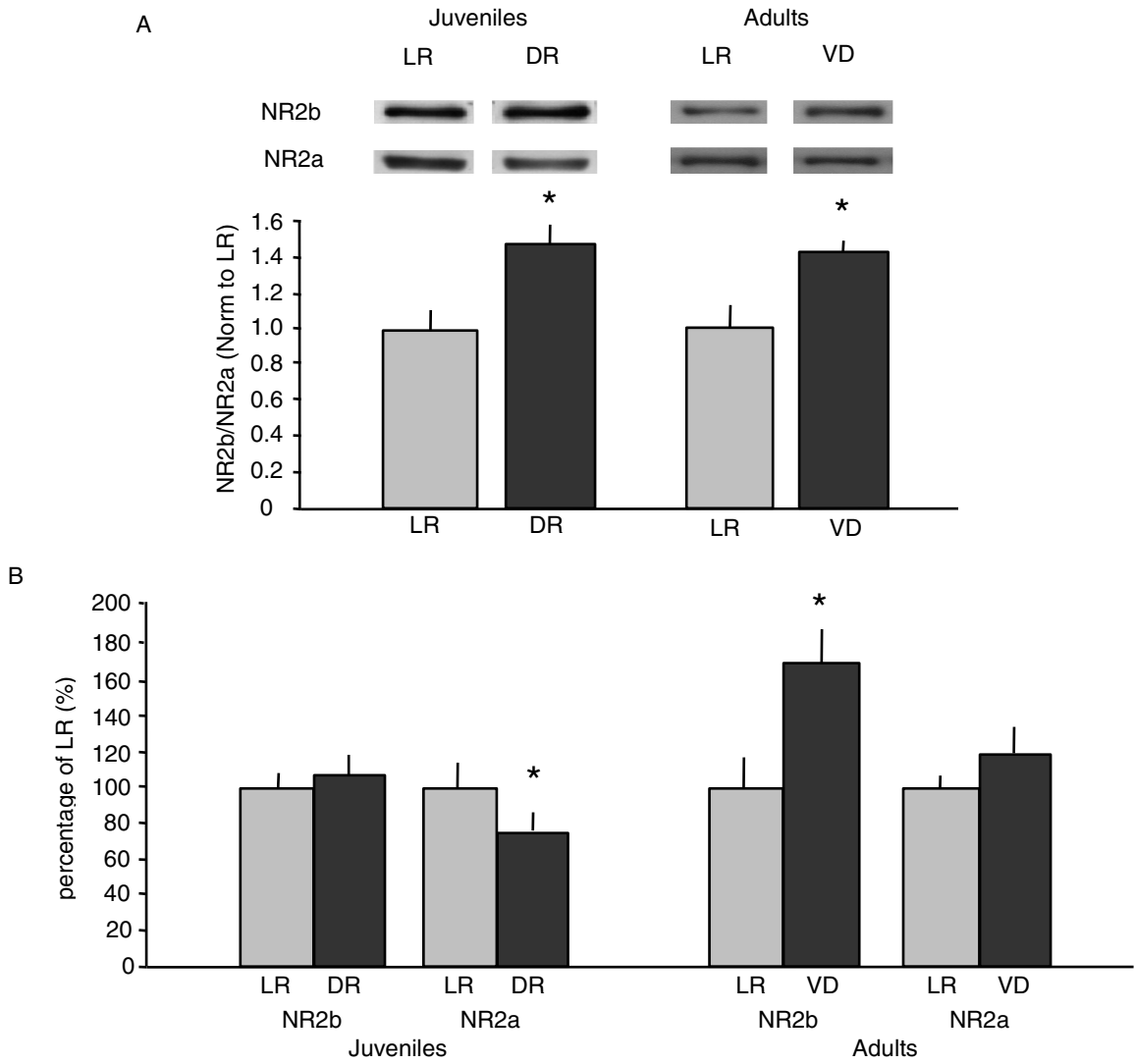
**Figure 11**



### **3.3.6 Visual deprivation induces an increase in the ratio of NR2b to NR2a in synaptoneurosomes prepared from visual cortices in adults**

Another well-documented consequence of experience-dependent cortical maturation is the decrease in the level of NR2b- relative to NR2a-containing NMDA receptors in the visual cortex. Quantitative immunoblotting for NR2b and NR2a was performed on synaptoneurosomes prepared from the visual cortices of dark-reared juveniles (P28) or visually deprived adults (P70) and light-reared age-matched controls. We see a significant increase in NR2b relative to NR2a in dark-reared vs. light-reared juveniles (LR=0.85±0.09, n=12; DR=1.20±0.13, n=13; p<0.02, unpaired student's t-test, Figure 12A). The increase in the ratio is due to a decrease in NR2a (average±SEM (norm to standard) LR=0.64±0.06; DR=0.49±0.06; p<0.05, unpaired student's t-test) rather than an increase in NR2b (LR=0.51±0.04; DR=0.56±0.06; p>0.05, unpaired student's t-test, Figure 12B). In addition we see a significant increase in NR2b relative to NR2a in visually deprived vs. light-reared adults (LR=0.82±0.11, n=6; VD=1.20±0.07, n=9; p<0.01, student's unpaired t-test, Figure 12A). Interestingly, the change in the ratio of NR2b relative to NR2a in visually deprived adults is due to an increase in NR2b (average±SEM (norm to standard): LR=0.62±0.10; VD=1.04±0.12; p<0.02, unpaired student's t-test) rather than a decrease in NR2a (LR=0.75±0.06; VD=0.89±0.12; p>0.05, unpaired student's t-test, Figure 12B). We see no significant difference in NR2b relative to NR2a in the frontal cortex of these subjects (LR=0.86±0.12, n=6; VD=1.03±0.06, n=9; p>0.05, unpaired student's t-test), demonstrating the effect of visual deprivation on NMDA receptor composition is restricted to the visual cortex.

**Figure 12. Visual deprivation regulates the composition of NMDA receptors in binocular visual cortex in both juveniles and adults.** A. Summary data demonstrate that visual deprivation induces a significant increase in the ratio of NR2b to NR2a in both juveniles and adults (\*:  $p < 0.05$ , unpaired t-test). Inset: representative immunoblots for NR2a and NR2b from juvenile and adult visual cortical synaptoneurosome preparations. B. Non-ratioed data show that the increased ratio of NR2b over NR2a observed in dark-reared juveniles is due to a significant decrease in the level of NR2a. In contrast, the increased ratio of NR2b over NR2a observed in visually-deprived adults is due to a significant increase in the level of NR2b (\*:  $p < 0.05$ , unpaired t-test). Data normalized to LR controls for display.



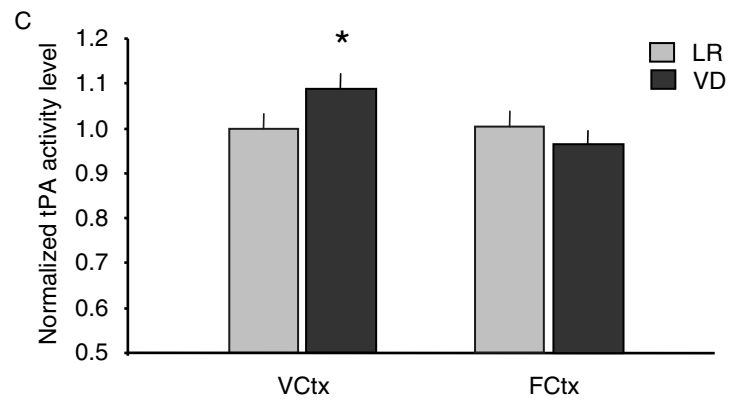
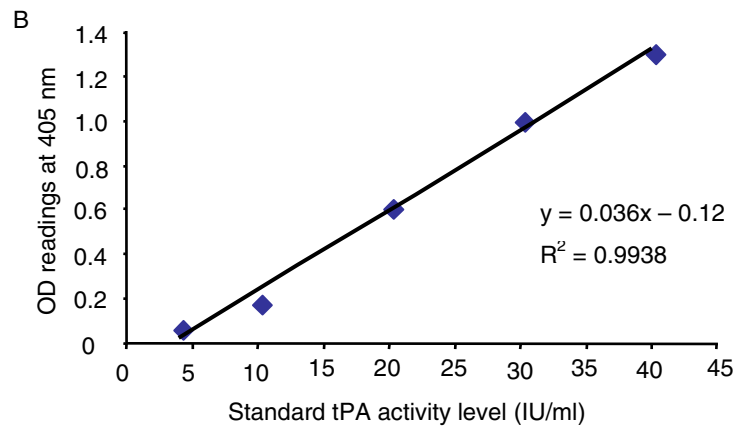
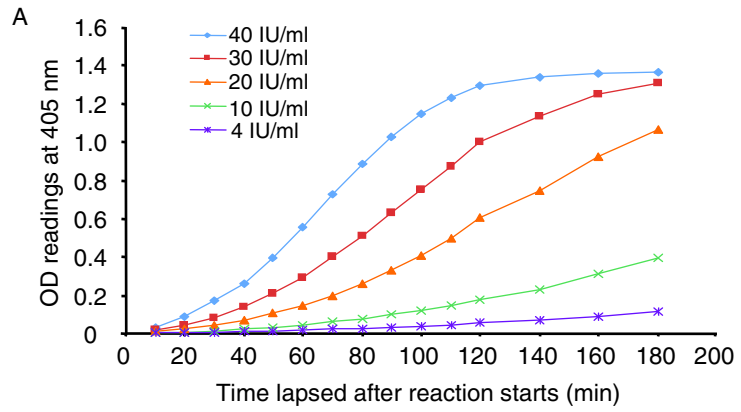
**Figure 12**

### **3.3.7 tPA activity is observed to increase in adult visual cortex following visual deprivation**

Tissue plasminogen activator (tPA) is a serine protease that cleaves plasminogen into the active form plasmin. The tPA-plasmin enzymatic system has been proposed to affect the synaptic plasticity by several mechanisms including degrading the extracellular matrix proteins (Mataga et al., 2001; Oray et al., 2004) and regulation of neurotrophins (Pang and Lu, 2003). The tPA chromogenic assay measures tPA activity in brain tissue homogenate. In the presence of exogenous fibrin, which acts as a catalyst, endogenous tPA in the homogenate prepared from visual cortex will convert plasminogen to plasmin, which in turn cleaves the chromogenic substrate resulting in a colored reaction product. The level of the final product is measured by the absorbance at 405nm, which is proportional to the amount of tPA activity in the sample within the linear range of the reaction (Figure 13A, B).

tPA activity in the cortical tissue measured by the chromogenic assay shows that the tPA activity level is significantly increased following 10 days of visual deprivation in the binocular visual cortex (normalized to the LR: VD =  $1.09 \pm 0.03$ , n=6; LR =  $1.00 \pm 0.04$ , n=6;  $p < 0.05$ , student's t-test, Figure 13C). The tPA activity is not affected by visual deprivation in frontal cortex dissected from the same set of subjects (normalized to LR: VD =  $0.92 \pm 0.06$ , n=6; LR =  $1.00 \pm 0.07$ , n=6;  $p > 0.05$ , student's t-test).

**Figure 13. Visual deprivation induces a significant increase in the level of tPA activity in adult visual cortex.** A. Concentration curves for the chromogenic reaction of tPA activity, using an exogenous purified single chain tPA standard. B. Linear regression for tPA concentration versus absorbance at 120 min time point, which was used for calculation of the tPA activity. C. Summary data show that visual deprivation induces a small but significant increase in the tPA activity level in the visual cortex (\*:  $p < 0.05$ , unpaired t-test). No changes in tPA activity level is observed in the frontal cortex following visual deprivation. Data normalized to average LR controls run on the same 96-well plate.



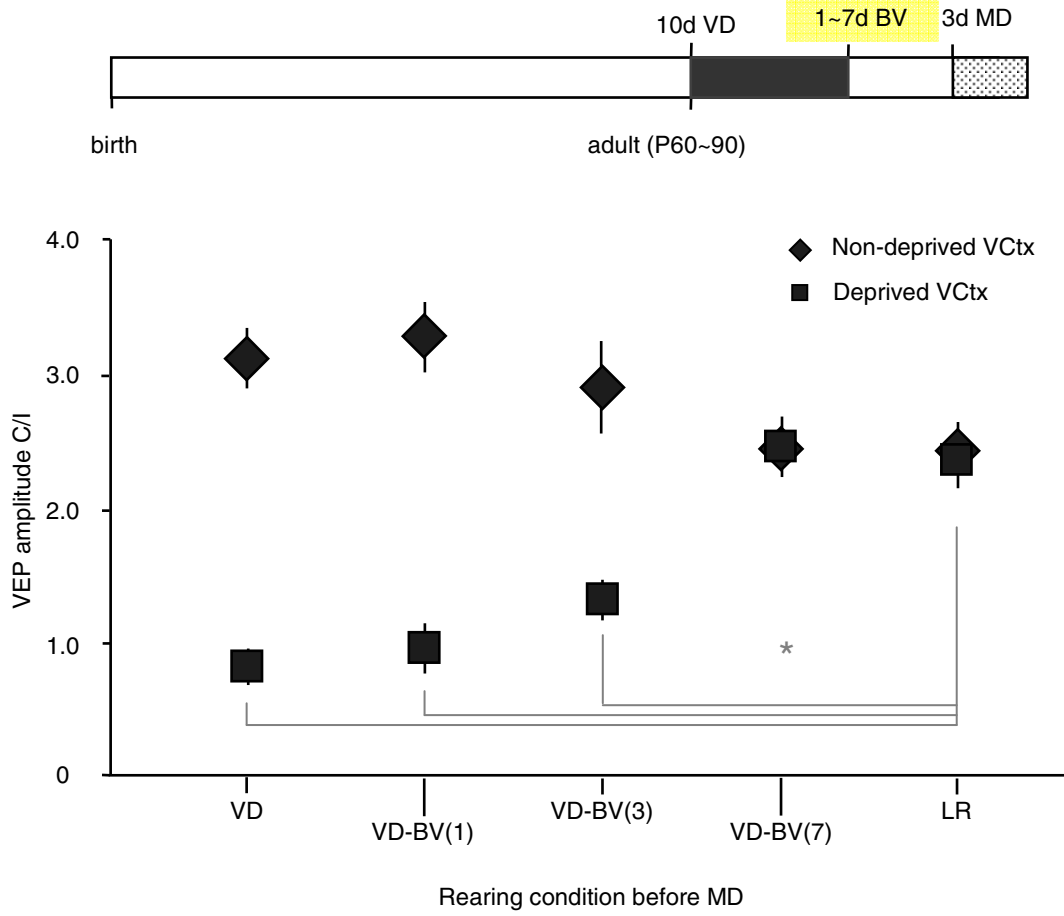
**Figure 13**

### **3.3.8 Visual deprivation reactivates persistent rapid ocular dominance plasticity in adults**

Visual deprivation reverses several molecular correlates of cortical maturation, suggesting that the visual cortex may be returned to a less stable, more plastic state by visual deprivation. To test the durability of the enhanced ocular dominance plasticity induced by visual deprivation, a period of normal binocular vision (BV) was imposed between visual deprivation and monocular occlusion. Remarkably, brief monocular deprivation continues to effectively induce an ocular dominance shift even when 1-3 days of binocular vision followed visual deprivation. Subjects receiving 7 days of binocular vision have a response to brief monocular deprivation that is indistinguishable from controls (VEP C/I in visual cortex contralateral to the deprived eye: VD=0.82±0.04, n=5; VD-BV1=0.94±0.05, n=5; VD-BV3=1.19±0.09, n=5; VD-BV7=2.86±0.57, n=4; LR=2.38±0.18, n=5; one-way ANOVA,  $F_{(4,16)}=8.858$ ,  $p<0.01$  with Fisher's PLSD post hoc analysis, Figure14). No difference is observed in contralateral bias of the visual cortex ipsilateral to the deprived eye across experimental conditions (VD=3.13±0.17, n=5; VD-BV1=3.32±0.08, n=5; VD-BV3=3.13±0.47, n=5; VD-BV7=2.76±0.38, n=4; LR=2.48±0.13, n=5; one-way ANOVA,  $F_{(4,16)}=0.516$ ,  $p>0.05$ ). This suggests that the reactivation of juvenile-like ocular dominance plasticity in adult visual cortex promoted by visual deprivation is persistent, and not easily reversed by normal visual experience.

**Figure 14. Visual deprivation induces persistent reactivation of rapid ocular dominance plasticity in adults.** Adults received brief MD immediately after 10 days of visual deprivation (VD) or when 1, 3 or 7 days of binocular vision was allowed following visual deprivation (VD-BV). VEPs amplitude C/I reveals that brief MD continued to effectively induce an OD shift in the visual cortex contralateral to the deprived eye, when 1 or 3 days, but not 7 days, of binocular vision follows visual deprivation (\*:  $p < 0.01$ , one-way ANOVA with Fisher's PLSD post hoc analysis). No significant difference is observed in the VEP C/I in visual cortex ipsilateral to the deprived eye.





**Figure 14**

## **3.4 Discussion**

### **3.4.1 Visually evoked potentials reliably assess the ocular dominance in rat visual cortex**

VEPs record the synchronized synaptic potentials from a large population of neurons in the visual cortex in response to visual stimulation. VEPs can be recorded within the primary visual cortex, on the dura surface covering the visual cortex, or over the occipital bone. Current source density analysis shows that the first positive peak in VEP waveform correlates with a current sink located about 400 $\mu$ m below the cortical surface (Porciatti et al., 1999; Sawtell et al., 2003), which is the location of layer IV. Layer IV is the recipient layer of thalamocortical projections, thus the primary component of the VEP waveform is believed to reflect the first cortical response to visual inputs. VEPs are widely used in evaluating visual cortical function in many species including human (Allen et al., 1986). VEPs can be used to examine many visual properties such as spatial acuity, contrast threshold and response timing. It has been shown that the amplitude of VEPs in response to pattern stimulation of contralateral and ipsilateral eye are a reliable index of the contralateral bias in rodent visual cortex (Dräger, 1978; Porciatti et al., 1999; Heynen et al., 2003; Sawtell et al., 2003; Pham et al., 2004; Ridder and Nusinowitz, 2006). Compared to single unit recordings, which is a traditional method to examine ocular dominance, VEPs have the advantage of being less subject to sampling bias and have the ability to yield quantitative data (Sawtell et al., 2003). In addition, the procedure is less invasive since it doesn't require the electrode to penetrate into the cortex and the recording can be done from the surface of the dura. Previous experiments have shown that VEP data

agree very well with results obtained by other methods such as single unit recording (Fagiolini et al., 1994; Gordon and Stryker, 1996), optical imaging (Cang et al., 2005; Hofer et al., 2005) and immediate early gene expression (Pham et al., 2004; Tagawa et al., 2005), and the precision of VEPs is sufficient for the purpose of OD detection (Porciatti et al., 1999; Heynen et al., 2003; Sawtell et al., 2003; Pham et al., 2004).

Curiously, ocular dominance shifts in response to prolonged monocular deprivation have been revealed in the adult visual cortex of mice by VEP analysis, optical imaging and immediate early gene expression (Sawtell et al., 2003; Pham et al., 2004; Tagawa et al., 2005; Hofer et al., 2006), but have not been detected by single unit recordings. Emerging evidence suggests that ocular dominance plasticity in adults, but not juveniles, can be acutely masked by sodium pentobarbital (Pham et al., 2004), an anesthetic typically used for single unit recordings. VEPs are performed under urethane anesthesia (Guidel stage III-3, characterized by 0.5-3Hz spontaneous activity), which may allow the detection of synaptic changes that are masked by sodium pentobarbital. Developmental changes in the sensitivity of GABA<sub>A</sub>Rs to the agonist properties of sodium pentobarbital may underlie these differences and explain the success of the VEP method for the study of adult ocular dominance plasticity

### **3.4.2 The enhanced ocular dominance plasticity induced by visual deprivation is juvenile-like**

The shift in ocular dominance induced by monocular deprivation is a sensitive index of the synaptic plasticity available in the binocular visual cortex. In juveniles, the response to monocular deprivation is characterized by two limbs: a rapid

depression in the response to stimulation of the deprived eye, followed by a slowly emerging potentiation of the response to stimulation of the non-deprived eye (Frenkel and Bear, 2004). In adults, brief monocular deprivation is ineffective. However, a longer period of monocular deprivation (>5 days) can induce an ocular dominance shift in adult mice, which is due to potentiation of the response to stimulation of the non-deprived eye (Sawtell et al., 2003; Hofer et al., 2006). Nonetheless, the rapid depression in the response to stimulation of the deprived eye following brief monocular deprivation is only seen in juveniles, and can be deemed as a hallmark of the juvenile form of ocular dominance plasticity.

Here we show that persistent, juvenile-like ocular dominance plasticity can be restored in the adult visual cortex by a period of visual deprivation. In visually deprived adults, brief monocular deprivation induces both limbs of responses as seen in juveniles: a rapid depression in the response to stimulation of the deprived eye, as well as an acceleration of the potentiation of the response to stimulation of the non-deprived eye. This non-invasive manipulation provides further evidence that the adult visual cortex maintains significant potential for synaptic plasticity and demonstrates that ocular dominance plasticity can be enhanced by visual deprivation throughout life.

### **3.4.3 Visual deprivation induces a decrease in inhibition relative to excitation, which may be required to lower the threshold for activity-dependent remodeling of the visual cortical circuits**

Increasing evidence suggests that a balance between the level inhibition and excitation (I/E) in the cortex may determine the level of activity-dependent synaptic plasticity available to cortical circuits (Hensch et al., 1998; Fagiolini and Hensch, 2000; Morales et al., 2002; He et al., 2004). The developmental increase in inhibition relative to excitation observed in the visual cortex coincides with the abrupt decline of the ocular dominance plasticity at the end of classical critical period (Rozas et al., 2001). Very early postnatally, the level of cortical inhibition is very low and the excitation level is very high. Monocular deprivation at this age does not shift ocular dominance, suggesting that there is an optimal range for inhibition over excitation during which the level of synaptic plasticity is maximized.

Genetically knocking out the synaptic isoform of GABA synthesizing enzyme, glutamic acid decarboxylase (GAD65), in mice resulted in an abnormally prolonged discharge of excitatory neurons in response to repeated presynaptic stimulation. This reduced level of inhibition is maintained in the transgenic mice into adulthood. As a result, GAD65 knock out mice retain a very low level of ocular dominance plasticity that can only be revealed by extra long-term (>15 days) monocular deprivation (Hensch et al., 1998). The lack of rapid ocular dominance plasticity could be rescued by application of GABA agonist diazepam at any point during the lifetime (Fagiolini and Hensch, 2000). These data indicate that a minimum level of inhibition is needed for the ocular dominance plasticity.

We have shown that deafferentation-induced remapping of somatosensory cortex involves multiple adjustments in the ratio of inhibition over excitation in the deafferented cortex of adult raccoons (He et al., 2004). The low level of GABA<sub>A</sub>Rs

relative to AMPARs that we observe in the visual cortex of juveniles dark-reared from birth is consistent with small IPSCs and large EPSCs recorded from visual cortical pyramidal neurons in dark-reared rats (Morales et al., 2002; Desai et al., 2002).

The decreased inhibition relative to excitation level following visual deprivation agrees with the prediction of the theory of synaptic scaling (Turrigiano and Nelson, 2000). Reduction in the activity level by deprivation scales up cortical excitation and scales down inhibition. The decrease in inhibition relative to excitation helps to maintain the homeostasis of visual cortical activity. Sensory deprivation by dark rearing has also been reported to scale up excitability in the deprived visual cortex with a concomitant compensatory scaling down of excitability in other non-deprived barrel (somatosensory) cortex (Goel et al., 2006). This bidirectional cross-modal modification of synaptic functions is associated with enhanced postsynaptic AMPA receptors function.

How does the level of inhibition relative to excitation modulate the level of synaptic plasticity? One possibility is through the regulation of NMDA receptors (Bear, 2003). Since the level of inhibition relative to excitation directly determines the level of neuronal excitability, a reduction in inhibition would result in an increase in excitability, which may allow stronger depolarization facilitating NMDA receptor activation. Another candidate mechanism is related to the regulation of tPA activity. A decrease in inhibition relative to excitation is correlated with an increase in tPA activity level, which will be discussed further in the section below.

The low level of GABA<sub>A</sub>Rs relative to AMPARs observed in the adult visual cortex following visual deprivation suggests that even in adulthood, visual deprivation can return the visual system to a more immature state. A low level of inhibition relative to excitation may be required to allow the visual cortex to respond rapidly to manipulations of visual input, particularly facilitating the rapid depression in response to the deprived eye after brief monocular deprivation.

#### **3.4.4 Increased NR2b over NR2a level induced by visual deprivation may facilitate the potentiation of response to the non-deprived eye following monocular deprivation**

Activation of the NMDA receptor is required for many forms of activity-dependent synaptic plasticity, including deprivation-induced ocular dominance shifts (Bear et al., 1990; Sawtell et al., 2003). Here we show that visual deprivation results in NMDA receptors with an immature composition, characterized by a high level of the NR2b subunit relative to NR2a. However, in juveniles, visual deprivation appears to prevent the insertion of NR2a-containing NMDARs into the synapse (Quinlan et al., 1999), while in adults, visual deprivation appears to stimulate the delivery of NR2b-containing NMDARs to the synapse. In both cases, the deprivation-induced regulation of NMDAR subunit composition would significantly impact the characteristics of NMDAR-dependent synaptic plasticity. NR2a knockout mice show a defect in the maturation of orientation selectivity, and a decreased ocular dominance shift in response to 4-5 days of monocular deprivation in critical period, suggesting that the shortening in NMDAR current duration by insertion of NR2a subunit is

essential for the precise refinement of receptive field (Fagiolini et al., 2003). Interestingly, the NR2a knockout mice have a normal developmental onset and termination for the critical period of ocular dominance plasticity, suggesting that other mechanisms might be involved in regulation of the timing of the traditional critical period.

It has been previously proposed that the ratio of synaptic NR2b- to NR2a-containing NMDARs determines the threshold for NMDAR-dependent long-term potentiation (Abraham and Bear, 1996; Quinlan et al., 1999; Tang et al., 1999). In the juvenile visual cortex, when NR2b-containing NMDARs prevail, the threshold for NMDAR-dependent LTP is low. Over the course of development, there is an experience-dependent decrease in NR2b-containing NMDARs and an associated increase in the threshold for LTP (Kirkwood et al., 1996). The correlation between high levels of NR2b-containing NMDARs and a low threshold for LTP has also been demonstrated following learning (Quinlan et al., 2004), following transgenic over-expression of NR2b (Tang et al., 1999) and following cortical lesions (Huemmeke et al., 2004). Therefore, we propose that the increase in the ratio of NR2b to NR2a observed in the visually deprived adults lowers the threshold for activity-dependent synaptic potentiation, which facilitates the rapid potentiation in response to stimulation of the non-deprived eye following brief monocular deprivation in the visually deprived animals.

In recent years, there has been a heated debate on the specific role of NR2a and NR2b-containing NMDA receptors in the induction of LTP and LTD. Some reported that NR2b specifically mediates induction of LTD, while NR2a specifically mediates



induction of LTP (Liu et al., 2004; Massey et al., 2004). Others say that the direction of synaptic modification (LTP or LTD) is not exclusively linked to any one of NMDA receptor subunits (Berberich et al., 2005; Zhao et al., 2005). Recently, experiments carried out simultaneously at three labs (Morishta et al., 2006) show that at least the induction of LTD is not completely dependent on NR2b-containing NMDA receptors, since blocking NR2b with two different antagonists doesn't abolish the induction of LTD.

Another question is the whereabouts of increased NR2b-containing NMDA receptors. It has been reported that NR2b-containing NMDA receptors may be actively mobilized between synaptic and extrasynaptic sites in mature neurons (Li et al., 1998; Tovar and Westbrook, 2002). Electrophysiological recording in slices prepared from visually-deprived adult mice shows that visual deprivation modifies synaptic transmission by increasing the rate of depression. In addition there is an enhancement of temporal summation of NMDA receptor mediated current, possibly through increased NR2b-containing NMDA receptors at peri/extrasynaptic sites (Yashiro et al., 2005). Because our preparation of synaptoneurosomes does not exclude peri-synaptic structures, we cannot rule out the possibility that the observed increase in NR2b occurs in a peri-synaptic population.

### **3.4.5 tPA plays an important role in allowing the structural changes following monocular deprivation**

The extracellular proteolytic cascade initiated by tPA activity has been reported to be involved in many brain functions, such as learning, memory and the

regeneration of the nervous system after injury (Madani et al., 1999; Seeds et al., 1995; Calabresi et al., 2000; Siconolfi and Seeds, 2001). All of these functions might involve remodeling of neural circuits through axon growth, cell migration, or dendrite pruning or formation (Seeds et al., 1997). Changes in neuronal responsiveness that have been observed following monocular deprivation are also likely to involve rewiring of the neuronal circuits (Antonini et al., 1999; Trachtenberg and Stryker, 2001). In fact, the increased density of the extracellular matrix in adult visual cortex is thought to exert a constraining force on the MD-induced changes. Application of an exogenous proteolytic enzyme chondroitinase A in adult rat visual cortex facilitates MD-induced ocular dominance shifts (Pizzarusso et al., 2002). On the other hand, blocking tPA activity in kitten visual cortex eliminates ocular dominance plasticity (Mataga et al., 1996; Muller and Griesinger, 1998). Not surprisingly, knocking out tPA also impairs ocular dominance plasticity in the visual cortex, while application of tPA rescues the response to MD (Mataga et al., 2002). An elevation of endogenous tPA activity has been observed following monocular deprivation in juvenile mice accompanied by increased spine motility in both the visual cortex contralateral and ipsilateral to the deprived eye (Mataga et al., 2004). This may help degradation of the extracellular matrix to allow morphological changes for ocular dominance shift. The correlation between tPA activity and spine dynamics is further supported by the fact that applying tPA to the visual cortex increases spine motility and this effect is occluded by monocular deprivation (Oray et al., 2004).

It is not clear which signaling pathway(s) is involved in the regulation of endogenous tPA activity. Recent evidence suggests that tPA activity might be

downstream of the regulation of inhibition relative to excitation. In GAD65 knock out mice, where ocular dominance plasticity is thought to be abolished due to an inadequate level of inhibition, the increase in cortical tPA activity induced by 2 days of monocular deprivation is also abolished (Mataga et al., 2002). On the other hand, application of diazepam, a GABA<sub>A</sub> receptor agonist, which rescues ocular dominance plasticity in GAD65 knock out mice, does not rescue ocular dominance plasticity in tPA knock out mice. Another molecule that has been implicated in the regulation of tPA activity is brain-derived neurotrophic factor (BDNF). BDNF has been reported to regulate ocular dominance plasticity (Huang et al., 1999; Gianfranceschi et al., 2003). Over expression of BDNF accelerates visual cortical development and induces an early onset and early closure of ocular dominance plasticity (Huang et al., 1999). It seems that a high level of BDNF could substitute for visual experience. Indeed, dark rearing from birth has no effect on BDNF over expressing mice. The transgenic animal appears completely normal in many of visual cortical functions including acuity, receptive field size, critical period for ocular dominance plasticity, and cortical inhibition (Gianfranceschi et al., 2003). An increase in both BDNF and tPA has been reported in late-phase LTP, which is believed to be a mechanism for long-term memory storage (Pang and Lu, 2004). In mouse hippocampal slices, the tPA-plasmin system cleaves pro-BDNF to mature BDNF, which is necessary for the expression of late-phase LTP (Pang et al., 2004). Since BDNF has also been shown to increase the level of cortical inhibition (Huang et al., 1999; Cotrufo et al., 2003), the two pathways might be involved in the same signaling pathway that leads to activity-dependent changes in tPA activity.

### **3.4.6 Visual deprivation is a non-invasive way to enhance OD plasticity in adults**

Ocular dominance plasticity in adults has previously been shown to be facilitated by enzymatic degradation of extracellular matrix components (Pizzorusso et al., 2002), demonstrating that the potential for synaptic plasticity in the visual cortex persists throughout life. Another successful effort in enhancing synaptic plasticity in adult cortex targeted an axonal growth inhibitor protein found in the oligodendrocytes, Nogo-A (Liu et al., 2006). Nogo-A is known to inhibit axon regeneration in the adult central nervous system. Genetically knocking out the ligand Nogo, or mutating its receptor NogoR, retains the juvenile level of ocular dominance plasticity in adult visual cortex (MacGee et al., 2005). Here we show that 10 days of visual deprivation is sufficient to reactivate rapid, juvenile-like ocular dominance plasticity. The enhanced ocular dominance plasticity that we observe following visual deprivation persists, even after 3 days of binocular vision. Such a non-invasive method to restore rapid ocular dominance plasticity holds potential therapeutic promise, because the ability to reverse the visual deficits in human amblyopia decreases with age (Mitchell and MacKinnon, 2002). In addition, the fact that visual deprivation reverses the developmental changes of some cortical molecules suggests the intriguing possibility that sensory deprivation can “reset” the visual cortex, rendering it with a juvenile-like potential for remodeling (Karmarkar and Dan, 2006).

## **CHAPTER 4: VISUAL DEPRIVATION IN ADULTHOOD**

### **PROMOTES RECOVERY FROM LONG-TERM MONOCULAR DEPRIVATION**

#### 4.1 Introduction

##### **4.1.1 The effectiveness of treatments for deprivation amblyopia decreases with age**

The ability of the mammalian cortex to reorganize in response to changing patterns of afferent input is highest during an early postnatal critical period and is significantly restricted in the adult. One of the most dramatic examples of reduced cortical plasticity is the decline in ocular dominance plasticity seen over development. During early postnatal development, relatively brief monocular deprivation induces a shift in the ocular preference of binocular neurons toward the non-deprived eye. This manipulation is ineffective in adults, consistent with the decrease in ocular dominance plasticity over development. The decrease in experience-dependent synaptic plasticity over development is likely to ensure necessary structural and functional stability to cortical circuitry in adults, but it also imposes a restriction on the potential for functional recovery from abnormal patterns of afferent input during development. This is what has been observed in the clinical treatment of stimulus deprivation amblyopia in humans.

The term ‘amblyopia’ originates from the Greek words ‘amblyos’, which means ‘blunt’, and ‘opia’, which means vision. It is used to refer to loss of monocular or

binocular vision that is associated with occlusion of the visual axis (Creig, 2005). A major cause of human amblyopia is a congenital unilateral cataract (Mitchell and Mackinnon, 2002). A cataract is a pathological opacity of the crystalline lens. Some cataracts are static, and some are progressive, with the latter being even more difficult to diagnose at birth. Presence of a cataract causes deprivation of patterned vision but not diffuse light. As a result, visual acuity deteriorates in the occluded eye. The loss of symmetric binocular inputs to the visual cortex also causes deficits in depth perception. Surgically removing the cataract and replacing the damaged lens restores the peripheral inputs for visual detection. Restoration of the cortical visual functions such as acuity and depth perception also requires correction of the cortical connections. Since occlusion of the bad eye occurs during the critical period for ocular dominance plasticity, the anatomy and physiological properties of the visual cortex shift to be dominated by the good eye. Occlusion of the good eye (reverse occlusion) following removal of cataract has been used clinically to force the visual cortex to respond to inputs from the previously bad eye. Since the level of synaptic plasticity declines over the development, the later the surgery is done, the less recovery of visual function could be achieved in the deprived eye. Mitchell and MacKinnon (2002) reviewed the outcomes of 11 clinical studies including 71 human cases, and found that the recovery of acuity declined dramatically when cataract removal surgery and therapeutic intervention were applied later than 150 days of age. Many patients with cataract removal after 150 days of age only recovered a rudimentary form vision in the bad eye. The decrease in the success of the therapy for deprivation amblyopia is similar to the critical period for ocular dominance plasticity

that has been defined by susceptibility to monocular deprivation in animal studies (Hubel and Wiesel, 1970; Mitchell, 1988; Fagiolini et al., 1994).

#### **4.1.2 Animal models for deprivation amblyopia and reverse occlusion**

To mimic the presence of a congenital unilateral cataract in experimental animals, one eye can be deprived of vision by lid suturing in early postnatal life. Similar to a congenital unilateral cataract, eyelid suturing results in a deprivation of patterned vision without blocking the detection of diffuse light. The consequence of early postnatal monocular deprivation includes deterioration of acuity in the deprived eye and a loss in the number of cortical neurons responding to stimulation of that eye (Hubel and Wiesel, 1968). This model can then be used to develop therapeutic strategies for recovery from deprivation amblyopia. Patching the non-amblyopic eye after removal of the cataract (reverse occlusion) was first advocated by du Buffon and has been an essential component of human amblyopic therapy for more than two hundred years (Mitchell and MacKinnon, 2002). Like cataract removal in humans, in animals monocularly deprived from birth, reverse occlusion has to be done early in development, within the critical period, to be effective (Blakemore et al., 1978; Mitchell, 1988; Liao et al., 2004; Prusky and Douglas, 2003).

In Chapter 3 we showed that a period of visual deprivation in adulthood can reinstate juvenile-like ocular dominance plasticity in the adult visual cortex, rendering the primary visual cortex sensitive to a rapid ocular dominance shift in response to brief monocular occlusion (He et al., 2006). Here we demonstrate that the same manipulation, visual deprivation in adulthood, allows for the recovery of function in an eye deprived of vision from birth.

## 4.2 Material and Methods

### Animal care and treatments:

To mimic the presence of a congenital unilateral cataract, Long Evans rats received monocular deprivation (for details, see Chapter 3 methods) from eye opening (~P13) and were raised with 12 hr light:12 hr dark /day until adulthood (>P70). Five experimental groups were used to explore methods to recover function in the deprived eye (Figure 14). In MD-BV group, chronically deprived subjects were given 7-14 days of normal binocular vision (BV, opening the deprived eye). In MD-RO group, chronically deprived subjects were given 7-14 days of reverse occlusion (RO, closing the initially open eye and opening the initially deprived eye). In MD-VD group, chronically deprived subjects received 10 days of visual deprivation (VD, putting into light-tight dark room). In MD-VD-BV and MD-VD-RO groups, subjects were visually deprived for 10 days before receiving either BV or RO treatment.

### VEP recording and spatial acuity test:

Visually evoked potentials (VEPs) were recorded with tungsten microelectrodes (0.1M $\Omega$ , Microprobe, MD) relative to a ground screw in the frontal bone and a reference electrode in dorsal neck muscle (as described in Chapter 3 methods). In short, the dura covering the binocular visual cortex (OC1b; ~7.0mm posterior to Bregma and 5 mm lateral to the midline) was exposed through a hole (~ 3mm in diameter) in the skull following urethane anesthesia (1.6g/kg i.p.). Electrode placement on the binocular visual cortex was confirmed by capturing a VEP in response to stimulation of the ipsilateral eye. Visual stimuli, 1Hz full screen vertical



square wave gratings with 96.28% maximal contrast, were presented on a CRT computer monitor 25cm from eyes, in a darkened room.

The amplitude of the primary positive component of the VEP (~150msec latency) was amplified (1000X), filtered (0.5-300Hz band pass filter followed by 60Hz low pass digital filter) and averaged in synchrony with the stimulus using ENFANT software (100 trials). VEP amplitudes were normalized to noise recorded at the same location in response to blank screen, which did not differ across experimental groups (one-way ANOVA,  $p>0.50$ ).

For the test of spatial acuity, a series of visual stimuli with spatial frequencies ranging from 0.04 cpd to 1.31 cpd were presented to the subject in random order. Each stimulus block contained 100 trials. The amplitude of the VEP (normalized to the maximal response recorded within the same session) was plotted against the log of spatial frequency. Visual acuity was estimated as the spatial frequency extrapolated to zero amplitude of the linear regression through the last 5-8 data points above noise (Figure 19; Porciatti et al., 1999).

## 4.3 Results

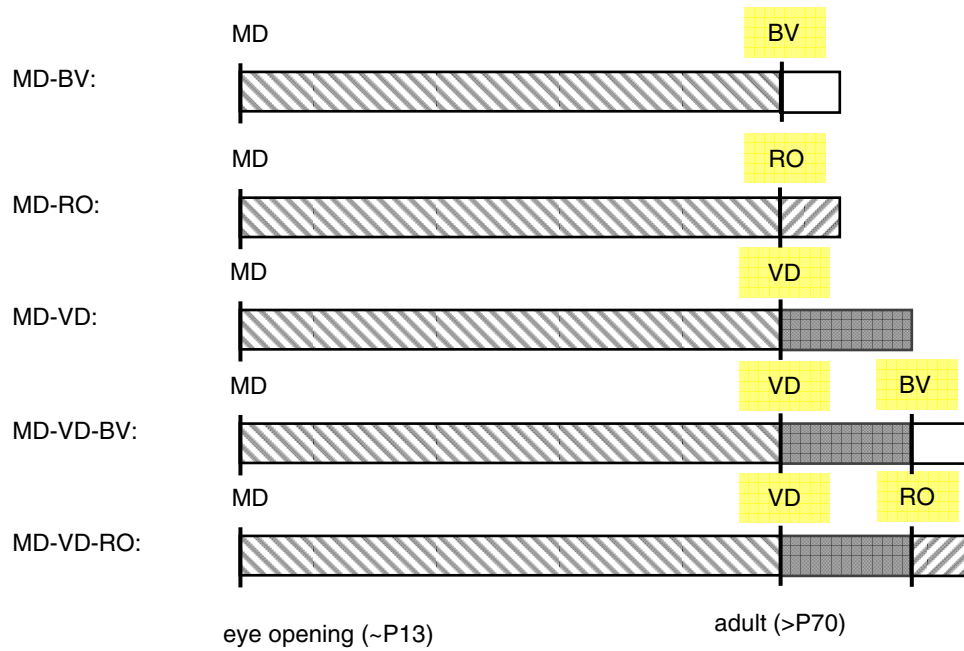
### 4.3.1 Effects of chronic monocular deprivation from eye opening

To mimic the effect of a congenital unilateral cataract, we performed monocular deprivation upon eye opening (~P13) and allowed the animals to reach adulthood with monocular vision. VEPs were used to evaluate the effect of chronic monocular deprivation on the ocular dominance of the binocular neurons in the primary visual cortex. Chronic MD induces a significant ocular dominance shift, as evidenced by a significant difference in the contralateral bias across the two cortical hemispheres (Figure 15b). Chronic MD results in a significant reduction in the contralateral bias of the deprived (contralateral to the deprived eye) hemisphere (VEP C/I: deprived VCtx=0.76±0.10) and a significant increase in the contralateral bias of the non-deprived (ipsilateral to the deprived eye) hemisphere (non-deprived VCtx=6.32±0.83; n=12) as comparing to normal control (gray bar, VEP C/I: right VCtx=2.25±0.19, left VCtx=2.22±0.18, n=6; p<0.01 for each hemisphere, unpaired student's t-test).

The reduced contralateral bias observed in the deprived visual cortex is due to a significant decrease in the response to stimulation of the deprived eye (the contralateral eye, Figure 15c left, normalized VEP amplitude: NR control=5.09±0.60; MD=3.35±0.53, p<0.05, unpaired student t-test) and a significant increase in the response to stimulation of the open eye (the ipsilateral eye, Figure 15c right, NR=2.36±0.34; MD=5.84±1.38, p<0.05, unpaired student t-test).

The increased contralateral bias seen in the non-deprived visual cortex resulted from a significant decrease in response to stimulation of the ipsilateral deprived eye

**Figure 15. Experimental design for chronic monocular deprivation and subsequent manipulations.** Subjects were monocularly-deprived (MD) from eye opening (~P13) and raised in a normally lighted environment (12hr light:12hr dark/day) until adulthood with monocular vision(>P70). In the MD-BV group, chronically deprived subjects were given 7-14 days of normal binocular vision (BV, opening the deprived eye). In the MD-RO group, chronically deprived subjects were given 7-14 days of reverse occlusion (RO, closing the initially open eye and opening the initially deprived eye). In the MD-VD group, chronically deprived subjects received 10 days of visual deprivation (VD, putting into light-tight dark room). In the MD-VD-BV and the MD-VD-RO groups, subjects were visually deprived for 10 days before receiving either BV or RO treatment.



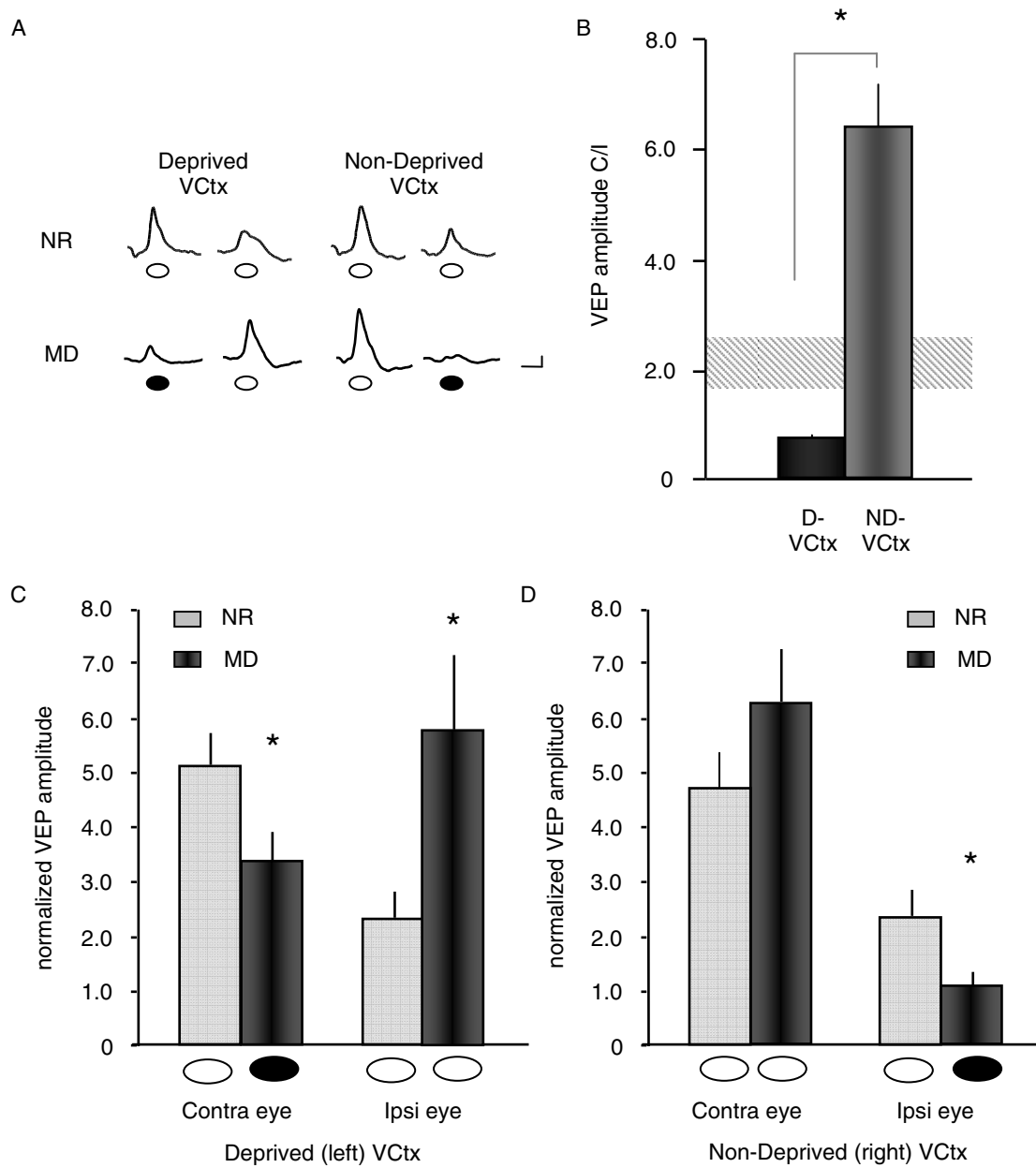
**Figure 15**

**Figure 16. Chronic monocular deprivation significantly shifts ocular dominance by decreasing the response to stimulation of the deprived eye and increasing the response to stimulation of the non-deprived eye.** A. Representative VEP waveforms recorded in the binocular region of the Visual cortex in each hemisphere in response to stimulation of each eye. NR: normal reared; MD: chronic monocular deprived. Open oval: open eye; filled oval: deprived eye. Scale bar: 100msec, 100 $\mu$ v.

B. Chronic MD results in a significant difference in the VEP C/I between the deprived and non-deprived visual cortex. The VEP C/I in the deprived visual cortex is significantly lower than in the age-matched normal reared controls (gray bar). The VEP C/I in the non-deprived visual cortex is significantly higher than in the normal reared controls (gray bar). \*:  $p < 0.001$ , Deprived visual cortex vs. Non-Deprived visual cortex, unpaired student's t-test.

C. The ocular dominance shift observed in the deprived visual cortex following chronic MD is due to a significant decrease in the VEP amplitude in response to stimulation of the deprived (contralateral) eye, and a significant increase in the VEP amplitude in response to stimulation of the non-deprived (ipsilateral) eye. \*:  $p < 0.01$ , MD vs. NR, unpaired student t-test.

D. The increased VEP C/I observed in the non-deprived visual cortex following chronic monocular deprivation is due to a significant decrease in the VEP amplitude in response to stimulation of the deprived (contralateral) eye. The VEP response to stimulation of the non-deprived (ipsilateral) eye is not significantly changed. \*:  $p = 0.15$ , MD vs. NR, unpaired student's t-test.



**Figure 16**

(Figure 15d right, normalized VEP amplitude: NR control= $2.06 \pm 0.25$ ; MD= $1.05 \pm 0.19$ ,  $p < 0.01$ , unpaired student's t-test). The VEP amplitude in response to the open eye in the non-deprived Visual cortex is not statistically different between the chronic MD and NR controls (Figure 15d left, normalized VEP amplitude: NR control= $4.56 \pm 0.62$ ; MD= $6.23 \pm 1.06$ ;  $p = 0.15$ , unpaired student's t-test), probably because the open eye was already dominant in this hemisphere.

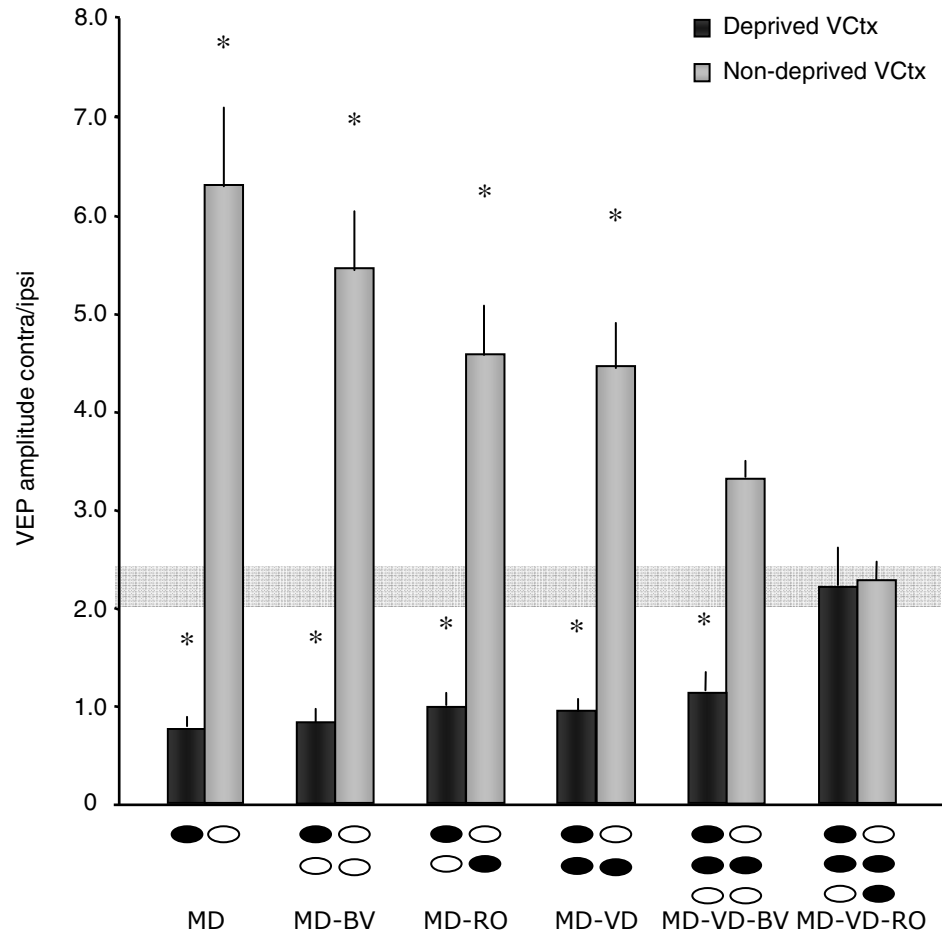
#### **4.3.2 Visual deprivation facilitates recovery of the normal contralateral bias in both hemispheres**

Due to the developmental loss of OD plasticity, the ability to recover function in adulthood in a chronically deprived eye is very limited. To ask if visual deprivation can reactivate sufficient OD plasticity to allow recovery of function following chronic deprivation, we tested the effect of different treatments for amblyopia with or without visual deprivation. Reopening the occluded eye to allow subjects 7-14 days of binocular vision did not return the VEP C/I to control values (Figure 17, MD-BV: deprived VCtx= $0.85 \pm 0.10$ ; non-deprived VCtx= $5.45 \pm 0.56$ ;  $n=3$ ). Similarly, performing a reverse occlusion, by opening the occluded eye and closing the previously open eye for 7-14 days did not return the VEP C/I to control values (MD-RO deprived VCtx= $0.97 \pm 0.12$ ; non-deprived VCtx= $4.57 \pm 0.54$ ;  $n=9$ ). Visually depriving the subjects for 10 days also has no effect on the shifted contralateral bias (MD-DE: deprived VCtx= $0.94 \pm 0.12$ ; non-deprived VCtx= $4.45 \pm 0.46$ ;  $n=9$ ). However, if binocular vision was preceded by 10 days of visual deprivation, a return to the normal contralateral bias is observed in the non-deprived hemisphere (VEP C/I:

MD-VD-BV: deprived VCtx=1.16±0.23; non-deprived VCtx=3.31±0.11, n=6). If reverse occlusion was preceded by 10 days of visual deprivation, a return to the normal contralateral bias is observed in both the deprived and non-deprived hemisphere (MD-VD-RO: deprived VCtx=2.21±0.38; non-deprived VCtx=2.28±0.21, n=11). This suggests that the enhanced synaptic plasticity in visual cortex observed following visual deprivation in adulthood is sufficient to allow recovery of the normal contralateral bias after chronic MD.



**Figure 17. Visual deprivation in adulthood allows recovery of the normal contralateral bias in subjects monocularly deprived from birth.** A significant shift in ocular dominance is induced in both the deprived and non-deprived visual cortex following chronic monocular occlusion (from eye opening to >P70). The shaded horizontal stripe marks the normal contralateral bias of age-matched controls. Subsequent binocular vision, reverse occlusion or visual deprivation had no effect on the contralateral bias of the deprived or non-deprived visual cortex. However, significant recovery of normal contralateral bias is observed in the non-deprived visual cortex when visual deprivation precedes binocular vision or reverse occlusion. In the deprived VCtx, significant recovery in the contralateral bias is observed only when visual deprivation precedes reverse occlusion. \*: One-way ANOVA ( $F_{(6, 30)} = 9.284, p < 0.001$  on deprived VCtx;  $F_{(6, 30)} = 6.297, p < 0.001$  on non-deprived VCtx), with Fisher's PLSD post hoc against NR. Ovals represent the history of visual experience of the primary input to that hemisphere. Filled oval represents deprivation by monocular deprivation, reverse occlusion or visual deprivation.

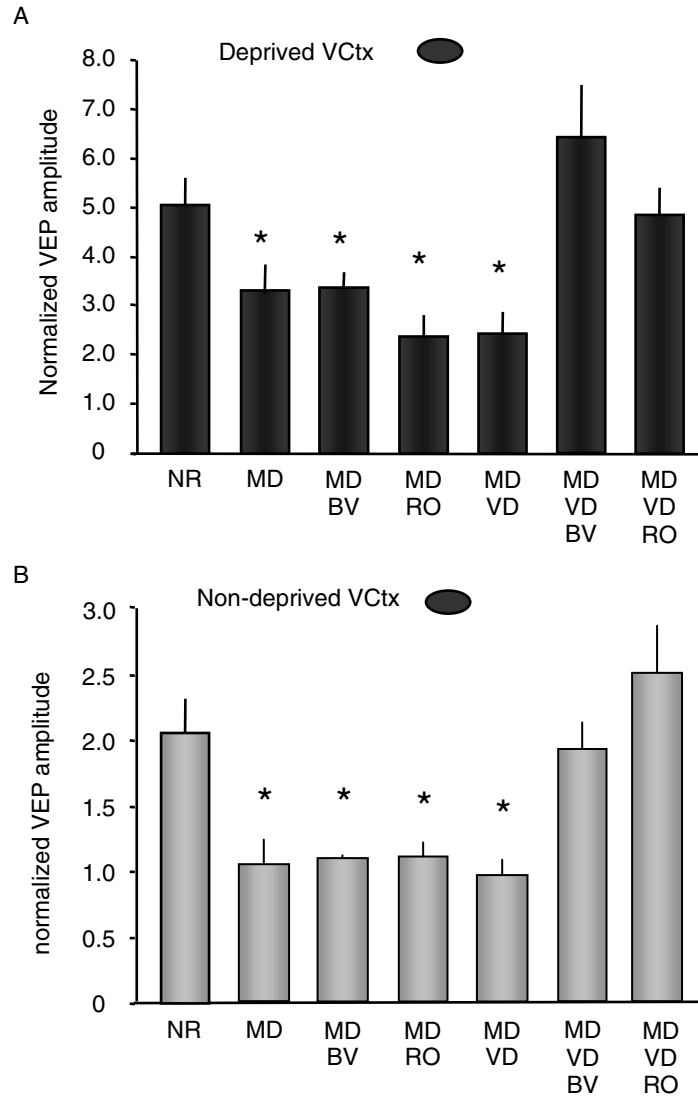


**Figure 17**

### **4.3.3 Visual deprivation facilitates recovery of the cortical response driven by the deprived eye**

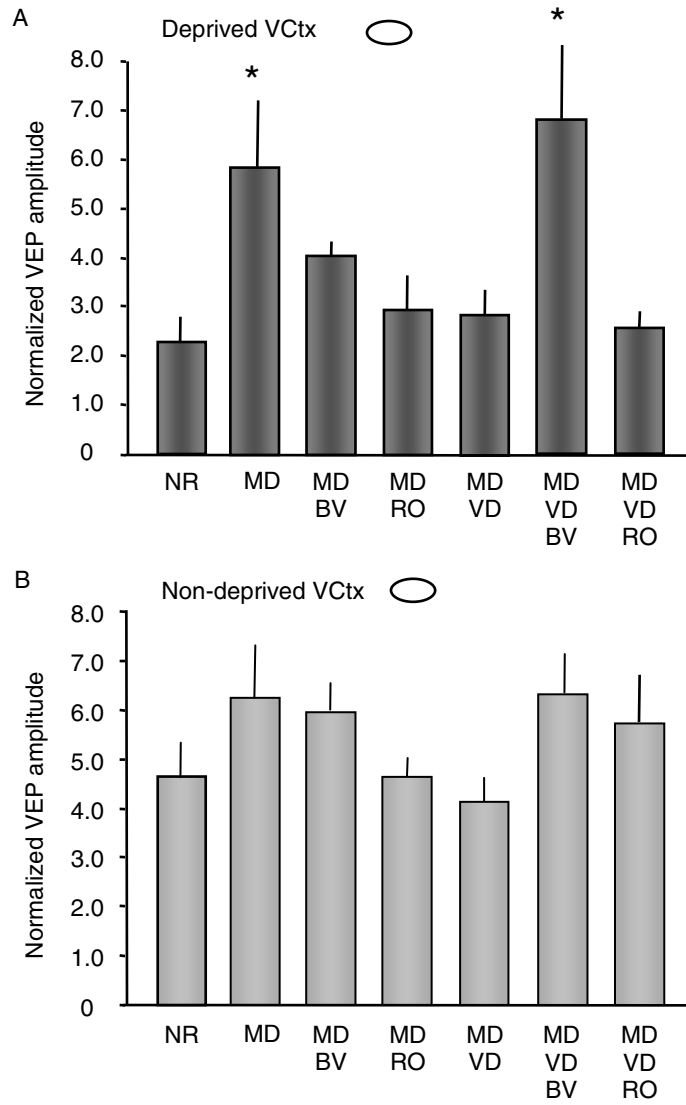
To ask if visual deprivation in adulthood can also reinstate the ability of the deprived eye to effectively drive cortical responses, we compared the VEP amplitudes in response to stimulation of each eye across all experimental groups. In the deprived visual cortex, the VEP amplitude in response to stimulation of the deprived eye is significantly reduced following chronic MD. Subjects that received binocular vision, reverse occlusion or visual deprivation alone showed no change in the VEP amplitude produced in response to stimulation of the deprived eye (Figure 18A, normalized VEP amplitude: NR control= $5.09 \pm 0.60$ ; MD= $3.35 \pm 0.53$ ; MD-BV= $3.44 \pm 0.29$ ; MD-RO= $2.43 \pm 0.43$ ; MD-VD= $2.46 \pm 0.42$ ). However, a significant increase in VEP amplitude is seen when visual deprivation precedes binocular vision or reverse occlusion (Normalized VEP amp: MO-VD-BV= $6.51 \pm 0.99$ ; MO-VD-RO= $4.87 \pm 0.58$ ). A similar trend is seen in the non-deprived Visual cortex, where the ability of the deprived eye to drive VEPs is also significantly reduced after chronic MD. No change in the VEP amplitude in response to stimulation of the deprived eye was observed in subjects that received binocular vision, reverse occlusion or visual deprivation alone. However, a significant increase in VEP amplitude in response to stimulation of the deprived eye is observed when binocular vision or reverse occlusion were preceded by visual deprivation (Figure 18B, normalized VEP amplitude: NR control= $2.06 \pm 0.25$ ; MD= $1.05 \pm 0.19$ ; MD-BV= $1.10 \pm 0.02$ ; MD-RO= $1.11 \pm 0.11$ ; MD-VD= $0.97 \pm 0.11$ ; MO-VD-BV= $1.94 \pm 0.20$ ; MO-VD-RO= $2.54 \pm 0.34$ ).

**Figure 18. Visual deprivation promotes the experience-dependent strengthening of the cortical response to an eye deprived of vision from birth.** A. In the deprived visual cortex, normalized VEP amplitudes reveal a significant reduction in the response to stimulation of the occluded eye following chronic monocular deprivation. Binocular vision, reverse occlusion and visual deprivation do not affect the VEP amplitude. If visual deprivation precedes reverse occlusion or binocular vision, there is a significant increase in the VEP amplitude in response to stimulation of the chronically-occluded eye (One-way ANOVA ( $F_{(6, 50)} = 6.045$ ,  $p < 0.001$ ), \*:  $p < 0.05$ , with Fisher's PLSD post hoc test against NR). B. In the non-deprived visual cortex, normalized VEP amplitudes reveal a significant reduction in the response to stimulation of the occluded eye following chronic monocular deprivation. Binocular vision, reverse occlusion and visual deprivation do not affect the VEP amplitude. If visual deprivation precedes reverse occlusion or binocular vision, there is an increase in the VEP amplitude in response to stimulation of the chronically-occluded eye (One-way ANOVA ( $F_{(6, 50)} = 8.167$ ,  $p < 0.0001$ ) \*:  $p < 0.01$ , with Fisher's PLSD post hoc vs. NR).



**Figure 18**

**Figure 19. The enhanced response to stimulation of the non-deprived eye following chronic MD is decreased by competition from the deprived eye.** A. In the deprived visual cortex, normalized VEP amplitudes reveal a significant enhancement in the response to stimulation of the non-occluded eye following chronic monocular deprivation. Binocular vision, reverse occlusion, visual deprivation and visual deprivation preceding reverse occlusion return the VEP amplitude in response to stimulation of the non-occluded eye to the normal level. Visual deprivation preceding binocular vision induces a repotentialization of the amplitude of the VEP in response to stimulation of the non-occluded eye (One-way ANOVA ( $F_{(6, 50)} = 3.066, p < 0.05$ ). \*:  $p < 0.05$ , with Fisher's PLSD post hoc test against NR). B. In the non-deprived visual cortex, no significant difference is observed in response to stimulation of the non-occluded eye across all experimental groups. One-way ANOVA, ( $F_{(5, 45)} = 1.055, p = 0.40$ ).



**Figure 19**

In the deprived Visual cortex, a significant potentiation of VEP amplitude is seen in response to stimulation of the non-occluded eye following chronic MD. To our surprise, most of our experimental manipulations, including binocular vision, reverse occlusion or visual deprivation, and visual deprivation followed by reverse occlusion returned VEP amplitude to the normal control levels (Figure 19A, Normalized VEP amp: NR=2.36±0.34; MD=5.84±1.38; MD-BV=4.05±0.19; MD-RO=2.97±0.76; MD-VD=2.85±0.51; MD-VD-RO=2.59±0.38). In contrast, the VEP amplitude produced in response to stimulation of the non-occluded eye was not different from chronic monocular deprivation alone when binocular vision follows visual deprivation, suggesting a repotentialization of the ipsilateral (open eye) projections when binocular vision follows visual deprivation (Normalized VEP amp: MD-VD-BV=6.87±1.56). In the non-deprived Visual cortex, there was no significant difference in the VEP amplitude in response to the non-occluded eye across all experimental groups (Figure 19B, NR control=4.56±0.62; MD=6.23±1.06; MD-BV=5.97±0.53; MD-RO=4.64±0.37; MD-VD=4.14±0.46; MD-VD-BV=6.35±0.51; MD-VD-RO=5.72±0.95), suggesting that the dominant input is relatively stable.

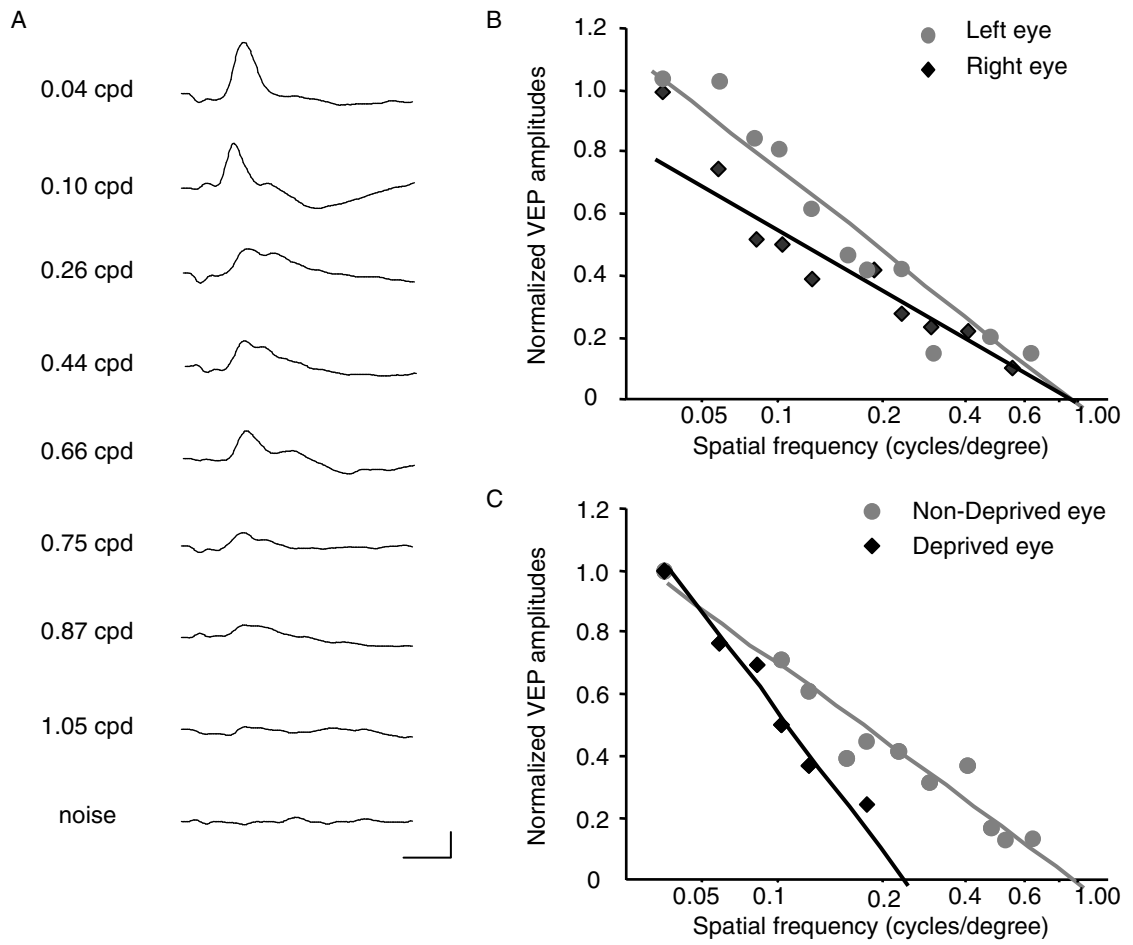


#### **4.3.4 Visual deprivation in adulthood promotes recovery of spatial acuity in the eye deprived of vision from birth**

Visual deprivation followed by binocular vision or reverse occlusion in adulthood can recover the ability of the deprived eye to drive cortical responses, suggesting that these manipulations may help to restore functional vision of an eye deprived of vision from birth. To assess the functional recovery of the chronically deprived eye, we used VEPs to estimate spatial acuity. For testing spatial acuity, VEPs were always recorded from the binocular region of the contralateral Visual cortex. VEP amplitudes are plotted against the log spatial frequency, and decrease as the spatial frequency of the visual stimulus increases (Figure 20A). Visual acuity is determined by extrapolating the linear regression of the last 5-8 points to zero (Porciatti et al., 1999). This method reveals that normally-reared Long Evans adult rats have a spatial acuity of 1.14 cpd (spatial acuity: NR control (grey bar)= $1.14 \pm 0.09$  cpd, n=5; Figure 20B, for summary data see Figure 21A), which is consistent with other physiological and behavioral estimates of adult rat spatial acuity (Fagiolini et al., 1994; Prusky et al., 2000; Pizzorusso et al., 2006; Iny et al., 2006). Chronic monocular deprivation results in a significant decrease in the spatial acuity of the deprived eye (spatial acuity: MD= $0.30 \pm 0.04$  cpd, n=8; Figure 19C, for summary data see Figure 21A). The acuity of the deprived eye is not improved if the subjects are treated with 7-14 days of binocular vision, 7-14 days of reverse occlusion or 10 days of visual deprivation alone (spatial acuity: MD-BV= $0.43 \pm 0.12$ cpd, n=3; MD-RO= $0.38 \pm 0.14$ cpd, n=4; MD-VD= $0.39 \pm 0.09$ cpd, n=4). However, when visual deprivation (10 days) precedes binocular vision (7-14 days) or reverse occlusion (7-

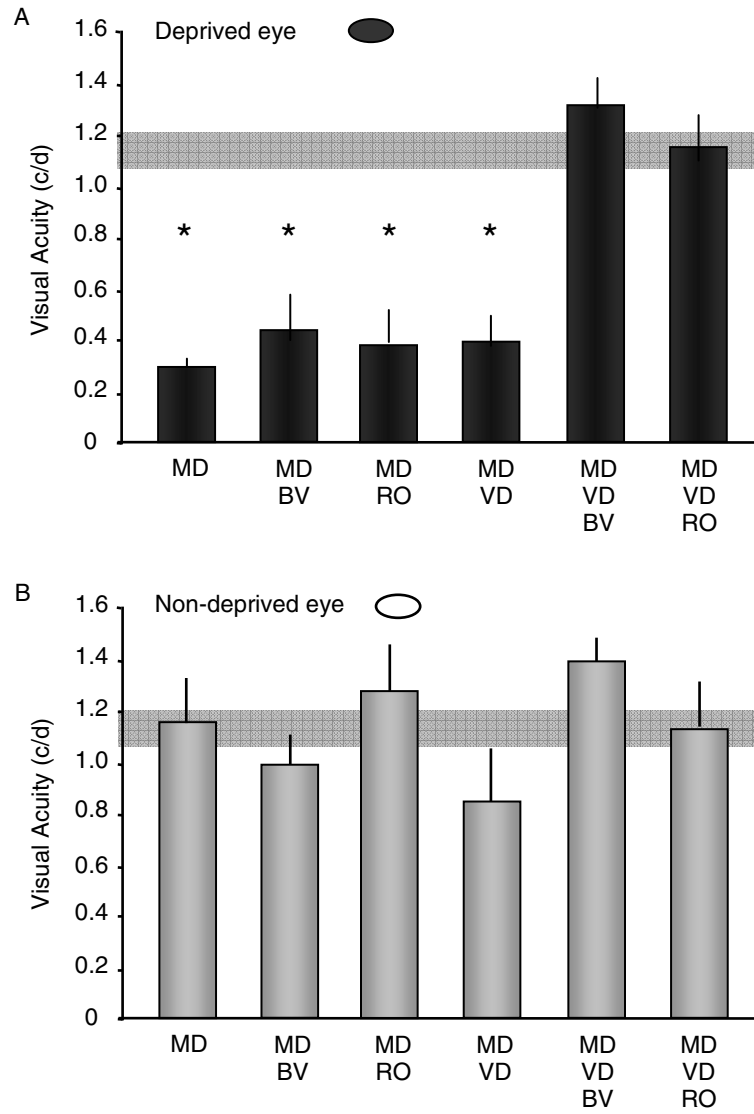
14 days), the spatial acuity in the chronically deprived eye recovers to normal (spatial acuity: MD-VD-BV=1.32±0.11, n=5; MD-VD-RO=1.14±0.11, n=8). The visual acuity of the non-deprived eye does not change significantly following any of the experimental manipulations (Figure 21B; spatial acuity: MD=1.17±0.17cpd; MD-BV=0.99±0.09cpd; MD-RO=1.28±0.16cpd; MD-VD=0.83±0.16cpd; MD-VD-BV=1.42±0.06; MD-VD-RO=1.14±0.14). These results demonstrate that the enhanced synaptic plasticity induced by visual deprivation in adulthood is sufficient to facilitate the recovery of cortical function in an eye deprived of vision from birth.

**Figure 20. Determination of spatial acuity using VEPs.** A. Representative VEP waveforms recorded from the binocular region of Visual cortex in response to stimulation of the contralateral eye in a normal reared adult. The amplitude of the primary response decreases as the spatial frequency of the visual stimulus increases. Noise is the response recorded to a blank screen of equal luminance. Scale bar: 100msec, 100 $\mu$ v. B. An example of spatial acuity assessment in a normal reared adult rat. The amplitude of the VEP (normalized to the maximal response recorded within the same session) is plotted against the spatial frequency log. Visual acuity is estimated as the spatial frequency extrapolated to zero amplitude of the linear regression through the last 5-8 data points above noise. C. An example of spatial acuity assessment in a chronically monocularly deprived rat. Note that the deprived eye has a lower spatial acuity than the non-deprived eye.



**Figure 20**

**Figure 21. VEPs reveal that visual deprivation promotes recovery of spatial acuity in the chronically deprived eye.** A. Chronic monocular deprivation from eye opening to adulthood results in a significant reduction in spatial acuity in the deprived eye. Binocular vision, reverse occlusion or visual deprivation in adulthood does not restore the spatial acuity of the chronically occluded eye. A significant recovery in spatial acuity is observed when binocular vision or reverse occlusion follows visual deprivation (One-way ANOVA ( $F_{(6, 30)} = 17.546$ ,  $p < 0.0001$ ), \*:  $p < 0.001$ , with Fisher's PLSD post hoc vs. NR). B. Chronic monocular deprivation or subsequent manipulation of binocular input does not change the spatial acuity of the non-deprived eye.



**Figure 21**

## **4.4 Discussion**

Chronic monocular deprivation initiated early during postnatal development induces significant changes in the organization and function of the visual cortex that are difficult to reverse in adulthood (Smith 1981; Smith and Holdefer 1985; Sparks et al., 1986; Mitchell 1988; Mitchell and MacKinnon, 2002; Iny et al., 2006; Prusky and Douglas, 2003; Pizzorusso et al., 2006). Here we show for the first time that following a period of visual deprivation, it is possible to recover function in an eye deprived of vision from birth by means of reverse occlusion or binocular vision in adulthood. The recovered function is mainly due to a potentiation of cortical response to stimulation of the deprived eye, resulting in a normal contralateral bias, normal VEP amplitudes and normal spatial acuity.

### **4.4.1 Factors affecting the functional recovery following chronic monocular deprivation**

A primary determinant of the level of ocular dominance plasticity available in the binocular region of the visual cortex is the history of visual experience. In many species, including the humans, monkeys, cats, ferrets and rodents, the degree of ocular dominance plasticity induced by monocular deprivation significantly decreases with age (Mitchell and MacKinnon, 2002; Hubel and Wiesel, 1968; Issa et al., 1999; Fagiolini et al., 1994; Guire et al., 1999; Sawtell et al., 2003). Visual deprivation, from birth or in adulthood, can enhance the level of synaptic plasticity available in the visual cortex (Cynader 1983; Mower 1991; Fagiolini et al., 1994; He et al., 2006). On the other hand, early visual experience, whether binocular, monocular, normal or

abnormal, shapes the anatomical connections within the visual system and constrains subsequent plasticity in adulthood. One exception is that if an ocular dominance shift has been induced by monocular deprivation before, it is easier to induce a second ocular dominance shift in adulthood following monocular deprivation of the same eye (Hofer et al., 2006). In this case, previous monocular experience seems to facilitate subsequent ocular dominance plasticity. However, this phenomenon is more likely to be a memory of previous experience that can be retrieved by similar manipulation, rather than a general facilitation of synaptic plasticity.

Chronic monocular deprivation induced early in postnatal development results in a significant loss of function in the deprived eye, including a reduction in the number of cortical neurons dominated by the deprived eye, as well as significant decrease in the acuity of the deprived eye. These phenomena have been reported in many species, such as rats (Iny et al., 2006; Prusky and Douglas, 2003; Pizzorusso et al., 2006), cats and monkeys (Smith 1981; Smith and Holdefer 1985; Sparks et al., 1986; Mitchell 1988). In addition to the functional decrement, there are also anatomical changes that accompany monocular deprivation, such as a decrease in the terminal arborization of thalamo-cortical axons serving the deprived eye (Antonini and Stryker 1993). Nonetheless, the connection between the deprived eye and binocular visual cortex must persist, to allow the recovery of function in juveniles following removal of the occlusion either by reverse occlusion or binocular vision.

Closing or removal (enucleation) of the initially non-deprived eye (reverse occlusion) has been used to reverse the effects of chronic monocular deprivation. Reverse occlusion helps the initially deprived eye to compete for postsynaptic cortical



neurons by suppressing activity from the strong eye and forcing the visual system to use the weak eye. Physiological and behavioral improvements have been obtained by reverse occlusion especially when the intervention is applied early in life (Movshon, 1976; Dräger, 1979; Blakemore et al., 1978; Swindale et al., 1981). A confound, though, has been reported in kittens, in that the visual function of the initially strong eye is deteriorated after reverse occlusion (Movshon, 1976).

Binocular vision, which increases the level of correlated activity from the weak eye but does not suppress inputs from the strong eye, has also been used to reverse the effects of chronic MD. Interestingly, in juveniles, the effectiveness of binocular vision is reported to be faster than reverse occlusion, and when combined with reverse occlusion, the recovery is more stable and longer lasting (Olson and Freeman, 1978; Mitchell et al., 2001; Kind et al., 2002). An explanation for this is that binocular vision allows the visual cortex to receive correlated activity from both eyes, which is important for recovery of function of binocular neurons. However, the effectiveness of binocular vision seems to differentiate between species. It works well in kittens, but in monkeys, the initially deprived eye cannot regain control over the cortical neurons unless the initially open eye is occluded (Blakemore et al., 1981). Moreover, reverse occlusion yields better final recovery of function in the deprived eye than binocular vision even in kittens (Mitchell et al., 2001). By all means, the recovery of visual function following chronic monocular deprivation is very primitive in adults, following either reverse occlusion or binocular vision (Dräger, 1978; Iny et al., 2006).

Another factor that might also affect the recovery from chronic monocular deprivation is the visual experience before the deprivation. In ferrets, it has been reported that recovery from chronic monocular deprivation is better in animals that have had some normal vision before the onset of deprivation than those that received monocular deprivation before eye opening (Liao et al., 2004). The facilitation of recovery by previous normal vision resembles the facilitated response to monocular deprivation by prior monocular deprivation reported by Hofer et al. (2006). Previous experience might have left some anatomical substrate in the cortex that can be retrieved by similar experience later in life. Some very limited recovery of spatial acuity in the chronically deprived eye has also been reported following normal binocular vision in adult rats that had received monocular deprivation a few days after eye opening, and thus had some normal vision in the deprived eye before deprivation (Iny et al., 2006). In contrast, enucleation of the strong eye does not induce functional improvement in the deprived eye of mice monocularly deprived from eye opening to adulthood (Dräger, 1978). To be as strict as possible, we chose to start the chronic monocular deprivation from eye opening, which would impose the most constraint on the recovery of function in the deprived eye in adulthood. Despite the extra difficulty, visual deprivation followed by reverse occlusion or binocular vision successfully recovers visual function in the eye deprived of vision from birth.

#### **4.4.2 Visual deprivation facilitates potentiation of the cortical response to stimulation of the initially deprived eye**

The recovery of function following juvenile monocular deprivation is likely due to an activity-dependent potentiation of the residual weak inputs that couple the deprived eye to the visual cortex (LeVay et al., 1980; Swindale et al., 1981; Blakemore et al., 1982; Freeman and Ohzawa, 1988). In adults, a gain of function following either reverse occlusion or binocular vision has been slow and modest, suggesting a developmental constraint in the ability to potentiate the residual inputs (Smith 1981; Mitchell 1988; Iny et al., 2006). In contrast, we demonstrate that a significant recovery of function can be achieved in adulthood following chronic monocular deprivation if the occlusion is removed after a period of binocular visual deprivation.

The functional recovery following chronic monocular deprivation involves an initial decrease in the cortical response to the non-deprived eye induced by visual deprivation, followed by an increase in the cortical response to the deprived eye induced by removal of the occlusion. A similar two-stage response to recovery from chronic monocular deprivation has been observed in kittens following reverse occlusion (Mitchell, 1991) and in adult rats following prolonged binocular vision (Iny et al., 2006). Interestingly, while a decrease in the potentiated response to the non-deprived eye may be necessary to enable potentiation of the response to the chronically deprived eye, it is not sufficient. Although binocular vision, reverse occlusion and visual deprivation all decrease the response to stimulation of the non-deprived eye, we observed no recovery in ocular dominance or spatial acuity of the deprived eye. The recovery of function in the chronically deprived eye is likely due to synapse-specific synaptic strengthening of the inputs serving the deprived eye, which

are enabled by visual deprivation, and proceed in the absence (as in reverse occlusion) or presence (as in binocular vision) of subsequent input from the non-deprived eye. Visual deprivation also allows repotentialization of the response to stimulation of the non-deprived eye when binocular vision is given following visual deprivation.

Pharmacological and genetic manipulations have implicated the developmental expression of particular molecules in mediating the developmental constraint in OD plasticity (Pizzorusso et al., 2006; McGee et al., 2005). However, the invasive nature of these manipulations, as well as the difficulty to use these methods to target a specific cortical region, may limit the applicability to other species, such as humans. Here we show that a relatively non-invasive manipulation, several days of visual deprivation in adulthood, allows for a rapid recovery of function in an eye deprived of vision from birth. Such a non-invasive method to restore rapid OD plasticity holds potential therapeutic promise, as the potential to reverse the visual deficits in human amblyopia decreases with age (Mitchell and MacKinnon, 2002).

## **Chapter 5: GENERAL DISCUSSION**

The level of synaptic plasticity at synapses in the mammalian cortex decreases over development. However, in adult cortex, large scale plasticity can be revealed by deafferentation or deprivation of the peripheral inputs. What happens following deafferentation of a single digit in the adult raccoon or complete visual deprivation in the adult rat that endows the cortex with higher level of plasticity? Are there different mechanisms for enhancing plasticity involved in these two situations? Or can they be explained by the same set of theory? In this chapter, I will discuss the general mechanisms underlying this deafferentation/deprivation-enhanced synaptic plasticity in adult cortex.

Following removal of either partial peripheral inputs, as seen in the single-digit deafferentation, or total peripheral inputs, as seen in visual deprivation, there is a decrease in the level of synaptic input activity in the cortical neurons dominated by the deprived input. First, BCM theory predicts that decrease in the level of input activity would slide the synaptic modification threshold of the deprived neurons to the left, enabling the potentiation of the weak synapses (Bienenstock et al., 1982). Second, the decreased modification threshold, together with redistribution of synaptic activity would result in Hebbian-like readjustment of the strength of synapses serving deprived and non-deprived inputs. This process can be viewed as a two-part problem, as there are two groups of synapses on the deprived neurons. On one hand, the synapses serving the deprived inputs, which are also the principal inputs of the

deprived cortical neurons, will be depressed. On the other hand, the weaker synapses serving the non-deprived inputs will be potentiated by enhanced activity in the non-deprived pathways.

### **5.1 Synaptic plasticity in response to single digit deafferentation in adult somatosensory cortex**

Cortical neurons in the adult somatosensory have restricted receptive fields. The anatomical basis of these restricted receptive fields is explained in part by the circuitry in the adult mammalian cortex. Thalamocortical projections send excitatory inputs to the layer IV excitatory neurons, and at the same time synapse onto inhibitory interneurons. The inhibitory interneurons provide feed forward inhibition that shut off the secondary excitatory inputs that originate from adjacent areas projecting onto the same postsynaptic neurons.

In the case of single-digit deafferentation, the first change following removal of the peripheral inputs is a disruption of the correlated pre- and postsynaptic activity in synapses on the deafferented cortical neurons. This de-correlation could lead to synaptic depression possibly by two means: 1. the firing frequency of the postsynaptic neurons evoked by presynaptic neurons falls below the modification threshold, thus would cause low frequency stimulation induced synaptic depression (Kirkwood et al., 1993); 2. The spikes of the presynaptic thalamocortical synapses lag behind the spikes of layer IV neurons evoked by the top-down feedback inputs from layer II/III, which would result in spike-timing dependent synaptic depression in layer IV neurons (Markram et al., 1997; Bi and Poo, 1998; Allen et al., 2003). Both

mechanisms will lead to a decrease in the efficacy of the synapses serving the deafferented inputs.

In contrast, on the same cortical neurons, the weak secondary synapses coming from the non-deprived inputs will be strengthened due to the lowered modification threshold initiated by deprivation of the primary input. The increase in AMPA receptors in the deafferented cortex might reflect the potentiation of the secondary synapses. It has also been suggested that sprouting from the non-deprived thalamocortical projections onto the deprived cortical neurons might also be involved in the deafferentation-induced receptive field reorganization (Buonomano and Merzenich, 1998). However, our biochemical data did not reveal an increase in NMDARs in the deafferented cortex, suggesting that there was not an increase in the number of glutamatergic synapses. The potentiation of the non-deprived synapses then brings the total activity input back to normal and eventually reaches a new balanced modification threshold.

## **5.2 Synaptic plasticity in response to visual deprivation and subsequent manipulation of visual experience in adult visual cortex**

In the case of visual deprivation, the primary input to the whole visual cortex is down regulated, with no competing inputs from within the same sensory module left. Although BCM theory predicts a lowered modification threshold, no change would be expected in the relative synaptic strength among all synapses due to the lack of binocular competition. Therefore, homeostatic mechanisms such as what described in synaptic scaling is more likely to be the predominant mode of the synaptic plasticity

(Turrigiano and Nelson, 2004). To maintain the output of visual cortical neural network, the excitability of the cortical neurons goes up. This is supported by our observation that the inhibition relative to excitation level decreased in the adult visual cortex following visual deprivation.

Other mechanisms that have been proposed to be underlying the sliding of modification threshold such as changes in NMDA receptors (Carmignoto and Vicini, 1992; Quinlan et al., 1999) subunit composition are also observed following visual deprivation in adults. The level of NR2b to NR2a ratio in synaptoneurosomes prepared from the binocular visual cortex is increased after visual deprivation. Increased NR2b/2a allows a longer time window for coincidence detection, which agrees with the prediction of BCM theory, indicating a lowered synaptic modification threshold for potentiation. Activation of NMDA receptors could also be facilitated by the increased excitability of neurons due to the decrease in inhibition over excitation. In addition, visual deprivation induces an increase in the level of tPA activity, which may initiate an enzymatic cascade through plasmin and cleave extracellular matrix substrates. A reduction in the ECM density would further facilitate morphological plasticity that might be needed for changes in the size and number of synapses. tPA-plasmin system is also known to process pro-BDNF into mature BDNF, the latter has been shown to facilitate potentiation of the synapses (LTP) (Bai et al., 2004). A working model for the molecular mechanisms involved in visual deprivation-enhanced plasticity consistent with our data is proposed in figure 22.



**Figure 22. Molecular mechanisms underlying visual deprivation enhanced plasticity.** Decreased activity due to visual deprivation induces a decrease in inhibition and a scaling up of excitation, which in turn induces NMDA receptor subunit switch and increases tPA activity. Increased NR2b relative to NR2a level allows enhanced Calcium influx into the postsynaptic neuron when activated. Increase tPA activity helps degrading the extracellular matrix (ECM) and increases processing pro-BDNF into mature-BDNF. All of these changes, as well as other unknown factors, lead to changes in the threshold for synaptic modification.

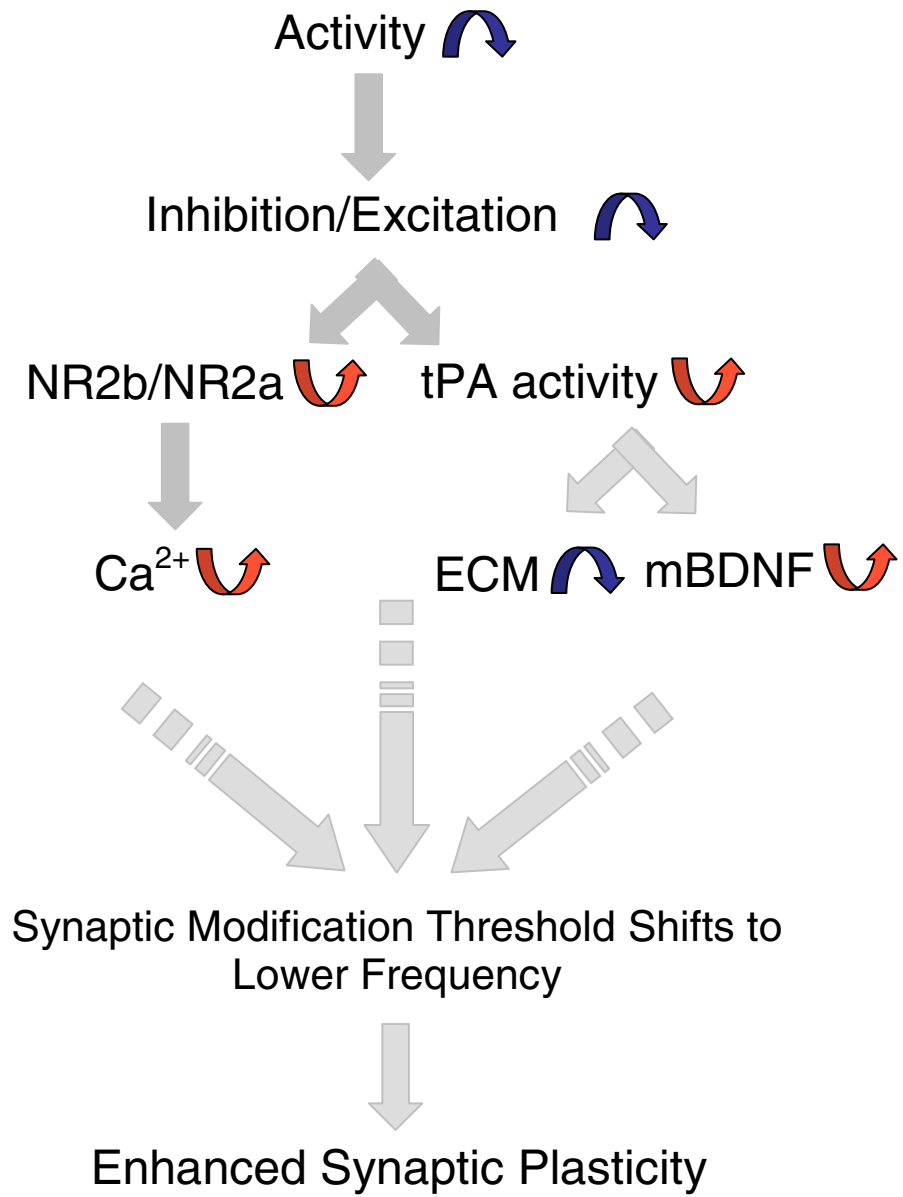


Figure 22

Visual deprivation in adults induces metaplastic changes, including a sliding of the modification threshold of visual cortical neurons to the left. This response to visual deprivation is thought to bestow the visual cortex with higher level of synaptic plasticity as demonstrated by a recovery of ocular dominance shift in response to 3-day monocular deprivation. The response of binocular neurons to the brief monocular deprivation following visual deprivation could be explained by Hebbian-learning. Deprivation of visual input in one eye (or allowing only one eye patterned vision after visual deprivation) disrupts the competitive balance between inputs from the two eyes. Synapses serving the deprived eye were de-correlated and then depressed, while synapses serving the non-deprived eye were potentiated as a result of the lowered threshold and subsequent experience. It is also possible that the depressed response to the deprived eye is a remnant of the effect of the visual deprivation. As shown in the chronic monocular deprivation experiments, visual deprivation itself was sufficient to depotentiate the level of response to the originally non-deprived eye in the ipsilateral visual cortex. Subsequent visual experience in the eye following visual deprivation potentiates the synapses serving that eye regardless of experience in the other eye.

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## Chapter 6: CONCLUSIONS

Synaptic plasticity is robust in juveniles, but significantly constrained in adults. Despite the decline in synaptic plasticity in adult cortex, it can be revealed by sensory deprivation by two means: 1) directly inducing reorganization of cortical circuits by changing the pattern of peripheral sensory inputs, such as deafferentation by single digit amputation and 2) increasing the level of synaptic plasticity available to the cortical circuits and facilitation of subsequent experience-dependent synaptic modifications, as seen in visual deprivation. Deafferentation employs a partial deprivation of the sensory cortex and shifts the balance between competing peripheral inputs. Removal of the primary inputs unmask the secondary weak inputs and resulted in receptive field reorganization. In contrast, visual deprivation completely deprives the visual system of its sensory input, resulting in a dramatic reduction in the total amount of activity. This induces metaplastic mechanisms and enhances the level of synaptic plasticity in the cortex that can respond to subsequent experience.

Using single digit deafferentation in adult raccoons, I have shown progressive elevation in the levels of glutamate and GABA receptors in the deafferented somatosensory cortex, which suggests progressive elevation in excitation and inhibition following deafferentation. The transient increase in AMPARs occurs immediately after deafferentation, and correlates with the expansion of the receptive fields. The delayed increase in GABA<sub>A</sub>Rs correlates with the refinement of the newly expanded receptive fields.

Visual deprivation presumably causes a reduction in the total activity level in the visual cortex. Using visual deprivation in adult rats, I have shown a significant decrease in the level of GABA receptors relative to glutamate receptors in the visual cortex, which is likely to result in a significant decrease in the inhibition relative to excitation level. The decreased inhibition over excitation level would not only help to maintain the total output level of the visual cortex but also lowers the threshold for activity-dependent remodeling of cortical circuits.

The changes in excitation over inhibition observed in both model systems suggest that this balance plays a critical role in allowing plastic changes in adult cortical circuits. How does the level of inhibition over excitation modulate the level of synaptic plasticity? One possible target is the regulation of NMDA receptor activation. Since the level of inhibition relative to excitation directly determines the level of excitability of the neurons, a reduction in this value would increase excitability, which would allow stronger depolarization, and facilitating NMDA receptor activation. Another possibility is that the inhibition over excitation balance regulates tPA activity. I have shown that the decrease in the level of inhibition over excitation in the adult visual cortex following visual deprivation is correlated with an increase in tPA activity level. Increased tPA activity could activate a proteolytic enzymatic cascade, which could either degrade extracellular matrix proteins to allow experience-dependent morphological (structural) changes, or cleave pro-BDNFs to BDNF and activate other downstream reactions that interfere with synaptic plasticity. Other work in the lab also supports the role of tPA in regulating synaptic plasticity.

I have also shown a return to the juvenile form of NMDA receptor subunit composition (with a higher NR2b/NR2a) following visual deprivation. The increase in NR2b/NR2a level is expected to slide the LTP/LTD threshold to the left along the frequency axis and would lower the threshold for LTP induction thus facilitates the potentiation of the synapses serving the open eye.

I have demonstrated that visual deprivation can enhance ocular dominance plasticity in adult rat visual cortex. Analysis of the cortical response to brief monocular deprivation following visual deprivation in adults demonstrated that the enhanced synaptic plasticity is rapid and juvenile-like. I further explored the potential of utilizing visual deprivation for therapeutic strategy, by demonstrating that visual deprivation can promote recovery of function in an eye that is deprived of vision from birth until adulthood.

This work shows that ocular dominance plasticity can be reversibly regulated by experience throughout life, and proposes a non-invasive method to enhance synaptic plasticity in adult cortex. Future studies will focus on the effects of direct manipulations of the molecules implicated on the response to visual deprivation, and the efficacy of visual deprivation in reversing effect of chronic monocular deprivation in other species such as cats and monkeys.

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