MOLECULAR MECHANISM OF LONG-TERM DEPRESSION AND ITS ROLE IN EXPERIENCE-DEPENDENT OCULAR DOMINANCE PLASTICITY OF PRIMARY VISUAL CORTEX

by

WEI XIONG

B. Medicine, Tongji Medical University, 1998

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY in THE FACULTY OF GRADUATE STUDIES (Neuroscience)

THE UNIVERSITY OF BRITISH COLUMBIA (Vancouver)

December 2008

© Wei Xiong, 2008
ABSTRACT

Primary visual cortex is a classic model to study experience-dependent brain plasticity. In early life, if one eye is deprived of normal vision, there can be a dramatic change in the ocular dominance of the striate cortex such that the large majority of neurons lose responsiveness to the deprived eye and, consequently, the ocular dominance distribution shifts in favor of the open eye. Interestingly, the visual experience dependent plasticity following monocular deprivation (MD) occurs during a transient developmental period, which is called the critical period. MD hardly induces ocular dominance plasticity beyond critical period. The mechanisms underlying ocular dominance plasticity during the critical period are not fully understood. It has been proposed that long-term depression (LTD) may underlie the loss of cortical neuronal responsiveness to the deprived eye. However, discordant results have been reported in terms of the role of LTD and LTP in visual plasticity due to the lack of specific blockers. Here we report the prevention of the normally-occurring ocular dominance (OD) shift to the open eye following MD by using a specific long-term depression (LTD) blocking peptide derived from the GluR2 subunit of the a-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid receptor (AMPAR). We were able to prevent the shift of OD to the open eye with systemic or local administration of the GluR2 peptide. Both electrophysiological and anatomical approaches were taken to demonstrate the peptide effect. Moreover, enhancing LTD with D-serine, a NMDA receptor co-agonist, brought back the ocular dominance plasticity in adult mice subject to four-day MD and, therefore, reopened the critical period. Our data indicate that LTD plays an essential role in visual plasticity
during the critical period and the developmental regulation of LTD may account for the closure of critical period in adult.

In an additional study, we have found anisomycin, a protein synthesis inhibitor, produces a time-dependent decline in the magnitude of the field EPSP (fEPSP) in mouse primary visual cortex and that this anisomycin-mediated fEPSP depression occludes NMDA receptor dependent LTD. In contrast, another two protein synthesis inhibitors, emetine and cycloheximide, have no effect either on baseline synaptic transmission and or on LTD. We propose that anisomycin-LTD might be mediated by p38 MAP kinase since anisomycin is also a potent activator of the P38/JNK MAPK pathway. In agreement with notion, the decline of the fEPSP caused by anisomycin can be rescued by the application of the P38 inhibitor SB203580, but not by the JNK inhibitor SP600125. The occlusion of LFS-LTD by anisomycin-induced fEPSP decline suggests that common mechanisms may be shared between the two forms of synaptic depression. Consistent with this view, bath application of the membrane permeant peptide discussed above, which specifically blocks regulated AMPA receptor endocytosis, thereby preventing the expression of LFS-LTD, prior to anisomycin treatment significantly reduced the anisomycin-induced decline of the fEPSP. In conclusion, this study indicates that anisomycin produces long-lasting depression of AMPA receptor-mediated synaptic transmission by activating P38 MAPK-mediated endocytosis of AMPA receptors in neonatal mouse visual cortex.
# TABLE OF CONTENTS

ABSTRACT .......................................................................................................................... ii

TABLE OF CONTENTS ........................................................................................................ iv

LIST OF FIGURES ............................................................................................................ vii

LIST OF ABBREVIATIONS ................................................................................................ viii

ACKNOWLEDGEMENTS ..................................................................................................... x

CO-AUTHORSHIP STATEMENT ........................................................................................ xi

CHAPTER I  Introduction ........................................................................................................ 1

1.1 Discovery of experience dependent visual plasticity .................................................. 2
1.2 Anatomy and physiology of primary visual cortex ..................................................... 8
1.3 Critical period .............................................................................................................. 15
1.4 Importance of understanding visual plasticity .......................................................... 22
1.5 Mechanism of ocular dominance plasticity ............................................................... 24
1.6 Hypotheses and objectives ......................................................................................... 40
1.7 References ................................................................................................................. 41

CHAPTER II  Selective LTD blockade prevents experience-dependent plasticity in the visual cortex during the critical period .............................................. 57

2.1 Introduction .............................................................................................................. 58
2.2 Materials and methods .............................................................................................. 61
2.3 Results ..................................................................................................................... 68
2.4 Discussion ............................................................................................................... 93
2.5 References ................................................................................................................. 101

CHAPTER III  Anisomycin activates p38 MAP kinase to induce LTD in mouse primary visual cortex ........................................................................................................... 108

3.1 Introduction .............................................................................................................. 109
3.2 Materials and methods .............................................................................................. 111
3.3 Results ..................................................................................................................... 115
3.4 Discussion ............................................................................................................... 130
3.5 References ................................................................................................................. 137
| 4.1 | LTD in ocular dominance plasticity during the critical period | 143 |
| 4.2 | Timing of the critical period of ocular dominance plasticity | 145 |
| 4.3 | Clinical implications | 149 |
| 4.4 | P38 MAP kinase mediates anisomycin-LTD in primary visual cortex | 150 |
| 4.5 | LTD/LTP is not the only means by which experience modifies neuronal network | 151 |
| 4.6 | Future directions | 152 |
| 4.7 | References | 154 |
LIST OF FIGURES

Figure 1.1 First study of ocular-dominance distribution by Hubel and Wiesel……………5

Figure 1.2 First study to show ocular-dominance plasticity………………………………………6

Figure 1.3 Monocular deprivation altered the ocular dominance columns in cat…………7

Figure 1.4 The visual pathway .................................................................................................11

Figure 1.5 Structure of primary visual cortex.........................................................................12

Figure 1.6 Ocular dominance columns.......................................................................................13

Figure 1.7 Orientation columns................................................................................................14

Figure 1.8 Ocular dominance plasticity in mouse visual cortex..............................................21

Figure 1.9 NMDA receptor dependent LTD is mediated by clathrin-dependent

          endocytosis of AMPA receptor....................................................................................32

Figure 1.10 Synthesized peptide derived from GluR2 subunit of AMPAR: a specific

          blockade for LTD.............................................................................................................35

Figure 2.1 GluR2₃₃ peptide blocks LTD in vitro.................................................................69

Figure 2.2 Biotinylation assay of surface AMPARs in the presence of TAT-

          GluR2₃₃ during LTD.....................................................................................................72

Figure 2.3 GluR2₃₃ peptide prevents the ocular dominance shift in vivo...........................75

Figure 2.4 Acute application of Tat-GluR2₃₃ did not affect spontaneous and

          evoked activities in primary visual cortex..................................................................78

Figure 2.5 Local infusion of Tat-GluR2₃₃ in the primary visual cortex blocked the
Figure 2.6 Data summary of the binocularity index results in the various conditions studied.

Figure 2.7 Zif268 staining confirmed the prevention of the OD shift by Tat-GluR23Y.

Figure 2.8 Developmental regulation of LTD.

Figure 2.9 D-Serine facilitates LFS-induced LTD in primary visual cortex of adult mouse.

Figure 2.10 D-serine treatment reopens ocular dominance plasticity in adult mice.

Figure 3.1 Anisomycin induced LTD in the primary visual cortex and occluded the LFS induced LTD.

Figure 3.2 Anisomycin induced LTD is mediated by the activation of P38/JNK MAPK pathway rather than its ability to inhibit protein synthesis.

Figure 3.3 Anisomycin induced LTD requires activation of P38 MAPK.

Figure 3.4 Activation of p38 induced endocytosis of surface AMPA receptors.

Figure 4.1 Schematic describing the molecular mechanism underlying ocular dominance plasticity.
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AMPA</td>
<td>a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid</td>
</tr>
<tr>
<td>AP2</td>
<td>Activating Protein 2</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BZ</td>
<td>binocular zone</td>
</tr>
<tr>
<td>CBI</td>
<td>contralateral bias index</td>
</tr>
<tr>
<td>D-APV</td>
<td>D-enantiomer</td>
</tr>
<tr>
<td>dLGN</td>
<td>Dorsal lateral geniculate nucleus</td>
</tr>
<tr>
<td>Egr-1/ZIF 268</td>
<td>Growth Response Protein 1</td>
</tr>
<tr>
<td>EPSCs</td>
<td>excitatory postsynaptic currents</td>
</tr>
<tr>
<td>EPSP</td>
<td>extracellularly recorded excitatory postsynaptic potentials</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GAD65</td>
<td>glutamic acid decarboxylase 65</td>
</tr>
<tr>
<td>GDI</td>
<td>guanyl nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GluR</td>
<td>glutamate receptor</td>
</tr>
<tr>
<td>GSK3β</td>
<td>glycogen Synthase Kinase 3 beta</td>
</tr>
<tr>
<td>Ip</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>Irβ</td>
<td>Insulin receptor beta</td>
</tr>
<tr>
<td>JNK</td>
<td>C-jun N-terminal kinase</td>
</tr>
<tr>
<td>LFS</td>
<td>low frequency stimulation</td>
</tr>
<tr>
<td>LGN</td>
<td>Lateral geniculate nucleus</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>LTD</td>
<td>long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen-activated protein</td>
</tr>
<tr>
<td>MD</td>
<td>monocular deprivation</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptors</td>
</tr>
<tr>
<td>MZ</td>
<td>monocular zone</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NR</td>
<td>NMDA receptor</td>
</tr>
<tr>
<td>NSF</td>
<td>N-ethylmaleimide sensitive fusion protein</td>
</tr>
<tr>
<td>OD</td>
<td>Ocular dominance</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase 1</td>
</tr>
<tr>
<td>Rap</td>
<td>receptor-associated protein</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to acknowledge my supervisor and mentor Dr. Max Cynader for his guidance, advice, and enthusiasm. Dr. Cynader offered me incredible opportunities and freedom to explore the experience-dependent brain plasticity. I am also grateful for his understanding and tremendous support during my application for residency training. With his selfless help, I am now able to continue my medical career and achieve the goal to be a physician scientist.

I would like to thank my supervisory committee members Dr. Joanne Matsubara, Dr. Nick Swindale, and Dr. Yutian Wang for their advice and encouragement. I am impressed and encouraged by their dedication, curiosity, and passion in pursuit of science.

I would like to give special thanks to Dr. Kojic Luba for his persistent support during my training at UBC. I would also love to acknowledge all my colleagues in Dr. Cynader’s lab, including Dong Qiang, Stephani Thompson, Wendy Wen, Ainsley Conquinco, Shanshang Zhu, and Guang Yang. It is a great pleasure to work with them.
CO-AUTHORSHIP STATEMENT

I was responsible for experimental design, data collection, statistical analysis and interpretation of all results, as well as manuscript preparation for studies in this thesis. Dr. Max Cynader, Dr. Yutian Wang, Dr. William Jia, and Dr. Luba Kojic revised the manuscripts and provided very valuable comments.

The experiment described in chapter 2, “Figure 2.2 Biotinylation assay of surface AMPARs in the presence of TAT-GluR23Y during LTD”, was completed in collaboration with Changiz Taghibiglou from Dr. Yutain Wang’s laboratory.

The experiment described in chapter 2, “Figure 2.4 Acute application of Tat-GluR23Y did not affect spontaneous and evoked activities in primary visual cortex”, was completed in collaboration with Dr. Luba Kojic. He set up the system which allowed us quantify the neuronal activity.
Chapter I

Introduction
1.1 Discovery of experience dependent visual plasticity

The mammalian cortex is organized anatomically into discrete areas, which receive, process, and transmit neural signals along functional pathways. The ability of the nervous system to wire and rewire itself in response to lasting changes in experience has become known as experience-dependent plasticity (Berardi, Pizzorusso et al. 2000; Hensch 2005). The brain is able to remodel its connections in order to adjust its responses to changing conditions. Particularly during early development, the brain shows the greatest plasticity when connections between neurons are being made and broken for the first time (Giza and Prins 2006; Horng and Sur 2006). For example, it is much easier to learn a language in childhood than in adulthood. Studying brain plasticity can help us understand how the complex neuronal networks wire up through development and refine their connections into adulthood. Furthermore, understanding developmental brain plasticity may make it possible to reawaken the critical period mechanism in the adult brain to help patients recover from pathological conditions, such as stroke trauma and neurodegenerative diseases (Hannan 2004; Nithianantharajah and Hannan 2006; Kleim and Jones 2008).

Primary visual cortex has been one of the classic models to study experience dependent brain plasticity for decades. Clear evidence for experience dependent visual plasticity has been found in the primary visual cortex of several species (Hubel and Wiesel 1963; Hubel, Wiesel et al. 1977; Bear and Colman 1990; Bear 2003; Hensch 2004). Over forty years ago, the classic studies of Wiesel and Hubel first established that
most cortical neurons, in normal kittens, respond to stimulation of either eye with varying
degrees of ocular dominance (Hubel 1963) (Figure 1.1). If an animal is allowed to
mature in a normal visual environment, these binocular connections in the cortex are
retained and normal visual receptive fields are formed for each eye. However, if one eye
is deprived of normal vision during the first few months of life, there can be a dramatic
change in the ocular dominance of the striate cortex such that the large majority of
neurons lose responsiveness to the deprived eye and, consequently, the ocular dominance
distribution shifts in favor of the open eye (Hubel and Wiesel 1963) (Figure 1.2).

In addition to the remarkable changes in physiological responses following
monocular deprivation, the anatomical connections of visual cortex are also altered by
manipulating visual experience (Shatz and Stryker 1978). In cat and monkey, visual
cortical neurons with similar ocular dominance are clustered together to form a radial
column running from pia to white matter. The columns alternatively receive
thalamocortical projections from either eye and can be visualized by injection of
transneuronal radioactive dye into one eye. Thirty years ago, two groups for the first
time demonstrated that monocular deprivation in early life was able to shrink the columns
serving the deprived eye and expand the normal eye columns in cat and monkey (Hubel,
Wiesel et al. 1977; Shatz and Stryker 1978) (Figure 1.3). Consistent with the findings in
cat and monkey, ocular dominance plasticity has also been discovered in other species,
such as rodents (Gordon and Stryker 1996; Antonini, Fagiolini et al. 1999), ferret (Issa,
Trachtenberg et al. 1999), baboon (Hendrickson, Wilson et al. 1978), Chimpanzee
(Tigges and Tigges 1979), sheep (Pettigrew, Ramachandran et al. 1984), rabbit(Hollander
and Halbig 1980), and human (Hitchcock and Hickey 1980; Horton and Hedley-Whyte 1984).

Therefore, both physiological and anatomical findings indicate the presence of experience dependent brain plasticity in primary visual cortex. Furthermore, the convenience of manipulating visual experience and the presence of only two discrete inputs make the visual cortex an excellent model to study the mechanism underlying experience dependent brain plasticity.
Figure 1.1 First study of ocular-dominance distribution by Hubel and Wiesel

A, Hand-made reconstruction of a microelectrode penetration through the postlateral gyrus of the cortical hemisphere in kitten. Adapted from Wiesel & Hubel, J Neurophysiol, 1963; 26: 1003-1017

B, Ocular-dominance distribution of 233 cells recorded from striate cortex of cat. Group 1 was driven only by contralateral eye while group 7 was only driven by ipsilateral eye. Contralateral dominance was marked for group 2 and was slight for group 3. Ipsilateral dominance was marked for group 6 and was slight for group 5. Group 4 equally responded to either eye. Adapted from Wiesel & Hubel, J Neurophysiol, 1963; 26: 1003-1017
Figure 1.2  First study to show ocular-dominance plasticity.

A, 6 months old kitten with right eye closed at 9 weeks. Ocular dominance shifted in favor of the open eye. Adapted from Wiesel & Hubel, J Neurophysiol, 1963; 26: 1003-1017.

B, Adult cat with right eye closed for 3 months. As in normal cat, the contralateral eye dominated. Ocular dominance shift was not observed. Adapted from Wiesel & Hubel, J Neurophysiol, 1963; 26: 1003-1017.
**Figure 1.3 Monocular deprivation altered the ocular dominance columns in cat.**

**A, B,** Cells of layer IV in normal cat were clustered according to ocular dominance. White patches represent the columns serving the eye through which the radioactive label was injected.

**C, D,** Columns that serve the deprived eye significantly shrunk following early MD.

**E, F,** Columns that serve the open eye significantly expanded following early MD.

Adapted from Shatz and Stryker, J Physiol. 1978 Aug;281:267-83.
1.2 Anatomy and physiology of primary visual cortex

The primary visual pathway is composed of retina, dorsal lateral geniculate nucleus (dLGN), and visual cortex (Figure 1.4). Retinal ganglion cells from each eye send visual information to separate layers of the LGN; layer IV of the primary visual cortex receives the geniculocortical axons from LGN and relays the input to extragranular layers (layer II, III, V, and VI) (Figure 1.5). In fact, primary visual cortex is the first site at which visual information acquired from each eye is integrated to form the basis of binocular visual perception (Antonini and Stryker 1993; Antonini, Gillespie et al. 1998).

1.2.1 Functional columns of primary visual cortex

Every horizontal cortical layer contains different cell types, such as pyramidal cell and basket cells, and forms different types of connections with other neurons. However, a strong vertical organization is also apparent. For example, neurons stacked on top of each other through the depth of the cortex tend to be connected and share similar ocular dominance and orientation selectivity despite residing in different layers. The ocular dominance columns of primary visual cortex were first described by Hubel and Wiesel in 1963 in cat and subsequently discovered in monkey (Hubel, Wiesel et al. 1977; Hubel 1988) (Figure 1.6). More interestingly, the size of ocular dominance column can be altered by manipulating the visual experience during critical period (Hubel, Wiesel et al. 1977; Fagiolini, Pizzorusso et al. 1994) (Figure 1.6). Another type of columnar organization observed in the visual cortex is orientation columns (Bosking, Zhang et al. 1997). Many neurons in visual cortex respond best to an edge or bar of light at a specific orientation. This preferred orientation remains roughly constant through the depth of the
cortex but varies mostly smoothly across the surface of the cortex (Figure 1.7). A notable feature is the presence of pinwheels, point singularities around which all orientations are represented in a radial pattern. Superimposing the ocular dominance and orientation maps from the same animal, one observes regular geometric relationships between the two columnar systems in monkeys (Bosking, Zhang et al. 1997). For instance, ocular dominance and orientation columns tend to meet at right angles, and orientation pinwheels tend to lie at the center rather than at the borders of ocular dominance columns. In addition, several other types of functional columns are also present in primary visual cortex, such as position columns, columns with preference for a particular spatial frequency of a stimulus, columns with preference for the direction of movement of a stimulus, and columns representing disparity of inputs from the two eyes (Ben-Shahar, Huggins et al. 2003; Lund, Angelucci et al. 2003; Tanaka 2003; McClelland, Garcia et al. 2006). Moreover, similar functional columns have also been discovered in primary auditory cortex, somatosensory cortex, and motor cortex (Mountcastle 1957; Sutter and Schreiner 1995).

1.2.2 Physiological importance of cortical columns

The presence of a columnar organization in various regions of the cortex of many mammalian species has suggested that columns form the basic information processing elements of the cortex, with each column being responsible for analyzing a small range of stimuli, and the same modular unit being repeated multiple times to span the entire range of stimuli (Szentagothai 1978). Therefore, it has been hypothesized that columns represent a fundamental functional unit for perception, cognition, memory and even
consciousness (Szentagothai 1978; Eccles 1981). However, at present there is no general agreement to account for the existence of columns. One argument is that such columnar structure has not been found in some mammalian species, such as rodents (Drager 1978; Purves, Riddle et al. 1992; Gordon and Stryker 1996). The primary visual cortex of rodent can be divided into a monocular zone serving only the contralateral eye and binocular zone serving both eyes, but no columnar structure has been identified (Gordon and Stryker 1996). Thus, the columnar organization of the cortex may not always imply a functional module (Swindale 1990; Purves, Riddle et al. 1992). In particular, it has been suggested that production of iterated patterns of circuitry might be an incidental consequence of the activity-dependent elaboration of synaptic connections and be of little significance to cortical function (Purves, Riddle et al. 1992).
Figure 1.4 The visual pathway

Visual information is converted into an electric signal in the retina and relayed to lateral geniculate nucleus (LGN). The LGN further relays the information to primary visual cortex located in the dorsal occipital lobe. Adapted from lecture notes of Anatomy 530a, University of Western Ontario.
Figure 1.5 Structure of primary visual cortex

In the visual cortex, the cell bodies of the neurons are stratified into six layers that typify the primate neocortex. In this 2 mm thick grey matter, the six layers are numbered from I to VI, starting from the outside (the layer in contact with the pia mater) to the bottom (the layer in contact with the white matter). Each layer is distinguished both by the type of neurons that it contains and by the connections that it makes with other areas of the brain. Adapted from online resource at The Brain From Top to Bottom, Mcgill University.
**Figure 1.6 Ocular dominance columns**

Ocular dominance columns in a monkey. Each afferent axon from LGN ascends through the deep layers of V1 (layers 5, 6) subdividing repeatedly and terminating in layer 4C in a couple of 0.5mm-wide clusters separated by 0.5mm-wide gaps (approximately). Axons from the two eyes alternate, leading to ocular dominance columns in 4C. The presence of horizontal connections and the arborizations between different layers bring about overlapping and blurring of ocular dominance columns beyond layer 4: the ocular dominance of a given cell varies then between pure monocularity and pure binocularity. Adapted from Hubel, D. H. (1988). Eye, Brain, and Vision. Number 22 in Scientific American Library.
Figure 1.7 Orientation columns

The orientation map in primary visual cortex of a tree shrew. The different colours represent patches that have different orientation preferences. The detail shows a pinwheel, where the orientation preference changes by 180° along a closed path around the center.

1.3 Critical Period

1.3.1 Critical period of ocular dominance plasticity

Interestingly, the visual experience dependent plasticity following monocular deprivation occurs during a transient developmental period, which can be measured by single-unit electrophysiology (Hubel 1970; Prusky and Douglas 2003) or anatomical methods (Fagiolini, Pizzorusso et al. 1994; Tagawa, Kanold et al. 2005). In all cases, plasticity wanes gradually rather than ceasing abruptly (Mower 1991). For instance, visual plasticity of kitten is very low at 3 weeks, rises sharply at 6 weeks, and gradually declines over the next 10 weeks (Mower 1991). This transient period in which visual plasticity is easily detected is called the critical period. Beyond the critical period, it becomes very difficult to alter the ocular dominance of visual cortical neurons, even with extended periods of MD (Hubel 1970; Gordon and Stryker 1996). Therefore, the critical period reflects an extreme form of sensitivity that the brain has in response to external stimuli during development (Hensch and Stryker 2004). In addition, the duration of the critical period is tightly correlated with average life expectancy. In rodents and cats, for example, plasticity is low at eye opening, peaks around four weeks of age, and declines over several weeks to months (Hubel 1970; Fagiolini, Pizzorusso et al. 1994; Daw, Reid et al. 1995; Gordon and Stryker 1996). In humans, the critical period for amblyopia is set by the age of eight to ten years (Daw, Reid et al. 1995).

1.3.2 Experience shapes neuronal network in the critical period

Notably, the critical period is not a simple, genetically determined, age dependent maturational process, but is rather a series of events itself controlled in a use-dependent
manner. The onset and duration of the critical period are regulated by experience-dependent development of the neuronal network rather than age. For instance, animals reared in complete darkness from birth express a delayed onset of the visual plasticity persisting into adulthood (Mower 1991; Fagiolini, Pizzorusso et al. 1994; Iwai, Fagiolini et al. 2003). Furthermore, Mower showed that dark rearing slowed the entire time course of the critical period. In dark reared cats, plasticity rose steadily over the first 12 weeks, and was maintained at 16 weeks (Mower 1991). Interestingly, the delayed critical period in dark-reared rats can be brought back to nearly normal when rats grow in conditions of environmental enrichment (large cages with running wheels and toys) (Maffei, Nelson et al. 2004). More interestingly, an early onset of the critical period can be induced by overexpression of BDNF (Huang, Kirkwood et al. 1999). In the transgenic mouse model, accelerated postnatal rise of brain-derived neurotrophic factor (BDNF) results in a precocious onset and an earlier termination of the critical period for ocular dominance plasticity (Huang, Kirkwood et al. 1999). These observations suggest that two players are present in the critical period. One is the basic structure and function of nervous system, as determined by individual genome. The other is the external environment to which the individual is exposed to. The dynamic interaction between both sides tailors the neuronal network to best fit the individual need.

1.3.3 Physiological importance of the critical period

What impact does the normal visual experience have on the development of visual cortex during the critical period? The mammalian primary visual cortex is largely immature at the time of eye opening (Blakemore and Van Sluyters 1975; Sherman and
Spear 1982; Boothe, Dobson et al. 1985; Fagiolini, Pizzorusso et al. 1994). For example, visual acuity is much less than that of the adult level. Moreover, selectivity of visual cortical neurons for orientation and movement direction of visual stimuli is poor. Ocular dominance and binocular vision are rudimentary (Blakemore and Van Sluyters 1975; Sherman and Spear 1982; Boothe, Dobson et al. 1985; Fagiolini, Pizzorusso et al. 1994). The ability to see higher resolution, focusing ability (accommodation), eye muscle coordination (aiming or alignment), and stereopsis are partially developed by 6 months of age in humans. Under the guidance of normal visual experience, visual cortex is able to form proper connections which allow the messages from the two eyes to be coordinated and allow us to see in depth. On the other hand, the binocular cells in the visual cortex are sensitive to small disparities in the visual scene projected onto the retina at the back of the two eyes. If the two eyes are slightly out of alignment (strabismus) or one eye is covered or has a cataract, the binocular connections do not form properly and stereoscopic depth perception does not develop (Hubel and Wiesel 1998). Therefore, it is believed that the appropriate development of the binocular system is the main purpose of the critical period in development (Hensch 2005).

1.3.4 Multiple critical periods in visual system

The critical period of ocular dominance is the focus of my thesis. However, it is worth noting that different aspects of visual function have different critical periods. For example, Harwert investigated the effects of monocular deprivation on all types of visual information processing (Harwerth, Smith et al. 1986). The basic spectral sensitivity functions of rods and cones have relatively short critical periods of development spanning
between 3 and 6 months. In contrast, the more complex functions, such as monocular spatial vision or resolution and binocular vision, have critical periods of 25 months or even longer (Harwerth, Smith et al. 1986). Therefore, there are multiple, partially overlapping sensitive periods of development and the critical period for each specific visual function is probably different. In general, there is a logical sequence of critical periods, ending earlier for functions dealt with at lower levels of the system (Daw and Beaver 2001). Moreover, different structures in the visual pathways also have different critical periods. This is evident, for example, from studies showing that many years of monocular deprivation has little functional consequence for neurons in the lateral geniculate nucleus of monkeys (Levitt, Schumer et al. 2001), yet has devastating consequences for neurons in primary visual cortex (Hubel, Wiesel et al. 1977; Kiorpes and Movshon 2004).

1.3.5 Critical periods of other systems

Other than the visual system, the critical periods have also been found in other systems. A good example is the cortical “barrels” map of rodent cortex that represents rodent whisker mosaics in a point-to-point manner on the somatosensory cortex surface. During the critical period (< 7 days of age), sensory deprivation induced by damaging whisker follicles can produce gross changes in the nascent barrel field maps. The anatomical shrinkage of cortical barrels is strictly limited to the one serving the deprived whisker (Van der Loos and Woolsey 1973). In auditory system, another sensory system, selective exposure to a particular frequency during a critical period (postnatal 3-7 weeks)
generates an overpresentation in the final tonotopic map. The tonotopic critical period can also be delayed by exposing animal in a uniformly noisy environment (Chang and Merzenich 2003). In the motor system, appropriate critical period development is a prerequisite to proper motor function later in life. At the neonatal neuromuscular junction, motor axons compete for a single target muscular fiber before postnatal 12 days. This process of refinement can be slowed or accelerated by manipulating neuromuscular activity (Thompson 1985; Sanes and Lichtman 1999). Not surprisingly, there is also critical period for more advanced functions, such as language. The cumulative critical period for language ends with the ability to properly discriminate subtle grammatical errors by the age of 12 years (Newport and Aslin 2004; Newport, Hauser et al. 2004). Other linguistic features, such as semantics, can be learned throughout life. The existence of critical period in multiple systems suggests that understanding the critical period of visual plasticity may help us gain insightful understating of others.

1.3.6 Ocular dominance plasticity in mouse

The higher mammals in which these phenomena have been studied are, unfortunately, unsuitable for genetic manipulation. For this reason, the mouse has attracted increasing interest as a species for study of the molecular and cellular machinery responsible for plasticity (Gordon and Stryker 1996; Hensch, Fagiolini et al. 1998; Hensch, Gordon et al. 1998). Developmental plasticity in the mouse primary visual cortex has been well characterized (Gordon and Stryker 1996; Antonini, Fagiolini et al. 1999). Owing to the lateral position of the eyes, only about a third of the mouse visual
cortex receives input from both eyes, but many cells in this region are binocular (Gordon and Stryker 1996). Unlike higher mammals, the binocular region of the mouse primary visual cortex lacks clear OD columns but contains mostly binocular cells that on average have a preference for the contralateral eye (Hofer, Mrsic-Flogel et al. 2006) (Figure 1.8 a). During the critical period, closure of one eye is able to shift the responses of cells in favor of the non-deprived eye as seen in higher mammals (Figure 1.8 b). It has been reported that 4 d of MD at the peak of the critical period are necessary and sufficient to shift OD toward the non-deprived eye (Gordon and Stryker 1996). The critical period for the effects of MD in mouse begins around p19, peaks near P28, and ends quickly after P32 (Gordon and Stryker 1996). In addition to physiological studies, anatomical studies have also demonstrated a reorganization of cortical connections in mouse primary visual cortex following MD (Antonini, Fagiolini et al. 1999; Tagawa, Kanold et al. 2005). Therefore, OD plasticity in mouse is comparable to those of higher mammals and proves itself a versatile method to study the experience dependent visual plasticity (Hofer, Mrsic-Flogel et al. 2006). In the future, the development of genetically encoded sensors for calcium, other ions, or membrane voltage will allow us to probe the mouse visual cortex with even higher specificity.
Figure 1.8 Ocular dominance plasticity in mouse visual cortex.

(a) An illustration of the mouse visual system. The major part of the primary visual cortex is dominated by the contralateral eye. The binocular region is at the lateral third and is innervated additionally by ipsilateral projections. bV1=binocular V1; mV1=monocular V1.

(b) 4 days of MD during the critical period shifts the OD distribution in favor of the non-deprived eye in mouse.

Adapted from Hofer et al. 2006, Current Opinion in Neurobiology, 16(4): 451-459.
1.4 Importance of understanding visual plasticity

1.4.1 Amblyopia

The selective depression of visual responsiveness to the deprived eye causes the animal to lose visual capability in the deprived eye (Wiesel 1982). Similar forms of deprivation-induced visual depression have been observed in many species, including monkeys (Hubel, Wiesel et al. 1977), rats (Fagiolini, Pizzorusso et al. 1994), and mice (Gordon and Stryker 1996). As a direct consequence of the shift in cortical ocular dominance, the eye with the weakened input becomes amblyopic, that is, visual acuity and contrast sensitivity are strongly reduced even when no physical damage to the retina exists (Dews and Wiesel 1970; Daw, Reid et al. 1995; Daw 1998; Maurer, Lewis et al. 1999). This pathological condition, unfortunately, occur in humans (Daw 1998; Daw 1998; Sakai, Bi et al. 2006). In young children, optical abnormalities, such as congenital cataract, strabismus (ocular misalignment), and anisometropia (a difference in refractive index between the two eyes), are well known to cause amblyopia (also called lazy eye), which has a prevalence of 2% in the whole population (Sakai, Bi et al. 2006; Webb, McGraw et al. 2006).

Amblyopia can be defined as diminished visual acuity in one eye in the absence of organic eye disease. Central vision develops from birth to age 7 or 8; if vision has not developed by then, there is little or no chance that it will develop later. Amblyopia is an important socioeconomic problem. Studies have shown that it is the number one cause of monocular vision loss in adults (Sakai, Bi et al. 2006). Furthermore, persons with amblyopia have a higher risk of becoming blind because of potential loss to the sound
eye from other causes (Flynn 1991). The diagnosis of amblyopia is based on clinical history, visual acuity tests, contrast sensitivity tests, and a full eye examination to rule out ocular pathology.

Current treatments for amblyopia are limited. The most important treatment for amblyopia is to remove the obstacle to vision. For example, cataracts must be removed in the first 2 months of life, and aphakic correction must occur quickly to minimize visual impairment. Treatment of anisometropia and refractive errors must occur next. The next step is to reinforce the use of the amblyopic eye by occlusion therapy. Occlusion therapy of the normal eye has been the mainstay of treatment since the 18th century. Patching, opaque contact lenses, occluders mounted on spectacles, and adhesive tape on glasses have been used for occlusion. In addition, penalization therapy is also an option to treat amblyopia. In this therapy, atropine is instilled in the nonamblyopic eye to blur its vision and force the use of amblyopic eye (Flynn 1991; Flynn 2000; Holmes, Beck et al. 2003; Holmes, Beck et al. 2004)

The idea behind the current treatment of amblyopia is to balance visual inputs from both eyes and subsequently minimize the competition for ocular dominance between the two eyes. However, either patching or penalization therapy needs to be carried on for several years. It is commonly seen that children are poorly compliant with the treatment and hardly tolerate the patch or medication. More insightful understanding of the molecular mechanisms underlying visual plasticity is important to develop better treatments for amblyopia. To address this issue, one of my projects is to provide a theoretical basis for a new treatment of amblyopia. This will be further discussed in chapter three.
1.5 Mechanism of ocular dominance plasticity

Accumulating evidence suggests that the loss of visual responsiveness following MD is not simply due to any damage to the retina or its target, LGN (Reiter and Stryker 1988; Hata and Stryker 1994; Hata, Tsumoto et al. 1999; Hensch 2005; Rittenhouse, Siegler et al. 2006; Cnops, Hu et al. 2008). It is now clear that the primary visual cortex is the site of the changes that underlies amblyopia (Cynader and Mitchell 1977; Gordon, Cioffi et al. 1996; Hensch 2005). Therefore, research has been focused on the mechanism underlying the intracortical reorganization of the primary visual cortex following MD in the past decades.

The fundamental units of the neuronal network are the neurons (Greek: nerve cell) and its connections to other neurons. Hence, the crucial question to understand brain plasticity is how experience modifies neuronal activity. It has been found that short term MD (1-4 days) decreases synaptic responses to visual stimulation without detectable morphological change to the neuronal connections in rodent (Gordon and Stryker 1996; Bear 2003). Moreover, it takes longer period of time for MD (>7days) to cause significant retraction of the geniculocortical axon branches (Antonini and Stryker 1993; Antonini and Stryker 1996; Gordon and Stryker 1996). In contrast, binocular deprivation (BD) produces weaker synaptic depression than does monocular deprivation and does not alter the distribution of OD (Wiesel and Hubel 1965). These observations give rise to the concept that the ocular dominance of cortical neurons is maintained by a process of activity-dependent competition between the synapses serving the two eyes (Heynen, Yoon et al. 2003). According to the nature of competition, it is reasonable to think that total retinal inactivity, which eliminates the spontaneous activity of retina, will cause
more dramatic ocular dominance shift than eye lid suture. However, visual plasticity is apparently more complicated than that. Rittenhouse et al. demonstrate that eye lid suture causes a significantly greater ocular dominance shift in kitten visual cortex than does total blockade of retinal activity with intraocular treatment of tetrodotoxin. More surprisingly, the open eye even loses the competition to the deprived eye if postsynaptic activity of cortical neurons is suppressed by intracortical infusion of a GABA receptor agonist (Reiter and Stryker 1988) or a NMDA receptor antagonist (Bear, Kleinschmidt et al. 1990). Taken together, these data suggest that the correlation between retinal input and the activity status of cortical neurons is crucial to drive the ocular dominance plasticity. At this frontal line, two hypotheses have been postulated to explain the mechanism of ocular dominance plasticity: these include synaptic mechanism and inhibitory-excitatory balance.

1.5.1 Synaptic mechanism

Sixty years ago, Hebb described the basic mechanism of synaptic plasticity: "any two cells or systems of cells that are repeatedly active at the same time will tend to become 'associated', so that activity in one facilitates activity in the other." (Hebb 1949). This prediction was verified decades later with the discovery of long-term potentiation (LTP) (Bliss and Lomo 1973). Apparently, Hebbian theory is only half of the story since it does not explain synaptic weakening. Not surprisingly, long-term depression (LTD), a long lasting synaptic weakening, was reported several years later (Levy and Steward 1979). This bidirectional modification of synaptic plasticity has generated tremendous interest in the past three decades.
Intensive studies in hippocampus have proposed that LTD and LTP are two fundamental models for synaptic modification (Bliss and Collingridge 1993; Bear 2003; Collingridge and Isaac 2003; Collingridge, Isaac et al. 2004; Wang 2008). LTD refers to a long lasting synaptic depression induced by patterns of weak stimuli; in contrast, LTP refers to a long lasting synaptic potentiation induced by a variety of strong stimuli. It is worth noting that two forms of LTD are present: homosynaptic LTD and heterosynaptic LTD. Homosynaptic LTD requires a weak presynaptic activity correlated with weak postsynaptic response (Mioche and Singer 1989; Frenkel and Bear 2004). Heterosynaptic LTD occurs at a synapse in which the presynaptic input remains inactive, and postsynaptic activity is driven by other converging afferents. The remarkable conceptual similarity between MD-induced visual loss and homosynaptic LTD implies that a common molecular mechanism is present in both conditions. Thus, homosynaptic LTD may prove itself as a valuable model for ocular dominance plasticity. For the remainder of this thesis, LTD refers to homosynaptic LTD, unless otherwise stated.

1.5.1.2 LTD in ocular dominance plasticity

Many studies have been done to explore the role of LTD in ocular dominance plasticity. In the past decade, accumulating evidence suggests an important role of LTD in the loss of visual responsiveness in MD (Berardi, Pizzorusso et al. 2003; Heynen, Yoon et al. 2003; Hensch 2004). First, homosynaptic LTD can be reliably induced between layer IV and II/III in the primary visual cortex during the critical period (Kirkwood, Rioult et al. 1996). Although LTD can be induced by various types of conditioning stimulation, a common protocol is low frequency stimulation (LFS; 1Hz for
900 stimuli) with stimulation intensity which is subthreshold for evoking synaptically induced action potentials. **Second**, LTD is most pronounced during the critical period for visual dominance plasticity (Kirkwood, Silva et al. 1997; Sermasi, Tropea et al. 1999; Xiong, Kojic et al. 2004). It becomes difficult to induce LTD in primary visual cortex of mouse beyond 3 months. **Third**, binocular deprivation induced by dark-rearing reduces the magnitude of LTD during the critical period (Kirkwood, Rioul et al. 1996; Sermasi, Tropea et al. 1999). **Fourth**, MD with eye suture causes a significantly stronger depression of deprived-eye responses in kitten visual cortex than does MD combined with intraocular tetrodotoxin treatment (Rittenhouse, Shouval et al. 1999). This result suggests that the basal activity is required for the synaptic plasticity. This finding also confirms that monocular deprivation is not simply a result of retinal inactivity. **Fifth**, LTD is occluded in the binocular zone of the primary visual cortex in MD (Heynen, Yoon et al. 2003). **Sixth**, both LTD and MD require the activation of NMDA receptor to depress synaptic response (Bear and Colman 1990; Kirkwood, Dudek et al. 1993; Xiong, Kojic et al. 2004). **Seventh**, one day MD induces dephosphorylation of the AMPAR GluR1 subunit, phosphorylation of AMPAR GluR2 subunit, and a decrease in the cell surface AMPARs, similar to the molecular changes in electrically induced LTD (Heynen, Yoon et al. 2003). **Eighth**, brief MD results in a loss of protrusions on apical dendrites of typical layer II/III pyramidal neurons in the binocular zone of mouse visual cortex only during the critical period (Gordon and Stryker 1996; Fagiolini and Hensch 2000; Mataga, Mizuguchi et al. 2004). This result indicates that the critical period is not only for the modification of synaptic strength but also for an anatomical alteration of the neuronal network. Last but not least, a chronic recording of visual evoked potential (VEP) in
awake mice reveals a rapid decrease in the magnitude of VEP to the deprived eye in the
first three days of MD (Frenkel and Bear 2004).

Although all the evidence suggests a correlation of LTD with the processes
involved in the ocular dominance plasticity, there is still no direct evidence to support
that LTD has a causative role for MD induced visual depression. The missing link would
be the evidence that selective blocking LTD, without affecting LTP and general neuronal
responsiveness, can prevent the loss of visual responsiveness following MD during the
critical period. In fact, studies have been done to examine OD plasticity under a variety
of conditions in which LTD mechanism is impaired (Hensch, Fagiolini et al. 1998;
Hensch, Gordon et al. 1998; Huang, Kirkwood et al. 1999; Bartoletti, Cancedda et al.
2002; Renger, Hartman et al. 2002; Hensch 2003; Shimegi, Fischer et al. 2003; Fischer,
Beaver et al. 2004). However, the lack of specificity to LTD associated with these
manipulations has generated equivocal and discordant results. For instance, endogenous
brain-derived neurotrophic factor (BDNF) prevents LTD in the visual cortex (Jiang,
Akaneya et al. 2003) but does not block the loss of deprived-eye input in transgenic mice
overexpressing it (Hanover, Huang et al. 1999). Transgenic mice with overexpression of
calcineurin lose ocular dominance plasticity while LTD is intact (Yang, Fischer et al.
2005). Other results have however resulted in the opposite direction, as mentioned
earlier. Therefore, a precise targeting of LTD with minimal influence on other systems is
required to examine the causative role of LTD in OD plasticity.
1.5.1.3 Molecular mechanism of LTD

1.5.1.3.1 Induction of LTD

The primary excitatory neurotransmitter in brain is glutamate. Glutamatergic transmission is mediated by ionotropic and metabotropic classes of glutamate receptor. Ionotropic glutamate receptors are subdivided into three groups: a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), N-methyl-D-aspartate (NMDA) and kainate receptors (Dingledine, Borges et al. 1999). Metabotropic glutamate receptors (mGluRs) are divided into eight known subtypes (mGluRs 1–8) and three groups based on sequence homology, second messenger coupling and pharmacology (Dingledine, Borges et al. 1999; Anwyl 2006). The stimulation protocols required for LTD vary from one region to another, on the primary visual cortex and the hippocampus, prolonged LFS is often required (900 pulses; 1-5 Hz) (Bliss and Collingridge 1993; Bear and Malenka 1994; Bear 2003).

Currently, our mechanistic understanding of LTD is based primarily on the studies in area CA1 of hippocampus because it is probably the most studied region. LTD typically requires NMDA receptor activation, postsynaptic Ca\(^{2+}\) influx and activation of a phosphatase cascade (Bear and Malenka 1994; Mulkey, Endo et al. 1994; Malenka and Bear 2004). However, the quantitative characteristic of Ca\(^{2+}\) signal required to induce LTD remains poorly known. In addition, LTD independent of postsynaptic NMDARs is also present in many regions of the brain (Anwyl 2006).
NMDA receptors is composed of two essential NR1 subunits and two or three NR2 subunits. There are four distinct types of NR2 subunits: NR2A–D (Cull-Candy, Brickley et al. 2001). NR2A and NR2B predominate in the forebrain, and NR2 subunits determine the receptors characteristics and recruit different intracellular signaling molecules (Cull-Candy, Brickley et al. 2001). There is evidence that during postnatal development, there is a change not only in expression, from NMDA receptors containing the NR2B subunit to those containing NR2A (Sheng, Cummings et al. 1994; Quinlan, Philpot et al. 1999), but also an additional shift in synaptic localization, with NR2A-containing NMDA receptors preferentially located synaptically and NR2B-containing receptors at extrasynaptic sites (Stocca and Vicini 1998; Rumbaugh and Vicini 1999; Philpot, Sekhar et al. 2001; Philpot, Espinosa et al. 2003). NR2B containing NMDA receptors have recently been implicated in hippocampal LTD in vitro (Liu, Wong et al. 2004) and in vivo (Fox, Russell et al. 2006) and a similar role for NR2B containing NMDA receptors in LTD has been shown to exist in perirhinal cortex (Massey, Johnson et al. 2004). However, this still remains a controversial issue (Bartlett, Bannister et al. 2007; Morishita, Lu et al. 2007).

Besides NMDARs, it has been well accepted that LTD induction requires activation of calcineurin or protein phosphatase 1 (PP1) in both hippocampus and primary visual cortex (Kirkwood and Bear 1994; Mulkey, Endo et al. 1994). In addition to phosphatase, several kinases are also required for certain types of LTD. For example, p38 MAP kinase is required for LTD induced in the hippocampus by Rap, a small GTPase (Zhu, Qin et al. 2002) and mGluR dependent LTD in CA1 (Bolshakov, Carboni
et al. 2000). In cerebellum, LTD requires activation of PKC (Linden 1994). In primary visual cortex, the role of kinase activation in LTD has not been well examined. In chapter, we will demonstrate that activation of p38 MAP kinase alone is sufficient to induce LTD but is not required for LFS-induced LTD.

1.5.1.3.2 Expression and maintenance of LTD

The expression of NMDA receptor dependent LTD is mediated by a rapid removal of AMPA receptors from the postsynaptic neuronal membrane as well as the modulation of AMPARs at the synapse; in addition, the maintenance of the change in synaptic function requires protein synthesis (Lee, Barbarosie et al. 2000; Malinow and Malenka 2002; Collingridge and Isaac 2003; Collingridge, Isaac et al. 2004). The synaptic trafficking of AMPARs is triggered by the activation of NMDA receptor (Malinow and Malenka 2002; Collingridge, Isaac et al. 2004). The influx of calcium through NMDARs activates a signaling cascade that involves a variety of kinases and phosphatases (Collingridge, Isaac et al. 2004) and, consequently, alters the phosphorylation status of AMPAR subunits to induce clathrin-dependent internalization (Beattie, Carroll et al. 2000; Ehlers 2000; Heynen, Quinlan et al. 2000; Lee, Liu et al. 2002; Ahmadian, Ju et al. 2004; Collingridge, Isaac et al. 2004; Wang 2008) (Figure 1.9). Although the precise mechanisms remain uncertain, studies have showed that the endocytosis of AMPARs depends on the interaction of the intracellular C-terminal tail of AMPAR subunits with proteins that direct them to extrasynaptic site of plasma membrane or to suborganelles inside the neuron (Malinow and Malenka 2002; Collingridge, Isaac et al. 2004).
Figure 1.9 NMDA receptor dependent LTD is mediated by clathrin-dependent endocytosis of AMPA receptor.

Calcium influx through NMDA receptor activates a signal cascade transduction. Recruitment of AP2 to the GluR2 subunit of AMPAR initiates clathrin-dependent endocytosis of AMPARs. Constitutive cycling of AMPAR is not regulated by neuronal activity.
AMPARs are composed of the different subunits GluR1–GluR4 (Hollmann and Heinemann 1994). Each receptor complex is composed of four subunits (Rosenmund, Stern-Bach et al. 1998). The predominant species of AMPARs are receptors composed of GluR1/GluR2 and GluR2/GluR3 (Wenthold, Petralia et al. 1996). GluR2 and GluR3 subunits have short cytoplasmic tails and tend to cycle continuously between non-synaptic and synaptic sites (Ahmadian, Ju et al. 2004). This constitutive cycling of GluR2 subunit depends on their interaction with NSF (N-ethylmaleimide sensitive fusion protein) and is not regulated by neuronal activity (Lee, Simonetta et al. 2004). In terms of the regulated endocytosis of AMPARs associated with LTD, several lines of evidence have pointed to the GluR2 subunit of AMPAR as a key regulator. The recruitment of the clathrin adaptor protein complex AP2 to a membrane-proximal region of the GluR2 subunit is apparently necessary to initiate clathrin coat assembly and receptor endocytosis (Lee, Liu et al. 2002; Collingridge, Isaac et al. 2004). Still on GluR2 C-terminal tail, association of the more distal site with PDZ domain-containing proteins GRIP and PICK1 are also important for LTD (Collingridge, Isaac et al. 2004). Furthermore, a recent study demonstrates that three tyrosine residues located near the end of the C-terminal tail of GluR2 subunit are required for insulin- and NMDAR-dependent LTD, whereas these residues do not influence the constitutive cycling of AMPARs (Ahmadian, Ju et al. 2004). A synthesized peptide containing these three tyrosine residues, which is derived from carboxyl tail of GluR2 subunit, has been shown to specifically block the regulated endocytosis of AMPARs in hippocampal slices and neuronal culture (Ahmadian, Ju et al. 2004) (Figure 1.10). The probable mechanism is that the GluR23Y peptide prevents the phosphorylation of three tyrosine residues on the carboxyl tail of
GluR2 subunit and subsequently blocks LTD expression (Ahmadian, Ju et al. 2004). In chapter two, we will examine the causative role of LTD in OD plasticity using this GluR2\textsubscript{3Y} peptide.
Figure 1.10 Synthesized peptide derived from GluR2 subunit of AMPAR: a specific blockade for LTD

A. The cartoon shows the GluR2 subunit of AMPAR. The expanded region shows the amino-acid sequence of C-terminal tail. A short peptide is derived from nine amino acids of C-terminal tail that is highlighted in green color. The AP2 and PDZ binding sites of the GluR2 subunit are also underlined and labelled. Adapted from Ahmadian, Ju et al. *Embo J* 23(5): 1040-50

B. The synthesized peptide (GluR2_{3Y}) derived from GluR2 subunit of AMPAR is able to specifically block the regulated endocytosis of AMPARs in hippocampal slices. Adapted from Ahmadian, Ju et al. *Embo J* 23(5): 1040-50

IEFCYKSRAEAKRMKVAKNPQINPSSQSQNFAKYNVYGIESV

AP2  PDZ
1.5.2 Cortical GABAergic system in visual plasticity

GABAergic circuitry represents the primary inhibitory system of the central nervous system. The cortical GABAergic system consists of an array of interneuronal cell types that display distinct morphology, physiological properties, and synaptic connectivity patterns (Acsady, Gorcs et al. 1996). The maturation of GABAergic system takes place during the critical period of ocular dominance plasticity and this process can be attenuated by rearing animal in the dark (Gordon, Kinch et al. 1997). Interestingly, it has been demonstrated that synaptic inhibition matures later than excitatory transmission in visual cortex (Benevento, Bakkum et al. 1992; Murphy, Beston et al. 2005). Thus, the discrepancy in the maturation of excitation and inhibition may define a window of opportunity for activity dependent plasticity to occur in early life. The basic idea is that there exist an optimal balance between excitation and inhibition that promotes plasticity and this is best exemplified during the critical period. In agreement with this notion, pharmacological activation or blockade of cortical GABA receptors during the critical period can dramatically alter the outcome of monocular deprivation (Ramoa, Paradiso et al. 1988; Reiter and Stryker 1988; Hata, Tsumoto et al. 1999). For instance, local infusion of the GABA$_A$ receptor agonist, muscimol, into primary visual cortex prevents the ocular dominance shift in MD cat during the critical period (Reiter and Stryker 1988; Hata, Tsumoto et al. 1999). More strikingly, accelerating the maturation of the GABAergic system by overexpressing BDNF shifts the critical period to an earlier age and shortens its length (Huang, Kirkwood et al. 1999). Consistent with this finding, the infusion of benzodiazepine, a use-dependent agonist of GABA$_A$ receptor, also triggers premature ocular dominance plasticity (Fagiolini, Fritschy et al. 2004). On the other
hand, the onset of the critical period can be delayed indefinitely, as seen in dark reared animals, if presynaptic release of GABA is kept low by gene-targeted disruption of the synaptic isoform of its synthetic enzyme, glutamic acid decarboxylase 65 (GAD65) (Hensch, Fagiolini et al. 1998).

The delayed visual plasticity in GAD65 KO mouse and a premature visual plasticity in BDNF overexpressing mouse suggest that OD plasticity may require a minimal level of inhibition or a threshold in the balance of inhibition/excitation. In vitro experiments have shown that the activity dependent synaptic plasticity can be restored in primary visual cortex by enhancing the GABAergic system (Kirkwood, Lee et al. 1995). Moreover, LTD is impaired in condition in which inhibitory strength is reduced, such as dark rearing or in GAD 65 KO mouse (Choi, Morales et al. 2002). Interestingly, the chronic administration of benzodiazepines for several days restores OD plasticity (Hensch, Gordon et al. 1998) and LTD (Choi, Morales et al. 2002) in GAD 65 KO mice. In contrast, acute exposure to benzodiazepines is not able to rescue LTD in GAD 65 KO mice. Thus, a sustained minimum level of inhibition appears to be required to support the induction of LTD and hence the elimination of deprived inputs following MD. Considering that LTD is the most easily induced before the onset of the critical period (Xiong, Kojic et al. 2004), we postulate that GABAergic system may play a “permissive” role in visual plasticity and control the beginning of the critical period.

The precise mechanism by which GABAergic system participates in visual plasticity remains unknown. The fact that the basket subtype of GABAergic cell is coupled to a local group of 40-50 cells raises the possibility that it is able to detect the
synchrony of inputs with this cluster (Hensch 2004). Thus, a minimum of inhibition could be required to differentiate the neural activity produced by deprived and non-deprived inputs. Detection of asynchronized information will finally result in a selective elimination of the deprived input. Another possible mechanism involves controlling backpropagation of action potential to dendrite of layer II/III pyramidal cells (Dan and Poo 2004). Recent work suggests that the precise timing between the arrival of backpropagating spikes and synaptic activation determines the sign and magnitude of synaptic changes (Dan and Poo 2004; Glantz and Schroeter 2004; Constantine-Paton 2006; Mu and Poo 2006). Consequently, changes in the efficacy of inhibition impinging on the dendrites can potentially affect the induction of plasticity by altering the integrative properties of the cells.

Although the above evidence may suggest that maturation of GABAergic system is tightly correlated with the timing of the critical period, it is worth noting that all these manipulations can also affect excitatory transmission. For example, dark rearing maintains the NMDA receptor composition and function in a juvenile state (Carmignoto and Vicini 1992; Quinlan, Philpot et al. 1999; Chen and Bear 2007) and enhances the AMPA receptor function (Desai, Cudmore et al. 2002). BDNF can enhance LTP and impair LFS-induced LTD in visual cortical slices (Akaneya, Tsumoto et al. 1997; Kinoshita, Yasuda et al. 1999). Moreover, it is still debated whether GABAergic inhibition controls the onset of critical or also its termination. Steps need to be taken to elucidate the precise role of GABAergic system in ocular dominance plasticity.
1.6 Hypotheses and objectives

1.6.1 Hypothesis 1: Long-term depression is the causative mechanism underlying the ocular dominance plasticity following monocular deprivation in mouse primary visual cortex.

1.6.1.1 Aim 1: Examine the specificity of GluR2$_{3Y}$ peptide in blocking LTD in primary visual cortex in vitro.

1.6.1.2 Aim 2: Examine the effect of GluR2$_{3Y}$ peptide on ocular dominance plasticity in primary visual cortex in vivo.

1.6.1.3 Aim 2: Facilitation of LTD reopens ocular dominance plasticity in adult animal.

1.6.2 Hypothesis 2: p38 MAP kinase mediates the anisomycin induced LTD in primary visual cortex

1.6.2.1 Aim 1: Demonstrate the synaptic depression induced by anisomycin and whether it is dependent on NMDA receptor.

1.6.2.2 Aim 2: Pharmacologically examine the role of p38 MAP kinase in anisomycin induced LTD.

1.6.2.3 Aim 3: Test whether anisomycin induced LTD requires regulated endocytosis of AMPARs.
1.7 Reference:

Acsady, L., T. J. Gorcs, et al. (1996). "Different populations of vasoactive intestinal polypeptide-immunoreactive interneurons are specialized to control pyramidal cells or interneurons in the hippocampus." Neuroscience 73(2): 317-34.


Akaneya, Y., T. Tsumoto, et al. (1997). "Brain-derived neurotrophic factor enhances long-term potentiation in rat visual cortex." J Neurosci 17(17): 6707-16.


Mulkey, R. M., S. Endo, et al. (1994). "Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression." Nature 369(6480): 486-8.


Chapter II

Selective LTD blockade prevents experience-dependent plasticity in the visual cortex during the critical period*

* A version of this chapter has been submitted for publication. Wei Xiong, Luba Kojic, Changiz Taghibiglou, Yingru Liu, Guang Yang, Ainsley Conquinco, Shiv Prasad, Yutian Wang, Max Cynader. Selective LTD blockade prevents experience-dependent plasticity in the visual cortex during the critical period.
2.1 Introduction

It has long been known that the environment in which the mammalian cerebral cortex finds itself early in postnatal life has the potential to alter the structure, function, and its connections with other parts of the brain (Bear 2003; Hensch 2004). The strongest evidence in support of experience-dependent modification of brain circuitry during a well-defined critical period of postnatal development comes from studies of vision (Gordon and Stryker 1996; Heynen, Yoon et al. 2003). The synaptic modification induced by monocular deprivation (MD) may reflect a process that normally refines the neuronal network during development (Bear 1996; Bear 2003; Heynen, Yoon et al. 2003; Hensch and Fagiolini 2005). Therefore, understanding the synaptic basis of the visual dominance plasticity will help us gain insight into the process, as well as to design better strategies, by which the older brain can regain its capacity of plasticity.

The mechanisms underlying the OD shift following MD remain uncertain. Long-term depression (LTD), a proposed cellular substrate for learning and memory, may play a role in the ocular dominance plasticity (Bear and Colman 1990; Kirkwood, Rioult et al. 1996; Rittenhouse, Shouval et al. 1999; Heynen, Yoon et al. 2003; Frenkel and Bear 2004). Since both LTD and MD show a similar decrease in responsiveness to a weak pattern of synaptic stimulation (Mioche and Singer 1989; Frenkel and Bear 2004), we surmised that LTD may play a key role in ocular dominance plasticity. In fact, several studies have been performed to examine the role of LTD and LTP in visual plasticity with discordant results (Hensch, Gordon et al. 1998; Rittenhouse, Shouval et al. 1999;
Heynen, Yoon et al. 2003; Frenkel and Bear 2004). Until now, the lack of specific blockers for LTD or LTP has hindered research in this area.

A large body of evidence accumulated in recent studies has suggested that the expression of LTD in hippocampus is clathrin-dependent and requires the GluR2-dependent endocytosis of postsynaptic AMPARs (Malinow and Malenka 2002; Brebner, Wong et al. 2005). The clathrin-dependent endocytosis of AMPA receptors requires phosphorylation of GluR2 subunit of AMAPR (Collingridge, Isaac et al. 2004). These studies have led to the development of a number of GluR2 derived peptides that can specifically prevent the expression of LTD in many areas of the brain including hippocampus (Ahmadian, Ju et al. 2004), cerebellum (Xia, Chung et al. 2000; Steinberg, Huganir et al. 2004), and nucleus accumbens (Brebner, Wong et al. 2005). In previous report, it has been demonstrated that a peptide derived from GluR2 carboxyl tail (GluR23Y; 869YKEGYNVYG877) was able to inhibit the regulated internalization of AMPARs from the cellular surface as well as LTD induction (Ahmadian, Ju et al. 2004). Moreover, this peptide has no effect on the constitutive AMPAR endocytosis and the basal synaptic transmission. Therefore, GluR23Y could be the specific blocking agent for LTD and could be utilized as a tool to study the role of LTD in visual plasticity.

In the present study, we used a LTD-blocking peptide derived from the mouse GluR2 carboxyl tail of the AMPAR (Ahmadian, Ju et al. 2004; Brebner, Wong et al. 2005) to determine whether visual cortical LTD can contribute to MD in mice. We were able to demonstrate that the GluR2 peptide blocked LTD in primary visual cortex via a
postsynaptic mechanism. To study the role of LTD in OD plasticity, we administered the GluR2 peptide systemically or locally in the primary visual cortex during a 4 day period of MD near the height of the critical period. Single unit recording was used to measure the OD distribution at the end of MD. A complementary anatomical measurement of the OD shift was also taken by examining the visually evoked expression of Zif268. The results presented here indicate that specific blocking of LTD prevents the OD shift following MD during the critical period.
2.2 Materials and Methods

2.2.1 Peptide synthesis

GluR23Y (YKECYNVYS), GluR23A (AKECANVAS), Tat-GluR23Y (YGRKKRRQRRRYKECYNVYS), Tat-GluR23A (YGRKKRRQRRRAKECANVAS), and FITC-conjugated Tat-GluR23Y peptides were synthesized by the Nucleic Acid and Peptide Service Centre at the University of British Columbia.

2.2.2 Monocular Deprivation

3% isofluorane (Abbott, North Chicago, IL) in oxygen was used for anesthesia. Lid margins were trimmed and antibiotic ophthalmic ointment (Vetropolycin, Pharmaderm) was applied to the eye. Three mattress stitches were placed using 6-0 vicryl, opposing the full extent of the trimmed lids. Mice were recovered by breathing room air and were monitored daily to be sure that the sutured eye remained shut and uninfected. Animals whose eyelids did not fully seal shut were excluded from further experiments. At the end of the deprivation period, mice were reanesthetized, stitches were removed, and lid margins were separated. Eyes were then flushed with sterile saline and checked for clarity under a microscope. Mice with corneal opacities or signs of infection were excluded from further study. In every case, MD was initiated within the critical period for mouse OD (p25–p30) and maintained for 4 days. All protocols for animal experiments were in accordance with National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care Center, University of British Columbia.
2.2.3 Surgical Implantation of Minipumps

Mice were anesthetized with 3% isofluorane in oxygen and mounted in a stereotaxic frame that allowed unobstructed vision. Ophthalmic lubricant was applied to protect the eyes, and body heat maintained at 37°C with a heating pad. For infusion experiments, Alzet osmotic minipumps (1007D, Alzet) were filled either with Tat-GluR23Y (2 μM resolved in saline) or Tat-GluR23Y (2 μM resolved in saline) and attached to 30G stainless steel cannulae. Under aseptic conditions, a longitudinal incision was made in the scalp over the mid-sagittal sinus and the portions of the skull overlying occipital and frontal portions of the brain were cleaned and dried. To avoid any damage to the binocular zone of the primary visual cortex, the location of the infusion was centered in the cortical monocular zone, approximately, 1 mm lateral to the midline and 1 mm rostral to lambda. A small hole (D<0.5 mm) was drilled through the skull above the occipital cortex contralateral to the deprived eye. The cannula was inserted to a depth of 1 mm under the surface of the skull and secured with cyanoacrylate adhesive (Alzet). The attached minipump was placed in a subcutaneous pocket at the nape of the neck. The scalp was closed over the implant, and the animal returned to its home cage. Minipump implantation was performed on the day prior to MD, and infusion continued for the duration of the MD, for a total of 5 days of infusion.

To estimate the diffusion range of the peptides, two mice were implanted with minipumps filled with FITC-conjugated Tat-GluR23Y and were sacrificed 24 h after infusion began. Fresh visual cortex slices (50 μm thick) were collected after transcardial
perfusion with 0.9% saline. Slices were subsequently examined under a fluorescent microscope to estimate the spread of the peptide under the infusion conditions used.

2.2.4 Slice preparation

Slices were prepared from C57BL/6 mice (Charles River, Quebec, Canada) at postnatal day (PD) 22–28. Animals were anesthetized with urethane (5mg/kg). After decapitation, coronal slices containing primary visual cortex (350 µM thick) were prepared using a vibrating blade microtome (Leica, Germany) in ice-cold artificial CSF (ACSF) containing (in mM): 126 NaCl, 5 KCl, 1 MgSO4, 2 CaCl2, 1.25 NaH2PO4, 26 NaHCO3, and 20 glucose that was bubbled with carbogen (95%O2/5% CO2) to adjust the pH to 7.4. Brain slices were placed in a submersion recording chamber with carbogenated ACSF and allowed to recover at 30°C for ~1h prior to recording.

2.2.5 In vitro Electrophysiological recording

Electrophysiological recordings were conducted in the chamber described above with continuous infusion of carbogenated ACSF at a rate of 1.5ml/min at 30°C. Extracellular field potential recordings were made from layers 2/3 with glass electrodes (1MΩ) filled with 1M NaCl. Field potentials were evoked via electrical stimulation through a concentric bipolar stimulating electrode (CBBRC75; FHC, Bowdoinham, ME) placed in the center of the cortical thickness that corresponded to layer 4. Slices were evaluated for responsivity every 15 seconds with a constant current pulse of 100 µs duration and 100-200 µA of current, chosen to yield a half-maximal response. This fEPSP was mainly mediated via AMPA receptors as it was completely abolished by the AMPAR antagonist DNQX (20 µM, Sigma). After a stable baseline was achieved, LTD
was induced using a low frequency stimulation protocol (LFS) consisting of 900 stimuli at 1Hz. For experiments with Tat-GluR23Y (0.4 μM), Tat-GluR23Y (0.4 μM), or DAPV (50 μM; Sigma), bath application of reagents was started at least 20min prior to LFS. Whole-cell recordings of visual cortical neurons in brain slices were performed using the “blind” method with a MultiClamp 700B amplifier. Recording pipettes were filled with solution containing (mM) 132.5 Cs-gluconate, 17.5 CsCl, 2 MgCl₂, 0.5 EGTA, 10 HEPES, 4 ATP, and 5 QX-314, with pH adjusted to 7.2 by CsOH. For experiments to test the effect of the GluR2 peptide on LTD, GluR23Y or GluR23A (100 μg/ml) was also included in the recording pipettes. EPSCs were evoked similarly to fEPSPs and recorded while visual cortical neurons were voltage clamped at –60 mV. Synaptic responses were evoked at 0.05 Hz except during the induction of LTD, which was triggered by delivering low frequency stimulation (300 pulses at 1 Hz) while the recorded cell was voltage clamped at -45 mV. Induction of LTD was performed within 10 min after the establishment of the whole cell configuration to avoid washout of intracellular contents.

2.2.6 In vivo Recordings

Electrophysiological recordings were performed under urethane (50 mg/ kg, ip, Sigma) anesthesia. Atropine (20 mg/Kg s.c., Optopics) was injected to reduce secretions and parasympathetic effects of anesthetic agents; and dexamethasone (4 mg/Kg s.c., American Reagent Laboratories) was administered to reduce cerebral edema. Mice were placed in a stereotaxic device, and a craniotomy was performed over the right visual cortex. Agar was applied to enhance recording stability and prevent desiccation. The eyelids were resected, and corneas were protected thereafter by frequent application of
Ringer’s solution. Body temperature was maintained at 37°C using a homeostatically controlled heating pad (Harvard). Heart rate was monitored continuously with EKG needles. Four to six sites (at least 100 µm apart) through the full thickness of the cortex were evaluated in each of four to six penetrations spaced evenly (at least 200 µm apart) crossing the binocular region (RF center azimuths < 25 degrees from the vertical meridian) of area 17 to avoid sampling bias. In some cases, we were able to isolate large individual neuronal responses, while other sites yielded multiunit activity. Sites were assigned to OD categories according to the seven-category scheme of Hubel and Wiesel (Gordon and Stryker 1996). OD histograms were constructed and CBI scores were calculated for each mouse using the formula: CBI = [(n1 - n7) + (2/3)(n2 - n6) + (1/3)(n3 - n5) + N]/2N, where N = total number of cells and nx = number of cells with OD scores equal to x. Response quality was assessed by rating the level of visually driven and spontaneous activity, each on a three-point scale (1 = low, 3 = high).

2.2.7 Quantitative Measurement of Single Unit Responses

A total of six mice (three Tat-GluR23Y-infused and three saline-infused) were used for quantitative analyses of visual responses. Single unit responsiveness was assessed using computer-driven stimulus presenting on 21 inch CRT monitor (Viewsonic G220F) and spike collection system with customized software from Dr. Nicholas Swindale (Department of Ophthalmology, University of British Columbia). Stimulus-driven neuronal responses were elicited using a high contrast low spatial frequency (0.05 cyc/deg) grating. Evoked responses and spontaneous activity were assessed issuing quantitative methods as described elsewhere (Giaschi, Douglas et al. 1993). Responses in mice treated with saline were compared with mice treated with Tat-GluR23Y; and
stimulus–driven neuronal responses and spontaneous activities were quantified at 1h and 2h after administration.

2.2.8 Cortical slice biotylination and immunoblotting following chemical LTD

Cortical slices containing visual cortex were pretreated with 1 µM TAT-GluR23Y or TAT- GluR23A for 30 min. After washing with ACSF, slices were subsequently treated with 10 µM NMDA and 10 µM glycine in the presence of 1 µM strychnine for 10 minutes. Slices were then washed with cold ACSF for 10 minutes to stop receptor trafficking. This was followed by 30 min incubation in cold ACSF containing 10mg/ml biotin (EZ-link, sulfo-NHS-SS-Biotin, Perce). After three washes with cold ACSF, binocular zone of the primary visual cortex from both sides of cortexes was dissected under microscope and homogenized in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris pH 8.0). The protein concentration in the supernatant was measured after centrifugation. 280 mg protein was incubated with 60 ul avidin-coated beads (Cat: A-9207, Sigma) at 4°C overnight. Biotylinated protein was spun down and washed with RIPA buffer for 3 times and was subsequently boiled in sampling buffer for 5 min. The biotylinated protein and total protein was loaded onto a 10% SDS gel. An antibody recognizing the N-terminal domain of mouse GluR2 (Chemicon, 1:1000) was used to detect the surface or total GluR2. On the same membrane, anti GSK-3β antibody (Sigma, 1:200) was subsequently used to confirm that biotin does not bind to intracellular protein. In addition, anti insulin receptor β antibody (Sigma, 1:500), anti GABA_A receptor β2/3 subunit (Sigma, 1:500), and anti NMDA NR1 subunit antibody (Sigma, 1:500) were applied as negative controls. Blots were developed
using enhanced chemiluminescence detection (Amersham) and the signal was quantified with Quantity One software (BIO-RAD). The ratio of the surface GluR2 protein over insulin receptor β was calculated. Surface GluR2 protein was compared between chemical LTD and control.

2.2.9 Immunofluorescent examination of Zif 268 in primary visual cortex

In MD mice treated with Tat-GluR23V or Tat-GluR23A for 4 days, the sutured eyelid was opened and the previously open eye was enucleated. Mice then remained in a dark room for 12 h and were subsequently exposed to light for another 2 h. The brains were then harvested after transcardial perfusion with 40 ml saline and subsequent 40 ml 4% paraformaldehyde in 0.1 M phosphate buffer. After overnight post-fixation and dehydration with 15% and 30% sucrose in 0.1 M phosphate buffer, the brains were sectioned at 14 μm using a cryostat. Sections were then mounted on slides and probed with rabbit anti-mouse ZIF 268 antibody (Santa Cruz, 1:200) for 2 h at room temperature. A secondary fluorescence-conjugated goat anti-rabbit antibody (Invitrogen, 1:500) was used for detection. Slices were examined with fluorescent microscopy (Axiovert 200, Zeiss) and photographs were acquired with North Eclipse software. The exposure time, offset, and amplification were the same for all pictures. Images were analyzed with ImageJ (NIH).
2.3 Results

2.3.1 Blocking LTD with GluR2 peptide in brain slice recording

We first investigated whether the GluR23Y peptide can function as a specific inhibitor of LTD in the visual cortex as it does in other brain areas (Ahmadian, Ju et al. 2004; Brebner, Wong et al. 2005; Fox, Russell et al. 2007). The peptide was rendered membrane permeable by fusing it to the cell membrane transduction domain of the HIV-1 Tat protein (YGRKKRRQRRR) to generate Tat-GluR23Y as previously described (Brebner, Wong et al. 2005). As shown in Figure 2.1A, low-frequency-stimulation (LFS) reliably induced LTD of extracellularly recorded excitatory postsynaptic potentials (EPSPs) in mouse visual cortex slices (the magnitude of the EPSPs at 30 min was reduced to $80 \pm 3\%$ of the baseline ($n = 5$; $p = 0.008$ compared with the baseline recorded 5 min prior to the start of LFS). This LTD was N-methyl-D-aspartate (NMDA) receptor dependent because it was blocked by the NMDA receptor antagonist D-APV at 50 µM (Figure 2.1 A, C, D). Bath application of the Tat-GluR23Y peptide (0.4 µM) for 20 min prior to LFS abolished LTD (96 $\pm 2\%$ of baseline, $n = 7$; $p = 0.012$ when compared to control LTD; Figure 2.1 A, C, D). In contrast, the control peptide, Tat-GluR23A (Tat-AKEGANVAG, in which the three critical tyrosine residues were replaced with alanines (Ahmadian, Ju et al. 2004) at the same concentration did not significantly alter the magnitude of LTD (87 $\pm 6\%$ of baseline, $n = 4$; $p = 0.23$; Figure 2.1 A, C, D).

To further confirm that the Tat-GluR23Y peptide interfered with the regulated endocytosis of postsynaptic AMPARs, we applied the membrane impermeable form of the peptides (GluR23Y or GluR23A; 100 µg/ml) into postsynaptic neurons under whole-cell recording conditions by including them in the
Figure 2.1

**Figure 2.1 GluR23Y peptide blocks LTD in vitro.**

**A,** Bath application of Tat-GluR23Y peptide blocked NMDA receptor dependent LTD of field recordings in primary visual cortex. Red diamond, control peptide (GluR23A); green circle, wild-type peptide (GluR23Y); blue circle, control LTD; light blue triangle, D-APV treatment.

**B,** Inclusion of GluR23Y in patch electrodes blocked LTD in whole-cell recordings. Control LTD recorded without peptide: 64±7%, n=7; GluR23Y: 84±5%, n=7, t-test: p=0.008 compared to control LTD; GluR23A: 63±3%, n=7, t-test: p=0.53 compared to control LTD.
C. Representative field excitatory postsynaptic potential (fEPSP) and excitatory postsynaptic current (EPSC) taken from time points 1 and 2 labeled in figure 1A and 1B.

D. Summary of results obtained under field recording and whole-cell recording conditions. Each bar represents the average normalized fEPSP or EPSC recorded in the last 5 min under conditions as labeled on X axis. Asterisk, p<0.05. Error bar represents one s.e.m.
patch electrodes. Consistently, GluR2$_{3Y}$, but not GluR2$_{3A}$, significantly reduced the LFS-induced LTD of excitatory postsynaptic currents (EPSCs) (Figure 2.1 B, C, D). We observed no significant change in basal synaptic transmission with either peptide under either field or whole cell recording conditions.

In addition to electrophysiological study, we also performed biochemical assay to semi-quantify the surface AMPARs in the presence of Tat-GluR2$_{3Y}$ during chemical LTD induced by NMDA as previously described (Heynen, Yoon et al. 2003). Slices containing visual cortex were incubated with 1 µM TAT-GluR2$_{3Y}$ for 30 minutes. Chemical LTD was then induced using 10 µM NMDA and 10 µM glycine in the presence of 1 µM strychnine for 10 minutes. After biotinylation, western blotting was used to measure the density of surface AMPARs. In the absence of TAT-GluR2$_{3Y}$, there was a 33% reduction of surface GluR2 subunit 10 min following NMDA treatment. In contrast, there was only an 11% reduction of surface GluR2 in slices treated with TAT-GluR2$_{3Y}$. The TAT-GluR2$_{3Y}$ peptide itself did not alter the density of surface GluR2 without chemical LTD induction (Figure 2.2 A, B). Moreover, GABA$_{A}$ receptor and NMDA NR1 subunit on cell surface were not changed by the peptide treatment, which suggests that the peptide is highly specific on GluR2 subunit of AMPAR.

Taken together, our results confirm that, as in the hippocampus, LTD in the primary visual cortex is NMDA receptor dependent and is mediated by GluR2-dependent endocytosis of postsynaptic AMPARs. Moreover, our results indicate that by blocking the regulated endocytosis, the Tat-GluR2$_{3Y}$ peptide is an effective inhibitor of visual cortex LTD and can be used to probe the role of LTD in the OD shift following MD.
Figure 2.2 Biotinylation assay of surface AMPARs in the presence of TAT-GluR2<sub>3Y</sub> during LTD.
A, Representative immunoblots for GluR2 subunit from a variety of conditions, including input (total protein), control (surface GluR2 with no treatment), NMDA treated only, 3A-peptide (treated with TAT-GluR23A only), NMDA+SG (treated with 10 µM NMDA, 10 µM strychnine, and 10 µM glycine for 10 minutes), 3Y-peptide (treated with TAT-GluR23Y only), and 3Y-peptide+NMDA (treated with GluR23Y and 10 µM NMDA). IRβ (insulin receptor beta) is only present on cell surface and was used for normalization. GSK-3β (Glycogen Synthase Kinase 3) is an intracellular protein and was absent in our surface protein preparation. NR1 subunit of NMDA receptor and β2/3 subunit of GABA_A receptor were examined to rule out the possibility that the peptide may influence NMDA receptor and GABA_A receptor.

B, The density of GluR2 subunit was normalized against IRβ and the normalized value was compared to control. The final result for each experimental condition is presented in the bar chart. There was a 33% reduction of surface GluR2 subunit 10 min following NMDA treatment. In contrast, there was only an 11% reduction of surface GluR2 in slices treated with TAT-GluR23Y. The TAT-GluR23Y peptide itself did not alter the density of surface GluR2.
2.3.2 Prevention of OD shift following MD with systemic administration of the GluR2 peptide

We first tested whether Tat-GluR23Y had any adverse effect on the normal OD distribution and properties of the visual cortical neurons in vivo. The control non-deprived group (ND) displayed a contralateral biased distribution of OD scores (CBI=0.77±0.01, n=5; Figure 2.3 A). Tat-GluR23Y peptide (10 nmol/g) intraperitoneal (ip) administration alone for 4 days had no influence on OD distribution in ND mice (CBI=0.79±0.02, n=5; p=0.35 compared to ND mice; Figure 2.3 C).

We then investigated the potential role of LTD in mediating the OD shift following MD during the critical period by ip administering Tat-GluR23Y or Tat-GluR23A peptide in animals at the peak of the critical period (p25-p30) (Figure 2.3 D). A four day period of MD was performed to assure a maximal OD shift following MD (Gordon and Stryker 1996; Yang, Fischer et al. 2005). In MD mice without peptide treatment, 4 day MD shifted the OD toward the open ipsilateral eye (CBI=0.49±0.05, n=5; p=0.007 compared to ND; Figure 2.3 B). The administration of Tat-GluR23Y (10 nmol/g) in MD mice significantly prevented the OD shift (CBI=0.74±0.02, n=5; p=0.006 compared to MD mice and p=0.26 compared to ND mice; Figure 2.3 E). In contrast, the control peptide (Tat-GluR23A, 10 nmol/g), did not prevent the OD shift to the open eye following MD (CBI=0.49±0.01, n=5; t-test: p=0.42 compared to MD mice and p=0.005 compared to ND mice; Figure 2.3 F).
Figure 2.3

A. ND MICE

B. MD MICE

C. ND MICE + GluR2

D. Peptide i.p injection daily
   MD 4 days
   Recording

E. MD MICE + GluR2y

F. MD MICE + GluR2z

CBI = 0.77
111 cells
5 mice

CBI = 0.40
154 cells
5 mice

CBI = 0.79
121 cells
5 mice

CBI = 0.74
140 cells
5 mice

CBI = 0.49
125 cells
5 mice

Cells (%)
Figure 2.3 GluR2$_{3Y}$ peptide prevents the ocular dominance shift in vivo.

A, OD distribution in non-deprived mice demonstrated a biased distribution toward the contralateral eye (CBI= 0.77±0.01, n=5, 111 cells).

B, OD distribution was shifted toward the open ipsilateral eye following 4 days’ MD (CBI=0.49±0.05, n=5, 154 cells; p=0.007 compared to ND).

C, Tat-GluR2$_{3Y}$ peptide via ip administration had no influence on OD distribution in ND mice (CBI=0.79±0.02, n=5, 121 cells; p=0.35 compared to ND mice).

D, Tat-GluR2$_{3Y}$ or Tat-GluR2$_{3A}$ was administered daily during MD for 4 days. Single unit recording was performed to measure OD distribution at the end of 4 days.

E, MD mice treated with Tat-GluR2$_{3Y}$ did not shift the OD distribution toward the open ipsilateral eye (CBI=0.74±0.02, n=5, 140 cells; p=0.006 compared to MD mice and p=0.26 compared to ND mice).

F, MD mice treated with Tat-GluR2$_{3A}$ shifted OD distribution toward the open ipsilateral eye following MD (CBI=0.49±0.01, n=5, 125 cells; p=0.42 compared to MD mice and p=0.005 compared to ND mice).
In addition, we tested whether Tat-GluR2$_{3Y}$ peptide treatment could alter the spontaneous activity and the evoked response of cortical neurons in the binocular zone of primary visual cortex. Spontaneous activity and evoked response were quantified before peptide or saline treatment, 1 hour, and 2 hour after ip injection of saline or peptide. The spontaneous activity prior to treatment was 3±1 spikes/s and 5±1 spikes/s in saline group and peptide group, respectively (n=3; p=0.48; Figure 2.4 A); evoked response was 20±3 spikes/s and 26±6 spikes/s in saline group and peptide group, respectively (n=3; p=0.44; Figure 2.4 A). Spikes recorded at 1h and 2h after treatment were normalized against the control spontaneous activity and evoked response, in saline and peptide treated group, respectively. Again, there was no significant difference between peptide treated group and saline treated group (n=3; p>0.05; Figure 2.4 B). Thus, we concluded that acute application of Tat-GluR2$_{3Y}$ did not affect spontaneous and evoked activities in primary visual cortex.
Figure 2.4 Acute application of Tat-GluR2_{3Y} did not affect spontaneous and evoked activities in primary visual cortex

A, Numbers of spikes recorded prior to treatment (0h), 1h after treatment, and 2h after treatment are present in the bar chart. At 0h, there was no significant difference in spontaneous activity and evoked activity in saline group and peptide group (n=3; spontaneous activity, p=0.48; evoked activity, p=0.44). The spontaneous activity in control saline group was 5±2 spikes/s and 3±0 spikes/s at 1h and 2h, respectively; it was 5±2 spikes/s and 5±1 spikes/s in peptide group at 1h and 2h, respectively (n=3; p=0.78 for 1h; p=0.85 for 2h). The evoked activity in saline group was 23±4 spikes/s and 22±5 spikes/s at 1h and 2h, respectively; and it was 23±3 spikes/s and 25±4 spikes/s in peptide group at 1h and 2h, respectively (n=3; p=0.95 for 1h; p=0.75 for 2h). Error bar represents s.e.m.

B, Normalized spontaneous and evoked activities at 1h and 2h from both saline and peptide groups are present. The spontaneous activity in saline group was 1.46±0.12 and 1.15±0.32 at 1h and 2h, respectively; it was 1.29±0.25 and 1.37±0.26 in peptide group at 1h and 2h, respectively (n=3; p=0.6 for both 1h and 2h). The evoked activity in saline group was 1.13±0.07 and 1.12±0.22 at 1h and 2h, respectively; and it was 0.98±0.12 and 1.09±0.18 in peptide group at 1h and 2h, respectively (n=3; p=0.41 for 1h; p=0.94 for 2h). Error bar represents s.e.m.
2.3.3 Prevention of OD shift following MD with local infusion of GluR2 peptide

Since it is plausible that systemic administration of the experimental or control peptides could influence the thalamus or the retina, we also attempted to apply the peptide directly to the primary visual cortex. Osmotic minipumps (1007D, Alzet) were used to chronically deliver Tat-GluR23Y or Tat-GluR23A peptides into the primary visual cortex. To avoid any lesion on binocular zone of primary visual cortex, the minipump was implanted in the monocular zone (see methods for details). We first used FITC-conjugated Tat-GluR23Y (2 µM) to determine how long it would take to diffuse over the binocular zone. 24 h after the implantation, the fluorescent dye was observed in the binocular zone of the primary visual cortex (Figure 2.5 A). Thus, we began a 4 day period of MD 24 h after the implantation of the pumps containing Tat-GluR23Y or Tat-GluR23A (2 µM) (Figure 2.5 B). At the end of MD, single unit recording was performed to examine cortical OD. Again, the mice treated with Tat-GluR23Y did not show any OD shift towards the open eye (CBI=0.76±0.01, n=5; p=0.82 compared to ND mice and p=0.004 compared to MD mice; Figure 2.5 C). In contrast, the mice treated with the control Tat-GluR23A showed a significant OD shift to the open eye (CBI=0.46±0.01, n=5; p=0.005 compared to ND mice and p=0.33 compared to MD mice; Figure 2.5 D). These results were consistent with the data obtained after systemic peptide administration, suggesting that the effects were localized in the visual cortex.
Figure 2.5 Local infusion of Tat-GluR23Y in the primary visual cortex blocked the OD shift.

A, FITC conjugated Tat-GluR23Y (2 µM) was delivered using Osmotic minipumps. 24 hours later, the fluorescent dye was detected in the whole visual cortex, but not in the brain stem or on the contralateral side of the brain. Left panel was taken under transmitted light. Right panel was taken under fluorescence. Scale bar is illustrated at the bottom right.

B, Peptides were started 1 day prior to 4-day MD and lasted for 5 days.

C, Local infusion of Tat-GluR23Y prevented the OD shift following MD. Mice treated with Tat-GluR23Y did not show any OD shift towards the open eye (CBI=0.76±0.01, n=5, 134 cells; p=0.82 compared to ND mice and p=0.004 compared to MD mice).

D, Local infusion of Tat-GluR23A did not prevent the OD shift following MD. Mice treated with the control Tat-GluR23A showed a significant OD shift to the open eye (CBI=0.46±0.01, n=5, 125 cells; p=0.005 compared to ND mice and p=0.33 compared to MD mice).
2.3.4 Anatomical measurement of OD distribution with Zif268 staining following MD in mice treated with GluR2 peptide

Our results from single unit recording show that blocking LTD in mouse V1 is able to prevent the MD-induced OD shift that normally occurs during the critical period (Figure 2.6). To further confirm this observation, we performed a complementary anatomical measurement of the OD shift by looking at visually evoked expression of Zif268. Zif268 (or Egr-1) belongs to a family of DNA-binding proteins encoded by immediate-early genes (IEGs) that are characterized by their rapid, protein synthesis-independent induction in the nervous system by a wide range of stimuli (Chaudhuri and Cynader 1993; Chaudhuri, Matsubara et al. 1995; Caleo, Lodovichi et al. 1999; Heynen and Bear 2001). Several previous studies have demonstrated that the expression of Zif268 can be used as an anatomical marker of visually activated neurons in the primary visual cortex (Chaudhuri and Cynader 1993; Caleo, Lodovichi et al. 1999; Pham, Graham et al. 2004). In the present study, MD mice at PD 25-30 were treated with Tat-GluR2\textsubscript{3Y} or Tat-GluR2\textsubscript{3A} daily through an ip injection. After a 4 day period of MD, the sutured eye was reopened and the previously open eye was enucleated. Mice were kept in a dark room for 12 hours and subsequently exposed to a normally-lit environment for another 2 hours (Figure 2.7 A). Immunofluorescent staining demonstrated that nuclear labeling of Zif268 was equally distributed in the binocular and monocular zones of ND mice (Figure 2.7 B). Zif268 expression was significantly decreased in the binocular zone of the primary visual cortex contralateral to the deprived eye following 4 day MD. In MD mice treated with Tat-GLuR2\textsubscript{3Y}, the loss of Zif268 expression was prevented (Figure 2.7 C). However, administration of the control peptide failed to prevent the loss of Zif268.
expression (Figure 2.7 D). Thus, consistent with the results of single unit recording, our results further show that the GluR2_{SY} peptide is able to prevent the loss of contralateral eye responsiveness in the binocular zone of the primary visual cortex following MD.
Figure 2.6 Data summary of the binocularity index results in the various conditions studied. The dots represent individual animals. Horizontal bar represents the mean of CBI for each group.
Figure 2.7

A

B

Peptide i.p injection daily

12h dark+2h light

MD 4days

Deprived eyes reopend
Previously open eye closed

C

MD+CNT peptide

D

MD+peptide
Figure 2.7  Zif268 staining confirmed the prevention of the OD shift by Tat-GluR23Y.

A, Staining of ZIF268 in a mouse with normal visual experience. Right panel shows that the immunofluorescent signals in layers 2, 3, 4, and 6. The binocular zone (BZ) of V1 is marked with a white bar. The monocular zone (MZ) is medial to binocular zone (right in this case). Note the immunofluorescent signals were similar in the BZ and MZ. The right panel shows the same section taken under transmitted light.

B, The closed eyes were opened in the MD mice treated with peptides. The previously open eyes were enucleated. Mice were kept in a dark room for 12 h and then exposed to light for 2 h.

C, Zif268 immunoactivity decreased in BZ of V1 contralateral to the deprived eye in mice treated with Tat-GluR23A(n=2). This is evident by comparing BZ to MZ following MD. The bottom panel shows the same section taken under transmitted light.

D, Tat-GluR23Y prevented the loss of Zif268 immunoactivity in BZ of V1 contralateral to the deprived eye following MD (n=2). The bottom panel shows the same section taken under transmitted light. Scale bar is illustrated at the bottom right.
2.3.5 Facilitation of LTD in primary visual cortex reopens ocular dominance plasticity in adult mouse

We have showed that LTD is necessary for ocular dominance plasticity in primary visual cortex. It is now of great interest to know whether LTD also undergoes a developmental change in early life and how it is related to critical period. To answer this question, we examined LFS-induced LTD at different ages between P11 and P95: before critical period, during critical period, and after critical period. A gradual decline in the magnitude of LTD was observed in the first 3 months (Figure 2.8). The peak of LTD magnitude was in the second week (22±6%; n=6). At the end of 3 months, the magnitude of LTD went down to 0±5% (n=5). Thus, the decline of LTD magnitude coincides with the closure of critical period. Taken together with the role of LTD in visual plasticity during critical period, it is probable that the loss of LTD mechanism during development may account for the closure of visual plasticity in adult.

We then ask whether facilitation of LTD in adult primary visual cortex can reopen ocular dominance plasticity. A recent study reports that a NMDA receptor co-agonist, D-serine, substantially enhanced LTD, but not LTP or depotentiation, in hippocampal slices from adult mice and subsequently spatial reversal teaching (Duffy, Labrie et al. 2008). D-serine occurs primarily in the brain, with highest concentrations in regions enriched in NMDAR (Hashimoto, Nishikawa et al. 1993; Schell, Molliver et al. 1995; Schell, Brady et al. 1997). In these areas, immunohistochemical studies have localized D-serine to protoplasmic astrocytes, which ensheathe nerve terminals especially in areas of the brain enriched in NMDA receptors (Schell, Brady et al. 1997). The augment of LTD in adult
Figure 2.8 Developmental regulation of LTD

LFS-induced LTD was examined in a variety of age groups: before critical period (p11-p12; p16-17), during critical period (p22-p23; p27-p28; p41-43), and after critical period (p83-95). The magnitude of depression is presented in the bar chart. Number of slices in each group is showed on top of the bars. Error bar represents S.E.M. The trend line shows the gradual decline in LTD magnitude in the first 3 months. The magnitude of LTD for each age group is demonstrated in the table.
animal by D-serine may be mediated by the activation of NR2B-containing NMDAR (Duffy, Labrie et al. 2008). We then tested whether D-serine could also enhance LTD in primary visual cortex of adult mouse. Brain slices of adult mice (p90-p100) were incubated with D-serine at 20 µM for 20 min while baseline fEPSP was obtained at the same time. LFS was applied after 20 min baseline recording to induce LTD. In control group, there was no significant change in fEPSP 30 following the end of LFS (100±1%; n=3). In contrast, LTD was rescued in slices treated with D-serine (72±10%, n=3; p<0.01 compared to control group) (Figure 2.9).

If D-serine can enhance LTD in primary visual cortex of adult mouse, the next question is whether it can also reopen ocular dominance plasticity in adult mouse. We explored this possibility in adult MD mice (p90-110). Four day MD did not shift ocular dominance in adult mice treated with saline (CBI=0.78, 92 cells, 3 mice). In MD mice subcutaneously treated with D-serine at 600mg/Kg b.i.d, a significant shift of OD toward the open eye was observed (CBI=0.5, 69 cells, 3 mice; p<0.05 compared to control group) (Figure 2.10). Thus, our data indicate that enhancing LTD mechanism in adult mice can reopen critical period of ocular dominance plasticity. It also further supports that LTD is the mechanism underlying OD plasticity and its developmental change may account for the closure of critical period in adult animal.
Figure 2.9 D-Serine facilitates LFS-induced LTD in primary visual cortex of adult mouse

LFS was not able to induce LTD in primary visual cortex of adult mouse (100±1% of baseline, n=3, open circle). Cortical slices containing primary visual cortex from adult mice (p90-p100) was incubated with D-serine at 20 µM. LTD was present 30 min following LFS in D-serine treated group (72±10% of baseline, n=3; p<0.01 compared to control group; solid diamond).
Figure 2.10 D-serine treatment reopens ocular dominance plasticity in adult mice

A, Adult mice received 4 day MD and subcutaneous administration of D-serine or saline at the same time.

B, MD in adult mice treated with saline did not shift ocular dominance (CBI=0.78, 92 cells, 3 mice). D-serine treatment in adult MD mice shifted ocular dominance toward the open eye (CBI=0.5, 69 cells, 3 mice; p<0.05 compared to control group).
2.4 Discussion

As a classic model for experience-dependent neuronal plasticity, OD plasticity has been extensively studied in past decades. However, the molecular mechanisms remain poorly understood. In the study of visual experience-dependent brain plasticity, LTD has been proposed as the cellular substrate for the loss of visual responsiveness following MD during the critical period (Bear, Cooper et al. 1987; Bear 1996; Bear 2003; Rittenhouse, Siegler et al. 2006). However, the lack of specific inhibitor of LTD has been a hurdle to unambiguously test the role of LTD in ocular dominance plasticity. In the present study, our results provide direct evidence to support that LTD plays a crucial role in the MD-induced OD shift during the critical period. Using the GluR2$_{3Y}$ peptide, we specifically target the final step for expression of LTD, that is, the regulated endocytosis of postsynaptic AMPARs in primary visual cortex (Brebner, Wong et al. 2005). This specific blockade allows us to precisely disrupt the expression of LTD without interfering with normal physiological functions of the neuronal network. We have demonstrated that GluR2$_{3Y}$ peptide prevents the expression of visual cortical LTD without affecting basal synaptic transmission (Figure 2.1 A, B). Consistent with the proposed role of LTD in visual plasticity, administration of the membrane-permeant form of GluR2$_{3Y}$ (Tat-GluR2$_{3Y}$) locally or systemically prevents the shift in OD following MD (Figure 2.6). In addition, the results obtained with electrophysiological recording are confirmed by examining the anatomical distribution of activity-driven expression of Zif268. It is less likely that the rescue effect we observed represents a general disruption of the information processing within the visual pathway because we did not observe any significant influence of the peptide on the basal visual responsiveness (Figure 2.4).
further use D-serine to enhance LTD mechanism in adult primary visual cortex and subsequently reopen critical period of ocular dominance plasticity in adult mice. Our results, together with previous reports (Heynen, Yoon et al. 2003; Frenkel and Bear 2004), strongly support the notion that LTD is the underlying mechanism for the OD shift in the visual cortex during the critical period.

Intensive studies in the hippocampus have established two fundamental models for synaptic modification: Long-term depression (LTD) and Long-term potentiation (LTP) (Bliss and Collingridge 1993; Collingridge and Isaac 2003; Collingridge, Isaac et al. 2004). The expression of LTD and LTP is mediated by a rapid removal or insertion of AMPARs from the postsynaptic neuronal membrane as well as the modulation of AMPARs at the synapse (Lee, Barbarosie et al. 2000; Malinow and Malenka 2002; Collingridge and Isaac 2003; Collingridge, Isaac et al. 2004). The predominant species of AMPARs are composed of GluR1/GluR2 and GluR2/GluR3 (Wenthold, Petralia et al. 1996). GluR2 and GluR3 subunits have short cytoplasmic tails and tend to cycle continuously between extra-synaptic and synaptic sites (Ahmadian, Ju et al. 2004). This constitutive cycling of GluR2 subunit depends on its interaction with NSF (Lee, Simonetta et al. 2004). Moreover, several domains on GluR1 and GluR2 subunits are involved in the regulated endocytosis of AMPARs associated with LTD and OD plasticity. GluR23Y peptide is able to prevent the phosphorylation of three tyrosine residues on carboxyl tail of GluR2 subunit and subsequently blocks LTD in hippocampal slices and neuronal culture (Ahmadian, Ju et al. 2004). The phosphorylation is probably mediated by Src kinase (Ahmadian, Ju et al. 2004). Therefore, our data also suggest that
the phosphorylation of three tyrosine residues is required for the regulated endocytosis of AMPARs. At the same time, we have to emphasize that these three residues are not the only ones required for LTD expression and MD effect. The modulation of other residues, such as dephosphorylation of Ser880 on GluR2 subunit and phosphorylation of Ser845 on GluR1 subunit, are also specifically associated with the expression of LTD in hippocampus (Heynen, Yoon et al. 2003). Furthermore, MD can regulate the phosphorylation status of these two aminoacyl residues in the same manner as LTD does (Heynen, Yoon et al. 2003). It remains unknown how MD can induce LTD in the primary visual cortex. Probable mechanisms may involve NMDAR dependent LTD (Heynen, Yoon et al. 2003) or spike-timing depend LTD (Froemke and Dan 2002; Fu, Djupsund et al. 2002; Froemke, Tsay et al. 2006) following a weak input to primary visual cortex from the retina of the deprived eye.

In the current study, our in vitro results showed that GluR23Y inhibited LTD in layer 2/3 of primary visual cortex by interfering with the endocytosis of AMPARs. Interestingly, a recent paper showed that molecular mechanism of LTD was different between layer 2/3 and layer 4 of primary visual cortex (Crozier, Wang et al. 2007). It reported that endocytosis of AMPARs was required for LTD of layer 4 but not for layer 2/3 (Crozier, Wang et al. 2007). This apparent contradiction between Crozier’s and our studies may be explained by several possibilities. First, regulated endocytosis of AMPARs is a common final step in the expression of LTD in many areas of the brain. The detailed mechanisms that trigger this common step (i.e. the internalization motif involved) could vary depending on the area and developmental stage of the brain under
study and also the protocols used to induce LTD. Indeed, at least three distinct internalization signaling motifs have been identified in the carboxyl tail region of GluR2. Crozier et al used an interference peptide that we previously developed to specifically target the AP2-dependent internalization motif (Lee, Liu et al. 2002). However, in our current study, we utilized an interference peptide that specifically targets the tyrosine-containing motif (Ahmadian, Ju et al. 2004). We did not use the AP-2 interference peptide because of the potential for unwanted effects as a result of blocking other endocytotic processes mediated by the AP2 adaptor. We chose the GluR23Y interference peptide anticipating that, since this motif is unique to GluR2/3, the effect should be more specific. The contradictory results may therefore be due to the fact that the LTD in layer 2/3 cells is preferentially mediated by the tyrosine-containing motif over the AP2 motif, and hence our interference peptide would be more efficient in blocking LTD in these cells than the AP2 interference peptide used by Crozier and colleagues. Another reason could be the different concentrations used in the two studies. Interference peptides are designed as competitive inhibitors to disrupt protein-protein interactions and hence the effectiveness of these peptides is critically dependent on the concentration of the peptide relative to the native binding partners at the site of action. This is further complicated by the fact that peptide efficacy is influenced by the binding characteristics of the targeted protein-protein interaction versus the protein-peptide interaction (binding constants). In our study, a much higher concentration of interference peptide was used than what was used in Crozier’s study. We chose a high concentration because of the potential difficulty in achieving an effective concentration at the synapses, the primary site of action of these peptides, which are remote from the neuronal soma, the site of
introduction of the peptide via the recording pipette. Also, given the fact that the AP2 adaptor is a common adaptor for many clathrin-mediated endocytotic processes, it is possible that a much higher concentration of the interference peptide would be required to reach maximal effective inhibition of the process of interest. In contrast, the tyrosine-containing motif is unique to GluR2/3, so the peptide's effect should not be diluted by binding to off-target proteins, resulting in a lower concentration requirement. Therefore it is possible that, even if the LTD in layer 2/3 neurons utilizes both AP2 and tyrosine-containing motifs, the AP2 interference peptide might fail to block the LTD expression due to an insufficient concentration at the site of action. It is relevant to mention that as a control, Crozier et al. did show that the same concentration of the peptide can block LTD in layer 4 cells. However, given that layer 4 cells are much smaller than layer 2/3 cells, there remains the possibility of a concentration difference at the site of action in the two cell types. In summary, considering all available results, it is safe to say that, as in many other areas of the brain, the LTD in both layer 2/3 and 4 visual cortical cells is mediated by facilitated endocytosis, and the failure to block LTD in layer 2/3 cells in Crozier’s study is likely due either to the AP2 motif not being involved and/or insufficient concentration of the interference peptide at the site of action in that study.

Both excitatory and inhibitory neurons are present in neocortex (Hensch and Stryker 2004). It has been showed that excitatory and inhibitory synapses can undergo LTD and LTP in primary visual cortex (Yoshimura, Ohmura et al. 2003). Moreover, dendritic endocytosis of AMPARs also is observed in GABAergic neurons (Biou, Bhattacharyya et al. 2008). In current study, we show that GluR23Y peptide is able to
prevent the regulated endocytosis of AMPARs. However, it is plausible that this peptide also blocks AMPARs endocytosis/LTD in GABAergic neurons. Thus, question arising from this possibility is in which type of neurons, excitatory or inhibitory, LTD mediates the ocular dominance plasticity. A quantitative study indicates that the number of GluR2 subunits of AMPARs is significantly higher on pyramidal cells than that on GABAergic interneurons in primary visual cortex (He, Hof et al. 2001). Given that the peptide specifically targets GluR2 subunit, we speculate that the possibility for LTD in GABAergic neuron is low, if not unlikely. Having said this, future study is warranted to clarify this issue.

Recent work suggests that the opening and closure of the critical period for OD plasticity may involve different mechanisms. The maturation of GABAergic inhibitory system in cerebral cortex has been proposed to underlie the opening of the critical period (Hensch, Fagioliini et al. 1998; Hensch 2005; Hensch 2005; Hensch and Fagioliini 2005). Enhancing the GABAergic synaptic transmission with diazepam before p19 shifts the critical period to an early age and does not extend the duration of critical period (Fagioliini, Fritschy et al. 2004). In addition, glutamic acid decarboxylase 65 (GAD65) knockout mouse does not have a critical period for OD plasticity, which can be restored by benzodiazepine (Hensch, Fagioliini et al. 1998). On the other hand, it remains unclear what mechanisms are underlying the closure of critical period. Our unpublished data (Xiong, Kojic et al. 2004) and previous report (Kirkwood, Silva et al. 1997) have shown a decline in the magnitude of LTD in the primary visual cortex during development, especially a rapid decline in LTD around the end of the critical period. One probable
mechanism for the decline in LTD would be an experience-dependent modification in the ratio between the synaptic NR2A containing and NR2B containing NMDA receptors (Bear 2003). NR2B containing NMDARs are dominant at postsynaptic membrane in juvenile rodents while NR2A containing ones are dominant in adult animals. It has been reported that binocular deprivation for 3 days in adult can restore the NR2A/2B ratio to juvenile level (He, Hodos et al. 2006). More interestingly, this manipulation reopens the susceptibility for the ocular dominance shift following a short term MD in adult rodents. Taken together with our present data, we suggest that the closure of critical period may be mediated by the weakening of LTD mechanism in the visual cortex. If this is true, a possible explanation for the specific enhancement of LTD in adult animal by exogenous D-serine is that normal endogenous D-serine level is subsaturating in the proximity of NR2B containing NMDAR (extrasynaptic space), but is near saturation at NR2A containing NMDAR (intrasynaptic space). In addition, NR2B containing NMDAR has a higher affinity to D-serine than NR2A containing NMDAR (Priestley, Laughton et al. 1995). Therefore, exogenous D-serine may augment the activity of NR2B containing NMDAR and facilitates LTD in adult.

Our study demonstrates that LTD is an essential early event following MD. It remains unknown what occur after LTD is established. A recent study has reported an increase in the response to the open eye following 7 days’ MD (Frenkel and Bear 2004). This result suggests that the early homosynaptic LTD may be followed by a subsequent heterosynaptic LTP. It will definitely be interesting to examine the effect the GluR23Y in the long term MD. Although we have not done so, our current data at least support the
notion that LTD is one of the major mechanisms for OD plasticity. If the early MD-
induced LTD is necessary for the subsequent induction of heteroysnaptic LTP, we would
expect that the peptide can also prevent OD shift following a long term MD. If this is the
case, the peptide might be a potential target for drug development for the treatment of
amblyopia.

In conclusion, we have demonstrated that LTD plays an essential role in ocular
dominance plasticity of primary visual cortex. This experience-dependent synaptic
remodeling during critical period, in nature, reflects a general rule about how central
nervous system reacts to environmental change and maintains the adaptation to it.
Therefore, it is not surprising to find that LTD may underlie the mechanisms for other
forms of experience-dependent brain plasticity, such as visual recognition memory
(Griffiths, Scott et al. 2008) and stress-induced impairment of spatial memory retrieval
(Wong, Howland et al. 2007). There is no doubt that a further understanding in the
synaptic basis of the visual dominance plasticity will help us gain insight into the process,
as well as to design better strategies by which the older brain can learn (Hensch 2005).
2.5 References:


Yoshimura, Y., T. Ohmura, et al. (2003). "Two forms of synaptic plasticity with distinct dependence on age, experience, and NMDA receptor subtype in rat visual cortex."

Chapter III

Anisomycin activates p38 MAP kinase to induce LTD in mouse primary visual cortex *

3.1 Introduction

Long-term depression (LTD) refers to a lasting decrease in the amplitude of a synaptic response which follows low-frequency stimulation (LFS) of a presynaptic pathway. The early phase of LTD is postulated to be the result of the internalization of postsynaptic receptors; and the maintenance of LTD involves a series of mechanisms, with post-translational events typically underlying the early maintenance phase, and transcription- and translation-dependent events underling the late (>3hr) maintenance phase (Huang, Li et al. 1994; Bennett 2000; Luscher, Nicoll et al. 2000). Intensive studies have been performed to explore the protein synthesis dependent phase of LTD using protein synthesis inhibitors. Among the protein synthesis inhibitors, anisomycin has been widely used to study the late phase of LTP and LTD (Kauderer and Kandel 2000; Steward and Worley 2001). It binds to the 60 S ribosomal subunit in eukaryotic cells and inhibits the peptidyltransferase reaction (Jimenez, Sanchez et al. 1975; Middlebrook and Leatherman 1989). In hippocampus and cerebellum, anisomycin had no impact on basic synaptic transmission and did not influence the early phase of LFS-induced LTD (Linden 1996; Huber, Kayser et al. 2000; Sajikumar and Frey 2003). In addition, anisomycin at 10-30 µM had no effect on voltage-gated calcium channels and mGluR1 receptors in the cerebellum (Linden 1996).

In addition to its protein synthesis inhibition effect, anisomycin is a strong activator of the p38 and C-jun N-terminal kinase (JNK) MAPK pathway (Shifrin and Anderson 1999). P38 MAPK was initially characterized owing to its role in the response of cells to various adverse stimuli such as heat shock and bacterial endotoxin linked to
apoptosis. Once activated, it phosphorylates a variety of proteins and relay signals downstream. P38 MAPK is highly expressed in brain areas including cerebral cortex, hippocampus, cerebellum, and few nuclei of the brainstem (Zhu, Qin et al. 2002). Recently, emerging evidence suggests that p38 MAPK is also a key player in the regulation of synaptic plasticity. Provocative new results indicate a requirement for p38 activation in a different form of hippocampal LTD that depends on NMDAR activation (Schwarze, Ho et al. 1999). Moreover, p38 also appears to be crucial for metabotropic glutamate receptor (mGluR) dependent LTD since perfusion of an active form of p38 can mimic and occlude this mGluR dependent LTD form of plasticity (Bolshakov, Carboni et al. 2000).

In the primary visual cortex LTD has been postulated to play a crucial role in the experience-dependent visual plasticity during the critical period (Heynen, Yoon et al. 2003). In this study, we initially intended to study the late phase of LTD in primary visual cortex. Surprisingly, we found that anisomycin administered prior to LFS induced prolonged depression of basal synaptic transmission that occluded subsequent LFS-LTD. We were able to demonstrate that this anisomycin-LTD was primarily mediated via the p38 MAPK signaling pathway and, likewise LFS-induced LTD, required GluR2-dependent clathrin-mediated endocytosis of postsynaptic AMPA receptors.
3.2 Materials and Methods

3.2.1 Slice preparation

Slices were prepared from C57BL/6 mice (Charles River, Quebec, Canada) at postnatal day (PD) 22–28. The protocols for animal experiments were in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals and was approved by the Animal Care Center, University of British Columbia. Adequate measures were taken to minimize pain or discomfort. Animals were anesthetized with urethane (5mg/kg). After decapitation, coronal slices containing either primary visual cortex or hippocampus (350 µM thick) were prepared using a vibrating blade microtome (Leica, Germany) in ice-cold artificial CSF (ACSF) containing (in mM): 126 NaCl, 5 KCl, 1 MgSO4, 2 CaCl2, 1.25 NaH2PO4, 26 NaHCO3, and 20 glucose that was bubbled with carbogen (95%O2/5% CO2) to adjust the pH to 7.4. Brain slices were placed in a “submersion” recording chamber with carbogenated ACSF and allowed to recover at 30°C for a minimum of 60 min prior to recording.

3.2.2 Electrophysiological recording.

Electrophysiological recordings were conducted in a submersion-type chamber perfused with ACSF at the rate of 1.5ml/min at 30°C. For the visual cortex, extracellular field potential recordings were made from layers 2/3 with glass electrodes (1MΩ) filled with 1M NaCl. Field potentials were evoked via electrical stimulation through a concentric bipolar stimulating electrode (CBBRC75; FHC, Bowdoinham, ME) placed in the center of the cortical thickness that corresponded to layer 4. For the hippocampus, excitatory postsynaptic responses were evoked by stimulating the Schaffer collateral-
commissural pathway with the electrode as described above and recordings were made in
the stratum radiatum of the CA1 region at least 60-80 µm away from the cell body layer.
Slices were evaluated for responsivity every 15 seconds with a constant current pulse of
100 µs duration and 100-200 µA of current, which yielded a half-maximal response.
After a stable baseline was achieved, LTD was induced using a low frequency
stimulation protocol (LFS) consisting of 900 stimuli at 1Hz.

3.2.3 Reagents.

Chemicals used in this study were as follows: anisomycin (Sigma, St. Louis, MO), cycloheximide (Tocris, Ellisville, MO), Emetine dihydrochloride hydrate (Sigma, St. Louis, MO), 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580; Tocris, Ellisville, MO), anthra [1,9-cd] pyrazol-6 (2H)-one (SP600125; Tocris, Ellisville, MO). Tat-GluR23Y (YGRKKRRQRRRYKECVNYS) and Tat-GluR23A (YGRKKRRQRRRAKECANVAS) peptides were synthesized by Nucleic Acid and Peptide Service Centre at University of British Columbia. Anisomycin, SB203580, and SP600125 were dissolved in DMSO. The final concentration of DMSO was less than 0.1%. Cycloheximide, emetine, D-APV, and the synthesized peptide were dissolved in water. The peptides were prepared as stock solutions of 1mM stored at -80°C and diluted to 0.4µM with ACSF immediately before each experiment. Because emetine is sensitive to light, all experiments involving it were conducted in a darkened room.
3.2.4 Immunoblotting for p38 MAPK.

Slices (containing primary visual cortex) were maintained in a static incubation chamber in ACSF at 30°C and aerated with 95% O2–5% CO2 for at least 1 hour prior to drug treatment. 30 min after either anisomycin (20 μM) or DMSO (0.1%) treatment, the primary visual cortex was rapidly dissected in ice cold 0.1M PBS. For each experimental group, at least five slices were pooled and homogenized in lysis buffer containing 150 mM NaCl, 10 mM Tris aninomethane (PH 7.4), 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1% NP-40, 2 mM PMSF, 10 ug/ml leupeptin, 10 ug/ml aprotinin, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, and 2 mM sodium orthovanadate. Protein content was evaluated with the DC Protein Assay Kit (BIO-RAD, Hercules, CA). Samples containing 100 μg protein were resolved on 10% SDS-PAGE and transferred to nitrocellulose. Membranes were blocked and incubated with the antibodies at the appropriate dilutions overnight at 4°C. The immunocomplexes were detected with 1:5000 HRP-conjugated goat anti-mouse IgG (PerkinElmer Life Sciences, Boston, MA) followed by enhanced chemiluminescence (ECL detection reagents, Amersham Pharmacia Biotech). Primary anti-di-phospho p38 MAPK monoclonal antibodies from mouse (New England BioLabs, Beverly, Massachusetts) were diluted at 1:500. Primary anti-total p38 MAPK antibodies (mouse, 1:1000) were obtained from Santa Cruz Biotechnology (Santa Cruz, California). Primary anti-β actin antibodies from rabbit (Sigma, Oakville, Ontario) were diluted at 1:250. Densitometric quantification of immunopositive bands was done using Quantity One (Bio-Rad, Hercules, CA).
3.2.5 Data analysis.

Only experiments with stable baseline recordings (<5% change over the baseline period) were included in the statistical analysis. The magnitudes of the field potentials were normalized to baseline and expressed as mean±SEM. To calculate the magnitude of LTD, the fEPSP amplitude obtained during the last 5 min of the baseline recording period and the final 5 min following each LFS period were compared. A t-test was used to compare averaged normalized fEPSP obtained from slices under different experimental conditions. For western blotting data, P-p38 signal and p38 signal were normalized against β actin signal. The ratios of P-p38 over p38 were compared between the DMSO treated group and anisomycin treated group.
3.3 Results

3.3.1. Effect of anisomycin on synaptic transmission and long-term depression in the primary visual cortex

The initial aim of our study was to test the effect of anisomycin on the late phase of LTD in mouse primary visual cortex. It has been shown that anisomycin has no effect on baseline fEPSP and short term LTD induced by LFS in hippocampus and cerebellum (Linden 1996; Huber, Kayser et al. 2000). In the first experiment, we started recording fEPSP 60 min after incubating slices containing primary visual cortex in 20 µM anisomycin. LFS was delivered after minimal 15 min stable baseline. In this preparation, LTD could not be induced in the slices pre-treated with anisomycin (95±3% of the baseline recorded in the last 5 min prior to LFS; n=31; p<0.01 compared to DMSO treated slices), while normal LTD was induced in sliced pre-treated with 0.1% DMSO as a vesicle (85±1%; n=10) (Figure 3.1 A). Although previous reports have suggested that anisomycin at 20µM does not interfere with synaptic function other than with protein synthesis in the hippocampus (Iordanov, Pribnow et al. 1997) and the cerebellum (Linden 1996), we decided to look at the basal synaptic transmission during anisomycin treatment. After achieving 15 min baseline, we applied 20 µM anisomycin through bath perfusion. A gradual decline in the basal fEPSP was observed. The normalized fEPSP magnitude was 88±3% and 80±3% of baseline, 30 min and 60 min after the beginning of anisomycin perfusion respectively (n=11; p<0.01 compared to original baseline) (Figure 3.1 B). Consistent with the initial finding, in the same slices, subsequent LFS-induced LTD was significantly reduced 30 min after the end of LFS (92±3% of the baseline recorded in the last 5 min prior to LFS; n=11; p<0.05 compared to slices treated with DMSO) (Figure
3.1B). In contrast, slices treated only with 0.1% DMSO as a vesicle did not have a
decline in the baseline synaptic transmission (96±3% of baseline; n=3; p<0.05 compared
with slices treated with anisomycin) and showed a normal magnitude of LTD (83±2% of
baseline recorded 5 min prior to LFS; n=3; p>0.05 compared with control LTD; p<0.05
compared with slices treated with anisomycin). These results suggest that anisomycin is
able to induce LTD-like phenomenon that occludes the LFS-induced LTD in primary
visual cortex.

To confirm that anisomycin has no effect on baseline fEPSP in hippocampal
CA3-CA1 pathway, we applied anisomycin (20 µM) after obtaining a stable baseline. In
hippocampus, in contrast to primary visual cortex, anisomycin did not change the
amplitude of fEPSP (104±2% and 100±2% of baseline, 30 min and 60 min after the
beginning of anisomycin perfusion respectively; p>0.05 compared to original baseline;
n=4). In the same slices, a subsequent LFS, in the presence of anisomycin, induced LTD
that was 84±3% of the baseline (p>0.05 compared with DMSO control group; n=4)
(Figure 3.1 C).

In addition, we confirmed that the LFS-induced LTD that was occluded by
anisomycin was dependent on NMDA receptor activation since 50µM D-APV blocked
this LTD (97±2%; n=6; p<0.05 compared to control LTD) while the control group
showed a normal LTD (85±1%; n=17) (Figure 3.1 D). Interestingly, we found that the
magnitude of fEPSP reduction after 1-hour treatment with anisomycin was similar to the
magnitude of the normal LTD induced by LFS without anisomycin (p>0.05).
Figure 3.1 Anisomycin induced LTD in the primary visual cortex and occluded the LFS induced LTD.

A, pre-treatment of 20 μM anisomycin significantly decreased the magnitude of LTD (95±3% of the baseline; n=31). 0.1% DMSO had no influence on the LFS-induced LTD.

B, 20 μM anisomycin significantly decreased the baseline synaptic transmission, which measured 88±3% and 80±3% of baseline, 30 min and 60 min after the beginning of perfusion respectively (p<0.01 compared to original baseline; n=11). The following LFS led to a weak depression of fEPSP, which was 92±3% of the baseline recorded during 5 min prior to LFS (900 pulses at 1Hz) (p<0.05 compared with slices treated with DMSO). The control group treated with 0.1% DMSO did not have a decline in the baseline
synaptic transmission and the magnitude of fEPSP decreased to 83±2% 30 min after LFS (p<0.05 compared with anisomycin treated group; n=3). Representative fEPSPs averaged from 10 consecutive stimuli were taken at the time points indicated in the top graphs (1, 2, and 3).

C, In CA3-CA1 pathway of hippocampus, perfusion of anisomycin at 20 µM for 1 hour did not influence the baseline (100±2% of baseline at 60 min; p>0.05 compared with original baseline; n=4) and did not occlude LFS-induced LTD (84±3% of baseline 30 min after LFS; p>0.05 compared with slices treated with DMSO; n=4). Representative fEPSPs averaged from 10 consecutive stimuli were taken at the time points indicated in the top graphs (1, 2, and 3).

D, Low Frequency Stimulation (LFS) induces LTD in mouse primary visual cortex and requires the activation of NMDA receptor. LFS induced a LTD in layer II/III (85±1%; p<0.05; n=17) while D-APV (50 µM) significantly blocked LFS-induced LTD (97±1%; p<0.05; n=6).
3.3.2 Activation of p38 is required for anisomycin-induced decline of fEPSPs and LTD occlusion

Anisomycin is a potent activator of p38/JNK MAPK. Treatment with anisomycin at 3.8µM for 15 minutes is enough to activate p38/JNK MAPK pathway (Shifrin and Anderson 1999). To determine whether the activation of p38/JNK was the cause of the anisomycin-LTD or whether it resulted from a more general protein synthesis inhibition, two additional protein synthesis inhibitors, emetine (100µM) and cycloheximide (60µM) were used. Emetine can efficiently block protein synthesis at this concentration but does not activate p38/JNK (Iordanov, Pribnow et al. 1997). Cycloheximide is only a weak activator of p38/JNK even at higher concentrations (80µM) (Iordanov, Pribnow et al. 1997). Slices treated for 1 hour with either emetine or cycloheximide did not demonstrate any decline in the magnitude of fEPSP (p>0.05 compared with baseline; n=3) (Figure 3.2 A). Subsequent LFS still induced LTD, as measured 30 minutes after the end of LFS. There was no significant difference in the magnitude of LTD after emetine or cycloheximide compared with control LTD (emetine: 82±3%, p>0.05, n=14; cycloheximide: 80±2%, p>0.05, n=11) (Figure 3.2 B). The results suggest that anisomycin has another activity beyond its protein synthesis effect that occludes LTD.

Emetine competes with anisomycin for binding to the 60 S ribosomal subunit. It has previously been shown that pretreatment of cells with emetine abolishes the activation of the p38/JNK MAPK pathway by anisomycin (Iordanov, Pribnow et al. 1997). Hence, we asked whether emetine could rescue LTD in the presence of
Figure 3.2 Anisomycin induced LTD is mediated by the activation of P38/JNK MAPK pathway rather than its ability to inhibit protein synthesis.

**A**, brain slices were incubated in either cycloheximide (60 µM) or emetine (100 µM) for 1 hour. There was no decline in the baseline synaptic transmission in the cycloheximide group (103±9%; p>0.05 compared to baseline; n=3) and emetine group (100±7%; p>0.05 compared to baseline; n=3).

**B**, cycloheximide (60 µM) and emetine (100 µM) were applied to the slices 1 hour prior to LFS. Although both chemicals are strong protein synthesis inhibitors at the concentration used, they did not influence LFS-induced LTD. There was no significant
difference between LTD in the presence of either synthesis inhibitor and the control LTD (p<0.05; n=11 for cycloheximide, n=14 for emetine).

C, pre-incubation of emetine (100 µM) 1 hour prior to anisomycin (20 µM) significantly rescued the LTD following LFS (59±8%; n=8; p<0.05 compared to the group treated only with anisomycin).

D, application of anisomycin for 30 min activated P38. Visual cortex slices were incubated with either anisomycin (20 µM) or DMSO (0.1%) for half an hour. The average phosphorylated P38 was 168±3% compared with control group treated with DMSO (p<0.05; n=4). Representative fEPSPs averaged from 10 consecutive stimuli were taken at the time points indicated in the top graphs (1, 2 and 3).
anisomycin. We found that pretreatment with emetine (100µM) for 1 hour prior to anisomycin administration significantly increased LFS-induced LTD compared to the slices treated with anisomycin only (59±8%; n=8; p<0.05) (Figure 3.2 C). These results further suggest that the anisomycin effect on stress activated protein kinase rather than on protein synthesis inhibition mediates the occlusion of LFS-induced LTD.

To further determine whether p38 or JNK had an effect on baseline synaptic transmission, a specific inhibitor of p38 (SB 203580) and another specific inhibitor of JNK (SP 600125) were used. Application of 2µM SB 203580 (IC50=0.6 µM) for 2 hours had no significant effect on baseline synaptic transmission, which was measured at 95±4% of the original baseline level (n=7) (Figure 3.3 A). In the same slices, LFS following SB 203580 still induced LTD (82±6 % of baseline recorded during 5 min prior to LFS; p>0.05 compared with control LTD; p<0.05 compared to slices treated with anisomycin) (Figure 3.3 A). This result implies that SB203580 has no effect on basic synaptic transmission and p38 MAPK is not necessary for NMDA receptor dependent LTD in mouse visual cortex. In the slices treated with 2µM SB 203580 1 hour prior to the anisomycin, perfusion of anisomycin for 1 hour only caused a weak decline in baseline (93±4%; n=11; p<0.05 compared to the slices treated only with anisomycin) (Figure 3.3 B). Furthermore, in the same slices, LFS following the treatment with both SB 203580 and anisomycin induced LTD that was not significantly different from control LTD (82±4% of baseline recorded during 5 min prior to LFS; p>0.05; n=11). This result highly suggests that p38 MAPK mediated the anisomycin-induced decline in fEPSP. To prove that anisomycin does activate p38 MAPK in primary visual cortex, we carried out
western blotting to look at the level of phosphorylated p38. After treatment with anisomycin (20µM) for half an hour, the average level of phosphorylated p38 was 168±3% of control slices treated with DMSO (p<0.05; n=3) (Figure 3.2 D).

On the other hand, the JNK inhibitor (SP 600125 at 20µM, IC50=40nM) neither rescued the decline of baseline fEPSPs (81±4%; n=7; p>0.05 compared with slices treated only with anisomycin) nor abolished LTD induced by anisomycin (89±4% of baseline recorded during 5 min prior to LFS; n=7; p>0.05 if compared to anisomycin treated slices) (Figure 3.3 C). These results suggest anisomycin activation of JNK was not critical for the decline in baseline synaptic transmission and the impediment of LTD.

These results, all together, implied that p38 activation is not required for the induction of NMDA receptor dependent LTD in the mouse visual cortex. However, the anisomycin activation of p38 is sufficient to induce another form of LTD.
Figure 3.3
**Figure 3.3 Anisomycin induced LTD requires activation of P38 MAPK.**

*A,* P38 inhibitor, SB203580, had no effect on baseline synaptic transmission and LFS-induced LTD by itself. Incubation of SB203580 (2 µM) for 2 hours had no significant effect on baseline responses (95±4%; n=7) and LFS-induced LTD was not significantly different from control LTD (82±6% of baseline recorded during 5 min prior to LFS; p>0.05; n=11). There was no significant difference the group treated with SB203580 and the one treated with DMSO (p>0.05; n=3 for DMSO group).

*B,* incubation of SB203580 (2 µM) 1 hour prior to anisomycin (20 µM) dramatically rescued the decline of baseline caused by anisomycin. The baseline fEPSP measured 1 hour after application of anisomycin was 93±4% (n=11), which was significantly higher than the one measured in the group treated only with anisomycin (p<0.05) but similar to the group treated with SB203580 alone (p>0.05). In the same slices, subsequent LFS induced normal LTD (93±4%; p>0.05; n=11).

*C,* the JNK inhibitor, SP600125 (20 µM), did not significantly rescue the decline of baseline following application of anisomycin and did not prevent the occlusion of subsequent LTD (89±4%; n=7; p>0.05).


3.3.3 Activation of p38 induces endocytosis of surface AMPA receptors

Since the anisomycin induced decline of baseline synaptic transmission occluded the subsequent LFS-induced LTD, we suggested that common mechanisms were involved in both effects. To further explore this question, we took advantage of a newly developed GluR23Y peptide (YKEGYNVYG) derived from the sequence of the AMPA receptor GluR2 carboxyl terminus that, when applied intracellularly, specifically blocks regulated AMPA receptor endocytosis and prevents LFS-induced LTD in hippocampal slices (Ahmadian, Ju et al. 2004). The peptide, alone with its control GluR23A in which the three required tyrosine residues were replaced with alanines, were rendered cell-permeant by fusing it to the cell-membrane transduction domain of HIV-1 Tat protein (YGRKRRQKRRQK) (Schwarze, Ho et al. 1999 1999). Consistent with the hippocampus data, bath application of the Tat-GluR23Y peptide (0.4 µM) 60 min prior to LFS significantly decreased the magnitude of LTD induced by LFS in mouse visual cortex (96±2%, n=7, p<0.05 compared with control LTD), while having no effect on basal synaptic transmission. By contrary, the GluR23A control peptide (0.4 µM) had no effect on the LFS-induced LTD (87±6%; n=4; p>0.05 compared with control LTD) (Figure 3.4 A). This result suggests that LFS-induced LTD in mouse primary visual cortex is mediated by the regulated endocytosis of AMPA receptors. We then asked whether the anisomycin-p38 induced fEPSP depression shared a common mechanism with the LFS-LTD, which required GluR2-dependent AMPA receptor endocytosis. To answer this question, we tested if Tat-GluR23Y (0.4 µM) could block the anisomycin-induced decline of fEPSPs. Slices were treated with the wild type peptide for 1 hour and then subjected to the co-application of peptide and anisomycin for another hour. The magnitude of the
baseline response recorded 1 hour after the beginning of anisomycin was 99±2% (n=8),
which was significantly different from the slices treated with anisomycin alone (p<0.01)
(Figure 3.4 B). In great contrast, the control peptide was not able to rescue the decline in
the baseline synaptic transmission following anisomycin (p<0.05 compared with wild
type peptide treated group; p>0.05 compared with slices treated with anisomycin; n=3)
(Figure 3.4 B). Taken together, these results in the mouse primary visual cortex support
the notion that p38 activation by anisomycin induces an AMPA receptor endocytosis and
thus leads to a decline in the baseline transmission, which occludes subsequent LTD.
Figure 3.4 Activation of p38 induced endocytosis of surface AMPA receptors.

A, pre-incubation with synthesized wild type GluR2 C-tail peptide (0.4 µM) 1 hour prior to LFS blocked the LFS-induced LTD in V1 brain slices (p<0.05; n=7) while the mutated
peptide which served as a negative control had no effect on LFS-induced LTD (p>0.05; n=4).

B. Application of wild type peptide (1µg/ml) 1 hour prior to anisomycin (20 µM) completely abolished the effect of anisomycin on baseline. The fEPSPs measured 1 hour after beginning of anisomycin was 99±2% of the baseline response measured before anisomycin application (p<0.01; n=8). A subsequent LFS protocol confirmed that LTD was occluded by the peptide. The control peptide did not rescue the decline in the magnitude of the fEPSP following anisomycin (n=3). Representative fEPSPs averaged from 10 consecutive stimuli were taken at the time points indicated in the left graphs (1, 2 and 3).
3.4 Discussion

Anisomycin is a potent protein synthesis inhibitor. It binds to the 60 S ribosomal subunit in eukaryotic cells and inhibits the peptidyltransferase reaction (Jimenez, Sanchez et al. 1975; Middlebrook and Leatherman 1989). Independent of its ability to block translation, anisomycin initiates the activation of the stress-activated protein kinase p38/JNK although the mechanism of this activation is still unknown (Iordanov, Pribnow et al. 1997). As an efficient protein synthesis inhibitor, anisomycin has been widely used to study protein synthesis dependent synaptic plasticity (Heynen, Yoon et al. 2003 10, 13).

In the current study, we elucidate another role of anisomycin in synaptic plasticity. We find that anisomycin treatment (20µM for 1 hour) induces a LTD-like phenomenon in the mouse primary visual cortex. This anisomycin-LTD is mediated by the activation of the p38 MAP kinase pathway. Moreover, the LTD induced by anisomycin occludes NMDA receptor dependent LTD induced by Low Frequency Stimulation. Although the p38 activation is sufficient to induce LTD in primary visual cortex, it is not required for LFS-induced LTD since the p38 inhibitor (SB203580) does not block this LTD. To further explore the mechanism underlying the p38 mediated LTD, we show that a synthesized peptide derived from the AMPA receptor GluR2 C-tail blocks the LTD induced by anisomycin. This peptide is able to specifically prevent the phosphorylation of there tyrosine residues on carboxyl tail of the GluR2 subunit of AMPA receptor and in turn blocks the regulated clathrin-mediated endocytosis of postsynaptic AMPA receptors (Ahmadian, Ju et al. 2004). Our results suggest that “anisomycin LTD”, similar to the LFS-induced LTD, requires the regulated endocytosis of AMPA receptors. In addition, LFS produced a rapid reduction in fEPSP while anisomycin produced a gradual decline
of fEPSP. The major reason is that LFS appears to trigger a fast intracellular signaling cascade. In contrast, extracellular application of anisomycin induces a slow response perhaps because of the kinetics of its penetration. So far as we know, this is the first study that demonstrates a role for p38 MAPK in the synaptic plasticity of mouse primary visual cortex.

3.4.1 Role of p38 MAPK in anisomycin-induced LTD

Anisomycin-induced LTD is mediated by the activation of the p38 MAPK pathway rather than by its protein synthesis inhibition function. Three lines of evidence support this notion. First, two protein synthesis inhibitors at the concentrations that are sufficient to block protein synthesis, which either does not activate (emetine) or slightly activates p38 (cycloheximide), did not affect baseline synaptic transmission or occlude LTD. Second, pre-application of emetine that is able to abolish the activation of the MAPK pathway by anisomycin (Luscher, Nicoll et al. 2000) rescued LFS-induced LTD occluded by anisomycin. Third, SB203580, a p38 inhibitor, rescued both the anisomycin LTD and LFS-induced LTD occluded by anisomycin but JNK inhibitor did not. In terms of specificity, SB203580 is a potent inhibitor and highly specific for p38 MAPK at the concentration (2 µM) used in this study (Taha and Stryker 2002). Consistent with our findings, a previous report in hippocampus also showed that the p38 activation is sufficient to induce progressive depression of the amplitude of the EPSC (Bolshakov, Carboni et al. 2000).
One possible argument would be that the anisomycin-induced LTD observed in this study represents only a simple deterioration of the general health of the slices caused by protein synthesis blockade. We believe that this is not the case since slices treated with either SB203580 (p38 inhibitor) (Figure 3.3 B) or the synthesized GluR23Y peptide (Figure 3.4 B) prior to anisomycin did not show significant declines in the magnitude of fEPSP after 1 hour treatment of anisomycin. Furthermore, other protein synthesis inhibitors, emetine and cycloheximide, do not depress fEPSPs (Figure 3.2 A) at the concentrations that have been widely used to block protein synthesis (Luscher, Nicoll et al. 2000). Therefore, the depression of fEPSP following anisomycin in the visual cortex reflects a change in synaptic transmission rather than general health of the slices.

Cycloheximide, in our study, did not influence early LTD within 30 min after tetanus. However, it has been used to block ocular dominance plasticity in developing mouse visual cortex (Taha and Stryker 2002). We speculate that cycloheximide may inhibit the synthesis of proteins required for maintenance of late phase LTD to impair the ocular dominance plasticity. Further study is warranted to clarify this possibility.

3.4.2 Region differential role of p38 MAPK in LTD

The role of the MAPK pathway in synaptic plasticity has been intensively studied. P38 MAPK pathway is postulated to play an important role in LTD. In rat hippocampus, p38 is required for the mGluR dependent LTD in CA1 (Bolshakov, Carboni et al. 2000) and dentate gyrus (Rush, Wu et al. 2002) as well as NMDA receptor dependent LTD in hippocampal slice cultures (Zhu, Qin et al. 2002). Also, the active form of p38 is
sufficient to induce a decline in the amplitude of the EPSP in area CA1 (Bolshakov, Carboni et al. 2000). Our data provide evidences showing that the p38 MAPK pathway also plays a role in LTD in the primary visual cortex. We demonstrate that activation of p38 is sufficient to induce a depression of the baseline synaptic transmission in the mouse primary visual cortex. This role of p38 is supported by observation that a p38 MAPK inhibitor abolishes the LTD induced by anisomycin.

Interestingly, in hippocampal CA1, the application of anisomycin has no influence on baseline synaptic transmission at a concentration used in our and others studies (Jimenez, Sanchez et al. 1975; Iordanov, Pribnow et al. 1997). Anisomycin does, however, block the late phase of LTD in hippocampal organotypic culture (Kauderer and Kandel 2000). Several reasons may account for the discrepancy between findings in hippocampus and primary visual cortex. One possibility is the region specific distribution of p38 MAPK subtypes in the brain (Zhu, Qin et al. 2002). In addition, subcellular localization and downstream signaling pathways of p38 MAPK subtypes may also be region dependent. Another possibility is that anisomycin at the concentration used in this study may activate a higher portion of p38 MAPK in the visual cortex than in the hippocampus. The hippocampus may also require a greater degree of activation of p38 to trigger the regulated AMPA receptor endocytosis than the visual cortex. This region specific role of p38 MAPK in synaptic plasticity is further supported with our finding that p38 MAPK was not required for NMDA receptor dependent LTD in primary visual cortex while it was necessary for NMDA receptor dependent LTD in hippocampal neurons (Schwarze, Ho et al. 1999).
3.4.3 How does activation of p38 MAPK lead to depression of fEPSP?

The mechanism underlying the depression of the fEPSP by activation of p38 MAPK is still uncertain. Our data suggest that activated p38 MAPK induces regulated AMPA receptor endocytosis to decrease synaptic transmission. AMPARs are composed of the different subunits GluR1–GluR4 (Hollmann and Heinemann 1994). Each receptor complex is composed of four subunits (Rosenmund, Stern-Bach et al. 1998). The predominant species of AMPARs are receptors composed of GluR1/GluR2 and GluR2/GluR3 (Wenthold, Petralia et al. 1996). GluR2 and GluR3 subunits have short cytoplasmic tails and tend to cycle continuously between non-synaptic and synaptic sites (Ahmadian, Ju et al. 2004). This constitutive cycling of GluR2 subunit depends on its interaction with NSF (N-ethylmaleimide sensitive fusion protein) (Lee, Simonetta et al. 2004). Moreover, several domains on GluR2 subunit are involved in the regulated endocytosis of AMPARs associated with LTD. A recent study demonstrates that three tyrosine residues on the carboxyl tail of GluR2 subunit are required for insulin- and NMDAR- dependent LTD, whereas it does not influence the constitutive cycling of AMPARs (Ahmadian, Ju et al. 2004). A synthesized peptide containing these three tyrosine residues, which is derived from carboxyl tail of GluR2 subunit, is able to specifically block the regulated endocytosis of AMPARs in hippocampal slices and neuronal culture (Ahmadian, Ju et al. 2004). This peptide is able to specifically prevent the phosphorylation of these tyrosine residues on carboxyl tail of the GluR2 subunit of AMPA receptor and in turn blocks the regulated clathrin-mediated endocytosis of postsynaptic AMPA receptors (Ahmadian, Ju et al. 2004). By using this peptide, we
successfully blocked the LTD induced by anisomycin or LFS in mouse primary visual cortex. These results strongly suggest both anisomycin-LTD and LFS-LTD share the common pathway, which is to induce long-lasting depression of AMPA receptor-mediated synaptic transmission by clathrin-mediated GluR2-dependent endocytosis of postsynaptic AMPA receptors. Moreover, previous reports have shown that activation of group 1 metabotropic glutamate receptors can induce internalization of postsynaptic AMPAR (Snyder, Philpot et al. 2001) and that the mGluR-dependent LTD is mediated via the activation of p38 MAPK (Bolshakov, Carboni et al. 2000). Taken together with these findings, our results suggest that activation of the p38 MAPK pathway facilitates the phosphorylation of AMPAR GluR2 subunits and induces the internalization of AMPAR. Another rising question from this study is how p38 MAPK activation leads to the removal of surface AMPA receptors. A recent paper shows that p38 MAPK has the ability to accelerate the endocytosis trafficking by stimulating the activity of guanyl nucleotide dissociation inhibitor (GDI) in extracting Rab5 from endosomal membranes and forming a GDI-Rab5 complex (Cavalli, Vilbois et al. 2001). Furthermore, the GDI-Rab5 complex has been shown to be an important component of the machinery controlling the clathrin-coated endocytotic vesicle formation (McLauchlan, Newell et al. 1998). Taken together, we suspect that the probable downstream processing result of p38 MAPK activation might be the formation of the guanyl nucleotide dissociation inhibitor-Rab5 complex (Huang, You et al. 2004).

Our study suggests that precautions are needed when anisomycin is used to study protein synthesis dependent synaptic plasticity, especially in the mouse visual cortex.
Also, this study demonstrates the p38 MAPK is able to induce LTD in the mouse primary visual cortex of. Similar to NMDA receptor dependent LTD, this process also requires regulated endocytosis of AMPA receptors. Further experiments are needed to identify the molecular pathway of regulated endocytosis of AMPA receptor by p38 MAPK activity.
3.5 References:


Chapter IV

General discussion
The mammalian cortex is organized anatomically into discrete areas, which receive, process, and transmit neural signals along functional pathways. The ability of the nervous system to wire and rewire itself in response to lasting changes in experience has become known as experience-dependent plasticity (Berardi, Pizzorusso et al. 2000; Hensch 2005). The brain is able to remodel its connections in order to adjust its responses to changing conditions. Particularly during early development, the brain shows the highest plasticity when connections between neurons are being made and broken for the first time (Giza and Prins 2006; Horng and Sur 2006). Clear evidence for experience-dependent brain plasticity has been found in the primary sensory systems of several species (Hensch 2004). Animal models are now revealing, with higher resolution, the molecular, cellular and structural events that underlie experience-dependent circuit refinement.

Primary visual cortex has been one of the classic models to study experience-dependent brain plasticity for decades (Bear 2003). Deprivation studies, conducted first in carnivores and primates and later in rodents, have shown that the maturation of visual cortex requires normal visual experience during a brief postnatal critical period of enhanced plasticity (Wiesel and Hubel 1963; Hubel, Wiesel et al. 1977; Gordon and Stryker 1996). In rodents, for instance, a short term of MD (1-4 days) decreases neuronal response to the deprived eye and shifts the ocular dominance distributions toward the non-deprived eye (Gordon and Stryker 1996; Bear 2003). Longer period of MD (>7days) can even cause significant retraction of the geniculocortical axon branches representing the deprived eye in mouse primary visual cortex (Antonini and Stryker 1993; Antonini
and Stryker 1996; Gordon and Stryker 1996). In contrast, binocular deprivation induced by rearing animals in the dark does not alter the ocular dominance distribution (Wiesel and Hubel 1965; Gordon, Cioffi et al. 1996). These observations give rise to the concept that the ocular dominance of cortical neurons is maintained by a process of activity-dependent competition between the synapses serving the two eyes (Heynen, Yoon et al. 2003). This synaptic remodeling during the critical period, in nature, reflects a general principle as for how central nervous system reacts to environmental change and maintains its adaptation to it. Therefore, understanding the synaptic basis of the visual dominance plasticity will help us gain insight into the process, design better strategies by which the older brain can learn, and develop new treatment for neurological conditions, such as stroke and neurodegenerative diseases (Hannan 2004; Nithianantharajah and Hannan 2006; Kleim and Jones 2008).

In this thesis, our studies were intended: 1) to investigate the molecular mechanism of long-term depression in primary visual cortex of mouse; and 2) to examine the role of LTD in experience-dependent visual plasticity.

### 4.1 LTD in ocular dominance plasticity during the critical period

Studies have been performed to examine the role of LTD in the mechanisms underlying ocular dominancy plasticity (Hensch, Gordon et al. 1998; Bear 2003; Fischer, Beaver et al. 2004). While there is much data suggesting a role for LTD in OD plasticity, most, if not all, is correlative rather than causative. The lack of a specific LTD blocker in these studies has generated equivocal and discordant results. In this thesis, we have
examined the role of AMPA receptor internalization, and hence LTD, in ocular dominance plasticity. A synthetic GluR2 C-terminal peptide that blocks clathrin-mediated endocytosis of AMPA receptors was used for this purpose (Ahmadian, Ju et al. 2004). A mutant form of the peptide was used as a control. The peptides were made membrane permeable by fusing them to the membrane transduction domain of the HIV-1 Tat protein. Using the GluR23Y peptide, we specifically targeted the final step for expression of LTD, that is, the regulated endocytosis of postsynaptic AMPARs in primary visual cortex (Brebner, Wong et al. 2005). This blockade allows us precisely target the expression of LTD without interfering with normal physiological functions of the neuronal network. The GluR23Y peptide was able to block LFS-LTD in primary visual cortex. This was highly specific to regulated endocytosis of AMPARs since we did not observe any change in surface NMDA and GABA receptors following peptide treatment. In young mice subject to MD for 4 days, systemic ip administration of GluR23Y prevented the shift of ocular dominance toward to non-deprived eye. Consistent with the notion that OD plasticity occurs in primary visual cortex rather than the retina or LGN, local infusion of the GluR23Y peptide also abolished the MD effect in young mice. These observations are unlikely due to a general disruption of neuronal activity since acute administration of the peptide did not alter either spontaneous or evoked neuronal activity in primary visual cortex. In addition to electrophysiological measurements, we also anatomically demonstrated that neurons in the binocular zone of primary visual cortex remained connected to the deprived eye following four day MD in mice treated with GluR23Y peptide. Our studies suggest that LTD, the fundamental mechanism of synaptic modification is involved in experience dependent visual plasticity. Most
importantly, for the first time, it provides direct evidence that LTD is necessary for the OD shift induced by MD and plays a causative role in visual cortical plasticity.

Taken together with previous reports (Kirkwood, Rioult et al. 1996; Rittenhouse, Shouval et al. 1999; Heynen, Yoon et al. 2003; Frenkel and Bear 2004), we now can attempt to reconstruct a possible overall signaling pathway that is set in motion by monocular deprivation in primary visual cortex (Figure 4.1). The reduced retinal activity from the deprived eye is relayed to neurons of primary visual cortex via the LGN. In the binocular cortical neurons, this weak activity induces a smaller calcium influx through postsynaptic NMDA receptor than does the strong activity from the non-deprived eye. The moderate increase of intracellular calcium initiates a cascade of molecular signals, including activation of phosphatases and kinases, and consequently alters the phosphorylation status of several residues on c-terminal tail of GluR2 subunit, such as three tyrosine residues located at the distal C-tail. Subsequently, the clathrin-dependent endocytosis of AMPARs is initiated, which result in LTD and weakening of the synapses from the deprived eye. The synaptic competition for binocular neurons between the two eyes further contributes to the elimination of the weakened synapses and the synaptic reorganization in favor of the non-deprived eye.

4.2 Timing of the critical period of ocular dominance plasticity

The ocular dominance plasticity is easily detected in early life following monocular deprivation and is barely detectable in adulthood. Considerable evidence suggests that the maturation of GABAergic system may account for the opening of critical
Figure 4.1 Schematic describing the molecular mechanism underlying ocular dominance plasticity.

Monocular deprivation induces clathrin-dependent endocytosis of postsynaptic AMPARs by activating NMDARs. The expression of LTD consequently suppresses the visual responsiveness to the deprived eye. As a result, OD distribution shifts toward the non-deprived eye.
period of OD plasticity (Huang, Kirkwood et al. 1999; Fagiolini and Hensch 2000; Hensch 2004; Hensch 2005). We now know that LTD is the most easily inducible before onset of the critical period for ocular dominance plasticity. The reason that ocular dominance plasticity is not correlated with the peak for LTD may be explained by a the delayed maturation of GABAergic system (Hensch 2005). In rodents, the total number of GABAergic synapses, and the magnitude of the evoked GABAergic responses, undergo a sharp 3-fold increase during the period between eye opening (2nd week) and the end of the critical period (5th week) (Hanover, Huang et al. 1999; Choi, Morales et al. 2002). Thus, GABAergic system may play a “permissive” role on the onset of the critical period.

However, how the critical period ends remains poorly understood. In this thesis, our data suggest that LTD is a key synaptic substrate of the MD effect. Interestingly, it has been observed that low-frequency stimulation becomes progressively less effective during development at inducing NMDA receptor dependent LTD in primary visual cortex (Kirkwood, Silva et al. 1997; Xiong, Kojic et al. 2004). The similar developmental time course of LTD and OD plasticity implies that common mechanisms may be shared between LTD and ocular dominance plasticity. In agreement with this notion, we demonstrated that enhancing LTD capacity in primary visual cortex via D-serine was able to reopen the critical period in adult mice. This result further supports the essential role of LTD in OD plasticity and suggests that the mechanism involved in LTD decline during development may account for the closure of the critical period.
Intensive studies have showed that the NMDA receptor plays a crucial role in regulating synaptic plasticity (Malenka and Bear 2004; Massey, Johnson et al. 2004; Wang 2008). Studies in the hippocampus and the perirhinal cortex suggest that NMDA receptor dependent LTD is mediated via activation of NR2B subunit containing receptors (Liu, Wong et al. 2004; Massey, Johnson et al. 2004). Moreover, during development, there is a change in the expression from NMDA receptors containing the NR2B subunit to those containing NR2A (Sheng, Cummings et al. 1994; Quinlan, Philpot et al. 1999; Erisir and Harris 2003). An additional shift in synaptic localization also occurs, with NR2A-containing NMDA receptors preferentially located synaptically and NR2B-containing receptors at extrasynaptic sites (Philpot, Sekhar et al. 2001; Philpot, Espinosa et al. 2003). In addition to this developmental change in NMDARs, visual experience can also alter NMDAR subunit composition and localization. For instance, visual experience decreases the proportion of NR2B-only NMDA receptors, shortens the duration of NMDAR-mediated synaptic currents, and reduces summation of synaptic NMDAR currents. In contrast, a period of binocular deprivation elongates the duration of NMDAR-mediated EPSCs; and this change is rapidly reversed after restoration of normal vision (Philpot, Sekhar et al. 2001). This experience-dependent change in NMDAR properties may be explained by alterations in its subunit composition. Quinlan et al show that the receptors containing the NR2A subunit are delivered to synapses by visual experience, but are replaced by NR2B-containing receptors after a period of binocular deprivation. These changes in subunit composition may alter the affinity of the receptor for glutamate and, consequently, its physiological properties (Quinlan, Olstein et al. 1999). Taken together, the switch from NR2B containing NMDAR to NR2A
containing receptor at synapses during development may underlie the closure of the critical period. If this is true, a possible explanation for the specific enhancement of LTD in adult animal by exogenous D-serine is that normal endogenous D-serine level is subsaturating in the proximity of NR2B-containing NMDAR (extrasynaptic space), but is near saturation at NR2A-containing NMDAR (intrasynaptic space). In addition, NR2B-containing NMDAR may have a higher affinity to D-serine than NR2A-containing NMDAR (Priestley, Laughton et al. 1995). Therefore, exogenous D-serine may augment the activity of NR2B-containing NMDAR and thereby facilitates LTD in adult animals without affecting LTP.

4.3 Clinical implications

LTD has also been associated with several pathological conditions, such as addiction and stress-induced memory impairment (Brebner, Wong et al. 2005; Wong, Howland et al. 2007). Our study suggests that LTD might be the underlying mechanism for amblyopia, a pathological condition of the visual cortex caused by visual deprivation, strabismus, or anisometropia. Current treatments for amblyopia include treatment of the peripheral defect in the less effective eye, and also patching and penalization therapies, which basically impair the normal eye to minimize the competition between the two eyes and to increase the effectiveness of the deprived eye. However, it is commonly seen that children are poorly compliant with these treatments and do not tolerate the patch or medication well. In our study, the fact that specific blockade of LTD rescues visual loss of the deprived eye provides a theoretical basis for new treatment of amblyopia. In fact, any manipulation specifically targeting LTD mechanism may possess great potential for
amblyopia treatment. Instead of targeting the normal eye, an LTD inhibitor will directly rescue the connections from the amblyopic eye and this therapy could dramatically increase the quality of life for patients. In our study, however, only a short period of Glu23Y peptide administration (4 days) was tested. Future studies are needed to elucidate the long-term effect (>7 days) of the peptide in mouse model of amblyopia.

4.4 P38 MAP kinase mediates anisomycin-LTD in primary visual cortex

The role of p38 MAP kinase in synaptic plasticity has been studied in a variety of regions in the central nervous system (Bolshakov, Carboni et al. 2000; Rush, Wu et al. 2002; Zhu, Qin et al. 2002). However, it is not known whether p38 MAP kinase is involved in LTD in primary visual cortex. In this thesis, we have demonstrated that anisomycin-LTD is mediated by activation of p38 MAP kinase since it can be blocked by a specific p38 MAP kinase inhibitor. Similar to LFS-induced LTD, clathrin-dependent endocytosis of AMPAR is also required for anisomycin-LTD. Therefore, our results suggest that the downstream signaling pathway of p38 MAP kinase for LTD induction is shared between the visual cortex and the hippocampus (Bolshakov, Carboni et al. 2000). It is worth noting that p38 MAP kinase is not necessary for LFS-induced LTD since its blockade does not abolish LFS-LTD. Thus, p38 MAP kinase may not be a therapeutic target for amblyopia. In addition, protein synthesis inhibition has been used to study the maintenance of long-term synaptic plasticity (Huang, Li et al. 1994; Bennett 2000; Luscher, Nicoll et al. 2000). Our results suggest that precautions are warranted when anisomycin is used to explore synaptic plasticity. In visual cortex, another protein synthesis inhibitor, cycloheximide, has been used to block ocular dominance plasticity.
via local infusion into the visual cortex (Taha and Stryker 2002). However, 
cycloheximide does not block LTD in our preparation. The seeming disassociation of 
LTD from OD plasticity is probably caused by an impaired late phase LTD. It has been 
showed that late phase LTD is dependent on protein synthesis (Bennett 2000). Therefore, 
we speculate that cycloheximide blocks the synthesis of proteins that are required to 
consolidate LTD in the late phase. Further study is warranted to examine this possibility.

4.5 LTD/LTP is not the only means by which experience modifies neuronal network

In this thesis, we have demonstrated that LTD is one of the fundamental 
mechanisms underlying ocular dominance plasticity in the primary visual cortex. 
However, it is worth noting that LTD/LTP is not the only mechanism by which activity 
generated by experience can modify the neuronal circuitry. For instance, synaptic scaling 
is a homeostatic form of plasticity mechanism that adjusts all of a neuron’s synaptic 
weights up or down in response to altered activity to promote stability of the neuronal 
network (Turrigiano, Leslie et al. 1998). In primary visual cortex, synaptic scaling can be 
regulated by visual experience. Two days of monocular deprivation increases the 
amplitude of mEPSCs onto pyramidal neurons in the deprived hemisphere, while leaving 
the control hemisphere unaffected (Desai, Cudmore et al. 2002). Moreover, synaptic 
scaling in visual cortex is also developmentally regulated. 2 day MD beginning at P14 
(immediately before eye opening) scales up mEPSC amplitudes onto principle neurons in 
layer 4, but the same treatment has no effect when begun at P21. By contrast, mEPSCs 
onto layer 2/3 pyramidal neurons are unaffected by monocular deprivation beginning at 
P14, but are scaled up by monocular deprivation beginning at P21. Interestingly, scaling
in layer 2/3 is coincident with the opening of the critical period for ocular dominance plasticity in rodent (Desai, Cudmore et al. 2002). These data indicate that mEPSC amplitudes can be globally scaled up or down as a function of development and usage, and suggest that synaptic scaling may be involved in the activity-dependent refining of cortical connectivity (Turrigiano and Nelson 2004). In addition to synaptic scaling, other mechanisms, such as intrinsic neuronal excitability (Zhang and Linden 2003) and metaplasticity (Abraham and Bear 1996), have also been proposed to regulate experience-dependent plasticity.

4.6 Future directions

The research described here addresses the hypothesis that LTD is a key mechanism underlying ocular dominance plasticity following MD during the critical period. We were able to prevent the ocular dominance shift following MD via specific targeting the final step of LTD, regulated endocytosis of AMPARs. Furthermore, we have found that enhancing LTD in primary visual cortex with D-serine reopens the critical period in adult mouse. Therefore, our data indicate that LTD plays a fundamental role in ocular dominance plasticity. The developmental regulation of LTD may, account at least in part for the closure of the critical period. Future studies are required to elucidate the molecular mechanism by which LTD is developmentally regulated. Insightful understanding in this matter will provide a theoretical basis to develop new strategies to enhance plasticity of the brains of adults and seniors. In addition, our current study concentrates on the LTD in excitatory neurons. It is well established that
LTD also exists in cortical inhibitory system. Therefore, future study is warranted to
study LTD in inhibitory neurons and its relationship with ocular dominance plasticity.
In this thesis, we also elucidate the role of p38 MAP kinase in anisomycin-induced LTD
in primary visual cortex. In agreement with previous studies (Bolshakov, Carboni et al.
2000), we have showed that p38 MAP kinase also induces regulated endocytosis of
AMPARs in primary visual cortex. However, the precise pathways following p38 MAP
kinase activation remain unknown. It has been demonstrated that p38 MAP kinase may
activate guanyl nucleotide dissociation inhibitor (GDI) which binds to Rab5 (small
GTPase in endosome). This GDI-Rab5 complex is involved in clathrin-dependent
endocytotic vesicle formation (McLauchlan, Newell et al. 1998). Future studies to
examine the probable role of GDI-Rab5 complex in LTD of primary visual cortex may
provide a therapeutic target for amblyopia.

Acsady, L., T. J. Gorcs, et al. (1996). "Different populations of vasoactive intestinal polypeptide-immunoreactive interneurons are specialized to control pyramidal cells or interneurons in the hippocampus." Neuroscience 73(2): 317-34.


Akaneya, Y., T. Tsumoto, et al. (1997). "Brain-derived neurotrophic factor enhances long-term potentiation in rat visual cortex." J Neurosci 17(17): 6707-16.


20-1.

Daw, N. W. and C. J. Beaver (2001). "Developmental changes and ocular dominance

Daw, N. W., S. N. Reid, et al. (1995). "Factors that are critical for plasticity in the visual
cortex." Ciba Found Symp **193**: 258-76;discussion 322-4.


**41**(1): 28-42.

Duffy, S., V. Labrie, et al. (2008). "D-Serine Augments NMDA-NR2B Receptor-
Dependent Hippocampal Long-Term Depression and Spatial Reversal Learning."


Malinow, R. and R. C. Malenka (2002). "AMPA receptor trafficking and synaptic

Massey, P. V., B. E. Johnson, et al. (2004). "Differential roles of NR2A and NR2B-
containing NMDA receptors in cortical long-term potentiation and long-term

spines in visual cortex by tissue plasminogen activator." Neuron 44(6): 1031-41.

Maurer, D., T. L. Lewis, et al. (1999). "Rapid improvement in the acuity of infants after

nondominant temporal lobectomy in patients with mesial temporal sclerosis."
Epilepsia 47(8): 1337-42.


Mioche, L. and W. Singer (1989). "Chronic recordings from single sites of kitten striate
cortex during experience-dependent modifications of receptive-field properties." J


Mulkey, R. M., S. Endo, et al. (1994). "Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression." Nature 369(6480): 486-8.


Rush, A. M., J. Wu, et al. (2002). "Group I metabotropic glutamate receptor (mGluR)-dependent long-term depression mediated via p38 mitogen-activated protein
kinase is inhibited by previous high-frequency stimulation and activation of mGluRs and protein kinase C in the rat dentate gyrus in vitro."


Sherman, S. M. and P. D. Spear (1982). "Organization of visual pathways in normal and

stress response that activates c-Jun N-terminal kinase and p38 mitogen-activated

kinase does not prevent the reverse ocular dominance shift in kitten visual


Steinberg, J. P., R. L. Huganir, et al. (2004). "N-ethylmaleimide-sensitive factor is
required for the synaptic incorporation and removal of AMPA receptors during

Steward, O. and P. Worley (2001). "Localization of mRNAs at synaptic sites on


auditory cortex: single-neuron versus multiple-neuron recordings." *J


