Requirement for AMPA receptor endocytosis and long-term depression in ocular dominance plasticity

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Abstract

The classic example of experience-dependent cortical plasticity is the ocular dominance (OD) shift in visual cortex following monocular deprivation (MD). As in other mammals with binocular vision, MD in mice induces bidirectional plasticity: rapid weakening of responses evoked through the deprived eye is followed by delayed strengthening of open-eye responses. It has been proposed that these bidirectional changes occur through three distinct processes. First, deprived-eye responses rapidly weaken through homosynaptic long-term depression (LTD). As the period of deprivation progresses, the modification threshold determining the boundary between synaptic depression and synaptic potentiation becomes lower via a decrease in the ratio of NR2A to NR2B N-methyl-d-aspartate receptor (NMDAR) subunits. Facilitated by the decreased modification threshold, open-eye responses are strengthened via homosynaptic long-term potentiation (LTP).

One established LTD mechanism is a loss of sensitivity to the neurotransmitter glutamate caused by internalization of postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs). Although it has been shown that MD similarly causes a loss of AMPARs from visual cortical synapses, the contribution of this change to OD plasticity has not been established.

Here we develop a herpes-simplex virus (HSV) vector to express a peptide (G2CT) in visual cortical neurons designed to block AMPAR internalization by hindering the association of the C-terminal tail of the AMPAR GluR2 subunit with the AP2 clathrin adaptor complex. We find that G2CT expression interferes with NMDAR-dependent AMPAR endocytosis and LTD, without affecting baseline synaptic transmission. When expressed *in vivo*, G2CT completely blocks the MD-induced depression of deprived-eye responses after MD without affecting baseline visual responsiveness or experience-dependent response potentiation in layer 4 of visual cortex. Additionally, we find that OD plasticity in layer 2/3 can occur in the absence of plasticity in layer 4, demonstrating the independence of OD plasticity across laminae. These data suggest that AMPAR internalization is essential for the loss of synaptic strength in layer 4 of the visual cortex following MD. This finding illustrates a critical role for LTD mechanisms in the physiological response to sensory deprivation, thereby suggesting potential therapeutic strategies in the treatment of amblyopia.

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Chapter 1

Bidirectional synaptic mechanisms of ocular dominance plasticity in the visual cortex

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1.1 Abstract

As in other mammals with binocular vision, monocular lid suture in mice induces bidirectional plasticity: rapid weakening of responses evoked through the deprived eye followed by delayed strengthening of responses through the open eye. It has been proposed that these bidirectional changes occur through three distinct processes. First, deprived-eye responses rapidly weaken through homosynaptic long-term depression (LTD). As the period of deprivation progresses, the modification threshold determining the boundary between synaptic depression and synaptic potentiation becomes lower, favoring potentiation. Facilitated by the decreased modification threshold, open-eye responses are strengthened via homosynaptic long-term potentiation (LTP). Of these processes, deprived-eye depression has received the greatest attention, and although several alternative hypotheses are also supported by current research, evidence suggests that AMPA receptor endocytosis through LTD is a key mechanism. The change in modification threshold appears to occur partly through changes in NMDA receptor subunit composition, with decreases in the ratio of NR2A to NR2B facilitating potentiation. Although limited research has directly addressed the question of open-eye potentiation, several studies suggest that LTP could account for observed changes *in vivo*. This chapter will present evidence supporting this three stage model, along with outstanding issues in the field.

1.2 Introduction

The substrate for binocular vision in mammals is the convergence of retinotopically-matched inputs onto common postsynaptic neurons in the primary visual cortex. In humans, several conditions which degrade or unbalance vision prior to adolescence can result in lasting visual impairments (Doshi and Rodriguez, 2007). Strabismus, uncorrected refractive errors, and cataracts can each lead to amblyopia, a prevalent condition affecting between 1 and 4 percent of people in the United States (Doshi and Rodriguez, 2007). Amblyopia is also referred to as cortical blindness, because although the visual deficits arise following perturbations early in the visual pathway, the ultimate location of the lasting visual impairment resides within the brain. Thus even if the original problem is corrected through surgery or refractive lenses, visual deficits remain. Understanding the molecular mechanisms and consequences underlying this loss of visual responsiveness can suggest novel therapeutic strategies for the treatment of amblyopia.

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

The location of first convergence of retinotopically-matched eye-specific inputs

varies across species; in macaque monkeys and cats, where much of the pioneering research in ocular dominance was performed, monocularly driven layer 4 neurons converge to produce binocular neurons in layer 2/3 (Hubel and Wiesel, 1968, 1969; Shatz and Stryker, 1978). In these species, layer 4 neurons are anatomically organized similarly to the dLGN, with alternating regions of cortex receiving projections from only the contra- or ipsi- lateral eye (Hubel and Wiesel, 1968, 1969; Shatz and Stryker, 1978). In contrast, the convergence of monocular inputs occurs a synapse earlier in mice where the majority, if not all, of the neurons in layer 4 receive binocular inputs (Drager, 1974).

Development, refinement and maintenance of these binocular connections is highly dependent on the quality of visual experience. Degrading vision in one eye shifts the response properties of cortical neurons such that they cease to respond to stimulation of the deprived eye. Using acute extracellular recordings of individual cortical neurons in kittens, Hubel and Wiesel (Hubel and Wiesel, 1962) demonstrated that in animals with normal visual experience, approximately 80% of cortical neurons are binocular, driven to various degrees by both eyes with a slight majority responding more strongly to stimulation of the contralateral eye. If the contralateral eye is deprived of patterned vision by suturing the eyelid closed for a period of 2-3 months, a manipulation termed monocular deprivation (MD), responses to the deprived eye are markedly decreased, and the majority of neurons respond only to the open ipsilateral eye (Wiesel and Hubel, 1963).

The effects of unbalanced visual experience are not limited to the response properties of individual neurons; MD also has profound anatomical consequences, the most striking of which is the plasticity of ocular dominance (OD) columns in cats and

macaque monkeys. Neuroanatomical techniques that allow the labeling of eye-specific thalamocortical projections reveal that these projections are organized into alternating bands receiving input from only one eye (Hubel and Wiesel, 1969, 1972; Wiesel *et al.*, 1974). Prolonged MD initiated early in life leads to shrinkage in the size of columns receiving input from the deprived eye, which is accompanied by an expansion of columns serving the open non-deprived eye (Hubel *et al.*, 1977).

What are the features of MD that are responsible for driving this disconnection of deprived-eye inputs and the accompanying loss of responsiveness? The weakening of visual responses following MD is not merely a consequence of reduced visual input; rather a decorrelation of activity from the two eyes appears necessary to induce a shift in ocular dominance. Binocular deprivation, which reduces inputs to both eyes equally, fails to induce an OD shift (Wiesel and Hubel, 1965; Gordon and Stryker, 1996; Frenkel and Bear, 2004). In contrast, artificially misaligning the eyes produces a strong OD shift, with a loss of binocularly-driven neurons (Hubel and Wiesel, 1965). This result is especially telling because within each eye the correlations present in natural scenes are entirely preserved, whereas intra-eye correlations are destroyed. Coupled with the results of binocular deprivation, this finding clearly highlights the importance of intraocular interactions in driving OD plasticity. A recent result further demonstrates that it is the degraded quality of visual experience following MD and not the reduction in light intensity reaching the retina that is required for OD plasticity. Blurring visual images to one eye using a strong contact lens, which disrupts both within and intra-eye visual correlations, produces an equivalent OD shift to monocular lid suture (Rittenhouse *et al.*, 2006).

The significance of understanding the synaptic and molecular bases of OD plasticity cannot be overstated. First, the processes revealed by OD plasticity are likely to be the same as those that refine cortical circuitry in response to the qualities of sensory experience during development, and thus determine the capabilities and limitations on visual performance in adults. Second, rapid OD plasticity is an example of cortical receptive field plasticity, the most common cellular correlate of memory in the brain. It is therefore likely that understanding the mechanisms of OD plasticity will yield insight into the molecular basis of learning and memory. Third, the detailed understanding of how synaptic connections are weakened by sensory deprivation will suggest possible strategies to reverse such changes, and possibly overcome amblyopia. Finally, the detailed understanding of how synaptic connections are strengthened by experience will suggest possible strategies to augment such changes, and promote recovery of function after brain injury.

In recent years, mouse visual cortex has emerged as the favored preparation for the mechanistic dissection of OD plasticity. First, mice display robust OD plasticity in response to MD, and the kinetics and behavioral consequences of OD plasticity are very similar to those observed in others species. In the mouse, most neurons exhibit responses that are dominated by the contralateral eye, and prolonged periods of contralateral MD (6 weeks to 12 months) shift the distribution of responses towards domination by the open, ipsilateral eye (Drager, 1978). Prolonged MD also produces roughly similar structural changes in cats and mice, leading to reduced arborization of thalamocortical axons subserving the deprived eye (Antonini and Stryker, 1993, 1996; Antonini *et al.*, 1999). Shorter periods of MD ranging from 4 – 10 days produce strong OD shifts in young cats

(Olson and Freeman, 1975; Movshon and Dursteler, 1977), similar to what has been reported in mice (Gordon and Stryker, 1996; Frenkel and Bear, 2004). In fact, changes in OD can be observed very rapidly in both species, in as little as 6 – 17 hours in cats (Mioche and Singer, 1989) and 24 hours in mice (Liu *et al.*, 2008).

There are several additional advantages of the mouse preparation for studies of OD plasticity. First, the property of binocularity is established early in cortical processing by convergence of thalamic inputs onto layer 4 neurons, potentially simplifying the analysis of the underlying synaptic changes. Second, mice are genetically homogenous and plentiful, enabling rapid progress using coordinated biochemical and electrophysiological studies *in vitro* and *in vivo*. Third, the absence of a columnar organization makes feasible the use of chronic recordings from awake animals. Fourth, the fact that the mouse visual cortex is relatively undifferentiated (e.g., compared to monkey V1) suggests that insights gained here might apply broadly across species and cortical areas. Fifth, genes can be delivered or deleted in mouse visual cortex by genetic engineering or by viral infection. Finally, mice have emerged as valuable models of human genetic disorders, offering the opportunity to use the powerful paradigm of OD plasticity to understand how experience-dependent cortical development can go awry in genetic disorders and, hopefully, suggest ways that these disorders can be corrected.

1.3 Bidirectional kinetics of OD plasticity in the mouse

Responsiveness of mouse visual cortex to stimulation of the eye has been measured using a number of methods, including single-unit recordings, visually evoked potentials (VEPs), optical imaging of intrinsic signals related to oxygen utilization, immediate early gene (IEG) expression, and imaging of cellular calcium transients. Each

of these methods introduces a different bias. Recording of spiking activity is biased towards deep layers of cortex, where units are easiest to isolate from one another; VEP recordings reflect summed synaptic currents that are dominated by thalamocortical input to layer 4; IEG expression is an indirect measure of activity that is possibly more reflective of plasticity mechanisms than visual responsiveness; and both calcium imaging and intrinsic signal imaging are limited measuring activity within a few hundred microns of the cortical surface. These differences are important to recognize, as it has become clear that the mechanisms of OD plasticity vary according to layer. However, despite this caveat, all methods yield a consistent picture of what happens when one eyelid is closed.

Visual responses of mice raised in a normal (laboratory) visual environment are dominated by the contralateral eye. Even in the binocular segment, the contralateral eye response is approximately double the ipsilateral eye response (Gordon and Stryker, 1996; Frenkel and Bear, 2004). These physiological measures of OD correlate well with the anatomical projections from the dLGN, which also show an approximate 2:1 bias towards the contralateral eye (Coleman *et al.*, 2009). Closure of the contralateral eyelid at approximately 4 weeks of age rapidly shifts this OD. A shift of the contra / ipsi ratio is detectable with as little as 1 day of MD and reaches an asymptote by 3 days, when the contra / ipsi ratio is approximately 1 (Gordon and Stryker, 1996; Frenkel and Bear, 2004; Liu *et al.*, 2008).

The technique of recording VEPs has been particularly useful for understanding the processes that account for this shift. A practical advantage of the VEP is that it can be recorded with chronically implanted electrodes. Thus, before- and after-MD measurements can be made from the same mice, and the data can be collected from

awake alert animals. Applying this VEP technique to varying periods of MD in mice demonstrated that the observed OD shift towards the non-deprived eye results from the combination of two distinct processes: a weakening of deprived-eye responses and a strengthening of open-eye responses (Frenkel and Bear, 2004). Although both processes contribute to the overall OD shift, they do so with distinct timecourses (Frenkel and Bear, 2004). Three days of MD produces a strong shift in the contra / ipsi ratio by weakening the response of the deprived (contralateral) eye without affecting the non-deprived (ipsilateral) eye responses. In contrast, seven days of deprivation yields both deprived-eye depression and potentiation of open-eye responses. This pattern of rapid deprived-eye depression and delayed open-eye potentiation has also been noted in other species (Mioche and Singer, 1989).

Importantly, the bidirectional consequences of MD are each functionally significant. In rats, the deprived eye exhibits a dramatic reduction in visual acuity assessed through visually-guided behavior following MD (Prusky *et al.*, 2000; Iny *et al.*, 2006). In the same visually-guided task (Iny *et al.*, 2006), open-eye performance was enhanced following MD, indicating that the bidirectional plasticity of VEPs reflects functionally meaningful changes in sensory processing.

Based on the synthesis of theoretical and experimental work, a comprehensive model of OD plasticity has been proposed (Frenkel and Bear, 2004): (1) Deprived-eye responses depress via homosynaptic long-term depression. (2) The plasticity threshold, determining the boundary between potentiating and depressing input activity, becomes lower in response to the decreased cortical activity which follows monocular lid closure. (3) Open-eye responses potentiate via homosynaptic long-term potentiation due to the

lower threshold for synaptic potentiation. In the sections that follow, we will briefly present the data that support this model. Alternative hypotheses for deprived-eye depression and open-eye potentiation will be discussed later in this chapter.

1.4 LTD as a molecular mechanism of deprived-eye depression in the mouse

MD triggers response depression in cortex by degrading images on the retina, not by eliminating retinal activity (Rittenhouse *et al.*, 2006). Monocular lid suture, which produces deprived-eye depression, leads to a decorrelation of LGN activity, whereas monocular inactivation with intraocular TTX fails to decorrelate LGN activity (Linden *et al.*, 2009) and does not produce deprived-eye depression (Frenkel and Bear, 2004). Thus, the adequate stimulus for response depression appears to be weakly correlated afferent activity arising in the visually deprived retina, and relayed to cortex by the lateral geniculate nucleus (Bear, 2003; Blais *et al.*, 2008). Synaptic modifications that are driven by activity in the affected inputs are said to be "homosynaptic." Thus, there has been a search for mechanisms of homosynaptic LTD of excitatory synaptic transmission. This search has been aided by the development of LTD paradigms that use electrical stimulation of synaptic transmission in brain slices or *in vivo*. An important caveat is that it is abundantly clear that the mechanisms of LTD vary from one synapse type to the next, so one must be cautious in generalizing (Daw *et al.*, 2004; Malenka and Bear, 2004).

1.4.1 Mechanisms of "canonical" LTD in CA1

The initial demonstration of LTD occurred in the CA1 region of the hippocampus and opened the door to an impressive array of mechanistic studies establishing a

canonical molecular pathway involved in weakening synaptic transmission. Prolonged low frequency trains of subthreshold stimulation (LFS) delivered to CA1 produce a persistent homosynaptic decrease in the strength of synaptic transmission (Dudek and Bear, 1992). Although multiple forms of LTD exist in CA1 (Oliet *et al.*, 1997), over the years a consensus has emerged regarding the molecular pathways connecting LFS to synaptic depression (reviewed in (Malenka and Bear, 2004)). Weak activation of NMDA receptors leads to a modest rise in postsynaptic intracellular calcium concentration (Mulkey and Malenka, 1992) activating a protein phosphatase cascade involving PP1 and calcineurin (Mulkey *et al.*, 1993; Mulkey *et al.*, 1994). LTD is accompanied by characteristic changes in the phosphorylation states of AMPA receptor subunits, and leads to dephosphorylation of the GluR1 cytoplasmic tail at serine-845 (S845), without affecting phosphorylation at a neighboring position, S831, that is implicated in LTP (Lee *et al.*, 1998; Lee *et al.*, 2000). Additionally, GluR2 becomes phosphorylated during LTD at S880 (Kim *et al.*, 2001). Activation of protein phosphatases, possibly in concert with these changes in AMPAR phosphorylation, leads to AMPAR endocytosis and a loss of receptors from the synapse, thereby weakening synaptic transmission (Carroll *et al.*, 1999; Beattie *et al.*, 2000; Ehlers, 2000).

1.4.2 Induction of homosynaptic LTD in the visual cortex

The "canonical" LTD mechanism in the CA1 region of hippocampus was used to guide early studies of synaptic depression in the visual cortex. Homosynaptic LTD can be induced in the visual cortex through LFS, and requires activation of NMDARs, a rise in postsynaptic calcium concentration, and activation of protein phosphatases (Kirkwood *et al.*, 1993; Kirkwood and Bear, 1994a). The discovery that LFS could induce LTD of

evoked thalamocortical transmission *in vivo*, and that this was sufficient to produce depression of visual responses (Heynen *et al.*, 2003), led to a series of experiments examining whether MD induces a pattern of molecular changes similar to those observed during LTD. These changes in AMPAR phosphorylation and trafficking can be detected biochemically using phosphorylation site-specific antibodies and assays of receptor surface expression. Thus the biochemical signature of LTD can be used as a "molecular fingerprint" to ask if similar changes occur in visual cortex following a period of MD.

1.4.3 MD-induced changes mimic and occlude LTD

To date, the question of biochemical similarities between LTD and MD has only been examined in the rat, but the results support the hypothesis that MD induces this type of LTD in visual cortex. MD produces dephosphorylation of GluR1 at S845 and phosphorylation of GluR2 at S880, without affecting phosphorylation at GluR1 S831, mimicking the pattern observed following LTD (Heynen *et al.*, 2003). Similarly, MD leads to a reduction in the levels of surface-expressed AMPARs, another component of the molecular signature of LTD (Heynen *et al.*, 2003).

A second approach to address if LTD is induced by MD is to ask if naturally occurring synaptic depression *in vivo* occludes LTD *ex vivo*. As originally pointed out by Nigel Daw and colleagues, however, the mechanisms of LTD appear to vary according to cortical layer (Daw *et al.*, 2004). This issue was recently examined in the mouse by Crozier *et al*. (2007). Identical stimulation protocols applied to the radial inputs to layers 4 and 3 induced LTD via activation of postsynaptic NMDARs. However, only in layer 4 was the LTD mediated by clathrin-dependent AMPAR endocytosis. Conversely, only in layer 3 was the LTD sensitive to pharmacological blockade of cannabinoid receptors.

However, in both layers, the LTD measured in slices was reduced (occluded) by 3 days of MD *in vivo*. Thus, the evidence suggests that MD induces LTD in both layer 3 and layer 4, but by distinct molecular mechanisms (Figure 1.1)

1.4.4 Pharmacological or genetic manipulations affecting LTD and OD plasticity

The evidence is very strong that MD leads to LTD of synaptic transmission in visual cortex. However, the question of the relative contribution of this change to the functional consequences of MD (*i.e.*, cortical blindness) remains controversial. A common approach to this question has been to examine OD plasticity in genetically or pharmacologically modified mice. Deficits in OD plasticity have been reported in multiple lines of knockout or transgenic mice, including mice lacking tissue-type plasminogen activator or αCaMKII, or mice over-expressing calcineurin or a dominant-negative CREB (Gordon *et al.*, 1996; Mataga *et al.*, 2002; Mower *et al.*, 2002; Yang *et al.*, 2005). Additionally, OD plasticity was prevented by inhibition of ERK activation or administration of IGF1 (Di Cristo *et al.*, 2001; Tropea *et al.*, 2006). In contrast, normal OD plasticity was reported in mice lacking PSD-95 or heterozygous for BDNF, as well as after pharmacological inactivation of TrkB receptors (Bartoletti *et al.*, 2002; Fagiolini *et al.*, 2003; Kaneko *et al.*, 2008a).

These results indicate that many different molecules may play a role in OD plasticity, however it is not yet clear whether the variety of genes implicated is indicative of multiple parallel pathways or rather a single common molecular pathway for OD plasticity. Along these lines, many of the studies using genetically modified mice did not examine synaptic plasticity in the visual cortex using LFS-induced LTD, making it unclear whether the deficits in OD plasticity are related to deficits in LTD.

In several studies, plasticity of synaptic transmission and OD were both examined following genetic manipulation, allowing the correlation of deficits in LTD and OD plasticity. However, there are limitations inherent to this approach: that manipulations may affect only the stimulation requirements for LTD in brain slices, not the core mechanism; that compensatory adaptations may occur; and that plasticity may be disrupted *in vivo* by alterations in retina, thalamus or behavioral state. Furthermore, many such studies have not taken into account key features of visual cortical plasticity: *first*, that an OD shift can occur by deprived-eye depression, open-eye potentiation, or both (Sawtell *et al.*, 2003; Frenkel and Bear, 2004), and *second*, that the mechanisms of LTD (Crozier *et al.*, 2007) and OD plasticity (Liu *et al.*, 2008) vary according to layer (Figure 1.1).

In support of the link between LTD and OD plasticity, the OD shift assayed using single unit recordings (layers not specified) is disrupted in the glutamic acid decarboxylase 65 (GAD65) knockout mouse, which has impaired cortical inhibition (Hensch *et al.*, 1998b). Although drifting baseline recordings obscured the deficit in the original report (Hensch *et al.*, 1998b), layer 3 LTD is also clearly impaired in these mice (Choi *et al.*, 2002), possibly due to alterations in NMDAR subunit composition (Kanold *et al.*, 2009). Similarly, cannabinoid receptor blockade prevents both LTD (Crozier *et al.*, 2007) and deprived-eye response depression in unit recordings restricted to layer 3 (Liu *et al.*, 2008).

On the other hand, a dissociation of LTD and OD plasticity was suggested in several protein kinase A (PKA) mutants. For example, the RIβ knockout mouse reportedly has a deficit in layer 3 LTD but exhibits a normal OD shift after 4 days of MD

(Hensch *et al.*, 1998a). Unfortunately, the significance of the LTD deficit is unclear, as control recordings in WT mice were not performed under these experimental conditions. Two additional studies deleting either of the RII subunits of PKA further complicate the relationship between PKA, LTD, and OD plasticity. RIIα KO mice display normal LTD in layer 3, whereas both LTP in this preparation and OD plasticity were moderately reduced (Rao *et al.*, 2004). In contrast, RIIβ KO mice exhibit normal LTP at the same synapse, but lack both LTD and OD plasticity (Fischer *et al.*, 2004).

Additionally, several studies have examined the contribution of metabotropic glutamate receptors (mGluRs) to LTD and OD plasticity. Initial reports using the mGluR antagonist MCPG claimed that depotentiation in layer 2/3 was blocked in mouse cortical slices, whereas OD shifts in kittens following brief MD (5 days) occurred normally (Hensch and Stryker, 1996). Unfortunately, the relevance of these results to LTD is unclear as MCPG not only fails to antagonize the action of glutamate, the endogenous mGluR ligand, but also fails to block cortical LTD (Huber *et al.*, 1998). In related experiments, genetic deletion of mGluR2, a group II receptor expressed presynaptically, did not affect OD plasticity, yet did disrupt the induction of LFS LTD in layer 2/3 of visual cortex (Renger *et al.*, 2002). A further dissociation between LTD and OD plasticity was observed in mice heterozygous for the postsynaptically expressed group I receptor mGluR5, which fail to display OD plasticity following brief MD (Dolen *et al.*, 2007), despite normal NMDAR-dependent LFS-induced LTD in layer 2/3 (Sawtell *et al.*, 1999).

Given that many different plasticity mechanisms exist in the visual cortex (Daw *et al.*, 2004), it is likely that a large portion of these seemingly conflicting results may be

attributable to laminar differences between the molecular pathways supporting LTD and LTP. In mice, MD produces an OD shift simultaneously in layers 4 and 3 (Liu *et al.*, 2008), suggesting that the disruption of layer-specific plasticity mechanisms (Wang and Daw, 2003; Rao and Daw, 2004) will affect OD plasticity in a complex fashion. Many studies utilizing single-unit recordings pool neurons recorded across all layers, thereby preventing analysis of layer-specific deficits in plasticity. In addition, the use of acute single-unit recordings in many studies precludes the separation of mechanisms impacting the loss of deprived-eye responses from those affecting potentiation of the open-eye because eye-specific responses cannot be compared before and after deprivation. In genetically modified mice with abnormalities in both LTP and LTD this can be especially problematic, as it becomes impossible to determine which process contributes to the observed OD phenotype.

If we restrict consideration to layer 4, where VEP recordings are made, and to periods of MD $\leq$ 3 days, when the shift is dominated by deprived-eye depression, the data support the hypothesis that MD shifts OD via the loss of AMPARs at visually deprived synapses. However, it remains to be determined if this is the only—or most important—mechanism for deprived-eye response depression.

1.5 Metaplasticity during monocular deprivation

After approximately 5 days of contralateral eye MD, the ipsilateral (non-deprived) eye responses begin to grow. Because there has been no change in the quality of visual experience through this eye, there must be an adaptation in the cortex that allows response potentiation. A theoretical framework for this aspect of OD plasticity was provided by the influential BCM theory (Bienenstock *et al.*, 1982). According to this

theory, the reduction in overall cortical activity caused by closing the contralateral eyelid decreases the value of the modification threshold, θ_m, thereby facilitating potentiation of correlated inputs (reviewed by (Bear, 2003)).

In accordance with theoretical predictions, experiments utilizing a period of dark rearing to decrease activity in the visual cortex have demonstrated that the threshold level of stimulation required to induce LTD and LTP is modifiable by prior visual experience. In both rats and mice, ≥ 2 days of darkness is sufficient to shift θ_m, moving the boundary between LTP and LTD induction toward lower stimulation frequencies (Kirkwood *et al.*, 1996; Philpot *et al.*, 2003; Philpot *et al.*, 2007). Brief re-exposure to light rapidly reverses the effects of dark-rearing on the modification threshold (Kirkwood *et al.*, 1996; Philpot *et al.*, 2003).

Modifications of NMDAR function were proposed as a physiological mechanism for changing θ_m (Bear *et al.*, 1987; Abraham and Bear, 1996), and a number of recent studies have focused specifically on the ratio of NR2A to NR2B subunits (Bear, 2003). This ratio is developmentally regulated and can be rapidly modulated by alterations in visual experience (Yoshii *et al.*, 2003; van Zundert *et al.*, 2004). Rats that are dark reared or exposed to the dark for brief periods show reductions in the ratio of NR2A to NR2B proteins, which can be reversed rapidly upon re-exposure to light (Quinlan *et al.*, 1999a; Quinlan *et al.*, 1999b). Additionally, dark rearing increases the decay times of synaptic NMDA currents (Carmignoto and Vicini, 1992), while also increasing the sensitivity to NR2B selective antagonists and temporal summation of synaptic responses (Philpot *et al.*, 2001) (Figure 1.2).

These findings are consistent with an increased proportion of NR2B-containing

NMDARs at synapses, and demonstrate that the changes observed at the protein level have a meaningful effect on synaptic transmission. The longer decay kinetics of NR2B-containing receptors have been proposed to facilitate the summation of inputs and thereby promote coincidence detection, possibly facilitating LTP (Monyer *et al.*, 1994; Flint *et al.*, 1997). In addition, NR2B subunits may recruit LTP promoting proteins to the synapse (Barria and Malinow, 2005).

The mechanism by which the NR2A / B ratio changes is determined by the length of dark exposure: short periods shift the ratio through increasing NR2B levels, whereas with longer periods NR2B protein levels return to normal and NR2A levels decrease (Chen and Bear, 2007). To determine if plasticity of the NR2A / B ratio is required for a shift in θ_m, visual experience was manipulated in mice with a fixed ratio due to genetic deletion of NR2A (Philpot *et al.*, 2007). The deletion of NR2A was found to both mimic and occlude the effects of dark rearing on the amplitude, decay kinetics, and temporal summation of NMDA currents. Importantly, when θ_m was examined by testing the frequency dependence of LTP and LTD, dark rearing failed to produce a shift in mice lacking NR2A, demonstrating a critical role for NR2A and the NR2A / B ratio in governing θ_m (Philpot *et al.*, 2007).

If θ_m is modified via changing the NR2A / B ratio, and a change in θ_m is permissive for potentiation of the open eye, the timecourse of changes in the NR2A / B ratio (and therefore θ_m) should slightly lead the timecourse of open-eye potentiation. When the NR2A / B ratio was examined in mouse visual cortex following MD of the contralateral eye, a significant decrease in ratio was observed following 5 and 7 days of deprivation but not with shorter periods (Chen and Bear, 2007). Given that open-eye

potentiation during MD does not occur until after 5 days (Frenkel and Bear, 2004), the observed timecourse is precisely as would be predicted. The suggestion that a low NR2A / B ratio is permissive for open-eye potentiation is supported by the finding of precocious open-eye potentiation in NR2A knockout mice, which have an artificially low NR2A / B ratio (Cho *et al.*, 2009).

1.6 LTP as a mechanism for open-eye potentiation

Although the strengthening of inputs originating from the open eye has been documented for over 30 years, the molecular mechanisms underlying this process have received scant attention relative to those mediating deprived-eye depression. Nonetheless, the predictions from the BCM theory are clear: open-eye inputs to the cortex, which remain at their original activity level during the early stages of MD, potentiate via homosynaptic mechanisms once θ_m drops below this activity level. LTP has been demonstrated at multiple cortical synapses *ex vivo*, and although the mechanisms appear to vary across layers similar to LTD (Wang and Daw, 2003), homosynaptic NMDAR-dependent LTP has been shown at layer 3 synapses in the rat (Kirkwood and Bear, 1994b). Additionally, in rats, NMDAR-dependent LTP can be induced in layers 4 and 3 *in vivo* following tetanic stimulation of LGN, and this LTP is sufficient to increase the magnitude of visually evoked responses (Heynen and Bear, 2001). These results suggest that homosynaptic LTP, possibly at thalamocortical synapses, can mimic the effects of open-eye potentiation after long-term MD.

Many manipulations known to disrupt homosynaptic LTP have been applied during OD plasticity (Daw *et al.*, 2004; Hensch, 2005; Hooks and Chen, 2007), although many of these studies suffer from the inability of acute single unit recordings to isolate

changes in deprived-eye pathways from those serving the open eye. One example of this is the finding that OD plasticity is disrupted in mice with either disrupted αCaMKII autophosphorylation or lacking the protein entirely, which suggests a role for LTP (Gordon *et al.*, 1996; Taha *et al.*, 2002). Unfortunately, because all measures of OD in these studies were performed by comparing the relative drive from the deprived and non-deprived eyes, it is not clear which processes were disrupted.

Although data on the mechanisms underlying open-eye potentiation in juvenile mice remain scarce, several related experiments are suggestive. In juvenile mice, blockade of NMDARs during the last 4 days of a 7 day MD blocks the potentiation of open-eye responses (Cho *et al.*, 2009). Also, open-eye potentiation is absent in adult mice with a postnatal deletion of NR1 targeted to layers 2-4 (Sawtell *et al.*, 2003), further suggesting that NMDAR mediated plasticity plays a role. Additional data addressing potential mechanisms comes from the recently discovered phenomenon of stimulus-selective response potentiation (SRP). In juvenile mice, the magnitude of visually driven thalamocortical responses in layer 4 increases following repeated presentations of an oriented stimulus (Frenkel *et al.*, 2006). This potentiation is specific to both the trained eye and the trained orientation, and is dependent on NMDAR activation. The discovery of SRP demonstrates that physiologically relevant potentiation of visual responses can occur *in vivo*, and shares a requirement for NMDAR activation with LTP.

If open-eye potentiation during MD occurs through LTP-like mechanisms, it is likely to be expressed through the delivery of AMPARs to synapses (Malinow *et al.*, 2000), similar to the role we propose for AMPAR endocytosis and LTD in deprived-eye depression. It has been shown that expression of a region of the GluR1 C-terminal tail is

sufficient to both prevent delivery of GluR1 to synapses, and block LTP (Shi *et al.*, 2001). Additionally, several studies in the amygdala, as well as the somatosensory and visual cortices, have shown that GluR1 delivery is required for experience dependent plasticity occurring *in vivo* (Takahashi *et al.*, 2003; Rumpel *et al.*, 2005; Frenkel *et al.*, 2006). If a similar blockade of GluR1 delivery could prevent potentiation of open-eye responses following 7 days of MD without affecting the decrease in deprived-eye responses, it would demonstrate that AMPAR insertion, and therefore likely LTP, is a necessary component subserving open-eye potentiation.

1.7 Alternative hypotheses for deprived-eye depression

Our view is that deprivation induces response depression via the mechanisms of LTD in layers 4 and 3, and that delayed response potentiation occurs via the mechanisms of LTP after permissive adjustment of the modification threshold. However, several alternative hypotheses have also been advanced to account for the phenomenology of OD plasticity.

1.7.1 Degradation of ECM proteins

Brief MD has been shown to lead to increased motility and a loss of dendritic spines located in superficial layers belonging to layer 5 pyramidal neurons in mice (Mataga *et al.*, 2004; Oray *et al.*, 2004). In both of these studies, the effect of MD on dendritic spine dynamics was found to be dependent on the tissue-type plasminogen activator (tPA) / plasmin proteolytic cascade. Brief MD elevates tPA activity in the cortex, and genetic deletion of tPA both reduces the magnitude of the OD shift assayed through single unit recordings and prevents the loss of dendritic spines during MD (Mataga *et al.*, 2002; Mataga *et al.*, 2004). Furthermore, spine motility can be increased

with plasmin treatment, an effect which is occluded by prior MD (Oray *et al.*, 2004). Together, these findings suggest that degradation of the extracellular matrix (ECM) by the tPA / plasmin cascade is an essential component of OD plasticity, possibly due to its role in promoting dendritic spine dynamics.

It is important to recognize, however, that these structural responses to MD are entirely consistent with the hypothesis that deprived-eye depression occurs through LTD mechanisms. It has been shown that LTD is associated with structural reorganization and a retraction of dendritic spines (Nagerl *et al.*, 2004; Zhou *et al.*, 2004; Bastrikova *et al.*, 2008). Unfortunately, a significant limitation of current studies examining dendritic spines following MD is that it is unclear whether the observed changes are at spines receiving input from the open or deprived eye. Given that MD affects both deprived-eye as well as open-eye responses, it is critical to determine if ECM degradation and increased spine motility relate to the depression or potentiation of visual responses.

1.7.2 Increases in intracortical inhibition

A second proposed mechanism to account for the loss of deprived-eye responsiveness following MD during the critical period focuses on an increase in intracortical inhibition (Duffy *et al.*, 1976; Burchfiel and Duffy, 1981). In a recent study, Maffei *et al.* (2006) demonstrated using whole cell recordings from connected pairs of neurons in the rat visual cortex that brief MD from P21 to P24 increased the inhibitory tone in the visual cortex (Maffei *et al.*, 2006). This was achieved by reciprocally strengthening excitatory connections onto fast-spiking (FS) interneurons and inhibitory connections from FS cells onto pyramidal neurons. In the same study, it was found that this strengthening of inhibitory feedback could be achieved through a novel form of LTP

of inhibition (LTPi), which was occluded by prior MD. The increase in inhibitory drive following MD appears to be developmentally regulated, as deprivation in younger animals (P14 to 17) leads to decreased inhibition coupled with increased excitatory drive (Maffei *et al.*, 2004).

The relevance of these experiments to OD plasticity in mice is unclear as they were performed in the monocular zone of rat visual cortex, which lacks input from the open eye, and therefore binocular interactions. In the mouse, a period of MD that is sufficient to cause maximal deprived-eye depression in the binocular zone (3 days), has no effect on VEPs in the monocular segment (Frenkel and Bear, 2008). Similarly, 4 days of complete darkness has no effect on VEP amplitude in the binocular segments (Blais *et al.*, 2008) (Figure 1.3, A, B).

VEPs may reflect the strength of feed-forward geniculo-cortical transmission, and therefore be insensitive to intracortical modifications. However, it is still unclear how a rise in inhibitory tone could account for the specific weakening of deprived-eye responses in the binocular zone during MD. Given the lack of OD columns in rats, lateral inhibition of neighboring columns cannot occur, therefore such a model requires the existence of eye-specific inhibitory networks within layer 4 of the binocular zone. The existence of such networks is unlikely based on the mixing of eye-specific afferents in the binocular visual cortex of rodents—there are few if any neurons that receive input exclusively from the ipsilateral eye.

1.8 Alternative hypotheses for open-eye potentiation

Homeostatic mechanisms have long been thought to play a role in the response to altered sensory experience. In fact, the BCM sliding threshold model describes a means

to achieve the homeostasis of firing rates in the face of decreased synaptic drive as the modification threshold will adopt whatever position is required to maintain the firing rate by adjusting synaptic weights via LTP or LTD. In this model, prolonged MD leads to a decrease in θ_m, which facilitates LTP of open-eye inputs, thereby increasing synaptic drive and restoring post-synaptic firing rates closer to their original position.

An alternative mechanism of homeostatic regulation is synaptic scaling, which was first described in dissociated rat cortical cultures, where blockade of activity with TTX results in the global multiplicative scaling up of synaptic weights (Turrigiano *et al.*, 1998). It has been proposed that such a mechanism may account for the strengthening of open-eye responses following MD (Turrigiano and Nelson, 2004). Visual deprivation, either through monocular inactivation via intraocular TTX or dark rearing, increases mEPSC amplitudes recorded in layer 2/3 neurons in juvenile rats (Desai *et al.*, 2002; Goel *et al.*, 2006).

Recent work using *in vivo* calcium imaging to measure visual responses in mice has suggested that similar mechanisms are also invoked during MD. In addition to the expected shift in responses towards open-eye dominance, responses of cells driven exclusively by the deprived eye were larger following MD (Mrsic-Flogel *et al.*, 2007). The results of this study share a similar limitation with acute single unit recordings, namely that it is impossible to measure responses in the same cells before and after a manipulation, necessitating between-group comparisons and relative measures of responsiveness. Therefore, it is not clear if previously monocular cells driven by the deprived eye undergo response potentiation, or if originally binocular cells become increasingly monocular.

Given that both the BCM and synaptic scaling models describe homeostatic mechanisms for maintaining a given level of post-synaptic activity, we should move beyond the artificial distinction often seen in the literature between "Hebbian" and "homeostatic" plasticity mechanisms in the context of OD plasticity. A more useful distinction, which is supported by both theoretical and experimental work, is between homeostatic mechanisms expressed globally (synaptic scaling) as opposed to homosynaptically (BCM; Figure 1.4).

One possible way to distinguish between homosynaptic BCM and heterosynaptic scaling models is in the predicted response to binocular deprivation (BD). A synaptic scaling model predicts that the reduction in visual drive from both eyes should lead to potentiation of responses following BD, whereas no change in responsiveness is predicted by BCM based models (Blais *et al.*, 1999). Visual responses measured with *in vivo* calcium imaging in layer 2/3 were potentiated following BD (Mrsic-Flogel *et al.*, 2007), although a similar increase was not observed in previous studies using either single unit recordings across cortical layers (Gordon and Stryker, 1996) or chronic VEP recordings in layer 4 (Frenkel and Bear, 2004; Blais *et al.*, 2008) (Figure 1.3C).

The NMDAR dependence of open-eye potentiation may provide a second means to distinguish between synaptic scaling and LTP. Homosynaptic LTP at layer 4 to 2/3 synapses as well as at layer 2/3 to 5 synapses requires NMDAR activation (Kirkwood *et al.*, 1993; Wang and Daw, 2003), whereas synaptic scaling in culture does not (Turrigiano *et al.*, 1998; Turrigiano and Nelson, 2004). Open-eye potentiation is absent in mice lacking NR1 in layers 2-4 (Sawtell *et al.*, 2003), suggesting that homosynaptic LTP may underlie the strengthening of open-eye responses. A further potential

distinction between LTP and synaptic scaling is the requirement for synaptic insertion of GluR1, which is necessary for LTP (Shi *et al.*, 2001) but not scaling (Gainey *et al.*, 2009).

An additional method to distinguish these two models is through the use of genetically modified animals deficient in synaptic scaling. This approach was recently used by Kaneko *et al*. (2008b), who studied OD plasticity in mice lacking tumor necrosis factor-α (TNFα), which fail to show scaling-up of synaptic responses following decreased activity *in vitro*. Using repeated imaging of intrinsic optical signals it was found that open-eye potentiation similarly fails to occur in the absence of TNFα. The finding of normal LTP in layer 2/3 of these mice strongly suggests that synaptic scaling may drive open-eye potentiation. One problem with this interpretation, as noted previously (Aizenman and Pratt, 2008), is that deprived-eye responses did not increase proportionally to open-eye responses during the later stages of MD (Kaneko *et al.*, 2008b). Likewise, at the behavioral level, delayed increases in open-eye acuity are not accompanied by parallel increases in deprived-eye acuity (Iny *et al.*, 2006). A key feature of synaptic scaling is that *all* synapses are scaled up or down equally, a feature which is essential to prevent information loss and preserve the relative strengths of distinct inputs (Turrigiano *et al.*, 1998). Therefore, the disproportionate effect on open-eye responses during the later stages of MD argues against synaptic scaling as the sole mechanism of open-eye potentiation.

At this time, it seems reasonable to suggest that the discordant findings from imaging versus electrophysiology and behavior may arise from significant laminar differences in the cortical response to MD and BD. Layer 4 neurons receiving

convergent thalamocortical inputs that are dedicated to each eye might maintain homeostasis via a homosynaptic BCM-type rule, in which a loss of strength of one input is compensated for by an increase in strength by a competing input. Conversely, neurons in the superficial layers that do not receive segregated inputs from the two eyes might maintain responsiveness in the face of deprived-eye depression via a heterosynaptic scaling mechanism.

1.9 Outstanding issues in OD plasticity

A clear challenge still facing the field of OD plasticity is to demonstrate a causal role for specific molecular processes in the response to MD. Several mechanisms have emerged as potential contributors to deprived-eye depression, but it remains to be determined which, if any, are required. Similarly, evidence suggests that a decrease in the NR2A / B ratio may facilitate open-eye potentiation, but again a causal role has yet to be demonstrated. Viral-mediated overexpression of NR2A or RNAi knockdown of NR2B in the visual cortex specifically during the period of MD may be able to test this by preventing a decrease in the NR2A / B ratio.

LTP provides a tempting mechanistic framework for open-eye potentiation, but many questions remain. Local disruption of either NMDAR function or synaptic AMPAR insertion specifically during the later stages of MD may help distinguish processes serving deprived-eye depression from those involved in open-eye potentiation. Additionally, it is important to determine whether open-eye potentiation occludes subsequent LTP, and to probe the relative contributions of homo- and hetero- synaptic processes to open-eye potentiation.

It is essential that future studies of OD plasticity recognize the numerous

differences in synaptic plasticity across cortical layers. This is especially important when manipulating molecular pathways during MD, as those pathways may only be involved in OD plasticity in specific layers. Greater attention must be given to the laminar position of neurons recorded with single unit techniques, as grouping neurons across layers may obscure effects of manipulations that are layer specific.

Experimental techniques for characterizing OD plasticity have advanced greatly over the last four decades. For example, the longitudinal within-animal observations of visual responses afforded by chronic VEP recordings have greatly added to the understanding of the kinetics of OD plasticity. Currently all available experimental techniques have significant limitations: single-unit recordings and calcium imaging offer single-cell resolution, but do not allow for chronic recordings, whereas VEP recordings allow for chronic measurements, but lack single cell resolution. Recent advances in transgenic calcium sensors (reviewed in (Knopfel *et al.*, 2006)) may provide a solution, allowing large numbers of neurons to be observed repeatedly over the course of MD. The combination of transgenic calcium sensors with virally-mediated disruption of specific molecular pathways should allow the investigation of whether individual neurons in a cortical network respond to MD in a cell-autonomous manner.

An additional longstanding challenge in the field has been to clearly label eye-specific inputs into the cortex in living tissue. Without this information it is difficult to interpret many of the results in the literature. For example, MD produces changes in dendritic spines (Mataga *et al.*, 2004; Oray *et al.*, 2004), but it is unclear whether these changes are restricted to spines receiving input from a particular eye. Likewise, it remains to be determined whether the occlusion of LTD by prior MD (Crozier *et al.*,

2007) is restricted to deprived-eye but not open-eye inputs into layer 4, as would be predicted.

1.10 Critical periods for OD plasticity

In this review we have focused on the mechanisms underlying the physiological response to MD, and have not yet addressed the developmental regulation of OD plasticity. Classically, OD plasticity has been described as developmentally restricted to a critical period in early post-natal life, and evidence still supports this view in many species, including rats, cats, and monkeys. In mice, on the other hand, OD plasticity has been demonstrated throughout adulthood (Tagawa *et al.*, 2005; Frenkel *et al.*, 2006; Hofer *et al.*, 2006a), indicating that the critical period concept is not applicable to all species. Although the response to MD occurs more slowly in adult mice, this plasticity appears qualitatively indistinguishable from that in juvenile animals (Frenkel *et al.*, 2006).

However, the potential for adult OD plasticity is definitely not restricted to mice. For example, recent work has shown that under certain conditions rats, which have a critical period, can exhibit rapid and robust OD plasticity as adults. Degradation of chondroitin sulphate proteoglycans in the ECM can restore a rapid OD shift in adult rats (Pizzorusso *et al.*, 2002), as can oral administration of the antidepressant fluoxetine (Maya Vetencourt *et al.*, 2008). Even subtle manipulations of experience are sufficient: both brief dark exposure (He *et al.*, 2006; He *et al.*, 2007) and environmental enrichment (He *et al.*, 2007; Sale *et al.*, 2007) are able to restore OD plasticity in adult rats. These newer findings build on seminal work performed in cats almost 30 years ago showing that OD plasticity can be restored by local infusion of norepinephrine into adult visual

cortex (Kasamatsu *et al.*, 1979). The restoration of plasticity in adult animals is especially significant from a therapeutic perspective, as dark rearing, environmental enrichment, and fluoxetine all promote the recovery of vision following chronic deprivation amblyopia.

The findings of OD plasticity in adult animals raises two important questions. First, are the mechanisms qualitatively different from those active in juveniles? There is some evidence that the molecular consequences of MD vary between juveniles and adults, suggesting that distinct pathways may be involved (Scott *et al.*, 2009). Second, is there a final common pathway (e.g., altered inhibition or NMDAR subunit composition) affected by the manipulations that promote plasticity in adult animals? Answering these two questions will represent a major advance in the treatment of amblyopia, and may provide insights into other forms of developmentally regulated plasticity, including learning and memory.

1.11 Conclusion

Advances in recording techniques, coupled with the development of molecular tools and transgenic mice have led to the identification of many of the molecular pathways involved in OD plasticity. Based on currently available evidence, we favor a three phase model of OD plasticity in layer 4 of mice: deprived-eye responses rapidly weaken following MD through homosynaptic LTD, while over a longer time period the threshold for synaptic modification is lowered, facilitating the strengthening of open-eye responses via homosynaptic LTP. In addition to presenting several directly testable hypotheses concerning OD plasticity in mice, this model suggests potential therapeutic strategies for amblyopia in humans.

In the subsequent chapters, I will describe efforts to test the necessity of LTD for depression of deprived-eye visual responses during MD in juvenile mice. As previously noted, there are several lines of evidence which suggest that LTD is induced at multiple cortical synapses by MD, although the requirement for these processes has not been determined. Given the diversity of pathways contributing to LTD induction, we chose to address this by focusing on a common expression mechanism involved in multiple forms of LTD: AMPA receptor endocytosis. In Chapter 2, I will describe experiments testing our ability to block NMDA-stimulated AMPAR endocytosis and LTD in the visual cortex. Experiments examining the consequences of blocking AMPAR endocytosis on OD plasticity will be presented in Chapter 3. In Chapter 4, I present data arguing against an explicit deterministic link between NR2B-containing NMDARs and LTD, and discuss the relationship of these findings to metaplasticity in the visual cortex. Finally, I will discuss the implications of these results for the understanding of OD plasticity, and address in detail several outstanding issues in the field.

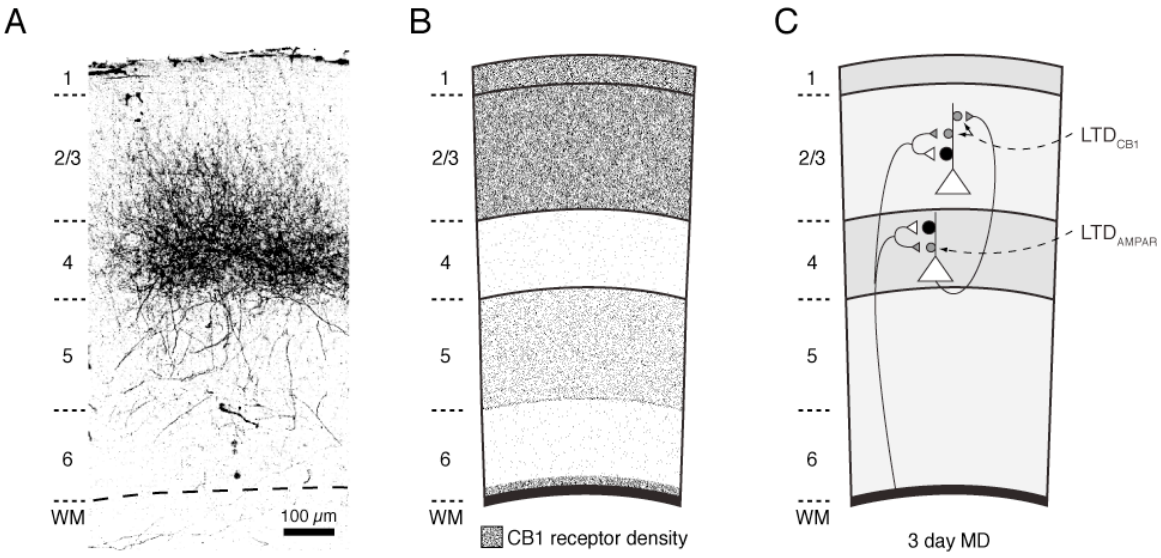


Figure 1.1 – Mechanisms of deprived eye depression vary across cortical layers

A. Thalamocortical axons project to both layer 4 and layer 3 of the mouse visual cortex. Axons were labeled with biotin-conjugated dextran (3,000 MW) injected into binocular dLGN of a P28 mouse. Imaging took place 4 days later from fixed coronal sections containing visual cortex. Laminar borders were determined based on Nissl staining. Image courtesy of J. Coleman. B. Schematic showing CB1 receptor density variations across cortical layers, with high levels of expression in supragranular layers and limited expression in layer 4. Drawing based on Deshmukh *et al.*, (2007). C. Three days of MD produces depression of deprived-eye responses in both layer 3 and layer 4 through distinct mechanisms. In layer 3, both LTD and deprived-eye depression require CB1 activation, whereas LTD is independent of post-synaptic AMPAR internalization. The absence of high CB1 expression in layer 4 correlates with the lack of a requirement for CB1 activation in both LTD and deprived-eye depression. In contrast, LTD in layer 4 requires AMPAR endocytosis, suggesting that deprived-eye depression at this synapse may also occur through AMPAR internalization.

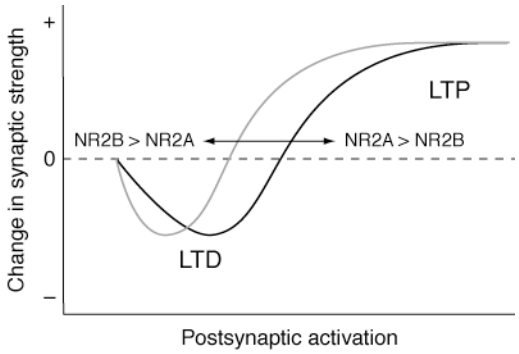


Figure 1.2 – NR2A / B ratio may act as a molecular mechanism for the sliding modification threshold

Model for the relationship between the strength of postsynaptic activation and long-lasting changes in synaptic strength. Black line depicts the modification curve relating the level of postsynaptic activation to synaptic plasticity, where high levels of activity promote synaptic strengthening by inducing LTP, whereas lower levels of activity decrease synaptic strength via LTD. Decreases in the ratio of NR2A to NR2B NMDAR subunits facilitates synaptic potentiation at intermediate levels of postsynaptic activation and is depicted as a leftward shift in the modification curve (gray line). Figure adapted from Philpot *et al*. 2001, based on data from Philpot *et al.* 2003 and 2007.

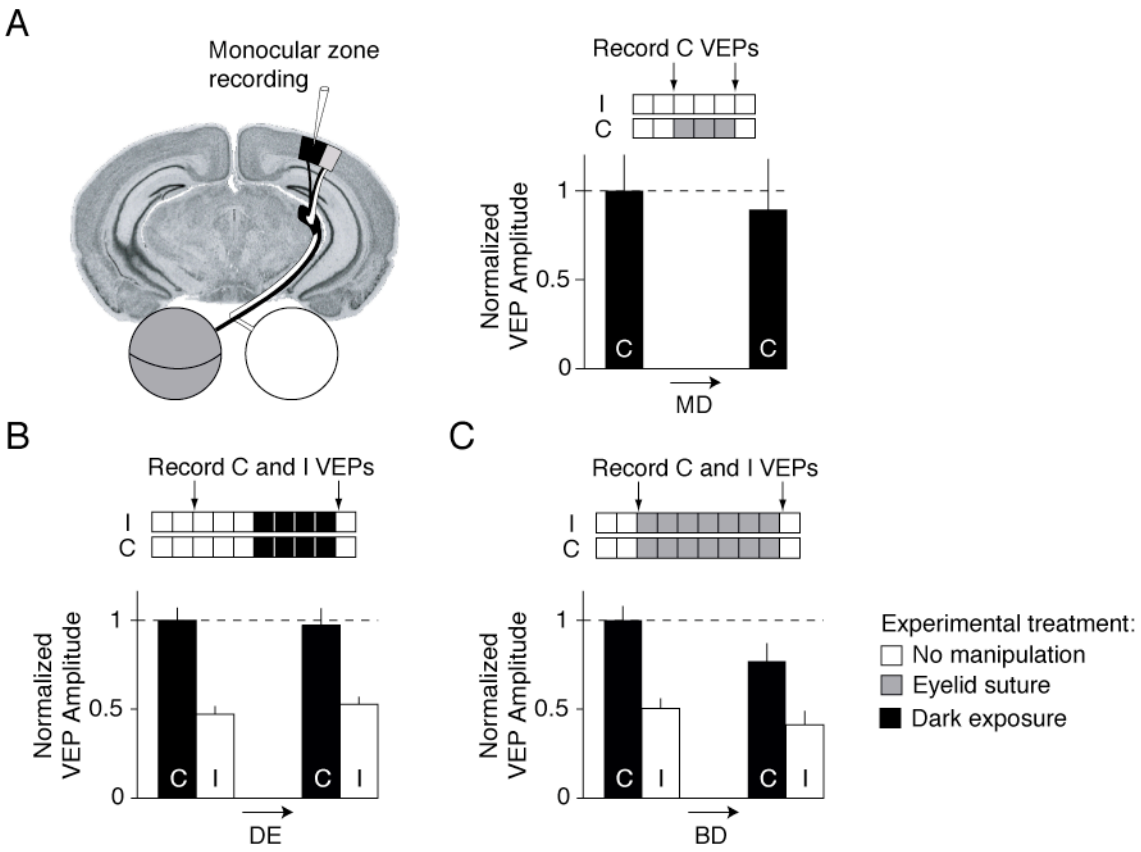


Figure 1.3 – Layer 4 VEPs display binocular competition but not synaptic scaling

A. MD fails to elicit changes in responses recorded in the monocular zone. B. 4 days of complete darkness fails to modify responses in the binocular zone; showing that deprived-eye depression is impaired in neurons which do not experience binocular competition. C. Prolonged binocular deprivation fails to produce scaling up of VEPs recorded in layer 4 of the binocular cortex. Responses to stimulation of the contralateral (C) and ipsilateral (I) eyes were recorded prior to and following 7 days of binocular lid suture. No change in the response to either eye was observed, contrary to the prediction that synaptic scaling would lead to increased responses following decreased input activity. Data are replotted from: (A) (Frenkel & Bear 2008); (B) (Blais *et al.* 2008); (C) (Frenkel & Bear 2004).

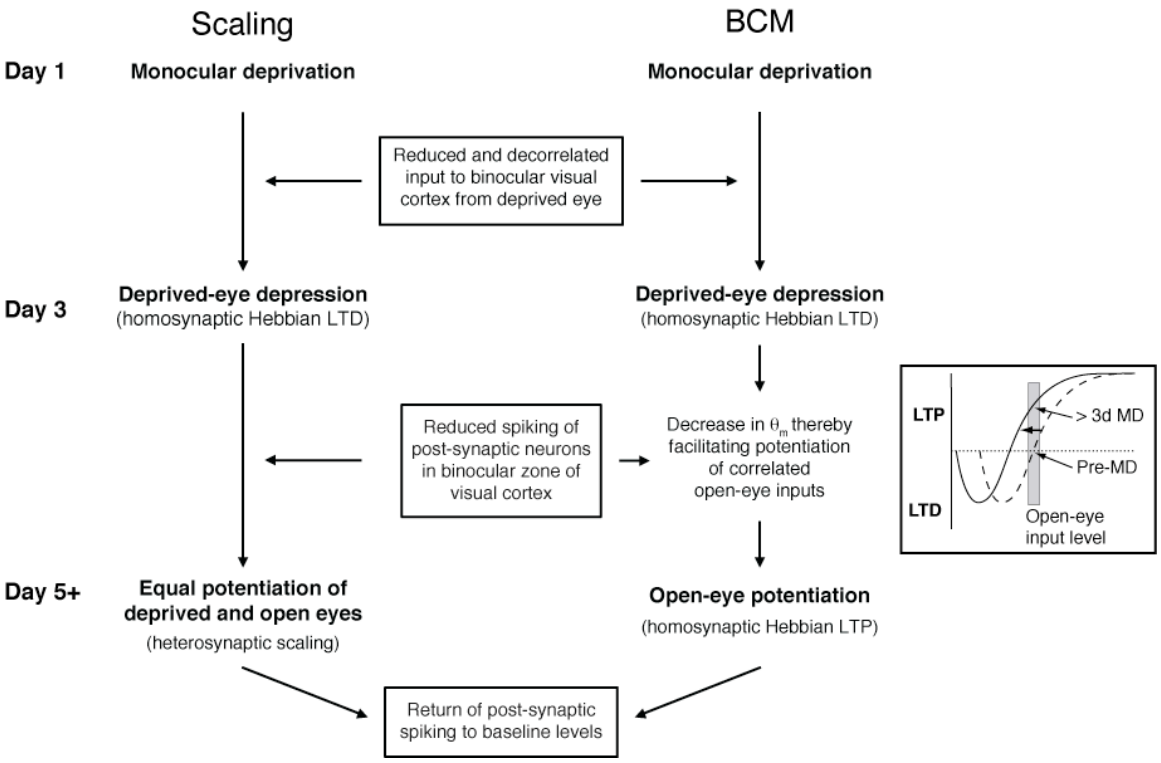


Figure 1.4 – Alternative mechanisms for achieving open-eye potentiation and homeostasis following MD

Under both the synaptic scaling and BCM hypotheses, the initial response to MD is the depression of deprived-eye responses, which results from the decorrelation of deprived-eye inputs following lid suture. This decorrelation of input activity drives homosynaptic LTD in the binocular visual cortex. A consequence the degraded input coupled with the weakening of synapses via LTD is reduced spiking activity of neurons in the binocular visual cortex, which, after several days leads to compensatory changes. The nature of these changes differs between the two models of open-eye potentiation. According to the synaptic scaling model, neurons respond to reduced spiking activity by globally scaling up synaptic weights, thereby increasing incoming drive and returning spiking to baseline levels. In contrast, under the BCM model, neurons respond to decreased spiking activity by lowering the modification threshold (θ_m), thus promoting LTP across a larger range of inputs. The inset shows that prior to MD the boundary between potentiating and depressing inputs is equal to the open-eye input level, thereby maintaining stable open-eye responses if visual experience is not manipulated. Following 3d of MD, the boundary shifts to the left, and open-eye inputs to the cortex (the strength of which has not changed as visual experience through the open eye has remained constant) now induce homosynaptic potentiation. This increase in drive from the open eye in turn elevates post-synaptic spiking to baseline levels. Therefore both the synaptic scaling and BCM models can account for both the potentiation of open-eye responses and the output homeostasis of visual cortical neurons. The difference lies in the behavior of the deprived-eye inputs during the later stages of MD. Synaptic scaling is heterosynaptic, therefore both open- and deprived- eye inputs are predicted to increase equally. In clear

contrast, BCM-mediated homeostasis occurs via homosynaptic mechanisms, thus only open-eye responses potentiate. Current data suggest that the deprived eye does not potentiate proportionally to the open eye, suggesting that global scaling of responses is not occurring in response to MD.

Chapter 2

Blockade of NMDA-stimulated AMPA receptor endocytosis and LTD in visual cortex by HSV-G2CT

Portions of this chapter were published together with Dr. Bong-June Yoon, Dr. Arnold J. Heynen, Dr. Rachael L. Neve, and Dr. Mark F. Bear in Proceedings of the National Academy of Sciences of the United States of America (2009) Vol. ***106****, pp. 9860-9865.*

2.1 Abstract

The classic example of experience-dependent cortical plasticity is the ocular dominance shift in visual cortex following monocular deprivation. The experimental model of homosynaptic long-term depression was originally introduced to study the mechanisms that could account for deprivation-induced loss of visual responsiveness. One established LTD mechanism is a loss of sensitivity to the neurotransmitter glutamate caused by internalization of postsynaptic AMPA receptors. Although it has been shown that MD similarly causes a loss of AMPARs from visual cortical synapses, the contribution of this change to the OD shift has not been established. Using a herpes-simplex virus (HSV) vector, we expressed in visual cortical neurons a peptide (G2CT) designed to block AMPAR internalization by hindering the association of the C-terminal tail of the AMPAR GluR2 subunit with the AP2 clathrin adaptor complex. We found that G2CT expression specifically interferes with NMDA receptor-dependent AMPAR endocytosis without affecting baseline AMPAR surface expression in cortical cultures. *In vivo* expression of G2CT produced no evidence of toxicity or alterations in baseline synaptic transmission, but blocked NMDA-stimulated AMPAR endocytosis and electrically-induced LTD in acute cortical slices from infected animals. These results demonstrate that HSV-mediated delivery of G2CT *in vivo* is sufficient to block LTD in layer 4 of visual cortex. Furthermore, they suggest that HSV-G2CT is a suitable reagent for assaying the requirement for AMPA receptor endocytosis and LTD in the loss of visual responsiveness following MD.

2.2 Introduction

The earliest and most dramatic effect of MD is the severe loss of visual cortical responsiveness to the deprived eye (deprivation amblyopia). Over the past 20 years, the hypothesis has been advanced that untimely or weak activation of cortical neurons by deprived-eye inputs is a trigger for synaptic depression in visual cortex (Bear *et al.*, 1987; Bear, 2003). This idea received strong support with the discovery of homosynaptic long-term depression, a persistent modification of transmission at synapses activated by various types of electrical stimulation. LTD has been studied intensively to uncover the mechanisms that make synapses weaker (Malenka and Bear, 2004). In sensory cortex, most forms of LTD require activation of NMDARs (Kirkwood and Bear, 1994a; Markram *et al.*, 1997; Feldman *et al.*, 1998; Crozier *et al.*, 2007) and, indeed, blockade (Bear *et al.*, 1990; Daw *et al.*, 1999) or genetic deletion of NMDARs (Roberts *et al.*, 1998) prevents the OD shift after MD. However, NMDAR blockade affects multiple forms of plasticity. Thus, it has been difficult to test the specific hypothesis that LTD mechanisms *per se* contribute to the OD shift, a problem compounded by the fact that it is now clear that there are multiple mechanisms that could compensate for one another (Rao and Daw, 2004; Crozier *et al.*, 2007).

The most advanced understanding of a mechanism for NMDAR-dependent LTD comes from studies of hippocampus (Malenka and Bear, 2004). Prolonged low frequency trains of subthreshold stimulation delivered to CA1 produce a persistent homosynaptic decrease in the strength of synaptic transmission (Dudek and Bear, 1992). Weak activation of NMDARs produces a modest rise in postsynaptic intracellular calcium concentration (Mulkey and Malenka, 1992), activating a protein phosphatase

cascade involving PP1 and calcineurin (Mulkey *et al.*, 1993; Mulkey *et al.*, 1994), leading to dephosphorylation of PKA substrates (Kameyama *et al.*, 1998). Importantly, one such substrate, S845 on the GluR1 AMPAR subunit, is dephosphorylated during LTD (Lee *et al.*, 1998; Lee *et al.*, 2000), which reduces transmission by decreasing channel open probability (Banke *et al.*, 2000). Dephosphorylation of S845 during LTD is dependent on PP1 and calcineurin (Beattie *et al.*, 2000; Ehlers, 2000), and occurs in the absence of phosphorylation changes at S831, which is phosphorylated by CaMKII following induction of LTP (Lee *et al.*, 2000).

These processes appear to set in motion a complex signaling cascade which ultimately results in the removal of AMPARs from the synapse, producing a decrease in the efficacy of synaptic transmission (Song and Huganir, 2002; Malenka and Bear, 2004; Kennedy and Ehlers, 2006). LTD leads to the loss of AMPARs from synapses via clathrin-mediated endocytosis (Carroll *et al.*, 1999; Man *et al.*, 2000). How does calcium influx through NMDARs signal the specific internalization of AMPA receptors? Formation of clathrin-coated endocytic vesicles requires a series of protein-protein interactions, ultimately leading to the detachment of a clathrin-coated vesicle from the plasma membrane (Slepnev and De Camilli, 2000). The AMPA receptor subunit GluR2 interacts with the AP2 clathrin adapter complex by binding to the μ2-adaptin subunit (Man *et al.*, 2000; Kastning *et al.*, 2007). AP2 is capable of nucleating clathrin polymerization and coat formation (reviewed in (Slepnev and De Camilli, 2000)). The GTPase dynamin is required to pinch off clathrin-coated vesicles (Hinshaw and Schmid, 1995; Takei *et al.*, 1995), and is recruited to assembling vesicles through interactions with the adaptor protein amphiphysin, which also binds AP2 (Wigge *et al.*, 1997; Wigge

and McMahon, 1998).

Many of these interactions appear to be regulated by intracellular signaling cascades also implicated in LTD. Phosphorylation of dynamin prevents its interaction with amphiphysin, and phosphorylation of amphiphysin prevents binding to AP2 and clathrin (Wilde and Brodsky, 1996; Slepnev *et al.*, 1998). Dephosphorylation of both proteins facilitates clathrin coat formation and endocytosis and is mediated by calcineurin (Bauerfeind *et al.*, 1997), the inhibition of which also prevents LTD. Furthermore, calcineurin can bind dynamin directly in a calcium-dependent manner, and this interaction is required for clathrin-mediated endocytosis (Lai *et al.*, 1999).

Additionally, several aspects of the endocytic machinery have been directly implicated in LTD. Blockade of dynamin – amphiphysin interactions with a dominant-negative form of amphiphysin prevents LFS-induced LTD in CA1 (Man *et al.*, 2000). Similarly, interactions between AP2 and GluR2 are required for hippocampal LTD (Lee *et al.*, 2002).

Another avenue by which AMPA receptor endocytosis appears to be regulated involves interactions between GluR2 and the PDZ domains of proteins localized to the postsynaptic density. Association of GluR2 with the postsynaptic anchoring protein GRIP is disrupted by phosphorylation of the GluR2 C-terminal tail at S880, thereby increasing binding with PICK, another PDZ containing protein, and promoting endocytosis (Matsuda *et al.*, 1999; Chung *et al.*, 2000; Kim *et al.*, 2001). Notably, this interaction is required for LTD and is blocked by NMDAR antagonists and protein-phosphatase inhibitors, which also block LTD (Kim *et al.*, 2001). Interestingly, the binding of PICK and GluR2 is also increased by modestly elevated calcium

concentrations, suggesting that activation of NMDARs may directly promote local endocytosis of AMPA receptors (Hanley and Henley, 2005; Hanley, 2008). Furthermore, tyrosine phosphorylation of GluR2 at a site (tyrosine 876) near the PDZ binding domain leads to decreased interaction with GRIP, without affecting the association with PICK (Hayashi and Huganir, 2004). Mutation of tyrosine 876 or expression of a peptide mimicking this region of GluR2 blocks induction of LTD, suggesting a critical role for this residue (Ahmadian *et al.*, 2004; Hayashi and Huganir, 2004).

In addition to NMDAR stimulated endocytosis, AMPARs also undergo constitutive endocytosis and recycling at synapses. This recycling is dependent on interactions between GluR2 and NSF, an ATPase implicated in membrane fusion events, which binds the GluR2 C-terminal tail at the same position as AP2 (Hay and Scheller, 1997; Lee *et al.*, 2002). Blockade of the NSF – GluR2 interaction produces rundown of synaptic transmission in CA1 neurons, which occludes LTD (Lee *et al.*, 2002).

Critically, the interaction between GluR2 and AP2 can be selectively disrupted without interfering with the binding of NSF by a peptide (KRMKLNINPS), which we term G2CT (Lee *et al.*, 2002). This peptide acts by mimicking the minimal GluR2 amino acid sequence required for AP2 interaction, with mutations that prevent the disruption of NSF activity (Figure 2.1A). Lee *et al.* showed that G2CT has remarkable selectivity when introduced postsynaptically in CA1, blocking NMDAR-stimulated AMPAR endocytosis and hippocampal LTD without affecting baseline transmission or AMPA-stimulated AMPAR internalization.

Although initial reports of LTD in the visual cortex suggested great similarity to CA1 (Kirkwood *et al.*, 1993; Kirkwood and Bear, 1994a), recent work has shown that

there are significant laminar differences in the mechanisms of both LTD and OD plasticity in mouse visual cortex (Daw *et al.*, 2004; Crozier *et al.*, 2007; Liu *et al.*, 2008). However, LTD of the radial input to layer 4 closely resembles that in hippocampal area CA1, notably including sensitivity to intracellularly loaded G2CT (Crozier *et al.*, 2007). We therefore reasoned that if the G2CT peptide could be introduced intracellularly *in vivo*, it would provide a method to assess the contributions of both NMDAR-dependent AMPAR internalization and LTD within layer 4 to the depression of deprived-eye responses following MD.

Here we show that it is possible to deliver the G2CT peptide to neurons using an HSV vector. We find that virally expressed G2CT blocks NMDA-stimulated AMPAR endocytosis in cortical neurons and synaptically induced LTD in layer 4 of visual cortex. Our results demonstrate that G2CT is a valuable reagent to test the requirement for LTD in MD-induced response depression.

2.3 Results

2.3.1 Viral expression of G2CT blocks NMDA-stimulated AMPAR internalization in cultured visual cortical neurons

We used an HSV vector (Neve *et al.*, 2005; Frenkel *et al.*, 2006) to deliver green fluorescent protein (GFP) and G2CT under separate promoters (HSV-G2CT) (Figure 2.1B). A virus expressing GFP alone (HSV-GFP) was used in several experiments to control for non-specific effects of viral infection or GFP expression.

We chose HSV-mediated gene delivery over other approaches for several reasons. First, HSV readily infects differentiated cells, and shows a preference for neurons over other CNS cell types, thus avoiding confounding effects due to expression of G2CT in glia (Neve *et al.*, 2005). Secondly, recent developments in packaging techniques have

greatly reduced cytotoxicity associated with HSV infection. Early studies reported unacceptable levels of toxicity (Isacson, 1995), which generally arises from three sources: reversions to wild-type lytic virus, high levels of helper virus (which is required for packaging), or contamination by toxic factors in crude cell lysates (Neve *et al.*, 2005). Genetic modifications in the helper virus and the complementing host cell reduce revertants, and multiple passages followed by sucrose gradient purification reduce helper virus levels and purify virus particles away from cell lysates (Lim *et al.*, 1996; Lim and Neve, 2001). Using this approach, HSV has been used successfully in the mammalian brain without apparent toxicity (Carlezon *et al.*, 1997; Carlezon *et al.*, 1998).

An additional advantage of HSV is its ability to successfully drive expression of multiple gene products from a single virus (Clark *et al.*, 2002). This property is desirable for expression of small interfering peptides such as G2CT (10 aa), given the strong possibility of steric hindrances in a GFP fusion protein. Lastly, for studies of OD plasticity we wanted to restrict expression of G2CT as closely as possible to the period of MD, thereby allowing normal development of the visual system to occur in the presence of a full complement of plasticity mechanisms. The rapid onset of gene expression following infection (as little as 2 hours in organotypic culture) makes HSV well suited to this task (Bahr *et al.*, 1994).

To confirm that the G2CT peptide could be expressed in infected neurons at a level sufficient to block NMDAR-dependent AMPAR endocytosis, we incubated cultured occipital cortical neurons with HSV vectors and, 24 hours later, examined the effect of brief NMDA treatment (20 μM, 5 min) on surface-expressed AMPARs. We found that ~90% of the cultured neurons expressed GFP at 24 hours post-infection (Figure 2.2A),

and that HSV-G2CT infected neurons exhibited normal morphological characteristics, including well defined dendritic spines (Figure 2.2B). In accordance with previous reports, we found that NMDA treatment significantly reduced the number of AMPARs present on the plasma membrane in uninfected control cultures as well as in cultures infected with HSV-GFP (uninfected: 76.2 ± 6.1% of untreated; $n = 8$, $p < 0.05$; HSV-GFP 82.4 ± 5.0% of untreated; $n = 8$; $p < 0.05$). However, HSV-G2CT infection completely blocked the NMDA-stimulated loss of surface AMPARs (98.6 ± 2.6 % of untreated; $n = 8$; $p > 0.5$) (Figure 2.3). Quantification of the level of surface expressed AMPARs under baseline (non NMDA-treated) conditions showed no significant difference between non-infected, HSV-GFP and HSV-G2CT infected cultures (100 ± 6.5, 137.7 ± 21.4, 120.1 ± 16.1 %, respectively), suggesting that expression of the G2CT peptide does not affect basal AMPAR trafficking and surface expression. The ability of G2CT to interfere with the stimulated endocytosis of GluR1 is likely due to an effect on heteromeric GluR1/GluR2 receptors.

The observed inhibition of AMPAR endocytosis by the G2CT peptide could result from a general disruption of clathrin-mediated endocytosis as opposed to a specific disruption in the association between AP2 and AMPARs. We therefore examined the AP2 and clathrin-dependent endocytosis of transferrin receptors (TfRs) (Motley *et al.*, 2003). Following transferrin application, robust internalization of TfRs was observed in non-infected, HSV-GFP, and HSV-G2CT infected cultures, and the levels of internalized receptors were similar across groups (Figure 2.4).

2.3.2 Viral expression of G2CT blocks NMDA-stimulated AMPAR internalization in visual cortical slices

We next examined the *in vivo* efficiency of HSV-mediated infection in mouse

visual cortex and tested the ability of HSV-G2CT to block NMDAR-dependent AMPAR endocytosis in acutely prepared slices. To test our ability to effectively deliver HSV-G2CT to the visual cortex *in vivo*, we performed a series of experiments in which HSV-G2CT was infused into the visual cortex of juvenile mice (P23 - 25), and animals were prepared for histology at various times post infection. Robust and sustained GFP expression was observed following infections targeted to layer 4 of visual cortex (Figure 2.5A). GFP expression was detected as early as 1 day post-infection and expression was maintained at high levels through at least 6 days post-infection. To determine if HSV infection under these conditions produced neurotoxic effects, we performed Nissl and Hoechst counterstaining. Infected regions of cortex did not show any abnormalities in the patterns of Nissl or Hoechst staining, nor was there any evidence of gliosis (Figure 2.5B). Apart from the presence of GFP+ neurons, infected areas of cortex were largely indistinguishable from neighboring uninfected regions.

In order to determine which cell types were infected, we conducted a series of experiments in which GFP+ cells were immunocytochemically double-labeled with a neuronal marker (Neu-N) or a marker of inhibitory interneurons (γ-amino butyric acid, GABA). 89% of GFP+ cells (n = 446) were positive for NeuN, confirming previous observations that HSV is highly neurotropic (Neve *et al.*, 2005). Interestingly, even though the infected volume contained many GABA+ cells (n = 165), very few of these also contained GFP (3 of 165; 1.8 %) (Figure 2.6). This unexpected difference in infectivity suggests that the effects of G2CT expression are primarily due to disruptions in AMPAR endocytosis in excitatory cells.

To assess if G2CT expression following *in vivo* infection was sufficiently strong

to interfere with activity-dependent AMPAR endocytosis, slices prepared 48 h post-infection were treated with NMDA (100 μm, 15 min; Figure 2.7A). In non-infected visual cortex, NMDA produced a significant reduction of surface-expressed AMPARs (GluR1: 77.3 ± 6.6 % of control, n = 5; $p < 0.05$; GluR2/3: 84.7 ± 3.5 % of control, n = 5; $p < 0.05$, Figure 2.7B), whereas internalization was blocked in HSV-G2CT infected cortex (GluR1: 91.0 ± 9.4 % of control, n = 5; $p > 0.40$; GluR2/3: 105 ± 12.4 % of control, n = 5, $p > 0.69$).

2.3.3 Pairing-induced LTD in layer 4 neurons is blocked by viral expression of G2CT

We next determined if viral expression of G2CT was sufficient to interfere with LTD of excitatory postsynaptic currents (EPSCs) evoked in layer 4 neurons by white matter stimulation, a process known to be sensitive to the G2CT peptide (Crozier *et al.*, 2007). HSV-G2CT was injected into one hemisphere of visual cortex and slices were prepared 48-72 h post-infection from both hemispheres, with the uninfected cortex serving as a control (Figure 2.8A, B). Pairing 1 Hz presynaptic stimulation with modest postsynaptic depolarization reliably elicited LTD in uninfected neurons (71.8 ± 2.2% of baseline, n = 5 cells from 5 mice, $p < 0.05$, paired t-test), consistent with previous findings (Crozier *et al.*, 2007). However, LTD was absent in G2CT-expressing cells (96.7 ± 10.1% of baseline, n = 5 cells from 5 mice, $p > 0.2$, paired t-test; Figure 2.8C). We therefore conclude that infection of visual cortex *in vivo* with HSV-G2CT is sufficient to block synaptic depression arising from AMPAR endocytosis.

It is possible that infected neurons fail to exhibit synaptic plasticity because they are injured. However, intrinsic electrophysiological properties, such as membrane potential (V_m), membrane resistance (R_m), and membrane capacitance (C_m), of non-

infected control cells (n = 12) and HSV-G2CT infected neurons (n = 8) were indistinguishable (V_m: -68.3 ± 3.0 vs. -68.4 ± 4.9 mV, $p > 0.5$; R_m: 256.3 ± 30.0 vs. 292.0 ± 31.0 MΩ, $p > 0.4$; C_m: 61.6 ± 5.2 vs. 61.9 ± 8.7 pF, $p > 0.5$). Similarly, when input-output curves and AMPA / NMDA ratios were examined in control and G2CT-expressing cells, no significant differences were observed (input-output: n = 5 control; 4 G2CT; repeated measures ANOVA, $p > 0.5$; Figure 2.9A; AMPA / NMDA: control: 100 ± 12.5% of control, n = 7; G2CT: 82.8 ± 24.5% of control, n = 6; $p > 0.5$; Figure 2.9B, C). Thus, the impairment of LTD cannot be explained by altered excitability or reduced NMDAR function in the infected neurons.

2.4 Discussion

Although it has been shown previously that MD can cause internalization of postsynaptic AMPARs and LTD (Heynen *et al.*, 2003; Crozier *et al.*, 2007), it remains unclear whether AMPAR endocytosis is required for the loss of deprived-eye responses following MD. The endocytosis of AMPA receptors during LTD is a complex process, regulated at multiple levels, but appears to critically depend on the molecular interaction of the clathrin adaptor complex AP2 with the GluR2 C-terminal tail (Lee *et al.*, 2002). Here we show that the G2CT peptide, which blocks this interaction, can block NMDA-stimulated AMPAR internalization and LTD when expressed in the visual cortex through HSV-mediated gene delivery.

The weakening of synaptic transmission via LTD is a complex process involving multiple signaling cascades affecting the phosphorylation and localization of many proteins. Further complicating the issue is evidence that the molecular pathways and expression mechanisms of LTD differ amongst synapses, especially in the cortex (Daw *et*

al., 2004). In a detailed study, Crozier *et al.* (2007) examined LTD at white matter – layer 4 synapses in comparison with layer 4 to layer 2/3 synapses in the visual cortex. In both layers delivery of a LFS-pairing protocol similar to that reported here produced LTD of synaptic transmission that was blocked by bath application of APV or post-synaptically loaded MK-801 (Crozier *et al.*, 2007). Downstream of NMDAR activation, the layer-specific mechanisms of LTD appear to diverge. The endocannabinoid receptor antagonist AM251 blocks LTD in layer 2/3 neurons but not 4 neurons, whereas LTD in layer 4 but not 2/3 was sensitive to inhibition of PKA or AMPAR endocytosis.

In a complementary set of experiments, LTD from 4 to 2/3 or from layer 2/3 to 5 was blocked by APV but not the broad acting mGluR antagonist LY341495 (Sawtell *et al.*, 1999; Rao and Daw, 2004). Furthermore, LFS induced LTD in layer 2/3 is unaffected by genetic deletion of mGluR5 (Sawtell *et al.*, 1999). In contrast, layer 2/3 to 6 LTD is insensitive to NMDAR blockade, but does require activation of group I mGluRs (Rao and Daw, 2004). Taken together, these results suggest that the mechanisms of LTD in layer 4 of the visual cortex are highly similar to those in CA1, making the G2CT peptide well suited to investigating the contribution of layer 4 LTD to OD plasticity.

The G2CT peptide was designed by Lee *et al.* (2002) to interfere with the association of the AP2 clathrin adaptor complex with the GluR2 AMPAR subunit. These authors found that expression of G2CT in hippocampal neurons blocked NMDA-stimulated AMPAR endocytosis but had no effect on AMPA-stimulated AMPAR endocytosis. A selective effect of G2CT on NMDAR-regulated AMPAR trafficking was further suggested by the finding that intracellular loading of the peptide into CA1 pyramidal neurons via the recording pipette blocked LTD without affecting baseline

synaptic transmission. Similarly, we failed to observe an increase in basal expression of AMPARs on the surface of cultured cortical neurons 24 hours after infection with HSV-G2CT. Furthermore, we saw no changes in EPSC amplitudes recorded from infected visual cortical neurons studied *ex vivo*, nor did we observe changes in the ratio of AMPA / NMDA receptor mediated responses. Thus, our data extend the previous conclusion that constitutive endocytosis of AMPARs is not affected by the G2CT peptide. Yet, neurons expressing the peptide fail to support NMDA-stimulated AMPAR endocytosis and do not exhibit NMDAR-dependent LTD in layer 4.

The G2CT peptide used in our study has been shown to be the minimal sequence needed to block GluR2-AP2 interactions (Lee *et al.*, 2002). As evidenced by our experiments showing unaffected AP2 and clathrin-mediated endocytosis of transferrin receptors, this peptide does not affect the interaction of AP2 with all endocytic complexes and thus likely does not act by sequestering AP2 from the plasma membrane. The basis for the remarkable selectivity of G2CT for NMDAR-regulated AMPAR endocytosis is currently unknown. The clathrin adapter AP2 is a complex heterotetramer comprised of two large subunits (α and β2) and two small subunits (μ2 and σ2) (reviewed in (Slepnev and De Camilli, 2000)). The β2 subunit of AP2 binds clathrin and promotes clathrin polymerization (Owen *et al.*, 2000), whereas target specificity for AP2 and clathrin mediated endocytosis appears to be conveyed by the μ2 subunit (Ohno *et al.*, 1995). μ2 is capable of directly binding GluR2, in addition to other transmembrane receptors including the transferrin receptor (Motley *et al.*, 2003; Kastning *et al.*, 2007). Notably, the recognition sequences for these cargos appear distinct, with TfR containing the classical μ2 recognition sequence (YXXΦ – where X is any amino acid and Φ is a bulky

hydrophobic residue) (Ohno *et al.*, 1995; Traub, 2003), whereas μ2 interacts with GluR2 via an atypical recognition site on the C-terminal tail of the receptor (Kastning *et al.*, 2007). It is therefore possible that G2CT specifically blocks the binding of GluR2 with AP2 without affecting other AP2 binding events. Indeed, a peptide inhibitor of GluR4-AP2 interaction has been shown to be insufficient in blocking GluR2-AP2 interaction *in vitro* (Lee *et al.*, 2002). Whatever the explanation, our experiments show empirically that G2CT has no observable effect on the fairly extensive range of neuronal properties we examined, with the exception of NMDAR-dependent synaptic depression.

Our experiments with G2CT focused primarily on LTD in layer 4 evoked through white matter stimulation. Given that LTD at layer 4 to layer 2/3 synapses was previously shown to be insensitive to G2CT delivered through the recording pipette (Crozier *et al.*, 2007), we did not examine the effect of HSV-expressed G2CT in layer 2/3. The concentration of peptide delivered directly in prior experiments (10 μM) is likely much higher than the concentration present in infected neurons in this study after taking into account solution exchange with the standard internal solution in our recording pipette. Thus it is extremely unlikely that HSV-delivered G2CT would succeed in blocking LTD in layer 2/3 where intracellularly loaded G2CT failed.

HSV has been successfully used for *in vivo* gene delivery in a number of previous studies, targeting a variety of brain regions, including the visual cortex (Mower *et al.*, 2002; Frenkel *et al.*, 2006). Our experiments using micropipette injections in the visual cortex of juvenile mice confirm previous reports that HSV can rapidly drive expression of exogenous genes with limited cytotoxicity. In contrast to other forms of virally-mediated gene delivery such as lentivirus or adeno-associated virus (AAV), HSV-

mediated expression does not persist indefinitely and transgene expression tends to decrease over time (Carlezon *et al.*, 1997; Carlezon *et al.*, 1998; Neve *et al.*, 2005). Although the transient expression of HSV-delivered genes can be beneficial in some studies (Mower *et al.*, 2002), it could potentially present a confound for our experiments. If HSV-G2CT is to be a viable reagent for assessing the contribution of AMPAR endocytosis to deprived-eye depression, expression of the peptide must persist for a sufficient period to allow surgical implantation of electrodes, followed by recovery from surgery and a period of MD sufficient to induce a loss of deprived-eye responsiveness. Our finding of robust GFP expression 6 days following infection suggests that HSV-G2CT is capable of driving expression for a period sufficient to address this question.

An interesting and unexpected finding was the preferential infection of excitatory neurons by HSV-G2CT. The source of this specificity is unclear, but may be related to specializations of the extracellular matrix (ECM) associated with inhibitory neurons. Perineuronal nets form primarily around inhibitory neurons and are present by age P14 in the mouse (Bruckner *et al.*, 2000). These dense clusters of ECM proteins may prevent HSV from contacting the plasma membrane, thereby reducing infectivity of inhibitory neurons. Regardless of the reason, our data indicate that any effects of HSV-G2CT on visual cortical plasticity are primarily due to the blockade of AMPAR endocytosis and therefore LTD in excitatory neurons.

In summary, HSV-G2CT represents a specific reagent to disrupt AMPAR endocytosis and LTD in the visual cortex, without affecting cell health or basal synaptic transmission. As such, HSV-G2CT is well suited to determining the functional requirement for LTD and AMPAR internalization during OD plasticity.

2.5 Methods

Subjects

Male C57Bl/6 mice were used for all experiments. Animals were group housed and kept on a 12hr light/dark cycle. All animals were treated in accordance with National Institutes of Health and Massachusetts Institute of Technology guidelines.

HSV vector construction and packaging

The p1005+ HSV-amplicon bicistronic plasmid (Clark *et al.*, 2002) was used as a backbone to generate both the HSV-GFP and HSV-G2CT viral vectors. In addition to a HSV-1 origin of replication and a packaging signal, p1005+ contains a first cistron comprised of the native HSV IE4/5 promoter, a multiple cloning site (MCS) and an SV40 polyA signal, while the second cistron is composed of a complete CMV-GFP expression cassette. To generate HSV-G2CT, a 50 bp fragment encoding the G2CT peptide (KRMKLNINPS) was amplified using PCR and two overlapping primers (5-ctcgaggcatgaaacgaatgaagctgaatattaaccc-3', 5'-accggtctaagatgggttaatattcagc-3'). The resultant PCR product was then cloned into the MCS, downstream of the HSV IE 4/5 promoter. HSV-GFP was generated using the unaltered p1005+ plasmid. Viral packaging was carried out as previously described (Neve *et al.*, 2005). Briefly, 2-2 cells were transfected with either the GFP or G2CT amplicon, then superinfected with 5*dl*1.2 helper virus. Resulting virus particles were passaged 3 times, then purified on a sucrose gradient to remove cell lysate contaminants.

Preparation of cortical cultures

For assays with dissociated neurons, cortical cultures were prepared from P0-1 C57Bl/6 mouse pups. The procedure was similar to that previously described for rat cortical cultures (Chen and Bear, 2007), with the following modifications. Neurobasal

media (Invitrogen) was used for plating and feeding, supplemented with 2% B27 supplement and 0.5 mM L-glutamine. Neurons were dissociated by incubation with 7 units / mL papain for 1 hr at 37 ºC followed by trituration, then plated. Cultures were maintained in a 37 ºC incubator with 5% CO_2, and one-half of the feeding media was changed twice weekly to replenish nutrients.

Infection of cortical neurons and imaging

Cultures were infected at 14-18 DIV with HSV vectors (1-2 multiplicity of infection, MOI). Infection efficiency was examined by DIC and epifluorescence imaging. To examine dendritic spines, infected neurons were imaged using a 2-photon laser-scanning microscope (Ultima, Prairie Technologies, Madison, WI) with the excitation wavelength set to 930 nm and with a 60x-0.90NA water-immersion objective.

For *in vivo* infections with HSV, a small volume of virus (~1 µl total) was injected into the binocular visual cortex 450 µm below dural surface at a rate of 0.1 µl/min.

Histology

At various periods following cortical injection of HSV-G2CT, mice were sacrificed with an overdose of sodium pentobarbital, and transcardially perfused with 4% paraformaldehyde. 50 µm sections were collected and counterstained with fluorescent Nissl stain (Molecular Probes) and Hoechst 33342 (Invitrogen). Stained sections were imaged using either a standard epifluorescence microscope and CCD camera (Hamamatsu) or a laser-scanning confocal microscope (Olympus). To determine the cell type of infected neurons, sections from additional infected mice were incubated with antibodies to GABA (rabbit polyclonal, 1:1000, Sigma) and NeuN (mouse monoclonal, 1:500, Chemicon). After incubation with the appropriate secondary antibodies, sections

were mounted on microscope slides and imaged. For quantitation, single XY scans containing infected fields of view were taken using a confocal microscope, and neurons were counted manually using ImageJ software.

Biochemical measurement of surface receptors

For disassociated cortical cultures, biotinylation experiments were performed 20-24 hrs following infection. Cultures were rinsed with warm DMEM and treated with NMDA (20 µM, 3 min). After brief rinses with DMEM, conditioned medium was added back and plates were incubated at 37°C with 5% CO_2 for 30 min. Surface receptors were biotinylated with 1.5 mg/ml biotin (Sulfo-NHS-SS-biotin, Pierce) in ACSF (in mM: NaCl 124, KCl 5, NaH_2PO_4 1.23, $NaHCO_3$ 26, $CaCl_2$ 2, $MgCl_2$ 1 and D-glucose 10, saturated with 95% O_2 / 5% CO_2) (20 min) on ice. Cultures were rinsed 3 times (1x with TBS and 2x with ACSF), and solubilized with RIPA buffer (1% Triton X-100, 0.5% SDS, 0.5% deoxycholic acid, 50 mM NaPO4, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, and 1 mg/ml leupeptin). Homogenates were centrifuged (14,000 x g) for 15 min at 4°C. Supernatants were collected and protein concentration was measured using a DC protein assay kit (Bio-rad). Extracts containing 50 µg of protein were incubated with Immobilized Neutravidin agarose (80 µl, Pierce) overnight at 4°C, washed (3x; RIPA buffer), then resuspended in SDS sample buffer (40 µl) and boiled.

Quantitative immunoblotting was performed on biotinylated surface proteins using primary antibodies to TfR (Chemicon) and to Glur1 (Upstate). An antibody to the GluR1 subunit was used in these experiments due to the limited amounts of sample making it necessary to strip and re-probe immunoblots following visualization of TfR protein levels. It should be noted that previous research has shown that activity-

dependent changes in GluR2/3 and GluR1 subunit levels within the visual cortex mirror one another and are highly correlated (Heynen *et al.*, 2003).

TfR internalization assays were performed as previously described (Ehlers, 2000) with minor changes. Briefly, cultures were pre-incubated with leupeptin (100 μg/ml; 30 min) prior to biotinylation. After 3 rinses with D-PBS, cells were incubated with 1 mg/ml Sulfo-NHS-SS-biotin in D-PBS on ice (20 min). Remaining biotin was quenched with 50 mM glycine (3 x 5 min). While control samples remained on ice, experimental samples were treated with 40 μg/ml transferrin in D-PBS (30 min, 37^{o}C). Biotinylated receptors remaining or recycled back to the surface were stripped with glutathione buffer (100 mM glutathione, 75 mM NaCl, 10 mM EDTA, 1 % BSA, 150 mM NaOH to pH 8.6) for 15 minutes (2x) and quenched with iodoacetamide (5 mg/ml; 3x5 min). Cells were lysed with RIPA buffer and biotinylated proteins were purified with Neutravidin agarose. To control for nonspecific binding and entrance of biotin into cells, data from transferrin-treated samples were normalized to control samples maintained at 4^{o}C.

For biochemical assays using cortical slices, viral vectors were unilaterally injected into visual cortex 2 days prior to biochemical assay. Visual cortical slices were then prepared from both hemispheres as previously described (Heynen *et al.*, 2003). After recovery (1-2 hrs) in ACSF, slices were transferred to a NMDA (100 μM) containing chamber and incubated (15 min). Slices were transferred back to a recovery chamber and incubated for 20 minutes. HSV-infected areas (visualized through GFP fluorescence) and corresponding control areas from the opposing hemisphere were microdissected, homogenized in RIPA buffer and processed as described above using antibodies to the GluR1 and GluR2/3 subunits of AMPARs (Upstate).

In vitro slice electrophysiology

Mice were injected in the visual cortex unilaterally with HSV-G2CT as described above, and acute slices were prepared 48 – 72 hours later as described (Crozier *et al.*, 2007). Slices recovered in ACSF containing (in mM): NaCl 124, KCl 5, NaH_2PO_4 1.23, $NaHCO_3$ 26, $CaCl_2$ 2, $MgCl_2$ 1 and D-glucose 10 (saturated with 95% O_2 / 5% CO_2) for 0.5 h at 32 ºC, and transferred to room temperature for an additional 0.5 h prior to recording. Recordings were made in ACSF at 30 ºC. Layer 4 pyramidal neurons were identified under IR-DIC and examined for GFP fluorescence. GFP-positive cells and neurons from the control non-infected hemisphere were patched under IR-DIC. Somatic whole cell recordings were made using 4-6 MΩ pipettes filled with (in mM): D-gluconic acid 103, CsOH 103, HEPES 20, TEA-Cl 5, NaCl 2.8, EGTA 0.2, MgATP 4, NaGTP 0.3, Na-phosphocreatine 10, QX-314 5, and 0.2% biocytin (290-300 mOsM, pH adjusted to 7.2 with CsOH). A liquid junction potential (approx. -14 mV) was corrected and series resistance (R_s) was monitored throughout. Experiments showing a greater than 20% change in R_s were excluded, as were cells with a resting membrane potential less negative than –50 mV. When possible, attempts were made to record from control and G2CT expressing neurons in slices prepared from the same animal on the same day. Baseline EPSCs were evoked (0.05 Hz) under voltage clamp (-65 mV) with a bipolar stimulating electrode (FHC, Bowdoinham, ME) placed in the white matter. Following a stable baseline, input-output curves were recorded and a stimulation intensity yielding a half-maximal response was chosen for further study. For LTD experiments, a stable baseline was recorded for 10 min, and LTD was induced by pairing 600 pulses at 1 Hz with depolarization of the post-synaptic neuron to -40 mV (Crozier *et al.*, 2007).

AMPA / NMDA ratios were determined essentially as described (Myme *et al.*,

2003) (Fig. S2). AMPA mediated EPSCs were recorded at -90 mV in ACSF supplemented to (in mM) 4 $CaCl_2$, 4 $MgCl_2$, and 25 D-glucose. Mixed AMPA and NMDA EPSCs were then recorded at +40 mV. A 1 ms time window, set flanking the peak of the AMPA mediated response, was used to determine the AMPA component of the mixed EPSC. The NMDA component of the mixed EPSC was determined using a 1 ms time window set 45 ms following the stimulus artifact, a period at which the AMPA mediated response at -90 mV has decayed to baseline. At each holding potential 4 – 6 responses were recorded at 0.05 Hz, responses showing polysynaptic activity were excluded from analysis. The ratio of AMPA to NMDA amplitude was calculated for HSV-G2CT infected neurons and normalized to the ratio obtained in control neurons.

Following recording, slices were fixed in 4% paraformaldehyde, and stained using Alexa-555 conjugated streptavadin (Molecular Probes) and deep-red Nissl stain (NeuroTrace 640/660, Molecular Probes). All recorded neurons were spiny, and located within layer 4.

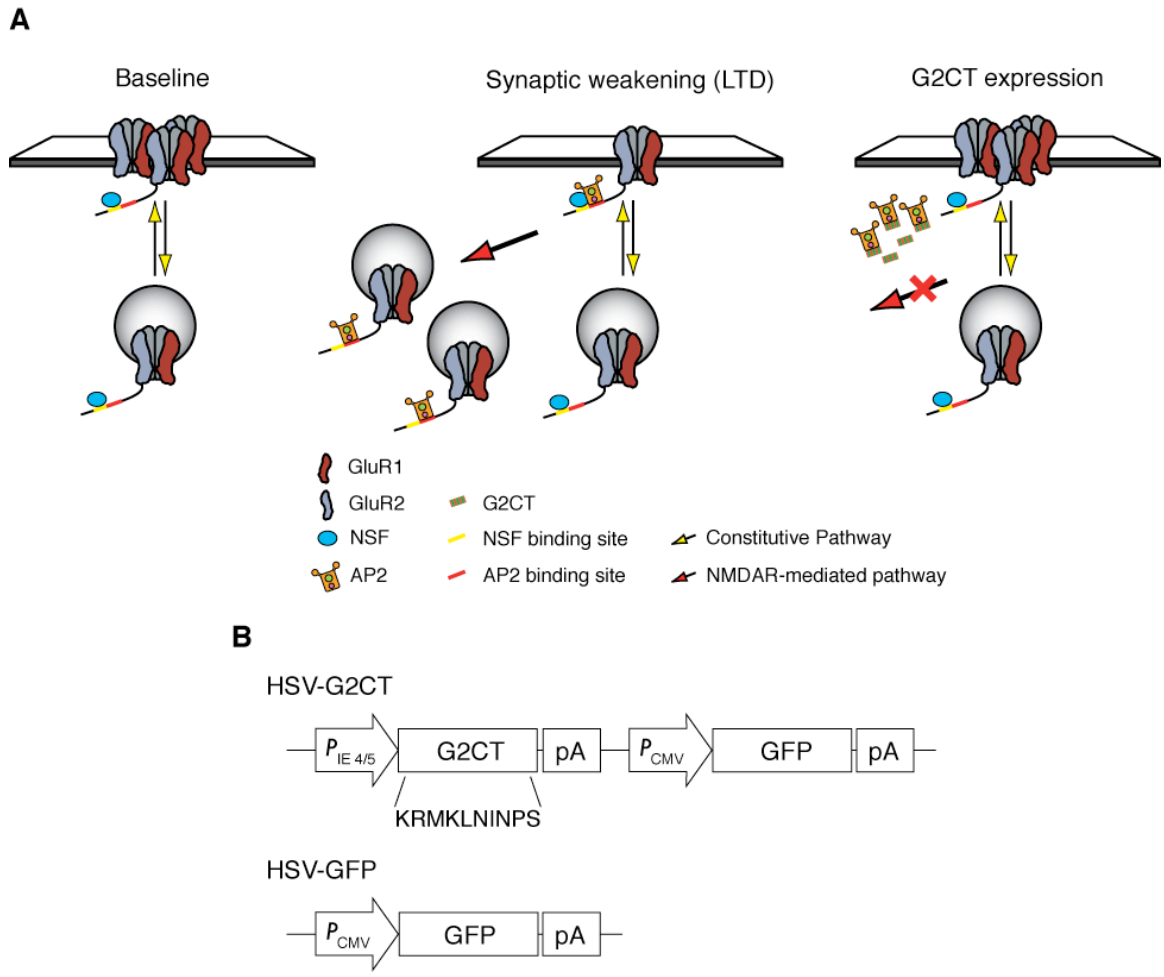


Figure 2.1 – Design of G2CT and HSV expression vector

A. Schematic illustrating mechanism through which G2CT blocks NMDA-stimulated AMPAR endocytosis. Activation of NMDARs leads to association of AP2 with GluR2 C-terminal tail, initiating clathrin-dependent endocytosis of the receptor. In the presence of G2CT, which mimics the AP2 binding site, NMDAR activation leads instead to AP2 binding G2CT, preventing endocytosis of AMPARs. Constitutive cycling of AMPARs requires the binding of NSF to GluR2, which is unaffected by G2CT. B. Schematic diagram of HSV-G2CT and HSV-GFP expression vectors. The G2CT peptide (KRMKLNINPS) is expressed off the first cistron by the native IE4/5 promoter, and the second cistron is a complete CMV-GFP expression cassette.

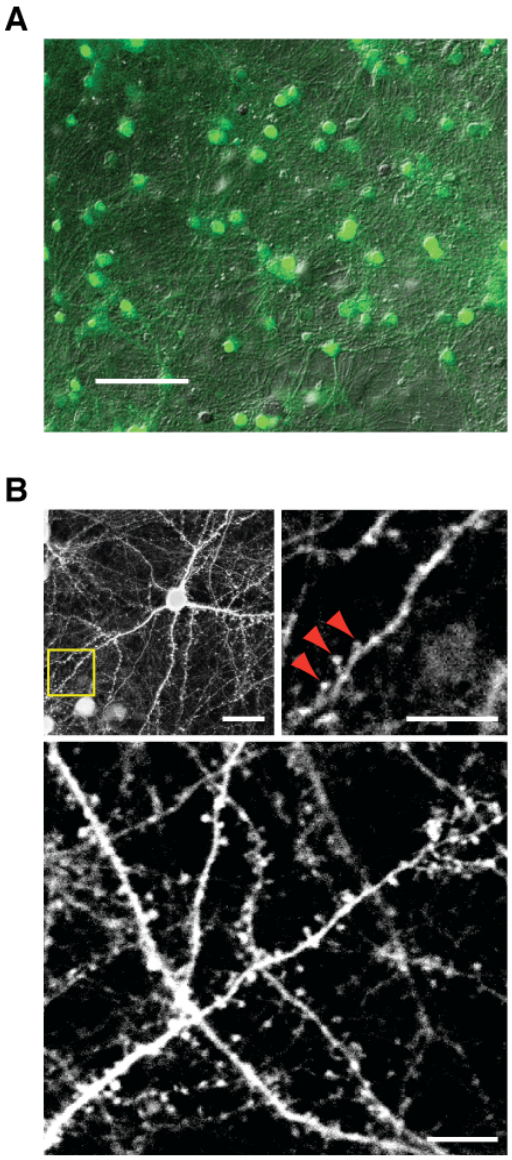


Figure 2.2 – HSV-G2CT expression in cortical cultures

A. HSV-G2CT readily infects cultured visual cortical neurons. The photomicrograph of cultured visual cortical neurons demonstrates that > 90 % of the neurons expressed GFP within 24 hours following incubation of virus (gray-scale DIC image overlayed with the GFP channel in green). Scale Bar 50 µm. B. HSV-G2CT infected neurons exhibit normal cell morphology. *Top left*: Deconvolved maximum-intensity projection (15 µm total using a 1.0 µm Z-step) of an isolated neuron with spiny dendrites. The yellow box indicates region with spines visible on distal dendrites. *Top right*: Close-up, single XY-scan image of the highlighted field in top left panel showing spines located on a distal dendritic segment (red arrowheads). *Bottom*: Single XY-scan image from a different area of the same culture showing a dense field of distal dendritic segments with numerous spines (scale bars: top right 25 µm; top left and bottom 10 µm).

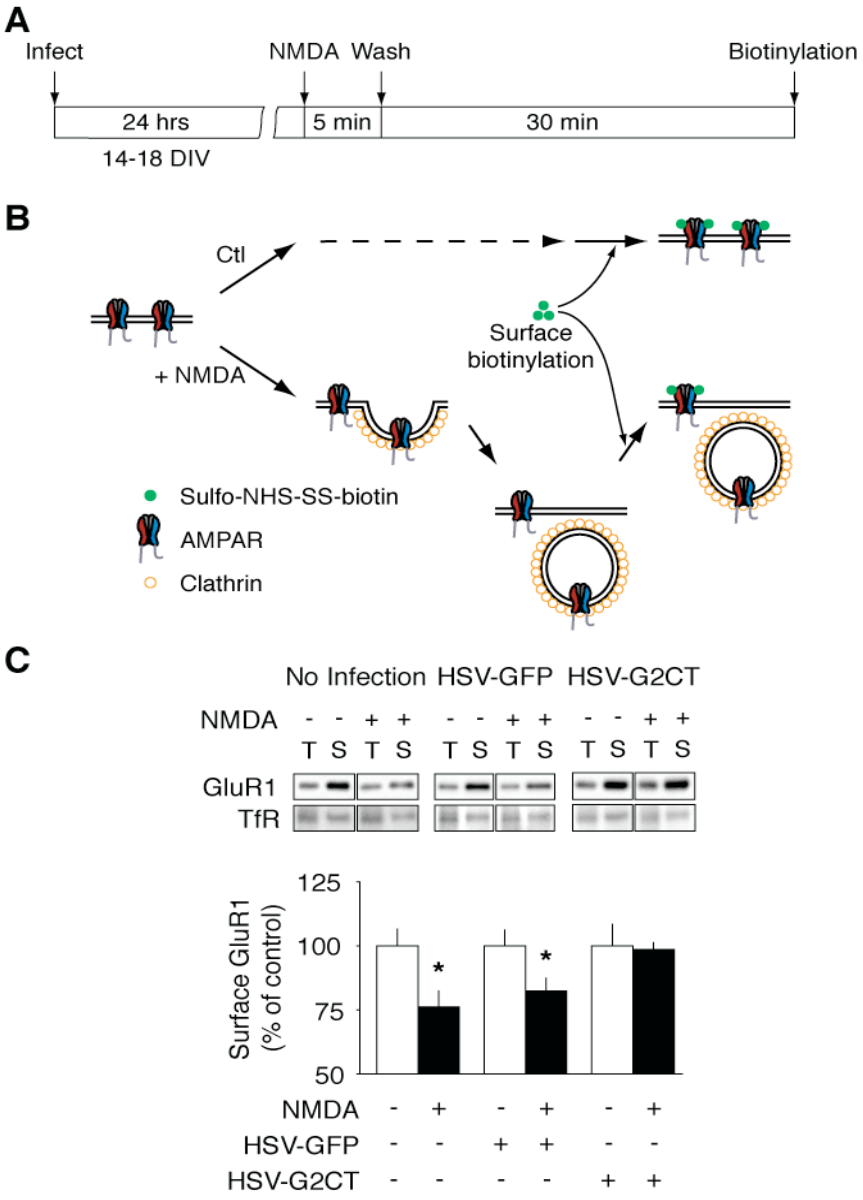


Figure 2.3 – Viral expression of G2CT blocks NMDA-stimulated AMPAR internalization in visual cortical cultures

A. Timecourse for AMPAR internalization experiments. B. Schematic of experimental design. Receptors remaining on the surface after treatment were labeled and visualized on western blots. C. HSV-G2CT infection prevents NMDA-stimulated AMPAR internalization. NMDA treatment produced a significant reduction of surface-expressed GluR1 in non-infected cultures (mean ± SEM of untreated control: 76.2 ± 6.1%; $n = 8$, $p < 0.05$) and HSV-GFP infected cultures (82.4 ± 5.0%; $n = 8$; $p < 0.05$), but not in HSV-G2CT infected cultures (98.6 ± 2.6 %; $n = 8$; $p > 0.5$). Note that surface GluR1 levels under control conditions (no infection $n = 8$, HSV-GFP $n = 12$, HSV-G2CT $n = 6$) were comparable between all groups, indicating that the G2CT peptide does not affect basal AMPAR trafficking (T: total; S: surface).

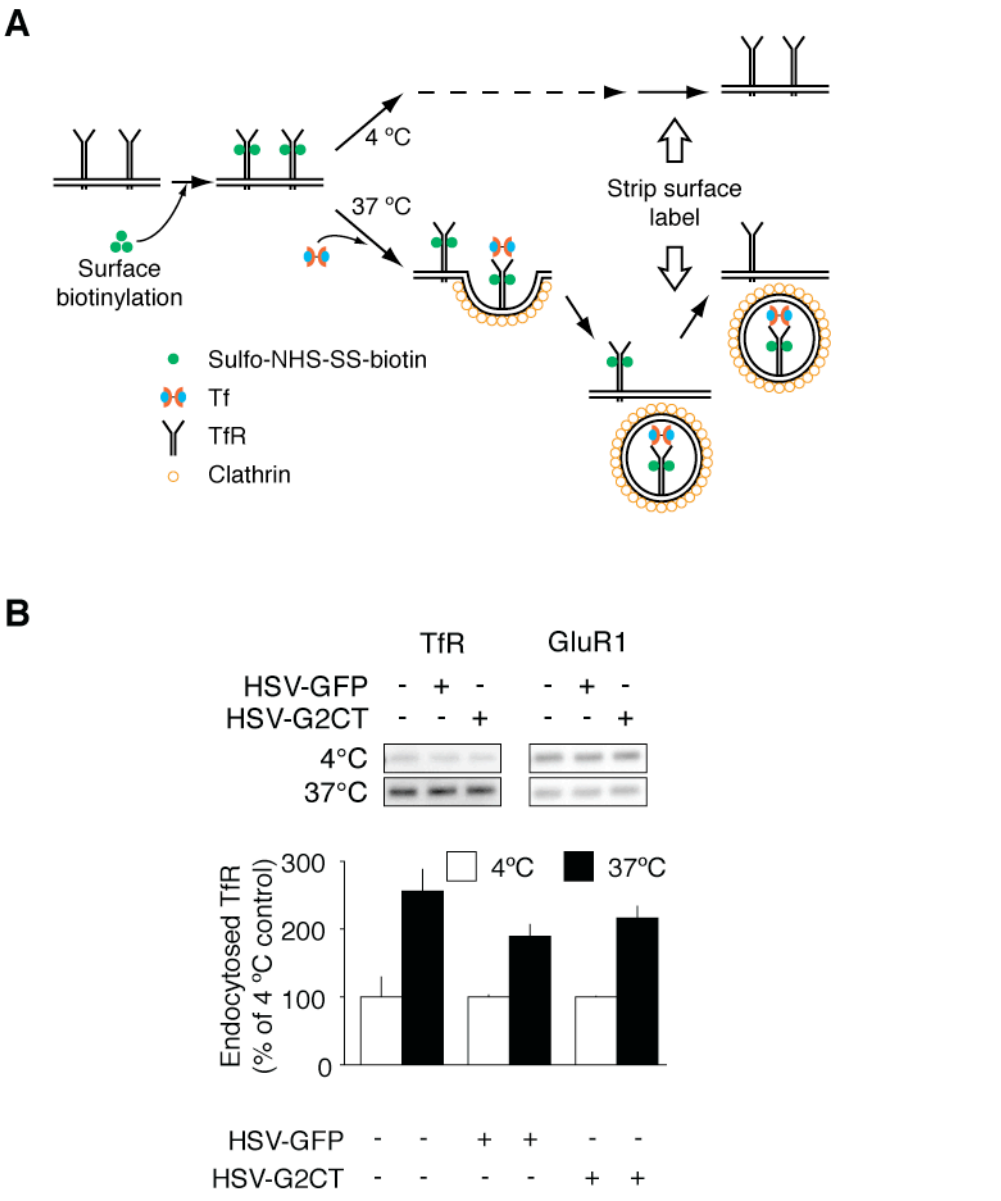


Figure 2.4 – Viral expression of G2CT does not block transferrin receptor endocytosis

A. Schematic of experimental design. Surface transferrin receptors (TfRs) were labeled prior to internalization and visualized on western blots after stripping label from non-internalized receptors. B. Viral expression of G2CT does not affect transferrin receptor (TfR) endocytosis. To rule out a nonspecific effect on AP2 and clathrin-mediated endocytosis, the level of surfaced expressed TfR was measured following transferrin application. Levels of internalized TfR at 37°C were comparable across cultures and non-specific GluR1 internalization was not observed.

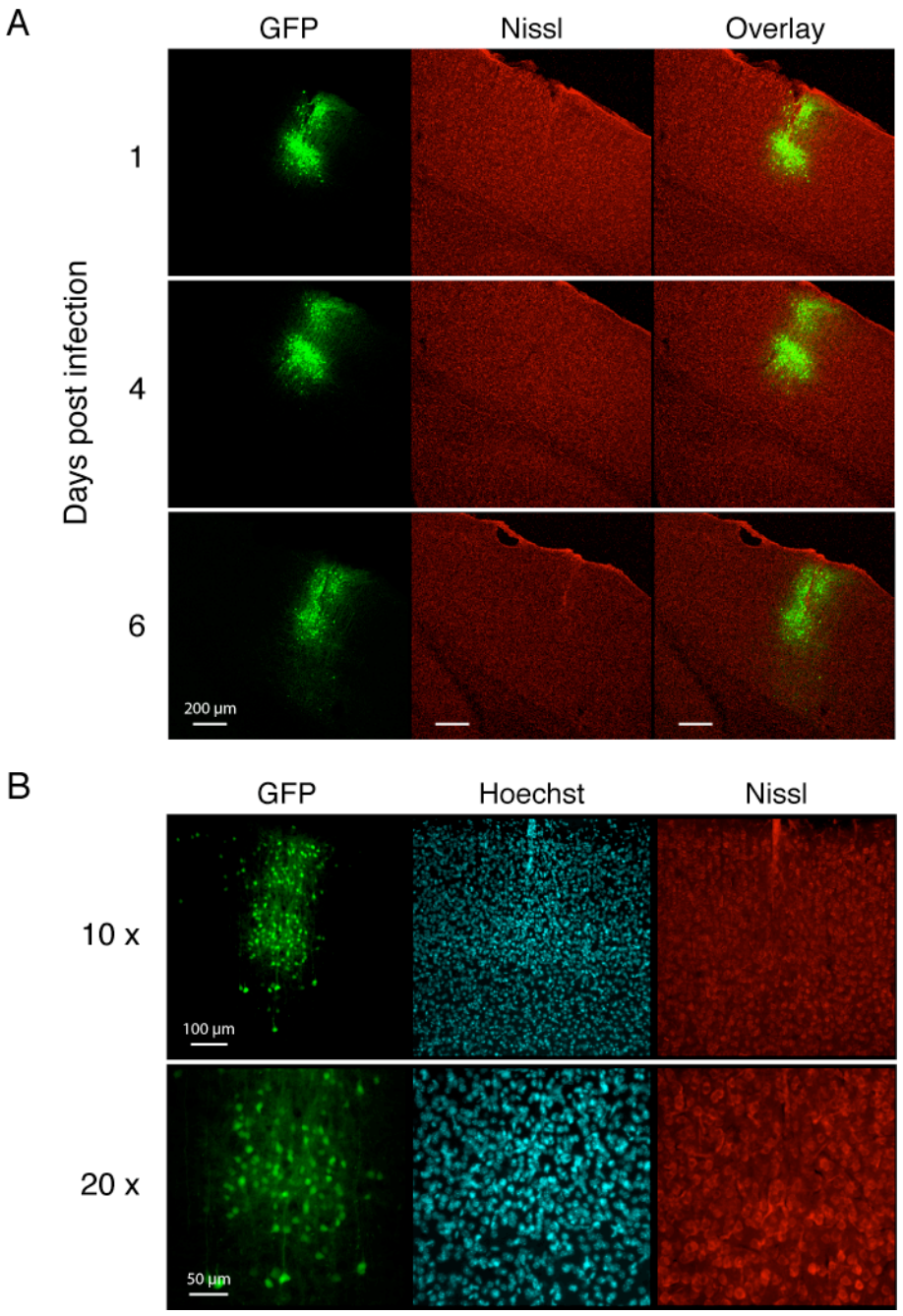
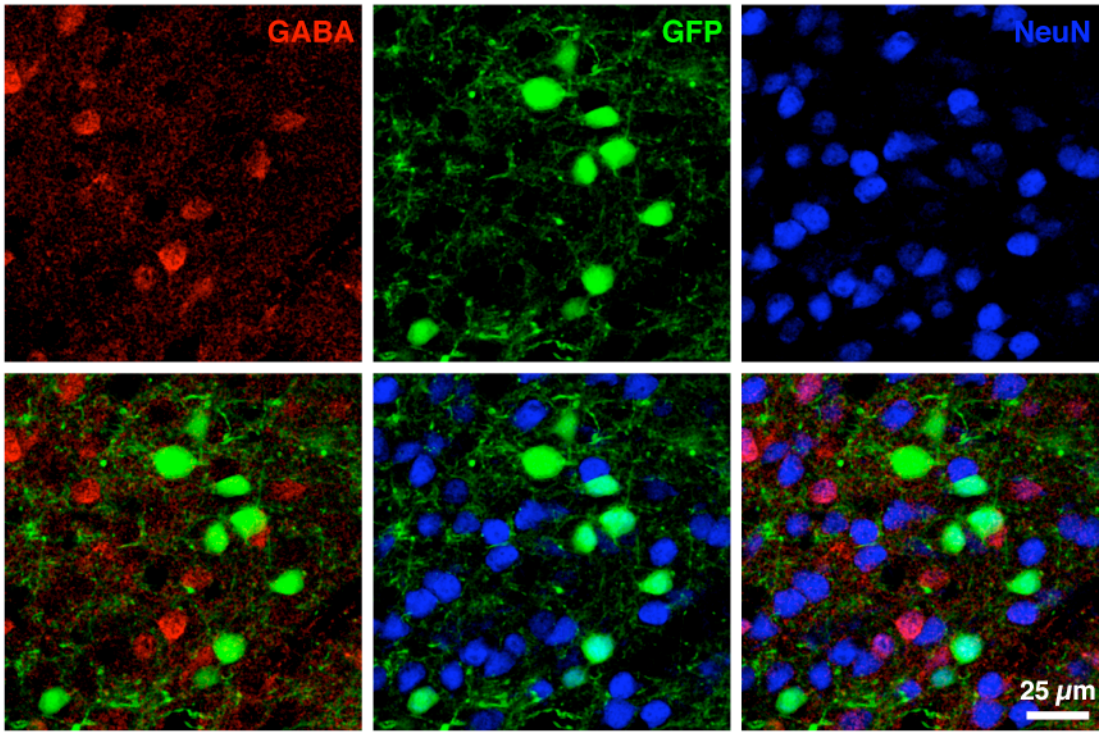


Figure 2.5 – Infection with HSV produces robust and sustained expression *in vivo*

A. GFP expression in neurons following injection of HSV-G2CT targeted to layer 4 of visual cortex is apparent as little as 24 hr post-infection and persists for at least 6 days. Targeted injections to layer 4 frequently produced GFP expression predominantly in layer 4 neurons and their apical processes, although GFP expression was often also observed slightly beyond the boundaries of layer 4. B. Infection of visual cortex with HSV-G2CT did not appear to produce neurotoxicity, as visualized through altered patterns of Hoechst or Nissl staining.

A



B

Identity of infected cells

GFP+ cells in FOV	GFP+ & NeuN+ cells	% GFP+ also NeuN+	GFP+ / GABA+ cells	% GFP also GABA+	# FOV
322	279	**86.7**	3	**0.9**	16

GABA+ cells in FOV	GFP+ & GABA+ cells	% Infected GABA+ cells	# FOV
165	3	**1.8**	16

C

Infection density

NeuN+ cells in FOV	GFP+ cells in FOV	% Infected neurons	# FOV
276	188	**68.1**	12

Figure 2.6 – HSV-G2CT primarily infects excitatory neurons

A. Co-localization of GFP+ (green cells) and GABA+ (red cells) was rarely observed following cortical infusion of HSV-G2CT, whereas GFP+ cells frequently co-localized with NeuN+ cells (blue). These results indicate that HSV infection is restricted primarily to excitatory neurons. B. Quantitative analysis of HSV-G2CT infections in visual cortex. 86.7 % of GFP+ cells were also NeuN+, but only 1.8 % of GABA+ cells were GFP+. C. Examining only the densest area of infection suggests that less than 70 % of neurons within this region are infected.

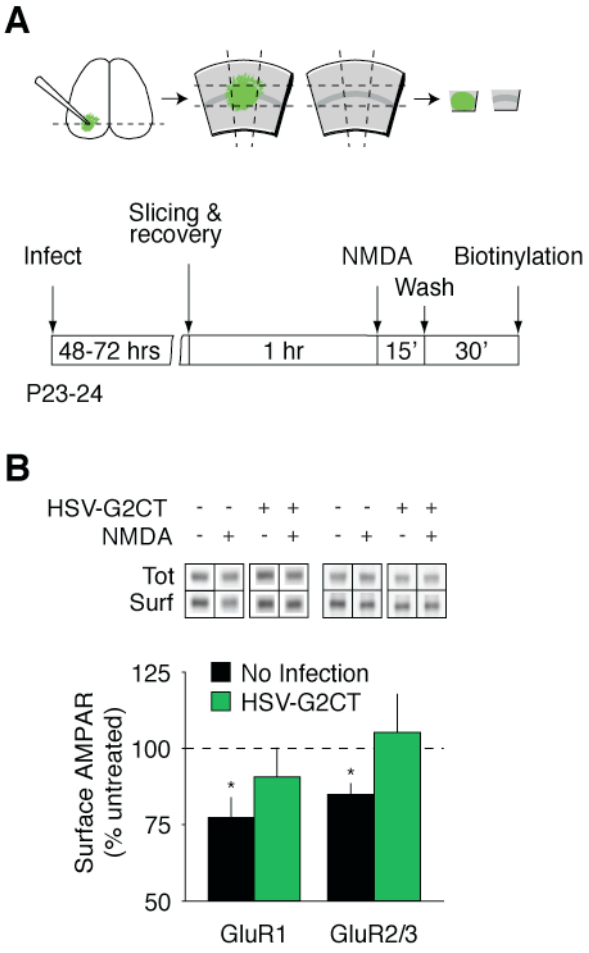


Figure 2.7 – *In vivo* infection with HSV-G2CT blocks NMDA-stimulated AMPAR endocytosis

A. Experimental design for experiments testing NMDA-stimulated internalization in the acute slice preparation. Visual cortical slices were prepared 48-72 hrs after *in vivo* infection and a region in the middle of the cortical thickness, corresponding to layer 4, was microdissected from both the HSV-infected and contralateral (non-infected) visual cortices. Following recovery, slices were treated with NMDA (100 µM; 15 min) and 30 min later surface proteins were biotinylated and immunoblot analysis performed.
B. NMDA treatment produced a significant reduction of surface-expressed AMPARs in non-infected visual cortex (GluR1: 77.3 ± 6.6 % of control, $n = 5$; $p < 0.05$; GluR2/3: 84.7 ± 3.5 % of control, $n = 5$; $p < 0.05$), whereas internalization was blocked in HSV-G2CT infected cortex (GluR1: 91.0 ± 9.4 % of control, $n = 5$; $p > 0.40$; GluR2/3: 105 ± 12.4 % of control, $n = 5$, $p > 0.69$).

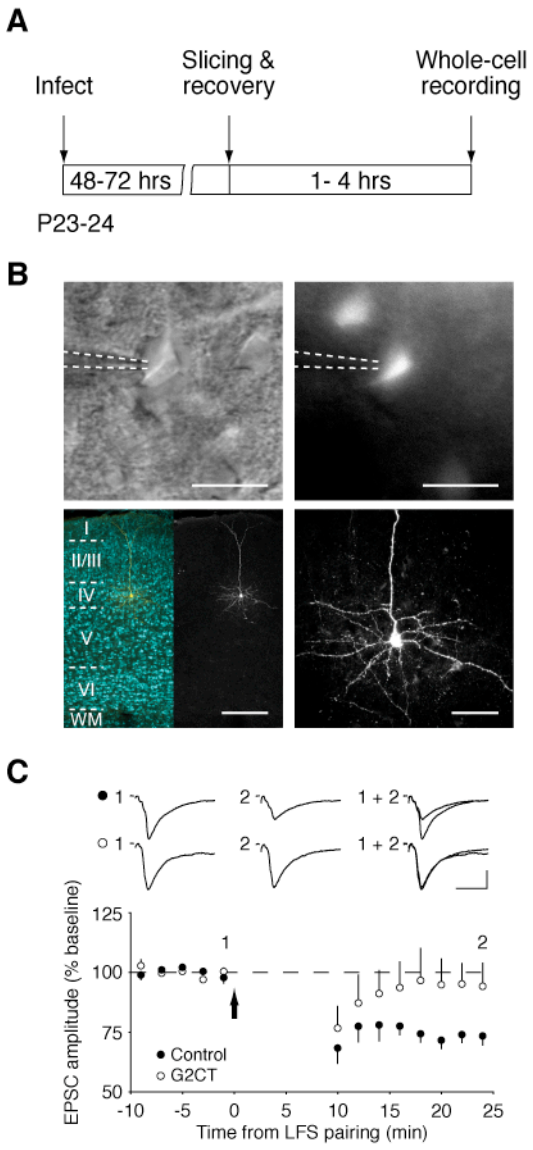


Figure 2.8 – Expression of G2CT blocks pairing-induced LTD in layer 4 of the visual cortex

A. Experimental timeline. B. Recordings were made from infected GFP positive cells in layer 4 of visual cortex. *Top:* IR-DIC (*left*) and GFP (*right*) images showing target cell prior to seal formation with recording pipette outlined in white. *Bottom left:* Nissl stained section indicating laminar borders. A biocytin filled and stained neuron is shown overlying the Nissl stain in yellow, and alone to the right. *Bottom right:* Higher magnification image of cell shown in bottom left. Note prominent apical dendrite and descending basal dendrites, features indicative of a layer 4 star pyramidal neuron. Scale bars: 25, 25, 200, 50 µM for top left to bottom right, respectively. C. Pairing-induced LTD is blocked in neurons infected with HSV-G2CT. Traces are representative EPSCs averaged over 6 consecutive responses recorded at times shown in summary graph below. Scale bars: 10 ms, 100 pA. Lower graph shows the time course of average EPSC

amplitude in control and G2CT expressing cells. A 1 Hz pairing protocol was delivered at time indicated by arrow. LFS pairing resulted in significant LTD in control (71.8 ± 2.2% of baseline, $n = 5$, $p < 0.05$, paired t-test) but not G2CT expressing (96.7 ± 10.1% of baseline, $n = 5$, $p > 0.2$, paired t-test) cells. A significant between group difference in LTD magnitude is also observed ($p < 0.05$, unpaired t-test).

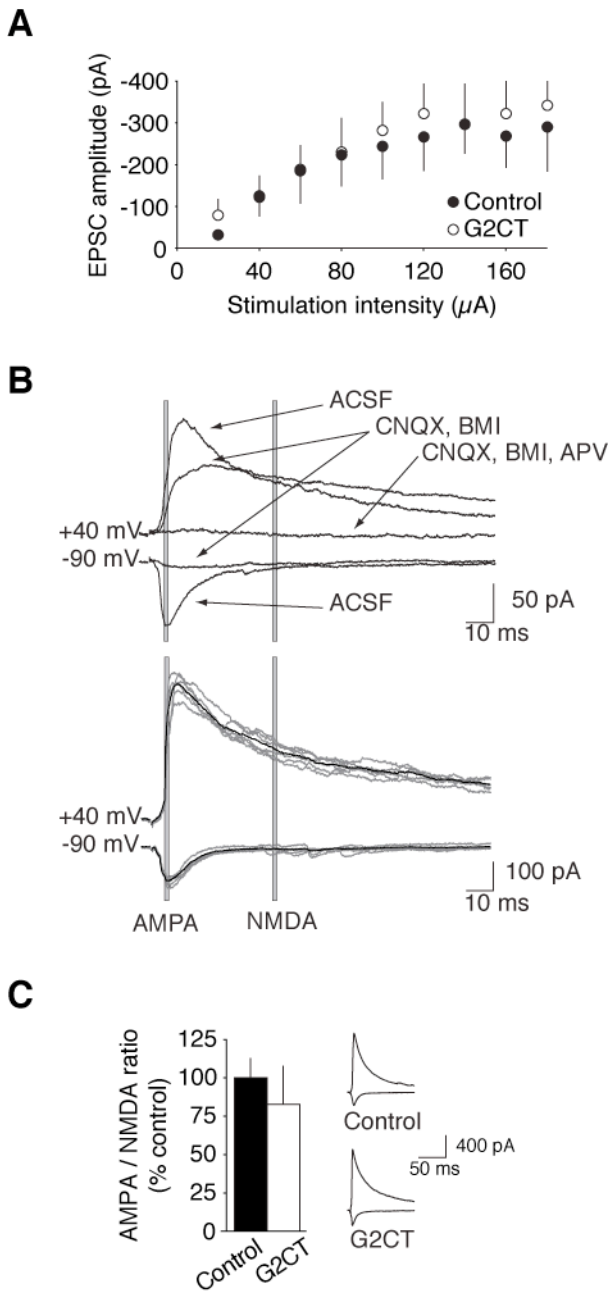


Figure 2.9 – HSV-G2CT does not affect basal synaptic transmission or AMPA / NMDA ratios in acutely prepared slices

A. G2CT expression does not affect basal synaptic transmission from white matter to layer 4 assayed via input-output relationships. B. AMPA / NMDA ratio analysis. *Top:* Pharmacological isolation of AMPA and NMDA mediated EPSCs. Rectangles indicate time windows used below. *Bottom:* Representative examples of AMPA mediated EPSCs recorded at -90 mV or mixed AMPA and NMDA EPSCs recorded at +40 mV. Gray traces are responses to individual stimuli, and the average trace is shown in black. AMPAR contribution to the mixed response was taken during a 1 ms window centered at

the peak of the AMPA-only response recorded at -90 mV. NMDAR contribution was measured during a 1 ms window 45 ms after stimulation. C. AMPA / NMDA ratios in G2CT expressing neurons are comparable to non-infected controls. Traces at right show representative EPSCs recorded at +40 mV (upward going) or -90 mV (downward going). Histogram presents average AMPA / NMDA ratios, normalized to control. No significant difference was observed between groups (control: 100 ± 12.5%, n = 7; G2CT: 82.8 ± 24.5%, n = 6; $p > 0.5$).

Chapter 3

Essential role for AMPA receptor endocytosis in MD-induced loss of visual responsiveness

*Portions of this chapter were published together with Dr. Bong-June Yoon, Dr. Arnold J. Heynen, Dr. Rachael L. Neve, and Dr. Mark F. Bear in Proceedings of the National Academy of Sciences of the United States of America (2009) Vol. **106**, pp. 9860-9865*

3.1 Abstract

Monocular deprivation produces profound changes in the responses elicited in the cortex by visual stimulation. Since the discovery of NMDAR dependent LTD in the hippocampus, this mechanism has been proposed to account for the loss of responsiveness to the deprived eye following MD. Previous work has shown both that MD mimics LTD with respect to AMPAR internalization and that prior MD occludes subsequent LTD in the visual cortex. Using HSV-mediated expression of G2CT, a peptide which blocks AMPAR receptor endocytosis and LTD in layer 4 of visual cortex, we now show that AMPAR endocytosis is required for deprived-eye depression in this layer. The effects of G2CT are specific to MD-induced depression in layer 4 as baseline visual responses and experience-dependent response potentiation were not affected. Additionally, we show using HSV-G2CT that OD plasticity in layer 2/3 of the visual cortex can occur in the absence of plasticity in layer 4, demonstrating that OD plasticity can occur independently across laminae. These data suggest that AMPAR internalization is essential for the loss of synaptic strength in layer 4 of the visual cortex caused by sensory deprivation.

3.2 Introduction

In animals with binocularly overlapping visual fields, the relative strength of eye-specific inputs into the visual cortex is highly susceptible to manipulations of sensory experience. An extensive literature has shown in many species, including monkeys, cats, ferrets, rats and mice, that a period of monocular visual deprivation shifts the balance of eye-specific responses away from the deprived eye and towards domination by the open eye. This classical shift in OD could be achieved through changes to either the numerator or the denominator of the OD ratio: deprived-eye responses could become smaller following deprivation or open-eye responses could become larger.

Since the discovery of OD plasticity over 40 years ago, it has become clear that both processes do in fact occur and contribute to the observed shift in OD. Changes in both deprived and non-deprived eye responses have been reported in chronic single-unit recordings in monocularly deprived kittens (Mioche and Singer, 1989). Furthermore, the anatomical effects of MD in kittens are striking: LGN axons subserving the deprived eye show reductions in cortical arborization, whereas open-eye thalamocortical arbors are extended (Antonini and Stryker, 1993). These changes can be observed on a large scale in the respective shrinkage and expansion of deprived- and open-eye OD columns (Hubel *et al.*, 1977; Shatz and Stryker, 1978).

Given that both deprived-eye depression and open-eye potentiation *can* occur following MD, it remained largely unclear under what circumstances each of these distinct processes becomes engaged by MD. Recent work using chronic recordings in the mouse has allowed disambiguation of MD induced changes in OD ratios by allowing the comparison of absolute changes in the visual response driven by each eye (Frenkel and

Bear, 2004). Brief MD (3d) produces depression of deprived-eye responses without affecting responses driven by the open eye. Extending the period of MD to 7d produces both deprived-eye depression as well as potentiation of open-eye responses.

Since its discovery, homosynaptic LTD has been hypothesized to account for the loss of deprived-eye responses following MD (Bear, 2003) and multiple correlative studies have shown that MD engages molecular mechanisms shared by LTD. In rats, brief MD produces a pattern of AMPAR phosphorylation and a loss of surface-expressed receptors, which mimics that induced through LTD of synaptic transmission (Heynen *et al.*, 2003). Furthermore, 3 days of MD in mice occludes the subsequent induction of LTD in both layer 4 and layer 2/3, suggesting a common saturable expression mechanism shared between these processes (Crozier *et al.*, 2007). As with most other forms of LTD reported in the sensory cortices (Kirkwood and Bear, 1994a; Markram *et al.*, 1997; Feldman *et al.*, 1998), LTD at these synapse in visual cortex requires activation of NMDARs (Rao and Daw, 2004; Crozier *et al.*, 2007). One approach to demonstrate a requirement for LTD in OD plasticity is therefore to block LTD via inhibition of NMDARs. Indeed, blockade (Bear *et al.*, 1990; Daw *et al.*, 1999) or genetic deletion of NMDARs (Roberts *et al.*, 1998) prevents the OD shift after MD. However, NMDAR blockade affects multiple forms of plasticity, including many forms of LTP, and could therefore achieve blockade of OD plasticity by blocking potentiation of the open eye.

In recent years, large advances have been made in the understanding of the molecular mechanisms underlying AMPAR trafficking and endocytosis in response to both NMDAR activation and LTD (discussed in Chapter 2). These results provide an opportunity to directly test whether AMPAR endocytosis, which is the endpoint of LTD

signaling cascades and directly leads to the weakening of synaptic transmission (Malinow and Malenka, 2002), is a requirement for deprived-eye depression following MD. Both NMDA-stimulated endocytosis of AMPARs and LTD depend on the interaction of the clathrin adaptor complex AP2 with the GluR2 C-terminal tail, and can be blocked with a peptide mimicking the AP2 binding site of GluR2 (KRMKLNINPS – termed G2CT) (Lee *et al.*, 2002). When loaded intracellularly through the recording pipette, G2CT blocks LTD at white matter – layer 4 synapses in the visual cortex (Crozier *et al.*, 2007). We therefore reasoned *in vivo* delivery of G2CT would provide a means to directly test the contribution of AMPAR endocytosis and LTD to MD-induced depression of visual responses.

We have previously described in Chapter 2 the generation of a HSV vector capable of driving the expression of G2CT in cortical neurons. We found that HSV-G2CT infection blocked NMDA-stimulated AMPAR endocytosis in both cortical cultures and slices acutely prepared from infected mice. Importantly, HSV-G2CT also blocked LTD in layer 4 without affecting baseline synaptic transmission or neuronal health.

Here we show that expression of G2CT in layer 4 neurons *in vivo* is sufficient to completely prevent depression of deprived-eye responses after brief MD, while leaving intact the mechanisms of experience-dependent synaptic enhancement. We conclude that AMPAR endocytosis plays a critical role in the loss of visual responsiveness after MD.

3.3 Results

3.3.1 Infection with HSV-G2CT does not affect baseline visual responses

Our prior results (Chapter 2) suggested that it would be feasible to use HSV-mediated delivery of G2CT to test the hypothesis that OD plasticity requires AMPAR internalization. Prior to performing these experiments, however, we first ensured that HSV-G2CT infection does not alter the baseline amplitude of VEPs *in vivo*. We targeted layer 4 of visual cortex with HSV-GFP (n = 14) or HSV-G2CT (n = 17) injections and implanted an electrode at the same site through which we could monitor VEPs prior to random assignment of animals to either the "MD" or "SRP" groups (see below) (Figure 3.1A, B). This analysis revealed no significant differences between HSV-GFP and HSV-G2CT infected groups in either the response to contralateral eye stimulation (HSV-GFP 174.7 ± 13.5 μV *vs*. HSV-G2CT 175.1 ± 10.9 μV, t-test: $p > 0.9$) or ipsilateral eye stimulation (HSV-GFP 98.7 ± 8.4 μV *vs*. HSV-G2CT 98.7 ± 7.7 μV, t-test: $p > 0.9$; Figure 3.1C). Furthermore, no systematic difference in the morphology of VEP waveforms was observed between groups, and waveforms were highly similar to those observed in control uninfected mice in prior studies (Sawtell *et al.*, 2003; Frenkel and Bear, 2004). In conjunction with the finding that HSV-G2CT does not affect basal AMPAR surface expression in cortical cultures or basal synaptic transmission in acute slices (Chapter 2), these results indicate that HSV-G2CT does not affect AMPAR trafficking under basal conditions.

3.3.2 G2CT expression blocks MD-induced depression of VEP responses

In a subset of infected animals, we monitored VEPs before and after 3 days of MD (Figure 3.2A, B). Within this subset of mice, as with the larger data set, there was no significant difference in pre-MD baseline VEP amplitudes between HSV-GFP and

HSV-G2CT animals (deprived-eye $p > 0.5$; non-deprived eye $p > 0.8$; unpaired t-test). Previous experiments using this chronic VEP method have shown that closing the contralateral eyelid for 3 days is sufficient to saturate deprived-eye response depression without affecting open-eye responses (Frenkel and Bear, 2004; Frenkel *et al.*, 2006). Consistent with these findings, we observed that 3 days of MD in HSV-GFP infected animals resulted in a significant decrease in deprived-eye responses (baseline 184.9 ± 23.2 *vs*. post-3d MD 119.3 ± 15.3 μV, $p < 0.01$, paired t-test, $n = 7$), whereas non-deprived-eye responses remained unchanged (baseline 109.6 ± 10.3 *vs*. post-3d MD 104.2 ± 15.1 μV, $p > 0.5$, paired t-test; Figure 3.2B, C). These findings demonstrate that viral infection *per se* does not impede normal OD plasticity. However, we found that viral infection with HSV-G2CT completely blocked the deprived-eye depression associated with 3 days of MD (baseline 167.4 ± 14.8 *vs*. post-3d MD 155.6 ± 21.7 μV, $p > 0.5$, paired t-test, $n = 10$; baseline HSV-G2CT vs. HSV-GFP $p > 0.13$, unpaired t-test; Figure 3.2B, C), while once again non-deprived-eye responses remained unchanged (baseline 111 ± 8 vs. post-3d MD 108 ± 9 μV, $p > 0.5$, paired t-test).

3.3.3 G2CT expression blocks the OD shift in layer 4 neurons

The traditional measure of ocular dominance is the ratio of spiking activity evoked by visual stimulation of either eye recorded from individual cortical neurons. A disadvantage of this technique is that unlike with VEP recordings, is exceedingly difficult to apply to chronic preparations, and thus cannot provide clear information on absolute changes in responses. Despite this, the technique of recording single units has the advantage of being able to isolate the evoked responses from individual neurons within well-defined laminar positions.

We therefore implanted multi-channel bundle electrodes in layer 4 at the time of injection with either HSV-GFP or HSV-G2CT. Following recovery from surgery, well isolated units were recorded, most of which showed clear responses to visual stimulation (Figure 3.3). We found that the distribution of OD scores for neurons recorded prior to MD did not differ between mice infected with HSV-GFP or HSV-G2CT (Mann-Whitney U test, $p > 0.45$). After 3 days of MD, the distribution of OD scores in HSV-GFP infected mice displayed a significant shift in OD, away from the deprived eye and towards domination by the open eye ($n = 6$ animals, $n = 18$ neurons pre-MD, 14 post-MD, Mann-Whitney U test, $p = 0.01$, Figure 3.4A). In contrast, MD failed to elicit an OD shift in layer 4 neurons recorded from HSV-G2CT infected mice ($n = 3$ animals, $n = 10$ neurons pre-MD, 14 post-MD, Mann-Whitney U test, $p > 0.8$; Figure 3.4B). These results confirm the observations made using VEP recordings and demonstrate that OD plasticity in individual layer 4 neurons requires AMPAR endocytosis.

3.3.4 OD plasticity in layer 4 is not required for an OD shift in layer 2/3

The most parsimonious explanation for our results is that expression of G2CT in layer 4 neurons prevents deprivation-induced depression of synapses impinging on the dendrites of these cells. It is possible however that the effects of HSV-G2CT infection on OD plasticity are indirect, reflecting a disruption of feedback from layer 2/3, particularly since the infections targeting layer 4 occasionally spread to layer 2/3 (Figure 2.5). Arguing against this possibility is the fact that OD shifts in layer 4 can occur independently of OD shifts in layer 2/3 (Liu *et al.*, 2008). This study found that treatment with the CB1 antagonist AM251 prevented OD plasticity in layer 2/3, whereas the OD shift in layer 4 occurred normally.

We therefore attempted to address whether the converse is also true: that OD plasticity in layer 2/3 is independent of plasticity in layer 4. Additionally these experiments served to test the possibility that G2CT blocks OD plasticity in layer 4 via an indirect disruption of plasticity in supragranular layers. Because the VEP method is not sensitive to changes in layer 2/3 (discussed below), we used multi-channel bundle electrode recordings of units in layer 2/3 before and after 3 days of MD in animals with HSV-G2CT infections targeted to layer 4. The results clearly show an OD shift after MD in layer 2/3 neurons in animals infected with HSV-G2CT ($n = 5$ animals, $n = 15$ neurons pre-MD, 10 post-MD, Mann-Whitney U test, $p < 0.05$; Figure 3.4C), in contrast to the blockade of OD plasticity observed in layer 4 recordings (Figure 3.4B). Thus, OD plasticity can occur independently in layers 4 and 2/3, and the effects of layer 4 infections with HSV-G2CT appear to be largely restricted to layer 4 neurons.

3.3.5 G2CT expression does not affect experience-dependent synaptic enhancement

OD plasticity requires activation of NMDARs (Bear *et al.*, 1990). Although our *in vitro* experiments revealed no defect in NMDAR-mediated synaptic transmission following infection with HSV-G2CT (Chapter 2), the possibility remained that other plasticity mechanisms triggered by NMDAR activation might be non-specifically disrupted by G2CT and thereby account for the observed absence of deprived-eye depression in HSV-G2CT infected animals. Therefore in a final series of experiments, we tested whether G2CT infection affects a newly characterized form of experience-dependent synaptic strengthening in visual cortex, termed stimulus-selective response potentiation (Frenkel *et al.*, 2006). SRP is induced by repeatedly presenting awake mice with high-contrast grating stimuli of a particular orientation. Over the course of 3-4 days,

the visual responses evoked by this grating potentiate relative to responses evoked by gratings of other orientations. SRP requires activation of NMDARs in layer 4 of visual cortex, and is believed to be mediated by delivery of AMPARs to activated thalamocortical synapses in a manner similar to LTP. SRP is therefore a powerful assay to determine the selectivity of G2CT in disrupting visual cortical plasticity.

Three groups of animals (non-infected, n = 10; HSV-GFP, n = 7; and HSV-G2CT, n = 7) were implanted with VEP electrodes following viral injection. VEPs were recorded daily in response to monocular visual stimulation with a grating stimulus of constant orientation delivered independently to each eye (Figure 3.5A). We found that in all groups, repeated presentation of a grating stimulus resulted in the potentiation of VEPs to that stimulus during subsequent recording sessions (Figure 3.5B-D), approaching saturation after three to four sessions. In each group the response evoked by the trained stimulus on day 3 was significantly larger than on day 0 ($p < 0.01$), and was also larger that evoked by a novel stimulus of orthogonal orientation ($p < 0.05$) (Table 3.1). Additionally, no significant between group differences in SRP expression were observed for either eye (repeated measures ANOVA, $p > 0.8$). These results further confirm that the blockade of OD plasticity with the G2CT peptide is unlikely to be the result of a non-specific interruption of all experience-dependent modifications.

3.4 Discussion

The results reported here address a longstanding hypothesis describing the mechanisms by which altered sensory experience produces a loss of physiological response. Although it has previously been shown that MD can cause internalization of postsynaptic AMPARs and LTD (Heynen *et al.*, 2003; Crozier *et al.*, 2007), the relative

contribution of this change to the OD shift was unknown. While multiple mechanisms may participate in the modification of cortex by sensory experience (discussed in Chapters 1 and 4, see, *e.g.,* (Maffei *et al.*, 2006; Crozier *et al.*, 2007; Gandhi *et al.*, 2008; Yazaki-Sugiyama *et al.*, 2009)) the present results suggest that NMDAR-dependent AMPAR internalization is essential for the plasticity of binocular connections in layer 4.

Layer 4 receives the bulk of the geniculocortical input that brings information from the retina into the visual cortex (Antonini *et al.*, 1999). We have used VEP recordings as the primary means to assay visual responsiveness in layer 4. Phase reversal of a sinusoidal grating stimulus at low frequency (0.5 – 1 Hz) drives VEPs, which display maximum amplitude when recording electrodes are placed in layer 4 (Porciatti *et al.*, 1999; Sawtell *et al.*, 2003). The robust VEP measured in this layer primarily reflects summed synaptic currents flowing into radially oriented dendrites (Porciatti *et al.*, 1999; Logothetis, 2003). Current source density (CSD) analysis of VEP responses reveals an initial early sink in layer 4, corresponding to direct activation by thalamocortical afferents (Sawtell *et al.*, 2003). Furthermore, experience-dependent changes in the VEP reflect, in large part, the modification of thalamocortical synapses (Sawtell *et al.*, 2003; Frenkel *et al.*, 2006; Liu *et al.*, 2008).

Relative to other measures of cortical activity (*e.g.* single unit recordings, optical imaging, or IEG expression), VEP recordings offer several advantages. First, because VEPs reflect population responses averaged from a large pool of neurons, they are less susceptible to the sampling biases common to single unit recordings. Neurons with large cell bodies are preferentially recorded, thereby biasing samples towards neurons in the supra- and infragranular layers (Towe and Harding, 1970; Logothetis, 2003). The

population nature of the VEP response also makes these recordings less susceptible to small movements of the electrode, leading to recordings that are highly stable over time (Frenkel and Bear, 2004). Achieving similar stability is exceedingly difficult with single unit recordings, where small movements can greatly affect the ability to repeatedly record from an individual neuron (Mioche and Singer, 1989). By facilitating chronic recordings, VEPs confer the additional advantage of allowing absolute measures of visual responsiveness (Sawtell *et al.*, 2003), an attribute used with great success to resolve the bidirectional nature of OD plasticity in juvenile mice (Frenkel and Bear, 2004).

In addition to these advantages, as a population measure VEP recordings also have limitations. The precise origin of the response is difficult to discern as many distinct classes of neurons likely contribute. Furthermore, the observed response at any given cortical depth reflects not only activity at that site, but also at other locations distributed across cortical depth (Mitzdorf, 1985). CSD analysis localizes the initial strongly negative component of the VEP to a sink in layer 4, presumably in response to thalamocortical activation (Sawtell *et al.*, 2003), demonstrating that VEPs can readily resolve activity in this layer. However, VEPs appear less suitable for examining responses in supragranular layers. Current sinks are difficult to resolve in layer 2/3, likely due to the longer latency of these events, making them more susceptible to the canceling effects of simultaneously occurring currents in other layers (Liu *et al.*, 2008).

For these reasons, we supplemented our experiments using VEPs with single unit recordings in layer 4, which allowed us to confirm the blockade of OD plasticity by G2CT in individual neurons. Although absolute measures of eye-specific responses are not possible unless a single neuron can be demonstrably held across days (a nearly

impossible task), we were able to assay the relative responsiveness of neurons to each eye and determine OD. In addition to serving a confirmatory role for the effects of G2CT on OD plasticity in layer 4, this technique allowed us to assess the effects of MD in layer 2/3 and demonstrate that an OD shift in layer 4 neurons is not required for an OD shift in layer 2/3 neurons.

As discussed previously, it is clear from prior studies that the mechanisms of LTD and OD plasticity vary across cortical laminae (Daw *et al.*, 2004; Crozier *et al.*, 2007; Liu *et al.*, 2008). Our decision to focus on the contribution of LTD mechanisms in layer 4 to mouse OD plasticity was motivated by the finding that the initial OD shift apparent in layer 4 VEPs is entirely the product of a weakened response to the deprived eye (Frenkel and Bear, 2004). Because VEPs allow the clear separation of effects on both the deprived and non-deprived pathways, they are well suited to investigating the specific effect of G2CT on deprived-eye depression. Additionally, unlike cats (Trachtenberg *et al.*, 2000), the OD shift in mice occurs simultaneously in layer 4 and 2/3 (Liu *et al.*, 2008) suggesting that the earliest response to MD may include weakening of thalamocortical transmission to layer 4. Lastly, the occlusion of LTD at white matter – layer 4 synapses by prior MD suggests that LTD is a potentially important component of OD plasticity in this layer.

Given that the focus of this study was the contribution of LTD via AMPAR endocytosis to OD plasticity in layer 4, our experimental design was influenced by two previous findings. First, LTD in layer 4 but not layer 2/3 is blocked by intracellular loading with the G2CT peptide (Crozier *et al.*, 2007), and second, VEPs are a useful measure of plasticity in layer 4 but not layer 2/3 (Liu *et al.*, 2008). We discovered, using

both VEPs and unit recordings, that the OD shift in layer 4 is prevented by expression of the G2CT peptide in layer 4 neurons.

Although we cannot rule out the possibility that chronic expression of G2CT *in vivo* produces an adaptive neuronal response that indirectly interferes with OD plasticity via unknown mechanism(s), several lines of evidence argue against this possibility. The G2CT peptide designed by Lee *et al*. (2002) and used in our study is the minimal sequence needed to block GluR2-AP2 interactions. These authors found a selective effect of G2CT on NMDAR-regulated AMPAR expression and LTD in CA1, without affecting baseline synaptic transmission. Previously, we showed similar selectivity for HSV-expressed G2CT in the visual cortex (Chapter 2). We have now demonstrated that expression of G2CT does not produce an increase in the magnitude of baseline VEPs measured *in vivo*, extending the previous conclusion that constitutive endocytosis of AMPARs is not affected by the G2CT peptide. Yet, neurons expressing the peptide fail to respond to MD with a loss of visual responsiveness. Additionally, if an adaptive response to G2CT does occur, it does not affect all forms of synaptic plasticity equally, as evidenced by the normal expression of SRP.

One of the more interesting aspects of our results is the finding that OD plasticity in layer 2/3 can occur in the absence of ongoing plasticity in layer 4. The question of separable laminar specific responses to OD was suggested by experiments in kittens showing that MD-induced changes in OD occur first in extragranular layers, followed later by layer 4 (Trachtenberg *et al.*, 2000). This response to OD appears to differ across species, as 24 hours of MD in mice produces equivalent shifts in OD distributions in both layers 2/3 and 4 (Liu *et al.*, 2008). Notably this rapid shift in layer 4 can proceed under

conditions in which the shift in layer 2/3 is blocked (Liu *et al.*, 2008). It appears likely that the source of this laminar independence is the direct thalamocortical innervation of both layers that is present in the mouse (Figure 1.1 and (Antonini *et al.*, 1999)). These direct afferents would presumably carry the depressing stimulus – decorrelated LGN activity (Linden *et al.*, 2009) – independently to both cortical layers where LTD-like mechanisms are engaged: NMDA-dependent AMPAR internalization in layer 4 and CB1 mediated synaptic depression in layer 2/3. Whereas the previous finding suggested that functional deprived-eye input could bypass layer 4 and drive unchanged responses in layer 2/3 (Liu *et al.*, 2008), the current results coupled with the anatomy indicate that the depressing stimulus can also bypass layer 4 to drive response depression in layer 2/3.

Importantly, it is difficult to argue that the significant OD shift observed in layer 2/3 from mice with HSV-G2CT infections targeted to layer 4 is the result of recording from layer 2/3 neurons that receive input predominantly from cells in layer 4 which were not actually infected. Electrodes were implanted in layer 2/3 directly above the site of infection, thus, given the columnar architecture of the cortex the recorded neurons are likely to receive inputs from infected cells. Additionally, VEP recordings in layer 4 demonstrate that OD plasticity is blocked in a large population of neurons, making the existence of a functionally significant pool of uninfected neurons exhibiting normal plasticity and driving a corresponding normal OD shift in layer 2/3 highly unlikely.

We targeted our infections to layer 4 of the visual cortex, and did not directly address whether HSV-G2CT infection of layer 2/3 prevents OD plasticity in those cells. As noted above, the goal of this study was to determine the requirement for LTD mediated through AMPAR endocytosis to OD plasticity in layer 4. The G2CT peptide

used in this study blocks LTD in layer 4 neurons (Figure 2.8), but has no effect on LTD in layer 2/3 (Crozier *et al.*, 2007). It is therefore highly unlikely that infection with HSV-G2CT would impact expression of LTD in layer 2/3. Furthermore our experiments were designed to utilize the advantages inherent to VEP recordings, which are ideally suited to isolating the effects of MD on the deprived-eye response from the open-eye response (Frenkel and Bear, 2004). As VEPs are unsuited to measuring OD in supragranular layers (Liu *et al.*, 2008), we decided to focus our analysis to the effects of expressing G2CT in layer 4 neurons.

The current findings bridge multiple levels of analysis to establish that one molecular mechanism for LTD is functionally significant in the brain. Although it has been known for almost 2 decades that NMDARs are critical for visual cortical plasticity (Bear and Coleman, 1990; Bear *et al.*, 1990), their precise contribution had not been pinpointed. The G2CT peptide is far more selective than NMDAR blockade or genetic deletion, but the effect on OD plasticity is just as robust. Thus, our findings provide strong support for the "LTD hypothesis" of visual cortical plasticity—that weak activation of NMDARs by poorly correlated inputs from the deprived eye is a key trigger for the loss of visual responsiveness following MD (Bear *et al.*, 1987).

3.5 Methods

Subjects

Male C57Bl/6 mice were used for all experiments. Animals were group housed and kept on a 12hr light/dark cycle. All animals were treated in accordance with National Institutes of Health and Massachusetts Institute of Technology guidelines.

HSV vector construction and packaging

HSV-GFP and HSV-G2CT were generated from the p1005+ HSV-amplicon bicistronic plasmid as described previously (Chapter 2). Briefly, a 50 bp fragment encoding the G2CT peptide (KRMKLNINPS) was cloned into the first cistron, downstream of the IE4/5 promoter. Viral packaging was carried out as previously described (Neve *et al.*, 2005).

Surgery

Procedures for viral delivery, electrode implantation, monocular deprivation, and SRP induction were performed essentially as described (Frenkel and Bear, 2004; Frenkel *et al.*, 2006). Mice were anesthetized with 50 mg/kg ketamine and 10 mg/kg xylazine i.p. and a local anesthetic, 0.1% lidocaine, was injected under the scalp. A head post was attached to the skull slightly anterior to bregma with cynoacrylate glue and a reference electrode was implanted in prefrontal cortex. A small craniotomy (<1 mm) was made over binocular visual cortex (3 mm lateral to lambda), and a glass micropipette attached to a syringe pump (WPI, Inc) was inserted to a depth of 450 mm. To protect the cortex during the infusion of virus, the dural surface was then covered by a thin layer of NeuroSeal (NeuroNexus Technologies, MI) or low-melt agarose (1.5% in ACSF). A small volume (~1 μL) of HSV-GFP or HSV-G2CT was injected at a rate of 0.1 ml/min, after which the injection pipette was held in place for approximately 5 minutes before

being withdrawn.

For VEP recordings, following viral delivery a tungsten microelectrode (FHC, Bowdoinham, ME) was implanted at the site of infection, 450 μm below the dural surface, and secured in place with cyanoacrylate adhesive. Following implantation, the entire exposure was covered with dental cement. Animals were monitored postoperatively for signs of infection or discomfort, and were allowed at least a 24 hour recovery before habituation to the restraint apparatus.

For single unit recordings, a custom-made multichannel bundle consisting of eight tungsten microwires (California Fine Wire, Grover Beach, CA) was implanted. To improve bundle implantation and to reduce the splaying of wires while lowering, the bundle was dipped in a 40 % sucrose solution then allowed to dry prior to implantation. This treatment greatly improved the efficiency of bundle electrode implants.

Monocular deprivation

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

Visual stimuli

Stimuli consisted of full-field sine-wave gratings of 100% contrast, square-reversing at 1Hz, and presented at 0.05 cycles/degree. Stimuli were generated either by a VSG2/2 card (VEP experiments) (Cambridge Research System, Cheshire, UK) or Psychophysics Toolbox extensions (Brainard, 1997; Pelli, 1997) running in custom MATLAB software (single unit experiments) (The Mathworks, Natick MA) and presented on a CRT computer monitor suitably linearized by γ correction. Visual responses were elicited by either horizontal or vertical oriented gratings. Orientation of stimuli during the first recording was randomized, but to avoid the confound of SRP in MD experiments, the stimuli presented to the same animal before and after deprivation were always orthogonal to each other (Frenkel *et al.*, 2006). The display was positioned 20 cm in front of the mouse and centered at the vertical meridian, occupying 92º x 66º of the visual field. Mean luminance was 27 cd/m^2.

In vivo Electrophysiology

All recordings were conducted in awake mice. The animals were alert and head-restrained during recording. Following postoperative recovery, the animals were habituated to the restraint apparatus for 30-60 min over 1-2 days. For recording sessions visual stimuli were presented to left and right eyes randomly. A total of 200 to 300 stimuli were presented per each condition. VEP amplitude was routinely quantified by measuring peak negativity to peak positivity of the response amplitude. For SRP experiments 200 - 400 stimuli were randomly presented daily to each eye (Frenkel *et al.*, 2006). At the end of experimentation, animals were euthanized and brains removed for histological analysis.

For single unit recordings, spiking activity was evoked in awake mice as above

and recorded using commercially available hardware and software (Plexon, Inc., Dallas TX). Output from each recording electrode was split, directed to pre-amplifiers, bandpassed filtered for spikes (300-3000Hz) and for local field potentials (1-300Hz), and sent to a PC running the data acquisition software. For recorded spikes, offline discrimination of single unit activity was based on waveform shape while multiunit activity was excluded. Spike trains were smoothed by convolution with a Gaussian kernel and spontaneous activity (recorded during viewing of a blank screen) was subtracted. The maximum response to stimulation of each eye (IE: ipsilateral eye; CE: contralateral eye) above spontaneous firing rate was used to calculate the OD score, defined as (IE – CE)/(IE + CE) (Rittenhouse *et al.*, 1999; Liu *et al.*, 2008).

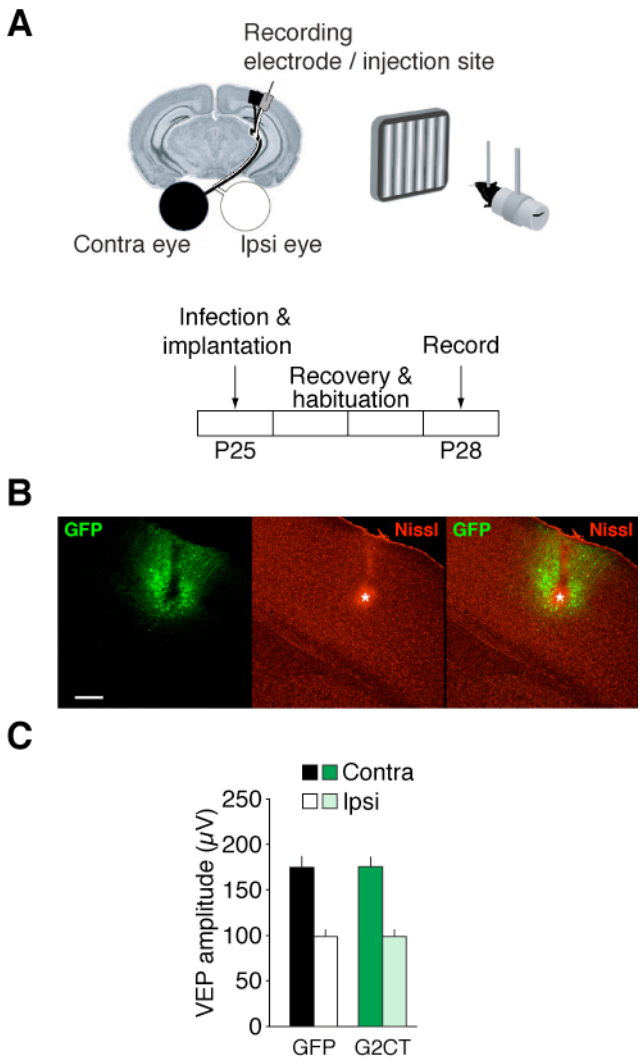


Figure 3.1 – HSV-G2CT does not affect baseline visual responses

A. Experimental design for viral infection and VEP recordings. Viral infusion and implantation of the recording electrode are performed in the binocular visual cortex, contralateral to the eye that will undergo subsequent MD. For recording, awake animals are head restrained in front of a video monitor, and VEPs are evoked through phase-reversing sinusoidal grating stimuli. B. Histological example verifying placement of recording electrode tip (electrolytic lesion indicated with asterisk) within the HSV infected cortical area. Scale bar: 200 µm. C. HSV-G2CT does not alter baseline VEP amplitudes. Animals were infected with HSV-GFP (n = 14) or HSV-G2CT (n = 17) and baseline VEPs were measured. No significant differences were observed between groups (contralateral eye response, HSV-GFP 174.7 ± 13.5 µV *vs*. HSV-G2CT 175.1 ± 10.9 µV, t-test: $p > 0.9$; ipsilateral eye response, HSV-GFP 98.7 ± 8.4 µV *vs*. HSV-G2CT 98.7 ± 7.7 µV, t-test: $p > 0.9$).

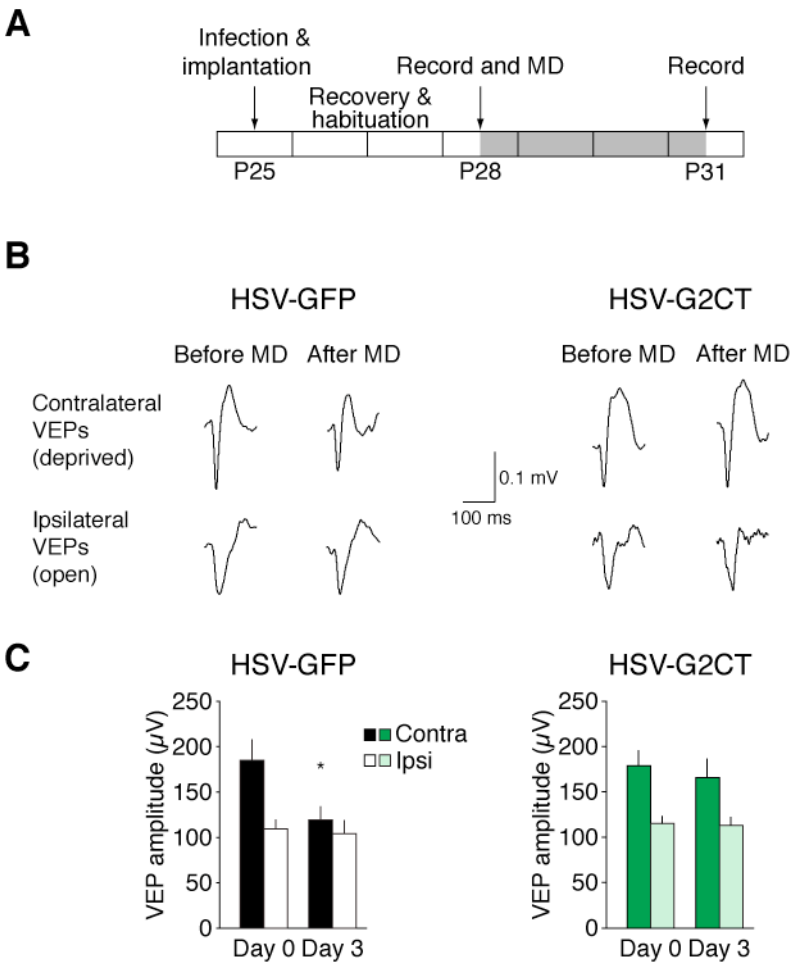


Figure 3.2 – HSV-G2CT blocks MD induced deprived-eye depression

A. Experimental timeline for monocular deprivation and VEP recordings. B. Example VEP responses recorded from HSV-GFP and HSV-G2CT infected mice both before and following 3 days of MD. VEPs evoked through the contralateral (deprived) eye decrease in amplitude following MD in HSV-GFP animals, but not in animals infected with HSV-G2CT. Ipsilateral (open) eye VEPs are unaffected by 3d MD. C. HSV-G2CT infection blocks MD-induced synaptic depression. A significant decrease in contralateral eye VEP amplitude is observed following 3d of MD in control (HSV-GFP) animals (baseline 184.9 ± 23.2 vs. post-3d MD 119.3 ± 15.3 μV, $p < 0.01$), whereas this decrease is blocked in animals infected with HSV-G2CT (baseline 168 ± 15 μV vs. post-3d MD 156 ± 21.7 μV, $p > 0.5$).

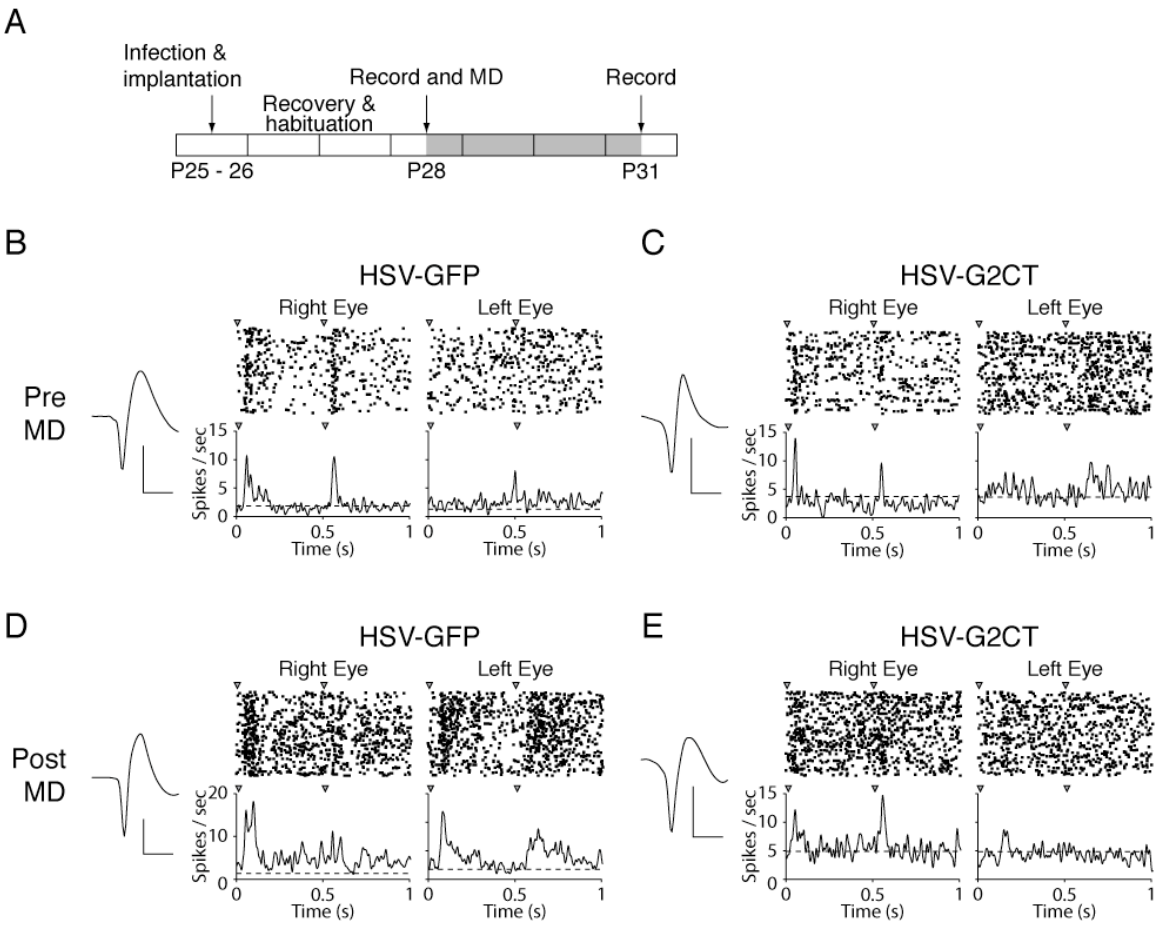


Figure 3.3 – Visually driven responses in single units recorded from HSV-GFP and HSV-G2CT infected mice

A. Experimental design for single unit recordings. B-E. Responses to visual stimulation for representative individual neurons recorded either before (B, C) or following MD (D, E) from animals infected with HSV-GFP (B, D) or HSV-G2CT (C, E). Raster plots and PSTHs are shown for each neuron in response to stimuli of either the right (contralateral) or left (ipsilateral) eye. Phase reversal of the grating stimulus occurred every 0.5 sec and is indicated by triangles. Dashed line on PSTH plots indicates the average spontaneous firing rate recorded during viewing of a blank screen. Scale bars for spike waveforms: 500 μs and 25, 50, 100, 50 μV for B-E, respectively.

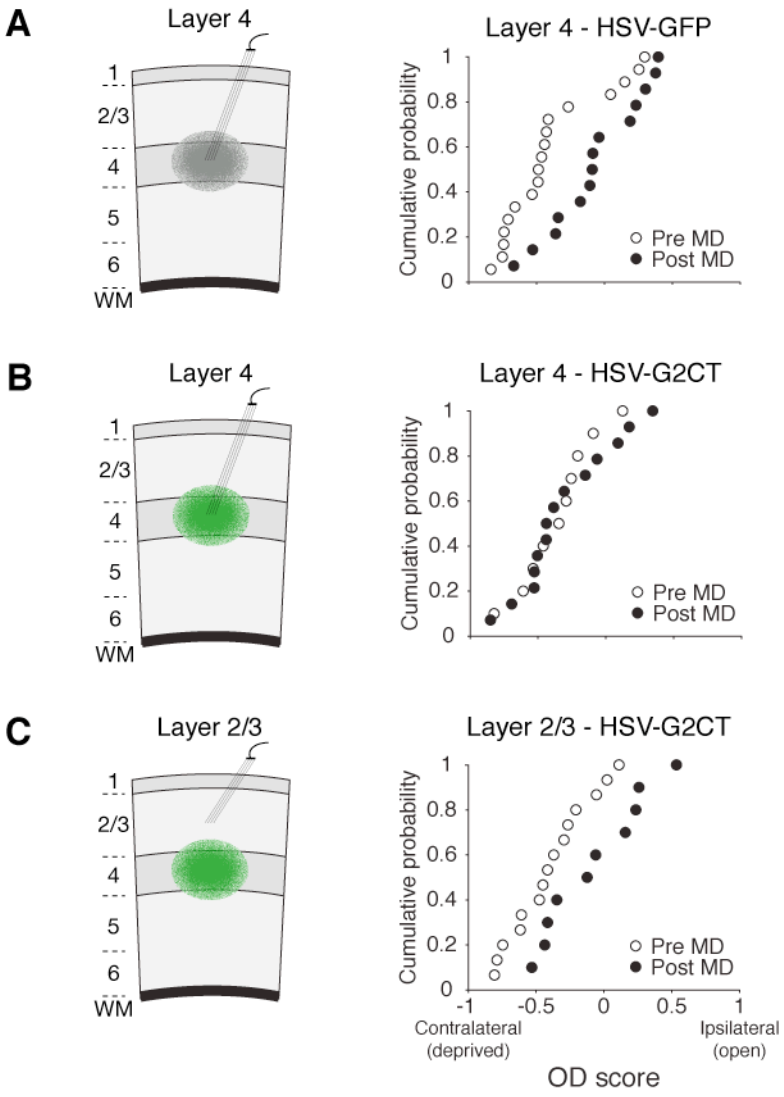


Figure 3.4 – Layer 4 infection with HSV-G2CT blocks OD plasticity in layer 4 neurons but not layer 2/3 neurons

A-C. *Left:* Schematics showing position of multi-channel recording electrode in layer 4 (A, B) or layer 2/3 (C), and the targeted site of infection with HSV-GFP (C) or HSV-G2CT (A, B). *Right:* Distribution of OD scores for neurons recorded either pre- or post-MD for the corresponding condition. A. A significant OD shift is observed in layer 4 single units from animals infected with HSV-GFP (n = 6 animals, n = 18 neurons pre-MD, 14 post-MD, Mann-Whitney U test, p = 0.01). B. The distribution of OD scores of neurons recorded in layer 4 from HSV-G2CT infected animals is not significantly different following 3d of MD (n = 3 animals, n = 10 neurons pre-MD, 14 post-MD, Mann-Whitney U test, p > 0.8). C. MD produces a significant shift in the distribution of OD scores of layer 2/3 neurons recorded following infection with HSV-G2CT targeted to layer 4 (n = 5 animals, n = 15 neurons pre-MD, 10 post-MD, Mann-Whitney U test, p < 0.05).

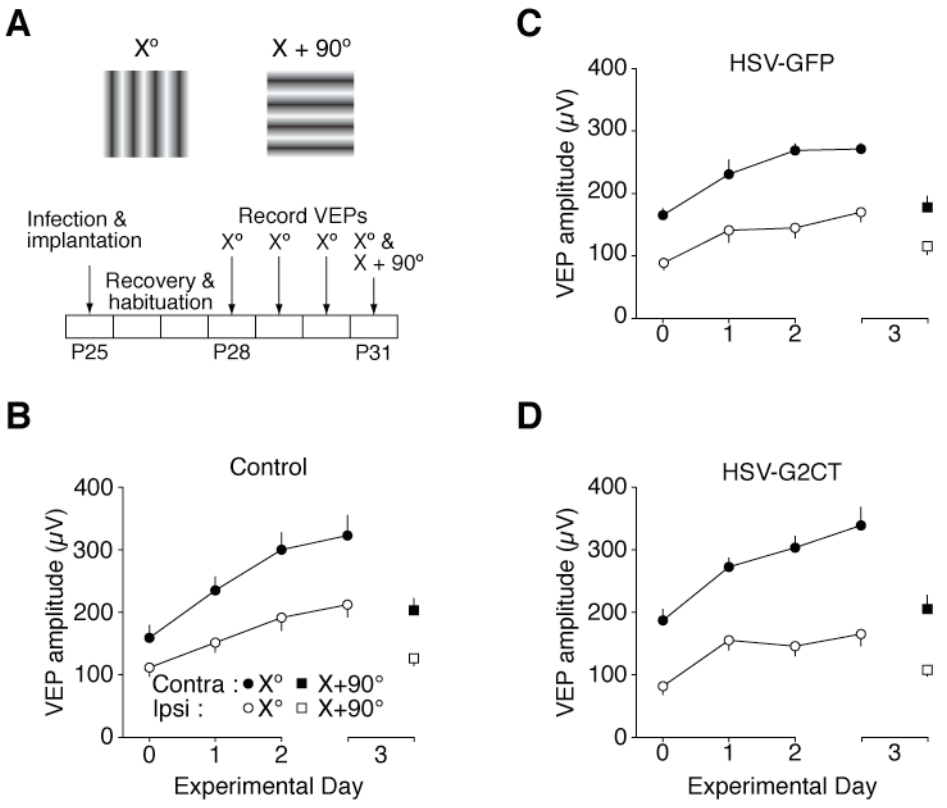


Figure 3.5 – Stimulus-selective response potentiation is unaffected by HSV-G2CT

A. Experimental design for SRP experiments. VEPs were recorded in response to grating stimuli of a single orientation (Xº) for 4 days, then in response to the orthogonal orientation (X + 90º). (B-D) Significant potentiation of responses evoked by the trained orientation was observed in non-infected control (B), HSV-GFP (C), and HSV-G2CT (D) infected animals (day 3 Xº *vs.* X + 90º, paired t-test, $p < 0.05$ for both contralateral and ipsilateral eyes under all infection conditions). Levels of SRP expression in all three groups are comparable (repeated measures ANOVA, $p > 0.5$).

		Day 0 X°	Day 3 X° (trained)	Day 3 X + 90° (novel)
Control	Contra	158.4 ± 20.1	321.9 ± 31.7 *+	202.7 ± 18.8 ‡
	Ipsi	110.8 ± 13.4	211.5 ± 19.9 *+	125.6 ± 11.8 #
HSV-GFP	Contra	164.4 ± 10.3	270.1 ± 8.6 *+	176.5 ± 18.1 #
	Ipsi	87.7 ± 10.8	169.0 ± 15.0 *+	114.7 ± 13.0 •
HSV-G2CT	Contra	186.1 ± 17.6	337.9 ± 29.5 *+	203.9 ± 22.8 #
	Ipsi	80.6 ± 13.1	164.4 ± 19.3 *+	107.0 ± 9.6 ‡

Table 3.1 – VEP amplitudes for SRP experiments

VEP amplitudes (in μV) for control (uninfected), HSV-GFP infected, and HSV-G2CT infected mice on day 0 and day 3 for both the trained and novel stimulus orientations (see figure 3.5). * $p < 0.01$, vs Day 0. + $p < 0.05$ vs Day 3 novel. •, ‡, # $p > 0.1, 0.15, 0.45$, respectively, vs Day 0. All comparisons were made within animal using paired *t*-tests.

Chapter 4

Activation of NR2B-containing NMDARs is not required for LTD

*Portions of this chapter were published together with Dr. Wade Morishita, Dr. Wei Lu, Dr. Roger A. Nicoll, Dr. Mark F. Bear, and Dr. Robert C. Malenka in Neuropharmacology (2007) Vol. **52**, pp. 71-76*

4.1 Preface

Monocular deprivation in mice produces a bidirectional and temporally distributed shift in OD: rapid weakening of deprived-eye inputs to the cortex is followed several days later by strengthening of inputs subserving the open-eye (Frenkel and Bear, 2004). It has been proposed that the critical event permissive for open-eye potentiation is a decrease in the modification threshold between depressing and potentiating stimuli (Bienenstock *et al.*, 1982; Bear, 2003; Frenkel and Bear, 2004). A decrease in the modification threshold would allow open-eye activity, which remains unchanged after closure of the other eye, to now induce potentiation.

A number of studies have demonstrated that the modification threshold can be affected by the history of sensory experience, with dark rearing shifting the boundary between LTD and LTP, facilitating potentiation with induction protocols of intermediate strength (*e.g.* a 5 Hz tetanus; reviewed in Chapter 1; (Kirkwood *et al.*, 1996; Philpot *et al.*, 2003; Philpot *et al.*, 2007)). The subunit composition of NMDARs has been proposed as a molecular correlate of the modification threshold, as manipulations that decrease the boundary between LTD and LTP also decrease the ratio of NR2A to NR2B subunits (Quinlan *et al.*, 1999a; Quinlan *et al.*, 1999b; Philpot *et al.*, 2001). In this model, the higher proportion of NR2B-containing receptors at synapses would favor LTP and open-eye potentiation due to the relatively greater temporal summation of NMDAR currents (Monyer *et al.*, 1994; Flint *et al.*, 1997) and preferential association with LTP-promoting proteins (Barria and Malinow, 2005) when compared to NR2A containing-receptors.

This model was challenged by findings explicitly linking the activation of NR2B-

containing NMDARs in CA1 to LTD, and NR2A-containing receptors to LTP (Liu *et al.*, 2004). Blockade of NR2B prevented LTD without affecting LTP, whereas NR2A blockade prevented LTP without affecting LTD. Extending this strict requirement for NR2B activation in the induction of LTD to the visual cortex challenges the interpretation of prior experiments with dark-reared animals, as these results suggest that a decrease in the NR2A / B ratio would promote LTD at the expense of LTP. However, dark rearing decreases both the NR2A / B ratio and the modification threshold required to induce LTP (Kirkwood *et al.*, 1996; Quinlan *et al.*, 1999a; Quinlan *et al.*, 1999b; Philpot *et al.*, 2003; Philpot *et al.*, 2007).

Importantly, the experiments performed by Liu *et al.* (2004) were based on the use of subunit-selective NMDAR antagonists to differentiate the contribution of NR2A and NR2B to LTP and LTD. Shortly after these results were published, several groups questioned the specificity of NVP-AAM007, the drug used by Liu *et al.* (2004) to block NR2A-containing receptors, and challenged the requirement for NR2A receptor activation in LTP (Berberich *et al.*, 2005; Weitlauf *et al.*, 2005).

We therefore felt it was important to revisit the question of whether LTD in CA1 could be induced without activation of NR2B-containing receptors. While we were performing these experiments, we became aware of similar efforts underway in the laboratories of Roger Nicoll at UCSF and Robert Malenka at Stanford. The following chapter contains the results of experiments conducted independently in each lab and published jointly. The contents of the joint publication are followed by an extended discussion relating these results obtained in CA1 to the metaplastic changes induced by MD in visual cortex.

4.2 Abstract

The triggering of both NMDA receptor-dependent long-term potentiation and long-term depression in the CA1 region of the hippocampus requires a rise in postsynaptic calcium. A prominent hypothesis has been that the detailed properties of this postsynaptic calcium signal dictate whether LTP or LTD is generated by a given pattern of synaptic activity. Recently, however, evidence has been presented that the subunit composition of the NMDA receptor determines whether a synapse undergoes LTP or LTD, with NR2A-containing NMDARs triggering LTP and NR2B-containing NMDARs triggering LTD. In the present study, the role of NR2B-containing synaptic NMDARs in the induction of LTD in CA1 pyramidal cells has been studied using the selective NR2B antagonists ifenprodil and Ro25-6981. While both antagonists reduced NMDAR-mediated synaptic currents, neither prevented induction of LTD. These results demonstrate that activation of NR2B-containing NMDARs is not an absolute requirement for the induction of LTD in the hippocampus.

4.3 Introduction

Although the phenomena of long-term potentiation and long-term depression have been studied for well over 15 years, much remains unknown about the detailed mechanisms responsible for their triggering and expression. In the CA1 regions of the hippocampus, it is well accepted that the triggering of the prototypic forms of LTP and LTD requires activation of postsynaptic NMDA receptors and the consequent rise in intracellular calcium concentration (Malenka and Nicoll, 1993; Malenka and Bear, 2004). Several lines of evidence suggest that the degree of NMDAR activation, and as a consequence the quantitative differences in the magnitude and perhaps timecourse of postsynaptic calcium elevation, dictates whether LTP or LTD is elicited (Malenka and Nicoll, 1993; Bear and Malenka, 1994; Cummings *et al.*, 1996; Yang *et al.*, 1999). Recently, an alternative hypothesis for the triggering of LTP and LTD has been suggested: that the subunit composition of NMDARs dictates whether LTD or LTP is generated (Liu *et al.*, 2004; Massey *et al.*, 2004). Specifically, it has been proposed that activation of NR2B-containing NMDARs leads to LTD whereas activation of NR2A-containing NMDARs leads to LTP. The evidence in support of this hypothesis primarily comes from pharmacological experiments. Selective antagonists of NR2B-containing NMDARs were reported to block LTD, but not LTP, in the CA1 region of the hippocampus (Liu *et al.*, 2004) and the perirhinal cortex (Massey *et al.*, 2004) while antagonists of NR2A-containing NMDARs had the opposite effects, blocking LTP but not LTD.

Several findings that involve molecular manipulations are difficult to reconcile with this NMDAR subunit specificity of LTP and LTD induction. Overexpression of

NR2B led to enhanced LTP in the hippocampus (Tang *et al.*, 1999) and activation of NR2B-containing NMDARs could generate LTP in mice lacking NR2A (Kiyama *et al.*, 1998; Weitlauf *et al.*, 2005) or with impaired NR2A-mediated signaling (Kohr *et al.*, 2003). The ability of NR2B-containing NMDARs to trigger LTP could be attributed to compensatory mechanisms caused by the prolonged molecular manipulations. More recently, however, the specificity of the drug used to block NR2A-containing NMDARs, NVP-AAM077, has been questioned as has the critical importance of these NMDARs in LTP induction (Berberich *et al.*, 2005; Weitlauf *et al.*, 2005).

Here, three independent groups have examined the LTD component of this hypothesis; that NR2B-containing NMDARs play a critical role in the triggering of LTD. All three groups independently find that in the CA1 region of the hippocampus the well-accepted selective antagonist of NR2B-containing NMDARs, ifenprodil, at concentrations that clearly reduce NMDAR-mediated synaptic responses, has no significant effect on LTD. These results are difficult to reconcile with the hypothesis that NR2B-containing NMDARs play a critical, obligatory role in the triggering of NMDAR-dependent LTD.

4.4 Results

4.4.1 Stanford Group

In previous work, the NR2B antagonists ifenprodil (3 μM) and Ro-25-6981 (0.5 or 3 μM) were found to block LTD in the CA1 region of the hippocampus (Liu *et al.*, 2004) and perirhinal cortex (Massey *et al.*, 2004). Surprisingly, however, we found that exposure of hippocampal slices for prolonged periods (30-120 min) to either of these NR2B antagonists had no detectable effect on the ability to elicit robust LTD when

compared to interleaved control slices. In fact, consistent with a previous study (Hendricson *et al.*, 2002) the magnitude of LTD was larger in the presence of either ifenprodil (2.5-5.0 μM) or Ro25-6981 (0.5 or 5.0 μM) (Figure 4.1A) (LTD in interleaved control slices, 75 ± 6.3% of baseline, n = 7; LTD in ifenprodil; 2.5 μM, 52%, n = 1; 3.0 μM, 62 ± 2%, n = 4; 5 μM, 45%, n = 1; LTD in Ro25-6981; 0.5 μM, 66 ± 16%, n = 2; 5.0 μM, 66 ± 6%, n = 4). The inability of the antagonists to block LTD was not due to lack of efficacy of the drugs on NMDARs as NMDAR EPSCs were clearly reduced following bath application of either antagonist (Figure 4.1B) (ifenprodil, percent reduction, 34 ± 13.4, n = 6; Ro25-6981, percent reduction: 33 ± 18.5, n = 2).

4.4.2 UCSF Group

Field potential recordings were made from the CA1 region of the hippocampus to assess the effects of the NR2B selective antagonist ifenprodil on LTD. Figure 4.2A shows a summary of a series of control (open circles) and interleaved experimental slices which were incubated in ifenprodil (3 μM) for at least 2 hours (closed circles). No obvious difference in the magnitude of LTD was seen between the two groups of slices (LTD in interleaved control slices, 76.9 ± 3.8%, n = 7; LTD in ifenprodil, 71.7 ± 1.9%, n = 8). This raises the question of whether the 3 μM ifenprodil used in these experiments was effective at blocking NMDAR-mediated currents. To address this issue we carried out whole cell recording of NMDAR EPSCs. The AMPAR component of the EPSC was blocked by NBQX (25 μM) and cells were held at +40 mV to relieve the voltage-dependent Mg^{2+} block of the NMDARs. As shown in Figure 4.2B, application of ifenprodil caused a reduction of the NMDAR EPSC (58.5 ± 9.2% of baseline, n = 6), similar in magnitude to that reported previously (Liu *et al.*, 2004). Thus despite the

ability of ifenprodil to antagonize NMDAR-mediated currents, it failed to affect LTD.

4.4.3 MIT Group

To assess the role of NR2B subunits in hippocampal LTD, we attempted to block induction of LTD using the selective antagonist ifenprodil. We found using field potential recordings that LTD induced by LFS was unaffected by treatment with ifenprodil (3 μM) (Figure 4.3A). Both treated slices and interleaved controls showed significant depression from baseline (average of last 5 minutes as percent of baseline: 82.6 ± 7.0%, n = 5, control; 85.8 ± 3.0%, n = 9, ifenprodil). Furthermore, the magnitude of depression did not differ significantly between groups (unpaired t-test, $p > 0.1$). As a further test of the possibility that LTD induction requires NR2B activation, we next asked if LTD induced using whole cell recordings and LFS paired with depolarization could be blocked by ifenprodil (3 μM). However, drug treated slices still showed significant LTD compared to baseline (average of last 5 minutes as percent of baseline: 66.1 ± 4.6%, n = 6), and once again the magnitude of LTD did not differ significantly from interleaved control slices (67.9 ± 7.2%, n = 6; Figure 4.3B). The failure of ifenprodil to block LTD was not due to a lack of drug efficacy on NMDARs, as isolated NMDAR currents were significantly reduced by application of ifenprodil (34.8 ± 2.9 % reduction; paired t-test, $p<0.05$, n=5; Figure 4.3C).

4.5 Discussion

We have presented results from three independent groups that normal LTD can be generated in the CA1 region of the hippocampus in the presence of selective antagonists of NR2B-containing NMDARs. All three groups also examined the effects of the antagonists on NMDAR-mediated synaptic responses and found that the concentrations

of antagonists used were sufficient to clearly reduce NMDAR EPSCs. These latter findings provide direct evidence that the antagonists reached synaptic NMDARs at effective concentrations. Importantly, these experiments were performed by labs with extensive experience in the study of LTP and LTD in hippocampal slices.

While it is conceivable that differences in experimental conditions might account for the discrepancy between our results and those previously published (Liu *et al.*, 2004), on the surface this seems unlikely. The same rat species (Sprague Dawley) at approximately the same age (3-5 weeks) were used in all experiments; the composition of the extracellular perfusing medium was very similar; the frequency of stimulation during recording of baseline synaptic responses was the same or similar; and the induction protocols were identical. However, we cannot exclude a contribution of other variables that can affect LTD thresholds, such as the rearing conditions of the animals (Xu *et al.*, 1997; Philpot *et al.*, 2003; Sawtell *et al.*, 2003).

Our results provide strong evidence that NR2B-containing NMDARs are not required for the triggering of LTD and therefore that NR2A-containing NMDARs are in fact sufficient to generate this form of plasticity. Combined with the recent work strongly questioning the critical importance of NR2A-containing NMDARs in the triggering of LTP (Berberich *et al.*, 2005; Weitlauf *et al.*, 2005) we conclude there is little support for the hypothesis that the molecular stoichiometry of synaptic NMDARs directly specifies the polarity of synaptic plasticity in CA1. The results to date continue to support the hypothesis that the calcium-influx through synaptic NMDARs with different subunit compositions can activate signaling cascades involved in either LTP or LTD, the critical variable being the magnitude and / or time course of the postsynaptic calcium

signal. Because NMDAR biophysical properties vary with subunit composition (Monyer *et al.*, 1992) and the different intracellular C-terminal tails of NR2A and NR2B subunits may serve as scaffolds for different intracellular signaling cascades (Sprengel *et al.*, 1998; Barth and Malenka, 2001; Kohr *et al.*, 2003), we favor an alternative hypothesis; that activity-dependent changes in the NR2A / B ratio are instead involved in the metaplastic regulation of LTD / LTP thresholds (Philpot *et al.*, 2001; Chen and Bear, 2007).

4.6 Extended discussion: Relationship to OD plasticity

This work clearly demonstrates that LTD in rat CA1 does not strictly depend on the activation of NR2B-containing NMDARs. When taken together with the finding that LTP also lacks a corresponding strict dependence on NR2A-containing receptors (Berberich *et al.*, 2005; Weitlauf *et al.*, 2005), it is clear that the NR2 subunit composition of NMDARs does not directly determine the polarity of synaptic plasticity as previously claimed (Liu *et al.*, 2004). Although we were ultimately interested in the requirements for synaptic plasticity in the visual cortex, we chose to use a hippocampal slice preparation in these experiments for several reasons. First, electrophysiology in the CA1 region is comparatively simple and has been extensively studied. Secondly, the results in the original paper (Liu *et al.*, 2004) rapidly became controversial with regards to the specificity of the reagents used and their effect on LTP (Berberich *et al.*, 2005; Weitlauf *et al.*, 2005). We therefore felt that addressing the effect of NR2B antagonism on LTD in rat CA1 would provide the best opportunity to compare our results with the original report.

Importantly, there is ample evidence to suggest that the lack of NMDAR subunit-

specific plasticity we observed in CA1 is also present in mouse visual cortex. Both LTP and LTD can be induced in layer 4 of mice either heterozygous or homozygous for the deletion of NR2A, demonstrating that there is not an explicit link between NMDAR subunit composition and the direction of plasticity (Cho *et al.*, 2009). Notably, although NR2A gene dosage did not determine the ability to induce either LTP or LTD, it did affect the optimal stimulation frequency for plasticity. Complete deletion of NR2A lowers the modification threshold sufficiently such that 1 Hz LFS, which reliably induces LTD in wild-type mice, produces synaptic potentiation (Cho *et al.*, 2009).

The evidence therefore suggests that in the visual cortex, like the hippocampus, the direction of plasticity is not tied directly to NMDAR subunit composition. However, it is clear that the ratio of NR2 subunits can affect the ability to induce LTP or LTD with a given stimulation frequency, with a decreased NR2A / B ratio favoring synaptic potentiation (Kirkwood *et al.*, 1996; Philpot *et al.*, 2003; Philpot *et al.*, 2007; Cho *et al.*, 2009). This relationship appears to result from the different biophysical properties and C-terminal interacting partners of NR2A and NR2B containing NMDARs (Monyer *et al.*, 1994; Flint *et al.*, 1997; Barria and Malinow, 2005). It has been proposed that differential patterns of postsynaptic calcium dynamics distinguish LTP and LTD, with modest slow rises in calcium inducing depression, whereas large rapid increases yeilding potentiation (Bear *et al.*, 1987; Lisman, 1989; Artola and Singer, 1993). The slower decay kinetics of NR2B-containing receptors would therefore favor potentiation following weaker input activity by facilitating the temporal summation of calcium transients (Monyer *et al.*, 1994; Flint *et al.*, 1997). Notably, dark rearing, which decreases the NR2A / B ratio and lowers the modification threshold, also increases

temporal summation of NMDAR currents (Philpot *et al.*, 2001).

The data of Liu *et al.* (2004) would have forced a significant revision of the model we have described to explain the delayed potentiation of open-eye responses during MD. This model requires a decrease in the modification threshold which occurs through a reduction in the ratio of NR2A to NR2B NMDAR subunits. Based on the data presented in this chapter, we continue to favor this model, in which the potentiation of open-eye responses occurs through LTP-like mechanisms facilitated by a decreased NR2A / B ratio.

4.7 Methods

4.7.1 Stanford Group

Transverse hippocampal slices (400 μm thick) were prepared from 3-5 week old Sprague-Dawley rats. Slices were cut in ice cold sucrose solution containing (in mM): sucrose 238, KCl 2.5, NaH_2PO_4 1, $NaHCO_3$ 26.2, $CaCl_2$ 1, $MgSO_4$ 2 and D-glucose 11 (saturated with 95% O_2 / 5% CO_2) and transferred to a holding chamber filled with external solution consisting (in mM): NaCl 119, KCl 2.5, NaH_2PO_4 1, $NaHCO_3$ 26.2, $CaCl_2$ 2.5, $MgSO_4$ 1.3 and D-glucose 11 (saturated with 95% O_2 / 5% CO_2). Slices were allowed to equilibrate at 28-30^oC for at least 1.5 h before being transferred to a recording chamber that was superfused (2 ml/min) with warmed (28-30^oC) external solution containing 50 μM picrotoxin. Prior to recording, the CA3 region was removed to reduce transmission of picrotoxin-induced epileptiform bursting to CA1.

Dendritic field EPSPs (fEPSPs) were recorded in the CA1 region with a patch pipette filled with external solution. Basal fEPSPs were evoked at 0.05 Hz with a monopolar stimulating electrode positioned in the stratum radiatum. LTD was induced by a 1 Hz, 15 min LFS, delivered through the stimulating electrode. Whole-cell voltage-clamp recordings were obtained from CA1 neurons. The patch pipette solution contained (in mM): $CsMeSO_4$ 117.5, HEPES 10, TEA-Cl 10, CsCl 15.5, NaCl 8, EGTA 0.25, MgCl 1, MgATP 4, NaGTP 0.3, QX-314 5 (295-300 mOsM, pH adjusted to 7.3 with CsOH). NMDAR-mediated EPSCs were evoked at a holding potential (V_H) of -40 mV in the presence of 5 μM NBQX. Input and series resistance were measured from a −4 mV, 70 ms voltage pulse elicited prior to each evoked EPSC and monitored throughout each experiment. Series resistance ranged between 7-20 MΩ. Experiments in which the series

resistance varied by more than 20% were not used in the study.

Field and whole-cell recordings were filtered at 1 and 2 KHz, respectively and digitized at 5 KHz with an A/D board (National Instruments) driven by custom acquisition software designed to run on IGOR pro. The magnitude of LTD was determined by averaging the slope of fEPSPs collected 40-45 min after the LFS. Statistical significance of data was determined using a Student's t-test with a level of significance, $P < 0.05$. Drugs were obtained from Tocris (NBQX, ifenprodil) or Sigma RBI (picrotoxin, Ro25-6981).

The NR2B antagonists, ifenprodil (2.5-5 μM) and Ro25-6981 (0.5, 5 μM) were present throughout the duration of the LTD experiments (70-80 min). In some experiments (n=4), slices were pre-incubated with ifenprodil (3 μM) for at least 1 hr to ensure maximum exposure of slices to the NR2B antagonist. LTD experiments utilizing NR2B antagonists were inter-leaved with control experiments, which were performed on the same day.

4.7.2 UCSF Group

Transverse hippocampal slices for fEPSPs recording (400 μm thick) were prepared from 20- to 28-day-old Sprague-Dawley rats in ice-cold ACSF, containing (in mM): NaCl 119, KCl 2.5, $CaCl_2$ 2.5, $MgSO_4$ 1.3, NaH_2PO_4 1.0, $NaHCO_3$ 26.2, and glucose 11 and sucrose cutting solution, containing (in mM): NaCl 87, KCl 2.5, $CaCl_2$ 0.5, $MgCl_2$ 7, NaH_2PO_4 1.0, $NaHCO_3$ 25, glucose 10 and sucrose 75. For whole cell recording experiments the slices (300 μm) were cut in the following solution (in mM): NaCl 87, KCl 2.5, $CaCl_2$ 0.5, $MgCl_2$ 7, NaH_2PO_4 1.0, $NaHCO_3$ 25, glucose 10 and sucrose 75. Solutions were saturated with 95% O_2 and 5% CO_2. Freshly cut slices were

placed in an incubating chamber containing ACSF and recovered at 37°C for 1-1.5 hr. Slices were then maintained in ACSF or ACSF with 3 μM ifenprodil at room temperature (23°C-26°C) prior to recording.

fEPSPs were evoked in CA1 stratum radiatum by stimulation of Schaffer collaterals with ACSF filled glass pipettes, and were recorded with ACSF filled 3-6 MΩ glass pipettes using an Axopatch-1B amplifier (Axon Instruments). Test stimuli consisted of 100 μs pulses of constant voltage delivered by stimulus isolation units (Digitimer Ltd). The stimulation rate was 0.1 Hz for all fEPSP recording experiments. After obtaining a stable baseline for 10-15 min, LTD was induced by applying 900 pulses at 1Hz (LFS) using the same stimulating strength used during baseline recording. The NR2B antagonist ifenprodil was continuously present in ACSF before, during and after the induction of LTD. fEPSPs were filtered at 2 kHz, digitized at 10 kHz, and stored on computers using IgorPro (Wavemetrics Inc.).

Somatic whole cell voltage-clamp recordings in acute slices were obtained from visually identified CA1 pyramidal cells using 2.5-5 MΩ glass electrodes filled with (in mM): 100 $CsMeSO_4$, 5 QX-Cal, 10 HEPES, 4 Mg-ATP, 0.4 Na-GTP, 10 Cs_4BAPTA, 0.1 spermine, pH 7.2, 288 mOsm. The stimulation rate for whole cell recording was 0.0083-0.033 Hz to minimize the rundown of NMDARs-mediated EPSCs. Cells in which the series or input resistance varied by >25% during the duration of the experiment were discarded. Evoked NMDARs-mediated EPSCs were recorded at +40mV in the presence of 100 μM picrotoxin and 25 μM NBQX. Once stable EPSCs were obtained for 10-20 min, 3 μM ifenprodil was applied. Once EPSCs were stable in the presence of ifenprodil, 100 μM D-AP-5 was applied to confirm that evoked EPSCs were entirely mediated by

NMDARs. Student's t-tests were used to analyze differences in physiological parameters. Data are presented as mean ± SEM. Ifenprodil, NBQX and AP-5 were obtained from Tocris, UK. All other chemicals and drugs were obtained from Sigma.

4.7.3 MIT Group

Transverse hippocampal slices (350 μm thick) were prepared from 3-4 week old Sprague-Dawley rats in ice-cold dissection buffer containing (in mM): NaCl 87, Sucrose 75, KCl 2.5, NaH_2PO_4 1.25, $NaHCO_3$ 25, $CaCl_2$ 0.5, $MgSO_4$ 7, ascorbic acid 1.3, and D-glucose 10 (saturated with 95% O_2 / 5% CO_2). For field recordings, slices recovered in ACSF containing (in mM): NaCl 124, KCl 5, NaH_2PO_4 1.23, $NaHCO_3$ 26, $CaCl_2$ 2, $MgCl_2$ 1 and D-glucose 10 (saturated with 95% O_2 / 5% CO_2) for 0.5 h at 32 ºC, and were then transferred to room temperature for an additional 0.5 h prior to recording. For fEPSP measurements, slices were superfused with ACSF at 30 ºC. For whole-cell recordings, CA3 was removed immediately following slicing and the ACSF was modified as follows: KCl was reduced to 2.5 mM, and 10 μM bicuculline methiodide was included.

fEPSPs were recorded in CA1 stratum radiatum with extracellular electrodes filled with ACSF. Baseline responses were evoked at 0.033 Hz with a 2-contact cluster electrode (FHC, Bowdoin, ME). After recording a stable baseline for 20 min, LTD was induced with 900 pulses at 1 Hz (LFS), at baseline stimulation intensity. The initial slope of the response was used to assess changes in synaptic strength.

Somatic whole cell recordings of CA1 pyramidal neurons were made using 4-6 MΩ pipettes filled with (in mM): D-gluconic acid 103, CsOH 103, HEPES 20, TEA-Cl 5, NaCl 2.8, EGTA 0.2, MgATP 4, NaGTP 0.3, Na-phosphocreatine 10, QX-314 5 (290-

300 mOsm, pH adjusted to 7.2 with CsOH). Baseline EPSCs in response to stimulation of the stratum radiatum were collected at 0.05 Hz, at a holding potential of -70 mV. A stable baseline was recorded for 10 min, and LTD was induced by applying 300 pulses at 1 Hz while depolarizing the post-synaptic neuron to -40 mV (paired LFS). NMDA currents were isolated using whole-cell recordings in ACSF supplemented with BMI (10 μM) and CNQX (10 μM). Currents were recorded at -40 mV in response to groups of 3 stimuli (delivered at 0.05 Hz), repeated approximately every 10 minutes. This sparse stimulation protocol was used to minimize the use-dependent decline in NMDAR-mediated responses (Philpot *et al.*, 2001). A liquid junction potential (approx. -14 mV) was corrected. Series resistance was monitored continuously and experiments showing a greater than 20% change were excluded.

Recordings were filtered at 1 or 2 kHz (field and whole cell recordings, respectively), digitized at 10 kHz, and analyzed using pClamp9 (Axon Instruments). For drug treated slices, ifenprodil (3 μM) was present for the duration of the experiment, and slices were pre-incubated with ifenprodil for at least 30 min prior to inducing LTD. All reagents were purchased from Sigma, with the exception of ifenprodil (Tocris). All data are presented as mean ± SEM.

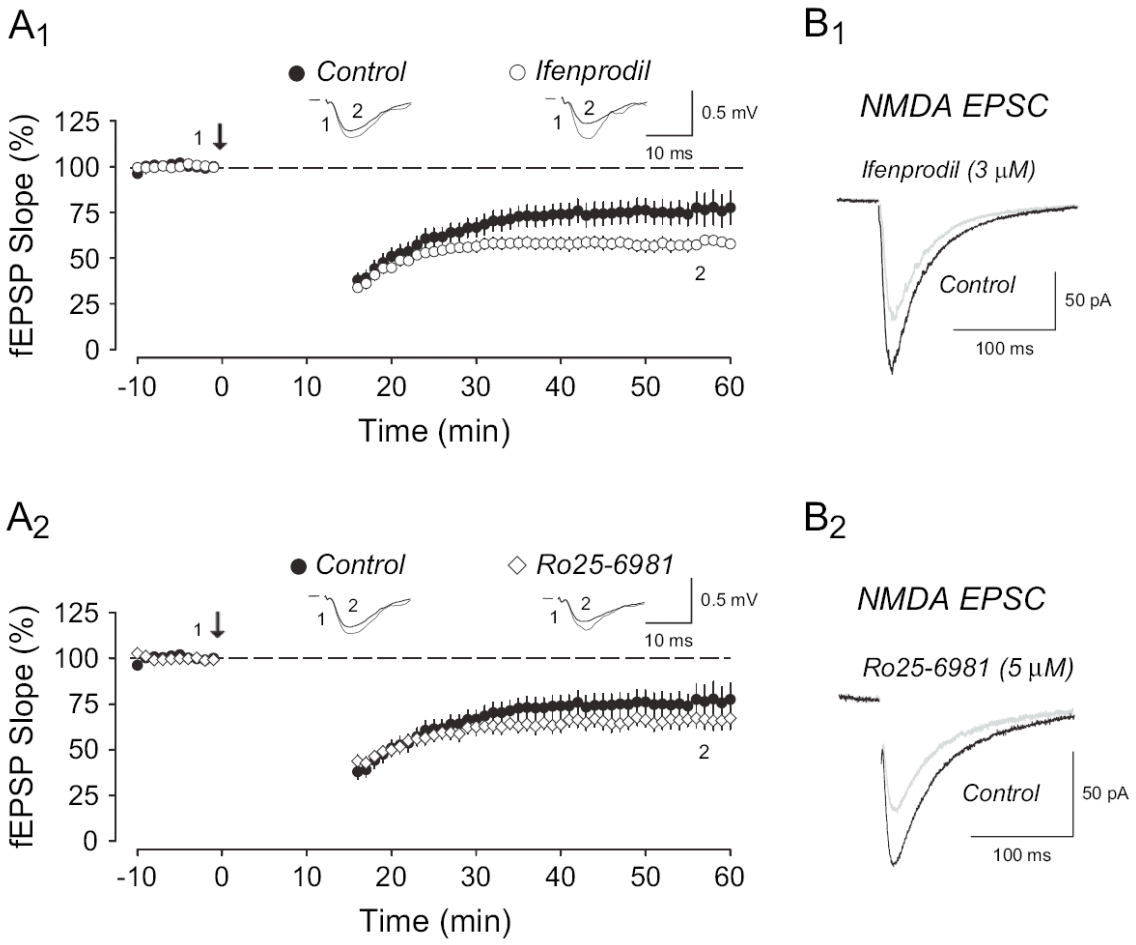


Figure 4.1 – NR2B antagonists do not block LTD in the CA1 region of the hippocampus (Stanford group)

$A_{1\text{-}2}$. Ifenprodil (2.5 – 5 μM, n=6, open circles, A_1) or Ro25-6981 (0.5, 5 μM n=6, open diamonds, A_2) do not block LTD when compared to inter-leaved controls (A_1, A_2 n=7, filled circles). LTD was induced with a 1 Hz, 15 min train (arrow). $B_{1\text{-}2}$. Both ifenprodil (3 μM, B_1) and Ro25-6981 (5 μM, B_2) reduce NMDAR-mediated EPSCs.

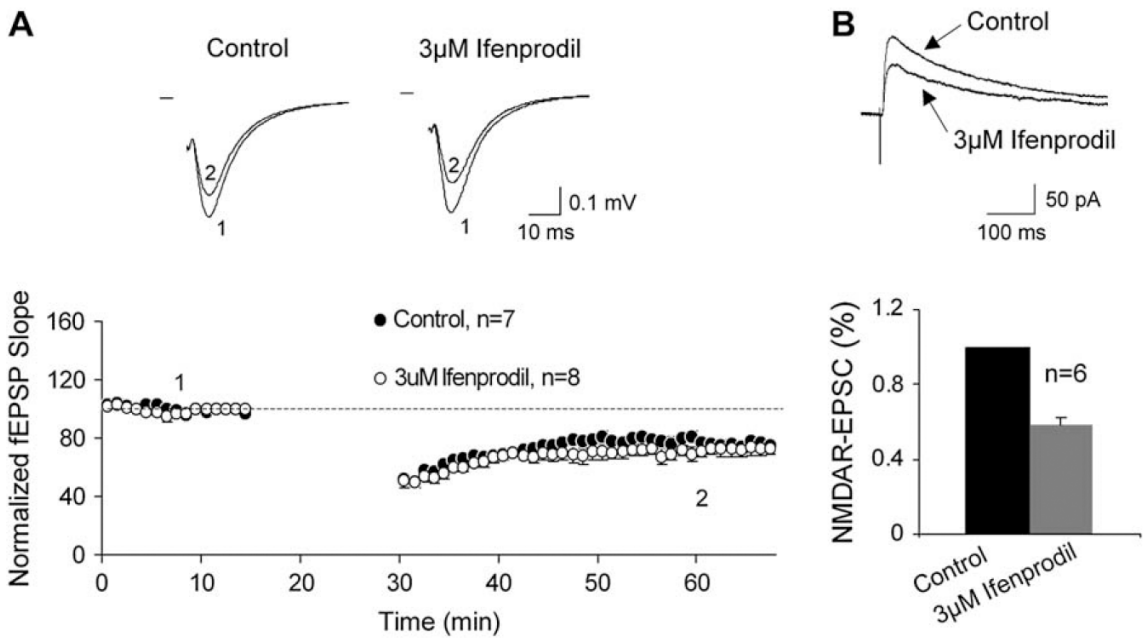


Figure 4.2 – Ifenprodil does not block induction of homosynaptic LTD by LFS (UCSF group)

A. Representative fEPSPs averaged from 30 consecutive stimuli were taken before and after LTD induction at the time points (1 and 2) indicated in the graph. Continuous bath application of NR2B antagonist ifenprodil (3μM) failed to prevent LTD induced by LFS. The average slope of fEPSCs after LTD induction (time point 2) is 76.9% ± 3.8 or 71.7% ± 1.9 of baseline (time point 1) in absence or presence of 3μM ifenprodil, respectively. B. Representative whole cell NMDAR-mediated EPSC traces were taken before (control) and after ifenprodil treatment, as shown above the bar graph. Ifenprodil (3μM) reduced the evoked NMDAR-mediated EPSCs in CA1 pyramidal neurons to 58.5±9.2% of control. These EPSCs were entirely mediated by NMDARs, because they were fully blocked by 100 μM AP-5 (data not shown).

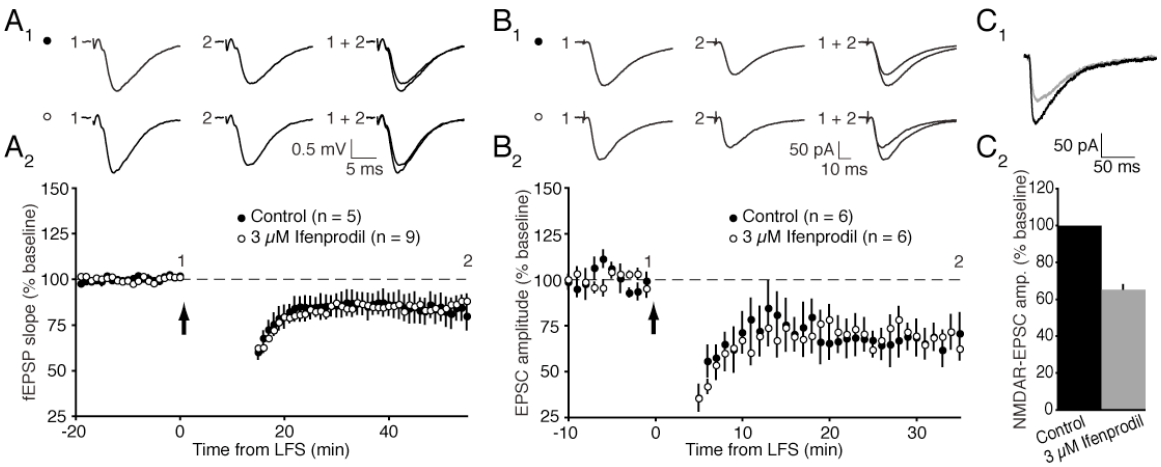


Figure 4.3 – Hippocampal LTD is not blocked by the NR2B antagonist ifenprodil (MIT group)

A. In field potential recordings, bath application of ifenprodil (3 μM) does not block LTD induced in CA1 stratum radiatum by LFS. A_1. Representative fEPSPs from control (closed circles) and ifenprodil treated slices (open circles) at the time points indicated in A_2; traces are averages of 4 consecutive responses. A_2. Timecourse showing averaged fEPSP slope measurements (mean ± SEM) for all slices. LFS was applied at the time indicated by the arrow. The average fEPSP slope over the last 5 min of recording for control and ifenprodil treated slices is 82.6 ± 7.0% and 85.8 ± 3.0% of baseline, respectively. B. Treatment with ifenprodil fails to block LTD induced in whole cell recordings using paired LFS. B_1. Representative EPSCs from the time points indicated in B_2; traces are the average of 6 consecutive responses. B_2. Timecourse of average EPSP amplitude in control and ifenprodil treated slices. Note that the average amplitude over the last 5 minutes of recording is 67.9 ± 7.2% of baseline for control slices and 66.1 ± 4.6% of baseline for ifenprodil treated slices. C. Ifenprodil reduces NMDAR-mediated EPSC's. C_1. Representative NMDAR-EPSCs obtained before (black) and following (grey) application of 3 μM ifenprodil. Traces are averages of 3 consecutive responses. C_2. Bar graph showing average amplitude of NMDA-EPSC following ifenprodil treatment as a percentage of pre-drug baseline. Ifenprodil significantly reduced NMDA-EPSCs to 65.2 ± 2.9 % of control (n=5).

Chapter 5

Implications and future directions

5.1 Introduction

The experiments in Chapters 2 and 3 of this thesis were designed to directly address the requirement of AMPAR endocytosis in the loss of deprived-eye responses following sensory deprivation. The NMDA-stimulated internalization of AMPA receptors is a critical expression mechanism for homosynaptic LTD at many synapses, including white matter – layer 4 synapses in the visual cortex. Thus, the ultimate goal of these experiments was to directly test the "LTD hypothesis" of deprived-eye depression in OD plasticity by determining whether the mechanisms of LTD are essential to response depression *in vivo*.

In Chapter 2, I have described our efforts to generate and validate HSV-G2CT as a reagent to specifically disrupt NMDA-stimulated AMPAR endocytosis and LTD in the visual cortex. In Chapter 3, I present evidence that *in vivo* infection with HSV-G2CT in layer 4 blocks MD-induced weakening of visual responses in that layer. Therefore, the experiments in this thesis demonstrate that a critical mechanism involved in homosynaptic LTD, the endocytosis of AMPA receptors, is essential for the response to sensory deprivation.

By definition, LTD is an artifact of specific patterns of stimulation (*e.g.* 1 Hz LFS) or drug application (*e.g.* brief NMDA treatment) delivered by the experimenter. These protocols reliably decrease the strength of synaptic transmission both *ex vivo* and *in vivo*, and provide opportunities to study the mechanisms underlying synaptic weakening. However, it is extremely improbable that outside of these experimental conditions neurons in the brain ever experience 15 minutes of 1 Hz activity or 5 minutes of drastically elevated global NMDAR activation. The value of studying LTD is

therefore the mechanistic framework it provides for understanding the role of synaptic weakening in the intact animal. As such, the experiments in this thesis can be taken as a demonstration of the validity of this approach. We have taken advances in the mechanistic understanding of LTD achieved *ex vivo* and shown their relevance to sensory plasticity *in vivo*.

5.2 Relationship to prior studies

Many studies have attempted to probe the contribution of LTD to OD plasticity by correlating the effects of pharmacological or genetic manipulations on both processes. As discussed in Chapter 1, these studies have reported seemingly conflicting results, with some finding correlated disruptions of LTD and OD plasticity whereas others describe double dissociations between these processes. We have previously argued that laminar differences in plasticity mechanisms may contribute in part to these discrepant findings. Our current results taken together with those of Liu *et al.* (2008) suggest an additional source of complexity: simultaneous and *independent* plasticity in layers 2/3 and 4. We demonstrate that blockade of OD plasticity in layer 4 by G2CT does not affect OD plasticity in layer 2/3, in complement to the finding that the CB1 antagonist AM251 blocks the OD shift in layer 2/3 but not in layer 4 (Liu *et al.*, 2008). If blocking plasticity in a given layer is insufficient to prevent other layers from exhibiting an OD shift, then pooling single unit recordings performed across layers is likely to obscure deficits in OD plasticity.

The intracellular signaling mechanisms contributing to LTP and LTD are complex and not fully elucidated (Malenka and Bear, 2004). It is possible that manipulations which disrupt LTD in cortical slices do so by altering the induction requirements *ex vivo*

without affecting the underlying processes, thus leading to an apparent loss of LTD with intact OD plasticity. Clearly the converse is also possible: the sensitivity to LTD-inducing stimuli in slices may be unaffected, whereas the effects of potentially more subtle stimuli could be lost *in vivo*. We have attempted to address this issue by utilizing a manipulation that specifically blocks AMPAR endocytosis, the ultimate expression mechanism of many forms of NMDAR-dependent LTD (Carroll *et al.*, 1999; Ehlers, 2000; Lee *et al.*, 2002; Malinow and Malenka, 2002).

Two confounds of studies using transgenic or knockout mice to study OD plasticity are first, potentially abnormal function in the visual pathway prior to the cortex, such as the retina or LGN; and second, the potential for the appearance of unexpected compensatory mechanisms. The use of HSV to drive G2CT expression in the current study seeks to mitigate these factors. We were able to inject HSV-G2CT stereotaxically into the visual cortex, targeting layer 4 and eliminating any potential for effects in pre-cortical areas. Additionally, rapid expression of transgenes following HSV infection (Figure 2.5A and (Neve *et al.*, 2005)) allowed delivery of G2CT closely timed to the onset of MD, thereby limiting the length of time available for developmental compensation to occur.

5.2.1 Other peptides which interfere with AMPAR trafficking

In addition to the G2CT peptide used here, several other peptides which mimic regions of the C-terminal tails of AMPAR subunits have been used to manipulate receptor trafficking *in vivo*. The C-terminal tail of GluR2 contains a region with three tyrosine residues (Y_{869}KEGY_{873}NVY_{876}G) located close to the C-terminal PDZ binding site, and tyrosine phosphorylation of this region by Src-family kinases has been

implicated in both NMDA- and AMPA-stimulated AMPAR internalization, as well as LFS-LTD (Ahmadian *et al.*, 2004; Hayashi and Huganir, 2004). A peptide mimicking this region ($GluR2_{3Y}$) blocks LTD in CA1 when loaded intracellularly through the recording pipette without affecting basal AMPAR surface expression (Ahmadian *et al.*, 2004; Wang *et al.*, 2004). Brebner *et al.* (2005) attempted to use this peptide to assess the contribution of LTD in the nucleus accumbens (NAc) to the expression of behavioral sensitization following amphetamine administration. To deliver $GluR2_{3Y}$ to neurons *in vivo,* these authors fused the peptide to the membrane transduction domain of the HIV-1 Tat protein. Either systemic (intravenous) injection or targeted infusion into the NAc blocked the expression of behavioral sensitization evoked by a challenge dose of amphetamine, thereby demonstrating a critical role for LTD in this process (Brebner *et al.*, 2005).

Relative to this prior work, we chose to use HSV mediated expression of the G2CT peptide for several reasons. First, we favored the G2CT peptide over $GluR2_{3Y}$ because although both peptides have been reported not to affect basal AMPAR levels or AMPA-mediated receptor internalization, phosphorylation of tyrosine-876 has been implicated in this process (Hayashi and Huganir, 2004). Thus, we felt that there was an increased probability of observing non-specific effects with the $GluR2_{3Y}$ peptide relative to G2CT. Secondly, the experimental design of Brebner *et al.* (2005) was aimed at determining the role of LTD in the expression of a phenomenon, and therefore exploited the advantages of delivering a peptide directly to neurons via fusion to a Tat sequence. This method led to extremely rapid rises in peptide concentrations, which allowed behavioral testing to occur within 90 minutes of delivery (Brebner *et al.*, 2005). In

contrast, we desired to block AMPAR endocytosis and LTD for the duration of sensory deprivation (3 days). We therefore felt that driving expression of the G2CT peptide within neurons would offer advantages in terms of maintaining peptide concentrations over time, and threrefore decided to pursue HSV-mediated delivery.

Additionally, several groups have successfully utilized viral expression of LTP blocking peptides to test the role of LTP mechanisms *in vivo*. Expression of the full length GluR1 C-terminal tail (GluR1CT) blocks LTP in the hippocampus and lateral amygdala (Shi *et al.*, 2001; Rumpel *et al.*, 2005). Viral expression of this peptide, either through lentivirus or HSV, has been shown to block a number of phenomena including fear conditioning (following infections of the lateral amygdala) and SRP (following infection of the visual cortex), thereby demonstrating a role for LTP mechanisms in these processes (Rumpel *et al.*, 2005; Frenkel *et al.*, 2006). The success of these prior studies suggested that analogous experiments utilizing HSV-expressed G2CT could succeed in testing a role for LTD in OD plasticity.

5.2.2 Future experiments addressing the role of AMPAR endocytosis in deprived-eye depression

A significant limitation of both the single-unit and VEP recording techniques used in this thesis is the inability to conclusively determine whether the recorded response originated from neurons infected with HSV-G2CT. Although the VEP is a population response, it is impossible to quantify what percentage of this response results from infected cells. Likewise the singe-unit experiments, while demonstrating that AMPAR endocytosis is a required component of deprived-eye depression in individual neurons, do not address whether infection with HSV-G2CT *per se* was required to block plasticity in the recorded neuron. This uncertainty is due to the inability to determine

electrophysiologically whether any given recorded neuron was actually infected.

In order to determine whether the effects of G2CT are cell autonomous or rather reflect alterations in the cortical network, I propose examining OD plasticity following HSV-G2CT infection using *in vivo* 2-photon imaging of calcium transients. This technique would allow the unambiguous identification of infected neurons within the cortex. An intriguing possibility is that OD plasticity in adjacent uninfected neurons is also blocked in HSV-G2CT infected animals. This would suggest that OD plasticity within individual neurons is highly influenced by the response properties of the surrounding cortical network.

Identification of infected neurons could be achieved via spectral separation of GFP from the calcium indicator OGB-1 as described previously (Gandhi *et al.*, 2008). Alternatively, we have produced a second version of HSV-G2CT in which GFP has been replaced with the red fluorescent reporter td-tomato. Although *in vivo* calcium imaging has generally been restricted to superficial layers, advances in optical components coupled with the shallowness of mouse cortex (layer 4 is only approx. 450 μm below the cortical surface) allow imaging of visual responses in layer 4 (Mrsic-Flogel *et al.*, 2007), facilitating comparison with our prior experiments. Most published reports using this technique utilize multi-cell bolus loading of calcium dyes (Stosiek *et al.*, 2003), which is only amenable to acute experiments and thereby necessitates ratiometric determinations of OD and across group comparisons. The development of genetically encoded calcium sensors (reviewed in (Knopfel *et al.*, 2006)) offers the potential to perform longitudinal studies of OD in identified populations of neurons (*e.g.* (Mank *et al.*, 2008)). Additionally, these experiments would ideally be performed in awake mice as recently

demonstrated (Dombeck *et al.*, 2009), thereby removing any effects of anesthesia on visual responses.

5.3 Alternatives to the LTD hypothesis of deprived-eye depression

Broadly speaking, there are two categories of changes that could account for the observed reduction in deprived-eye responses which represents the initial effect of MD. First, excitatory drive into the cortex from the deprived eye could become weakened, thereby decreasing responses driven by the deprived eye. Second, intracortical inhibition of deprived-eye inputs could increase following MD, thereby suppressing or "masking" visual responses evoked by the deprived eye.

A large body of work in kittens has supported the former idea, demonstrating that MD leads to a reduction in cortical innervation by excitatory thalamocortical axons subserving the deprived eye (Shatz and Stryker, 1978), which is accompanied physiologically by considerable weakening of deprived-eye inputs (Singer, 1977; Tsumoto and Suda, 1978; Mitzdorf and Singer, 1980). The alternative hypothesis, that the OD shift observed following MD results from altered intracortical inhibition, was originally suggested over 30 years ago, again based on experiments in kittens (Duffy *et al.*, 1976; Burchfiel and Duffy, 1981). Local iontophoretic administration of bicuculline, a $GABA_A$ receptor antagonist, restores binocularity and deprived-eye responses in cortical neurons following MD (Burchfiel and Duffy, 1981). However, attributing these results to MD-induced plasticity of inhibitory networks is difficult, as blockade of GABA receptors in normal cats both broadens receptive field properties and unmasks previously subthreshold responses (Sillito, 1975). A similar unmasking was also observed for responses evoked through either the deprived or non-deprived eye in MD animals (Sillito

et al., 1981).

As rodents have emerged as the preferred system for studies of visual cortical plasticity, questions of the relative contribution of MD-induced changes in excitation and inhibition have been re-addressed with new approaches. The finding that brief MD leads to reduced surface expression of AMPARs and occludes subsequent induction of LTD (Heynen *et al.*, 2003; Crozier *et al.*, 2007) coupled with the experiments in this thesis showing that blockade of OD plasticity by G2CT argues that LTD at excitatory synapses plays a central role in the weakening of visual responses.

Additional work has shown that cortical inhibitory networks can also be strongly affected by MD. Brief periods of deprivation induce potentiation of feedback inhibition involving fast-spiking basket cells in layer 4 of the monocular visual cortex in rats studied *ex vivo* (Maffei *et al.*, 2006). Additionally, two recent studies used calcium imaging in mice *in vivo* and found MD-induced OD shifts in both excitatory and inhibitory neurons (Gandhi *et al.*, 2008; Kameyama *et al.*, 2010). Thus, both the inputs to and outputs from inhibitory neurons can be modified by deprivation.

In a recent study, Yazaki-Sugiyama *et al.* (2009) used *in vivo* intracellular recordings from layer 2/3 to assay the OD of individual excitatory or inhibitory neurons in mouse cortex. The reported response to MD in pyramidal neurons is highly similar to previous studies (Gordon and Stryker, 1996; Frenkel and Bear, 2004): an initial contralateral-eye bias in visual responses is lost following brief MD and neurons become increasingly binocular, without a further shift if the period of MD is extended to 14d (Yazaki-Sugiyama *et al.*, 2009). Interestingly, however, intracellular recordings from FS interneurons, putatively parvalbumin-positive large basket cells, exhibited strongly

binocular responses prior to MD. Brief MD leads FS cells to develop a contralateral (deprived) eye bias in OD, which reverses with 14d MD to favor the open eye.

In a technically challenging investigation of the functional consequences of this bidirectional inhibitory plasticity, Yazaki-Sugiyama *et al.* (2009) performed a series of recordings from pyramidal neurons in which the $GABA_A$ antagonist picrotoxin (PTX) was slowly infused intracellularly through the recording pipette. Nominally, this technique allows the examination of OD in the same neuron both prior to and following intracellular blockade of $GABA_A$ receptors, thereby isolating the response to excitatory inputs. In non-MD control mice, PTX greatly increased the binocularity of visually driven responses. Notably, binocularity was increased in both neurons with an initial contralateral bias (the majority of cells) and those with an initial ipsilateral bias. In short-term MD animals, infusion of PTX did not change the overall degree of binocularity, but rather produced an inversion of OD for each neuron. Cells exhibiting a contralateral bias prior to $GABA_A$ blockade became dominated by the ipsilateral eye following intracellular PTX application, and vice versa.

Although there are multiple caveats to these findings (discussed in detail in Appendix 1), the results of Yazaki-Sugiyama *et al.* (2009) provide novel evidence for a complex role for FS interneurons in OD plasticity and raise several interesting questions regarding the roles played by excitation and inhibition in shaping OD. One puzzling result is the dramatic increase in the binocularity of pyramidal cell responses recorded from non-MD animals following intracellular blockade of inhibitory inputs. This result suggests that excitation onto pyramidal cells is binocularly balanced, with the maintenance of OD biases dependent on asymmetric inhibition. The finding that FS cells

lack such a bias and are in fact strikingly binocular (Yazaki-Sugiyama *et al.*, 2009) suggests that monocularly-biased inhibition relies on other interneuron subtypes and that these subtypes play a greater role in modulating OD than previously thought (Hensch, 2005; Katagiri *et al.*, 2007). Notably, the binocularly balanced excitatory drive contrasts with the anatomical bias of feed-forward (excitatory) projection neurons in the LGN (targeting layer 4 and deep layer 2/3 in mice), which favors the contralateral eye by approximately a 2:1 ratio, equivalent to the physiological response ratio recorded in layer 4 (Frenkel and Bear, 2004; Coleman *et al.*, 2009). Additionally, these results argue that decreases in AMPAR-mediated synaptic transmission are insufficient to account for OD plasticity. This interpretation contrasts with the results presented in this thesis showing that blockade of AMPAR endocytosis in excitatory neurons prevents MD-induced deprived-eye depression.

The plasticity of excitatory and inhibitory neurons was addressed in a second recent study that used calcium imaging to study the effects of MD on genetically indentified inhibitory neurons (Kameyama *et al.*, 2010). These experiments found that both excitatory and inhibitory neurons on average display OD biases strongly favoring the contralateral eye. However, by examining the OD scores obtained from individual neurons, the authors found that the OD distributions were different between cell types, with the population of inhibitory neurons exhibiting a tighter distribution that was more centered around binocular responses. Following brief MD, significant OD shifts away from the deprived eye were observed in both excitatory and inhibitory neurons, suggesting that the loss of deprived-eye responses may also involve OD shifts in inhibitory cells (Kameyama *et al.*, 2010). Arguing against the idea that inhibition plays a

critical role in the expression of MD-induced changes is the finding that intracortical infusion of bicuculline following MD does not affect the observed shift in OD ratio (Khibnik, L.A., Cho, K.K., and Bear, M.F., *submitted*).

5.3.1 Future experiments addressing the contribution of intracortical inhibition to OD plasticity

Clearly further experimentation will be required to conclusively establish the role for inhibitory changes in OD plasticity. Recent advances in extracellular recording techniques facilitate the distinction between excitatory and inhibitory neurons in mice (Niell and Stryker, 2008). Applying this approach to OD plasticity would allow confirmation of the bidirectional shift in FS interneurons reported by Yazaki-Sugiyama *et al.* (2009), while also facilitating the extension of these results from layer 2/3 to deeper cortical layers. Additionally, a recently generated parvalbumin-Cre mouse (Hippenmeyer *et al.*, 2005) can be used to genetically label parvalbumin-positive neurons for *in vivo* calcium imaging experiments, thereby restricting analysis to a particular class of inhibitory cells. Similar experiments examining OD plasticity in other classes of genetically identified interneurons could determine the source of monocularly-biased inhibition required to explain the loss of pyramidal cell bias reported by Yazaki-Sugiyama *et al.* (2009) following delivery of intracellular PTX. Lastly, the discovery of genetic means to transiently silence populations of neurons (*e.g.* allatostatin receptors or halorhodopsin (Lechner *et al.*, 2002; Zhang *et al.*, 2007)) combined with advanced recording techniques (Niell and Stryker, 2008) suggests that it is ultimately possible to determine the contribution of inhibitory inputs from specified cell types to the response properties of both excitatory and inhibitory neurons across cortical layers.

5.4 Mechanisms of open-eye potentiation

In mice, MD produces a biphasic response in the visual cortex. Initial changes in OD occur through the weakening of deprived-eye responses, whereas longer periods of deprivation also drive open-eye potentiation. As discussed in Chapter 1, we favor a mechanism for the delayed appearance of open-eye potentiation based on the BCM theory and the molecular mechanisms of LTP. As the length of MD increases beyond 3 days, the sliding modification threshold determining the boundary between depressing and potentiating stimuli would shift to favor response potentiation, likely through a decrease in the ratio of NR2A to NR2B NMDAR subunits (Chen and Bear, 2007). In Chapter 4, we present evidence refuting an explicit link between NR2B and LTD, and argue that a decrease in the NR2A / B ratio in fact favors the induction of synaptic potentiation. As activity from the open eye remains unchanged during MD, this change in the modification threshold would then be permissive for the induction of LTP-like mechanisms at cortical synapses receiving open-eye inputs.

An alternative mechanistic framework for the potentiation of open-eye responses is provided by synaptic scaling. Reductions in the activity level of neurons can lead to global upregulations in synaptic strength, allowing for the homeostatic elevation of output activity (Turrigiano *et al.*, 1998). In the context of MD, the reduction in cortical activity following closure of the previously dominant contralateral eye would induce the upwards scaling of all synaptic weights, thereby strengthening open-eye responses (Turrigiano and Nelson, 2004).

A third possibility is suggested by a recent study showing that callosal inputs play a functionally significant role in the OD of neurons with receptive fields close to the vertical meridian (Restani *et al.*, 2009). Using 7d MD in rats, this study found that

blocking callosal activity via muscimol infusions into the visual cortex contralateral to the recording site restored binocularity in deprived animals. As this duration of MD has been shown to produce both deprived-eye depression and open-eye potentiation in mice (Frenkel and Bear, 2004), it is important to determine if the effects of blocking callosal activity occur through changes to the deprived- or open-eye response. Interestingly, when this was examined, the authors found opposite contributions of callosal inputs prior to and following MD. In non-MD animals, silencing the contralateral cortex increased binocularity of single unit responses by decreasing the strength of the response from the ipsilateral eye (Restani *et al.*, 2009). This result suggests that a large component of the ipsilateral-eye response is driven by activity that first crosses the midline at the optic chiasm (and drives the dominant response in the contralateral cortex), which then re-crosses the midline via trans-callosal projections. In contrast, the blockade of callosal activity following MD has no effect on ipsilateral-eye responses, but instead leads to enhanced responses evoked through the deprived eye.

These results require a complex inversion in the cortical circuitry following MD, with callosal projections having a net excitatory effect prior to MD, which changes after deprivation to primarily modulating activity indirectly via feedforward inhibition (Restani *et al.*, 2009). In this study the determination of the relative contribution of callosal activity to contralateral- or ipsilateral-eye responses relies on firing rate measures from single unit recordings as well as absolute VEP amplitudes. The manner in which these techniques were implemented in this study leads to several potential confounds. The authors used acute single unit recordings, making several penetrations with a recording electrode both before and after delivery of muscimol (or saline in control

animals). Thus, no attempt was made to record from the same cells before and after silencing callosal activity. Although such an experiment is technically very challenging, it would strengthen the conclusions concerning the opposing effects of callosal activity before and after MD. Additionally, in this study VEP recordings were made from superficial cortical depths, making the laminar origin of the response difficult to determine (discussed in detail in Chapter 3). Lastly, as these experiments were conducted under urethane anesthesia, it is important to determine whether a similar contribution of callosal activity is present in awake animals.

Nonetheless, the results of Restani *et al.* (2009) clearly demonstrate that callosal projections are involved in OD plasticity, and suggest the possibility that potentiation of open-eye responses may involve the strengthening of callosal projections subserving the open eye. Support for this idea comes from the finding that MD can affect OD not only in the cortex contralateral to the deprived eye (where the experiments described in this thesis were conducted), but also in the hemisphere ipsilateral to the deprived eye (He *et al.*, 2007).

5.4.1 Future experiments examining the mechanisms of open-eye potentiation

These three mechanisms proposed to account for open-eye potentiation – homosynaptic LTP of thalamocortical afferents subserving the non-deprived eye, heterosynaptic scaling of responses, or potentiation of trans-callosal drive dominated by the ipsilateral-eye – are not mutually exclusive and all three processes may be induced following prolonged MD.

The differential contributions of LTP and synaptic scaling to open-eye potentiation can be distinguished on the basis of several factors, including NMDAR

dependence and the effects of binocular deprivation. Both cortical LTP and open-eye potentiation are blocked by NMDAR antagonists (Kirkwood *et al.*, 1993; Wang and Daw, 2003; Cho *et al.*, 2009), whereas synaptic scaling is NMDAR independent (Turrigiano *et al.*, 1998; Turrigiano and Nelson, 2004). Additionally, BD, which presumably reduces cortical activity to a greater degree than MD and therefore should more readily induce scaling, does not produce potentiation of responses measured through layer 4 VEPs (Frenkel and Bear, 2004; Blais *et al.*, 2008). In contrast to these results, mice lacking TNFα have normal cortical LTP, but are deficient in synaptic scaling and lack open-eye potentiation (Kaneko *et al.*, 2008b).

A key future question is therefore to systematically extend these results to determine the relative contribution of LTP, synaptic scaling, and callosal projections to open-eye potentiation in the awake animal. The advantages of VEP recordings in mice, which easily permit chronic recordings from awake animals and thereby allow the separation of effects on the open-eye pathway from those on deprived-eye responses, suggest that this model system would be ideal to answer these questions. To assay the contribution of callosal inputs to OD plasticity, Restani *et al.* (2009) silenced the cortex contralateral to the recording site through infusion of muscimol. The conclusions of this study depend on the effective silencing of the entire contralateral visual cortex, which is difficult to demonstrate unequivocally. A better approach might be to remove visual drive from the contralateral hemisphere by infusing muscimol into the LGN contralateral to the recording site, while continuously recording VEP responses. Due to its small volume when compared to the cortex, drug application to the LGN is more likely to result in complete silencing of activity. Additionally, performing these experiments in awake

mice removes potential confounds related to anesthesia. If results similar to those reported previously in rats are observed in these experiments, it will be important to determine if they also occur in animals with disrupted open-eye potentiation, such those lacking NR1 or TNFα (Sawtell *et al.*, 2003; Kaneko *et al.*, 2008b).

In addition to the distinctions described above, LTP and synaptic scaling also display differential requirements for the insertion of GluR1 containing AMPARs at synapses. In a series of experiments analogous to those reported in this thesis utilizing HSV-G2CT to probe the role of LTD in deprived-eye depression, we have begun to use HSV-mediated expression of the GluR1CT peptide to determine the contribution of LTP mechanisms in open-eye potentiation. GluR1CT blocks LTP by preventing the delivery of GluR1-containing AMPARs to synapses (Shi *et al.*, 2001) and has been used to demonstrate the involvement of LTP mechanisms *in vivo* (Rumpel *et al.*, 2005; Frenkel *et al.*, 2006). Additionally HSV-mediated delivery of GluR1CT in the visual cortex has been used successfully in this laboratory (Frenkel *et al.*, 2006). Importantly, synaptic scaling in the visual cortex depends on delivery of GluR2-containing AMPARs and is not blocked by GluR1CT (Gainey *et al.*, 2009). Therefore, if HSV-expressed GluR1CT blocks open-eye potentiation following 7d MD, this would strongly argue that LTP, as opposed to synaptic scaling, is the critical mechanism underlying this phenomenon.

It is clear that like LTD, the mechanisms of LTP vary across cortical layers (Wang and Daw, 2003). With regards to deprived-eye depression, these mechanistic differences lead to independent responses to MD in layers 4 and 2/3 (Chapter 3 and (Liu *et al.*, 2008)). What remains to be determined is whether open-eye potentiation can occur in a similarly independent manner. To address this, I propose to utilize layer-specific

expression of the GluR1CT peptide based on the endogenous expression pattern of *RORß,* a gene encoding a brain-expressed nuclear receptor (Carlberg *et al.*, 1994). Importantly, the expression of *RORß* is restricted to layer 4 of the visual cortex (Allen Mouse Brain Atlas, 2009, http://mouse.brain-map.org), an expression pattern mimicked in BAC transgenic mice expressing GFP from the *RORß* locus (GENSAT project; (Heintz, 2004)). Given the success of the BAC transgenic strategy with regards to GFP expression, we will attempt to drive Cre recombinase from the same BAC, thereby restricting Cre expression to layer 4. Due to potential developmental or compensatory effects resulting from the prolonged disruption of LTP mechanisms, I propose to use viral infection of the visual cortex to deliver GluR1CT immediately prior to manipulating visual experience. In order to restrict expression of the peptide to Cre-expressing neurons, an inverted FLEX cassette will be used (Schnutgen and Ghyselinck, 2007). Because the peptide coding sequence is inverted using the FLEX system, this approach will eliminate off-target expression that can occur when using *loxP-STOP-loxP* systems. AAV-mediated gene delivery of FLEX constructs been successfully demonstrated *in vivo* (*e.g.* see (Atasoy *et al.*, 2008; Cardin *et al.*, 2010)), and therefore we will pursue a similar strategy. Notably, once validated, this approach could be easily extended to other mouse lines expressing Cre in restricted subsets of cells, such as parvalbumin-positive interneurons.

Given the differential timecourses of deprived-eye depression and open-eye potentiation following MD, it is possible that the prior weakening of deprived-eye inputs is required to allow subsequent potentiation to occur. Alternatively, input activity to the cortex may be sufficiently decreased following MD to allow changes in the modification

threshold in the absence of any decreases in synaptic strength. To address this question, we will utilize the G2CT peptide to block deprived-eye depression in layer 4 and test for changes in the open-eye response following 7d MD. Given that G2CT is ineffective in blocking LTD in layer 2/3 (Crozier *et al.*, 2007), a potential finding of open-eye potentiation measured through layer 4 VEPs in G2CT expressing animals could result from processes dependent on LTD in supragranular layers. This possibility could be addressed by pairing G2CT expression with AM251 to block LTD and deprived-eye depression in both layer 4 and layer 2/3 (Crozier *et al.*, 2007; Liu *et al.*, 2008).

5.5 Mechanisms of OD plasticity in adult mice

The work presented in this thesis focuses on the mechanisms of OD plasticity in juvenile mice. Early work in mice suggested that MD fails to shift OD when it is initiated after about 5 weeks of age (Gordon and Stryker, 1996), consistent with the well established concept of a critical period for visual cortical plasticity (Hubel and Wiesel, 1970; Morishita and Hensch, 2008). However there is now broad agreement from multiple labs utilizing multiple methods of analysis that adult (> P60) MD produces a robust OD shift in mouse visual cortex (Sawtell *et al.*, 2003; Lickey *et al.*, 2004; Pham *et al.*, 2004; Tagawa *et al.*, 2005; Frenkel *et al.*, 2006; Hofer *et al.*, 2006b; Fischer *et al.*, 2007; Sato and Stryker, 2008; Hofer *et al.*, 2009; Kameyama *et al.*, 2010). What remains unclear is whether these changes in OD reflect plasticity of both deprived- and non-deprived-eye inputs to the cortex in a manner similar to that in juvenile mice.

A number of the studies cited above measured OD acutely either through single-unit recording, visually-driven *Arc* induction, or intrinsic optical imaging (Tagawa *et al.*, 2005; Fischer *et al.*, 2007; Hofer *et al.*, 2009). Although all of these studies show shifts

in the OD ratio towards the open eye following MD, they do not clearly identify whether changes in the open- or deprived-eye pathways are responsible. In studies that did examine absolute changes in response following MD, all studies report potentiation of open-eye responses. Several of these also find depression of the deprived-eye (Frenkel *et al.*, 2006; Fischer *et al.*, 2007), whereas others report that adult OD plasticity is restricted to open-eye potentiation (Hofer *et al.*, 2006b; Sato and Stryker, 2008; Kameyama *et al.*, 2010). Regardless of the source of changes in OD, there is consensus that the effects of OD occur more slowly in adults relative to juveniles. In juvenile mice OD shifts have been reported in as little as 24 hours (Liu *et al.*, 2008), and strong shifts are seen after 3-4 days of MD (Gordon and Stryker, 1996; Frenkel and Bear, 2004; Sato and Stryker, 2008). In contrast, 7 days of MD is typically required to induce OD plasticity in adult mice (Frenkel *et al.*, 2006; Sato and Stryker, 2008). The mechanistic basis for this difference is unclear, but could potentially be related to a delayed timecourse of metaplasticity in the adult visual cortex.

5.5.1 Future experiments investigating mechanisms of OD plasticity in adult mice

The G2CT peptide presents an opportunity to directly test the contribution of deprived-eye depression to adult OD plasticity in mice. I propose to infuse HSV-G2CT into the visual cortex of adult (> P60) mice and monitor changes in OD plasticity induced by 7d MD through VEP recordings, in a manner analogous to that described in Chapter 3. Despite its limitations, the chronic VEP technique represents a proven and reliable method for clearly localizing the effects of MD to either the deprived- or non-deprived-eye response. If G2CT fails to block OD plasticity and deprived-eye depression is not observed, this would suggest that OD plasticity in adults is primarily mediated through

effects on the open-eye pathway. In contrast, the failure of G2CT to block deprived-eye depression in adults would indicate that mechanisms other than LTD in layer 4 are responsible for this process in adults.

5.6 Relationship of mouse OD plasticity to other species

I have argued previously that mice represent an advantageous model system for studying sensory plasticity. The relatively undifferentiated visual cortex in mice coupled with the early establishment of binocularity by the convergence of retinotopically-matched thalamic inputs onto layer 4 neurons facilitates recording of visual responses through VEPs. Additionally, the genetic tools available in mice have led to rapid advances in understanding the molecular mechanisms governing the strength and plasticity of synaptic transmission. We have harnessed these advantages in the experiments described in this thesis in order to demonstrate that AMPAR endocytosis, a key expression mechanism of LTD, is critically important to the loss of deprived-eye responses following MD.

However the question remains, are the mechanistic findings obtained in mice generalizable to other species? Perhaps the greatest difference relative to mice is the presence of anatomical OD columns in cats and monkeys resulting from the eye-specific segregation of thalamocortical axons in layer 4 (Hubel and Wiesel, 1972; Shatz and Stryker, 1978). MD leads to shrinkage of columns innervated by the deprived eye that is accompanied by expansion of open-eye columns; changes that are mirrored in the electrophysiological response properties of individual neurons in layer 4 (Hubel *et al.*, 1977; Shatz and Stryker, 1978). It would appear likely that deprived-eye depression and LTD are involved in the shrinkage of deprived-eye columns, while open-eye potentiation

and LTP correspondingly mediate the expansion of open-eye columns. The successful demonstration that HSV-G2CT blocks deprived-eye depression in mice suggests a means of testing this hypothesis using G2CT and GluR1CT to block LTD and LTP, respectively.

If the findings from mice presented in this thesis extend to other species, it suggests that these mechanisms are also involved in the pathology of amblyopia in humans. Amblyopia can result from a number of conditions, including strabismus, childhood cataracts, and unbalanced astigmatism (Doshi and Rodriguez, 2007). It can also develop if one eye must be covered with a patch during childhood. It is therefore possible that briefly blocking the molecular mechanisms of LTD, possibly through weak NMDAR antagonists such as FDA-approved memantine (Rogawski and Wenk, 2003), in conjunction with applying an eye patch could prevent the development of amblyopia. Furthermore, understanding the processes involved in the development of amblyopia will lead to possible approaches to reverse these processes. Thus, a mechanistic understanding of OD plasticity in mice may eventually lead to the treatment of amblyopia and thereby potentially cure an important cause of blindness in humans.

Appendix 1

Bidirectional ocular dominance plasticity of inhibitory networks

This appendix was prepared as a Commentary for publication in Cell Science Reviews together with Dr. Mark F. Bear.

A1.1 Abstract

Monocular visual deprivation (MD) produces profound changes in the ocular dominance (OD) of neurons in the visual cortex. MD shifts visually evoked responses away from the deprived eye and towards domination by the open eye. Over thirty years ago, two opposing theories were proposed to account for these changes: either through effects on excitatory visual drive, thereby shifting the balance of excitation in favor of the open eye, or through effects on intracortical inhibition, thereby suppressing responses from the deprived eye. In the intervening years, a scientific consensus emerged to localize the effects of MD to plasticity at excitatory connections in the visual cortex. A recent study by Yazaki-Sugiyama and colleagues challenges this idea and reports a functionally significant bidirectional OD shift in fast-spiking inhibitory interneurons.

A1.2 A brief history: Inhibition, ocular dominance and ocular dominance plasticity

Ocular dominance plasticity is the preeminent model of experience dependent plasticity in the mammalian nervous system. Retinotopically-matched inputs from both eyes converge on individual cortical neurons to produce binocular vision. The strength and persistence of these connections is highly susceptible to manipulations that asymmetrically degrade the quality of visual experience, such as monocular deprivation (Wiesel and Hubel, 1963). Awake recordings in juvenile mice have shown that a brief period of monocular lid suture produces an OD shift solely through the loss of responsiveness to the deprived eye (Frenkel and Bear, 2004). Broadly speaking, there are two categories of changes that could account for the observed reduction in deprived-eye responses, which are not mutually exclusive. First, excitatory drive into the cortex from the deprived eye could become weakened, thereby decreasing responses driven by the deprived eye. Second, intracortical inhibition of deprived-eye inputs could increase following MD, thereby suppressing or "masking" visual responses evoked by the deprived eye.

A large body of work in kittens has supported the former idea, demonstrating that MD leads to a reduction in cortical innervation by excitatory thalamocortical axons subserving the deprived eye (Shatz and Stryker, 1978), which is accompanied physiologically by considerable weakening of deprived-eye inputs (Singer, 1977; Tsumoto and Suda, 1978; Mitzdorf and Singer, 1980). The alternative hypothesis, that the OD shift observed following MD results from altered intracortical inhibition, was originally suggested over 30 years ago, again based on experiments in kittens (Duffy *et al.*, 1976; Burchfiel and Duffy, 1981). Local iontophoretic administration of bicuculline,

a $GABA_A$ receptor antagonist, restores binocularity and deprived-eye responses in cortical neurons following MD (Burchfiel and Duffy, 1981). However, attributing these results to MD-induced plasticity of inhibitory networks is difficult as blockade of GABA receptors in normal cats both broadens receptive field properties and unmasks previously subthreshold responses (Sillito, 1975). A similar unmasking was also observed for responses evoked through either the deprived or non-deprived eye in MD animals (Sillito *et al.*, 1981).

In recent years rodents have emerged as the preferred system for studies of visual cortical plasticity, and questions of the relative contribution of MD-induced changes in excitation and inhibition have been readdressed with new approaches. Several lines of evidence demonstrate that the mechanisms of homosynaptic long-term depression play a central role in the weakening of visual responses (reviewed in (Smith *et al.*, 2009)). Brief MD leads to reduced surface expression of AMPA-type glutamate receptors and occludes subsequent induction of LTD (Heynen *et al.*, 2003; Crozier *et al.*, 2007). Furthermore, preventing a weakening of excitatory transmission in the cortex by blocking AMPA receptor endocytosis completely prevents the loss of deprived-eye responses following MD (Yoon *et al.*, 2009).

Additional work has shown that cortical inhibitory networks can also be strongly affected by MD. Brief periods of deprivation induce potentiation of feedback inhibition involving fast-spiking (FS) basket cells in layer 4 of the monocular visual cortex in rats studied *ex vivo* (Maffei *et al.*, 2006). Additionally, two recent studies used calcium imaging in mice *in vivo*, and found MD-induced OD shifts in both excitatory and inhibitory neurons (Gandhi *et al.*, 2008; Kameyama *et al.*, 2010). Thus, both the inputs

to and outputs from inhibitory neurons can be modified by deprivation.

In a recently published article in the journal *Nature*, Yazaki-Sugiyama *et al.* (2009) take a direct approach to study plasticity of inhibitory interneurons and describe a novel and surprising response of these cells to MD. The authors used *in vivo* intracellular recordings in mice to assay the OD of individual cortical neurons. This approach enabled them to directly compare OD and the consequences of MD in pyramidal neurons and FS interneurons. In addition, by slowly infusing picrotoxin into pyramidal neurons, they could examine the contribution of somatic inhibition to OD and the expression of OD plasticity. This approach enabled the investigators to address a number of interesting questions, discussed below.

A1.3 How does OD differ in excitatory and inhibitory cells, and how is it shaped by inhibition?

In pyramidal neurons recorded from control (non-deprived) mice, visually-evoked spiking displays a biased response, with greater firing elicited by stimulation of the contralateral eye. The degree of contralateral bias observed with intracellular recordings (roughly 2:1) is similar to what has been observed previously using either extracellular single-unit recordings or visually-evoked potentials (Gordon and Stryker, 1996; Frenkel and Bear, 2004), and matches the anatomical density of projection neurons serving the two eyes in the LGN (Coleman *et al.*, 2009). Interestingly, however, intracellular recordings from FS interneurons, putatively parvalbumin-positive large basket cells, exhibited strongly binocular responses, without bias towards either eye (Yazaki-Sugiyama *et al.*, 2009) (Figure A1.1A, left column).

The observed binocularity of FS cells differs from two other recent reports, both of which found contralaterally dominated responses in inhibitory neurons. In

parvalbumin-positive interneurons from normally reared rats, visually-evoked c-Fos induction showed a 2:1 contralateral bias (Mainardi *et al.*, 2009). Additionally, using GAD67-GFP knock-in mice and *in vivo* calcium imaging, Gandhi *et al.* (Gandhi *et al.*, 2008) report similar OD distributions for excitatory and inhibitory neurons in control mice, with both populations showing contralaterally biased responses.

The basis for these differences remains to be determined. Yazaki-Sugiyama *et al.* discuss two potential causes. First, GAD67-GFP knockin mice have reduced levels of GABA (Tamamaki *et al.*, 2003), which may impact the normal development of OD (Hensch, 2005). However, Kameyama *et al.* (2010) performed similar experiments in VGAT-Venus mice, which have normal cortical GABA levels. These authors also show a strong contralateral bias in the OD distribution of inhibitory cells. Secondly, *in vivo* calcium imaging is restricted to superficial layers. However, this second potential confound is unlikely to be meaningful given that the cells recorded by Yazaki-Sugiyama *et al.* were also located in layer 2/3. A third possibility which remains to be addressed is that the high spontaneous firing rates of FS neurons (~ 4x higher than pyramidal neurons) reported by Yazaki-Sugiyama *et al.* obscure visually-evoked responses and lead to unreliable or inaccurate measurements of binocularity (discussed further below).

To demonstrate the functional consequences of somatic inhibition on the OD of excitatory neurons, Yazaki-Sugiyama *et al.* performed technically challenging recordings from pyramidal neurons in which the $GABA_A$ antagonist picrotoxin (PTX) was slowly infused intracellularly through the recording pipette. This technique allows the examination of OD in the same neuron both prior to and following intracellular blockade of $GABA_A$ receptors, thereby isolating the response to excitatory inputs. In non-MD

control mice, PTX greatly increased the binocularity of visually driven responses (Figure A1.1A, left column). Notably, binocularity was increased in both neurons with an initial contralateral bias (the majority of cells) and those with an initial ipsilateral bias.

This result suggests that excitation onto pyramidal cells is binocularly balanced, with the maintenance of OD biases dependent on asymmetric inhibition. This interpretation requires the existence of biased inhibitory networks, which might be expected to include FS cells due to their apparently central role in OD (Hensch, 2005; Katagiri *et al.*, 2007). The finding that FS cells lack such a bias and are in fact strikingly binocular (Yazaki-Sugiyama *et al.*, 2009) suggests that monocularly-biased inhibition relies on other interneuron subtypes and that these subtypes play a greater role in modulating pyramidal cell responses than previously thought. Alternatively, the apparent binocularity of FS cell spike-bias might actually reflect the averaging of two populations of neurons, each slightly biased towards one eye.

The findings are surprising because feed-forward (excitatory) projections from the LGN (targeting layer 4 and deep layer 2/3 in mice) show an anatomical bias favoring the contralateral eye by approximately a 2:1 ratio, equivalent to the physiological response ratio recorded in layer 4 (Frenkel and Bear, 2004; Coleman *et al.*, 2009). Thus, it has been assumed that the contralateral bias of cortical responses simply reflects excitatory innervation density, an idea well supported by recordings from species with anatomically defined OD columns, such as the cat and monkey. The model of balanced ocular dominance in excitatory neurons of mouse visual cortex requires new assumptions about how inputs from the two eyes target excitatory and inhibitory neurons, and how these cells interconnect.

The possibility needs to be considered that the apparent loss of biased responses in pyramidal cells following PTX infusion is caused by saturation of pyramidal cell firing rates, which might clip the dominant contralateral eye response and obscure eye-specific differences in excitatory input. Previous results have shown elevated spontaneous firing rates using this technique (Nelson *et al.*, 1994), and although there is a trend towards elevated firing rates following PTX in the current study, the difference is not significant. Perhaps this concern could be addressed in future studies by varying the contrast of the visual stimulus or by passing hyperpolarizing current into the neuron before and after PTX infusion.

A1.4 How does brief (3 day) MD shift OD in excitatory and inhibitory cells, and how is expression of the shift shaped by inhibition?

Three days of contralateral eye MD produces a shift in the OD of visual cortical neurons away from the previously dominant contralateral eye and towards the ipsilateral non-deprived eye (Gordon and Stryker, 1996; Frenkel and Bear, 2004). In measurements of spike bias from pyramidal neurons following MD, Yazaki-Sugiyama *et al.* found a similar loss of contralateral bias, with the average response across neurons having become highly binocular. In contrast FS cells, which had binocular responses in control mice, were reported to acquire biased responses and shift *towards* the deprived (contralateral) eye following MD (Figure A1.1A, middle column).

On the surface, this observation in FS cells again disagrees with Ghandi *et al*. (2008) who find that the OD of inhibitory neurons in the GAD67-GFP mice does not change from baseline after 3 days of MD. However, recall that the inhibitory neurons studied by Ghandi *et al*. in non-deprived mice were, like the excitatory neurons, initially biased toward the contralateral eye. Thus, both studies are actually in agreement that

inhibitory neurons show a contralateral-eye bias after 3 days of MD. The question is whether this reflects a *shift* in OD, or is rather the maintenance of the baseline, non-deprived state.

An interesting clue to resolving the difference is the finding by Yazaki-Sugiyama *et al.* that spontaneous activity in FS neurons is dramatically (> 5x) reduced by MD. As mentioned previously, high spontaneous activity could mask the contralateral-eye bias of FS cells in non-MD control animals. The apparent shift in OD of FS cells observed by Yazaki-Sugiyama *et al.* could possibly be explained by an unmasking of biased inputs due to the reduction in spontaneous activity.

An additional recent study in VGAT-Venus mice found that inhibitory neurons show a strong initial contralateral bias that shifts towards the open eye after 2 days of MD (Kameyama *et al.*, 2010). Unlike GAD67-GFP mice, cortical GABA levels are normal in these animals, which may partly explain the contrasting results. Additional differences in aesthesia and recording technique (intracellular electrodes *vs*. calcium imaging) may also contribute to the variety of results regarding the response of inhibitory cells to short (2-3 day) periods of MD.

Yazaki-Sugiyama *et al.* used intracellular PTX to block somatic inhibitory inputs and isolate excitatory drive onto pyramidal cells following brief MD (Figure A1.1A, middle column). Infusion of PTX did not change the overall degree of binocularity, suggesting that on a population level, changes in inhibition make a minor contribution to expression of the OD shift. Interestingly, however, PTX infusion produced an inversion of OD for each individual neuron: those exhibiting a contralateral bias prior to $GABA_A$ blockade became dominated by the ipsilateral eye following intracellular PTX

application, and vice versa.

These results suggest that monocularly biased excitation, which was not observed in non-MD control neurons with PTX, emerges following visual deprivation. In addition, the conversion of open-eye dominated responses to deprived-eye dominated responses following PTX suggests the presence of inhibitory networks biased to the open-eye after 3 days of MD (Figure A1.1B). Given that open-eye dominated FS cells were not observed after short-term MD, it appears that there must be a key role for other inhibitory cell types in shaping OD biases.

A1.5 How does long-term (14 day) MD shift OD in excitatory and inhibitory cells?

To determine the effect of prolonged MD, Yazaki-Sugiyama *et al.* extended the period of deprivation to 14 days (LTMD). In pyramidal neurons LTMD did not produce any further change in OD, largely consistent with prior results (Gordon and Stryker, 1996; Frenkel and Bear, 2004). However, the FS cells again yielded surprises. LTMD led to responses dominated by the ipsilateral non-deprived eye, a reversal of the contralateral bias seen after 3d MD. Thus Yazaki-Sugiyama *et al.* describe a bidirectional response to MD in FS cells: initially binocular neurons acquire contralateral (deprived) eye bias during the early phases of MD, which reverses to ipsilateral (non-deprived) eye bias with longer periods of deprivation (Figure A1.1A, top row).

The finding of a strong OD shift away from the deprived eye in FS neurons is consistent with the observations of Ghandi *et al.* in the GAD67-GFP mice (Gandhi *et al.*, 2008). In contrast, parvalbumin positive cells failed to exhibit a shift in OD following 120 days of MD when assayed via visually-evoked c-Fos in rats (Mainardi *et al.*, 2009). These discrepant findings may be the result of varying plasticity among interneuron

subtypes or species-specific mechanisms.

A1.6 OD plasticity in adults

Early work in mice suggested that MD fails to shift OD when it is initiated after about 5 weeks of age (Gordon and Stryker, 1996), consistent with the well established concept of a critical period for visual cortical plasticity. However there is now broad agreement from multiple labs utilizing multiple methods of analysis that adult (~ P60) MD produces a robust OD shift in mouse visual cortex (Sawtell *et al.*, 2003; Lickey *et al.*, 2004; Pham *et al.*, 2004; Tagawa *et al.*, 2005; Frenkel *et al.*, 2006; Hofer *et al.*, 2006b; Fischer *et al.*, 2007; Sato and Stryker, 2008; Hofer *et al.*, 2009; Kameyama *et al.*, 2010). Notably, the pre-PTX spike bias data from adult MD mice reported by Yazaki-Sugiyama *et al*. also support the existence of adult OD plasticity. In non-MD mice, neurons with ipsilaterally-dominated responses were not observed, whereas MD progressively increased the percentage of cells dominated by the non-deprived eye such that after LTMD, 50% of neurons exhibited an apparent OD shift and were now dominated by the non-deprived ipsilateral eye (Figure A1.2). Although summary data and statistical analysis of adult OD plasticity were not included in the paper, these results support the idea that robust OD plasticity lacks a critical period and continues into adulthood, where it is expressed through changes in excitatory neurotransmission.

Therefore, it is puzzling that Yazaki-Sugiyama *et al*. used OD plasticity in adults as a control experiment to demonstrate the relevance of bidirectional changes in intracortical inhibition to the consequences of MD. The authors appear to argue that the absence of changes in spike bias following PTX infusion in adults is evidence that inhibitory network changes are critical to OD plasticity because OD shifts are only

observed at ages when these changes occur. The repeated finding that OD plasticity is in fact induced by adult MD suggests instead the opposite conclusion: reorganization of inhibitory function is not a necessary component of OD shifts, but rather may be a secondary effect to changes in excitatory inputs that is restricted to juvenile animals.

A1.7 Caveats and unresolved issues

The finding of a bidirectional response to MD in FS cells suggests a complex role for inhibition in shaping OD. To suggest a mechanism supporting this bidirectional plasticity and accounting for the seemingly paradoxical shift towards the deprived eye, the authors present modeling data showing that a spike-timing-dependent plasticity (STDP) rule, if properly constructed, predicts a bidirectional OD shift in accordance with the observed data. However, it is not clear whether this STDP rule has a physiological basis in layer 2/3 FS cells in mouse visual cortex. First, Yazaki-Sugiyama *et al.* show that the STDP rule is by itself insufficient to account for the eventual loss of deprived-eye responses and reversal of spike bias observed with prolonged MD because the rule favors elimination of active synapses over inactive ones. To compensate, the STDP rule must be supplemented by the slow elimination of inactive synapses. Second, the shape of the STDP rule used in the model is based on recordings from rat somatosensory cortex and includes regions in which synaptic weights are potentiated, which were not observed in the experimental data (Lu *et al.*, 2007).

A further caveat concerns the use of intracellular PTX, which allowed Yazaki-Sugiyama *et al.* to determine the functional consequences of inhibitory inputs and their role in shaping pyramidal cell OD. This manipulation aims to remove inhibitory inputs from only the recorded cell, leaving inhibitory networks within the cortex intact.

Therefore, other pyramidal cells synapsing onto the recorded neuron are themselves still subject to normal inhibitory influences and still have their spike outputs, which comprise a large extent of the recorded cell's excitatory inputs, modulated by inhibition. Additionally, to demonstrate the efficacy of intracellular PTX on blocking inhibitory inputs, the authors examined mIPSCs using whole-cell patch recordings with internal solution containing PTX. Unfortunately, given the dramatically slower diffusion of compounds from sharp microelectrodes, this experiment is an inadequate control for the intracellular delivery of PTX *in vivo*. Demonstrating the timecourse for the blockade of inhibitory currents evoked through electrical stimulation or GABA iontophoresis by PTX delivered through sharp electrodes could address this issue more clearly.

A related concern is that the use of a cesium acetate based internal solution in the PTX experiments as opposed to the potassium acetate solution used in prior experiments may impact the observed spike biases. Cesium is a well-known blocker of a variety of potassium channels, and as such can have effects on membrane depolarization and spike output. This may account for the apparent reduction in contralateral bias in non-MD animals prior to PTX (average bias approx. 0.13) when compared to the average bias reported from experiments using potassium acetate filled electrodes (average bias approx. 0.3).

A key result of Yazaki-Sugiyama *et al.* is the contrasting initial OD and subsequent response to MD in pyramidal and FS neurons. This finding critically depends on the ability to unequivocally identify these respective neuronal cell types. Although examples of biocytin filled neurons are presented, it is unclear to what extent cell-type classifications were confirmed morphologically.

Lastly, it must be noted that the use of barbiturate anesthesia by Yazaki-Sugiyama *et al.* could potentially confound the results by accentuating inhibition within the cortex. Previous work has shown that administration of barbiturates can in some circumstances alter OD (Pham *et al.*, 2004). The choice of anesthetic used while measuring OD may contribute in part to the contrasting reports of OD plasticity in inhibitory cells. Gandhi *et al*. (2008) used isoflurane in combination with chlorprothixene for *in vivo* calcium imaging and found contralaterally biased inhibitory cells showing an OD shift after 7 but not 2 days of MD. In contrast, anesthetic was not used during c-Fos induction experiments in parvalbumin-positive cells which showed contralaterally biased responses that remained unaffected by >120d MD (Mainardi *et al.*, 2009).

The results presented by Yazaki-Sugiyama *et al.* raise several interesting questions about the role of inhibitory neurons in OD that need to be addressed with future experiments. The initial binocularity of FS neurons and their paradoxical shift towards the deprived eye have not been observed previously (e.g. (Gandhi *et al.*, 2008; Mainardi *et al.*, 2009)). Recent advances in extracellular recording techniques facilitate the distinction between excitatory and inhibitory neurons in mice (Niell and Stryker, 2008). Applying this approach to OD plasticity would allow both confirmation of the paradoxical shift in FS neurons and the extension of these results to deeper cortical layers. Additionally, a recently generated parvalbumin-Cre mouse (Hippenmeyer *et al.*, 2005) can be used to genetically label parvalbumin-positive neurons for *in vivo* calcium imaging experiments, thereby restricting analysis to a particular class of inhibitory cells while avoiding the confound of reduced GABA levels in GAD67-GFP knock-in mice (Gandhi *et al.*, 2008). Similar experiments examining OD plasticity in other classes of

genetically identified interneurons could determine the source of monocularly-biased inhibition required to explain the loss of pyramidal cell bias following delivery of PTX. Lastly, the discovery of genetic means to transiently silence populations of neurons (*e.g.* allatostatin receptors or halorhodopsin (Lechner *et al.*, 2002; Zhang *et al.*, 2007)) combined with advanced recording techniques (Niell and Stryker, 2008) suggests that it is ultimately possible to determine the contribution of inhibitory inputs from specified cell types to the response properties of both excitatory and inhibitory neurons across cortical layers.

Applying Occum's razor — the notion that the simplest solution is usually the correct one — to the problem of OD plasticity has always suggested that the initial contralateral bias and its subsequent loss after MD could both be well explained by the relative strength of eye-specific excitatory inputs from the thalamus to the cortex. In describing a novel role for the plasticity of inhibitory networks during MD, the results of Yazaki-Sugiyama *et al.* require far more complexity in the cortical circuit than previously assumed in order to explain both initial conditions and the response to MD, while raising many interesting questions for future study.

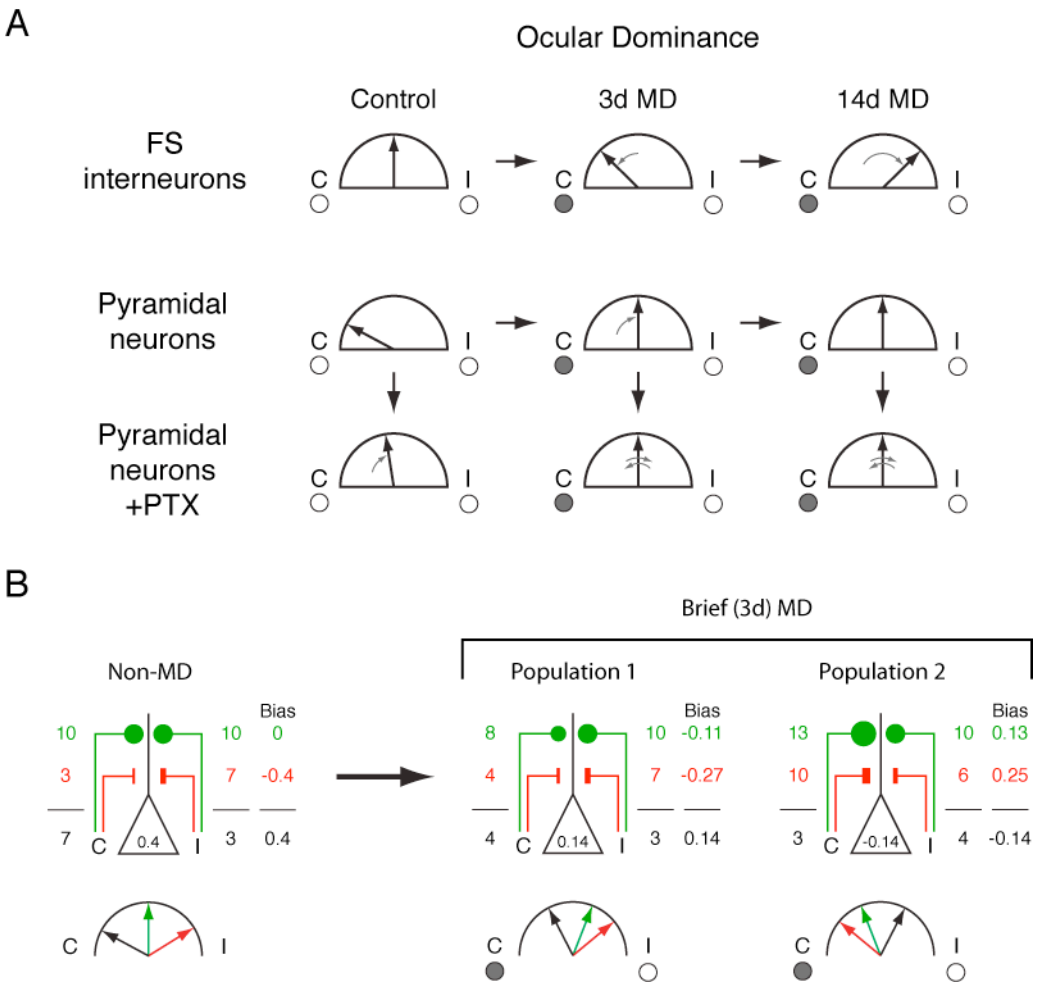


Figure A1.1 – Consequences of bidirectional OD plasticity in fast-spiking GABA circuits

A. Schematic summary of OD data for FS and pyramidal neurons reported by Yazaki-Sugiyama *et al*. Black arrows indicate OD bias under a given condition, ranging from purely contralateral-eye responses to purely ipsilateral-eye responses. Small grey arrows indicate changes relative to control (non-MD or no PTX conditions). *Top row:* In non-MD mice, FS cells exhibit binocular responses, which first shift towards the deprived eye after 3d MD, then become biased to the open eye after 14d MD. *Middle row:* Pyramidal neurons in non-MD mice display contralaterally biased responses, which become binocular following either 3 or 14d MD. *Bottom row:* Spike bias in pyramidal neurons after intracellular PTX application. In non-MD mice, responses become binocular with intracellular PTX. This result requires the existence of monocularly biased inhibitory neurons. Notably FS cells in non-MD animals are binocular. Either 3 or 14d MD induces OD shifts in pyramidal neurons, leading to binocular responses. After 3d MD, PTX leads to inversion of responses in each individual pyramidal neuron, requiring both that some cells develop contralaterally biased excitation following contralateral eye MD and the presence of open-eye dominated inhibition. B. Schematic representation of the relative strengths of excitatory and inhibitory inputs to produce the observed data.

Numbers represent relative excitatory (green) and inhibitory (red) synaptic strengths of inputs driven by stimulation of the contralateral or ipsilateral eye. Number in center of pyramidal neuron is the overall spike bias, and numbers to left and right represent the influences of contralateral and ipsilateral visual stimulation, respectively. Excitatory and inhibitory inputs sum to generate the overall response and resulting spike bias. *Left panel:* In order to produce contralaterally biased responses in neurons receiving binocularly balanced excitation, ipsilaterally dominated inhibitory inputs (and therefore ipsilaterally dominated inhibitory neurons) are required. *Right panels:* After MD excitatory neurons are on average binocular, comprised of two populations showing a modest bias to either eye. These overall biases invert in the absence of inhibition, thus the excitatory inputs have the opposite OD bias from the neuron itself.

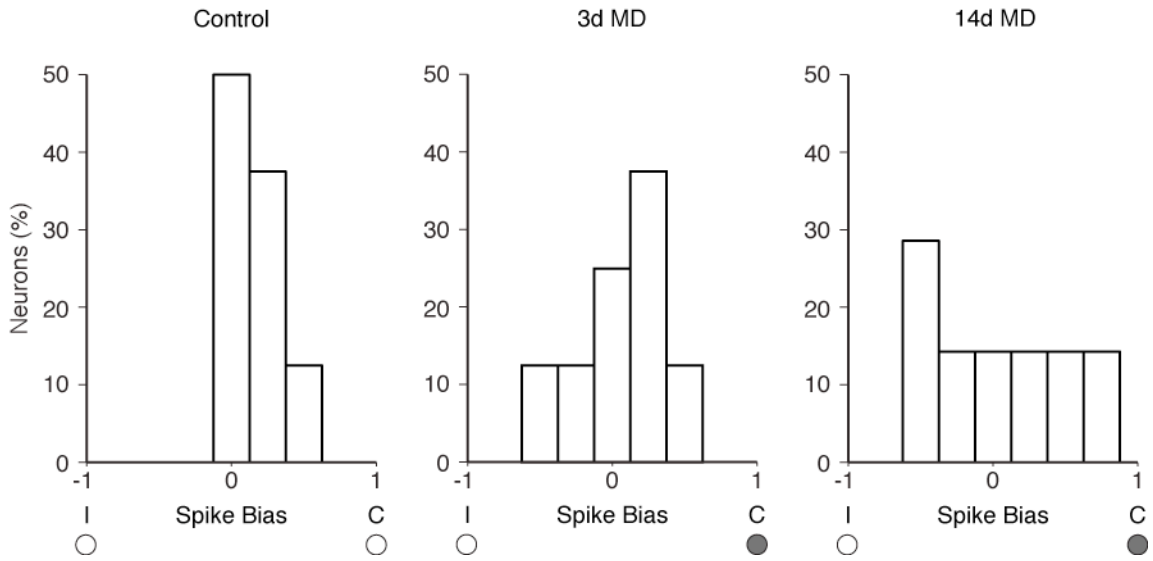


Figure A1.2 – OD plasticity in adult mice

Summary histograms based on spike biases reported by Yazaki-Sugiyama *et al.* in adult mice. In non-MD control animals, nearly all pyramidal neurons show contralaterally biased responses. 3d MD leads to an apparent OD shift, with some neurons now dominated by the ipsilateral non-deprived eye. Following 14d MD, over 50% of neurons now show open-eye dominated spike biases. These data support previous findings that mice lack a critical period and exhibit robust OD plasticity into adulthood. Data obtained from Yazaki-Sugiyama *et al.* (2009), figure 4b, before PTX condition.

Abbreviations

AAV – adeno-associated virus
ACSF – artificial cerebrospinal fluid
AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR – AMPA receptor
BAC – bacterial artificial chromosome
BD – binocular deprivation
BD – binocular deprivation
CSD – current source density
dLGN – dorsal LGN
ECM – extracellular matrix
EPSC – excitatory postsynaptic current
fEPSP – field excitatory postsynaptic potential
FS – fast spiking
GABA – γ-aminobutyric acid
GFP – green fluorescent protein
HSV – herpes simplex virus
IEG – immediate early gene
LGN – lateral geniculate nucleus
MD – monocular deprivation
NAc – nucleus accumbens
NMDA – N-methyl-d-aspartate
NMDAR – NMDA receptor
OD – ocular dominance
OGB1 – Oregon green BAPTA–1
PP1 – protein phosphatase 1
RORß – retinoid-related orphan receptor-ß
SRP – stimulus-selective response potentiation
TNFα – tumor necrosis factor-α
VEP – visually evoked potential

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