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Molecular substrates of plasticity in the developing visual cortex

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Molecular Substrates of Plasticity in the Developing Visual Cortex

by

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To my parents

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Molecular substrates of plasticity in the developing visual cortex

Sharif Taha

In the mammalian visual system, information from the two eyes does not converge until it reaches the primary visual cortex. This convergence creates the substrate for an extraordinary synaptic plasticity, one in which afferent activity is the engine which drives both physiological and anatomical change. Forty years of electrophysiological experiments have beautifully defined the rules which govern this plasticity, and shown that they largely conform to Hebb's postulate: effective, robustly depolarizing inputs are strengthened, and ineffective connections are weakened. More recent anatomical experiments have demonstrated that surprisingly quick anatomical changes contribute to plasticity in both the cortex and the thalamocortical afferents.

The intracellular signaling events which mediate these changes largely remain a mystery. The recent characterization of ocular dominance plasticity in the mouse, however, brings a genetically tractable model organism to bear on the problem. This thesis describes experiments in the mouse using both pharmacological and genetic approaches to understanding the molecular substrates of ocular dominance plasticity. In particular, the events underlying long-lasting, anatomical change were targeted for study. Chapter Two describes studies of plasticity in a mouse bearing a mutant form of the signaling molecule αCaMKII, which is known to be important for the induction of plasticity in

many systems, and has been proposed to underlie the maintenance of existing plasticity as well. Our data suggest that in ocular dominance plasticity, as in hippocampal long-term potentiation, a role for αCaMKII is likely to be confined to the initial, inductive stage of plasticity. Chapter Three describes studies of the role of protein synthesis in ocular dominance plasticity. *In vitro* models of plasticity suggest that protein synthesis serves as a marker for the transition from short- to long-term plasticity, possibly associated with rearrangements of anatomical connections. Our experiments reveal that ocular dominance plasticity is protein synthesis-dependent, and that this dependence arises surprisingly quickly. Moreover, there is a dissociation between requirements for protein synthesis in pre- and post-synaptic elements of the thalamocortical circuit: while protein synthesis inhibition in the cortex blocks ocular dominance plasticity, inhibition of thalamic protein synthesis does not.

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Chapter One:

Introduction

Overview

The brain is an organ of staggering complexity. Quantitative estimates of the interconnectivity of neurons provide a crude measure of the intricacy of the neural plexus. A cubic millimeter of cat visual cortex – which spans roughly the width of a quarter's thickness – contains about 50,000 neurons. Each of these neurons supports roughly 6,000 synapses, yielding a total of 300×10^6 synapses per square millimeter (Beaulieu and Colonnier, 1983). Extrapolating from these figures, one scientist estimates that a single human's neocortex houses on the order of 60×10^{12} connections (Shepherd, 1990) – granted, a number of such magnitude that it is difficult to marshal any true intuition of its size[1], but the point remains: the brain harbors an extraordinary number of connections in a singularly small volume.

As impressive as this intricate connectivity is, it merely underscores the extraordinary developmental processes that underlie the generation of the mature nervous system. Each of a given neuron's thousands of synapses are made with what appears to be remarkable precision. Alpha motor neurons, for instance, course from spinal cord to muscle, and make synapses onto muscle fibers that are at once organized with respect to the muscle innervated, the number of synapses per muscle fiber, and the total number of target cells innervated by a given motor neuron (Purves, 1988). The result is a finely tuned motor system, one that can flexibly adapt to the demands of grasping an eggshell or hoisting a

[1] Where intuition fails, emotion does not: Hebb laments the "appalling number of cells (in the brain)...and even more appalling number of possible connections between them". (D.O. Hebb, The Organization of Behavior, 1949).

sack of potatoes. The precisely assembled neural architecture which mediates these behaviors is, like countless other neural circuits, the product of the interplay between genetic and environmental forces.

The mammalian visual system is one such circuit, and the most extensively studied one to boot. In carnivorous species, thalamocortical afferents representing the two eyes arise in the lateral geniculate nucleus (LGN) of the thalamus, and converge on layer IV of the primary visual cortex. As in the case of the neuromuscular junction, the synapses made by thalamocortical afferents are tightly regulated to create a highly organized pattern of connectivity. Most famously, geniculate afferents from the two eyes are organized into largely divergent cortical domains known as ocular dominance columns: some patches of cortical tissue are driven primarily by the left eye, and others by the right. Together, these alternating columns tile the surface of the cortex (Hubel and Wiesel, 1962) like the squares on checkerboard (albeit a warped and stretched one).

From the time of their initial description by David Hubel and Torsten Wiesel, the developmental origin of these ocular dominance columns has been a subject of intense interest. Soon after the demonstration of their existence, Hubel and Wiesel went on to show that these neural circuits could be profoundly shaped by manipulations of the animal's environment. Experiments with lid suture in kittens demonstrated that the cortex is not composed of static neural circuits, irrevocably welded into place. Brief closure of one eye resulted in dramatic rearrangements of the connections subserving both the

deprived and the non-deprived eyes, with the columns devoted to the former shrinking, and those devoted to the latter expanding (Wiesel and Hubel, 1963).

This phenomenon, known as ocular dominance plasticity, is now perhaps the best characterized example of activity dependent plasticity – that is, synaptic form shaped by synaptic function -- in the nervous system. We posses a fairly comprehensive understanding of the rules which guide the plasticity and the nature of the changes that occur following monocular deprivation. The pioneering studies by Hubel and Weisel in the 1960s identified two central features of ocular dominance plasticity which have powerfully shaped our understanding of cortical plasticity, and laid the foundation for subsequent research.

First, activity dependent change in the cortex is *competitive*, pitting afferents representing the two eyes against one another in a battle for cortical territory. Where one eye gains (that is, expands its cortical representation), the other necessarily loses. This interaction occurs only where afferents subserving the two eye contest common cortical territory. In the monocular crescent, for example, afferents of the deprived eye are largely spared the effects of MD (Sherman et al., 1974).

Secondly, these competitive interactions are largely confined to a developmental *critical period*, a temporally circumscribed window in which synapses are exceptionally plastic. Brief monocular lid suture in kittens produces profound and lasting changes in cortical connectivity (and enduring impairments to visual function through the deprived eye:

Freeman and Olson, 1982). The identical manipulation in adult cats yields minimal cortical changes, and indeed, even very lengthy MD results in only modest rearrangements in the neuronal architecture (Jones et al., 1984; Daw et al., 1992).

Plasticity induced by MD is thought to capture the physiological elements which guide the course of normal development. Experimental and theoretical approaches suggest the arrangement of thalamocortical connections, including their hallmark columnar organization, arises as a consequence of activity dependent interactions (Miller et al., 1989; Katz and Shatz, 1996). Binocular inactivation of retinal ganglion cells prevents the formation of ocular dominance columns, demonstrating that retinal activity plays at least a permissive role in organizing the developing visual cortex (Stryker and Harris, 1986). This activity need not be visually driven. Indeed, it is clear that in some animals, such as macaques, ocular dominance columns are present *before* birth, and thus could never be the product of visually driven activity (Horton and Hocking, 1996).

Investigations of activity dependent plasticity during the critical period have geometrically expanded since 1963, with the original Hubel and Wiesel study published in that year serving as the bedrock for subsequent investigation. Experiments elaborating on these initial observations have painted a remarkably clear picture of the rules governing activity dependent competition in ocular dominance plasticity; a growing understanding of the molecular events which initiate synaptic plasticity; and very recently, insight into the mechanisms which control the onset and subsequent closure of the critical period itself.

The last of these two areas has been spurred in great part by the characterization of a mouse model for ocular dominance plasticity. Unlike larger mammals such as primates and cats, the mouse has been the subject of intense genetic analysis and manipulation. Technologies which enable the stable introduction of genes into the mouse genome (yielding transgenic mice), coupled with complementary protocols which enable discreet DNA sequences to be removed entirely from the genome (producing knockout (KO) mice: (Capecchi, 1989), have added immensely valuable tools to the arsenal of pharmacological, electrophysiological, and anatomical methods which have traditionally been used to study ocular dominance plasticity. Studies of transgenic and KO mice represent a dawning understanding of the cell biological mechanisms which underlie ocular dominance plasticity, enabling as they do a molecule by molecule examination of critical players in the events which transform electrical activity into enduring anatomical changes.

Mice differ from traditional models for ocular dominance plasticity in lacking columnar organization of receptive field properties such as ocular dominance or orientation. Indeed, orientation selectivity seems to be scarcely a feature of cortical receptive fields at all, as only a minority of cortical neurons show any bias for orientation selectivity (~25%: Gordon and Stryker, 1996; Hensch et al., 1998). It is possible other, unmapped receptive field structures are arranged in columnar fashion in rodents and this organization remains undetected. In any case, ocular dominance plasticity in mice is robust (the magnitude of plasticity following MD is comparable in mice and cats, with

cats showing effects that are about 15% larger than mice), rapid (with four days required for near-saturating shifts in mice, and two for cats), and regular (ocular dominance plasticity in mice is governed by the same rules which have been identified in carnivores: Gordon, 1996).

Already studies in transgenic mice have provided some insights into the signal transduction mechanisms which the cell employs to initiate synaptic change. As for other models of plasticity, intracellular kinases play a key role in this pathway to plasticity. Their downstream targets, which presumably include the molecules which lead to enduring alteration of a synapse, have been more challenging to identify. Consequently, the mechanisms by which a cell, having initiated plasticity, maintains a stably altered synaptic strength, remain poorly understood.

This thesis details two different approaches to elucidating the molecular mechanisms which underlie the stable transformation of synaptic strengths and architecture during ocular dominance plasticity. Both employ mouse models of ocular dominance plasticity, utilizing in the first experiments genetically mutant mice, and in the second, pharmacological approaches, to investigate molecular mechanisms underlying plasticity.

Chapter 2 describes experiments utilizing mutant mice harboring genetically altered αCaMKII, a protein which plays a central role in plasticity processes, including ocular dominance plasticity (Gordon, 1996). While the molecule's role in inducing plasticity has been well established *in vitro*, strong theoretical arguments, with some experimental

support, have advanced the hypothesis that αCaMKII is well suited to maintain previously potentiated synapses in their altered state, serving as a kind of molecular memory (Lisman, 1994; Lisman and Zhabotinsky, 2001). It is specifically the molecule's capacity for autophosphorylation which is thought to underlie this role. Clever biochemical and genetic manipulations have yielded a mutant mouse containing αCaMKII which is stripped of the ability to autophosphorylate, but is otherwise normal (Giese et al., 1998). This enables for the first time a test of the role of autophosphorylation in both the induction and the maintenance of *in vivo* plasticity.

Chapter 3 details experiments elucidating the spatial and temporal nature of a requirement for protein synthesis in ocular dominance plasticity. Though protein synthesis has long been known to play a role in the late phases of many models of synaptic plasticity, the nature of such a requirement in ocular dominance plasticity, if any, was unknown. Using pharmacological methods to target distinct brain nuclei for protein synthesis inhibition with cycloheximide infusion, I was able to establish a requirement for protein synthesis in ocular dominance plasticity, and to probe both the anatomical locus of this requirement and the timing of the requirement for protein synthesis. The results suggest that cortical changes guide subsequent thalamocortical changes, and that anatomical changes may underlie even the most rapid plasticity following monocular occlusion.

Background

The Rules Governing Ocular Dominance Plasticity

A coherent picture of the rules governing ocular dominance plasticity has emerged, including a broad understanding of the roles played by afferent information carried by the thalamocortical inputs, the importance of the relative timing of action potentials in these afferents, and the role of the post-synaptic cortical neurons. The locus and mechanism of the changes that follow monocular occlusion are part of a still developing story (discussed further below), one that may change our understanding of the details of how these rules are implemented, but the broad themes which govern their implementation seem well established.

Many of these themes were presciently anticipated by Donald Hebb (Hebb, 1949), who recognized the power of activity-dependent mechanisms as a neurophysiological substrate for associative learning. He proposed memories or "traces" might be stored as changes in synaptic strength, and specifically, that a metabolic or growth process which amplified efficacious inputs could be a neural substrate for learning. In the colloquial, this has been condensed to "neurons that fire together wire together"; appending Stent's elaboration might add "... and neurons that fire apart fall apart". This correlation-based process is the central rule which guides activity dependent plasticity in the visual cortex. It is remarkable that in his book The Organization of Behavior, Hebb introduced not one, but two powerful theories: his "dual trace mechanism" also seems to have been very

close to the mark. To account for both the rapid acquisition and the longevity of many memories, he proposed a rapid, labile, and non-structural stage accounting for the acquisition phase, and suggested the growth process outlined above might account for the longevity. This is an idea that will be revisited in both Chapters 2 and 3, in considerations of how synaptic change is stored at the synapse.

A central feature of the interaction of afferents subserving the two eyes is that is a competitive one, with the outcome of the competition determined by the relative levels of activity in the pathways subserving the two eyes. Monocular deprivation induces profound changes in the relative responsivity of the two eyes, with the strength of the deprived eye's input to cortex diminishing dramatically, and that of the non-deprived open eye increasing (Wiesel and Hubel, 1963). Binocular deprivation (BD), which of course effects the same suppression of light-driven retinal activity -- but in both eyes – results in a minimal loss of neuronal responsivity (Wiesel and Hubel, 1965). Together, these two experiments rule out the possibility that absolute levels of afferent activity are sufficient to initiate plasticity mechanisms engaged by monocular deprivation. A nice anatomical correlate of this competitive interaction was shown in studies of soma size in the lateral geniculate nucleus (Hickey et al., 1977). Following monocular deprivation, cell bodies in the deprived-eye laminae shrink, a change that may represent the loss of post-synaptic trophic support. Unlike electrophysiological measures of ocular dominance, which are simply between-eye comparisons of cortical input strength, measurements of soma size are absolute. Thus, they are well suited for assessing the affects of MD upon monocular regions of the LGN and cortex, and for assessing the

effects of manipulations that treat the two eyes equally, such as binocular deprivation. After MD, substantial shrinkage in cell soma in the deprived eye laminae occurs, but only in the binocular region of the cortex. Cells in the monocular segment of deprived eye layers are largely spared the effects of the MD.

The exemption from the punitive effects of MD in the monocular crescent seems to arise specifically from the lack of competing inputs in the region, and not from a distinct genetic or biochemical endowment. An artificial monocular zone can be created, by local mechanical destruction of retinal ganglion cells (Sherman et al., 1974). If this is followed by monocular deprivation of the contralateral eye, behavioral, physiological, and anatomical measures of the effects of MD show that deprived eye cortical inputs are spared – but only and precisely in the location of the scotoma created in the open eye.

A clever variant of the MD paradigm reiterates the importance of relative, rather than absolute levels of activity, in determining the outcome of ocular dominance plasticity. While a sutured eye will lose cortical input when paired with an open eye, if paired instead with a TTX injected eye, both electrophysiological and anatomical measures show the sutured eye emerges the victor (Chapman et al., 1986).

It is not simply the levels of activity carried by afferents subserving the two eyes, but also the timing of the action potentials occurring in afferent terminals, that regulates ocular dominance plasticity. Strabismus is a potent inducer of plasticity, though it need not act through differences in levels of activity between the two retinae. It disrupts synchronous

firing in the two eyes, however, and this is what sets plasticity in motion. Strabismus differs from MD in that it results in specifically the loss of binocular connections in the cortex, without reallocation of cortical territory to favor one eye's inputs (Hubel and Wiesel, 1965). Ocular dominance columns become more crisply segregated with the loss of convergent connections necessary for binocularly driven neurons (Blakemore, 1975). Alternating monocular deprivation, which explicitly and completely decorrelates driven activity between the two eyes, achieves the same end (Blasdel and Pettigrew, 1979). A more extreme, and perhaps cleaner, version of this manipulation was achieved in a developmental context by bringing patterns of afferent activity completely under investigator control, with the implantation of stimulating electrodes in each of the optic nerves (Stryker and Strickland, 1984). With endogenous retinal activity silenced through binocular TTX injections, the effects of synchronous nerve stimulation were compared with asynchronous stimulation. The results of asynchronous stimulation echo the effects of strabismus, as binocular summation is lost, and ocular dominance columns with especially crisp borders form.

The cortical neuron plays a central role in mediating the competition that is set in motion by imbalances in afferent activity. Pharmacological approaches have been the most illuminating experimental approach in demonstrating this role. Cortical infusion of TTX blocks the effects of MD, consistent with a post-synaptic, cortical role in arbitrating the effects of MD (Reiter et al., 1986). TTX effects are not limited to cortical elements of the circuit, however, and their effects could be explained as a pre-synaptic effect on thalamocortical afferents. The absence of $GABA_A$ receptors from thalamocortical

afferents (Needler et al., 1984) provides a means of untangling "pre" from "post": continuous infusion of the $GABA_A$ agonist muscimol pins cortical membrane potentials in a hyperpolarized state, without affecting activity in thalamocortical afferents. When combined with MD, the results are striking (Reiter and Stryker, 1988). Plasticity is not blocked, but reversed, such that the afferents serving the open eye lose input strength to the cortex, while those of the deprived eye increase their relative robustness. The results, which have been buttressed with studies demonstrating anatomical correlates of the effect, both with bulk label (Hata and Stryker, 1994) and single axon studies (Hata et al., 1999) place the post-synaptic neuron squarely in the role of arbitrator of the afferent competition, as well as providing the experimental confirmation of Stent's elaboration of the original Hebb hypothesis. It is interesting to note that while back-propagating action potentials (capable of invading dendritic arborizations) have been proposed to be the associational signal which triggers Hebbian strengthening of synapses (Magee and Johnston, 1997), the muscimol infusion experiments demonstrate explicitly that plasticity proceeds robustly in the absence of post-synaptic action potentials. Thus, while back-propagating action potentials may under normal conditions contribute the associational signal, they are not necessary.

Anatomical correlates of the changes occurring after MD have been extensively studied, particularly the structural changes which occur in thalamocortical arbors. Two main effects of MD are observed in neurons of the LGN which project to primary visual cortex: soma size changes, as outlined above, and dramatic rearrangements in afferents projecting to the primary visual cortex.

Bulk label of geniculocortical afferents first revealed an anatomical correlate of ocular dominance plasticity (Hubel et al., 1977; Shatz and Stryker, 1978). After MD, the area over which thalamocortical terminals serving the deprived eye arborize shrinks; the converse occurs for afferents serving the nondeprived eye. Subsequent technical advances illuminated the morphological changes occurring in a single geniculate neuron's terminal arborization (Antonini and Stryker, 1993) after monocular deprivation. The morphological changes that occur in single axons are consistent with the pictures painted by bulk label studies. Axons from neurons serving the deprived eye are stunted and withered, while those from the non-deprived eye flourish, increasing the expanse and complexity of their arbors. These dramatic structural rearrangements are striking, but equally surprising is the speed with which they occur. Changes are underway with four days of monocular deprivation, and largely consummated by seven days of monocular deprivation. This time course places rearrangements in the thalamocortical arbors just a step behind the emergence of plasticity measured electrophysiologically.

Post-synaptic anatomical plasticity has not received as much experimental attention, mainly because of technical difficulties associated with labeling single neurons and the added hurdle of gauging a neuron's position with respect to the map of ocular dominance. In a single study, the clever use of intracellular staining of cortical neurons combined with anatomical methods to delineate ocular dominance columns provided one solution to these problems (Kossel et al., 1995). As for the pre-synaptic thalamocortical afferents, it appears the dendritic arborizations of post-synaptic cortical neurons are dynamic players

in undergoing structural rearrangements. Layer IV cortical neurons largely confine their dendrites to one eye's ocular dominance column. Strabismus heightens this tendency and MD tends to weaken it, suggesting that these dendritic rearrangements partner with pre-synaptic changes in propelling ocular dominance plasticity.

The preceding paragraphs summarize work that has gone into elucidating the rules which govern ocular dominance plasticity. It is, in its broad strokes, a largely coherent story. Investigations of the development of visual cortex, on the other hand, are less well understood – one prominent point of contention being precisely when and where activity dependent plasticity begins, and activity independent mechanisms leave off.

In recent years, much attention has been focused on the role of supragranular cortical layers in shaping development and ocular dominance plasticity, and with good reason. With respect to plasticity, a recent experiment has shown that layer IV, though the direct recipient of thalamocortical input, is in fact the slowest layer to show a shift in ocular dominance following monocular occlusion (Trachtenberg et al., 2000). The result is surprising, given the long-held assumption that plasticity proceeds in a largely bottom-up fashion, starting with thalamorecipient layer IV and then progressing to other cortical layers. However, this finding is consistent with similar reports from studies in the barrel cortex (Diamond et al., 1994), and with studies of the critical period for ocular dominance, which indicate that extragranular layers have a much lengthier period of susceptibility to the effects of monocular deprivation than layer IV (Daw et al., 1992). Two explanations for these findings have been advanced. It is possible that the

extragranular layers simply exert a multiplicative effect upon the plasticity initiated in layer IV. Alternatively, it may be that the extragranular layers are truly in the driver's seat, and dictate the reorganization that follows MD.

The anatomical connections of the upper layers in particular, and the plasticity demonstrated by those connections, make the latter proposition an attractive one. Supragranular layers are known to harbor patchy lateral connections, which appear to link columns with similar receptive field qualities, including orientation and ocular dominance (Rockland and Lund, 1982; Gilbert and Wiesel, 1989). These horizontal connections follow Hebbian rules in undergoing activity-dependent changes: manipulations such as strabismus, which decorrelate firing in the two eyes, result in the loss of horizontal connections between contralateral and ipsilateral ocular dominance columns, and promote connections between same-eye ocular dominance columns (Löwel and Singer, 1992). Moreover, they do so extremely rapidly – two days of strabismus produces large-scale redistribution of these horizontal connections (Trachtenberg and Stryker, 2001), a time scale which is considerably more rapid than the redistribution observed in thalamocortical afferents following MD. Given the speed of these changes, and the horizontal connections' ability to integrate information over a large region of the cortex, it has been proposed that they may mediate the rapid extragranular changes which follow MD. However, a comprehensive theory of the rapid extragranular changes following MD may be difficult to reconcile with a primary role for horizontal connections, as the infragranular layers, which are not known to have extensive horizontal connections, also show rapid plasticity (Trachtenberg et al., 2000). Such a

theory must also incorporate species differences in anatomical connections: in Chapter 3, I discuss findings in mice which are consistent with an extragranular locus for the initial site of ocular dominance plasticity.

Studies in young cats and ferrets of the development of patchy horizontal connections buttress an organizing role for the upper layers. Patchy horizontal connections emerge in the upper layers before electrophysiological methods can be employed to reliably record single unit properties (Chapman and Stryker, 1993). Clever investigators have exploited the emergence of these patchy connections to assay for the first signs of cortical organizations, and to probe the proto-maps which portend the development of mature cortical maps. These patchy cortical connections, while requiring activity for their organization (TTX infused directly into the cortex prevents their appearance) are in fact *not* reliant upon action potentials from the retinae to support their development: binocular enucleation does not prevent patchiness from developing (Callaway and Katz, 1991; Ruthazer and Stryker, 1996). Having removed the eyes, it is not possible to assign a functional role to these columns: confirmation awaits the intrepid experimentalist who is willing to repeat the experiment with TTX inactivation. Nonetheless, these patches are evidence of cortical organization in the absence of retinal input, and have been construed as evidence of a self-organizing process in the cortex.

Molecular Mechanisms Underlying Ocular Dominance Plasticity

The successes of carnivore models in elucidating the rules of ocular dominance plasticity have not, for the most part, extended to uncovering the molecular mechanisms through which those rules are implemented. Pharmacological approaches, using direct brain infusion of bioactive molecules, have implicated some signaling pathways in ocular dominance plasticity, and will likely continue to yield useful information. However, the technique is one that is subject to substantial limitations, foremost amongst them difficulties in regulating concentrations of drugs with point source infusions, a problem that is compounded in the case of bulky or highly charged molecules, which do not diffuse far from the cannula. An additional hurdle is the unintended effects on cortical activity an infusion may produce – often, suppression of activity, which of course blocks plasticity. Genetically altered mice provide an alternative approach for studies of molecular mechanisms, one which complements pharmacological techniques. Knockout mice obviate problems of drug specificity and damage created by bulky cannula implantations (while of course bringing their own caveats to interpretation, including developmental compensation for congenital defects). Though the mouse model is a relatively new contributor to studies of ocular dominance plasticity, many of the studies described below have drawn heavily on the possibilities offered by this genetic model.

A cell biological view of the events leading to plasticity identifies three steps leading to synaptic change: 1) depolarization[2] of the post-synaptic neuron occurs, 2) second messenger signaling pathways are engaged, and 3) the action of downstream molecules, which likely includes both existing and newly translated molecules, causes synaptic change. Many of the important molecular players in steps 1 and 2 have been identified. Step 3 is of course a gross oversimplification, a conceptual grab-bag which includes many poorly understood events, including the actual mechanisms by which synapses are physically altered. Only a handful of experiments, including studies of molecules which may be involved in structural changes at the synapse, address mechanisms which fall into this category.

Of the receptors potentially mediating post-synaptic depolarization, the role of the N-methyl-D-aspartate (NMDA) receptor has by far received the most attention, because of its putative role as a "coincidence detector" which mediates associative strengthening of inputs. A beautiful study of synaptic maturation in the frog optic tectum is consistent with this role. Immature synapses contain primarily NMDA receptors which are silent at resting potential, while more mature synapses contain both α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and NMDA receptors (Wu et al., 1996); this is consistent with a scenario in which NMDA receptors mediate depolarization only in the presence of concurrent AMPA receptor driven depolarization. Studies of NMDA versus AMPA receptor mediated current flow in the cat visual cortex are perhaps consistent with a similar maturation process, as the NMDA receptor mediated component

[2] Failure to depolarize is likely just as important as depolarization in ocular dominance plasticity, as illustrated by muscimol infusion experiments (Reiter and Stryker, 1988). However, our understanding of

of monosynaptic EPSCs peaks in postnatal weeks 5-6 (Iwakiri and Komatsu, 1993), roughly concurrent with the peak of immunostaining for NMDA R1 subunit staining (Catalano et al., 1997), and with the peak of ocular dominance plasticity in the kitten, which occurs at ~p28 (Hensch, 1996). NMDA receptor kinetics evolve rapidly during this period of early postnatal development, showing a trend toward faster decay times in rat (Carmignoto and Vicini, 1992) and ferret (Roberts and Ramoa, 1999) visual cortex, though the interpretation of this change has been debated. These changes are sensitive to manipulation by afferent activity, as visual experience and dark rearing have opposite effects upon NMDA receptor maturation, with the latter prolonging the slow kinetics of the immature state, and the former leading to rapid, adult-like kinetics (Quinlan et al., 1999; Philpot et al., 2001). Thus, expression and kinetics of the NMDA receptor are dynamically regulated during the critical period, providing correlative evidence of a role in ocular dominance plasticity.

Despite this circumstantial evidence, solid evidence of a role for the NMDA receptor in activity dependent plasticity – apart from a simple role in basal synaptic transmission – was a long time in coming. While numerous pharmacological experiments showed blockade of the NMDA receptor blocked ocular dominance plasticity (Kleinschmidt et al., 1987; Gu et al., 1989; Bear et al., 1990), such manipulations have potent suppressive effects upon normal synaptic transmission (Miller et al., 1989) and even cause reverse plasticity effects similar to those observed with muscimol infusion (Bear et al., 1990). A technically challenging solution to this problem came in the form of infusion of anti-sense DNA directed against the NR1 subunit of the NMDA receptor, which substantially

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the molecular events which cause synaptic depression *in vivo* is very poor.

and selectively suppresses NMDA receptor transmission (Roberts et al., 1998), without affecting visually driven responses in the visual cortex. It also blocks the effects of monocular deprivation, suggesting that once the NMDA receptor's putative correlation detection role is carefully dissected from its role in normal synaptic transmission, the former may indeed exist, and be necessary for ocular dominance plasticity.

The roles played by the ionotropic AMPA receptor and the metabotropic glutamate receptors (mGlurR) in ocular dominance plasticity are less well-defined, though their actions in *in vitro* plasticity has been extensively examined (Selig et al., 1995; Huber et al., 1998; Balschun et al., 1999). Neither blockade of the AMPA receptor with the antagonist CNQX (DeFreitas and Stryker, 1990), nor blockade of mGluRs' function with the antagonist MCPG prevents ocular dominance plasticity (Hensch and Stryker, 1996); the latter finding has been challenged the on grounds that MCPG does not adequately block the effects of mGluR action in the visual cortex.

Downstream of the glutamatergic receptors, second messengers take over the task of transforming electrical activity into lasting synaptic change. In various forms of *in vitro* plasticity, second messengers target their effects in two directions -- back to the source of depolarization (the activated dendrite), and to the nucleus, presumable to initiate transcriptional events that ultimately contribute to more long-lasting forms of synaptic change. It is likely that ocular dominance plasticity works at least similarly. Monocular deprivation induces rapid transcriptional and translational changes (Chaudhuri et al., 1995; Pham et al., 2001), supporting the notion that nuclear events are involved in

plasticity induction; results discussed in Chapter 3 suggest these changes are needed for ocular dominance plasticity. The existence of an "early" plasticity stage which comes about through rapid modification of existing proteins is purely speculative in the case of ocular dominance plasticity, but it is suggestive that plasticity has been observed after as little as one hour of deprivation (Peck and Blakemore, 1975). This approaches the duration required for transcription and translation to occur (perhaps half an hour); if, additionally, the necessity of transporting these newly synthesized proteins to distant dendritic sites is considered, it may well be there simply isn't time for nuclear events to contribute to the first plasticity events.

Using pharmacological approaches, the second messengers protein kinase A (PKA) and extracellular signal-regulated kinase 1,2 (ERK) have been implicated as players in ocular dominance plasticity. PKA targets include both pre- and post-synaptic participants elements of the synapse, including GABAergic and glutamatergic channels, so it is well-positioned to exert effects on synaptic transmission (Rotenberg et al., 2000). Mice genetically deficient in the PKA R1β subunit are impaired in *in vitro* measures of plasticity, but show normal shifts in ocular dominance following monocular deprivation (Hensch et al., 1998). It is known that developmental compensation in this mouse leads to upregulation of the PKA R1α subunit, and this may allow plasticity to proceed. Pharmacological inhibition of PKA in the kitten blocks plasticity, with minor effects on neuronal responses (Beaver et al., 2001). A similar approach using infusion of ERK inhibitor blocks the effects of monocular deprivation, again with little or no effect on neuronal spiking, and also blocks layer II/III LTP (Di Cristo et al., 2001). Both PKA

and ERK are nodes in complicated signaling pathways, and the specific roles played by their substrate molecules remains to be deciphered.

The molecule α-calcium calmodulin kinase II (αCaMKII) is similar, in that it can initiate signaling in a number of divergent pathways, with divergent effects, in multiple locations. However, a prodigious amount of work has gone into characterizing the molecule and its targets, and so its actions are perhaps better defined than those of other second messengers. Moreover, the molecule itself has been proposed to be both activator and effector, constituting a "molecular memory" which can be maintained at the synapse, an idea is central to the motivation of the experiments outlined in Chapter 2.

αCaMKII is an abundant protein, comprising between 1 - 2% of the *total* protein in the forebrain (Bennet et al., 1983). Eight to twelve subunits of the molecule assemble into a rosette holoenzyme comprised of α and β subunits (in the forebrain the ratio of α to β subunits is about 3 to 1: Miller and Kennedy, 1985). Once activated by Ca^{2+}/calmodulin binding, αCaMKII can phosphorylate numerous substrate molecules, including the NMDA receptor (Omkumar et al., 1996) and the transcription factor cAMP response element binding protein (CREB: Dash et al., 1991). Importantly, αCaMKII can autophosphorylate at threonine 286. Autophosphorylation switches the molecule into a Ca^{2+} autonomous state, which allows the molecule to sustain Ca^{2+}-independent kinase activity – in effect providing a mechanism to turn transient Ca^{2+} influx into a sustained plasticity induction signal.

A necessary role for αCaMKII in plasticity mechanisms has been demonstrated both *in vivo* and *in vitro*. Blockade of αCaMKII activity, either through pharmacological or genetic means, prevents LTP induction in both hippocampal and cortical neurons (Silva et al., 1992; Kirkwood et al., 1997; Lisman et al., 1997). Moreover, overexpression of activated αCaMKII is sufficient to induce potentiation of excitatory post-synaptic potentials in hippocampal neurons, and to occlude further induction of LTP through tetanizing stimuli (Pettit et al., 1994). *In vivo* studies of mutant mice lacking αCaMKII have established a necessary role for the molecule for plasticity in the barrel field (Glazewski et al., 1996) and the visual cortex (Gordon et al., 1996). More recently, a specific role for αCaMKII autophosphorylation has been probed through the development of mutant mice carrying a single point-mutation (conversion of threonine 286 to alanine: T286A) which renders αCaMKII unable to autophosphorylate. These mice show impairments in both LTP induction and learning paradigms (Giese et al., 1998), as well as in hippocampal place cell firing (Cho et al., 1998) and barrel cortex plasticity (Glazewski et al., 2000).

Results from studies of these $\alpha CaMKII^{T286A}$ mice are consistent with either of two proposed roles. Autophosphorylation may be simply a facilitatory step in inductive mechanisms, which accelerates induction of plasticity mechanisms by allowing αCaMKII enzymatic activity to outlast the fleeting duration of the Ca^{2+} transients which activate the kinase. Autophosphorylation certainly prolongs αCaMKII activation to last over a period of minutes to hours (Barria et al., 1997), and possibly much longer, given

appropriate conditions (Lisman and Zhabotinsky, 2001), and its inductive role has been well established in LTP paradigms (Malenka et al., 1989; Malinow et al., 1989).

In addition to this inductive role, the molecule has also been proposed to play a role in maintaining existing synaptic changes (Lisman, 1985; Miller and Kennedy, 1986; Lisman, 1994; Lisman and Zhabotinsky, 2001), a function that has received limited experimental support (Feng, 1995). Studies of αCaMKII^{T286A} mice have been pivotal in highlighting a vital role for autophosphorylation in αCaMKII function, but they have not directly examined whether this role is confined to inductive or maintenance stages of plasticity (or involved in both). Experiments outlined in Chapter 2 provide the first direct *in vivo* examination of the hypothesis that αCaMKII plays a role in maintaining existing changes in synapses.

PKA and αCaMKII signaling ultimately contribute to changes in nuclear events, likely including CREB-mediated transcription. CREB has been implicated in mediating synaptic plasticity in evolutionarily divergent animals (*Aplysia*: Dash et al., 1990; *Drosophila*: Yin et al., 1994; mice: Pham et al., 1999) and widely divergent plasticity paradigms (late phase hippocampal LTP: Silva et al., 1999; courtship condition in *Drosophila*: Griffith et al., 1993; activity-dependent development: Pham et al., 2001). In a number of plasticity paradigms, the requirement for CREB activity seems to be confined to late phases of plasticity, which has prompted the suggestion that CREB activity is the molecular bridge linking short-term changes to long-lasting plasticity (Pittenger and Kandel, 1998). The strength of this hypothesis suffers in part from the

technical difficulties associated with studying long-lasting changes in preparations which are themselves not terribly long-lasting (slice preparations, for instance). More sophisticated genetic manipulations, which will enable better temporal control of gene expression *in vivo* (Bishop et al., 2000), will undoubtedly shed light on these questions.

Ultimately, intracellular communication through signaling pathways yields changes in the nuts and bolts of synaptic architecture – for many forms of plasticity, including ocular dominance plasticity, structural changes in synaptic connectivity are the endstage of mechanisms underlying long-lasting change. Our understanding of the molecules that underlie these changes is limited, but a handful of promising candidates in this category have been identified. The tissue plasminogen activator is one such molecule, which is perhaps unique in having been implicated in the degradative mechanisms which likely pave the way for synaptic rearrangements. Tissue plasminogen activator is necessary for ocular dominance plasticity in cats (Muller and Griesinger, 1998) and mice (Mataga et al., 1999), and may mediate a rise in proteolytic activity that accompanies monocular deprivation in mice (Mataga et al., 2000). Studies of the degradative activity of this molecule offer hope of gaining some understanding of molecular substrates of *competitive* interactions between the two eyes' inputs. Recruitment of the molecule during MD suggests a competitive mechanism which operates through conservation of the total strength of the two eyes' input, in which degradation of deprived eye inputs necessarily precedes their replacement by non-deprived eye inputs.

The neurotrophins are another class of molecule that may participate in structural change. They have potent effects upon the morphology of visual cortex neurons (McAllister et al., 1995), as well as upon electrophysiological and anatomical plasticity *in vivo* (Cabelli et al., 1995; Gillespie et al., 2000). An exciting and unexpected role for neurotrophins in controlling the timing of critical period plasticity has recently been identified: mice overexpressing the brain-derived neurotrophin factor (BDNF) demonstrate precocious visual cortical plasticity (Hanover et al., 1999). This precocious plasticity may be mediated by the effects of BDNF in promoting maturation of inhibitory interneurons (Huang et al., 1999), a mechanism that is consistent with recent demonstrations of inhibition-mediated control of the timing of the critical period (Fagiolini and Hensch, 2000).

Chapter 2

Autophosphorylation of αCaMKII is required for the induction, but not maintenance, of rapid ocular dominance plasticity

Summary

Studies of neuronal plasticity have distinguished between early, transient stages required for the induction of synaptic change, and subsequent long-lasting stages necessary for maintenance. The synaptic protein alpha calcium-calmodulin-kinase II (αCaMKII) is necessary for the induction of synaptic plasticity. Autophosphorylation of αCaMKII results in sustained, calcium-independent activity; this autophophorylated form of the molecule has been proposed to underlie the continued expression of synaptic changes. Using genetic tools, we have examined the role of autophosphorylation of αCaMKII in the induction and maintenance of *in vivo* visual cortical plasticity. Transgenic mice possessing mutant αCaMKII which cannot be autophosporylated are impaired in the induction, but not maintenance, of plasticity in primary visual cortex.

Introduction

The late stages of many forms of synaptic plasticity are sensitive to disruption by inhibitors of protein synthesis (Stanton and Sarvey, 1984; Frey et al., 1988; Otani et al., 1989). This provides a convenient biochemical assay for probing the constituent stages of plasticity, but raises questions about how a neuron manages events in the far-flung reaches of its thousands of dendrites. Of particularly concern is the manner in which synaptic weights can be adjusted in a dendrite-specific manner, a property known as input specificity. If translational events in the soma are indeed necessary for synaptic change, neurons must have some means of routing communications between the dendrite and the soma rather precisely, to ensure that gene products from the soma are delivered precisely and exclusively to activated dendrites targeted for synaptic rearrangements.

At least three proposals have been put forth to account for the molecular events which enable input-specific plasticity. The first of these holds that protein synthesized in the soma are ushered to dendrites which have been "tagged" for plasticity by an activity-dependent, protein synthesis-independent mechanism (Frey et al., 1988). This proposal has received support in the form of clever dual pathway LTP experiments. An LTP-inducing stimulus in one pathway allows a subsequent stimulus to induce LTP in a second, independent pathway, even in the presence of protein synthesis inhibitors (Frey and Morris, 1997). This result is consistent with the mechanism proposed: synaptic "tags" allow activated dendrites to capture somatically-synthesized proteins necessary for

plasticity. This scheme has the advantage of obviating the supposition of elaborate intracellular trafficking, which many have dismissed on the grounds that it is simply too complicated. On the other hand, it ignores evidence that such trafficking mechanisms indeed exist (discussed below). Moreover, it remains to be determined how such a synaptic tagging mechanism might be implemented *in vivo*, where activity and resultant plasticity occur in an ongoing fashion, rather than in the discrete, temporally-delimited manner of *in vitro* LTP induction.

The two remaining proposals in effect short-circuit the difficulties of accounting for complex soma-dendrite trafficking schemes. The first of these does so by arguing that protein synthesis occurs locally at dendrites, and therefore need not involve the soma. It has been clear for some time that ribosomal aggregations exist at the base of dendritic spines (Steward, 1982). Recent demonstrations of activity dependent dendritic translation of plasticity-related genes (Ouyang et al., 1999; Steward and Halpain, 1999) and the necessity of such dendritic translation in plasticity paradigms (Kang and Schuman, 1996; Huber et al., 2000), as well as insight into the mechanisms which regulate activity-dependent translation (Wu et al., 1998), have provided a framework for understanding how dendritic protein synthesis might contribute to input-specific plasticity. Local dendritic translation is an attractive mechanism for simultaneously dispatching the problems of supplying new proteins to dendrites undergoing plasticity, while accounting for input specificity. Ironically, it does not necessarily eliminate a cellular requirement for sophisticated trafficking mechanisms, as local translation assumes a highly organized means of routing mRNA (if not protein) to appropriate dendrites. In any case, it appears

that mechanisms capable of highly precise activity-dependent mRNA trafficking indeed exist, and that the signals controlling this trafficking resides in the mRNA (Mayford et al., 1996; Steward et al., 1998). In addition, it is worth pointing out that though local translation has been proposed as a means of eliminating the need for nuclear participation in dendritic plasticity (Aakalu et al., 2001), transcriptional inhibitors are known to disrupt late-stage plasticity in multiple systems (Bailey et al., 1992; Nguyen et al., 1994). It is possible that some combination of nuclear and non-nuclear macromolecular synthesis contribute to activity-dependent plasticity.

The third and final proposal holds synaptic plasticity can be induced and maintained entirely through covalent modifications of existing synaptic proteins, specifically αCaMKII (Lisman, 1985; Miller and Kennedy, 1986; Lisman, 1994). This proposal is motivated in part by concerns that demonstrations of a requirement for macromolecular synthesis in synaptic plasticity could arise from non-specific effects of transcriptional and translational inhibitors (Lisman, 1985). More centrally, however, it is derived from considerations of the unique structure and action of the αCaMKII molecule itself.

In its resting state, αCaMKII requires Ca^{2+}/calmodulin binding for activation (Bennet et al., 1983). However, the molecule is capable of autophosphorylation, a modification which renders its kinase action Ca^{2+}-autonomous (Miller and Kennedy, 1986; Thiel et al., 1988). This autonomous state prolongs the duration of the molecule's activated state, enabling kinase activity to outlast typically fleeting Ca^{2+} transients. Autophosphorylation occurs through an intra-molecular reaction between subunits, where

Ca^{2+}-autonomous subunits can phosphorylate neighboring subunits, provided they have bound Ca^{2+}/calmodulin (Hanson et al., 1994). These two features of the molecule -- the combination of 1) an activated, Ca^{2+}-autonomous state which can be generated through 2) an intra-molecular reaction – suggest that αCaMKII might act as a molecular "switch", which, once activated by Ca^{2+}, could be maintained in the "on" position through the positive feedback mechanisms provided by autophosphorylation. Modeling approaches suggest that such a switch could operate in an metabolically efficient manner, such that "on" and "off" states of the molecule could be stably maintained without sapping cellular energy reserves (Zhabotinsky, 2000; Lisman and Zhabotinsky, 2001).

In the context of synaptic plasticity, this suggests that the αCaMKII molecule, which is known to be necessary for *induction* of plasticity (Nicoll and Malenka, 1999), could play an additional role in *maintaining* plasticity. In this scenario, a potentiated synapse, having initiated plasticity through αCaMKII signaling, could maintain that potentiated state through a local population of permanently activated αCaMKII (Lisman et al., 1997; Lisman and Zhabotinsky, 2001). This mechanism provides an entirely local mechanism for effecting synaptic change, one which in addition is resistant to the degradative effects of phosphatases and protein turnover.

It is clear that autophosphorylation of αCaMKII plays a necessary role in plasticity. Mice homozygous for altered αCaMKII in which a single point mutation has been introduced (substitution of alanine for threonine 286: $\alpha CaMKII^{T286A}$ mutants), which prevents autophosphorylation from occurring, are impaired in hippocampal LTP (Giese et

al., 1998), cortical plasticity (Glazewski et al., 2000), and hippocampal place cell formation (Cho et al., 1998). It is not yet clear, however, if this impairment arises from a requirement for autophosphorylation in inductive or maintenance stages of plasticity. Limited experimental evidence supports a role for αCaMKII in maintenance stages of plasticity (Feng, 1995).

Monocular and binocular deprivation paradigms have allowed us to isolate induction and maintenance stages of ocular dominance plasticity in $\alpha CaMKII^{T286A}$ mutant mice, and assess the specific role of autophosphorylated αCaMKII in each of those stages. Our results support the conclusion that autophosphorylated αCaMKII plays a key role in induction of ocular dominance plasticity, but not in its maintenance. $\alpha CaMKII^{T286A}$ mice exhibit sluggish plasticity in response to monocular occlusion, but synaptic changes, once induced, are as stable as those occurring in wild-type mice.

Materials and Methods

Mouse generation. $\alpha CaMKII^{T286A}$ mice were generated (as described in Giese et al., 1998) and generously supplied by A. Silva.

Monocular and binocular deprivation. MD was performed according to published protocols (Gordon and Stryker, 1996), except 3% isofluorane (Abbott, North Chicago, IL) in oxygen was used for anesthesia. To assess rapid ocular dominance plasticity, MD was initiated within the critical period for mouse ocular dominance (p26-p30), and lasted for four days. Long-term MD (LTMD) was initiated during or prior to the critical period

and averaged 20 days in wild-type and heterozygous mice (min.=12 d, max=27 d), and 17 days in homozygous mutants (min.=10 d, max.=26 d). Mice in which LTMD was followed by binocular deprivation (BD) received MD and BD of variable lengths. LTMD and LTMD + BD durations are illustrated in Figure 2-3. For generation of the timeline of critical period plasticity shown in Figure 2-2A, four day MD was performed starting at ages which ranged from p22 to p37.

Single unit electrophysiology. Mice were anesthetized with Nembutal/chlorprothixene anesthesia and recording performed with standard techniques (Gordon and Stryker, 1996). Single-unit microelectrode recordings were made with sharp-tipped tungsten electrodes. In each mouse, units were recorded at intervals >60 microns in penetrations confined to the binocular zone of visual cortex. For most experiments, cells were hand-mapped on a tangent screen, and ocular dominance responses rated on a 1 (purely contralateral responses) to 7 (purely ipsilateral) scale. Results from each mouse were represented in a single contralateral bias index (CBI) score, which was calculated using the formula $CBI = [(n_1-n_7)+(2/3)(n_2-n_6)+(1/3)(n_3-n_5)]/2N$, where N is the total number of cells recorded, and n_x is the number in ocular dominance category x. Cells were also scored for response amplitude and habituation.

Results

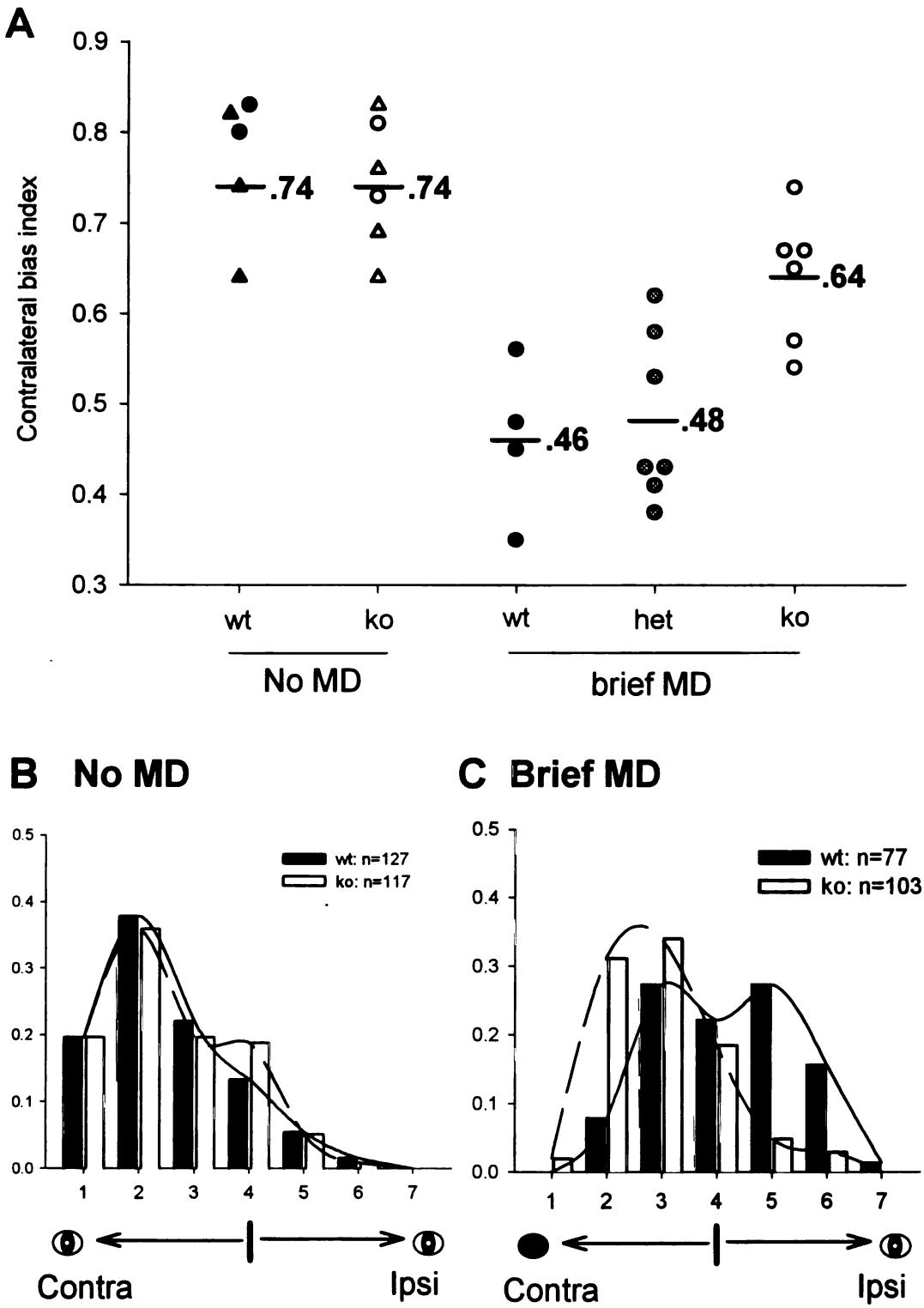
Brief MD (four days) revealed profound impairments in the cortical plasticity of homozygous mutant mice (figure 2-1). Both wild-type and KO mice show high nondeprived CBI scores (figure 2-1A), which is normal for mice. Brief MD drives robust

Figure 2-1. Rapid ocular dominance plasticity is impaired following brief MD in homozygous αCaMKII^{T286A} mice.

(A) Non-deprived wild-type (closed symbols) and homozygous KO (open symbols) mice show a similar bias toward the contralateral eye, as indicated by similarly high CBI scores (not significantly different, $p = 0.92$; t-test). Data from non-deprived critical period (circles) and older (> p40: triangles) mice are shown. Following brief four day MD, single units recorded in wild-type and heterozygous (shaded symbols) αCaMKII^{T286A} mice show a pronounced shift toward the open, ipsilateral eye (significantly different from both non-deprived groups; $p < 0.005$ for all comparisons; t-test). Units recorded from homozygous knock-outs remain primarily driven by the deprived contralateral eye, yielding a mean CBI which differs significantly from both deprived wild-type and heterozygote mice ($p < 0.01$ relative to both groups, t-test); deprived homozygote knockouts are also significantly different from both groups of non-deprived mice ($p < 0.05$ for both groups; t-test).

(B) Ocular dominance histograms of single unit responses from wild-type (closed bars) and αCaMKII^{T286A} homozygous (open bars) mice demonstrate a similar left-shifted distribution of responses in the two groups prior to MD. Lines (unbroken = wild-type, broken = homozygous knock-out) are smoothed fits plotted to highlight the profile of each distribution.

(C) Following brief four day MD, single unit responses recorded in wild-type animals shift toward the open ipsilateral eye, while responses in homozygous knock-outs remain primarily driven by the deprived contralateral eye.


A
Contralateral bias index
0.9
0.8
0.7
0.6
0.5
0.4
0.3
.74
.74
.46
.48
.64
wt
ko
wt
het
ko
No MD
brief MD
B No MD
C Brief MD
Percentage of total number of units
wt: n=127
ko: n=117
wt: n=77
ko: n=103
1 2 3 4 5 6 7
Contra
Ipsi

plasticity in wild-type mice, resulting in a relative increase in the strength of the non-deprived ipsilateral eye's input to cortex, and a lower CBI score. In contrast, brief MD induces only sluggish plasticity in KO mice. Examination of single unit responses (figure 2-1C) reveals that after MD, responses in homozygotes continued to be dominated by input from the deprived contralateral eye, while wild-type littermates showed robust shifts toward the nondeprived ipsilateral eye. In deprived wild-type mice, 45% of units recorded were predominantly driven by the nondeprived eye (figure 2-1C: OD categories 5, 6, and 7), while those categories accounted for fewer than 10% of responses in deprived homozygous mutants. Heterozygote mice showed plasticity comparable to wild-type levels (figure 2-1A), indicating that a single gene dosage of wild-type αCaMKII is sufficient to maintain normal function.

Apparent impairment of plasticity in homozygotes could be caused by changes in the timing of the critical period, rather than by inherent defects in plasticity mechanisms; this has been observed in other transgenic mice (Hanover et al., 1999; Huang et al., 1999). To rule out this possibility, we performed brief monocular deprivations before and after the normal peak (~p26-p30) of the critical period. Plasticity in homozygous mutants peaked around p27, within the normal developmental window for ocular dominance plasticity, indicating critical period timing was not altered in mutant mice (figure 2-2A: low CBI values indicate greatest plasticity).

Receptive field properties characterized in homozygous KO mice, including receptive field area and retinotopy (figure 2-2B and C), were similar to wild-type littermates,

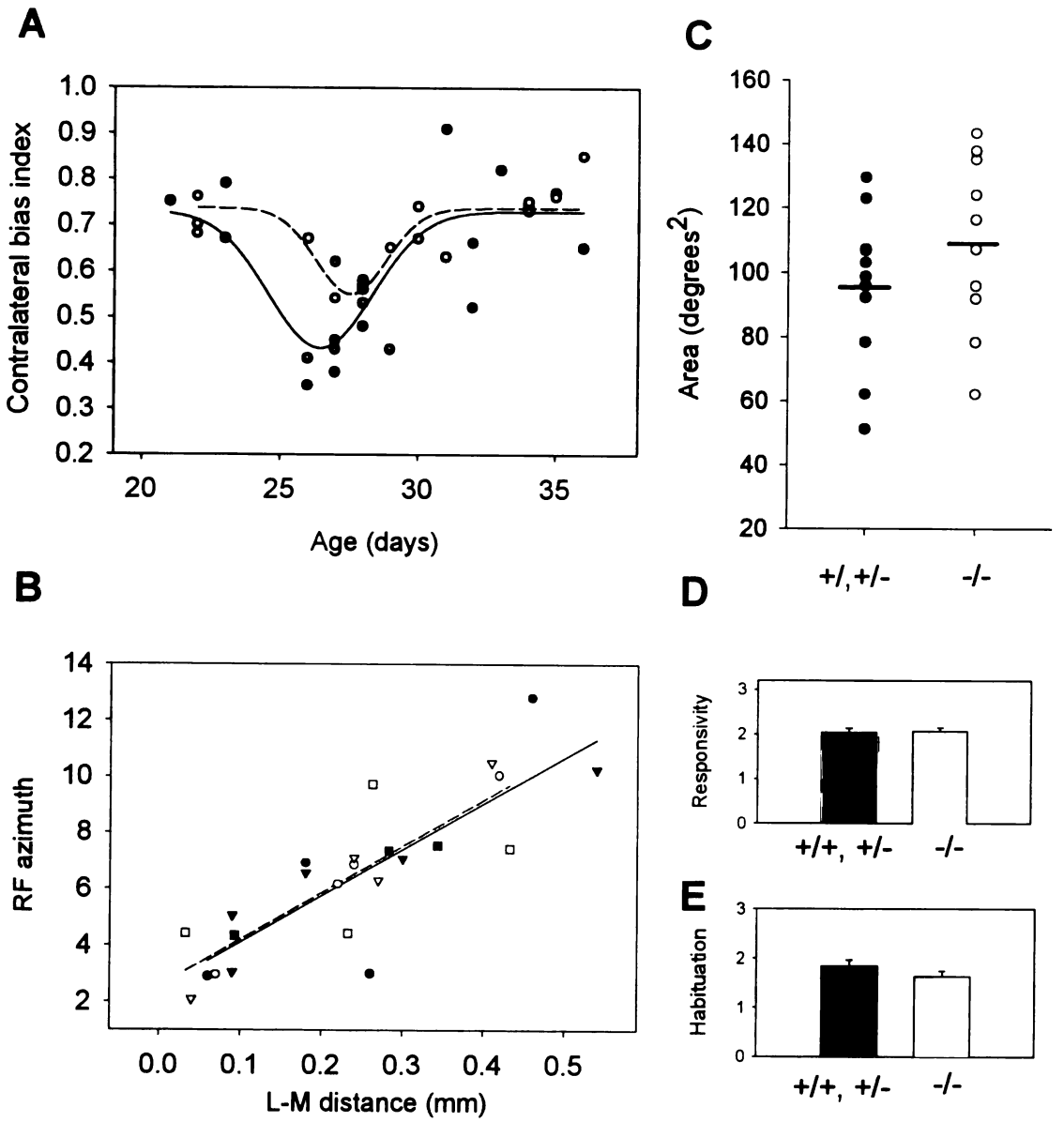
Figure 2-2 Timing of the critical period and response properties are not altered in αCaMKII^{T286A} mice.

(A) Four day monocular deprivations performed in animals ranging from p22 to p37 (MD start date) reveal that the peak of the critical period is not shifted in homozygous knockout animals (open symbols, fitted with broken line; n = 16 mice) relative to wild-type (closed symbols; n = 13) and heterozygous (shaded symbols; n = 9) littermates (data from wild-type and heterozygous animals fitted with unbroken line). Maximal plasticity for both groups falls within the peak of the critical period (Hanover et al., 1999).

(B) Retinotopy is not altered in homozygous knockout (open symbols, fitted with broken line) mice relative to wild-type/heterozygous littermates (closed symbols, unbroken line; p = 0.93, comparing slopes between groups; t-test). Different symbols represent data taken from different individual animals.

(C) Mean receptive field area does not differ (p = 0.19; t-test) between homozygous knockout animals (mean ± SEM = 110 ± 7.8 degrees^2) and wild-type/heterozygous (mean ± SEM = 95 ± 7.2 degrees^2) littermates.

(D) and (E) Responsivity (wt/het mean ± SEM = 2.0 ± 0.1; ko mean ± SEM = 2.1 ± 0.1; p = 0.84; t-test) and habituation (wt/het mean ± SEM = 1.8 ± 0.1; ko mean ± SEM = 1.6 ± 0.1; p = 0.20; t-test) are similar between wild-type/heterozygotes and knockout animals.


A
Contralateral bias index
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
20
25
30
35
Age (days)
C
Area (degrees^2)
160
140
120
100
80
60
40
20
+/,+/-
-/-
B
RF azimuth
14
12
10
8
6
4
2
0.0
0.1
0.2
0.3
0.4
0.5
L-M distance (mm)
D
Responsivity
3
2
1
0
+/+, +/-
-/-
E
Habituation
3
2
1
0
+/+, +/-
-/-

indicating that the gross organization of the visual system was not disrupted in mutant animals. Finally, the defect in plasticity could not be accounted for by reduced visual activity, since responsivity and habituation of single unit responses in wild-type and homozygous mice did not differ (figure 2-2D and E).

Though plasticity following brief MD of homozygous mutant mice was sluggish, activity-dependent plasticity was not absent (CBI scores of deprived KO mice significantly different from non-deprived mice, $p < 0.05$, t-test). This is especially apparent in examination of single unit responses of KO mice: responses driven exclusively by the contralateral eye were almost absent after brief MD (figure 2-1C, OD category 1), though they accounted for 20% of responses in nondeprived animals (figure 2-1B). Consequently, we explored the effects of long-term MD (LTMD) using the deprivation paradigms illustrated in figure 2-3. Indeed, substantially lengthening the period of deprivation was sufficient to drive robust plasticity in homozygous mutants, resulting in a substantial increase in the relative strength of the nondeprived eye's input to cortex (figure 2-4A). In heterozygote and wild-type littermates, extended periods of deprivation also caused additional plasticity, indicating the effects of brief MD were not saturating.

LTMD-induced plasticity in mutant mice allowed us to investigate the stability of induced changes. We reasoned that if autophosphorylated αCaMKII is required for the maintenance of synaptic changes, LTMD-induced plasticity in homozygous mutant mice

Figure 2-3. LTMD and LTMD + BD paradigm

Deprivation paradigm used for inducing plasticity, and assessing recovery from the effects of LTMD. Each group is rank ordered by length of MD (shortest to longest MD ranked from top to bottom). The gray vertical strip indicates the peak of critical period for ocular dominance plasticity. Mean wt/het LTMD = 19.8 d; mean ko LTMD = 17.1 d; mean wt/het LTMD + BD = 21.6 d + 22.8 d; mean ko LTMD + BD = 22.2 d + 21.0 d.

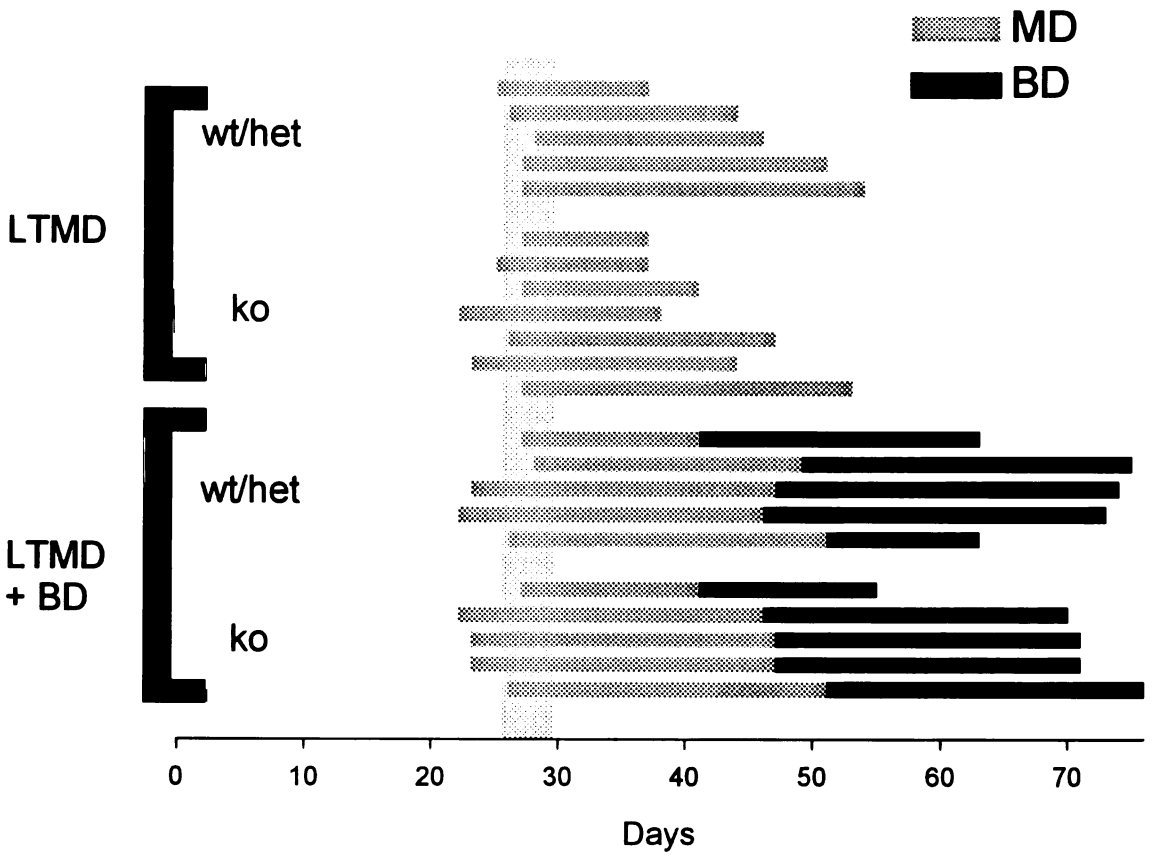
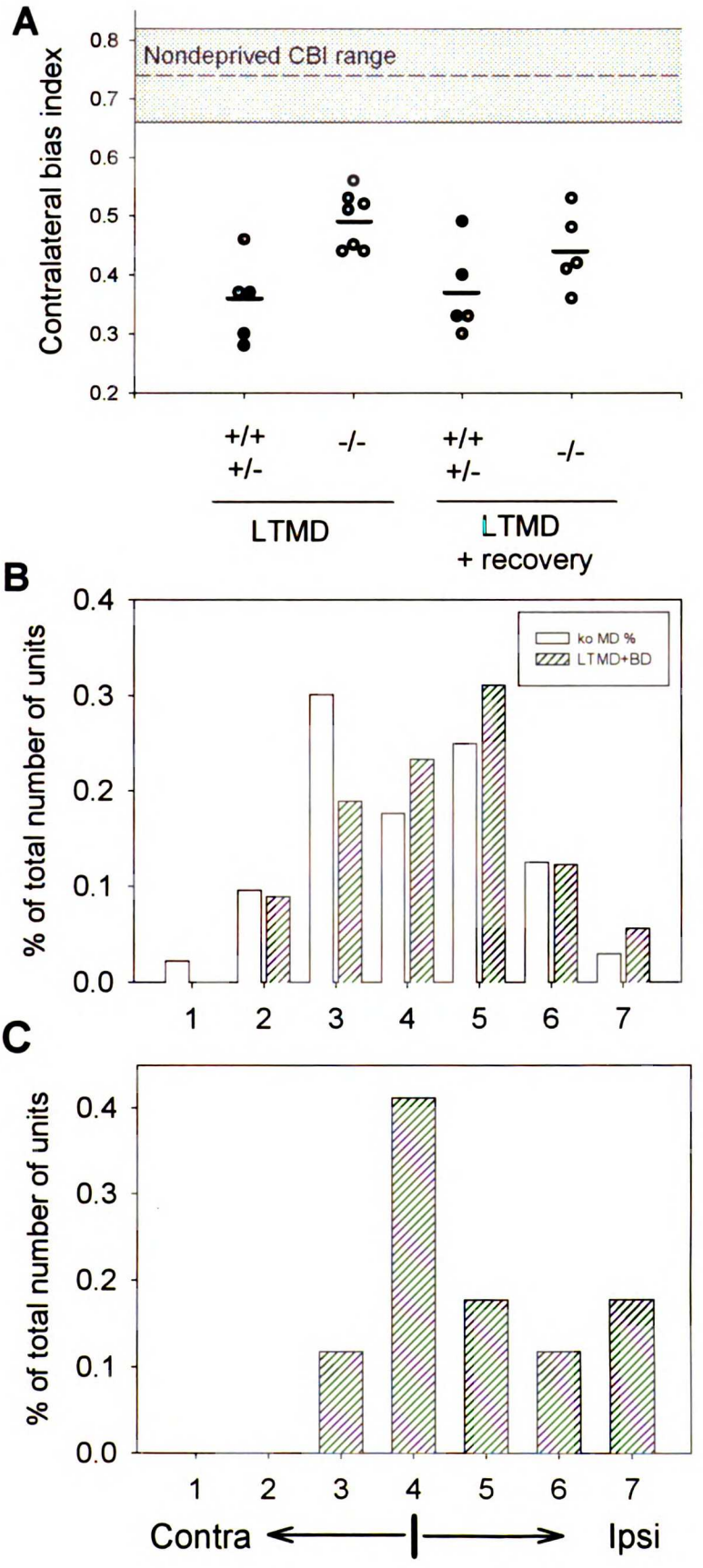

MD
BD
wt/het
LTMD
ko
wt/het
LTMD
+ BD
ko
0
10
20
30
40
50
60
70
Days

Figure 2-4. Long-term MD can drive plasticity in homozygous mutant mice, which cannot be reversed by BD.

(A) LTMD drives robust plasticity in homozygous mutant mice (open symbols). Wild-type (closed symbols) and heterozygous (shaded symbols) littermates show additional plasticity above and beyond that revealed with four day MD. Following LTMD, CBI scores of both wild-types/heterozygotes ($p < 0.001$, t-test) and homozygous ($p < 0.001$, t-test) mice are significantly different from non-deprived animals (gray strip shows non-deprived CBI range). If animals are subjected to LTMD followed by lengthy BD, ocular dominance scores show no signs of decaying to baseline CBI values. CBI scores after LTMD + BD do not differ from those resulting from LTMD alone for either group ($p = 0.81$ for wild-type/het mice; $p = 0.14$ for homozygous mice; t-test).

(B) The distribution of single unit CBI scores in homozygous knock-out mice following LTMD + BD (hatched bars; $n = 90$ units) remains similar to the distribution of scores observed after LTMD (open bars; $n = 136$) alone.

(C) Examination of single unit responses ($n = 17$ units) recorded in the homozygous mutant mouse which underwent the shortest period of LTMD (14 d), followed by lengthy BD (22 d), shows plasticity induced by LTMD is stable even when LTMD period is relatively brief.


A
Contralateral bias index
0.8
0.7
0.6
0.5
0.4
0.3
0.2
Nondeprived CBI range
+/+
+/-
-/-
+/+
+/-
-/-
LTMD
LTMD
+ recovery
B
% of total number of units
0.4
0.3
0.2
0.1
0.0
ko MD %
LTMD+BD
1
2
3
4
5
6
7
C
% of total number of units
0.4
0.3
0.2
0.1
0.0
1
2
3
4
5
6
7
Contra
Ipsi

would be fragile and subject to reversal. Ocular dominance plasticity is driven by unequal levels of activity in the two eyes. Therefore, after inducing plasticity with LTMD, we occluded vision in both eyes. If the plasticity induced by LTMD were labile, we would expect it to reverse during a recovery period of BD, when activity in the two eyes was equal.

In homozygous mice, extended periods of binocular deprivation failed to reverse the effects of prior LTMD. CBI values following LTMD + BD were similar to those found after LTMD alone (figure 2- 4A) for both wild-type/heterozygous and KO mice. Comparison of single unit responses from KO mice undergoing LTMD versus those undergoing LTMD + BD show that the addition of a BD period does not affect the distribution of ocular dominance scores resulting from LTMD alone (figure 2-4B); plasticity in homozygous mice was thus as stable as that in wild-type and heterozygote mice. Inspection of a single homozygous mouse which underwent the shortest LTMD, followed by lengthy BD, shows that even in conditions which minimized the duration of plasticity induction, synaptic changes were stable and had not relaxed to the nondeprived state favoring the contralateral eye (figure 2-4C). Thus, the absence of autophosphorylated αCaMKII in homozygous mutant mice does not impair the stability of existing plasticity, even when tested over a period of weeks.

Discussion

These results represent the first *in vivo* test of the role of αCaMKII autophosphorylation in induction and maintenance phases of cortical plasticity. We find that the induction of ocular dominance plasticity is impaired in homozygous mutant mice, suggesting an important role of autophosphorylated αCaMKII in this process . Once induced in mutant animals, however, plasticity is stable over a period of weeks, suggesting autophosphorylated αCaMKII is not required for the continued expression of synaptic changes.

In vitro studies show that αCaMKII can act directly on synaptic proteins, including the NMDA and AMPA-type glutamate receptors, and that these interactions may give rise to synaptic potentiation (McGlade-McCulloh et al., 1993; Omkumar et al., 1996; Barria et al., 1997). It is possible that similar interactions may contribute to ocular dominance plasticity. If this is the case, impaired induction of ocular dominance plasticity in homozygous KO mice could be accounted for by the disruption of such interactions, resulting from the inability of the T286A mutant αCaMKII to maintain a sustained "on" state.

Autophosphorylation of αCaMKII is not necessary for the stability of induced synaptic changes. Synaptic plasticity induced by LTMD in mice lacking autophosphorylated αCaMKII is stable over a period of weeks. This is not simply the consequence of a

general developmental arrest of plasticity, as BD periods overlapped with times when mouse V1 connections are still plastic (Antonini et al., 1999).

If autophosphorylated αCaMKII is not necessary for the long-term persistence of synaptic changes, how are these synaptic rearrangements maintained? As in wild-type mice, anatomical changes may underlie long-term storage of synaptic changes in homozygous mutant mice (Antonini et al., 1999).

Chapter 3

Rapid ocular dominance plasticity requires cortical, but not geniculate, protein synthesis

Summary

Synaptic plasticity is a multistep process, in which rapid, early phases eventually give way to slower, more enduring stages. Diverse forms of synaptic change share a common requirement for protein synthesis in the late stages of plasticity, which are often associated with structural rearrangements. Ocular dominance plasticity in the primary visual cortex (V1) is a long lasting form of activity-dependent plasticity comprised of well defined physiological and anatomical stages; however, the nature of the molecular events underlying these stages remains poorly understood. Using infusion of the protein synthesis inhibitor cycloheximide, we investigated a role for protein synthesis in ocular dominance plasticity. Suppression of cortical, but not geniculate, protein synthesis impaired rapid ocular dominance plasticity, while leaving neuronal responsiveness intact. These findings suggest that structural changes underlying ocular dominance plasticity occur rapidly following monocular occlusion, and that cortical changes guide subsequent alterations in thalamocortical afferents.

Introduction

The molecular events underlying different forms of activity-dependent synaptic change show remarkable stereotypy in their progression: plasticity is generally composed of distinct, often sequential, stages, in which the transition from early to late stages is marked by a dependence on protein synthesis (Bailey et al., 1996; Dubnau and Tully, 1998; Levenes et al., 1998; Mayford and Kandel, 1999). The sensitivity of late stages of plasticity to disruption by protein synthesis inhibitors was first demonstrated in studies of memory formation *in vivo*, where injections of puromycin in mice were shown to disrupt long-term, but not short-term, expression of learned maze sequences (Flexner et al., 1963). Subsequent investigations have shown that a similar requirement exists in the late stages of sensitization of the gill withdrawal reflex in *Aplysia* (Bailey et al., 1992) and multiple forms of long-term potentiation (LTP: Krug et al., 1984; Frey et al., 1988; Kurontani et al., 1996; Barea-Rodríguez et al., 2000) and long-term depression (Linden, 1996; Huber et al., 2000). In each case, short-term expression of plasticity is thought to require only covalent modifications of existing proteins, while long-term changes require new protein synthesis.

Protein synthesis is thought to be necessary for structural rearrangements at the synapse, and studies in systems that provide accessible anatomical preparations support this view. In *Aplysia*, for instance, the time course of the development of long-term sensitization of the gill withdrawal reflex is paralleled by an increase in the number of synaptic

varicosities in sensory neurons and structural rearrangements in synaptic active zones (Bailey and Chen, 1989). Moreover, these structural changes share with long-term sensitization a requirement for ongoing protein synthesis (Bailey et al., 1992).

The findings described above suggest that protein synthesis is a molecular gateway to long-term plasticity, driven by structural rearrangements at the synapse. The developing visual cortex is an experimental system in which the time course of distinct physiological and anatomical substrates of plasticity has been well-studied, and hence provides the opportunity to investigate the molecular correlates of these changes. During a discrete critical period in postnatal development, synapses in the primary visual cortex are exceptionally plastic, such that periods of monocular deprivation (MD) induce robust changes in the balance of the two eyes' input to cortex (Wiesel and Hubel, 1963; Olson and Freeman, 1980). In the mouse, the most rapid effects of MD are evident in electrophysiological recordings after two days of deprivation, and reach near saturating levels after four days of MD (Gordon and Stryker, 1996). Anatomical changes in thalamocortical arbors require substantially longer periods of deprivation for full expression (Antonini et al., 1999).

Little is known about the molecular mechanisms that underlie ocular dominance plasticity. In particular, the events required for initiating long-lasting changes at the synapse remain enigmatic. N-methyl-D-aspartate receptor function is required for ocular dominance plasticity (Bear et al., 1990; Roberts et al., 1998), as is the kinase action of the synaptic protein alpha calcium-calmodulin kinase II (αCaMKII; Gordon et al., 1996);

both molecules are likely to be involved in early stages of plasticity induction, given their well-defined roles in mediating neuronal calcium influx (Daw et al., 1993) and initiating calcium-dependent signaling (Soderling, 2000). Downstream signaling events are not well understood, but the many instances of activity-dependent protein synthesis documented suggest that translation of proteins is required for normal plasticity. Multiple plasticity-related genes show visually driven increases in protein levels, including αCaMKII (Wu et al., 1998) and the calcium/cyclicAMP-response element binding protein (Pham et al., 1999), and lengthy MD can alter gross levels of protein synthesis in the lateral geniculate nucleus (LGN: Kennedy et al., 1981). Neurotrophins and their receptors are regulated in an activity-dependent fashion *in vitro* and *in vivo* (Zafra et al., 1992; Schoups et al., 1995; Androutsellis-Theotokis et al., 1996; Lein and Shatz, 2000) and have potent effects upon synaptic plasticity in the visual cortex (Cabelli et al., 1995; McAllister et al., 1995; Hanover et al., 1999; Gillespie et al., 2000). To date, however, there has been no direct demonstration of a requirement for protein synthesis in ocular dominance plasticity.

To investigate this issue, we infused the protein synthesis inhibitor cycloheximide into distinct neural targets, while subjecting mice to concurrent MD. We demonstrate that intact levels of protein synthesis are necessary for ocular dominance plasticity, and that this requirement for translation arises rapidly after monocular occlusion. The locus of this requirement is cortical; while thalamic inhibition of protein synthesis leaves ocular dominance plasticity intact, cortical infusions of cycloheximide block the effects of MD.

Materials and Methods

Monocular deprivation. MD was performed according to published protocols (Gordon and Stryker, 1996), except 3% isofluorane (Abbott, North Chicago, IL) in oxygen was used for anesthesia. In every case, MD was initiated within the critical period for mouse ocular dominance (p26-p30), and lasted for four days.

Surgical implantation of minipumps. Mice were anesthetized with 3% isofluorane in oxygen, and mounted in a stereotaxic holder. Ophthalmic lubricant was applied to protect the eyes, and body temperature maintained with a heating pad maintained at 37°C. For infusion experiments, Alzet osmotic minipumps (Alza 1002, 0.25 ul/h) were filled either with cycloheximide (10 mg/ml; Sigma, St. Louis, MO) or with physiological saline for control experiments, and attached to 30 gauge stainless steel cannulae. Under aseptic conditions, a longitudinal incision was made in the scalp over the mid-sagittal sinus, and the skin retracted. The portions of the skull overlying occipital and frontal portions of the brain were cleaned and dried. To aid in securing the cannula, a single skull screw was implanted approximately 2 mm lateral of the midline and 2 mm caudal of bregma in the skull overlying the non-infused hemisphere. Cannulae were implanted using stereotaxic coordinates (V1 infusion coordinates: 1 mm lateral (midline), 1 mm rostral (lambda), 1 mm deep; LGN infusion coordinates: 1.3 mm lateral, 0.7 mm caudal, 2.5 mm deep) and were secured to the skull and skull screw with dental cement. The attached minipump was placed in a subcutaneous pocket at the nape of the neck. The scalp was closed over

the implant, and the animal returned to its home cage when sternal. For plasticity experiments, minipump implantation was performed on the day prior to MD, and infusion continued for the duration of the MD, for a total of five days of infusion. For autoradiography experiments and quantitative measurements of receptive field properties, implantation was performed in p25-p30 mice, and infusion lasted five days.

Single unit recording. All monocularly deprived mice were prepared for electrophysiology after four days of MD. Single units were recorded from normal, non-deprived mice which ranged in age from p30 to p77 (mean = p48). Mice were anesthetized for electrophysiological recording with a combination of Nembutal (50 mg/kg; Abbott) and chlorprothixene (0.2 mg; Sigma, St. Louis, MO) using standard protocols (Gordon and Stryker, 1996). In each mouse, single units were isolated at intervals of 60 microns or more, using lacquer coated tungsten electrodes in the binocular region of primary visual cortex contralateral to the deprived eye. A handlamp was used to project moving bars or squares onto a tangent screen to drive neuronal responses. The balance of the two eyes' input to each unit was scored on the 1 to 7 scale of Hubel and Weisel (Hubel and Wiesel, 1962) , where a value of 1 indicates complete domination by the contralateral eye, and 7 indicates input arises from the ipsilateral eye only. For each mouse, these ocular dominance scores were used to compute a single contralateral bias index (CBI) according to the formula:

$$CBI = \left[\frac{(n_1 - n_7) + \left(\frac{2}{3}\right)(n_2 - n_6) + \left(\frac{1}{3}\right)(n_3 - n_5) + N}{2N} \right]$$

where N = total number of cells, and n_x = number of cells with ocular dominance scores equal to x. Each unit was also scored for habituation, response amplitude, and the presence or absence of orientation bias. To assess the effects of cycloheximide infusion on neuronal activity, single unit responsiveness was assessed using computer driven stimulus presentation and spike collection. Stimulus-driven neuronal responses were assessed in response to a high contrast, low spatial frequency square wave grating presented at the optimal orientation.

^{3}H-leucine injection and slice preparation. Mice were anesthetized with 3% isofluorane in oxygen, and body heat maintained at 37°C with a heating pad. 100 μC of tritiated leucine (1 mCi/mL, NEN Life Sciences, Boston, MA) were injected into the tail vein. Anesthesia was discontinued, and mice were allowed to survive for 30 minutes after righting themselves. They were then deeply anesthetized with Nembutal, and perfused with cold 0.1 M phosphate buffer followed by 4% paraformaldehyde. The brain was removed and cryoprotected overnight in 4% paraformaldehyde/30% sucrose. 40 μm coronal slices were cut on a freezing microtome, mounted from 1% gelatin onto slides, dehydrated and defatted, and allowed to dry overnight.

Autoradiography. In the darkroom, sections were coated with a thin layer of emulsion (Kodak NTB-2, Kodak Scientific Imaging Systems, New Haven, CT) by dipping. They were allowed to dry for four hours in a humidified chamber, then transferred to dry, light-tight boxes to be stored at 4°C for exposure. Sections were developed by serial exposure to developer, water rinse, and fixative, and bisbenzimide-stained before dehydrating and cover slipping.

Quantification of protein synthesis inhibition and cell density. Silver grain puncta, representing incorporation of tritium-labeled leucine into nascent proteins, were counted to quantify the effects of cycloheximide infusion. All quantification of protein synthesis inhibition was carried out by comparing hemispheres (infused vs. uninfused) in a single section, and were therefore perfused identically and on slides exposed for the same length of time. Typically, sections were developed after 10 days, though exposures ranged in length from 6 to 21 days. To measure protein synthesis in the cortex, measurements were made in coronal sections containing portions of V1 corresponding to central areas of the visual field (~ 1 mm from the caudal pole of the brain). Digital photographs of fields ~ 350 x 450 μm in size were taken at approximately 1 mm intervals across the medial-lateral extent of the cortex. Silver grain puncta were counted using an automated particle counting function developed on the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) with the threshold set at an arbitrary level, which was identical for infused and uninfused hemispheres in each section. Cortical cell density was quantified using similar counting procedures in bisbenzimide-stained sections identical to those used for measuring protein synthesis inhibition. Measurements of protein synthesis inhibition in the LGN were made in a similar fashion, with two changes. Protein synthesis was quantified in serial sections of the LGN, and therefore included the entire volume of the LGN. Also, the area encompassed by the LGN in each section was identified using cytoarchitectonic cues, and the silver grain count restricted to that area.

Results

Inhibition of protein synthesis in visual cortex

Monocular deprivation in mice must be maintained over four days to induce expression of near saturating plasticity (Gordon and Stryker, 1996). Thus, it was necessary to establish a reliable means of inhibiting protein synthesis in the visual cortex over a period of days. We used osmotic minipumps to infuse cycloheximide directly into cortical tissue medial to V1, choosing a concentration that minimized neurotoxic effects of the drug, but that was sufficient to suppress protein synthesis several millimeters from the infusion site.

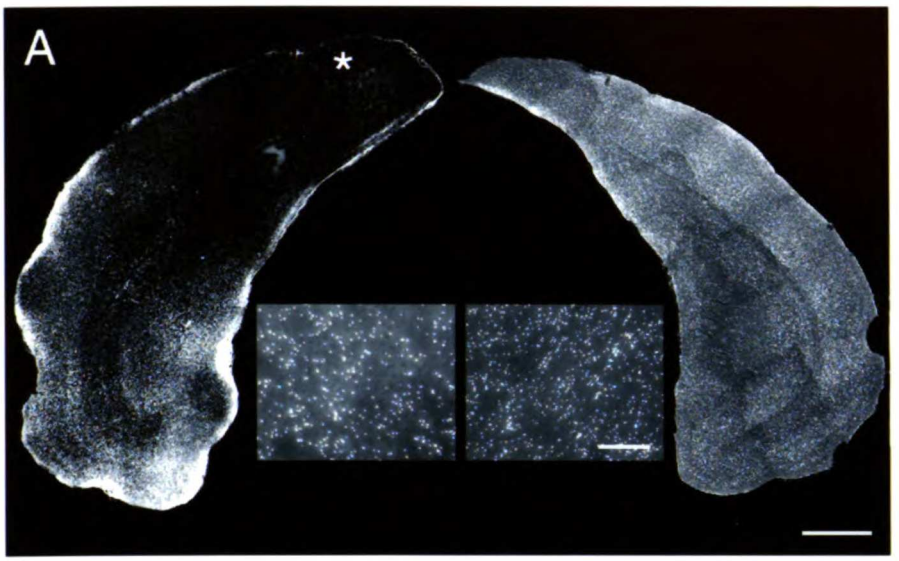
A typical example of the effects of extended (5 day) unilateral infusion of cycloheximide is shown in a coronal slice of mouse brain in Figure 3-1A. Protein synthesis in the region of the visual cortex, as measured by incorporation of the tritium-labeled amino acid leucine into nascent proteins (see Experimental Procedures), was substantially suppressed in the cycloheximide-infused hemisphere. Silver grain density decreased in a lateral to medial gradient in the cycloheximide-infused hemisphere, reflecting the medial placement of the infusion cannula. Protein synthesis levels in the uninfused hemisphere were uniformly high across the medial-lateral span of the cortex, indicating that little, if any, cycloheximide crossed the midline. High magnification insets show silver grain puncta in the region of the visual cortex.

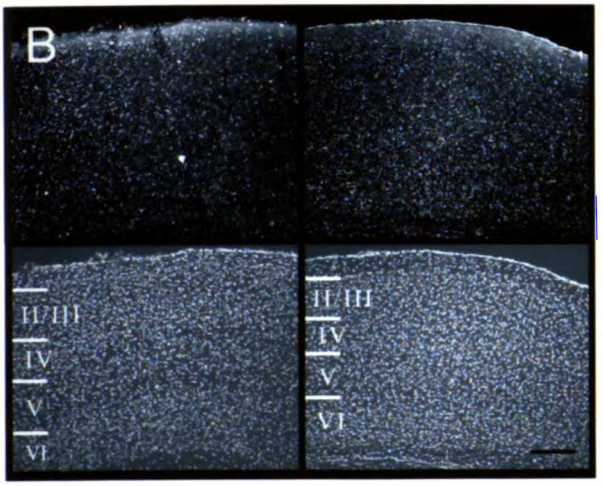
Figure 3-1. Cortical cycloheximide infusion blocks protein synthesis locally.

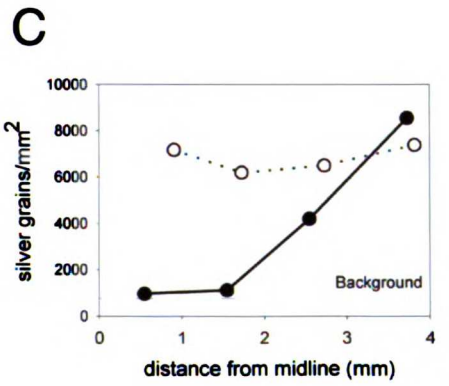
(A) Coronal sections through V1 show suppression of protein synthesis in the infused hemisphere (left, with mediolateral position of cannula indicated by the asterisk), but not the uninfused hemisphere (right). High-magnification insets show silver grains in the region of V1 (~2 mm from the midline in this section); the silver grain count in uninfused V1 (right inset) is approximately two-fold higher than that in cycloheximide-infused V1 (left inset). Scale bar, 1 mm; inset scale bar; 100 μm.

(B) Silver grains representing protein synthesis in cycloheximide-infused (top left) and uninfused (top right) coronal sections, with nuclear bisbenzimide staining from identical sections (below) showing cortical laminae in V1. Sections are the same as those shown in (A). Scale bar, 100 μm.

(C) Quantification of silver grain density over the medial-lateral extent of the cycloheximide-infused (closed symbols) and uninfused (open symbols) sections shown in (A).


A
*


B
II/III
IV
V
VI
II/III
IV
V
VI


C
10000
8000
6000
4000
2000
0
silver grains/mm2
Background
0
1
2
3
4
distance from midline (mm)

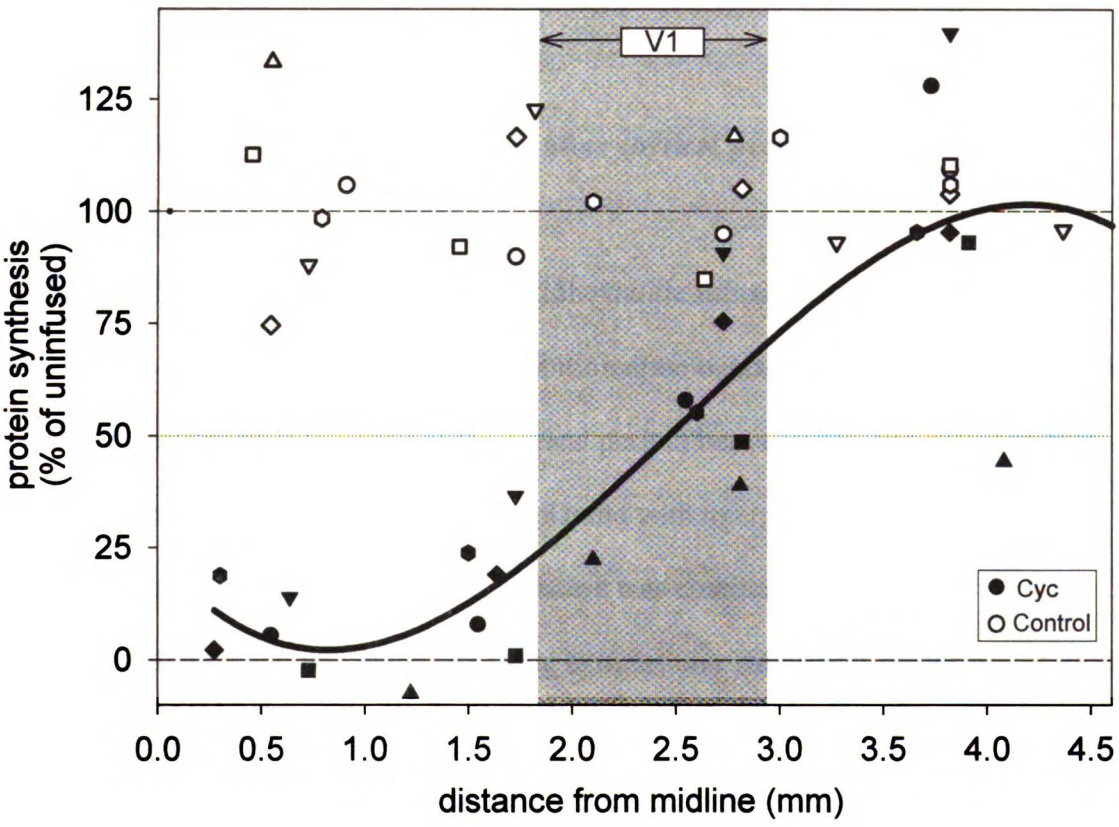
The spatial gradient of protein synthesis inhibition was quantified by sampling silver grain counts across the medial-lateral extent of the hemisphere (Figure 3-1C; results from section shown in Figure 3-1A). In the infused hemisphere, protein synthesis was reduced to near background levels close to the midline, remained substantially inhibited 2-3 mm away, and rose to control levels near 4 mm from the midline. Protein synthesis levels varied only slightly across the extent of the uninfused hemisphere.

A side by side comparison of infused and uninfused bisbenzimide-stained sections (Figure 3-1B) shows that cycloheximide infusion did not disrupt the normal laminar structure of the cortex, nor reduce cell density (cell density in infused V1 = 93 ± 6% of uninfused V1; $p = 0.42$; t-test) in the region of the visual cortex. These properties of normal cortical architecture were maintained despite substantial suppression of protein synthesis; in the region shown, silver grain density in the cycloheximide-infused hemisphere was reduced approximately 2-fold relative to the uninfused hemisphere.

Grouped results taken from multiple unilateral cortical infusions ($n = 6$ mice) are shown in Figure 3-2. Silver grain counts were normalized to the average silver grain density in all uninfused hemispheres and plotted as a function of distance from the midline. Using cytoarchitectonic cues, V1 was located in infused hemispheres: the gray strip indicates the position of V1 relative to the midline (medial margin of V1 = 1.8 ± 0.2 mm from midline; lateral margin = 2.9 ± 0.2 mm). On average, our infusion protocol reduced protein synthesis in V1 to less than half that measured in uninfused hemispheres (45 ± 12% at midpoint of V1, mean ± 95% confidence interval). Though there was variability

Figure 3-2. Cortical cycloheximide infusion reliably suppresses protein synthesis in V1.

Protein synthesis is significantly suppressed over the span of V1 (gray strip) in cycloheximide-infused hemispheres (n = 6; closed symbols) relative to protein synthesis levels in uninfused hemispheres (n = 6; open symbols). The regression line (black line; r^2 = 0.78) fitted to data from cycloheximide-infused hemispheres shows protein synthesis is significantly suppressed relative to saline-infused controls ($p<0.0001$). Different symbols represent data taken from individual animals.


V1
125
100
75
50
25
0
protein synthesis
(% of uninfused)
Cyc
Control
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
distance from midline (mm)

in the extent of protein synthesis inhibition, all infused hemispheres showed significantly reduced protein synthesis near the medial margin of V1 (avg. = 18 ± 5.1% of uninfused mean at 1.70 ± 0.09 mm from midline; $p < 0.001$; t-test). Near the lateral margin of V1, protein synthesis was somewhat higher (61 ± 7.7% at 2.7 ± 0.04 mm), but still significantly reduced relative to control levels ($p < 0.05$; t-test). Thus, our infusion protocol yielded a reliable means of suppressing protein synthesis in the region of the V1.

Blockade of ocular dominance plasticity after cortical protein synthesis inhibition

Having established the effectiveness of cycloheximide infusion in suppressing protein synthesis, we tested the effects of such inhibition upon ocular dominance plasticity. Lid suture was performed at the peak of the critical period, between p26 and p30, and MD lasted four days in all cases. Mice were implanted with minipumps for cycloheximide infusion one day prior to lid suture, and infusion was continued over the four days of MD. Ocular dominance was measured using extracellular single unit recordings of visual responses in binocular visual cortex. For purposes of comparison, units from each mouse were used to compute a single contralateral bias index (CBI), which reflects the relative strength of the contralateral eye's input to cortex.

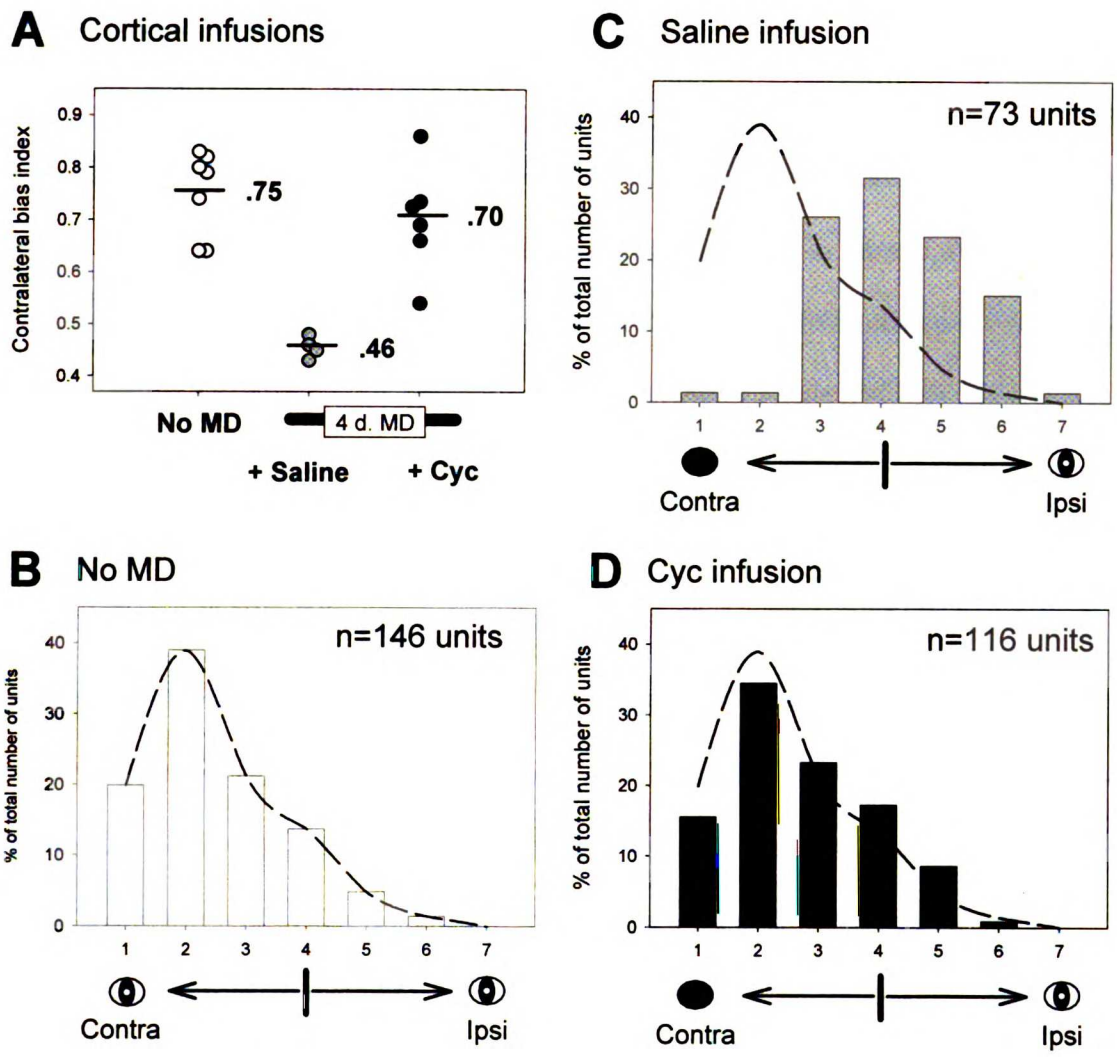
In normal mice, cortical responses are dominated by the contralateral eye. This is reflected in the high mean CBI of nondeprived mice (CBI = 0.75 ± 0.03, mean ± SEM; Figure 3-3A) and the left-shifted distribution of units in the ocular dominance histogram (Figure 3-3B). Control mice infused with physiological saline one day prior to, and

Figure 3-3. inhibition of cortical protein synthesis blocks ocular dominance plasticity.

(A) Normal, non-deprived mice show a strong contralateral bias (n = 7 mice; open symbols). After four day MD, saline-infused animals shift toward the open ipsilateral eye (n = 4; gray symbols), but cycloheximide-infused animals (n = 6; closed symbols) do not. Mean CBI for each group is indicated to the right of horizontal bars.

(B) The histogram of single units recorded from normal, non-deprived mice is weighted heavily toward the contralateral eye. A smoothed curve (broken line) was fitted to the data for purposes of comparison with (C) and (D) below.

(C) and (D) Histograms of saline- (C) and cycloheximide-infused (D) animals showing ocular dominance scores of single units recorded from animals shown in (A). The dashed line in both panels shows the distribution of ocular dominance scores for the nondeprived mice shown in A; the left-shifted peak reflects the contralateral bias found in normal mice. MD + saline infusion (gray bars) results in a relative increase in cortical responses driven by the ipsilateral eye and a pronounced shift toward higher ocular dominance scores. Plasticity is blocked in cycloheximide-infused animals (black bars); units recorded from these mice continue to be driven by the closed contralateral eye, and their distribution remains similar to non-deprived animals.


A Cortical infusions
Contralateral bias index
0.9
0.8
0.7
0.6
0.5
0.4
.75
.46
.70
No MD
4 d. MD
+ Saline
+ Cyc
C Saline infusion
n=73 units
% of total number of units
40
30
20
10
0
1 2 3 4 5 6 7
Contra
Ipsi
B No MD
n=146 units
% of total number of units
40
30
20
10
0
1 2 3 4 5 6 7
Contra
Ipsi
D Cyc infusion
n=116 units
% of total number of units
40
30
20
10
0
1 2 3 4 5 6 7
Contra
Ipsi

concurrent with four days of MD, displayed a robust shift in ocular dominance, such that the balance of neuronal responses came to favor the nondeprived ipsilateral eye (CBI = 0.46 ± 0.01; Figure 3-3A). The shift in the distribution of ocular dominance scores of individual units following saline + MD treatment can be seen in Figure 3-3C. The dotted line is a best fit for the distribution of ocular dominance scores in normal, nondeprived animals; following saline infusion + MD, responses shifted significantly ($p < 0.0001$; t-test) toward the open ipsilateral eye.

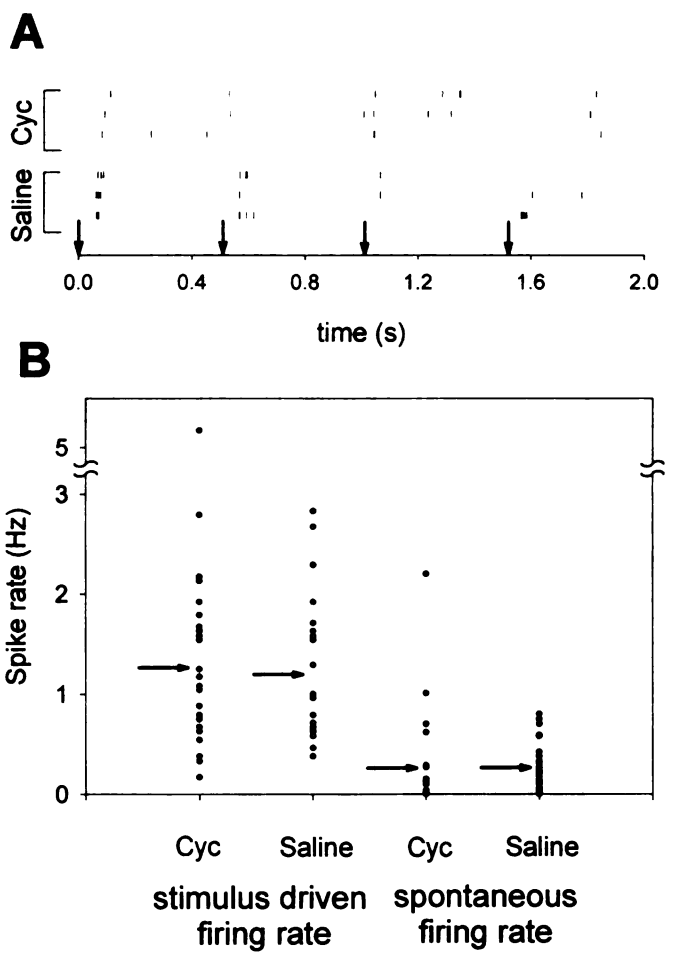
Infusion of cycloheximide blocked the normal effects of MD. The mean CBI of animals undergoing cycloheximide + MD treatment (CBI = 0.70 ± 0.04) was similar to that of nondeprived animals ($p = 0.36$; t-test), but significantly different from that of the saline + MD group ($p < 0.005$; t-test) . The majority of neurons in cycloheximide-infused animals continued to be driven by the deprived contralateral eye, and as can be seen in Figure 3-3D, the distribution of ocular dominance scores following cycloheximide infusion remained very similar to that observed in nondeprived animals.

Response properties of neurons in cycloheximide-infused V1

Ocular dominance plasticity is critically dependent upon cortical levels of neuronal responsiveness (Shaw and Cynader, 1984; Reiter et al., 1986; Reiter and Stryker, 1988; Hensch et al., 1998). To ensure that our results were not simply the consequence of suppressing neuronal responses, we characterized neuronal activity in cycloheximide-

Figure 3-4. Neuronal responses are normal in cycloheximide-infused mice.

(A) Neurons in both cycloheximide- (top) and saline-infused (bottom) cortices respond to visual stimulus presentation (arrows) at short latency. Vertical ticks represent the time of occurrence of action potentials from a single neuron over three stimulus presentations. (B) Mean (arrows) stimulus-driven and spontaneous firing rates did not differ between groups. Each symbol represents the firing rate for a single neuron (cycloheximide-infused, n = 29 units; saline-infused, n = 26 units).


A
Cyc
Saline
0.0
0.4
0.8
1.2
1.6
2.0
time (s)
B
Spike rate (Hz)
5
3
2
1
0
Cyc
Saline
Cyc
Saline
stimulus driven firing rate
spontaneous firing rate

and saline-infused animals. The raster shown in Figure 3-4A illustrates typical stimulus-driven neuronal responses. For both groups, flashed, high-contrast gratings elicited a response (often a single action potential, and occasionally a short burst of action potentials) at short latency. Population analysis of electrical activity (Figure 3-4B) shows that levels of stimulus-driven activity were similar in both groups of animals (cycloheximide-infused mean = 1.26 ± 0.19 spikes/s; saline-infused mean = 1.20 ± 0.13 spikes/s; $p = 0.77$; t-test). Spontaneous levels of electrical activity were also comparable between groups (cycloheximide-infused mean = 0.26 ± 0.08 spikes/s; saline-infused mean = 0.26 ± 0.05 spikes/s; $p = 0.98$; t-test). Thus, cycloheximide infusion under the conditions employed here did not suppress normal levels of neural activity. None of the other receptive field properties quantified, including retinotopy, orientation bias, receptive field area, and habituation, were affected by cycloheximide infusion (Figure 3-5; infused vs. uninfused cortex, all $p > 0.05$; t-test).

Intact ocular dominance plasticity following geniculate protein synthesis inhibition

Visual information from the retina is communicated to the visual cortex through the LGN of the thalamus. The thalamic neurons extending afferents to the visual cortex are not, however, passive relay elements. During MD, thalamocortical afferents undergo dramatic activity-dependent rearrangements, including extensive pruning of deprived eye arbors, and complementary expansion of non-deprived eye afferents (Antonini and Stryker, 1993). We performed a second set of experiments, then, in which we probed the

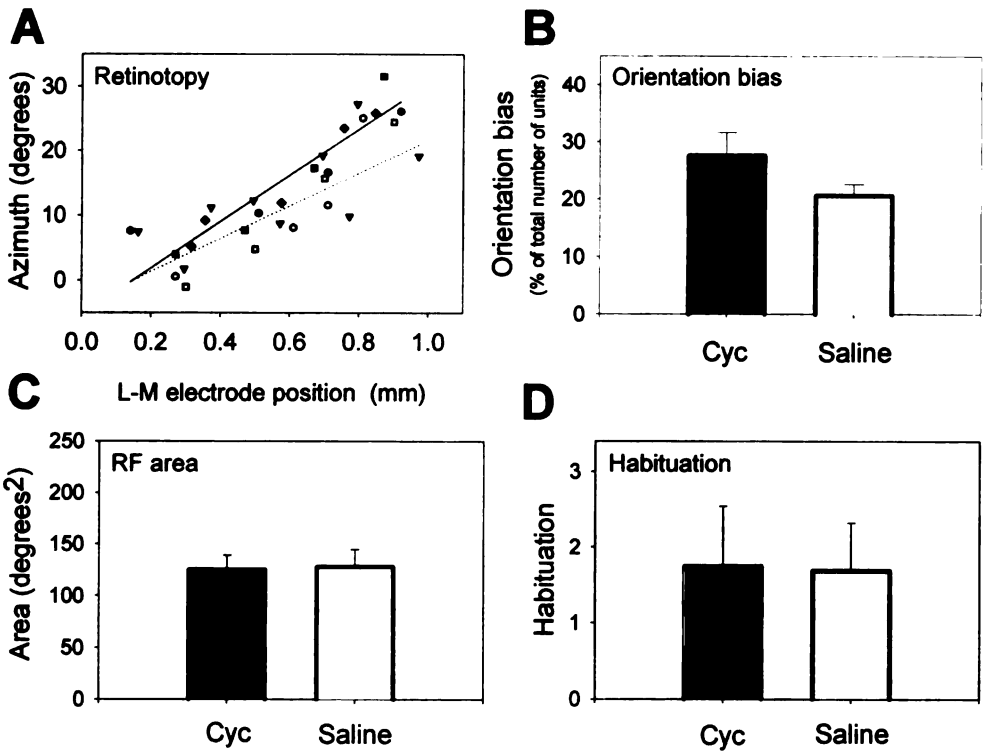
Figure 3-5. Receptive field properties are not altered by cycloheximide infusion.

(A) Retinotopy in cycloheximide- (n = 18 penetrations in 4 hemispheres; closed symbols; unbroken line is linear best fit) and saline-infused animals (n = 13 penetrations in 3 hemispheres; open symbols; dotted line is linear best fit) is similar (p = 0.52, comparing slopes between groups; t-test).

(B) Orientation bias in single units recorded in cycloheximide- (28 ± 4% of total number of units, 6 animals, n = 116 units) and saline-infused (21 ± 2%, 4 animals, n = 73 units) animals does not differ (p = 0.18; t-test).

(C) Receptive field area does not differ between groups (p = 0.89; t-test). Mean value ± SEM is 125.0 ± 14 degrees^2 in cycloheximide-infused animals (n = 14 units) and 128.1 ± 17 degrees^2 in saline-infused animals (n = 18 units).

(D) Habituation is similar between groups (cycloheximide-infused mean ± SEM: 1.75 ± 0.07; n = 116 units; saline-infused mean ± SEM: 1.69 ± 0.07; n = 73; p = 0.56; t-test).


A
Retinotopy
Azimuth (degrees)
30
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
L-M electrode position (mm)
B
Orientation bias
Orientation bias
(% of total number of units)
40
30
20
10
0
Cyc
Saline
C
RF area
Area (degrees2)
250
200
150
100
50
0
Cyc
Saline
D
Habituation
Habituation
3
2
1
0
Cyc
Saline

thalamic contribution to rapid plasticity induced by brief MD. Selective suppression of protein synthesis within the pre-synaptic element of the thalamocortical circuit was attained by infusing cycloheximide into the diencephalon near the LGN, enabling us to dissect the pre- and post-synaptic contributions to the earliest expression of ocular dominance plasticity.

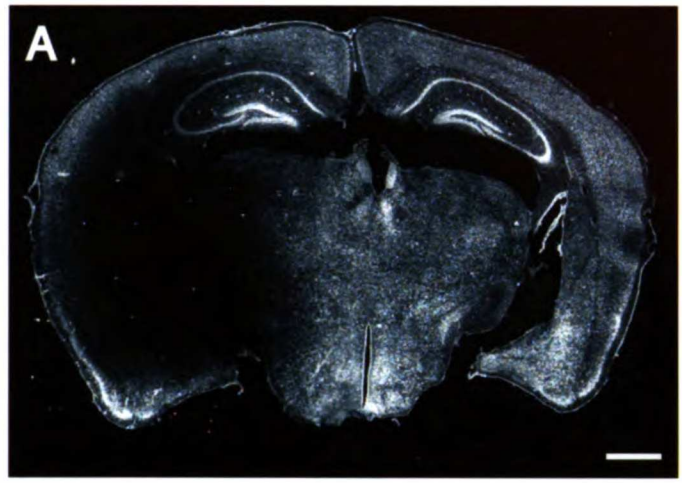
Cannulae infusing cycloheximide were placed anterior to the LGN, which allowed us to selectively suppress protein synthesis in the LGN without affecting translation in V1. Peri-thalamic infusion resulted in a large sphere of protein synthesis inhibition which included much of the LGN in the infused hemisphere, but did not extend into the overlying visual cortex or the contralateral thalamus (Figure 3-6A). Quantification of relative levels of protein synthesis in serial sections of infused hemispheres revealed substantially reduced protein synthesis levels, which averaged 36 ± 7% (relative to uninfused; significantly different from uninfused, $p < 0.0001$; t-test) over the entire volume of the LGN (Figure 3-6C). Though caudal sections of the geniculate showed less suppression of protein synthesis, the central core of the LGN, which projects to the binocular region of V1 investigated in this study, showed reliable inhibition. Protein synthesis levels in V1 of infused and uninfused hemispheres were comparable (protein synthesis in V1 of infused hemisphere = 106 ± 3.3% of uninfused hemisphere; $p = 0.14$; t-test), supporting the notion that thalamic cycloheximide infusion did not block protein synthesis in post-synaptic cortical neurons.

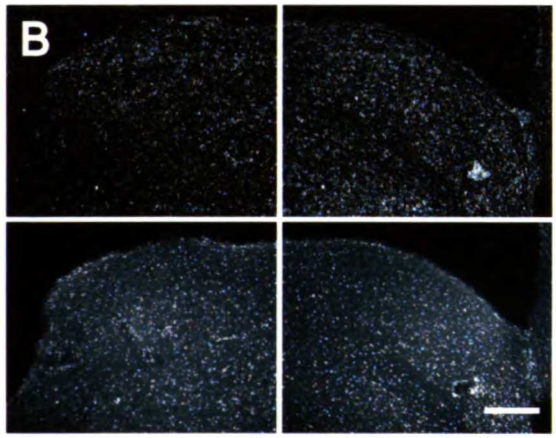
Figure 3-6. Thalamic cycloheximide infusion inhibits LGN protein synthesis.

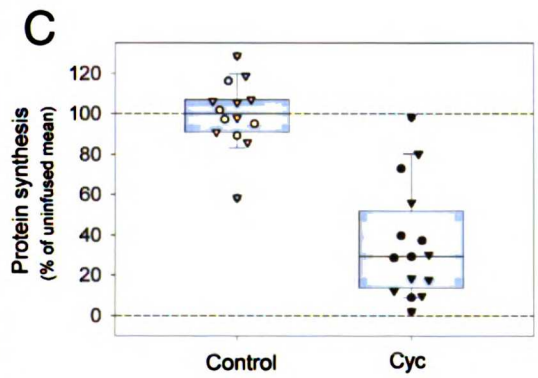
(A) Cycloheximide infusion suppresses LGN protein synthesis in the infused hemisphere (left hemisphere), but not ipsilateral V1 (not illustrated: caudal to section shown) or contralateral LGN (right hemisphere).

(B) High magnification photographs of silver grains representing nascent protein synthesis (top) in the LGN of the cycloheximide-infused hemisphere (left), and the contralateral LGN (right). Bisbenzimide staining in the same coronal sections is shown below.

(C) Quantification of protein synthesis inhibition in the LGN (uninfused, n = 14 sections, 2 hemispheres; cycloheximide-infused, n = 15 sections, 2 hemispheres). Each symbol represents a silver grain density derived from a single section; different symbols represent sections from different individual animals. Box plots represent median values, 10^{th}, 25^{th}, 75^{th}, and 90^{th} percentiles.


A


B


C
Protein synthesis
(% of uninfused mean)
120
100
80
60
40
20
0
Control
Cyc

Despite substantial inhibition of LGN protein synthesis, ocular dominance plasticity was normal in cycloheximide-infused animals. The mean CBI of cycloheximide-infused animals (CBI = 0.47 ± 0.02) following MD was not significantly different from that of control saline-infused mice (CBI = 0.41 ± 0.01, Figure 3-7A, $p = 0.09$; t-test), and both groups were significantly different from non-deprived mice (both $p < 0.001$; t-test). Ocular dominance histograms for both groups showed a pronounced shift in the distribution of single unit responses toward the open ipsilateral eye (Figure 3-7B and 3-7C). The results suggest an asymmetry in the contribution of pre- and post-synaptic elements to the expression of rapid plasticity; translation of novel proteins within cell bodies of the LGN is not needed for initial changes following brief MD.

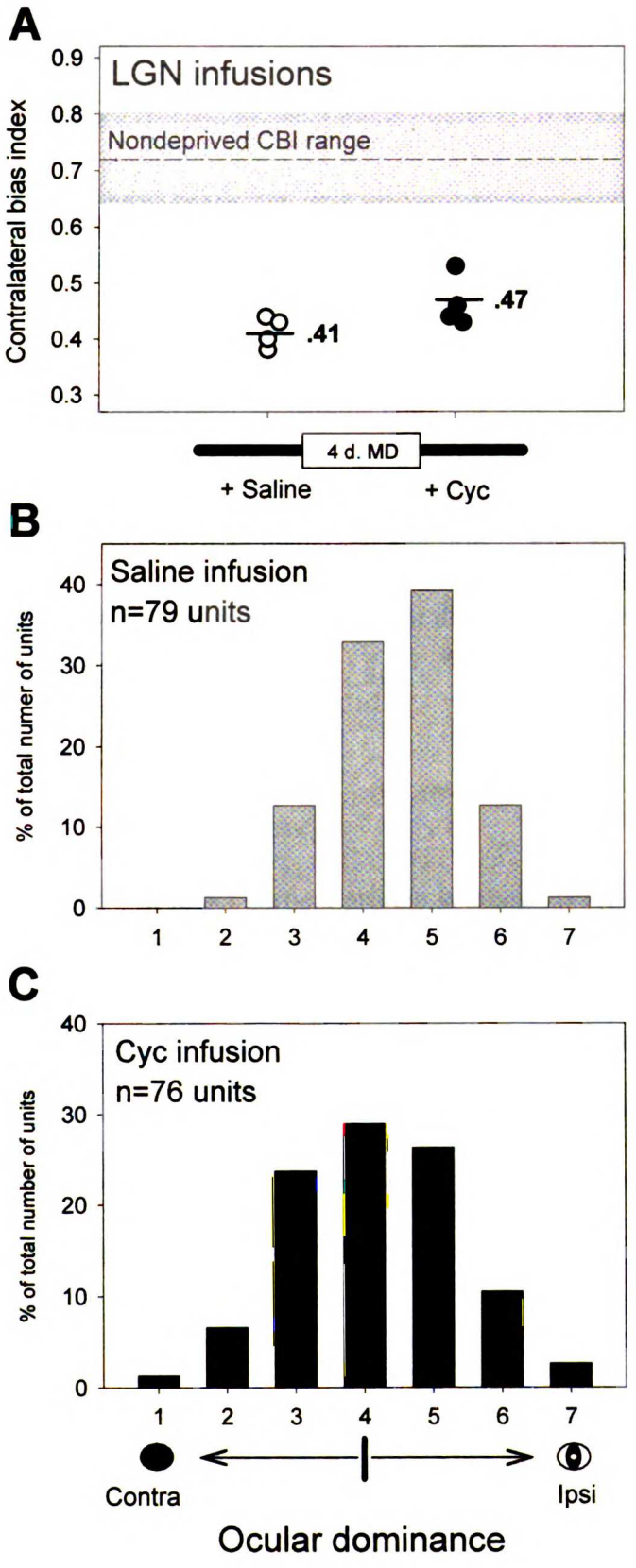
Normal plasticity in mice lacking dendritic αCaMKII mRNA

The selective blockade of ocular dominance plasticity with cortical, but not geniculate, cycloheximide infusion, suggests protein synthesis must be intact in cortical neurons for plasticity to proceed. The intracellular locus of that requirement, however, is unknown. Local dendritic translation is an attractive option in this regard, as it provides a simple explanation for the input specificity associated with many forms of synaptic change, including ocular dominance plasticity. Manipulations of the protein αCaMKII constitute a credible strategy for assaying the importance of dendritic translation, as αCaMKII shows activity-dependent dendritic translation and is known to be required for ocular dominance plasticity. We employed a genetic approach to test the possibility of a

Figure 3-7. Ocular dominance plasticity is intact after thalamic infusion of cycloheximide.

(A) CBI values well outside of the normal range for nondeprived animals (gray strip) indicates saline- (n = 4 mice) and cycloheximide-infused (n = 4) animals both show robust plasticity in response to four day monocular deprivation.

(B) and (C) Ocular dominance histograms for saline- (gray bars) and cycloheximide-infused (black bars) animals. As in Figure 3, the dotted curve is a best fit line for the ocular dominance distribution of units from normal, nondeprived mice; in both (B) and (C), the distribution of individual units is shifted toward the right, and toward the open ipsilateral eye.


A
LGN infusions
Nondeprived CBI range
Contralateral bias index
0.9
0.8
0.7
0.6
0.5
0.4
0.3
.41
.47
4 d. MD
+ Saline
+ Cyc
B
Saline infusion
n=79 units
% of total numer of units
40
30
20
10
0
1
2
3
4
5
6
7
C
Cyc infusion
n=76 units
% of total number of units
40
30
20
10
0
1
2
3
4
5
6
7
Contra
Ipsi
Ocular dominance

requirement for dendritic protein synthesis of αCaMKII in ocular dominance plasticity by assessing the effects of MD in mice lacking the 3' untranslated region (UTR) of the synaptic protein αCaMKII. Genetic ablation of this 3' UTR removes dendritic targeting sequences from the nascent RNA, and thus prevents accumulation of αCaMKII mRNA at dendrites.

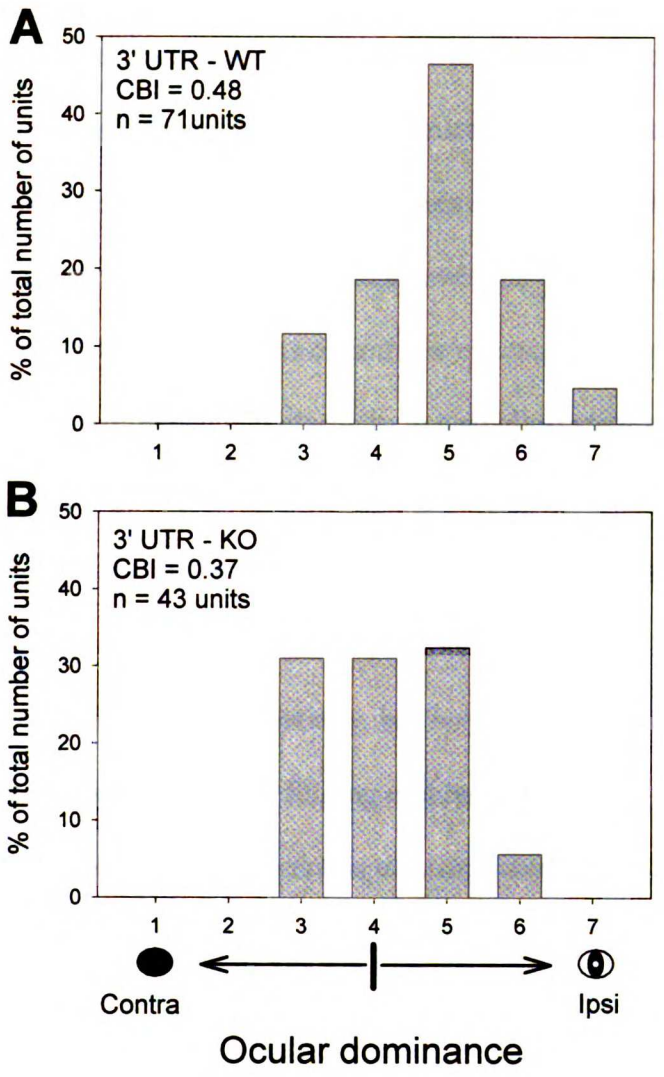
Plasticity in 3'-UTR homozygous knockouts and wild-type littermates was identical following four day monocular deprivation. Both groups showed a robust shift in neuronal responses toward the nondeprived ipsilateral eye (Fig. 3-8). Thus, dendritic translation of αCaMKII mRNA may be unnecessary for ocular dominance plasticity.

Discussion

We have demonstrated that intact cortical protein synthesis is required for ocular dominance plasticity. Moreover, the blockade of plasticity by cycloheximide is not the trivial consequence of suppressing neuronal responses, as single units recorded in cycloheximide-infused animals demonstrate normal levels of spontaneous and evoked neuronal firing. Two aspects of the requirement for protein synthesis – its timing and locus – have interesting implications for the molecular mechanisms which mediate ocular dominance plasticity.

Figure 3-8. Absence of dendritic aCaMKII mRNA does not impair ocular dominance plasticity.

Units from both wild-type (top) and mutant (bottom) mice lacking dendritic αCaMKII mRNA show reliable shifts toward the open ipsilateral eye after four day MD.


A
3' UTR - WT
CBI = 0.48
n = 71units
% of total number of units
50
40
30
20
10
0
1
2
3
4
5
6
7
B
3' UTR - KO
CBI = 0.37
n = 43 units
% of total number of units
50
40
30
20
10
0
1
2
3
4
5
6
7
Contra
Ipsi
Ocular dominance

Rapid structural change in ocular dominance plasticity

The timing of the requirement for protein synthesis following monocular occlusion is rapid, as suppression of cortical translation blocks the earliest plasticity apparent following monocular occlusion. While anatomical changes induced in thalamocortical arbors might well be expected to require protein synthesis for their expression, the finding that cortical protein synthesis blocks even the most rapidly expressed effects of MD is surprising. Characterizations of both the electrophysiological and anatomical dimensions of activity-dependent plasticity in a single system are rare, but evidence from studies of gill withdrawal sensitization in *Aplysia* (Bailey et al., 1992), hippocampal LTP (Engert and Bonhoeffer, 1999), and memory formation in chicks (Rose and Stewart, 1999) converge in suggesting that protein synthesis is required for structural changes which underlie "late" stages of plasticity, but *not* for the quick electrophysiological changes which precede them. Our finding of a requirement for protein synthesis in rapid ocular dominance plasticity suggests this electrophysiological plasticity may also have a structural substrate, and in this regard may be akin to the "late" stages of other forms of plasticity.

If anatomical changes indeed contribute to the rapid changes induced by MD, this must be reconciled with much evidence documenting the relatively slow emergence of anatomical changes which substantially lag electrophysiological plasticity. In the cat, electrophysiological plasticity is apparent after hours of MD (as few as 4-6 hours:

Movshon and Dursteler, 1977; Freeman and Olson, 1982; Frank et al., 2001), while a full week of MD is required for consistent expression of changes in thalamocortical arbors (Antonini and Stryker, 1993). Nor are more rapid changes evident in the fine structure of thalamocortical terminals: the density of thalamocortical terminals is not changed by MD (Silver and Stryker, 1999). In the mouse, the disparity in the timing of electrophysiological and anatomical change is even greater. Four days of MD drive electrophysiological plasticity to near saturating levels, but weeks of MD (between 20 and 40 days) are necessary for the emergence of significant changes in geniculocortical afferents of deprived mice (Antonini et al., 1999). Slow anatomical plasticity which greatly lags electrophysiological change is not necessarily an intrinsic property of anatomical plasticity, however. Recent experiments in the cat suggest that a rapid time course for structural change is plausible – dramatic anatomical changes in corticocortical connections are apparent with only two days of strabismus (Trachtenberg and Stryker, 2001). Thus, it seems likely that anatomical change can arise shortly after MD begins, and may contribute to rapid plasticity, as our data suggest. The notion of generally slow, lagging anatomical plasticity likely has much to do with assumptions about the locus of initial plasticity, which is discussed further below.

A cortical locus for rapid ocular dominance plasticity

Suppression of cortical protein synthesis blocks the effects of MD, while substantial reduction of protein synthesis in the LGN leaves ocular dominance plasticity intact. While cortical cycloheximide infusion would likely disrupt translation in thalamocortical

axon terminals, there is, to our knowledge, little evidence of protein synthesis machinery in the axon terminals of mammalian CNS neurons. Thus, the requirement for protein synthesis is likely to be confined to the post-synaptic, cortical element of the thalamocortical circuit. The implications of these findings extend beyond a simple demonstration of the necessity of protein synthesis in ocular dominance plasticity. Rather, they suggest that the most rapid effects of MD are subserved by structural changes in cortical neurons, which shape subsequent plasticity in thalamocortical afferents.

Two caveats to this interpretation bear consideration. First, we cannot rule out the possibility that transient, translation-independent changes in thalamocortical axon terminals contribute to the most rapid effects of MD. Second, it is possible that protein synthesis in the LGN is required for rapid ocular dominance plasticity, but the low levels of translation that persist following cycloheximide infusion into the LGN are sufficient to mediate plasticity. This appears unlikely, as geniculate cycloheximide infusions more fully blocked protein synthesis than did cortical infusions, which prevented ocular dominance plasticity. A more parsimonious explanation of our data suggests a cortical locus for the most rapid plasticity following MD.

Ocular dominance plasticity: balancing mechanisms for stability and speed

If cortical changes are indeed primary in mediating ocular dominance plasticity, then lagging changes in thalamocortical arbors are expected, and do not reflect any

intrinsically slow mechanisms subserving anatomical change. One possibility is that thalamocortical changes are *instructed* by cortical plasticity, and therefore necessarily lag cortical remodeling, which may itself by subserved by structural changes. In this model, thalamocortical arbors may be viewed as an enduring and stable base upon which rapid changes in cortical connections can be implemented. Ocular dominance plasticity would then be subserved by a two-tiered system of synaptic change, in which dynamic cortical networks promote rapid plasticity, while thalamocortical networks provide both stability and the means to amplify initial cortical changes. Data from a recent study of rapid physiological plasticity following MD in the cat provide strong evidence for this model, by showing that the earliest shifts in ocular dominance are confined to cortical layers outside thalamorecipient layer IV (Trachtenberg et al., 2000).

Activity-dependent dendritic translation

We have considered the locus of the requirement for translation primarily at a circuit level. The intracellular locus of this requirement is also of interest, as activity-dependent dendritic translation has been proposed to underlie input-specific plasticity. Though αCamKII is necessary for ocular dominance plasticity and known to undergo activity-dependent protein synthesis, ocular dominance plasticity in mice lacking dendritic αCamKII mRNA is normal. Thus, dendritic synthesis of αCamKII protein may not be required for ocular dominance plasticity. A caveat to this interpretation is that normal plasticity mechanisms may be altered in αCamKII 3'-UTR knockout mice as a consequence of compensatory developmental changes. In addition, our findings do not

exclude the possibility that dendritic translation of other transcripts contributes to ocular dominance plasticity.

Which proteins are translated?

Our results point to a central remaining question: namely, which proteins must be translated for structural rearrangements to occur in ocular dominance plasticity? Numerous transcription factors show activity-dependent regulation, and presumably some of their target molecules participate in altering synaptic architecture. A number of promising candidates in this group have been identified, and include the adhesion protein N-cadherin, which is upregulated by activity and necessary for late, but not early, phases of *in vitro* plasticity (Bozdagi et al., 2000), and the protease tissue plasminogen activator, which exhibits activity-dependent translation (Qian et al., 1993), and is required for ocular dominance plasticity in mice (N. Mataga and T.K. Hensch, personal communication) and cats (Muller and Griesinger, 1998).

Chapter Four:

Conclusions and Future Directions

Discussion

Synaptic plasticity requires not only mechanisms to induce change, but also mechanisms which stabilize those changes and ensure that they endure. Our understanding of the molecular mechanisms which are required for inducing synaptic plasticity is growing rapidly; however, the mechanisms which enable such plasticity to persist remain murky. Using a mouse model of ocular dominance plasticity, I have probed molecular mechanisms thought to contribute particularly to these long-lasting synaptic changes. There are two major conclusions from these studies. Studies of a genetically mutant mouse possessing αCaMKII which is unable to autophosphorylate show that αCaMKII autophosphorylation is required for rapid induction of ocular dominance plasticity, but suggest it is not necessary for those changes to endure over a period of weeks. Experiments examining a role for protein synthesis in activity-dependent development of the visual cortex reveal a requirement for translation in ocular dominance plasticity. Pharmacological blockade of translation demonstrates that protein synthesis in V1, but not in the LGN, is necessary for ocular dominance plasticity.

There are caveats to both of these interpretations, and future directions which ramify from these findings as well. For each study, these topics are discussed briefly below.

αCaMKII, phosphorylation, and translation

What role does αCaMKII autophosphorylation play in inducing ocular dominance plasticity?

The effects of brief and long-term MD demonstrate clearly that $\alpha CaMKII^{T286A}$ homozygous mice have impaired plasticity. What accounts for this impairment? A direct mechanism for αCaMKII-mediated potentiation of synaptic transmission has been documented using biochemical techniques (McGlade-McCulloh et al., 1993). AMPA-type glutamate receptors can be phosphorylated by αCaMKII, a modification that increases current flow through these receptors four-fold. This interaction occurs during LTP-inducing stimuli *in vitro* and provides a potent means of rapidly boosting synaptic transmission (Barria et al., 1997). This is not the only mechanism contributing to αCaMKII-mediated potentiation, however, as preventing this αCaMKII-AMPA receptor interaction does not block αCaMKII-mediated synaptic potentiation (Hayashi et al., 2000). αCaMKII catalyzes insertion of cytoplasmic AMPA receptors into the cell membrane, acting through an as yet undefined pathway that is not dependent on AMPA receptor subunit phosphorylation. Both of these mechanisms provide quick, local mechanisms for αCaMKII-mediated potentiation of synaptic transmission.

Though these findings provide a plausible mechanism to account for the plasticity deficits observed in $\alpha CaMKII^{T286A}$ homozygous mutants, it is not known whether these mechanisms operate *in vivo*, or whether they have a role in ocular dominance plasticity.

While it is difficult to dissect the biochemical pathways underlying plasticity in an *in vivo* model, the studies undertaken in this thesis show that the mouse model offers an accessible means of doing so. *In vitro* studies, such as those outlined above, are a rich source of hypotheses which are testable provided appropriate mutant mice are generated. While some of these genetic tools do not yet exist, a number do. AMPA receptor insertion into cell membranes at the synapse appears to rely on a PDZ-dependent interaction, a finding which spotlights a potential role for the post-synaptic density protein 95 (PSD-95), which harbors PDZ-domains and is known to interact with both αCaMKII and AMPA receptors. PSD95 knockout mice exist and offer one potentially fruitful avenue for elucidating a role for receptor trafficking in ocular dominance plasticity. The generation of additional mutant mice will no doubt soon offer the possibility of examining other important inter-molecular interactions, such as that between αCaMKII and the mGluR1 subunit of AMPA receptors, in ocular dominance plasticity.

What accounts for the residual plasticity observed in $\alpha CaMKII^{T286A}$ ko mice after LTMD?

αCaMKII autophophorylation allows the kinase to operate in a Ca^{2+}-autonomous fashion. *In vitro* experiments suggest that Ca^{2+}-autonomous activity can be maintained for at least an hour following LTP induction, and theoretical approaches suggest the state could persist much longer (Lisman and Zhabotinsky, 2001). Preventing autophosphorylation traps αCaMKII in a Ca^{2+}/calmodulin dependent state, and consequently the kinetics of

the kinase mimic those of the very rapid and fleeting Ca^{2+}-transients required for its activation. Preventing autophophorylation does not, however, completely block the enzymatic activity of the molecule. Mutant T286A αCaMKII can still phosphorylate substrate molecules, and translocate to the post-synaptic density following Ca2+ influx, albeit very transiently (Shen et al., 2000). Thus, one possibility accounting for residual plasticity in $\alpha CaMKII^{T286A}$ homozygous mice is that plasticity continues to be mediated by αCaMKII, but at a much reduced pace.

As with all knockout mice, it is also possible that developmental compensation accounts for the remaining plasticity in $\alpha CaMKII^{T286A}$ homozygotes, and this leaves open the possibility that this plasticity is somehow mechanistically different from the usual form of ocular dominance plasticity. This is a possibility that is difficult to address thoroughly, though some steps could be taken to ensure that the plasticity in $\alpha CaMKII^{T286A}$ homozygous mice follows the same rules that plasticity in wild-type mice does. In this regard, it is reassuring that BD does not cause a large decrement in cortical responsiveness; this suggests that competitive mechanisms are at work in the knockout mice. Pharmacological methods may provide the best complement to the genetic studies as they might provide a means of testing a role for αCaMKII – though not specifically autophosphorylation of the molecule – in different phases of ocular dominance plasticity. The lipophilic kinase inhibitor KN-93 shows high specificity for αCaMKII and could be delivered to the visual cortex by direct infusion. This provides a means of blocking αCaMKII function with temporal specificity, and allows the kinase's action to be

separately evaluated in induction (during four day MD) and maintenance phases (after MD) of ocular dominance plasticity.

How relevant is the "molecular memory" model for ocular dominance plasticity?

Given that the "molecular memory" model was proposed specifically as a mechanism to account for the persistence of synaptic *potentiation*, is assaying for the maintenance of ocular dominance plasticity in αCaMKII^{T286A} mice a fair test of the hypothesis? Studies in cats suggest that both synaptic potentiation and depression occur during ocular dominance plasticity (Mioche and Singer, 1989). Chronic recordings of single unit responses reveal that deprived eye responses disappear first, followed later by the emergence of non-deprived eye responses. Similar experiments have not been performed in the mouse. Anatomical studies, do, however, provide some information about the polarity of MD-induced changes in the mouse (Antonini et al., 1999), and suggest that MD may exert early growth potentiating effects on non-deprived eye arbors and later growth suppressing effects on deprived-eye thalamocortical arbors.

Taken together, these studies suggest that both potentiation and depression contribute to the effects of MD, and in as much as the "molecular memory" model purports to account for potentiation of synapses, ocular dominance plasticity provides a reasonable assay for testing the hypothesis. There are ways, however, to more cleanly dissociate *in vivo* potentiation from depression. Reverse suture paradigms provide a means of isolating the mechanisms underlying potentiation of initially deprived eye connections; if combined

with the pharmacological approach outlined above, using infusion of KN-93, a specific role for αCaMKII in potentiating synapses might be better assessed.

Finally, results from studies of barrel cortex plasticity in $\alpha CaMKII^{T286A}$ homozygous mutants are intriguing, in suggesting that cortical defects in plasticity are limited to impairments in potentiation, not in depression (Glazewski et al., 2000). In wild-type adolescent mice, whisker trimming paradigms cause barrel plasticity which results both from a depression of deprived whisker responses and potentiation of spared whisker responses. In adolescent $\alpha CaMKII^{T286A}$ mice, potentiation is blocked, but depression of deprived whisker responses proceeds normally. While not conclusive, the results support a cautious interpretation of the plasticity defects seen after MD as impairments in potentiation mechanisms, and lend credence to the validity of using ocular dominance as a model for synaptic potentiation.

Translation in ocular dominance plasticity

Is a requirement for protein synthesis evidence of structural/anatomical change?

As discussed in Chapter 3, evidence from other systems suggests that protein synthesis is required for anatomical rearrangements at the synapse. This is clearest from studies of the gill-withdrawal reflex in *Aplysia*, where protein synthesis is clearly required for structural changes in the sensory neuron mediating the reflex (Bailey et al., 1992). Data from other systems are consistent with this requirement (Engert and Bonhoeffer, 1999;

Rose and Stewart, 1999). In any case, it is reasonable to assume that the demands of altering neuronal morphology are in part met with resources marshaled from the nucleus – proteins synthesized through nuclear translation.

Of course, evidence that protein synthesis is required for anatomical plasticity does not demonstrate that a requirement for protein synthesis signifies anatomical plasticity. In this thesis I have outlined experiments which use inhibitors of protein synthesis as a pharmacological means of probing for structural changes in rapid ocular dominance plasticity. The results suggest anatomical changes occur rapidly following monocular occlusion, but they can do no more than suggest. Ultimately, the validity of this approach will be evaluated in light of evidence gathered from techniques which allow direct observation of ongoing morphological changes in neurons during plasticity paradigms. Happily, these techniques exist and are being put to use. Two photon microscopy has already been employed to assess the effects of whisker trimming upon plasticity of cortical neurons' dendrites (Lendvai et al., 2000). Similar studies could in principal be carried out in V1, to examine the rapidity with which morphological changes occur following monocular occlusion. While it might be difficult to assess ocular dominance of individual cortical neurons in rodents, comparisons of binocular and monocular regions of cortex might provide a statistical approach to circumvent that problem.

Finally, it is worth noting that anatomical changes can occur very rapidly, so it is not unreasonable to posit an anatomical substrate for the effects of four day MD. Cortico-

cortical plasticity has been shown to be particularly rapid (Trachtenberg and Stryker, 2001).

Can protein synthesis occur in axons?

Blockade of ocular dominance plasticity with cortical, but not geniculate, infusion of the protein synthesis inhibitor cycloheximide suggests that the cortex is the site of the first changes induced by MD. This interpretation rests squarely on the conviction that thalamocortical axons are unlikely sites for protein synthesis. A search of the literature reveals little evidence of protein synthetic machinery in axon terminals of mammalian CNS neurons. Evidence of protein synthesis in axons – but not axon terminals -- has been reported in invertebrates (Sotelo et al., 1999), and in the mammalian PNS (Koenig et al., 2000); these accounts seem especially to pertain to exceptionally long or large axons, which have metabolic demands not easily met by slow axonal transport. It remains to be seen whether these reports will be extended to axon terminals of the mammalian CNS, and furthermore whether such machinery could be engaged in an activity-dependent fashion. In contrast, there is a wealth of data which shows that protein synthesis machinery is present in dendrites (as well as the soma: Steward, 1982; Tiedge and Brosius, 1996), that this machinery can be regulated in an activity dependent fashion (Scheetz et al., 1998; Wu et al., 1998), and that translation of plasticity-related genes occurs in cortical neurons following monocular deprivation (Pham et al., 1999). These data support the view that cortical cycloheximide infusions exert their effects on

plasticity through inhibition of translation in cortical neurons, and not in thalamocortical axon terminals.

Why isn't protein synthesis required in the LGN?

Cortical, but not thalamic, protein synthesis is required for ocular dominance plasticity. At least two possibilities might account for this exclusively post-synaptic requirement. It is possible that changes in the cortex are necessary for subsequent thalamocortical changes to occur. Alternatively, plasticity could be initiated in both pre- and post-synaptic elements of the thalamocortical circuit simultaneously, but expressed more rapidly in post-synaptic cortical neurons than in pre-synaptic thalamocortical afferents. In this scenario, considerations of the transit time required for proteins produced in geniculate cell bodies to make their way to thalamocortical terminals – via slow axonal transport – provide a ready explanation for the delay between fast cortical and slow geniculate expression of plasticity.

While it is difficult to disentangle these alternatives, there is reason to believe that the first, and not the second, hypothesis account for the observed results. In cats, ocular dominance plasticity is expressed most rapidly *outside* thalamorecipient layer IV (Trachtenberg et al., 2000). If similar processes are at work in the mouse, there is no simple way to account for lagging thalamocortical plasticity by longer protein transport times. Instead, it becomes more likely that intrinsic properties endow cortico-cortical synapses with exceptionally rapid plasticity.

How is translation regulated by activity?

Neurons possess multiple mechanisms for activity-dependent regulation of translation. For instance, poly-adenylation mediated translation of the αCaMKII transcript is known to occur in the visual cortex as a consequence of visual activity (Wu et al., 1997). And NMDA receptor-dependent phosphorylation of the elongation factor 2 (EF-2), regulates translation by halting ribosomal translocation (Scheetz et al., 1997). While both of these mechanisms suggest means of controlling translation in ocular dominance plasticity, an important step is to demonstrate directly that patterns of activation associated with competition, and not simply activation, are sufficient to engage these regulatory pathways. Antibodies specific to phosphorylated EF-2 provide one means of assessing a role for that pathway in MD-induced translational changes; they enable MD-induced signaling through this pathway to be assessed during and after the critical period.

Which translated proteins are necessary for ocular dominance plasticity?

Demonstration of a requirement for protein synthesis in ocular dominance plasticity leads naturally to the question of which of the thousands of proteins expressed in a neuron are among those whose translation is required. Studies of knockout mice are making inroads sorting through the thousands of possibilities – and there are in fact many promising candidates -- but this is a slow process. Full realization of the promise of the mouse as a genetic model may well come about through its use in forward genetic screens, an

approach which in principal could aid in identifying a number of molecular candidates in one fell swoop. This approach requires, of course, significant time and resources to carry out, but its great advantage lies in uncovering previously unknown players in the plasticity process. While studies of knockout mice have been very fruitful, they lean heavily on *in vitro* studies to identify promising candidate molecules; forward screens of mice deficient in ocular dominance plasticity offer the hope of broadening our molecular vistas in an unbiased fashion.

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