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DEVELOPMENT AND PLASTICITY OF VISUAL CORTEX: A ROLE FOR INTRACORTICAL INTERACTIONS

by

Takao Kurt Hensch

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

NEUROSCIENCE

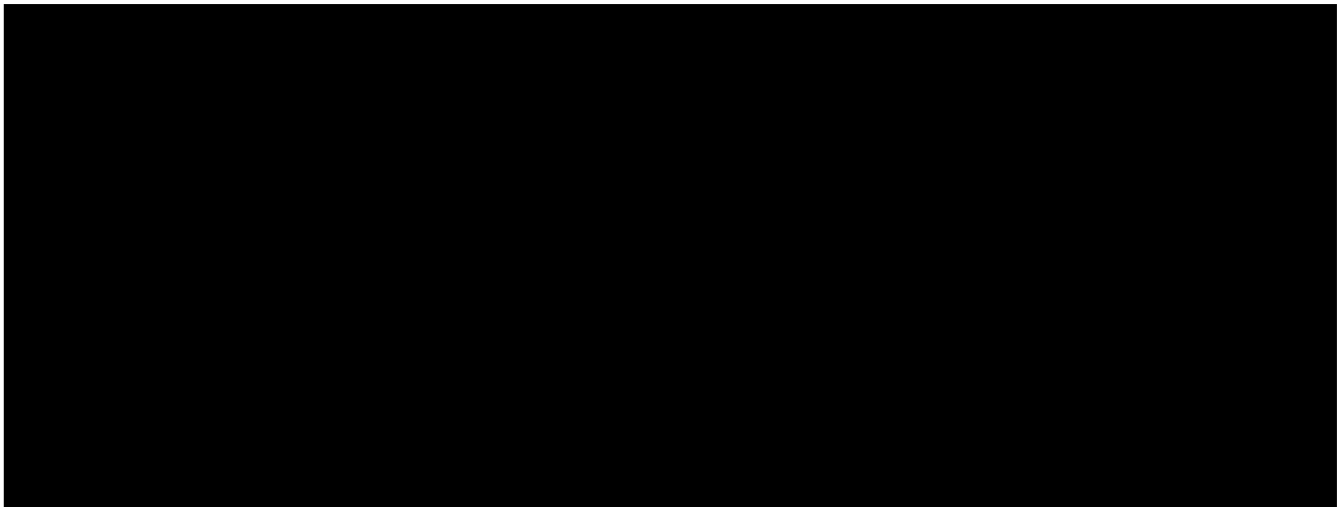
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Being at UCSF has made me feel truly blessed. Before coming to the Bay Area, I was given the precious opportunity to learn the way of the micro-electrode from Dr. Masao Ito and to enter the world of cortical plasticity with Dr. Wolf Singer. Yet, only here in San Francisco did I truly savor participation in a larger scientific community. This is indeed a very special place.

I am grateful to have had many interactions with Dr. Roger Nicoll, my thesis committee chairman. Roger graciously allowed me to join his lab meetings and showed me what it means to be the quintessential scientist. Dr. Ken Miller provided not only the theoretical motivation for this thesis, but also an untiring ear for questions and discussion. Dr. Larry Katz (Duke Univ.) has been a creative inspiration ever since I used carbachol-conjugated fluorescent microspheres as an undergraduate at Harvard. I thank him for flying up the coast to committee meetings from his sabbatical at Cal Tech.

Dr. Michael Stryker has been an excellent thesis advisor, giving me the freedom and support to pursue my interests. I sincerely hope that his hunger for knowledge will bring him to Japan often. I thank all the members of the Stryker lab who patiently taught and collaborated with me. Antonella, Gary, Yoshio, Ed, Deda, Josh, Silver, Mike C., and especially Sheri: I couldn't have done it without you! And Michela, I hope our collaboration is forever. Grazie, topo.

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Most of all, I thank my parents, Kurt and Taki Hensch. They have quietly encouraged me for thirty years to do my best, no matter what the undertaking. This thesis is dedicated to their unwavering commitment, which has been my soul's sustenance in difficult times.


ADVISOR'S STATEMENT

All physiological recordings of brief monocular deprivation effects reported in chapter 2 were made by Takao Hensch. Several members of the lab assisted at various stages (eyelid suture, animal handling, set-up) to ensure that he remained blind to which eye had been deprived. Optical imaging and rat recordings were made possible through the collaboration of Drs. Michael Crair and Michela Fagiolini, respectively.

Takao Hensch and Joshua Gordon, a graduate student in the laboratory, worked together on investigations of visual cortical development and plasticity in mice lacking PKC γ (chapter 2) and PKA RI β (chapter 4). Drs. Chong Chen and Susumu Tonegawa (MIT) produced the PKC mutant, and Drs. G. Stanley McKnight, Rejean Idzerda, and graduate student Eugene Brandon (Univ. of Washington, Seattle), supplied the PKA mutant. Takao performed all *in vitro* experiments, while Josh carried out the *in vivo* recordings with Takao's assistance (e.g. eyelid sutures, animal set-up). Chapter 4 was originally prepared for publication by both, then rewritten in its entirety and resubmitted by Takao following additional *in vitro* experiments. Takao's contribution reflects that of a standard thesis chapter.

Mice lacking the 65kDa isoform of the GABA-synthesizing enzyme, glutamic acid decarboxylase (GAD65), were generously provided by Drs. Shera Kash and Steinunn Baekkeskov of the Hormone Research Institute (UCSF). Takao Hensch and Dr. Michela Fagiolini collaborated on the *in vivo* experiments reported in chapter 5.

The work presented in chapter 3 has previously been published in *Science*, vol. 272: 554-557 (1996).


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Development and Plasticity of Visual Cortex: A Role for Intracortical Interactions

by

Takao Kurt Hensch

ABSTRACT

Columnar organization is the hallmark of cortical architecture. In the visual cortex, columns are sculpted from initially overlapping inputs serving the two eyes by a competitive, activity-dependent process. Intracortical interactions may participate in the refinement of ocular dominance columns via a rapid plasticity of excitatory connections and a regulatory role for local inhibitory circuits.

Afferent arbors in cortex can be rapidly remodeled early in life, as demonstrated by brief periods of monocular deprivation. Robust functional plasticity occurred even more quickly in both cats and rodents, justifying a comparison of ocular dominance plasticity with *in vitro* models of long-term potentiation (LTP) and depression (LTD). For example, mice lacking the γ isoform of protein kinase C expressed plasticity both *in vitro* and *in vivo*. Generally, however, neither LTP nor LTD of supragranular field potentials correlated with ocular dominance plasticity.

Pharmacological blockade of metabotropic glutamate receptors or genetic disruption of the regulatory I β subunit of protein kinase A (PKA RI β) prevented synapse weakening in cortical slices. A probable presynaptic signaling defect further abolished LTP by theta-burst stimulation in PKA RI β mutant mice. Nevertheless, clear plasticity was observed *in vivo*. Conversely, successful potentiation and depression of extracellular field potentials also failed to predict

the absence of ocular dominance shifts in mice lacking a synaptic isoform of the GABA synthesizing enzyme glutamic acid decarboxylase (GAD65). Thus, commonly studied models of LTP and LTD in brain slices may mislead us about the principles underlying ocular dominance plasticity.

GAD65 mutants, on the other hand, provide the first mouse model in which monocular deprivation consistently failed to induce plasticity. These mice may yield valuable insights into the role of intrinsic inhibitory circuits in the experience-dependent modification of visual cortex, since plasticity could be restored *in vivo* by enhancing postsynaptic GABA_A currents with diazepam. Given that intracortical inhibition may regulate plasticity, we locally infused diazepam into kitten visual cortex and observed the normal segregation of thalamocortical afferents. The final width of columns is defined by intracortical interactions in computational models of development. Our results supported this view, but in a direction opposite that of theoretical predictions. Enhancing intracortical GABA_A-mediated inhibition widened column spacing, remarkably similar to reports of raising animals with artificial strabismus.

Our results are consistent with the self-organization of thalamocortical connectivity and emphasize the need for a better cellular understanding of local circuit interactions in the developing cortex.

Roger Nicoll

TABLE OF CONTENTS

Acknowledgments	iii
Advisor's Statement	v
Abstract	vi
List of Tables and Figures	x

CHAPTER ONE	1
<hr/>	
Introduction	
Ocular Dominance Plasticity	5
Intracortical Interactions	15
PART I: EXCITATORY INTERACTIONS	20
CHAPTER TWO	21
<hr/>	
Rapid and Robust Physiological Plasticity in Cat and Rodent Primary Visual Cortex	
Summary	22
Introduction	23
Materials and Methods	25
Results and Discussion	27
CHAPTER THREE	50
<hr/>	
Ocular Dominance Plasticity Under Metabotropic Glutamate Receptor Blockade	
Summary	51
Introduction	52
Materials and Methods	53
Results	56
Discussion	67

CHAPTER FOUR	70
Visual Cortical Plasticity with Impaired Intracortical Signalling in PKA RIβ-Deficient Mice	
Summary	71
Introduction	72
Materials and Methods	75
Results	78
Discussion	98
PART II: INHIBITORY INTERACTIONS	105
CHAPTER FIVE	106
Inhibitory Circuits Regulate Visual Cortical Plasticity	
Summary	107
Introduction	108
Materials and Methods	110
Results	112
Discussion	121
CHAPTER SIX	129
Intracortical Inhibition Regulates Ocular Dominance Column Segregation	
Summary	130
Introduction	131
Materials and Methods	137
Results	143
Discussion	156
CHAPTER SEVEN	165
Conclusions and Future Directions	
References	173

LIST OF TABLES AND FIGURES

CHAPTER ONE

Figure 1-1: Development of Columnar Organization in Visual Cortex	4
---	---

PART I

CHAPTER TWO

Figure 2-1: Normal Ocular Dominance of Cat Visual Cortex	29
Figure 2-2: Effects of Brief Monocular Deprivation in Kitten Visual Cortex	31
Figure 2-3: Robust Plasticity Following 2 Day Deprivations in Kittens	34
Figure 2-4: Maximal Plasticity Requires 2 Days of Deprivation in Kittens	37
Figure 2-5: Physiological Plasticity Precedes Anatomical Rearrangement	39
Figure 2-6: Maximal Plasticity Requires 4 Days of Deprivation in Rodents	42
Figure 2-7: Intracortical Interactions in Visual Cortical Plasticity	45
Figure 2-8: Visual Cortical Plasticity <i>in vitro</i> and <i>in vivo</i>	48

CHAPTER THREE

Figure 3-1: mGluRs Mediate Synapse Weakening <i>in vitro</i>	59
Figure 3-2: Protocol for mGluR Blockade <i>in vivo</i>	62
Figure 3-3: Visual Responses and Plasticity Under mGluR Blockade	64
Table 3-1: mGluRs Do Not Mediate Ocular Dominance Plasticity	65

CHAPTER FOUR

Figure 4-1: Normal Morphology and Responses in PKA RI β - Cortex	81
Figure 4-2: Ocular Dominance Plasticity with LTD Defect in PKA RI β - Mice	85
Figure 4-3: Defective Extracellular LTP in PKA RI β - Visual Cortex <i>in vitro</i>	88
Figure 4-4: Reverse Suture Potentiates Responses in PKA RI β - Mice <i>in vivo</i>	91

Figure 4-5:	Intact Postsynaptic LTP Mechanisms in PKA RI β - Mice	94
Figure 4-6:	Impaired Intracortical Signalling in PKA RI β - Mice	97

PART II

CHAPTER FIVE

Figure 5-1:	Targeted Disruption of the Major Synaptic Isoform of GAD	114
Figure 5-2:	GAD-65 Regulates Rapid Ocular Dominance Plasticity	117
Figure 5-3:	Retinotopy and Receptive Field Size in Mice Lacking GAD-65	120
Figure 5-4:	Visual cortex can sustain plasticity in the absence of GAD65	123
Figure 5-5:	Schematic Role of Intracortical Inhibition in Cortical Plasticity	127

CHAPTER SIX

Figure 6-1:	Intracortical Interactions Predict Column Periodicity	133
Figure 6-2:	Benzodiazepine Agonists Enhance GABA _A Currents	136
Figure 6-3:	Protocol for Local Modulation of Inhibitory Circuits <i>in vivo</i>	139
Figure 6-4:	Quantification of Column Spacing	142
Figure 6-5:	Cortical Physiology Under Chronic Benzodiazepine Treatment	145
Figure 6-6:	Chronic Diazepam Widens Ocular Dominance Columns	148
Figure 6-7:	Graded Anatomical Effects of Diazepam Infusion	150
Figure 6-8:	Homogeneous Column Size Across Control Hemispheres	153
Figure 6-9:	Opposite Spacing Trend for Benzodiazepine Inverse Agonist	154
Figure 6-10:	Manipulations Affecting Column Spacing in Development	158
Figure 6-11:	“Strabismic” Ocular Dominance in Diazepam Kittens	160

CHAPTER SEVEN

Figure 7-1:	New Perspectives on Thalamocortical Interactions	168
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Chapter 1

Introduction

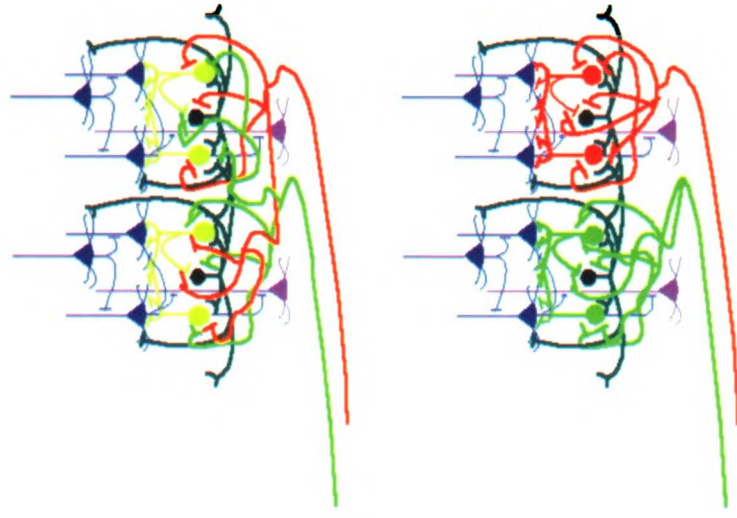
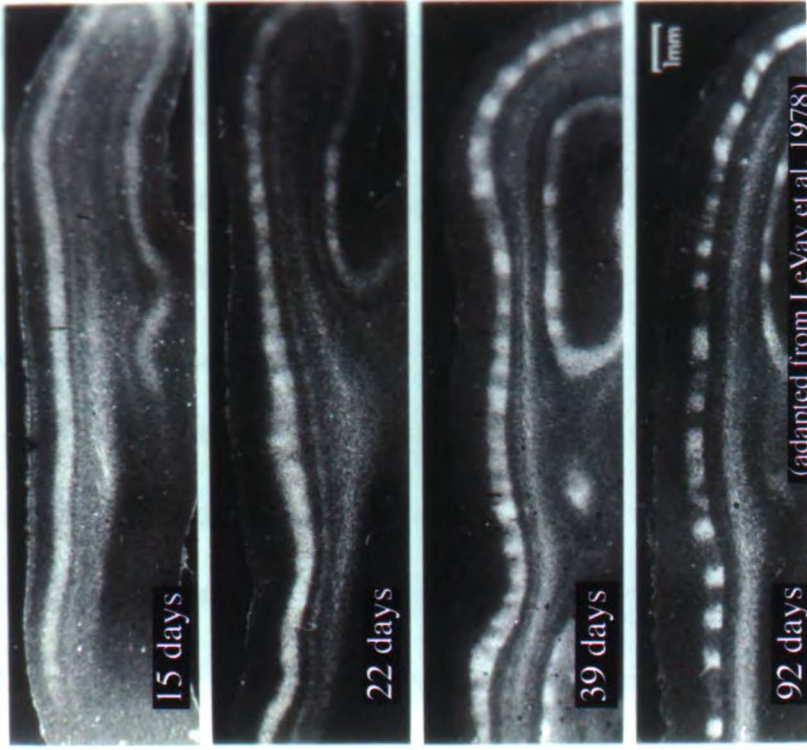
Columns are the hallmark of mammalian cortical architecture. First described by Mountcastle (1957) in the somatosensory cortex, neurons with similar functional properties have been found to cluster in such far-reaching areas as the prefrontal (Goldman-Rakic and Schwartz, 1982), medio-temporal (MT) (Albright et al., 1984), and infero-temporal (IT) (Fujita et al., 1992) cortices. Histologically-defined columns or modules have since confirmed that axonal projections throughout the cerebral cortex tend to be organized in vertically aligned groups or patches (Gilbert and Wiesel, 1983; Rockland and Lund, 1982). The primary visual cortex perhaps best exemplifies this ubiquitous characteristic of neocortical anatomy and physiology (LeVay and Nelson, 1991).

The pioneering work of Hubel (1988) and Wiesel (1982) identified many of the basic aspects of columnar organization and its development in the striate cortex. Afferents to area 17 endow cortical neurons with response properties specific to stimulus orientation, visual field position, and the eye being stimulated. The latter physiology is a direct consequence of the anatomy: thalamic axon terminals serving each eye define discrete "ocular dominance" columns.

Eye-specific delineations are, however, a feature of the mature cortex. In newborn kittens, for example, afferent arbors are extensively intermingled (Fig. 1-1; (LeVay et al., 1978)). Over the ensuing weeks, visual activity guides their segregation. An elucidation of the principles and mechanisms by which such

clustered structures emerge has fueled cortical research for the past thirty years. In this thesis, I examine the putative involvement of intrinsic cortical circuits in these developmental processes.

Fig. 1-1. Development of columnar organization in visual cortex. Left, Thalamocortical axons serving the two eyes are intermixed in cortical layer IV of early postnatal kittens. During subsequent weeks, they segregate into the right and left eye patches typically seen in mature animals. Thirty years of investigation have determined that ocular dominance columns form through a competitive process (adapted from LeVay et al, 1978). Right, Although monocular proline injections only label afferents in the left panel, these axons actually arrive into a highly interconnected network of cortical cells. How these circuits might influence the segregation process is the major theme of this thesis.



Ocular Dominance Plasticity

Manipulations of visual experience provided the first clue that cortical column formation may be activity-dependent. Depriving one eye of vision during the segregation process widens open eye columns at the expense of closed eye input (Shatz and Stryker, 1978). Consequently, the distribution of physiological responses from a sample of cortical neurons is also biased toward the non-deprived eye. Since monocular deprivation is effective and reversible only within a critical period early in development (Hubel and Wiesel, 1970; Olson and Freeman, 1980), the resultant ocular dominance plasticity has been thought to reflect an exaggeration of normal developmental rules. That is, the two eyes' inputs appear to compete for cortical space.

Visual experience *per se* does not appear to be required, since ocular dominance column formation is not prevented by binocular deprivation (Kalil, 1982; Mower et al., 1985). A further intraocular injection of the voltage-gated sodium channel blocker tetrodotoxin (TTX) into one eye, however, induces a physiological shift in favor of the non-silenced eye (Chapman et al., 1986). The demonstration that spontaneous retinal activity alone is sufficient to drive ocular dominance plasticity may explain how the primate critical period can occur prenatally, at a time when visual experience is non-existent (Rakic, 1976). Only binocular impulse blockade with TTX prohibits column segregation (Stryker and Harris, 1986). Interestingly, asynchronous electrical stimulation of the optic

nerves restores column formation under such conditions; whereas, synchronous stimulation does not (Stryker and Strickland, 1984). Patterns of afferent activity arriving in visual cortex, therefore, competitively refine ocular dominance columns in development.

In theoretical considerations of activity-dependent plasticity, the target structure also plays a crucial role in close collaboration with its presynaptic partner. As first postulated by Hebb (1949), and later refined by Stent (1973), the efficacy of a synaptic connection can be increased or decreased depending on the correlation between pre- and postsynaptic activation. Connections stabilize if the probability is high that presynaptic afferents and their postsynaptic targets are active in temporal synchrony; and they destabilize when the postsynaptic cell is strongly activated while the presynaptic terminal is silent, or if the postsynaptic terminal is silent while the presynaptic is active. Thus, in these models the postsynaptic neuron functions as a cellular "coincidence detector." Numerous investigations at several synapses have demonstrated the existence of Hebbian plasticity in the developing nervous system (Stryker et al, in preparation).

A gating function of postsynaptic activity in visual cortical plasticity was indirectly implicated by a clever experiment which took advantage of the cortical origin of orientation selectivity (Rauschecker and Singer, 1979). Restricting vision not only to one eye, but also to a particular orientation, shifts the physiological ocular dominance of only those cells responding to that preferred orientation. A direct infusion of TTX into the primary visual cortex of kittens

concurrent with monocular deprivation reversibly prevents the normal shift in ocular dominance in favor of the open eye (Reiter, et al. 1987). Results such as these, however, serve only as a preliminary indication that electrical activity in either the target, afferents, or both are required for precise connectivity. Ideally, one would like to analyze separately the involvement of postsynaptic versus presynaptic activity during development.

The absence of γ -amino-butyric acid type A (GABA_A) receptors on geniculo-cortical afferents (Needler et al., 1984) allows the selective inhibition of postsynaptic target neurons. Silencing cortical cells with the specific GABA_A receptor agonist muscimol at the peak of the critical period leads to an ocular dominance shift in favor of a closed, deprived eye (Reiter and Stryker, 1988; Hata and Stryker, 1994). This finding is in agreement with the Hebb/Stent model: connections from a less-active, deprived eye are stabilized because they are better correlated than open, active eye afferents with the silent target cells. Not merely a pharmacological curiosity, the postsynaptic control of experience-dependent plasticity is also manifest anatomically, leading to an expansion of deprived-eye afferents at the expense of open-eye inputs (Hata and Stryker, 1994). Moreover, muscimol infusion beginning two weeks earlier and extending through the end of the critical period disrupts the normal segregation of ocular dominance columns (Hata and Stryker, unpublished observations). Regulation of presynaptic arbor refinement by postsynaptic activity, therefore, appears to be

a common correlation-based rule for both the plasticity and initial formation of thalamocortical circuitry.

An alternative method of selectively silencing the target is to prevent the binding of presynaptically-released neurotransmitter to its postsynaptic receptor. Recent work at the neuromuscular junction elegantly demonstrates that correlations between transmission efficacy and presynaptic activity, rather than the overall amount of activity, determine the fate of inputs competing for synaptic space. Imaging single mature neuromuscular connections *in vivo*, Balice-Gordon and Lichtman (1994) partially blocked transmission along the end-plate by focal application of the postsynaptic nicotinic acetylcholine receptor antagonist, α -Bungarotoxin. The ensuing imbalance of muscle response to its single motor input introduced a competition within the same axon reminiscent of that occurring between multiple inputs onto immature muscle cells (for review see Hall and Sanes, 1993). In both the mature and developing case, active presynaptic arbors overlying silent synaptic sites destabilize and retract, suggesting that the context within which a synaptic site is inactive is critically important to its preservation.

Glutamate is the primary excitatory neurotransmitter in the central nervous system (Nicoll et al, 1994) and is also used by geniculo-cortical afferents (Baughman and Gilbert, 1980). Increasing postsynaptic activity by chronic infusion of glutamate itself - the converse of muscimol experiments described above - prevents ocular dominance plasticity, but is difficult to interpret (Shaw

and Cynader, 1984). Continuous spiking of cortical neurons could correlate almost perfectly even with deprived inputs and lead to a stabilization of afferents from both eyes. Alternatively, the signal-to-noise ratio of cortical responses may be reduced to a point insufficient for synaptic changes to occur at all. Like TTX, glutamate may also have both pre- and postsynaptic effects. Thus, it has been more fruitful to direct selective antagonists *in vivo* against the three major classes of glutamate receptor: ionotropic N-methyl-D-aspartate (NMDA)-preferring, non-NMDA, and metabotropic glutamate receptors.

Researchers of developmental plasticity have focused much attention on the NMDA glutamate receptor because of its unique voltage-dependent activation (for review see Constantine-Paton et al, 1990; Rauschecker, 1991). Since NMDA receptors open only above a certain threshold membrane potential, they appear ideally-suited as a molecular coincidence detector on postsynaptic cells responsible for the *in vivo* observations. Moreover, *in vitro* models of use-dependent plasticity such as Long-Term Potentiation (LTP) and Depression (LTD) of synaptic transmission in many systems critically depend on a second feature of the NMDA receptor, its calcium permeability (Bliss and Collingridge, 1993; Debanne and Thompson, 1994). The relevance of such models to experience-dependent development of connectivity *in vivo* is a major issue of this thesis.

In kitten visual cortex, NMDA receptor expression is developmentally-regulated: 70% of immature visual responses are blocked by the selective

antagonist 2-amino-5-phosphono-valerate (APV) while only 30% are affected in the adult (Fox et al., 1989; Hagihara et al., 1988; Tsumoto et al., 1987). Dark-rearing instead delays the loss of NMDA receptor function in kitten visual cortex (Fox et al., 1991). Receptor kinetics exhibit a dramatic change during the critical period for ocular dominance plasticity in the rat (Carmignoto and Vicini, 1992). NMDA-gated channel open times are prolonged in the immature rat visual cortex and gradually become briefer during the first postnatal month. Importantly, this developmental shift in kinetics -- which is highly analogous to the maturation of nicotinic acetylcholine receptors at the neuromuscular junction -- can also be delayed by eliminating visual experience via dark-rearing or intracortical activity with TTX injection (Carmignoto and Vicini, 1992).

Given the wealth of correlative evidence, it has still been difficult to assign the exclusive role of "coincidence detector" to the NMDA receptor in ocular dominance plasticity. NMDA receptor antagonists, indeed, prevent the shift in favor of the open eye following monocular deprivation (Rauschecker and Hahn, 1987; Kleinschmidt et al, 1987; Bear et al, 1990). Unfortunately, intracortical NMDA receptor blockade also profoundly interrupts normal transmission of sensory information (Miller et al, 1989). The effect of silencing cortex is already known from muscimol experiments (see above), and in fact, a reverse shift in favor of the deprived eye was also observed near APV infusion sites (Bear et al, 1990). Thus, whereas there is no evidence against a specialized role for NMDA receptors in deprivation-induced plasticity, APV experiments do not confirm it

(for review see Fox and Daw, 1993; Daw et al, 1994). It would be interesting to know whether there is also an anatomical effect on presynaptic arbors of NMDA receptor blockade during deprivation, as well as on their segregation into ocular dominance columns during normal development.

As mentioned earlier, a major property of the NMDA receptor which is important for *in vitro* models of plasticity is its calcium permeability (Bliss and Collingridge, 1993). Calcium can, however, also permeate non-NMDA receptors which lack the GluR2 subunit (Seeburg, 1993). Expression of the GluR2 subunit during development may, therefore, be an indicator of the critical period for plasticity (Pellegrini-Giampietro et al., 1992). The role of non-NMDA receptors in experience-dependent plasticity has briefly been addressed in kitten striate cortex. Intracortical infusion of the selective non-NMDA antagonist, 6-cyano-7-nitroquinoxaline dione (CNQX), does not silence cortex (as found for APV, Miller et al, 1989), but has no effect on ocular dominance plasticity (DeFreitas and Stryker, 1990). Alternatively, the indirect influx of calcium into postsynaptic cells via voltage-gated channels following synaptic depolarization has not been addressed, despite suggestions that these currents may also be developmentally regulated (Bode-Greuel and Singer, 1988; Geiger and Singer, 1986).

Glutamate released at central synapses also activates second messenger-linked receptors. Three classes of metabotropic glutamate receptor (mGluR) have been identified by molecular cloning (Nakanishi and Masu, 1994), two of which couple to the cyclic AMP cascade (postsynaptic Group II and presynaptic

Group III) and one to the phosphoinositide pathway (postsynaptic Group I). Group I receptors can indirectly raise intracellular calcium concentrations through release from intracellular stores. Thus, developmental peaks in Group I mGluR expression (Reid et al, 1995) or glutamate-induced phosphoinositide turnover (Dudek and Bear, 1989) have been correlated with the critical period for ocular dominance plasticity. The hypothesis that mGluR activation underlies ocular dominance plasticity is specifically addressed in chapter 3.

Attempts to go beyond receptors to the heart of postsynaptic coincidence detection mechanisms have long been hampered by the lack of good second-messenger antagonists which can be effectively administered *in vivo*. Fortunately, recent advances in molecular techniques now allow investigations at this level by avoiding many of the pitfalls of pharmacology. For example, the topographic refinement of retinal axons to lower visual centers such as the ferret LGN (Hahm et al., 1991), rat superior colliculus (Simon et al, 1992), and frog optic tectum (Cline et al., 1987) is also mediated by postsynaptic NMDA receptor activation. Direct transfection of a constitutively active form of α -calcium/calmodulin dependent kinase II (α CamKII) into the target tectum promotes the unmasking of "silent" NMDA only synapses (Wu et al., 1996) and disrupts the morphological maturation of its presynaptic inputs (Zou and Cline, 1996). The advent of techniques to record ocular dominance plasticity in mice carrying targeted disruptions of various enzymes has revealed a permissive role for α CamKII in visual cortical development (Gordon et al, 1996). Here, we

examined the possible involvement of protein kinase C (PKC) and protein kinase A (PKA) pathways in visual cortical plasticity in chapters 2 and 4, respectively.

The realization that postsynaptic processes can regulate the refinement of developing presynaptic inputs has naturally led to a search for intercellular messengers which might mediate retrograde communication. Recently, the membrane-permeable gas Nitric Oxide (NO: (Reid et al., 1996; Ruthazer et al., 1996)) and the polysialylated form of Neuronal Cell Adhesion Molecule (psaNCAM: Baker et al, 1994) have been ruled out as mediators of ocular dominance plasticity, despite their apparent involvement in LTP-like phenomena *in vitro* (Muller et al., 1996; Schuman and Madison, 1991). Proteases seem to facilitate the restoration of closed eye input following reversal of eyelid sutures, but curiously are not involved in the effects of the initial deprivation (Griesinger and Mueller, 1995). Even glial factors may play a part: the administration of immature astrocytes into visual cortex restores plasticity in cats past the critical period (Mueller and Best, 1989), and Schwann cell transplants into rat ventricles can prevent the effects of monocular deprivation (Pizzorusso et al., 1994) and promote the maturation of cortical receptive field properties in the absence of visual experience (Fagiolini et al, in press).

Neurotrophins are one of many factors secreted by glial cells which have received a great deal of attention recently. Of the three receptor tyrosine kinases (trks), only trkB activation consistently affects the morphological substrates of ocular dominance. Thus, columns desegregate in the presence of excessive

Neurotrophin-4/5 (NT4/5: (Cabelli et al., 1995)) or Brain-Derived Neurotrophic Factor (BDNF: Hata et al, 1996), as well as in the absence of endogenous NT4/5 (Cabelli et al, 1996). The normal shrinkage of thalamic cell bodies following monocular deprivation is also prevented by cortical NT4/5 administration (Riddle et al, 1995). Exogenous BDNF reportedly produces a reverse physiological shift in favor of the deprived eye (Galuske et al, 1996). All of these results with *trkB* ligands could be explained by analogy to muscimol due to their profound reduction of cortical responsiveness (Gillespie et al, 1996). However, an activity-dependent trophic effect is more likely, given the striking lamina-specific sprouting of basal dendrites in response to individual neurotrophins (McAllister et al, 1995). It will be interesting to observe the morphology of single thalamocortical arbors exposed to BDNF or NT4/5, especially since the precise localization of *trkB* receptors has remained unclear (Cabelli et al., 1996; Cellerino et al., 1996; Silver et al., 1996).

By contrast, rodent species, which do not exhibit gross anatomical ocular dominance columns, appear to be more sensitive to the *trkA* ligand Nerve Growth Factor (NGF). Administering NGF in rats, the Maffei laboratory (1991, 1992) reported the first evidence of a neurotrophic component to visual cortical development. A delayed onset of NGF's ability to prevent the physiological effects of monocular deprivation in mice suggests an indirect mechanism of action (Fagiolini and Stryker, 1996). Potent *trkA* phosphorylation in the basal

forebrain by NGF could enhance cholinergic mechanisms in the visual cortex (Gnahn et al., 1983; Holtzman et al., 1995).

Individual neuromodulators – such as acetylcholine (Gu and Singer, 1993), norepinephrine (Kasamatsu and Pettigrew, 1976), or serotonin (Gu and Singer, 1995) – have been reported to modestly regulate ocular dominance plasticity in kittens. Combined depletions of cholinergic and noradrenergic inputs to visual cortex appear to have more complete effects (Bear and Singer, 1986). NGF may, therefore, influence Hebbian mechanisms at cortical synapses via the depolarizing actions of elevated acetylcholine release (McCormick, 1992; Nicoll et al., 1990). The role of neuromodulatory systems on visual cortical development should be revisited in this context.

Intracortical Interactions

The preceding overview depicts a simple, three-component scheme of columnar development and plasticity in the visual cortex: afferents serving the two eyes compete for cortical space by activating individual target cells, which detect and reward co-active inputs with an intercellular messenger. However, in reality, developing thalamocortical axons arrive into a highly interconnected neuronal network (Fig. 1-1). Indeed, 75% of the synapses formed in cortex arise from the apparently random connections of one cell type, the cortical pyramidal

cell (Braitenberg and Schuez, 1991). The major focus of this thesis is on the potential influence these excitatory connections as well as local inhibitory interactions may have on the generation of cortical structures underlying ocular dominance.

A rich plexus of intracortical fibers seems to mature at the same time and according to the same activity-dependent principles as thalamocortical afferents (Katz and Callaway, 1992). Long-range horizontal connections span several millimeters in the cat and specifically link discrete columns of cells with similar orientation preference (Gilbert and Wiesel, 1989; Ts'o et al., 1986; Weliky et al., 1995). A divergent strabismus during development induces these patchy terminations within columns of like ocular dominance (Loewel and Singer, 1992). Binocular deprivations (Callaway and Katz, 1991) or enucleations (Ruthazer and Stryker, 1996) arrest the refinement of long-range, tangential arbors at an initial stage of crude clustering (Callaway and Katz, 1990; Luhmann et al., 1986). Moreover, even this modest organization achieved by spontaneous thalamic and cortical activity in the absence of vision can be prevented by intracortical TTX infusions (Ruthazer and Stryker, 1996). Thus, anatomical observations also seek Hebbian explanations at the cellular level for the development of long-range horizontal connections.

On the shorter dimensions of individual columns, relatively little is known about the functional organization of intracortical interactions. Two physiological approaches have been employed *in vivo*, both involving paired

extracellular recordings. In one paradigm, the region around a single site is focally stimulated (electrical: (Asanuma and Rosen, 1973; Ezure and Oshima, 1985), glutamate: (Hess et al., 1975)) or inactivated (GABA: (Eysel, 1992) as the second electrode is moved progressively farther away. Lateral synaptic inhibition appears to spread from 400 μ m out to several millimeters, as compared with GABAergic axons which typically extend less than one millimeter (Albus and Wahle, 1994). The diffusion of iontophoresed drugs or stimulation of axons of passage by electrical current generally prevents accurate measurement of synaptic interactions over short distances.

By contrast, cross-correlation analysis of spike discharges to natural visual stimuli enables measurement from neighboring cells. Horizontal functional excitation can range up to 400 μ m within mature columns (Ghose et al., 1994; Hata et al., 1991; Toyama et al., 1981). Interestingly, a transient coupling of cell pairs separated by up to 600-800 μ m is seen at the peak of the critical period (Hata et al., 1993). Unfortunately, the identity of cell types (excitatory versus inhibitory) is not revealed by extracellular recordings from the intact animal. Moreover, correlations of spike trains have only a limited capacity to detect inhibitory synaptic connections, as well as small slowly-rising potentials.

Slice preparations provide a higher level resolution of cortical circuits. Spike-triggered averaging (Komatsu et al., 1988) and minimal stimulation techniques (Stratford et al., 1996) detect sub-threshold excitatory, as well as inhibitory (Salin and Prince, 1996), inputs onto identified pyramidal cells. More

difficult intracellular recordings from connected pairs of cells are also beginning to reveal properties of individual neocortical synapses (Luebke et al., 1996; Stratford et al., 1996; Thomson and Deuchars, 1994). Fast optical imaging with voltage-sensitive dyes sheds new light on cortical networks (Albowitz and Kuhnt, 1992; Nelson and Katz, 1995; Tanifuji et al., 1994). An elegant converse method is the photostimulation of caged glutamate to activate cells synaptically-linked to the recorded neuron (Callaway and Katz, 1993; Dalva and Katz, 1994). Such maps of intracortical interactions in slices at different developmental ages have satisfyingly recapitulated the anatomical refinement of patchy, long-range horizontal connections.

We, therefore, adopted a combined *in vitro* and *in vivo* approach to address the role of intracortical interactions in ocular dominance plasticity and columnar development. In particular, we used LTP and LTD models in slices to assay the functional integrity of visual cortical circuits under various conditions of pharmacological or gene-targeted perturbations. Once characterized, the influence of these manipulations on local circuit excitation or inhibition was directly examined on experience-dependent processes in the developing animal. We found that extracellular LTP and LTD-like modifications of intracortical excitatory transmission did not necessarily correlate with ocular dominance plasticity. However, altering intrinsic levels of inhibition robustly regulated plasticity and column spacing *in vivo*. Our results provide strong support for the

PART I

Excitatory Interactions

Chapter 2

Rapid and Robust Physiological Plasticity in Cat and Rodent Primary Visual Cortex

Summary

Occluding vision through one eye during a critical period in early life results in a loss of responses to the deprived (D) eye in visual cortex. D-eye geniculocortical arbor rearrangement appears to account for some of this loss, since their size is reduced by about half following 6 days of monocular deprivation (MD). Anatomical effects of 4 days MD are less pronounced however, and varying degrees of plasticity in striate cortex have been noted for much shorter periods of deprivation. Rapid MD effects may reflect an important transitional state when D-eye afferents are anatomically present but functionally ineffective. We, therefore, investigated the minimum deprivation duration that consistently produces a loss of functional connections from the D eye to primary visual cortex at the peak of the critical period. Both single-unit recording and optical imaging of intrinsic signals revealed a robust ocular dominance plasticity following a deprivation of just 2 days in kittens and 4 days in rats that was indistinguishable from much longer MD effects. These results validate the use of *in vitro* rodent models of rapid synaptic plasticity to provide clues regarding the biochemical and cellular mechanisms underlying visual cortical plasticity *in vivo*.

Introduction

Prolonged monocular vision early in life produces both physiological and anatomical changes in the primary visual cortex (Wiesel, 1982). Although it has been disputed exactly when sensitivity to this manipulation is lost (Daw et al., 1992; Jones et al., 1984), most investigators agree that ocular dominance plasticity reaches its peak around one month after birth in kittens (Hubel and Wiesel, 1970; Olson and Freeman, 1980). Interestingly, rodent species, which exhibit no gross columnar segregation of right and left eye input, display a similar physiological sensitivity profile to monocular deprivation (Fagiolini et al., 1994; Gordon and Stryker, 1996). Determining how rapidly ocular dominance changes can be induced at the height of the critical period in these two species may, therefore, give us clues to the biochemical and cellular constraints on this process.

Bulk injections of tracers into one eye have demonstrated the widening of open eye columns at the expense of closed eye input following months of deprivation (Shatz and Stryker, 1978). Recently, elegant single axon reconstructions have shown that even one week of deprivation at the peak of the critical period is sufficient to produce a striking remodeling of terminal arbors (Antonini and Stryker, 1993). Active elimination of branches is a progressive morphological process, since afferents deprived of vision for only 4 days may be reduced in total axonal length, but their maximal density and area of coverage by higher density arbor are largely unchanged (Antonini and Stryker, 1996). Even

the atrophy of afferent cell bodies in the lateral geniculate nucleus is detectable after as few as four days of deprivation (Riddle et al., 1995), but the degree of shrinkage is less consistent than after long-term deprivation (Antonini and Stryker, 1996).

A number of studies have addressed the time course with which changes in ocular dominance can be measured by extracellular unit recordings. Both chronic (Mioche and Singer, 1989), as well as acute, anesthetized preparations (Movshon and Dursteler, 1977; Olson and Freeman, 1975; Peck and Blakemore, 1975; Van Sluyters and Freeman, 1977) have demonstrated an early loss of binocular summation within hours of monocular deprivation and noticeable shifts in ocular dominance histograms soon thereafter. Since these physiological changes could be induced rapidly, the underlying processes were proposed to be similar to those of other short-term neural events such as learning and memory (Blakemore and Mitchell, 1973; Kandel and O'Dell, 1992).

Unfortunately, most early reports were not systematic, often relying on anecdotal observations of single animals for each time point. We, therefore, revisited this issue to determine precisely when rapid, functional ocular dominance plasticity can be considered maximal using both single-unit electrophysiology and optical imaging of intrinsic signals. Sub-threshold activity, which constitutes the bulk of the optical signal (Toth et al., 1996), may respond to deprivation very quickly, given our recent knowledge of rapid morphological changes in afferent arbors.

We further determined a sensitivity profile for brief monocular deprivations at the peak of the critical period in rats. Rodents are often used to investigate properties of putative learning-related phenomena *in vitro*, such as Long-Term Potentiation (LTP) and Depression (LTD) (Tsumoto, 1992). Demonstrating a robust and rapid form of *in vivo* plasticity in these species is, therefore, prerequisite to studying LTP and LTD mechanisms as the cellular basis for experience-dependent development in the visual cortex.

Materials and Methods

Under brief halothane anesthesia, animals were deprived of vision through one eye by lid suture beginning at the peak of the critical period (cats: P27-40; rats: P26-29; mice: P24-28). Physiological recordings were carried out blind to which eye had been deprived. During 48 hours of MD, kittens were either immediately returned to their home cage or handled throughout the day to ensure that they remained alert. Rodents (Long-Evans hooded rats; C57Bl/6x129 F1 mice) were simply returned to their home cages in a 12:12 hour light:dark cycle. Mice lacking the γ isoform of Protein Kinase C (PKC γ) were generated by C. Chen and S. Tonegawa as described previously (Abeliovich et al., 1993).

Animals were prepared for acute single-unit recording using standard techniques (Fagiolini et al., 1994; Gordon and Stryker, 1996; Reiter et al., 1986). In brief, kittens were anesthetized and maintained with a combination of barbiturate infusion (Nembutal, 10mg/kg i.v.) and N₂O:O₂ (2:1) ventilation. Rodents were not ventilated, and rat anesthesia was induced and maintained with urethane (6 ml/kg, 20% solution in saline i.p.), while nembutal (50 mg/kg i.p.) with chlorprothixene (0.2mg, i.m.) was used for mice. Extracellular unit recordings were obtained from vertical penetrations spaced evenly at 400 μ m intervals along the medial bank of cat Area 17 or at 200 μ m intervals within the binocular zone of rodent V1 (ca. 4mm from the central fissure) with resin-coated tungsten microelectrodes (1-3M Ω ; (Hubel, 1957)). Intrinsic signals from the same regions of cortex illuminated with 610nm light were captured through a matching filter by a cooled slow-scan CCD camera (Princeton Instruments, NJ). Standard optical imaging techniques determined reflectance changes to monocular presentation of a moving (1 cycle/sec) oriented grating (0.1-0.2 cycles/deg), which were divided by the optical response to presentation of a blank (grey) screen (Bonhoeffer and Grinvald, 1993).

Light bar stimuli were swept across the receptive field of single units with a hand-held lamp to assign each cell to an ocular dominance group on the basis of Hubel and Wiesel's (1962) seven-point scale. An ocular dominance score of 1 indicates response to contralateral eye stimulation exclusively, and a score of 7 purely ipsilateral eye response. Intermediate scores (2-6) reflect a degree of

binocular responsiveness. The Contralateral Bias Index (CBI), a weighted average of the bias towards one eye or the other, was calculated for each hemisphere according to the formula: $CBI = [(n_1 - n_7) + (2/3)(n_2 - n_6) + (1/3)(n_3 - n_5) + N] / (2N)$, where N = total number of cells, and n_x = number within ocular dominance group X . An optical CBI was computed similarly by taking the difference in signal strength between the two eyes for the optimal orientation at each pixel in the image.

Results and Discussion

As first described by Hubel and Wiesel (1962), normal kitten visual cortex received predominantly binocular input (Fig. 2-1). Polar maps of the intrinsic signal image were equally bright for the right and left eye, indicating similar response strength. Ocular dominance distributions derived from each pixel in the image were gaussian in shape, as for single-unit recordings based on a subjective 7-point scale.

Just two days of deprivation at the peak of the critical period drastically reduced the luminance of the polar map obtained through the deprived eye (Fig.

Fig. 2-1. Normal ocular dominance of P30 kitten visual cortex. Top, optical imaging of intrinsic signals reflects both spiking and sub-threshold activity contributing to the orientation map. The polar map encodes the strength of the response in terms of luminance. Note that the map derived through either eye is similarly bright. Rostral, right; medial, up. Bottom, the ocular dominance distribution for the hemisphere can be determined on a pixel-by-pixel basis for the optical image (left), or by the traditional 7-point classification scheme of Hubel and Wiesel for single-unit recordings. The overall bias can be quantified as the weighted average of the distribution, the contralateral bias index (CBI). Single-unit CBI values near 0.5 indicate a binocular distribution.

Non-Deprived Control

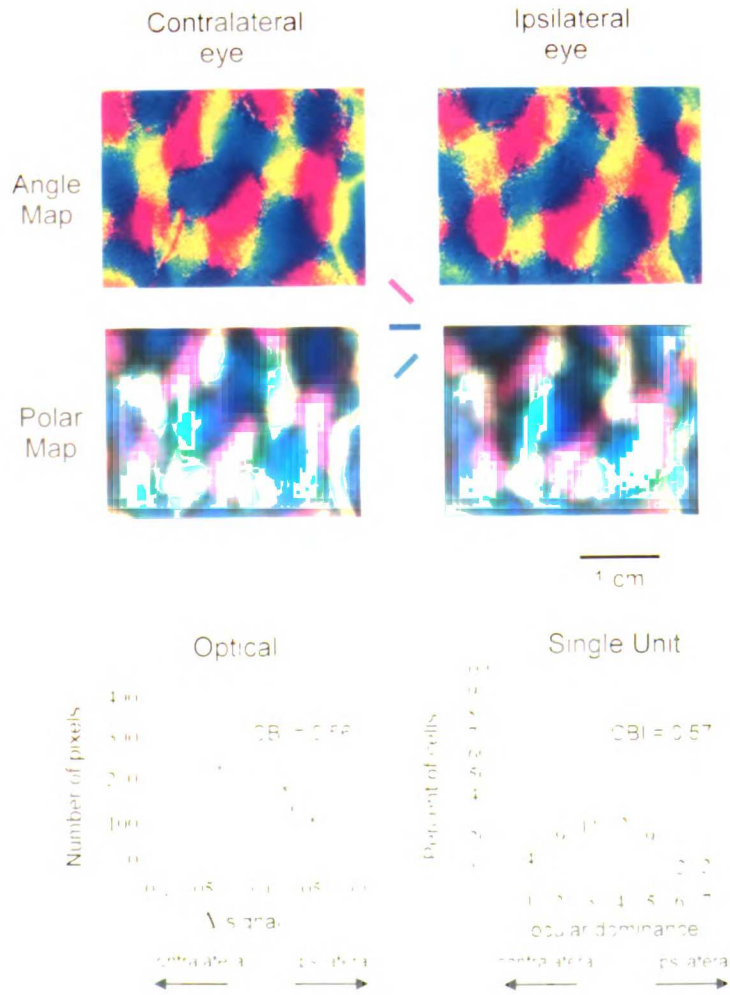
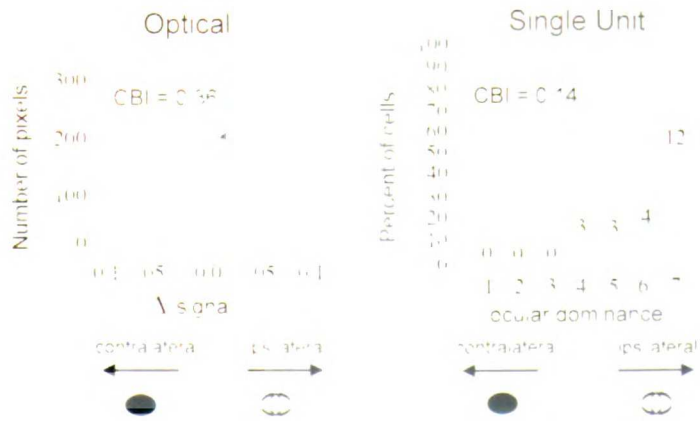
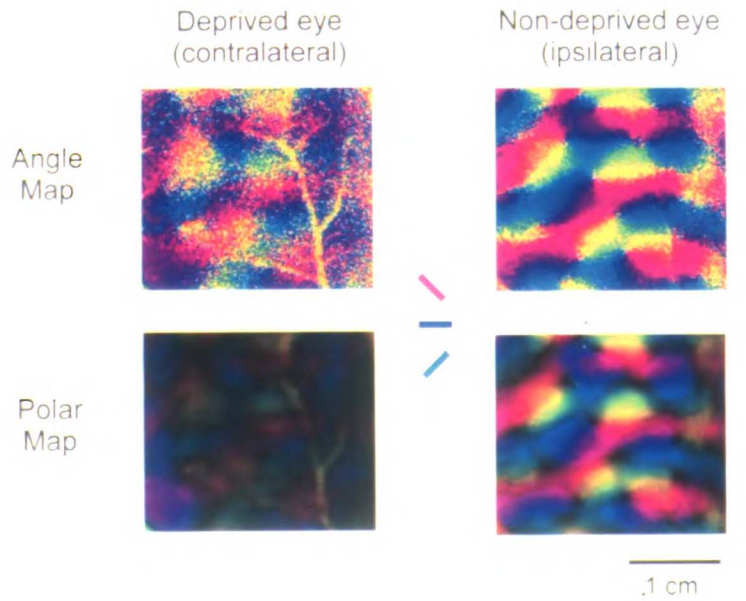


Fig. 2-2. Effects of brief monocular deprivation in kitten visual cortex. Top, robust orientation maps of intrinsic optical images appear when stimulating the non-deprived eye, but are profoundly reduced for the closed eye. The 'blackness' of the deprived eye polar map reflects a strong shift toward the open eye following just 2 days of deprivation at the peak of the critical period (P28-30). Bottom, the ocular dominance histograms reveal the skewed distribution in favor of the open ipsilateral eye. This is reflected in the low CBI value.

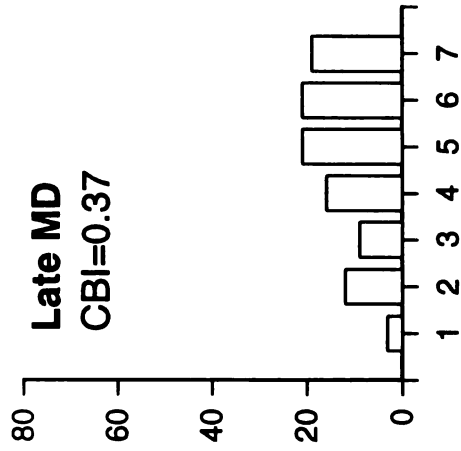
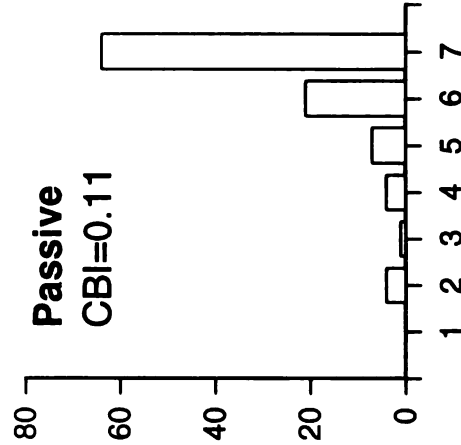
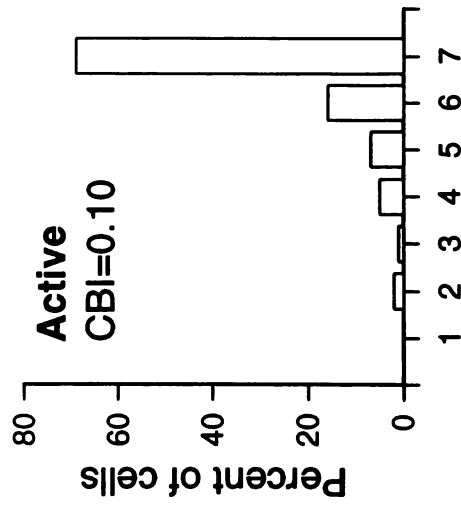
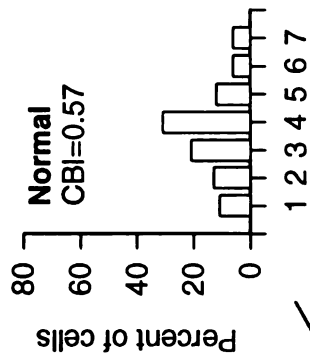
2 Day Monocular Deprivation



2-2). Earlier reports of reduced, but substantial, current source density profiles (Kossut and Singer, 1991) or synaptic inputs (Blakemore et al., 1982) evoked from the deprived eye following 2 to 7 day deprivations agree with our observation of some deprivation-resistant patches in the orientation maps (Crair et al., 1996). Nevertheless, both optical and single-unit ocular dominance histograms exhibited a significant bias in favor of the open eye. Note that a similar brief deprivation was much less effective late in the critical period (Fig. 2-3, right). This change was quantified in the contralateral bias index (CBI), which shifted from 0.57 in Fig. 2-1 to 0.10 in Fig. 2-2, but only to 0.37 in Fig. 2-3.

Ocular dominance plasticity has been thought to be effective only when young animals are visually alert, thus being conspicuously absent from anesthetized and paralyzed preparations (Freeman and Bonds, 1979; Imamura and Kasamatsu, 1988; Singer and Rauschecker, 1982). These results gave rise to the idea of consolidation processes which “lock-in” changes induced by imbalances in afferent activity. In support of this hypothesis, NMDA receptor blockade prevents ocular dominance plasticity in a time-delimited fashion, being effective at 1 hour but not 6 hours post-deprivation (Rauschecker and Hahn, 1987). Monitoring cells with chronic ‘floating’ electrodes, Mioche and Singer (1989) also observed a minimum time requirement for the manifestation of ocular dominance changes that could not be reduced by increasing visual exposure. Our 2-day deprivation effect was robust to active or passive use of the

Fig. 2-3. Robust plasticity following 2 day deprivations in kittens. Single-unit recordings reveal a profound shift of responsiveness toward the non-deprived eye following 2 days of monocular deprivation, regardless of whether the animals were continuously handled during the day (Active: n=177 cells, N=3 cats) or returned to their home cages (Passive: n=195 cells, N=5 cats). Two days deprivation is much less potent when initiated late in the critical period (late MD: P110-112, n=58 cells, N=1 cat). Non-deprived control data were taken from Stryker and Harris, 1983 (Normal: n=372 cells, N=6 cats).



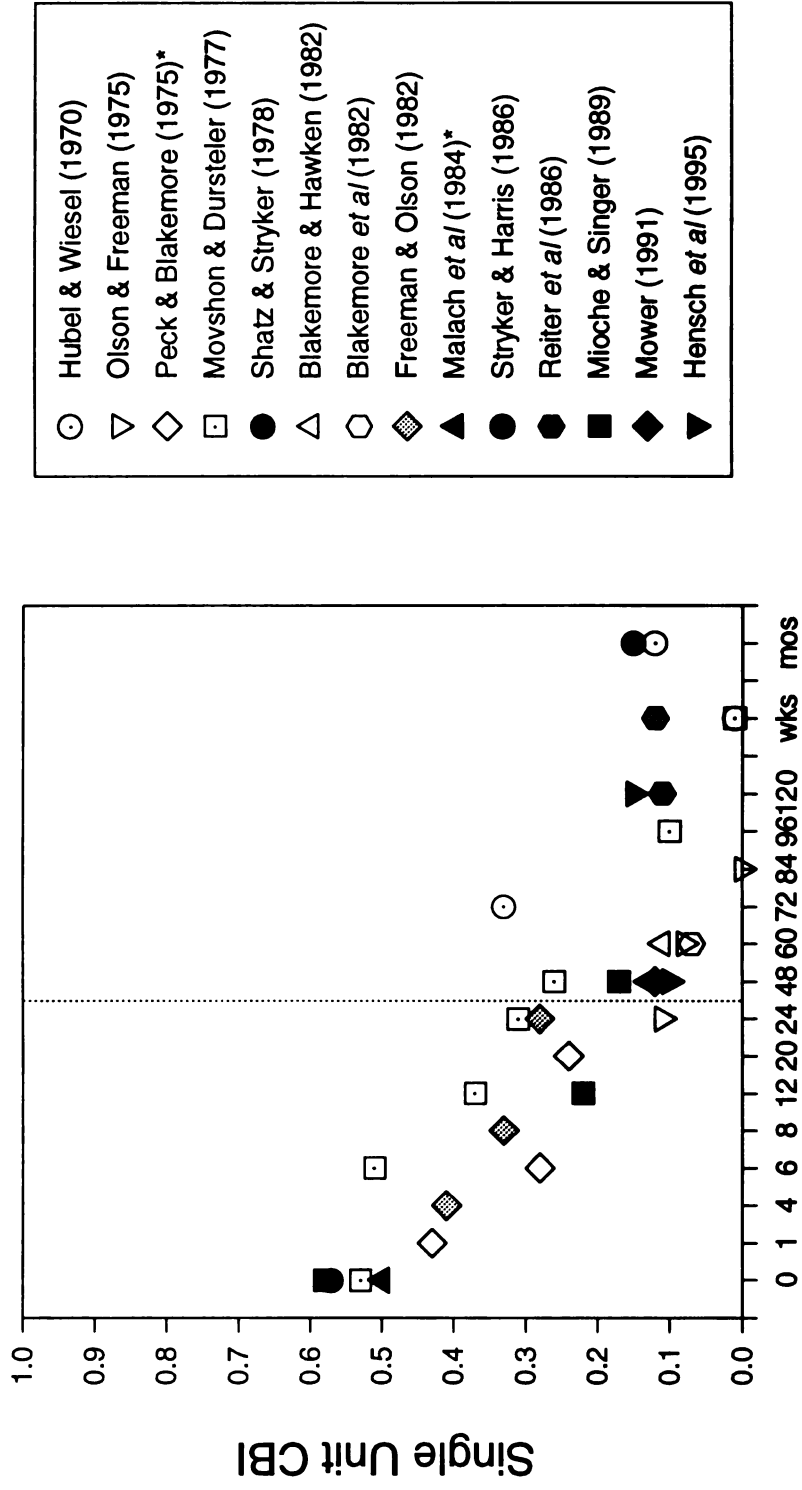
Ocular Dominance

open eye by gentle handling (Fig. 2-3), suggesting that any consolidation must have occurred within this time period.

In order to determine whether 2-day effects represented 'maximal' physiological responses to monocular deprivation, we calculated a CBI value according to our formula for all isolated cases we could find in the literature of brief deprivation at the peak of the critical period (P28-35). Plotting these data on the same curve, we found ocular dominance plasticity to be a gradual process, apparently reaching a plateau between one and two days (Fig. 2-4; (Mioche and Singer, 1989)). Directly comparing one- and two-day deprivations, we found a strong but more variable phenotype of the shorter manipulation (Fig. 2-5), which was also reflected in a less-dark polar map through the closed eye (data not shown). Curiously, one-day deprivations in our hands were more robust than those previously reported in the literature, which emphasize a loss of binocularity and a prominent residual monocular response via the deprived eye (Freeman and Olson, 1982; Freeman and Olson, 1979; Van Sluyters and Freeman, 1977).

Most importantly, we confirmed that rapid physiological plasticity clearly precedes large-scale anatomical reorganization of afferent arbors, which consistently accompanies week-long deprivations (Antonini and Stryker, 1996). Two days were as powerful as five days, or even months (Shatz and Stryker, 1978), of monocular vision when given at the right time in development (Fig. 2-5). Thus, *in vitro* models of learning and memory such as LTP and LTD may very

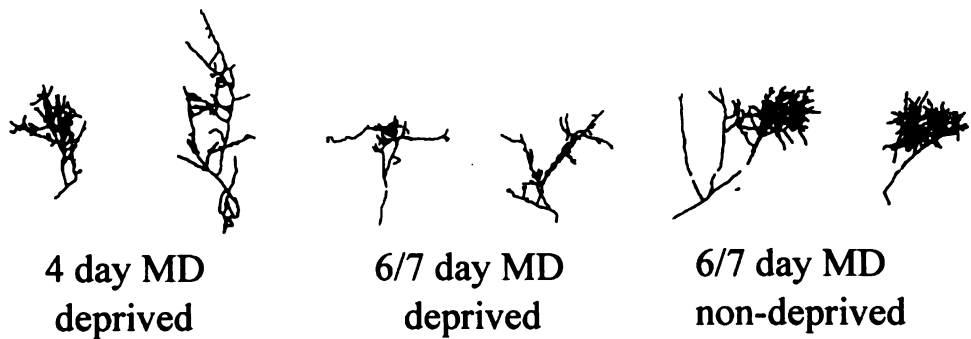
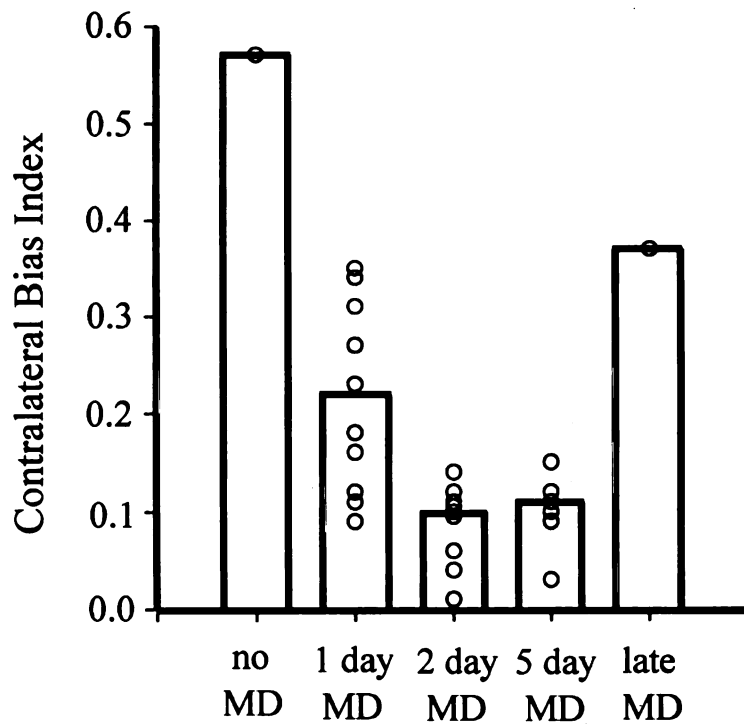
Fig. 2-4. Maximal plasticity requires 2 days of deprivation in kittens. The magnitude of ocular dominance plasticity is maximal by two days of deprivation. An analysis of results from the literature using our CBI summarizes data from the indicated references, including only those animals deprived during the peak of the critical period (P27-35). To analyze 5-point histograms a modified formula was used (Reiter et al, 1986). Filled symbols indicate grouped data, and hollow symbols represent single cases. Some authors (*) used a spaced deprivation paradigm involving timed light exposures interleaved with "consolidation" periods in total darkness.



Length of Deprivation (hours)

Fig. 2-5. Physiological plasticity precedes anatomical rearrangements. Top, Ocular dominance shifts are similarly reliable across animals deprived at the peak of the critical period for either two days (mean CBI=0.10, N=8 cats) or 5-7 days (mean CBI=0.11, N=7 cats). One-day deprivations are more variable and on the average less strong than at two days (mean CBI=0.22, N=9 cats, including 3 from literature). Columns indicate mean values, and hollow symbols represent individual cases. Normal and late MD data as in Fig. 2-3. Bottom, Maximal large-scale anatomical reorganization of thalamocortical arbors requires at least one week of deprivation. Note the greater variability in deprived-eye arbor reduction at 4 days versus 6/7 days (adapted from Antonini and Stryker, 1996).

Physiological plasticity precedes large-scale thalamocortical axon rearrangement



(adapted from Antonini & Stryker, 1996)

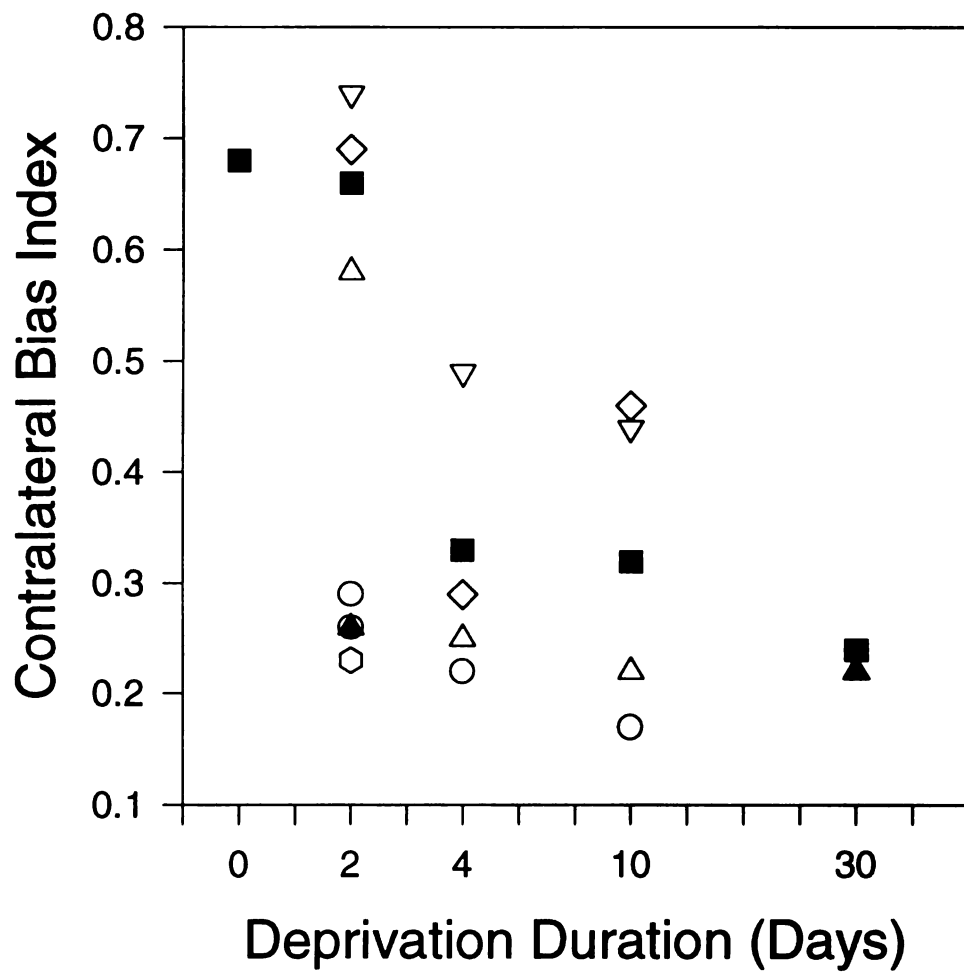
well underlie early stages of ocular dominance plasticity (Bear et al., 1987; Bear and Kirkwood, 1993; Kandel and O'Dell, 1992; Singer, 1995; Tsumoto, 1992). However, as almost all of these studies are conducted in neocortical slices from rodents, we decided to first better characterize the time course of *in vivo* plasticity in these species.

We found that ocular dominance plasticity was slower in rats than in cats (Fig. 2-6). Four days of deprivation were required before maximal physiological effects could be consistently obtained, as observed recently for the mouse (Gordon and Stryker, 1996). These changes were also manifest in optical images of intrinsic signals (M. Fagiolini, personal communication). Rodents lack a gross columnar segregation of right and left eye inputs within the binocular zone of primary visual cortex (Draeger, 1974). Perhaps in the absence of a gradually emerging anatomical substrate, purely physiological processes must shoulder the burden of plasticity for each synapse onto every cell before a saturating ocular dominance shift can be detected. Further work at the level of single axon reconstructions is required before such conclusions can be reached.

For whatever reason, the slowed monocular deprivation effect in rodents opens a longer window of analysis onto the plasticity process. For example, the presence of a consolidation step, which was difficult to detect in the rapid events of kitten cortex, may be evident at two days in the rat (Fig. 2-6). At this time point, half the animals shifted fully, whereas others remained at a non-deprived level. Determining what distinguishes these two groups will be very insightful.

Fig. 2-6. Maximal plasticity requires 4 days of deprivations in rats. Brief periods of monocular lid suture were initiated at the peak of the critical period (P26-29). The CBI of non-deprived animals is slightly higher in rodents than cats, but also decreases significantly with 4 or more days of deprivation. Note that at 2 days, when kitten effects are maximal, half of the rats have shifted fully while the others appeared non-deprived. Hollow symbols, individual cases; Filled symbols, mean values. Data for month-long and non-deprived animals taken from Maffei et. al. 1992.

Rat Brief MD



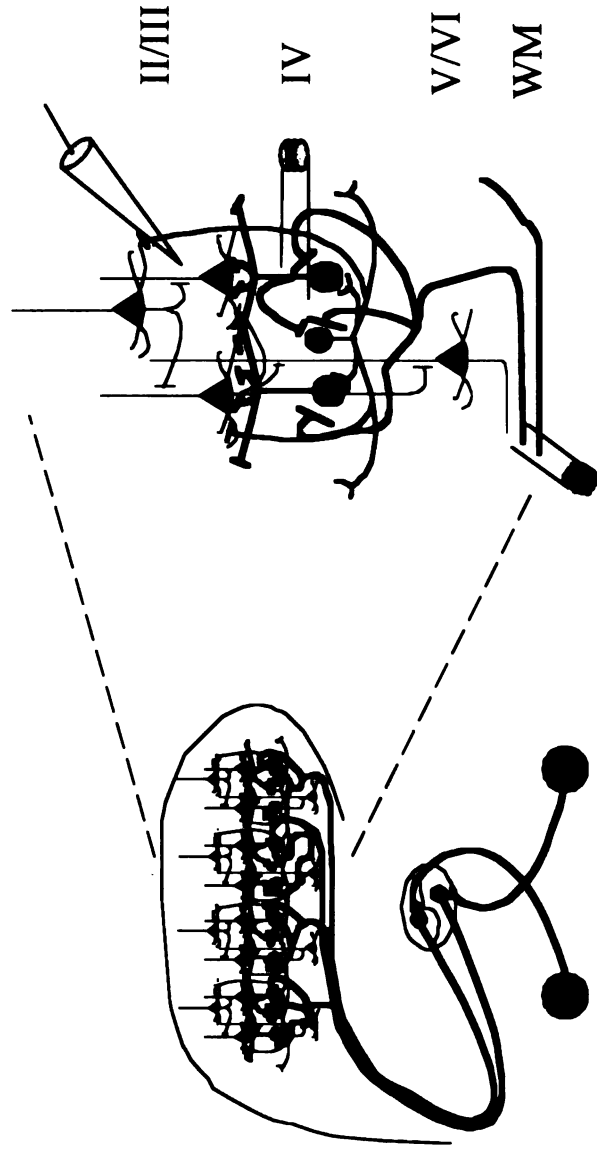
Moreover, we now have a lower limit on the time constant for biochemical and cellular processes underlying plasticity *in vivo*. It is now reasonable to make slice preparations of rodent cortex and directly compare rapid forms of synapse modification *in vitro* with the phenomenon in the intact brain (Fig. 2-7).

To illustrate this approach, we compared LTP of supragranular field potentials following theta-burst stimulation (TBS) of the white matter with monocular deprivation effects in mice. Ocular dominance changes are known to be progressively more pronounced in extragranular layers (Gordon and Stryker, 1996; LeVay et al., 1980; Shatz and Stryker, 1978) and not solely due to transmission failure at thalamocortical synapses (Kossut and Singer, 1991). In slices of kitten visual cortex, LTP of synaptic transmission is also greatest at intracortical synapses in layer II with respect to the other laminae (Toyama et al., 1991). Connections to layer II/III are conveniently preserved in coronal slices through the binocular zone of mouse primary visual cortex (Fig. 2-7). Thus, a contribution of plasticity within excitatory cortical circuits to ocular dominance plasticity may be observed in such an *in vitro* preparation by direct comparison.

The age-dependent ability to generate extracellular LTP in the upper cortical layers from the white matter has recently been correlated with the critical period (Kirkwood et al., 1995). This form of neocortical plasticity has also been suggested to operate according to the same principles as those found in mature hippocampal area CA1 (Kirkwood et al., 1993). We, therefore, chose to examine mice lacking the γ -isoform of protein kinase C (PKC γ). These mutants reportedly

Fig. 2-7. Intracortical interactions *in vitro*. Rapid synapse modification of excitatory transmission can be studied in coronal slices through the binocular zone of rodent primary visual cortex. This preparation preserves intracortical connections, whereas white matter stimulation activates a mixture of sub-cortical and cortical fibers. Ocular dominance plasticity beyond the input layer IV has been reported previously (Shatz and Stryker, 1978; LeVay et. al. 1980; Gordon and Stryker, 1978).

Intracortical Interactions *in vitro*



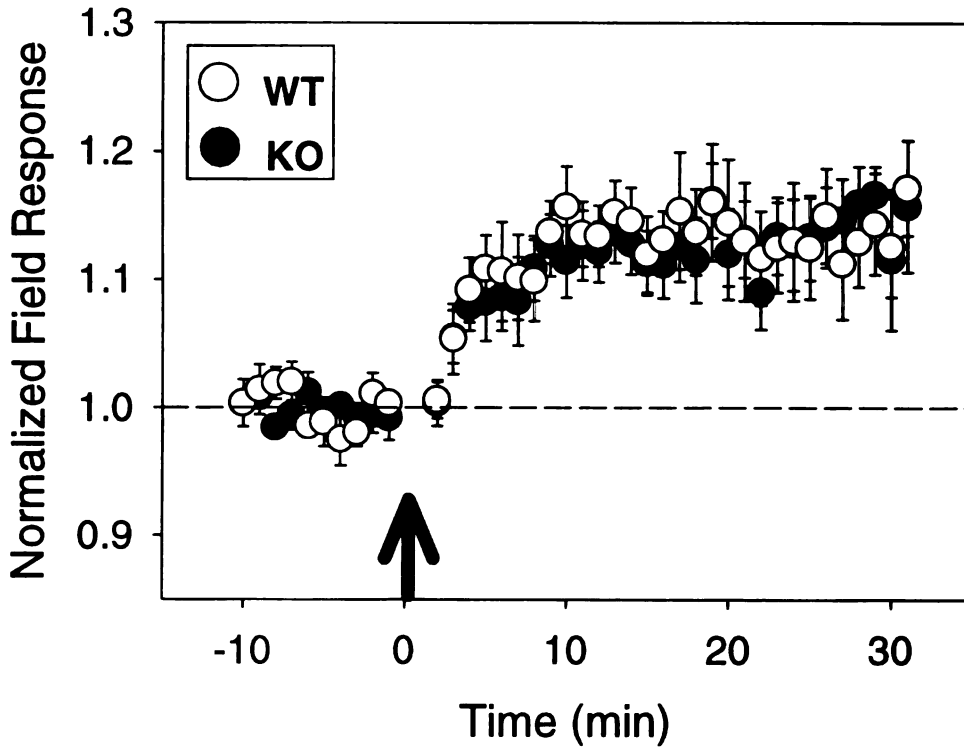
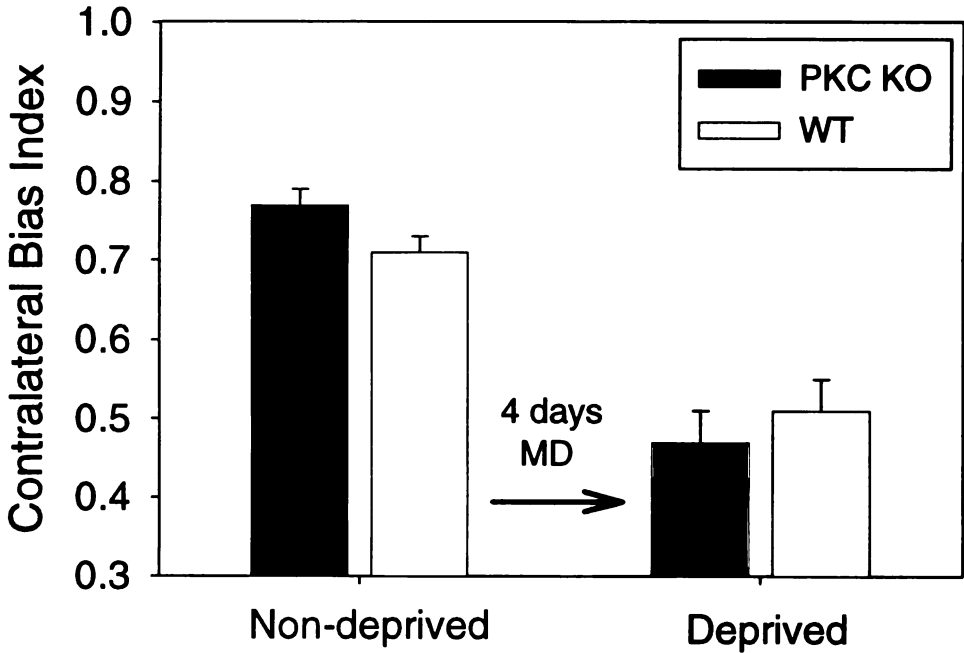
express a defect in CA1 plasticity requiring the weakening of synapses before LTP can be induced (Abeliovich et al., 1993). We found no evidence of such a defect in the binocular zone of visual cortex, where naïve supragranular field responses potentiated nicely following TBS of the white matter (Fig. 2-8b). Consistent with a role for LTP in ocular dominance plasticity, brief (4-day) monocular deprivations also produced a response shift in favor of the open eye for both mutant and wild type animals recorded blind to genotype (Fig. 2-8a).

Our results point out that principles of synaptic plasticity in the immature neocortex and adult hippocampus *in vitro* may not be the same. They also indicate that competitive synapse elimination machinery may differ between the visual cortex and cerebellum in the same brain. The well-known 1:1 relationship of climbing fibers onto Purkinje cells in the mature cerebellar cortex arises from a competitive removal of supernumerary climbing fibers by parallel fibers as they impinge onto the dendritic tree (Crepel, 1982). This is also an activity-dependent process, since NMDA receptor blockade can prevent the refinement (Rabacchi et al., 1992). Purkinje cells in mice lacking PKC γ retain multiple climbing fiber responses well into adulthood (Kano et al., 1995).

Thus, the combined use of *in vitro* and *in vivo* rodent preparations is likely to constrain and refine our thinking about the cellular mechanisms underlying visual cortical development. In the chapters that follow, I pursue this approach with a particular emphasis on the correlation between potentiation and depression of intracortical excitatory transmission and monocular deprivation

Fig. 2-8. Visual cortical plasticity in mice lacking protein kinase C γ . Top, Effects of brief (4 day) monocular deprivation are shown as mean CBI values \pm s.e.m. for wild type (white) and mutant (black) mice recorded blind to genotype. N=3 hemispheres from 2 non-deprived, and N=3 hemispheres from 3 deprived animals of each; n=23-36 cells per hemisphere. Bottom, Theta-burst stimulation (arrow) of the white matter induced similar magnitude LTP of supragranular field responses from slices of both wild type (white) and mutant (black) visual cortex. Thus, both *in vivo* and *in vitro* forms of rapid physiological plasticity are intact in the absence of this isoform of PKC.

Visual Cortical Plasticity in PKC γ -Deficient Mice



Chapter 3

Ocular Dominance Plasticity Under Metabotropic Glutamate Receptor Blockade

Summary

Occluding vision through one eye during a critical period in early life nearly abolishes responses to that eye in visual cortex, a phenomenon mimicked by long-term depression of synaptic transmission *in vitro* which may require metabotropic glutamate receptors (mGluRs) and is age-dependent. Peaks in mGluR expression and glutamate-stimulated phosphoinositide turnover during visual cortical development have been proposed as bases for the critical period. Pharmacological blockade of mGluRs with a broad spectrum antagonist specifically prevented synapse weakening in mouse visual cortical slices but did not alter kitten ocular dominance plasticity *in vivo*. Thus, mGluR activation cannot account for the critical period in development.

Introduction

Connections in the developing vertebrate visual system are sculpted by an activity-dependent competition between inputs for common postsynaptic neurons. Manipulations of visual experience, such as monocular deprivation (MD) during a well-defined critical period, regulate cortical physiology and ultimately lead to anatomical rearrangements (Shatz, 1990; Shatz and Stryker, 1978; Wiesel and Hubel, 1963). The biochemical basis for experience-dependent changes in visual circuitry remains largely unknown.

Metabotropic glutamate receptors (mGluR) are reported to play a role in the neural plasticity of several systems, including synapse strengthening in the hippocampus (Aiba et al., 1994a; Bashir et al., 1993; Conquet et al., 1994; Richter-Levin et al., 1994; Riedel et al., 1995; Riedel et al., 1994) and long-term depression (LTD), a form of age-dependent (Dudek and Bear, 1993; Dudek and Friedlander, 1996) synapse weakening in the hippocampus ((Bashir and Collingridge, 1994; Bashir et al., 1993; Bolshakov and Siegelbaum, 1994; Oliet et al., 1996; O'Mara et al., 1995; Yang et al., 1994; Yi et al., 1995); but see (Selig et al., 1995)), neocortex (Haruta et al., 1994; Kato, 1993), and cerebellum (Aiba et al., 1994b; Hartell, 1994; Shigemoto et al., 1994).

Expression of mGluRs (Catania et al., 1994; Reid et al., 1995) and glutamate-stimulated phosphoinositide (PI) turnover (Dudek and Bear, 1989; Jia et al., 1995) have both been shown to peak transiently during development of

primary visual cortex, concurrent with the height of sensitivity to visual deprivation. Thus, mGluR function is a candidate mediator of cortical plasticity, accounting for both the time course of the critical period and the loss of responsiveness from an eye deprived of vision. We have now examined developmental plasticity of primary visual cortex both *in vitro* and *in vivo* using the broad spectrum mGluR antagonist *R,S*- α -methyl-carboxyphenylglycine (MCPG).

Materials and Methods

Recordings were obtained from layer II/III in the binocular zone of 400 μ m-thick coronal slices of mouse primary visual cortex (C57Bl/6; <six weeks old) continuously superfused with oxygenated (95%O₂/5%CO₂) artificial cerebrospinal fluid (ACSF), containing (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO₄, 1.0 NaH₂PO₄, 26.2 NaHCO₃, 2.5 CaCl₂, 11 Glucose. Extracellular pipettes (1M NaCl, 1-3M Ω) monitored stable, half-maximal baseline field potentials evoked from layer IV by a bipolar Pt-Ir electrode delivering 100msec pulses at 0.1 Hz. Five episodes of 10 trains at 5 Hz of 4 pulses at 100 Hz (TBS) were given at 10 sec intervals to induce LTP before depotentiation was attempted with 900 pulses at 1 Hz (Kirkwood et al., 1993). All experiments were terminated by bath application of 10 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Tocris) and 50 μ M D(-)-2-

amino-5-phosphonopentanoic acid (D-APV, Sigma) to determine the synaptic component of the field response. Similar results were obtained by measurements of synaptic slope or peak amplitude normalized to the baseline period before TBS. Responses were re-normalized to the 10 minutes preceding 1 Hz stimulation to adjust for variable elapsed time post-TBS across experiments. Both LTP and depotentiation were prevented by the N-methyl-D-aspartate (NMDA) receptor antagonist APV (data not shown), as also reported for LTD of naive synapses (Kirkwood and Bear, 1994b). R,S- α -MCPG or (+)-MCPG (Tocris) were dissolved in 100mM NaOH at 50mM then diluted to 500 μ M in ACSF.

In visual cortical slices prepared and maintained as above, I_{AHP} currents were evoked by stepping the membrane potential of regular-spiking supragranular pyramidal cells (McCormick et al., 1985) from -50 to +40 mV for 100 msec in the whole-cell voltage clamp mode (Axoclamp-2B). The pipette solution contained (in mM): 122.5 KGlucuronate, 17.5 KCl, 10 HEPES buffer, 0.2 EGTA, 8 NaCl, 2.0 Mg-ATP, 0.3 Na₃-GTP (3-8M Ω , pH 7.2, 290-300mOsm). t-ACPD (Tocris) was dissolved in ACSF and bath applied.

Experiments were performed *in vivo* according to methods described previously (H.O. Reiter, D.M. Waitzman, M.P. Stryker, 1986). Infusion cannulae connected to osmotic minipumps (Alza model 2002) were implanted bilaterally into postnatal day 28 kitten striate cortex under sterile conditions (Fig. 3-2). One eyelid was sutured shut under brief halothane anesthesia on P30 and MD was verified for 5 days. R,S- α - or (+)-MCPG (25-50mM in 100mM NaOH), or vehicle

(100mM NaOH, pH 10) solution were delivered at a constant rate (0.5ml/hr) throughout the experiment. In some cases, MCPG solutions were first neutralized to physiological pH 7, which rendered the drug inactive on the *in vitro* I_{AHP} assay.

On P35, animals were prepared for acute single-unit recording using standard techniques in accordance with UCSF guidelines for animal care. In brief, kittens were anesthetized and maintained with a combination of barbiturate infusion (Nembutal 10mg/kg i.v.) and N₂O:O₂ (2:1) ventilation. Extracellular unit recordings were obtained immediately in front and no further than 1.5mm from each cannula with resin-coated tungsten microelectrodes (1-3MΩ) in vertical penetrations spaced evenly at 400μm intervals along the medial bank. Electrode tracks were reconstructed in Nissl-stained coronal sections to confirm sampling from all layers of visual cortex.

Light bar stimuli were swept across the receptive field with a hand-held lamp to assign each cell to an ocular dominance group on the basis of Hubel and Wiesel's seven-point scale (1962). Here an ocular dominance of seven represents complete dominance by the open eye. The contralateral bias index (CBI), a weighted average of the bias toward one eye or the other, was calculated for each treated hemisphere, separately and as a group, according to the formula: $CBI = [(n_1 - n_7) + (2/3)(n_2 - n_6) + (1/3)(n_3 - n_5) + N] / (2N)$, where N = total number of cells, and n_x = number within ocular dominance group x.

In each hemisphere, multi-barreled iontophoretic pipettes were lowered into striate cortex just beyond the most distant penetration site used for determination of ocular dominance (<1.8mm from cannula). Activation thresholds for kainic acid and t-ACPD (both 20mM in saline) were determined by gradually increasing iontophoretic ejection currents (WPI model 160) in 20nA steps once every 60 sec. Each round of iontophoresis was preceded by isolation of multiple units with visual stimulation and concluded by verifying the presence of the same visually-driven cells. Additional iontophoretic penetrations amidst the single-unit sites and well beyond (>3 mm) confirmed that ocular dominance recordings lay within a region in which MCPG was effective.

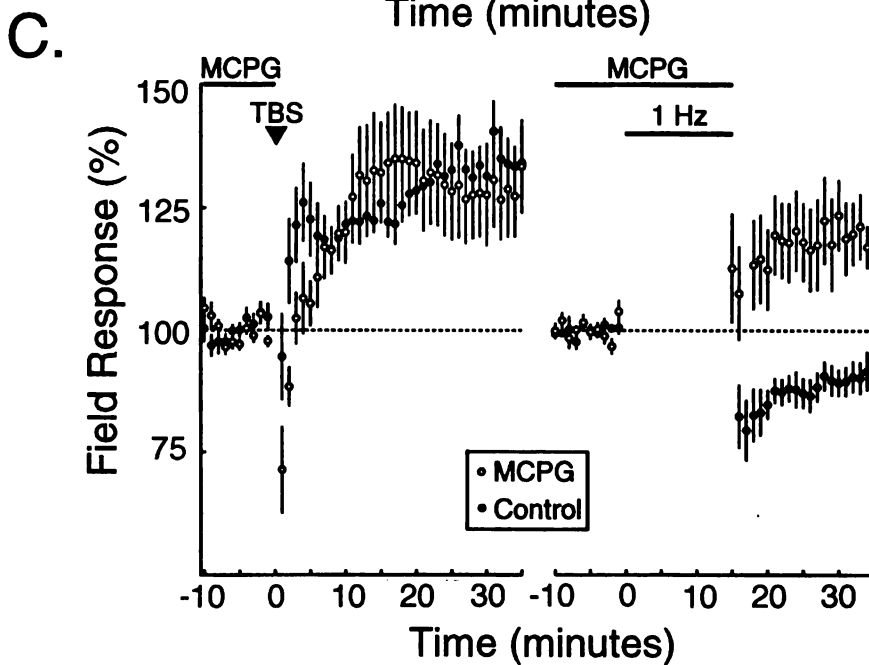
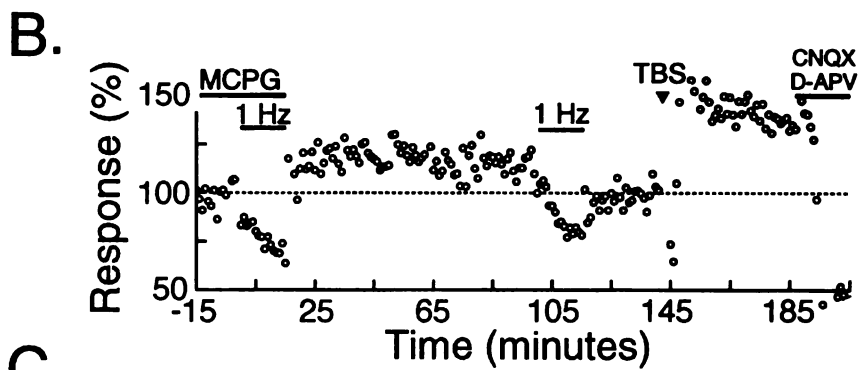
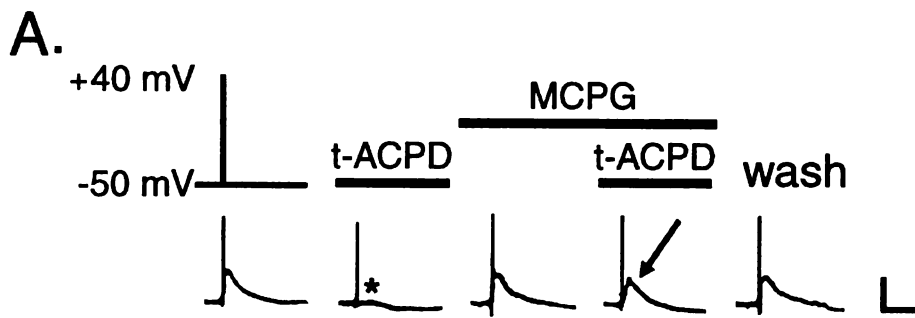
Results

It is generally believed that the mechanisms responsible for the formation of ocular dominance columns during normal development underlie the effects of monocular deprivation (MD) (Shatz, 1990). At the peak of the critical period in the cat (four weeks after birth), significant segregation of the afferent axons serving the two eyes has already taken place (Antonini and Stryker, 1993; LeVay et al., 1978). Thus, we chose to examine depotentiation of experimentally-potentiated responses, rather than depression of naive synapses, as the most appropriate *in vitro* model for the loss of responses through the deprived eye.

Theta-burst stimulation (TBS), to produce long-term potentiation (LTP), and low-frequency stimulation (1Hz), to elicit depression of transmission from layer IV to II/III in visual cortex *in vitro*, have been advocated as physiologically-relevant models for understanding naturally-occurring synapse modifications (Kirkwood and Bear, 1994a; Kirkwood et al., 1993; Kirkwood et al., 1996). Visual experience similarly regulates functional development of visual cortex across mammalian species (Draeger, 1978; Fagiolini et al., 1994; Gordon and Stryker, 1996; LeVay et al., 1980; Shatz and Stryker, 1978), and slices of mouse visual cortex also exhibited a TBS-induced LTP that could be depotentiated by 1Hz stimulation (Fig. 3-1).

Specific blockade of mGluR activation and depotentiation by MCPG was confirmed in developing mouse visual cortex *in vitro*. Activation of postsynaptic PI-linked mGluRs by the specific agonist 1S,3R-1-amino-cyclo-pentane-1,3-dicarboxylate (t-ACPD) depolarizes neurons by closing potassium channels, such as the calcium-activated after-hyperpolarizing potassium current (I_{AHP}) (Baskys, 1992; Charpak et al., 1990; Gereau and Conn, 1995; Stratton et al., 1990). MCPG prevented the t-ACPD effect on I_{AHP} in visual cortical pyramidal cells (Fig. 3-1A).

Fig. 3-1. Synaptic depotentiation in primary visual cortex via metabotropic glutamate receptors. A, Activation of post-synaptic mGluRs by the specific agonist t-ACPD (20 μ M) blocked the I_{AHP} potassium current (*), which is normally opened by calcium influx through voltage-gated calcium channels in response to membrane depolarization. Bath application of MCPG (500 μ M) had no effect on I_{AHP} itself, but prevented abolition of the potassium current by t-ACPD (arrow). B, When MCPG was present for at least 10 minutes before and during 1 Hz stimulation (bar), previously potentiated synaptic responses failed to depotentiate, but were clearly capable of depression and repotentialization once the drug had washed out. Baseline field potentials were first potentiated by TBS (not shown) and their synaptic component determined at the end of the experiment by ionotropic receptor antagonists (CNQX and D-APV). C, Ensemble averages of synaptic field potential slope measurements revealed that depotentiation was specifically prevented by mGluR blockade. TBS (arrowhead) induced LTP of similar magnitude in control (●) and 500 μ M MCPG (o) solutions (20 min. post-TBS = +29 \pm 3% vs. +33 \pm 10%, n=9 and 8, respectively; $p > 0.7$, Student's t-test). In contrast, subsequent 1 Hz stimulation (bar) depotentiated control responses, but unmasked a further persistent potentiation when delivered in the presence of MCPG (-10 \pm 3% vs. +19 \pm 6%, respectively, 20 min. post-1Hz; $p < 0.002$, Student's t-test).



Bath application of MCPG at least 10 minutes prior to and throughout 1Hz stimulation also reversibly prevented depotentiation *in vitro* (Fig. 3-1B). Instead, a robust and persistent potentiation was unmasked, unlike the depotentiation produced in control solutions. In contrast, TBS-induced LTP was not affected by MCPG in visual cortical slices (Fig. 3-1C). Potentiation under mGluR blockade following a stimulus that would normally induce LTD has also been observed in the hippocampus (Yang et al., 1994; Yi et al., 1995) and cerebellum (Hartell, 1994; Shigemoto et al., 1994).

Having demonstrated an essential role for mGluRs in synaptic weakening *in vitro*, we examined whether mGluR activation was necessary for the loss of functional inputs *in vivo* resulting from MD. Stereotaxically implanted cannulae delivered vehicle or MCPG solutions from osmotic minipumps to kitten striate cortex for one week including 5 days of MD at the peak of the critical period (Fig. 3-2). When extracellular unit recordings were made in Area 17 at the end of the week blind to the minipump contents, the ocular dominance of neuronal responses was strongly shifted in favor of the open eye (Fig. 3-3A). The loss of deprived-eye responses was indistinguishable across hemispheres treated with vehicle, inactive, or active MCPG solutions (Table 1).

To confirm the efficacy of drug treatment *in vivo*, iontophoretic electrodes were lowered into regions of cortex where ocular dominance had previously been mapped and shown to have shifted. Control visual responses were vigorous even within 50 μ m of the alkaline vehicle source, and cells were

Fig. 3-2. Protocol for mGluR blockade *in vivo*. Osmotic minipumps containing either vehicle or MCPG solutions were connected to cannulae implanted into kitten visual cortex at the peak of the critical period (P27). Drug concentrations were allowed to stabilize over a sufficient area for two days, then one eyelid was sutured shut for five days. Single-unit recordings were made just anterior to the infusion sites blind to the minipump contents. Ocular dominance histograms for both hemispheres were compiled before lowering multi-barreled iontophoretic pipettes **into the same regions of cortex.** Kainic Acid or t-ACPD were alternately applied to visually-isolated cells, and the threshold current to activate them within an arbitrary 10 second window was determined for each agonist.

Methods

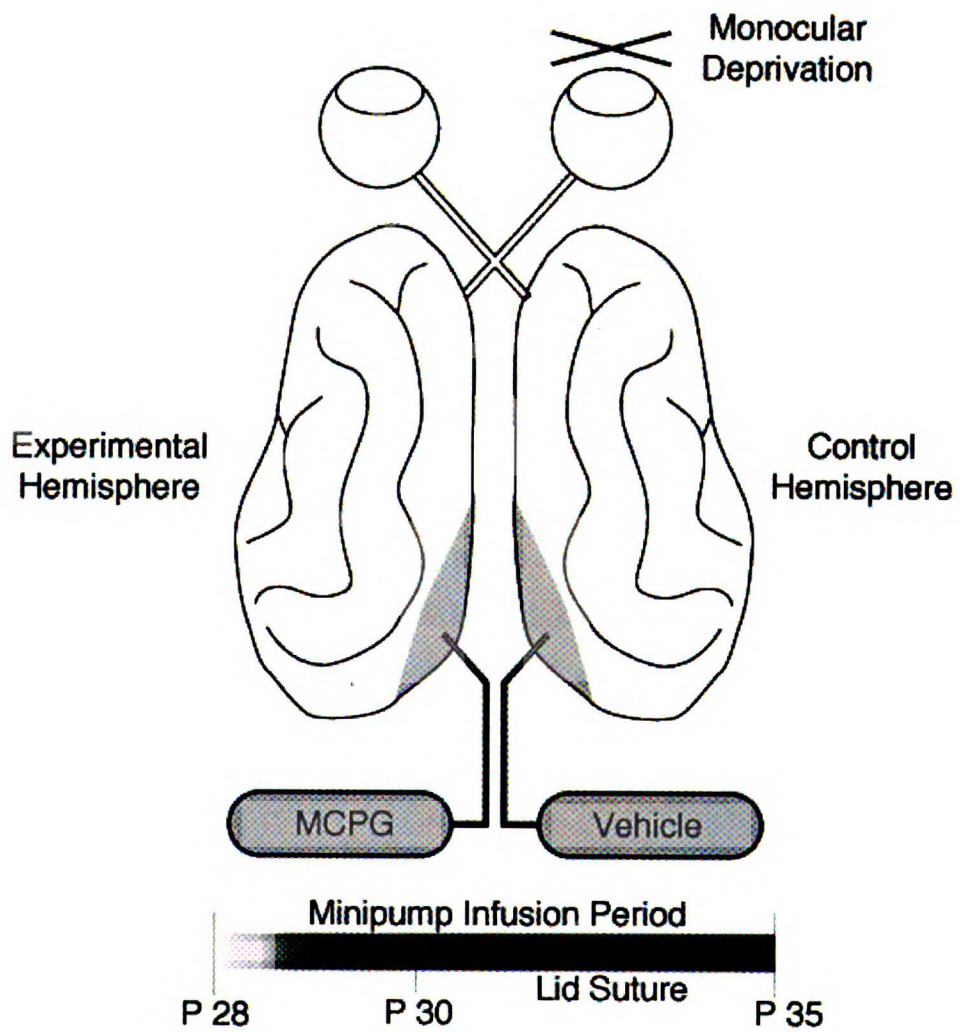


Fig. 3-3. Loss of deprived-eye responses following monocular deprivation during metabotropic glutamate receptor blockade. A, Ocular dominance of neuronal responses shifted profoundly in favor of the open eye in both hemispheres of each animal infused with vehicle (hollow bars) or active MCPG (black bars) solutions (CBI=0.14 vs. 0.11, n=108 and 171 cells, respectively, in 4 kittens; $p>0.7$, Student's t-test). B, Selective antagonism of metabotropic glutamate receptors by MCPG in kitten visual cortex *in vivo*. Control units either in vehicle-treated cortex (o) or in the same hemisphere distant from the MCPG infusion site (\diamond) had similar t-ACPD thresholds. Neurons in the presence of MCPG (\blacklozenge) were almost never activated by t-ACPD, despite normally low kainic acid thresholds, even when ejecting currents reached the limits of the iontophoresis machine (1000nA). C, The ionotropic glutamate receptor agonist kainic acid or the mGluR-specific agonist t-ACPD were alternately iontophoresed onto cells isolated by visual stimulation in regions of cortex where ocular dominance had previously been mapped. Activation thresholds for each drug were arbitrarily determined as the minimum ejecting current required to attain maximal cell firing within a ten second application period (note pulse offset artifacts). Kainic acid evoked brisk spikes in both vehicle- and MCPG-treated hemispheres with low threshold currents. Ejecting t-ACPD from a neighboring barrel at similar current levels depolarized control units to spike threshold. Although visually-driven, neurons in MCPG-treated cortex typically did not exhibit a response to the metabotropic agonist. The example illustrates rare activation of a nearby large unit just at the offset of a 1000nA t-ACPD pulse. Visual responsiveness of individual cells remained robust even after strong iontophoretic currents.

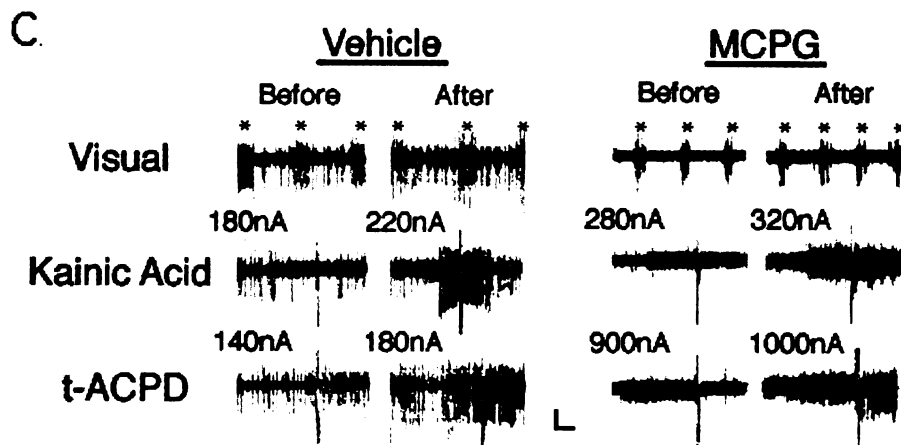
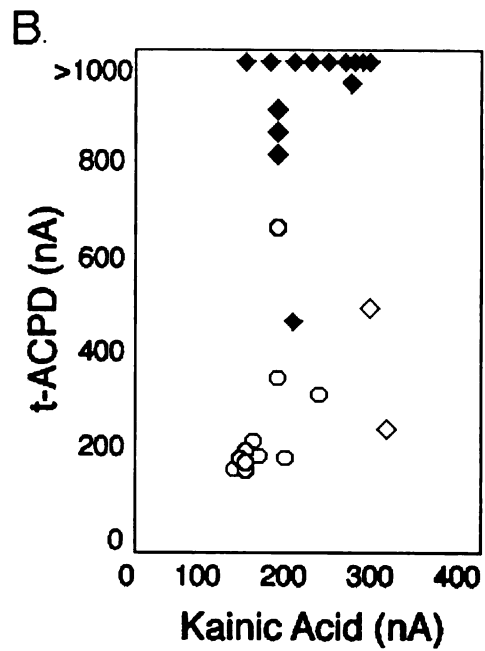
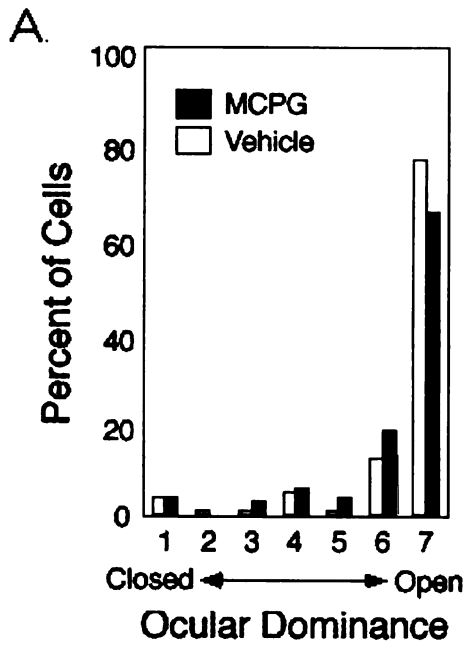


Table 3-1. Monocular Deprivation Effects in the Presence of MCPG. MD induced a profound loss of responsiveness from the deprived eye regardless of whether the hemisphere was infused with **vehicle, inactive, or active** MCPG solution ($p > 0.3$, Mann-Whitney U-test).

Experiment	MCPG		Vehicle		
Animal	Isomer	no. of cells	CBI*	CBI*	no. of cell
MUC 872	R,S	64	0.26	0.09	15
MUC 899	R,S	24	0.10	0.03	22
MUC 903	R,S	36	0.08	0.15	31
MUC 906	(+)	42	0.03	0.12	40
TOTAL	ACTIVE	171	0.11		
MUC 875	inactive	41	0.09	0.09	16
MUC 876	inactive	19	0.10	0.18	14
MUC 883	inactive	33	0.07	0.28	36
TOTAL	INACTIVE	93	0.09		
TOTAL	VEHICLE			0.15	174

* CBI = Contralateral Bias Index, where values of 1.00 and 0.00 represent complete dominance by the closed or open eye, respectively (Reiter et al, 1986).

activated by similar iontophoretic currents for both the ionotropic glutamate receptor agonist kainic acid and the mGluR-specific agonist t-ACPD (Fig. 3-3C, left). In contrast, neurons in the MCPG-treated hemispheres were rarely depolarized to spike threshold by t-ACPD, although they were strongly driven by visual stimulation and control-level kainic acid ejection currents (Fig. 3-3C, right).

Grouped data demonstrated a greater than 5-fold elevation in activation threshold specifically for t-ACPD as compared to control units recorded in the opposite hemisphere or more distant in the same hemisphere (Fig. 3-3B). Consistent with acute iontophoresis results in the cerebellum (Lingenhoehl et al., 1993), somatosensory cortex (Cahusac, 1994), and thalamus of rats (Salt and Eaton, 1994), chronic MCPG infusion *in vivo* selectively blocked postsynaptic effects of mGluRs in kitten striate cortex without altering visual responsiveness.

A reduction by about 50% in the size of deprived eye geniculocortical arbors accounts for much of the effect of one week MD (Antonini and Stryker, 1993). However, anatomical effects of four days MD are less pronounced (Antonini and Stryker, 1996), and varying degrees of plasticity in striate cortex have been noted for much shorter periods of deprivation (cf. Chapter 2). Rapid effects of MD, which may reflect an important transitional state when deprived eye afferents are anatomically present but functionally ineffective, also did not require mGluR activation. Ocular dominance plasticity produced by 2 days of

MD was similar in magnitude following 50mM MCPG infusion (CBI=0.12, n=51 cells) to control (CBI=0.10, n=284 cells).

Discussion

Postsynaptic mGluR blockade did not impede ocular dominance plasticity in primary visual cortex. Molecular cloning has revealed at least eight different subtypes of G protein-coupled mGluRs which can be classified into three subgroups (Nakanishi and Masu, 1994; Pin and Duvoisin, 1995). Of particular relevance to the present work, MCPG is known to block postsynaptic Group I (mGluR 1 and 5)-mediated inositol trisphosphate formation (Birse et al., 1993; Eaton et al., 1993; Hayashi et al., 1994; Thomsen et al., 1994), and subsequent calcium release from internal stores (Bashir et al., 1993; Saugstad et al., 1995).

Both racemic R,S-MCPG and the stereoselective isomer (+)-MCPG failed to block plasticity *in vivo* (Table 1), indicating that none of the known PI-linked mGluR splice variants underlies the developmental critical period (Brabet et al., 1995; Joly et al., 1995; Saugstad et al., 1995). Moreover, MCPG at the concentrations used *in vivo* is a broad spectrum antagonist of mGluR subtypes, including postsynaptic Group II (mGluR 2 and 3) (Hayashi et al., 1994) as well as presynaptic Group III (mGluR 6, 7, and 8) receptor coupling to the cyclic AMP signalling pathway (Jane et al., 1994; Manzoni et al., 1995). More selective and

potent reagents are necessary to dissect the contributions, if any, of group II and III mGluRS.

Certain forms of potentiation and depression of synaptic transmission in the neocortex *in vitro* have been proposed to underlie activity-dependent development in the intact animal (Kirkwood et al., 1993). The present results extend earlier findings that mGluRs specifically mediate LTD of naive synapses in rat visual cortex (Haruta et al., 1994; Kato et al., 1991) with no role in TBS-induced LTP, and suggest that neocortical depotentiation and LTD share similar mechanisms. Our data are also in agreement with evidence against mGluR involvement in hippocampal LTP induction (Chinestra et al., 1993; Hsia et al., 1995; Manzoni et al., 1994). An mGluR-mediated molecular "switch" of naive or depotentiated synapses to an mGluR-independent state has been proposed to explain the discrepant findings (Bortolotto et al., 1994). The present results show that experience-dependent changes in visual cortical circuitry do not pass through such mGluR-sensitive naive states. Developmental plasticity in cortex *in vivo* may differ from adult hippocampal plasticity *in vitro*, as shown previously for PKC γ mutant mice (cf. Chapter 2).

The electrophysiological changes in neuronal excitability assayed here are consistent with a blockade of at least the postsynaptic PI-coupled mGluRs (Figs. 3-1A, 3-3B) (Gereau and Conn, 1995; Stratton et al., 1990). Indeed, greater than 1 mM MCPG effectively antagonizes t-ACPD induced PI turnover and subsequent plasticity in the hippocampus *in vivo* (Riedel, 1996; Riedel et al., 1995).

Nevertheless, ocular dominance plasticity following MD in the presence of MCPG was normal. Thus, the peaks in mGluR expression (Reid et al., 1995) and receptor-mediated PI turnover (Dudek and Bear, 1989; Jia et al., 1995) at the height of the critical period do not underlie the activity-dependent refinement of connections in primary visual cortex.

Chapter 4

Visual Cortical Plasticity with Impaired Intracortical Signaling in PKA RI β -Deficient Mice

Summary

Developing sensory systems are sculpted by an activity-dependent strengthening and weakening of connections. Studies of long-term potentiation (LTP) and depression (LTD) *in vitro* have been thought to reveal the mechanisms underlying this experience-dependent development. In mice lacking the RI β regulatory subunit of protein kinase A (PKA), synaptic depression is defective in the mature hippocampus and was disrupted in visual cortical slices as well taken at the height of the developmental critical period *in vivo*. Extracellularly recorded synaptic responses in cortical layer II/III also failed to potentiate following high-frequency stimulation in RI β -deficient mice. However, the development and plasticity of responses *in vivo* were normal in these mutants. Postsynaptic mechanisms of LTP induction in individual pyramidal cells were also normal, suggesting that PKA RI β might be important specifically for presynaptic function in intracortical signaling. The absence of paired-pulse facilitation in mutant visual cortex supported this hypothesis. Activity-dependent plasticity in the intact neocortex can therefore proceed despite defects in commonly-studied synapse modification paradigms *in vitro*.

Introduction

Manipulations of visual experience during a critical period early in life have long been known to perturb the functional organization of connections in the mammalian visual cortex (Movshon and Kiorpes, 1990; Wiesel and Hubel, 1963). The cellular and molecular basis for this plasticity, however, remains largely unknown. Studies of learning and memory in mature animals provide several promising candidate factors that may contribute to developmental plasticity *in vivo* (Blakemore and Mitchell, 1973; Kandel and O'Dell, 1992).

Most notably, the cyclic AMP second messenger system has been implicated in such diverse systems as transient synaptic facilitation (Byrne et al., 1993; Ghirardi et al., 1992) and persistent structural changes in *Aplysia* (Glanzman et al., 1990; Schacher et al., 1993; Wu et al., 1995), synaptogenesis in the pond snail *Helisoma* (Funte and Haydon, 1993), olfactory associative learning in fruit flies (Davis, 1993; DeZazzo and Tully, 1995), LTP/LTD in the hippocampus (Brandon et al., 1995; Huang and Kandel, 1994; Huang et al., 1994; Qi et al., 1996; Weisskopf et al., 1994), and hippocampal learning behavior in vertebrates (Bourtchouladze et al., 1994; Wu et al., 1995).

In these systems, it is thought that an influx of calcium triggers calcium/calmodulin-sensitive adenylyl cyclases to produce cyclic AMP-dependent protein kinase (PKA) activation (Cooper et al., 1995). PKA is a pivotal second-messenger, serving two potentially crucial functions in plasticity.

Cytoplasmic PKA can phosphorylate targets such as ion channels and receptors to rapidly modulate synaptic efficacy (Blackstone et al., 1994; Colwell and Levine, 1995; Johnson et al., 1994), while protein synthesis-dependent growth processes may be initiated via the cyclic AMP response element binding protein (CREB) and a cascade of immediate early genes following the translocation of PKA to the nucleus (Spaulding, 1993).

A two-stage process also appears to dramatically reorganize kitten geniculocortical afferents serving the two eyes in response to a period of monocular deprivation during the critical period (Antonini and Stryker, 1993). Large-scale anatomical restructuring (Antonini and Stryker, 1993) is preceded by a more rapid physiological ocular dominance plasticity, which is detectable with extracellular recording techniques (Hensch et al., 1995; Mioche and Singer, 1989; Movshon and Dursteler, 1977). Efforts to interfere pharmacologically with cellular processes and thereby disrupt ocular dominance plasticity have yielded some insight into the role of electrical activity (Hata and Stryker, 1994; Reiter et al., 1986; Shaw and Cynader, 1984) and particular neurotransmitter receptors and neuromodulators (Bear and Singer, 1986; Hensch and Stryker, 1996; Kleinschmidt et al., 1987; Reid et al., 1996; Ruthazer et al., 1996). However, with regard to intracellular proteins such as kinases, the intracortical infusion approach is prohibitively restricted by the limited availability of effective and specific agonists and antagonists which can be functionally delivered *in vivo*.

In order to investigate a putative role for PKA in ocular dominance plasticity, we turned to a new class of tools provided by recent techniques for manipulating the mouse genome (Grant and Silva, 1994; Mayford et al., 1995). Rodent models of ocular dominance plasticity replicate the essential aspects found in other animals: visual experience modulates cortical responses via a correlation-based competition between inputs from the two eyes (Draeger, 1978; Fagiolini et al., 1994; Gordon and Stryker, 1996).

Here we analyze visual cortical plasticity in the binocular zone of primary visual cortex (V1) of mice carrying a targeted gene disruption of the RI β regulatory subunit of PKA (Brandon et al., 1995). Inactivation of the neuronal RI β subunit gene yields mice whose total PKA catalytic activity is unimpaired, apparently due to a compensatory up-regulation of the RI α subunit. Nevertheless, they show highly selective impairment in the ability to depress synaptic transmission in the dentate gyrus and CA1 region of hippocampus (Brandon et al., 1995); and they lack a presynaptic form of LTP in the CA3 region (Huang et al., 1995), suggesting an important role for the RI β isoform in these functions. Since the disruption of cellular processes is similar *in vitro* and *in vivo* in mutant mice, these animals also allow us to directly test whether simple assays of LTP and LTD in neocortical slices reveal normal mechanisms of experience-dependent changes in the developing mammalian brain (Kirkwood et al., 1995, 1996; Singer, 1995; Tsumoto, 1992).

Materials and Methods

***In vitro* recordings and analysis.** Mice carrying a targeted disruption of the PKA RI β gene were generated as described previously (Brandon et al., 1995). Coronal slices (400 μ m) through the binocular zone of the primary visual cortex (V1) were prepared blind to genotype from animals at the peak of the critical period for monocular deprivation effects (P24-33) and maintained at 27-29°C in oxygenated (95%O₂/5%CO₂) ACSF, containing (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO₄, 1.0 NaH₂PO₄, 26.2 NaHCO₃, 2.5 CaCl₂, 11 Glucose. Extracellular field potentials were recorded with a 1M NaCl (1-3M Ω) electrode inserted into layer II/III, and stable baseline responses were evoked from layer IV or the white matter at 0.1 Hz with a glass bipolar stimulating electrode.

To induce LTP, five episodes of TBS were applied (Kirkwood and Bear, 1994a). Each TBS consisted of 4 pulses at 100 Hz repeated 10 times at 5 Hz. LTD and depotentiation were assayed with low-frequency stimulation (900 pulses at 1 Hz; (Dudek and Bear, 1993)). Field experiments were terminated with a bath application of the non-NMDA and NMDA glutamate receptor antagonists CNQX (Tocris) and D-APV (Sigma) to confirm the synaptic nature of the extracellular response. Measurements of field EPSP amplitude were normalized to the baseline period before TBS or LFS and plotted against running time of the experiment.

Individual layer II/III cortical or hippocampal CA1 pyramidal cells were recorded with patch electrodes (5-8M Ω) in the whole-cell voltage clamp mode (Axoclamp-2B), using either the 'blind' technique or under direct visualization with infrared Nomarski DIC optics (Stern et al., 1992). The pipette solution contained (in mM): 122.5 Cesium or Potassium Gluconate, 17.5 Cesium or Potassium Cl, 10 HEPES buffer, 0.2 EGTA, 8 NaCl, 2.0 Mg-ATP, 0.3 Na₃-GTP, and 0.15% Biocytin (pH 7.2, 290-300mOsm). LTP was induced within 10 minutes of obtaining whole-cell access by pairing membrane potential depolarization to 0mV with 100 synaptic stimuli at 1 Hz, then monitored at a baseline holding potential of -70mV and stimulation at 0.1 Hz. Measurements of EPSC slope were normalized to the baseline period before pairing, and whole-cell input and series resistances were monitored for stability throughout the experiment.

***In vivo* recordings and analysis.** Electrophysiological procedures have been described in detail elsewhere (Gordon and Stryker, 1996). In brief, mice were anesthetized with 50 mg/kg Nembutal (Abbot) and chlorprothixene (0.2 mg, Sigma) and placed in a stereotaxic holder. The animal breathed a mixture of oxygen and room air through a trachea tube, and additional anesthetic doses (0.15 to 0.25 mg) were administered to maintain a heart rate of 6 to 9 Hz. A 5 x 5 mm portion of the skull was removed, exposing the visual cortex, and the intact dura was covered with agarose (2.8% in saline). The corneas were protected with silicone oil and optic disk locations projected onto a tangent screen to determine

the vertical meridian. Optic disk locations varied only slightly across animals (Mean \pm S.D.; Elevation = 33.6 ± 5.7 , Azimuth = 65.0 ± 6.0).

Resin-coated tungsten microelectrodes (2-4 M Ω) were used to record single units from primary (V1) visual cortex, as verified by electrode track reconstructions and histologic criteria. Data were obtained from the binocular zone, the region of V1 representing the central 25° of the upper portion of each visual hemifield. Receptive fields of isolated single units were plotted on a screen placed 30 cm from the animal, using a hand-held projection lamp. Cells were assigned ocular dominance scores according to the 7-point classification scheme of Hubel and Wiesel (1962), where a score of 1 indicates response to contralateral eye stimulation exclusively, and a score of 7 purely ipsilateral eye response. Intermediate scores (2-6) reflect a degree of binocular responsiveness. A weighted average of the bias toward one eye or the other, the contralateral bias index (CBI), was calculated for each hemisphere according to the formula: $CBI = [(n_1 - n_7) + (2/3)(n_2 - n_6) + (1/3)(n_3 - n_5) + N]/2N$, where N = total number of cells, and n_x = number of cells with ocular dominance scores equal to x.

For monocular deprivation experiments, eyelid margins were trimmed and the lids sutured under halothane anesthesia at the peak of the critical period (for 4 days beginning P25-27). All recordings were made from the binocular zone of V1 contralateral to the deprived eye, blind to the genotype of the animal. Some recordings were also made blind to deprivation status. For reverse suture experiments, initial deprivations were performed without trimming the eyelids

(for 5 days beginning P20-22), in order to facilitate reopening (for 4 to 8 days). Recordings were made blind to the order in which eyes were deprived. At the end of each experiment, an overdose of Nembutal was given and the animal perfused.

Histological analysis. For Nissl staining, mice were transcardially perfused with 0.5M phosphate-buffered saline (PBS) followed by 10% Formalin in PBS. After post-fixation, the brain was removed, cryoprotected in 30% sucrose-10%Formalin and cut into 40 μ m sections on a freezing microtome. Sections were mounted on slides, defatted, and stained with cresylecht violet (Schmid). For single-cell reconstructions following whole-cell patch clamp recordings, slices were fixed in 4% paraformaldehyde for at least 24 hours before cryoprotection and resectioning at 50 μ m on the freezing microtome. Sections were processed according to standard avidin-biotin complex (ABC) techniques (Vector) and biocytin label was visualized by the Nickel-intensified diaminobenzidine (Ni-DAB) reaction (Horikawa and Armstrong, 1988).

Results

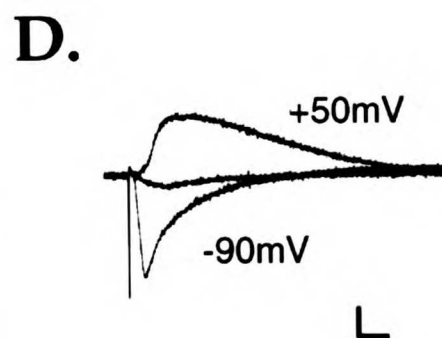
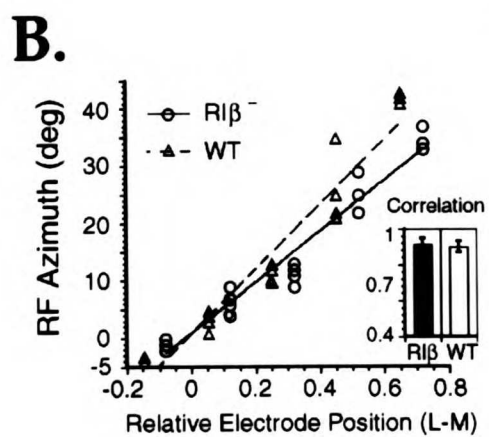
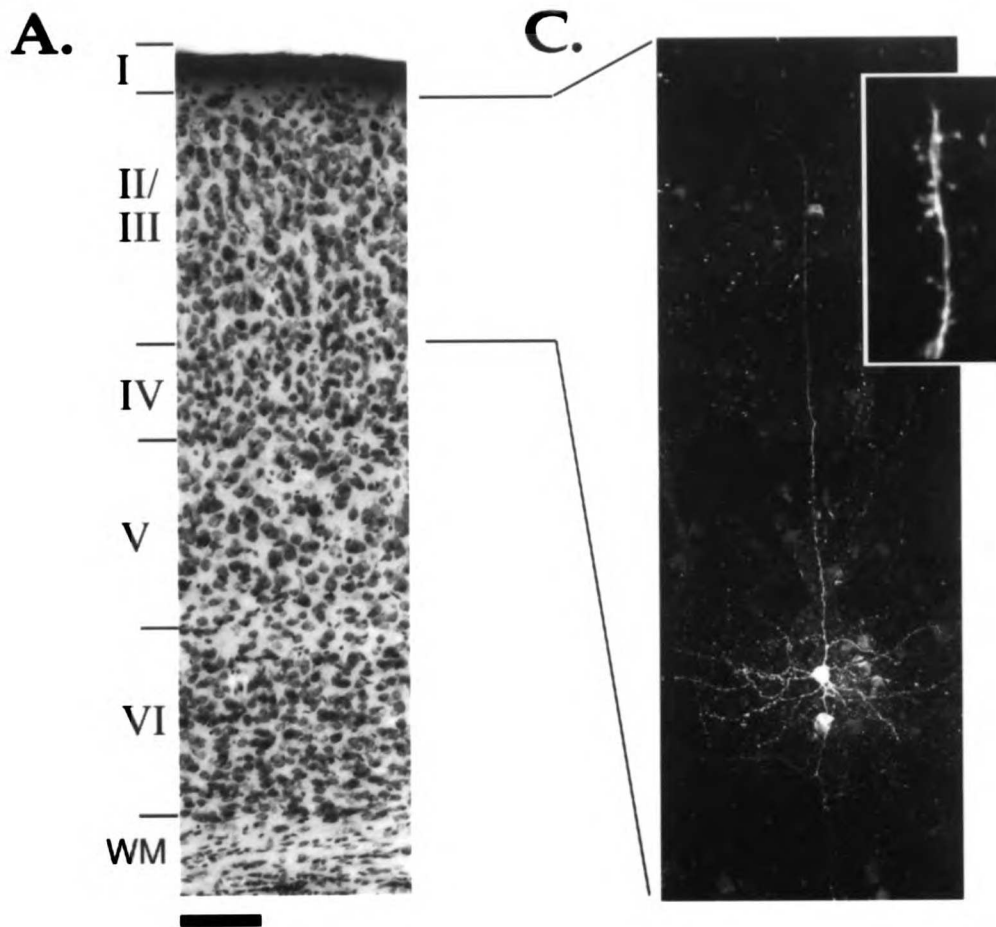
Normal visual cortical morphology and responses in the absence of RI β .

We first characterized the morphology and synaptic responses in the visual cortex of RI β -deficient (RI β -) mice. A normal six-layered binocular region of the

primary visual cortex was observed with typical pyramidal cells in the supragranular layers bearing many post-synaptic spines (Fig. 4-1A,C). Stimulation of the underlying layer IV evoked normal N-methyl-D-aspartate receptor-gated (NMDA_R) and non-NMDA_R-mediated synaptic currents in these cells, which could be blocked by 50 μ M D-APV and 10 μ M CNQX, respectively (Fig. 4-1D; Stern et al, 1992). The influx of calcium through NMDA_R channels on dendritic spines is thought to be essential for the induction of both LTP and LTD in the hippocampus and neocortex (Singer, 1995; Tsumoto, 1992). Thus, the post-synaptic structures required to initiate potentiation and depression *in vitro* appeared to be present in the visual cortex of RI β ⁻ mice.

Extracellular single-unit recordings were obtained *in vivo* from V1 of two RI β ⁻ and two wild type (WT) adult mice blind to genotype. A comparison of receptive field size, retinotopy, orientation selectivity, ocular dominance, and response strength revealed neuronal response properties in WT and RI β ⁻ V1 to be indistinguishable. Receptive field size distributions in RI β ⁻ and WT animals overlapped considerably, and the mean receptive field size from the RI β ⁻s did not differ significantly from WTs (5.9 \pm 0.1 and 6.4 \pm 0.3 degrees, respectively; $p=0.25$). A linear retinotopic arrangement was revealed by regression analysis of receptive field azimuth on electrode position for both RI β ⁻ and WT V1 (Fig. 1B).

FIG 4-1. Normal morphology and cortical synaptic responses in RI β - mice. A, Normal lamination identified in nissl-stained coronal sections through the binocular zone of primary visual cortex taken from animals at the peak of the critical period. Scale bar, 100 μ m. B, Visual responses were retinotopically organized in RI β - cortex. A series of evenly spaced microelectrode penetrations were made across a portion of the lateromedial extent of V1 in each animal. At least three and up to 7 cortical cell RFs were mapped in each penetration. RF-center azimuths were plotted vs. electrode position. Inset, Correlation coefficients of 3 RI β - and 4 WT regressions. C, A neuron filled with biocytin in the supragranular layers exhibited normal pyramidal morphology with a long apical dendrite extending to the pial surface and profuse basal processes. Numerous postsynaptic spines were readily visible (inset). Scale bar (cf. panel A): 45 μ m, 6 μ m inset D, Synaptic responses to underlying layer IV stimulation exhibited fast non-NMDA_R and slower NMDA_R-mediated components in supragranular pyramidal cells. Whole-cell voltage-clamp recordings at -90mV were blocked with 10 μ M CNQX and 10 μ M bicuculline methiodide to reveal NMDA_R-mediated responses at +50mV. The latter were blocked by 50 μ M D-APV (central trace).



Moreover, the scatter about this relationship was equally low for both genotypes, as demonstrated by the high correlation coefficients (Fig 4-1B, inset).

The distribution of ocular dominance scores of cells in the binocular zones of non-deprived RI β ⁻ and WT mice was similar (Fig. 4-2C). The Contralateral Bias Index (CBI), a measure of the degree to which the contralateral eye dominates the cortex, did not differ significantly for 3 RI β ⁻ and 4 WT hemispheres (mean CBI = 0.68 ± 0.05 and 0.66 ± 0.04 , respectively; $p=0.7$ t-test). Thus, the primary visual cortex of mice could develop normal gross morphology, synaptic currents, receptive field size, retinotopy and ocular dominance in the complete absence of the RI β subunit of protein kinase A.

Loss of deprived eye responses without LTD or depotentiation in RI β ⁻ mice. The original report of the PKA RI β knockout mice described an inability to generate LTD in the dentate gyrus and CA1 region of the hippocampus. It is generally believed that synaptic plasticity mechanisms are similar in both the hippocampus and neocortex (Kirkwood et al., 1993), and indeed we confirmed an impairment of synapse weakening in RI β ⁻ visual cortex *in vitro* (Fig. 4-2). A depotentiation protocol failed to significantly reduce field EPSPs in the supragranular layers following an earlier theta-burst stimulus (TBS) in mutants recorded blind to genotype (mean response 20 minutes post-conditioning= $80 \pm 5\%$ in WT vs. $100 \pm 4\%$ in RI β ⁻; $n=8$ and 11 slices, respectively from 6 mice each; $p<0.01$, t-test). Similarly, LTD of naïve, untetanized synapses by low-frequency

stimulation of layer IV was also absent in RI β ⁻ mice (98 \pm 3% field response 20 minutes post-LFS; n=5 slices from 3 mice; Fig. 4-2B).

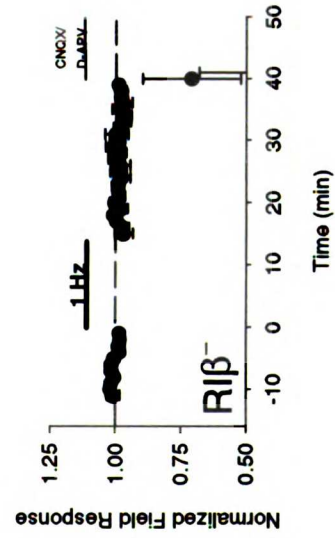
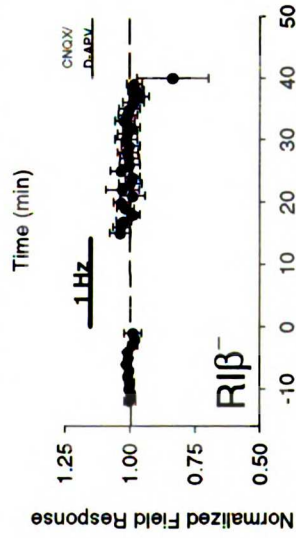
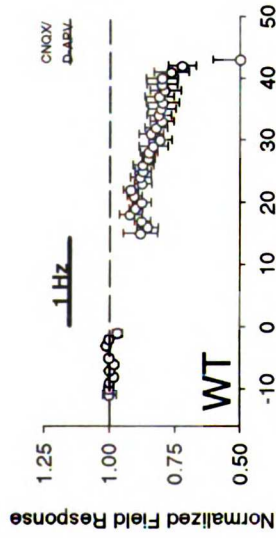
We then examined in V1 *in vivo* the effects of occluding vision through one eye. Three mutant and three WT mice underwent monocular deprivation by lid suture for 4 days beginning between P26 and P28. Ocular dominance distributions of neurons recorded blind to genotype from V1 contralateral to the deprived eye revealed significant shifts towards the open, ipsilateral eye in both WT and RI β ⁻ mice (Fig. 4-2C; deprived vs. non-deprived χ^2 -test, $p < 0.0005$ for each genotype). As a further control, five additional RI β ⁻ mice were studied blind to deprivation status. Ocular dominance distributions were normal as for either parental strain (mean CBI=0.68 \pm 0.06 for n=5 non-deprived hemispheres), unless the animals were deprived, in which case they shifted normally (mean CBI=0.41 \pm 0.03 for 2 deprived hemispheres; t-test, $p < 0.01$). Thus, a mechanism for the functional disconnection of input from a deprived eye *in vivo* remained intact despite the absence of LTD in PKA RI β ⁻ mice.

Potentiation of responses *in vivo* without TBS-induced LTP in RI β ⁻ mice. In the course of studying depotentiation, we were surprised to discover that TBS also consistently failed to induce LTP in the mutant mice. The ability to generate LTP in the supragranular layers of cortex from the white matter has been correlated with ocular dominance plasticity, since both phenomena appear to be regulated by age and visual experience (Kirkwood et al., 1995, 1996). A

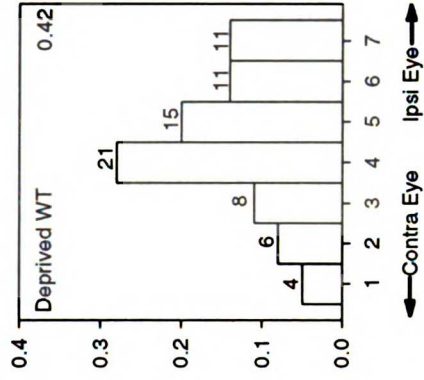
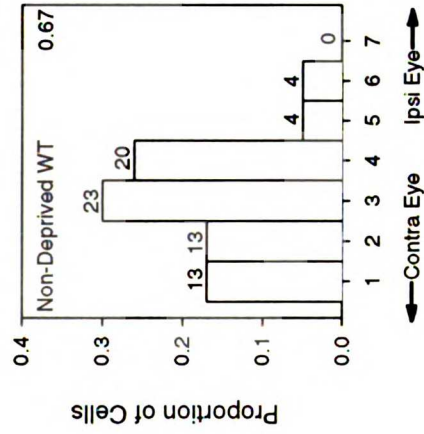
FIG. 4-2. Ocular dominance plasticity with defective synapse weakening *in vitro*. Low-frequency stimulation neither following a previous theta-burst (A) nor applied directly to naïve transmission from layer IV (B) could depress extracellular field potential amplitudes in layer II/III of RI β ⁻ mice (filled circles; open circles, WT). Representative traces 5 minutes before and 20 minutes after LFS are shown superimposed to the right of each graph. Scale bar: 0.3mV, 10ms.

C, Monocular deprivation led to a loss of deprived-eye responses in RI β ⁻ mice. Ocular dominance distributions from the binocular zone of two non-deprived WT (n=77 cells, hollow bars) and RI β ⁻ (n=75 cells, hatched bars) adult mice are shown with contralateral bias indices in the upper right hand corner of each graph (left column). Ocular dominance distributions from monocularly-deprived juvenile WT and RI β ⁻ animals are shown to the right (n=76 cells, WT; n=78 cells, RI β ⁻ from 3 mice each). Animals were deprived for 4 days beginning at P25-P27. *In vivo* and *in vitro* experiments were conducted blind to genotype.

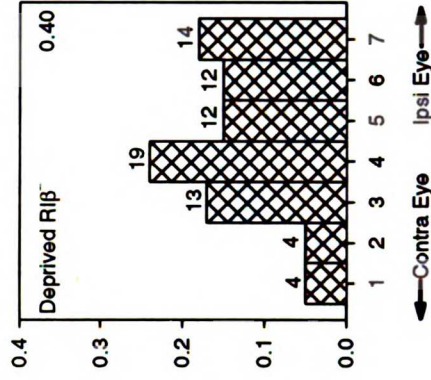
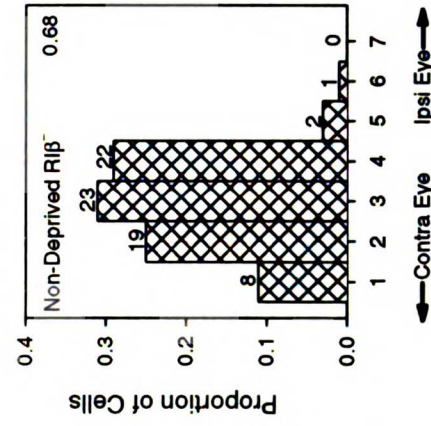
A.



C.



B.



theta-burst tetanus applied to the white matter of the binocular zone (Fig. 4-3A) potentiated layer II/III field EPSPs in WT ($123 \pm 6\%$ at 25 minutes post-TBS, $n=6$ slices from 4 mice) but not $RI\beta^-$ animals ($96 \pm 3\%$ at 25 minutes post-TBS, $n=5$ from 3 mice; $p < 0.01$, t-test).

Maturation of inhibition in the cortical circuit has been proposed to underlie the developmental regulation of LTP induction from the white matter (Kirkwood and Bear, 1994a). To circumvent this hypothetical "plasticity gate," we moved the stimulating electrode to layer IV (Fig. 4-3B). Once again TBS induced a robust potentiation in WT ($123 \pm 6\%$ at 25 minutes post-TBS, $n=8$ slices in 7 mice) but not in $RI\beta^-$ ($103 \pm 3\%$ at 25 minutes post-TBS, $n=11$ slices in 6 mice; $p < 0.01$, t-test). In order to assess the efficacy of TBS to induce LTP in neocortical slices, we applied a stronger tetanus which was previously found to be potent in $RI\beta^-$ mutant hippocampus (Fig. 4-3C; (Brandon et al, 1995)). Four bouts of 100 Hz stimuli for 1 second each produced only a brief post-tetanic potentiation which decayed back to baseline in both WT and $RI\beta^-$ mice (mean field EPSP 25 minutes post-100Hz = $102 \pm 3\%$ and $101 \pm 4\%$, respectively; $n=5$ slices from 3 mice each, $p > 0.1$, t-test). Thus, TBS was an effective tetanus protocol for neocortical potentiation *in vitro*, yet it failed to produce LTP in animals lacking PKA $RI\beta$.

The shift of ocular dominance in monocularly-deprived animals could in principle be mediated solely by depressant mechanisms in the deprived pathway, and need not involve an increase of responses to the open eye. We,

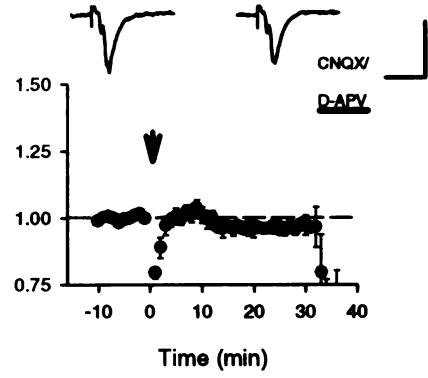
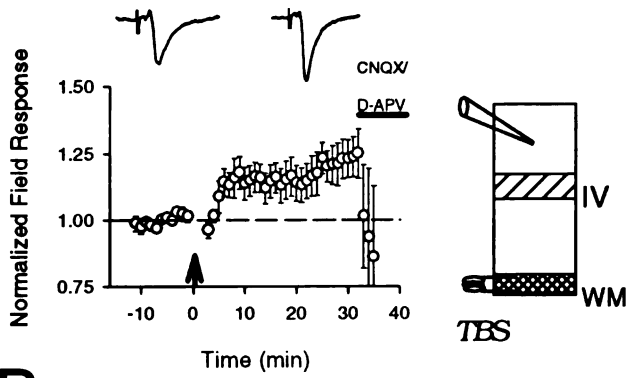
FIG. 4-3. Defective extracellular LTP in the visual cortex of PKA RI β ⁻ mice.

TBS (arrow) applied to the white matter (A), or directly to layer IV (B) potentiated supragranular field response amplitudes in WT (open circles) but not RI β ⁻ (filled circles) mice. C, More powerful tetani (four 1-second bursts of 100 Hz, arrow) failed to induce LTP in both WT and mutant slices. Representative traces 5 minutes before and 25 minutes after tetanus are shown above each graph. Sample traces during post-tetanic potentiation are also indicated in C. Scale bar: 0.3mV, 20ms for each.

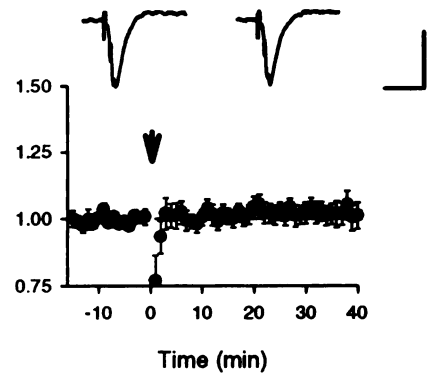
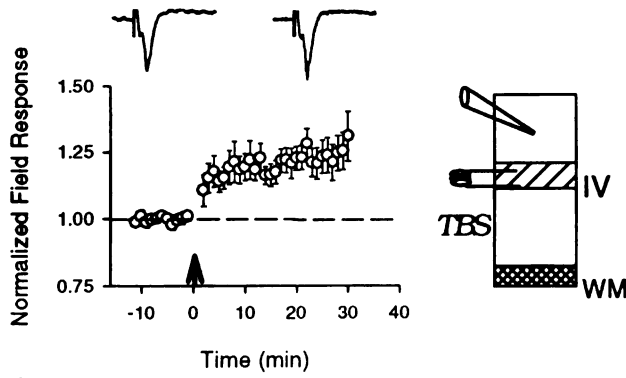
Wild Type

PKA RI β ⁻

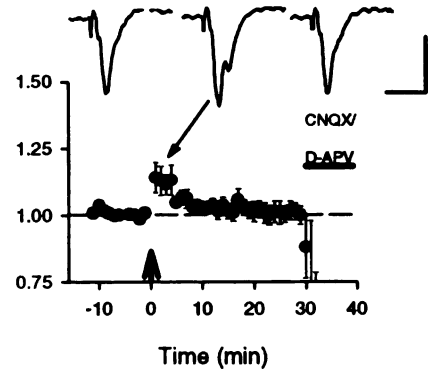
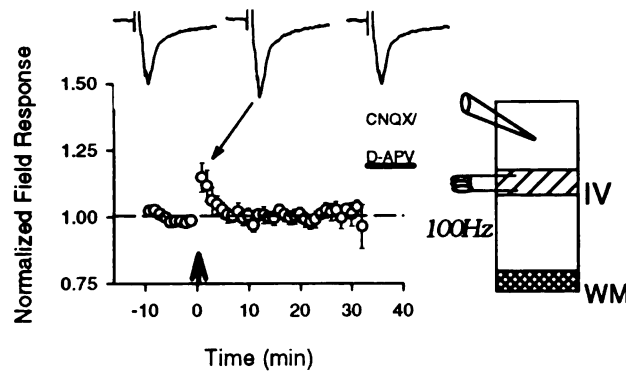
A.



B.



C.

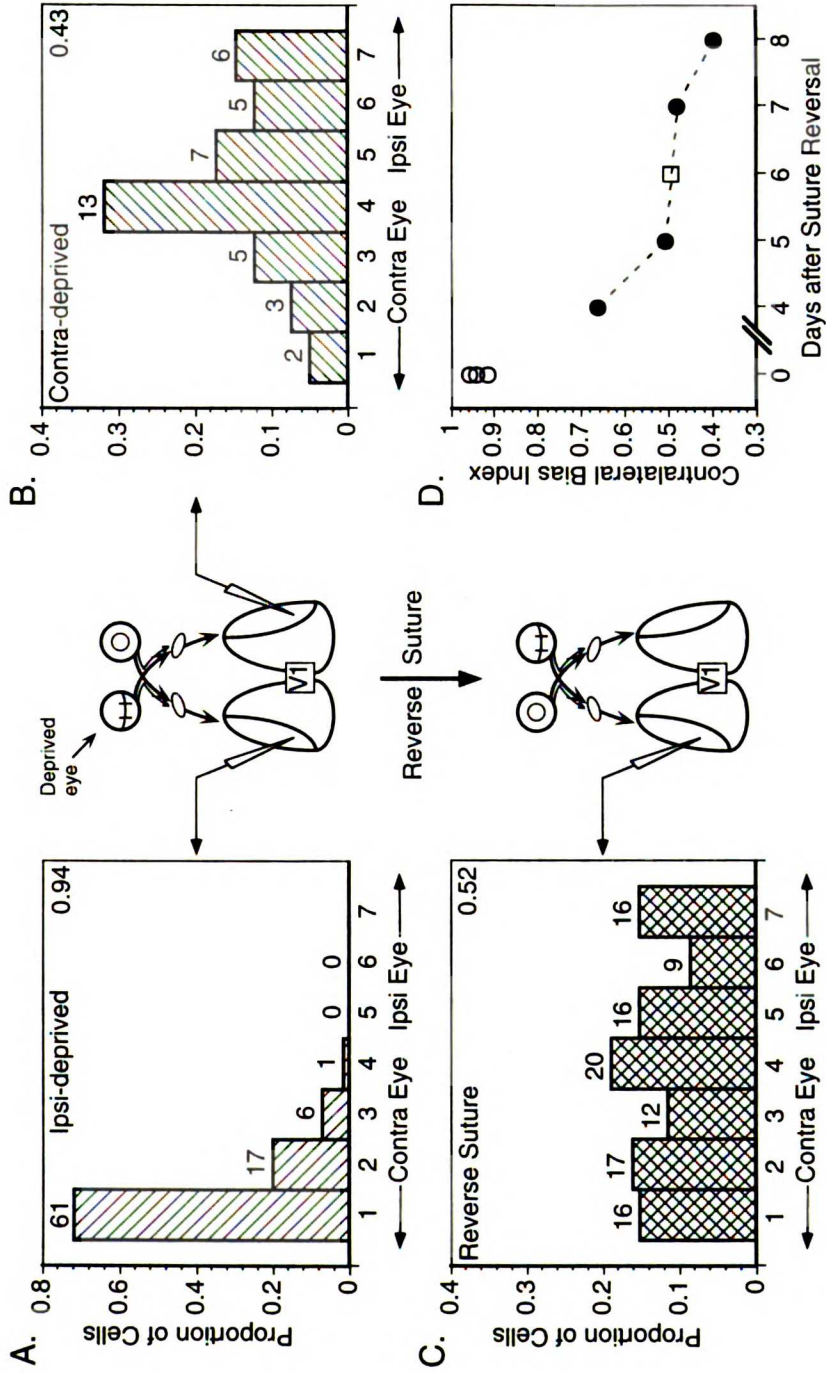


therefore, used a reverse suture paradigm to demonstrate whether inputs that had previously been made ineffective could again become dominant, requiring an *in vivo* mechanism similar to potentiation (Wiesel and Hubel, 1965). After an initial 5-day deprivation of the ipsilateral eye early in the critical period, few cortical cells responded at all to stimuli presented to the deprived eye (Fig. 4-4A; n=85 cells in 3 RI β ⁻ hemispheres). Responses to this initially-deprived eye reemerged once the eye was opened and the initially open eye was sutured shut for 4-8 days (Fig. 4-4C).

The ocular dominance distribution after reverse suture shifted significantly and normally back toward the initially-deprived eye (n=106 cells from 4 RI β ⁻ animals, and 26 cells from 1 WT mouse; $p < 0.0005$, χ^2 -test). Furthermore, the degree of recovery was greater with successively increasing periods following suture reversal (Fig. 4-4D). These data demonstrated a dramatic increase in efficacy of inputs from an initially-deprived eye and established the existence of a potent potentiation mechanism *in vivo* in RI β ⁻ mice, despite the absence of TBS-induced LTP.

Impaired intracortical signaling in PKA RI β ⁻ mice prevents TBS-induced LTP. Since NMDA_R-dependent LTP in the CA1 region of the RI β ⁻ hippocampus was reported to be intact (Brandon et al., 1995), we examined the cause of the potentiation defect in neocortex *in vitro*. TBS induced LTP in WT slices via NMDA_R activation, since the ability to generate LTP was reversibly blocked by the NMDA_R antagonist D-APV (Fig. 4-5A; Kirkwood and Bear, 1994).

FIG. 4-4. Response potentiation by reverse suture in RI β mice. A, Ocular dominance distribution of cells recorded ipsilateral to the deprived eye in 3 RI β mice. Mice were deprived for 5 days beginning at P20-P22. Because of the innate bias toward contralateral eye dominance in non-deprived animals, monocular deprivation of the ipsilateral eye resulted in nearly complete dominance of the cortex by the contralateral eye (compare with the less dramatic effect in the opposite hemisphere shown in panel B). C, Ocular dominance distribution of cells recorded ipsilateral to the initially deprived eye in 4 reverse-sutured RI β mice. Mice were initially deprived for 5 days beginning at P21; at P26 all mice underwent reverse suture for an additional 4-8 days. D, Individually calculated CBIs of ipsi-deprived and reverse-sutured animals shifted back toward the initially closed eye. Moreover, the degree of recovery was greater with increasing periods following suture reversal. Open circles, ipsi-deprived RI β mice (same animals as in A). Closed circles, reverse sutured RI β 's (same animals as in C). Open square, reverse-sutured WT.

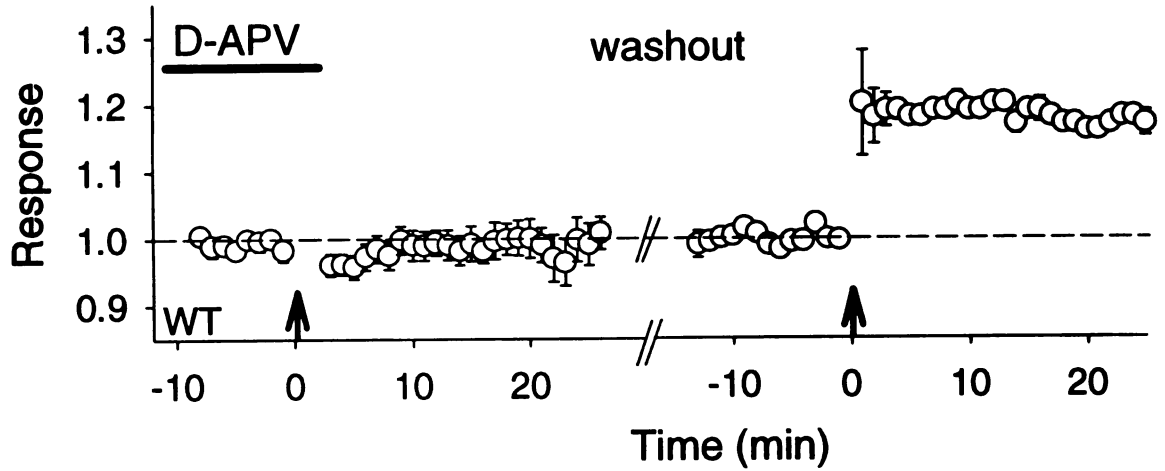


Having observed the presence of NMDA_R currents in supragranular RI β -pyramidal cells (Fig. 4-1D), we attempted to induce LTP by directly engaging the NMDA_R by pairing postsynaptic depolarization in whole-cell voltage clamp mode with low-frequency stimulation of synaptic inputs (Gustafsson et al., 1987; Kirkwood and Bear, 1994a; Yoshimura and Tsumoto, 1994). Robust LTP using this pairing protocol could be observed in RI β - mice in both the visual cortex and hippocampal CA1 region within the same slice (Fig. 4-5B, n=9 cells each from 7 mice), consistent with the original report of intact tetanus-induced LTP in CA1 (Brandon et al, 1995). Thus, postsynaptic mechanisms required for LTP induction were preserved in individual pyramidal cells lacking the RI β subunit of PKA.

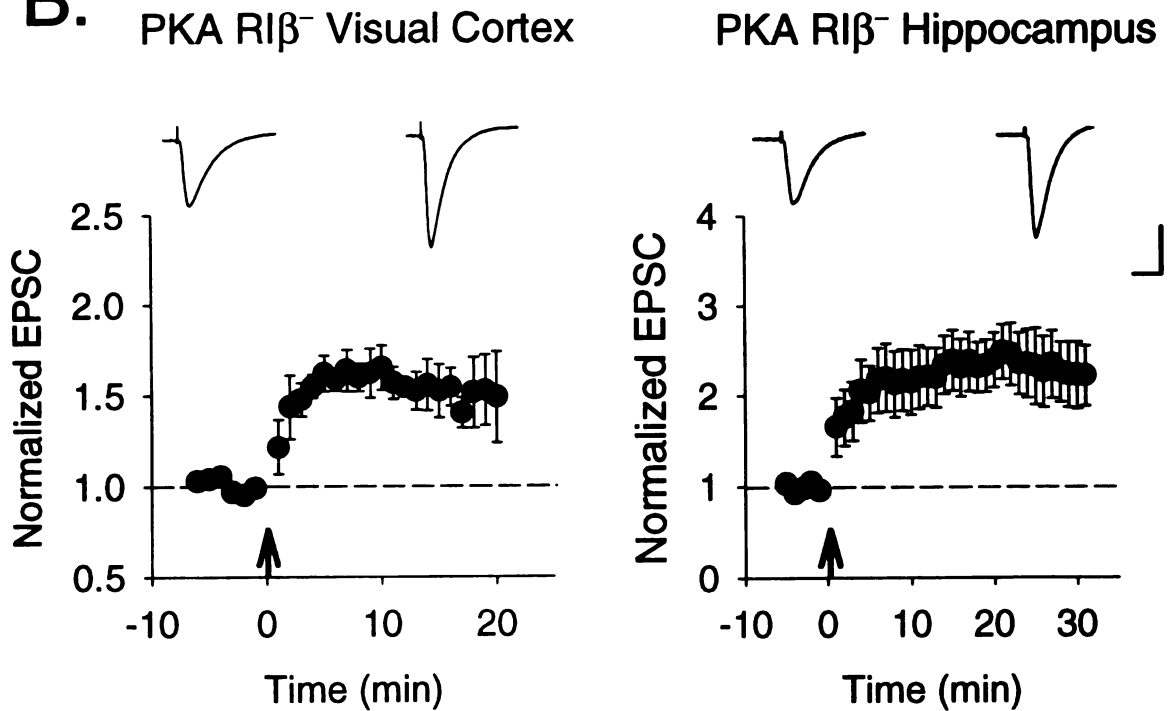
The failure of TBS to induce LTP in visual cortex may reflect an inability of the high-frequency stimulus to propagate through the cortical circuit. It has recently been reported that short-term changes in neocortical synaptic strength that occur during TBS are strongly correlated with the magnitude of LTP subsequently expressed (Castro-Alamancos and Connors, 1996). Consistent with this hypothesis, whole-cell current clamp responses of supragranular pyramidal cells during a theta burst exhibited a sustained facilitation of responses in WT, but decremented strongly in mutant slices (Fig. 4-6A). To confirm this qualitative impression, we examined paired-pulse facilitation (PPF) with whole-cell voltage clamp recordings (Andreassen and Hablitz, 1994). Ascending WT projections from layer IV to II/III exhibited a prominent facilitation only at the

FIG. 4-5. Intact postsynaptic LTP mechanisms in PKA RI β - mice. A, TBS induced LTP in wild type slices through NMDA_R activation. D-APV (50-100 μ M) reversibly prevents LTP. B, Engaging the NMDA_R by postsynaptic depolarization also elicited robust LTP in mutant visual cortex when paired with synaptic stimulation, just as in the hippocampus. Thus, postsynaptic LTP induction mechanisms were not perturbed in individual pyramidal cells lacking the RI β subunit of PKA. Scale bar: 100pA, 20ms.

A.



B.



shortest inter-stimulus intervals, in agreement with a recent description of intracortical connections in the cat (Stratford et al., 1996). PPF was pronounced at all intervals tested in mutant hippocampal area CA1, as expected from previous extracellular recordings (Brandon et al., 1995). In RI β -V1, however, little or no PPF was observed, even at the shortest theta-burst inter-stimulus intervals of 10ms (Fig. 4-6B, n=8 cells from 3 mice each; p<0.01 WT vs. RI β -cortex, t-test).

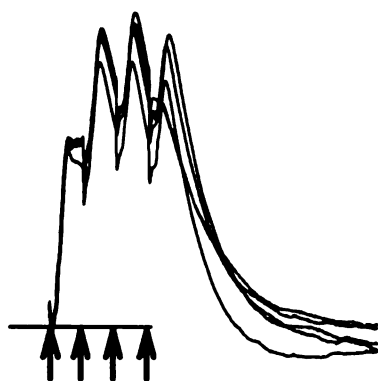
Thus, neocortical connections were more sensitive to PKA RI β gene deletion than Schaeffer collateral synapses in hippocampal area CA1. Since short-term plasticity is generally thought to be a property of neurotransmitter release machinery (Zucker, 1989), the pronounced impairment of PPF in RI β -cortex suggests an important presynaptic role for PKA in intracortical signaling. Alternatively, cortical inhibitory circuits could be compromised in RI β mutants, since the PKA pathway is also known to potentiate GABA_A receptor currents (Kano and Konnerth, 1992). We noted, however, that postsynaptic activation of PKA by norepinephrine abolished spike frequency adaptation normally in cells lacking RI β (data not shown; (Madison and Nicoll, 1982)). The disruption of rapid synaptic facilitation, by either cause, could have rendered TBS ineffective, even though intracellular postsynaptic LTP induction mechanisms remained intact in the absence of RI β .

FIG. 4-6. Synaptic facilitation defect may render TBS ineffective in RI β - mice.

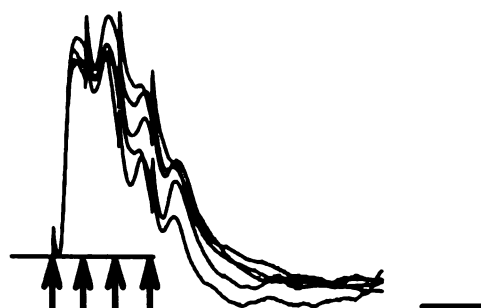
A, Whereas TBS produced a prolonged depolarization in a wild type pyramidal cell, a decrementing response was observed in the knockout. Whole-cell current clamp responses to the first bursts in five successive episodes of TBS to layer IV are shown superimposed. Arrows indicate the 4 stimulus pulses delivered at 10ms intervals. Scale bar: 5mV, 20ms. B, Paired-pulse facilitation was differentially perturbed in RI β - visual cortex and hippocampus. Ascending intracortical projections from layer IV showed a prominent facilitation only at 10ms interpulse intervals in WT supragranular pyramidal cells voltage-clamped to -70mV. Mutant V1 connections exhibited little or no facilitation, whereas PPF in RI β - CA1 was pronounced at all intervals tested (n=8 cells each; ** p<0.01, * p<0.05, t-test WT vs. RI β - cortex). Scale bar: 40pA, 20ms.

A.

Wild Type



PKA RI β ⁻



B.

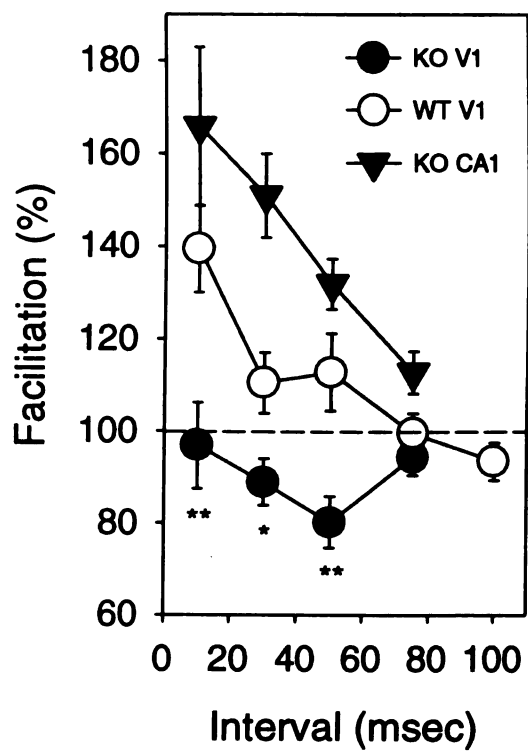
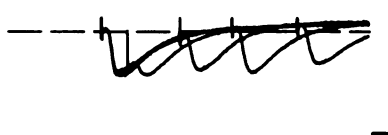
PKA RI β ⁻ Hippocampus



WT Visual Cortex



PKA RI β ⁻ Visual Cortex



Discussion

Our results indicate that the RI β regulatory subunit of protein kinase A is not required for the normal functional development and plasticity of the primary visual cortex *in vivo*, despite profound effects on synaptic plasticity in slices of V1. This does not exclude a role for PKA in ocular dominance plasticity *in vivo*. Indeed, the PKA holoenzyme is a tetramer composed of a regulatory subunit dimer, which contains the cyclic AMP binding sites, and a single catalytic subunit bound to each regulatory subunit (Spaulding, 1993). At least four regulatory (RI α , RI β , RII α , RII β) and two catalytic (C α , C β) subunits have been characterized in mice (Cadd and McKnight, 1989). Although the α subunits are ubiquitously expressed in neural and non-neural tissues, the β isoforms show a more restricted pattern of expression and are highly expressed in the nervous system. Since selective inhibitors of the PKA isoforms are not available, mice carrying deletions of other subunits or in combination should provide valuable insight into the roles of the various isoforms of PKA in experience-dependent development.

Dissociation of LTD from plasticity *in vivo* in PKA RI β - mice. The inability to weaken synapses *in vitro* in the absence of the RI β subunit is similar to the deficit first reported in the hippocampus (Brandon et al., 1995). By analogy to CA1 and the dentate gyrus, PKA may be playing a role in the postsynaptic induction mechanism of visual cortical LTD and depotentiation.

An apparent presynaptic defect in the mutant mice, as described above, may also contribute to the impairment, but is less likely, since low-frequency stimulation is used to induce LTD and would not be expected to engage the same disrupted processes as does high-frequency stimulation. Enhanced intracortical inhibition, however, potently prevents LTD (Dudek and Friedlander, 1996). Future work will need to determine the cause of the cortical signaling defect in PKA RI β mutants.

The finding that plasticity can occur *in vivo* in the absence of LTD in extracellular slice recordings is consistent with several earlier findings. We have recently shown that blockade of metabotropic glutamate receptors selectively prevents depotentiation in mouse visual cortical slices, but does not impede the loss of deprived-eye responses following monocular deprivation in kittens (Hensch and Stryker, 1996). Similarly, spatial learning *in vivo* has been dissociated from a metabotropic glutamate receptor-mediated form of LTD at hippocampal mossy fiber synapses (Yokoi et al., 1996). Indeed, the loss of LTD in the CA1 region of the same PKA RI β mice studied here, as well as mice lacking the C β catalytic subunit of PKA (Qi et al., 1996), does not correlate with their intact spatial learning abilities (Huang et al., 1995).

The NMDA receptor antagonist, APV, is reported to lead to a reverse shift in favor of the deprived eye near the infusion cannula (Bear et al., 1990). In this case, activity-dependent weakening of open eye afferents can occur in the absence of NMDA receptor-mediated processes, such as LTD induction via low-

frequency stimulation. Taken together, the results above suggest that other forms of depression *in vitro*, such as NMDA_R-independent homosynaptic LTD (Artola et al., 1990) or metabotropic glutamate receptor-independent heterosynaptic LTD as recently described in the hippocampus (Scanziani et al., 1996), may be more relevant to experience-dependent plasticity in sensory cortex.

Potentiation of visual responses and LTP in PKA RI β - mice. While the onset of the critical period for visual cortical plasticity *in vivo* is not related to the ability to generate LTP, the end of the critical period has recently been correlated with the capacity to potentiate supragranular layer field responses from the white matter (Kirkwood et al., 1995, 1996). The maturation of an inhibitory “plasticity gate” *in vitro* has been proposed to account for the end of the critical period (Kirkwood and Bear, 1994a; Kirkwood et al., 1995). By the same reasoning, one might propose that an inhibitory or hypothetical presynaptic “gate” is shut in RI β - mice; and yet monocular deprivation and reverse suture produce robust plasticity in the intact animal. We conclude that assaying extracellular field response changes by high-frequency stimulation of the white matter is a poor indicator of experience-dependent plasticity *in vivo*.

NMDA_R-dependent LTP could still play a role in neocortical plasticity, since the intracellular mechanisms to generate LTP in individual pyramidal cells are intact in RI β - mutants. A dissociation between the efficacy of tetanus and pairing protocols to induce LTP has previously been reported for the cortex (Sah and Nicoll, 1991). A pairing paradigm has recently provided direct evidence that

LTP is important for the activity-dependent formation of glutamatergic synapses in the hippocampus (Durand et al., 1996), whereas earlier attempts with tetanic stimulation suggested the erroneous view that LTP occurs in the hippocampus only at later stages of development (Battistin and Cherubini, 1994; Bekenstein and Lothman, 1991; Dudek and Bear, 1993; Dudek and Friedlander, 1996; Harris and Teyler, 1984; Jackson et al., 1993). At thalamocortical synapses of the barrel cortex, pairing low-frequency presynaptic stimulation with post-synaptic depolarization induces LTP only during the critical period for plasticity *in vivo* (Crair and Malenka, 1995).

Theta-burst stimulation apparently fails to generate cortical LTP in the absence of PKA RI β due to a defect in short-term plasticity. Paired-pulse facilitation is believed to be a purely presynaptic phenomenon, wherein lingering residual calcium produced by action potential invasion of the terminal bouton leads for a brief time to enhanced transmitter release to a closely following spike (Zucker, 1989). RI β is believed to be the regulatory subunit isoform of PKA that confers the greatest sensitivity to small changes in intracellular cyclic AMP concentration (Cadd et al., 1990), which can be tightly coupled to calcium influx.

In cortical presynaptic terminals lacking RI β , calcium-dependent facilitation mechanisms on the millisecond time-scale were disrupted (Fig. 4-6). Stronger tetani, however, were still capable of producing post-tetanic potentiation, a short-term presynaptic enhancement lasting a few minutes (Fig. 4-3C; Zucker, 1989), possibly due to the compensatory up-regulation of the RI α

subunit (Brandon et al., 1995), which is activated at 3-7 fold higher concentrations of cyclic AMP (Cadd et al., 1990). Recently, it has been shown that PKA-dependent synaptic facilitation is due to a direct modulation of the secretory machinery in hippocampal neurons (Trudeau et al., 1996).

A presynaptic locus of RI β action is consistent with the loss of LTP at the mossy fiber synapse in the CA3 region of the mutant hippocampus (Huang et al., 1995). Mossy fiber LTP has recently been shown to be a presynaptic phenomenon mediated by the cyclic AMP pathway (Huang et al., 1994; Weisskopf et al., 1994). Interestingly, decreased inflammation and pain behavior in the PKA RI β ⁻ mice are also best explained by a presynaptic reduction in neurotransmitter release from primary afferent terminals in the spinal cord and periphery (A.B. Malmberg, E.P.B., R.L.I., G.S.M., and A.I. Basbaum, in preparation). Unlike a long-lasting late stage of LTP (Huang and Kandel, 1994; Nguyen et al., 1994; Qi et al., 1996), the dependence of early phases of LTP in CA1 of the hippocampus on PKA is less clear (Blitzer et al., 1995; Weisskopf et al., 1994). We have observed a greater sensitivity to RI β gene deletion in ascending visual cortical layer IV projections than in synapses made by Schaeffer collaterals (Fig. 6). A simple extrapolation of findings from the hippocampal CA1 area to the binocular zone of visual cortex may, therefore, be misleading.

Indeed, LTP mechanisms may not be essential for developmental sensory plasticity, as indicated by recent evidence from α -calcium/calmodulin-dependent kinase II (α CaMKII) knockout mice. Barrel field reorganization is

intact (Glazewski et al., 1996) and ocular dominance plasticity is variably impaired in juvenile α CaMKII mutants (Gordon et al., 1996), while LTP induction is reported to be drastically reduced (Kirkwood et al., 1994). Similarly, recent studies have dissociated both NMDA_R-dependent LTP (Bannerman et al., 1995; Barnes, 1995; Nosten-Bertrand et al., 1996; Saucier and Cain, 1995), and presynaptic mossy fiber LTP in PKA RI β mutants (Huang et al., 1995) from hippocampal spatial learning behavior. Other mechanisms of plasticity perhaps unrelated to LTP, such as those involving neurotrophins (Thoenen, 1995), may need to be considered when trying to link plasticity *in vivo* to that studied *in vitro*.

It will be crucial to determine precisely which changes in synaptic contacts are responsible for experience-dependent plasticity during development. In kittens, geniculocortical afferents quickly and dramatically reorganize in response to MD (Antonini and Stryker, 1996). However, we are now aware that much more rapid physiological plasticity can occur prior to large-scale anatomical reorganization (cf. Chapter 2). Interestingly, physiological plasticity has been shown to be stronger in layer II/III than in layer IV, and strongest in infragranular cells (Gordon and Stryker, 1996; LeVay et al., 1980; Shatz and Stryker, 1978). A more precise investigation of the sites of plasticity in the visual thalamocortical circuit is required (Stryker, 1995). Through the combined use of a well-defined *in vitro* model and genetically-engineered mice lacking various

plasticity molecules, we can begin to approach a cellular/molecular understanding of experience-dependent plasticity *in vivo*.

PART II

Inhibitory Interactions

Chapter 5

Inhibitory Circuits Regulate Visual Cortical Plasticity

Summary

The primary inhibitory neurotransmitter in the brain, γ -amino-butyric acid (GABA), is synthesized by two isoforms of the enzyme glutamic acid decarboxylase (GAD65 and GAD67), each of which is encoded by a separate gene. To address the role of intrinsic inhibition in developmental plasticity, we examined mice lacking GAD65, which is normally highly concentrated in synaptic terminals. Neurons in mutant visual cortex failed to exhibit a bias in favor of the open eye following brief monocular deprivation at the peak of the critical period, as was observed for wild type controls recorded blind to genotype. An *in vitro* model of visual cortical plasticity - LTP and LTD - was intact in mutant slices and did not predict the plasticity defect *in vivo*. Intracerebroventricular injections of diazepam restored ocular dominance plasticity to mice lacking GAD65, suggesting a selective perturbation of the GABA_A system rather than a non-specific disruption of cortical development. Consistent with a reduction of rapid GABAergic transmission, prolonged neuronal discharges in the mutants increased their apparent cortical receptive field sizes, although gross retinotopy developed normally. We discuss these results in terms of an intracortical inhibitory regulation of visual cortical plasticity and propose experiments to test this hypothesis.

Introduction

An eye deprived of vision in early life extensively loses its ability to activate visual cortical cells. A reduction of geniculocortical arbor size can account for this weakened input into layer IV (Antonini and Stryker, 1993; Shatz and Stryker, 1978). However, an enhancement of intracortical inhibition via the non-deprived eye may underlie the further suppression of closed eye responses seen in extragranular layers (cf. Chapter 2; (Kossut and Singer, 1991)). Inactivating the open eye restores responsiveness to the deprived eye in many cortical units (Blakemore et al., 1982; Blakemore and Hawken, 1982; Kratz et al., 1976), as does the systemic (Duffy et al., 1976) or microiontophoretic (Mower and Christen, 1989) administration of the GABA_A receptor antagonist bicuculline. Moreover, the rapid physiological effects of monocular deprivation which precede large-scale anatomical rearrangement could be entirely mediated by functional changes in the GABA system (Skangiel-Kramska and Kossut, 1984).

Intracortical inhibitory interactions in normal animals appear to enhance the contrast between adjacent ocular dominance columns (Sillito et al., 1980). Given that the numerical density of GABAergic synapses in visual cortex is not altered by monocular deprivation (Bear et al., 1985), greatly expanded open eye columns may passively suppress the output from the remaining small islands of deprived eye afferents to layer IV (Sillito et al., 1981). A more active role for intracortical inhibition is suggested by reversal of the ocular dominance shift in

favor of the deprived eye with chronic infusions of the GABA_A receptor agonist muscimol (Hata and Stryker, 1994; Reiter and Stryker, 1988), or the prevention of plasticity by bicuculline infusion concurrent with monocular deprivation (Ramoia et al., 1988). Unfortunately, crude pharmacological manipulations either strongly shut down or hyperexcite the cortex and yield little insight into the role of endogenous inhibitory circuitry in visual cortical plasticity. We, therefore, adopted a genetic approach to address this issue.

The major inhibitory neurotransmitter in the brain, GABA, is synthesized by two molecular forms of glutamic acid decarboxylase (GAD), which are encoded by distinct genes (Erlander and Tobin, 1991). The 65 kDa isoform (GAD65) differs from the larger GAD67 (67 kDa) in several respects. In particular, GAD65 is localized to synaptic terminals (Kaufman et al., 1991) and constitutes the majority of GAD apoenzyme, serving as a reservoir of inactive GAD to be activated by the cofactor pyridoxal phosphate when additional GABA synthesis is required (Martin et al., 1991). Moreover, GAD65 mRNA levels are increased during periods of active synaptogenesis (Greif et al., 1991), and predominate in the visual system (Feldblum et al., 1993). By contrast, GAD67 is localized to somata and dendrites (Kaufman et al., 1991) and may provide a constitutive level of GABA throughout the cell by a transporter release mechanism in tonically active neurons (Feldblum et al., 1993).

We have, therefore, examined visual cortical plasticity in mice carrying a targeted disruption of the GAD65 gene, based on the hypothesis that GAD65

may be specialized to respond to rapid changes in demand for vesicular GABA release from synapses during alterations in neuronal activity.

Materials and Methods

Insertion of a neomycin-resistance cassette in exon one of the GAD65 gene created a nonsense mutation and functionally disrupted the protein in mice as described (Kash et al., 1996). Homozygous mutant animals were born at a ratio that did not suggest embryonic lethality. Single-unit recordings of visual responses were made *in vivo* using standard techniques (cf. Chapter 4; (Gordon and Stryker, 1996)). In brief, mice were anesthetized with Nembutal (50 mg/kg) and chlorprothixene (0.2 mg) and placed in a stereotaxic holder. The animal breathed a mixture of oxygen and room air through a trachea tube, and additional anesthetic doses (0.15 to 0.25 mg) were administered to maintain a stable heart rate (6-9 Hz). Receptive fields were plotted and optic disk locations projected onto a tangent screen placed 30 cm from the animal to determine the vertical meridian. Retinotopy was mapped in mature mice (>1.5 mos. old) by multiple electrode penetrations spaced at 200 μ m intervals along the medio-lateral axis through both the monocular and binocular zones of primary visual cortex (V1). Discharge profiles were acquired by a computer-based visual

stimulation system generating high contrast bars sweeping through the receptive field.

For monocular deprivation experiments, eyelid margins were trimmed and lids sutured under brief halothane anesthesia at the peak of the critical period (for 4 days beginning P25-27). All recordings were made blind to the genotype of the animal from the binocular zone contralateral to the deprived eye. We defined the binocular zone as the region of V1 representing the central 25° of the upper portion of each visual hemifield. In "rescue" experiments, either diazepam (3.5mM) or vehicle (20% propylene glycol) solution was injected intracerebroventricularly (1.5µl per side i.c.v.) twice daily beginning one day prior to and concurrent with four days of monocular deprivation.

Cells were assigned ocular dominance scores according to the 7-point classification scheme of Hubel and Wiesel (1962), where a score of 1 indicates response to contralateral eye stimulation exclusively, and a score of 7 purely ipsilateral eye response. Intermediate scores (2-6) reflect a degree of binocular responsiveness. A weighted average of the bias toward one eye or the other, the contralateral bias index (CBI), was calculated for each hemisphere according to the formula: $CBI = [(n_1 - n_7) + (2/3)(n_2 - n_6) + (1/3)(n_3 - n_5) + N]/2N$, where N = total number of cells, and n_x = number of cells with ocular dominance scores equal to x.

Coronal visual cortical slices were prepared and maintained as described previously (cf. Chapters 3 and 4, (Hensch and Stryker, 1996)). Long-term

potentiation (LTP) of supragranular field responses was monitored in response to theta-burst stimulation (TBS) of layer IV, followed by a depotentiation (LTD) protocol of low-frequency stimulation (cf. Chapter 4; (Kirkwood et al., 1993)). For GAD-6 immunostaining, mice were transcardially perfused with saline then 4% paraformaldehyde in 0.1M phosphate buffer. Brains were fixed in agar (5%) blocks and cut at 50 μ m on a vibratome. Sections were incubated at 4°C for 48 hours in mouse GAD-6 primary antibody (Developmental Studies Hybridoma Bank) and visualized with Cy3-donkey-anti-mouse IgG secondary (Jackson) on an epifluorescence microscope (Nikon).

Results

Disruption of the GAD65 gene eliminated the enzyme as detected by GAD-6 immunostaining (Fig. 5-1). Age and genetic background-matched wild type animals displayed a typical punctate pattern of staining, with higher intensity labeling in neocortical layer IV and str. pyramidale and str. lacunosum moleculare of the hippocampus. The latter regions in particular are rich in inhibitory connections, consistent with earlier studies localizing this isoform to

Fig. 5-1. Targeted disruption of the GAD65 gene. A, Wild type mice highly expressed GAD65 protein in areas of concentrated inhibitory synaptic termination, such as the stratum pyramidale and stratum lacunosum moleculare of the CA1 region of hippocampus. In the binocular zone of visual cortex, labeling was seen throughout all layers with a slightly higher intensity in layer IV. B, The mutant mice had no GAD65 protein as detected with the GAD-6 antibody.

GAD-6 Immunostaining



Wild Type

I
II/III
IV
V
VI
white matter
str. pyramidale
str. radiatum
str. lac. mol.



GAD-65 KO

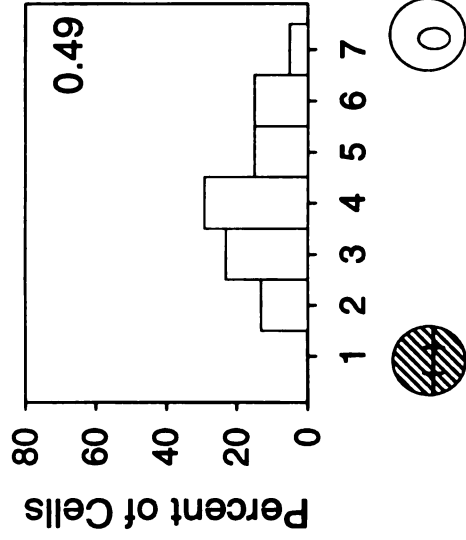
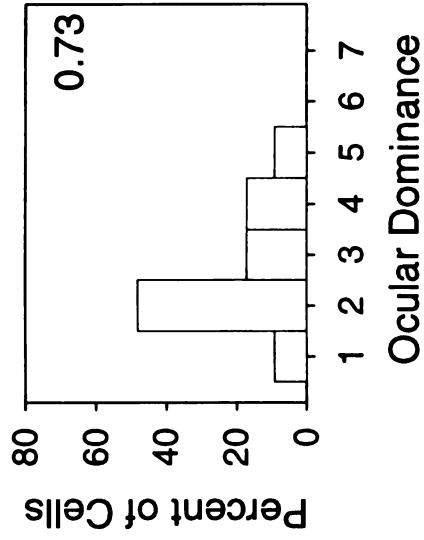
the synaptic terminal compartment (Esclapez et al., 1994; Kaufman et al., 1991; Vardi and Auerbach, 1995). Despite the absence of GAD65, animals remained viable, presumably because the GAD67 gene remained intact.

A brief (4 day) monocular lid suture paradigm was used to examine visual cortical plasticity at the peak of the critical period (P25-32). Wild type mice exhibited a robust shift in ocular dominance toward the open eye (Fig. 5-2B), as characterized previously (Gordon and Stryker, 1996). Mice lacking GAD65, however, showed no change in eye preference despite the deprivation, responding better to contralateral eye input as typically seen for animals raised with normal visual experience (Fig. 5-2A). An inability to express plasticity could, however, be a simple artifact of inappropriate cortical circuit development, although gross morphology and cortical lamination appeared to be normal in the mutants (data not shown). We, therefore, carried out several physiological experiments to determine whether primary visual cortex could sustain refinements of connectivity in the chronic absence of GAD65.

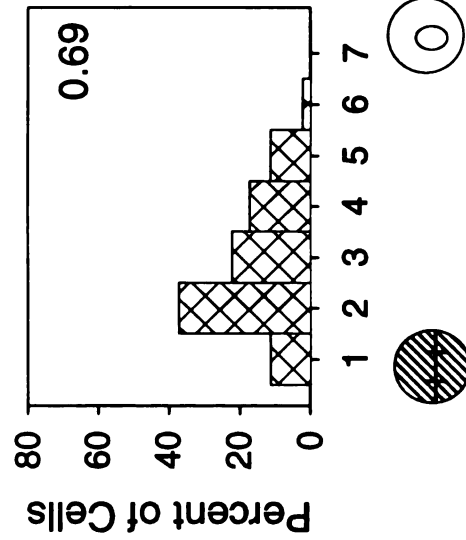
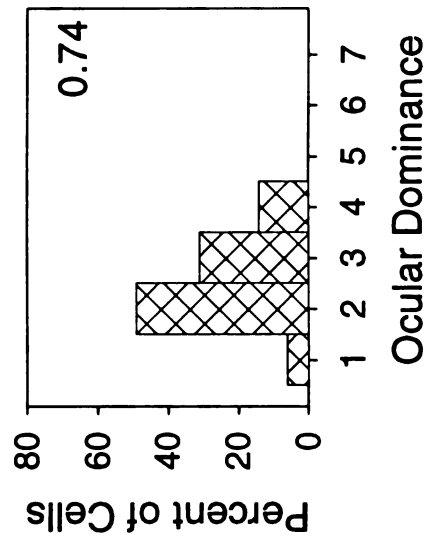
Mutant cortex developed a normal retinotopic organization. Receptive field locations moved linearly from peripheral to central visual regions with successive penetrations along the medio-lateral axis of V1, exhibiting high correlation coefficients for both wild type and GAD65 mutant cortices (data not shown). The onset of visually-driven responses was robust and easily identified in mutants, but precise receptive field dimensions were often difficult to

Fig. 5-2. Regulation of ocular dominance plasticity by GAD65. A, Despite four days of monocular lid suture, the ocular dominance distribution of cells (n=131 cells, N=6 mice) in mutant visual cortex was no different from non-deprived adults (p=0.8, t-test). B, In recordings made blind to genotype, wild type mice exhibited a typical shift of responses in favor of the open eye (n=132 cells, N=6 mice). The CBI of deprived animals was significantly different from that of normals (p<0.01, t-test).

Wild Type



GAD65 KO

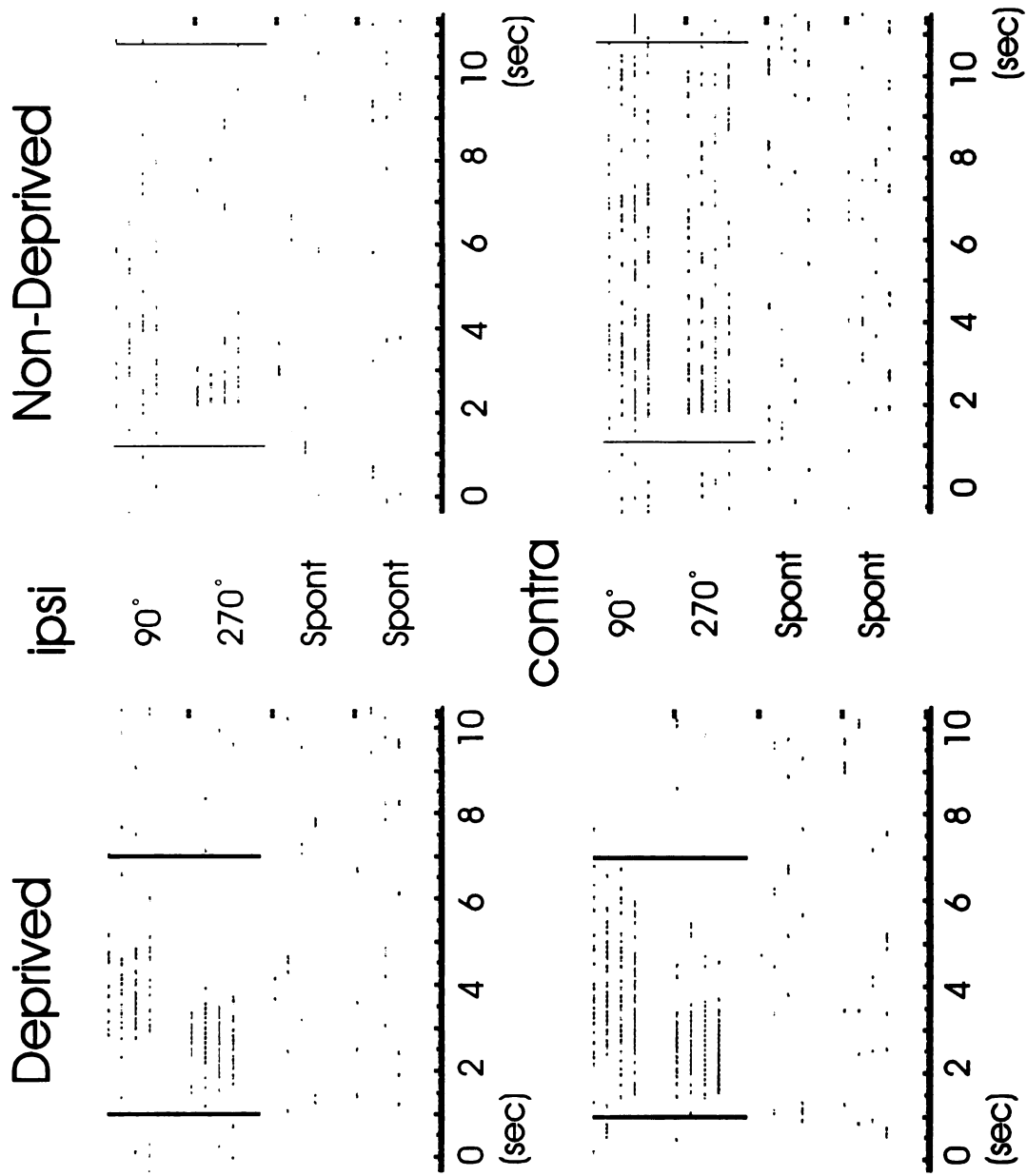


determine due to persistent spike firing as stimuli exited the presumptive field (Fig. 5-3). Prolonged discharges were more pronounced through the contralateral eye and observed in approximately 90% of all mutant cortical neurons recorded, regardless of deprivation status. Unlike evoked responses, however, levels of spontaneous activity were low and indistinguishable from wild type, suggesting that the physiological effects of GAD65 deletion only became apparent when the network was visually activated.

Synaptic transmission was further examined in slice preparations of visual cortex. The ability to generate Long-Term Potentiation (LTP: Kirkwood et al, 1995, 1996) or Long-Term Depression (LTD: Dudek and Friedlander, 1996) *in vitro* has been correlated with the critical period for ocular dominance plasticity *in vivo*. Mutant supragranular synaptic responses did not differ from wild type slices in size, shape, or threshold stimulation intensity. Moreover, robust LTP and LTD of extracellular field potentials could be expressed following theta-burst and low-frequency stimulation, respectively, in the visual cortex of mice lacking GAD65 (Fig. 5-4A). Thus, intracortical circuits were functional and malleable in mutant animals, however, these *in vitro* forms of plasticity failed to predict the deficits observed *in vivo*.

Given that certain synaptic modifications were supported with weakened inhibitory connections, we attempted to “rescue” ocular dominance plasticity by enhancing the remaining GABAergic synaptic transmission *in vivo*.

Fig. 5-3. Prolonged discharge to visual stimulation in mice lacking GAD65. Receptive fields (delimited by vertical lines) appeared large in mutants due to a prolonged discharge. Although an abrupt onset of spiking signalled the leading edge, firing often continued as the light bar stimulus passed through the presumptive receptive field. Well-delineated, smaller receptive field borders were more apparent through the ipsilateral eye regardless of whether the contralateral eye had been deprived (left) or not (right).



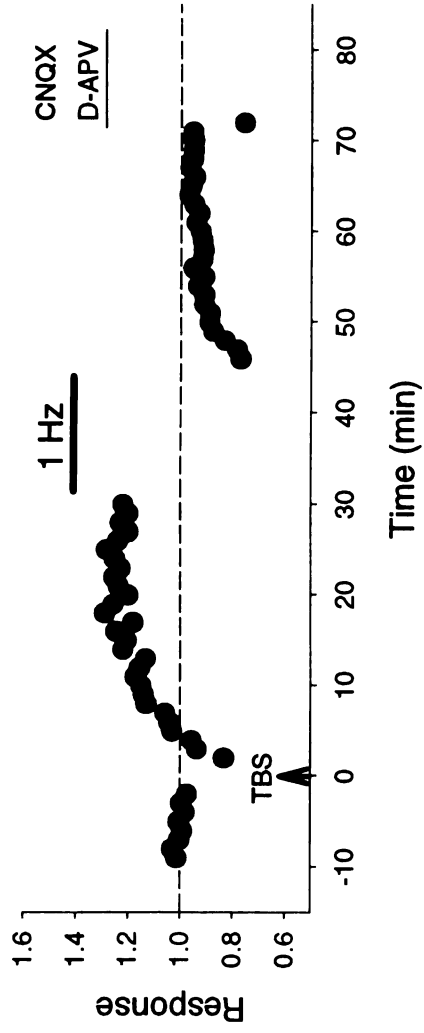
Administration of the benzodiazepine agonist diazepam (i.c.v.) restored the bias to GAD65 mutants in favor of the open eye following a concurrent brief monocular deprivation (Fig. 5-4B). Vehicle-treated mutants did not shift, as expected (compare Fig. 5-4A with 5-2A). Thus, the mechanisms for basic visual cortical plasticity remained intact in the absence of GAD65, however, their induction was prevented by selective impairment of intrinsic GABA_A receptor-mediated inhibitory circuits. Consistent with this view, diazepam treatment reduced the number of cortical units exhibiting prolonged discharges to about 60%.

Discussion

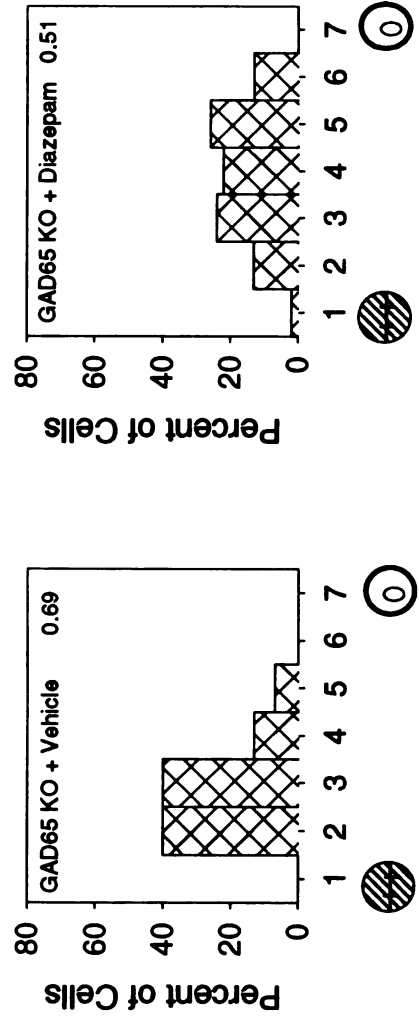
Based on its localization to synaptic terminals (Kaufman et al., 1991), high expression in phasically active neurons (Feldblum et al., 1993), presence as the major component of apoenzyme and activation by pyridoxal phosphate (Martin et al., 1991), GAD65 appears to play a pivotal role in rapid GABAergic transmission in the brain. Since visual stimulation releases copious amounts of GABA in the visual cortex (Shirokawa and Ogawa, 1992), it seems likely that intrinsic inhibitory circuits might regulate the activity-dependent plasticity process during the developmental critical period. We have now identified mice

Fig. 5-4. Visual cortex can sustain plasticity in the absence of GAD65. A, Theta-burst stimulation of layer IV robustly potentiated mutant supragranular field responses, which could be depotentiated by low-frequency stimulation, as shown in this example. Reduced intracortical inhibition did not affect these *in vitro* models of plasticity, suggesting that the chronic absence of GAD65 throughout development did not alter basic properties of intracortical connectivity. B, Enhancing the postsynaptic impact of reduced GABA release was able to restore ocular dominance plasticity to the mutants. Intraventricular administration of diazepam concurrent with a brief monocular deprivation at the peak of the critical period induced plasticity *in vivo* which was significantly different from unshifted vehicle-treated homozygous littermates (n=61 cells, N=3 mice; $p < 0.01$, t-test).

GAD65 KO exhibits robust LTP and LTD *in vitro*



Diazepam rescues GAD65 KO plasticity defect *in vivo*



lacking GAD65 as the first murine model which consistently fails to exhibit visual cortical plasticity in response to brief monocular deprivation.

Gene targeting eliminates the protein from the entire animal, yet we interpret our findings predominantly as a consequence of reduced intracortical inhibition. The effects of monocular deprivation are the result of a competitive interaction between left and right eye inputs which first converge at the level of the primary visual cortex. Although GAD65 expression is particularly high in the lateral geniculate nucleus, earlier work in cats and monkeys demonstrates that ocular dominance plasticity takes place as a change within cortex itself rather than at earlier stages along the visual pathway (Wiesel and Hubel, 1963).

We, therefore, propose a regulatory role for intrinsic intracortical inhibition during experience-dependent plasticity (Fig. 5-5A). In this scheme, strong visual activation of non-deprived afferents is further amplified by excitatory interactions within the cortex (Douglas et al., 1995; Douglas and Martin, 1991). Intracortical inhibition keeps cortical activity in check, improving the correlation between open eye inputs and their postsynaptic targets. Moreover, inhibitory circuits may further suppress the already weakened, uncorrelated input from the deprived eye. Hebbian processes would then normally shift cortical responses away from the closed eye.

In the absence of GAD65, however, coincidence detection mechanisms fail to discriminate between the two competing inputs (Fig. 5-5B). Reduced inhibitory synaptic transmission elevates both deprived and non-deprived eye

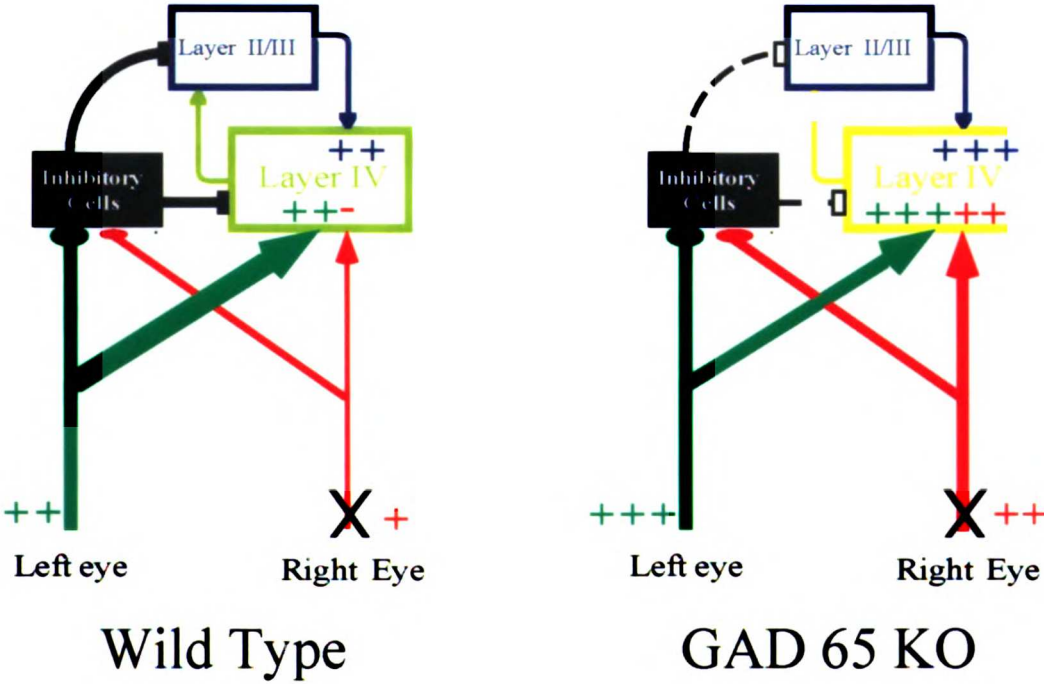
activity in the retina and thalamus, but most importantly permits unrestrained excitatory feedback within the cortex. Under such conditions, there is an unusually high probability of coincident activity between deprived afferents and cortical target cells. Thus, closed eye inputs are not lost and plasticity is not observed *in vivo*.

By contrast, reducing inhibition greatly enhances the ability to induce both LTP (Artola and Singer, 1987; Bear and Kirkwood, 1993; Bear et al., 1992; Kirkwood and Bear, 1994a) and LTD (Artola et al., 1990; Dudek and Friedlander, 1996) *in vitro*. We also found robust LTP and LTD in slices of visual cortex from GAD65 mutants. As we demonstrated in earlier studies disrupting metabotropic glutamate receptors (cf. Chapter 3) or the R1 β isoform of PKA (cf. Chapter 4), LTP and LTD of extracellular field potentials mislead us about the rules of ocular dominance plasticity in the intact brain, despite previous claims to the contrary (Kirkwood et al., 1995; Kirkwood et al., 1996).

A reduction of GABAergic synaptic transmission at cortical loci in GAD65 mutants is supported by two physiological findings. The enlargement of receptive field size in GAD65 mutants agrees with bicuculline iontophoresis studies which reported similar contributions of intracortical inhibitory mechanisms to the receptive field properties of the cat (Sillito, 1975). Previous work has shown that GAD65 mutants have a reduced seizure threshold in response to GABA_A receptor antagonists and leads to the premature spontaneous death of some animals (ca. 13%, <3 weeks postnatal;

Fig. 5-5. Hypothetical regulation of developmental plasticity by intrinsic inhibition. A, Intracortical interactions are represented as a balance between an inhibitory pool of neurons which normally restricts the positive feedback of an excitatory population (Douglas et al., 1995). Depriving one eye of vision produces an imbalance in afferent activity impinging on both pools of cortical cells. Interneurons in wild type animals gate recurrent excitation to match postsynaptic responses to open eye input levels, leading to their stabilization. Intracortical inhibition driven by the open eye may further reduce already poorly correlated deprived-eye responses, promoting synapse elimination. B, In the absence of GAD65, rapid GABA release is compromised during visual stimulation. A consistent phenotype is the prolonged spike discharge of mutant cells as light bar stimuli leave their presumptive receptive fields. Relatively uncontrolled recurrent excitation amplifies both deprived and non-deprived eye responses, increasing the likelihood that both inputs will coincide with postsynaptic activity. Hence, no competitive interaction occurs and all afferents are preserved despite the deprivation.

Regulation of Plasticity by Intrinsic Inhibitory Circuits



(Kash et al., 1996)). We note, however, that an epileptic pathology in those animals surviving to the peak of the critical period is unlikely to explain the disruption of ocular dominance plasticity. First, visual pathways had developed sufficiently well to express plasticity *in vitro*, as well as *in vivo* following restoration of inhibitory balance with benzodiazepine agonist treatment. Second, previous work has shown that the presence of seizure-like electrical activity *per se* does not disrupt ocular dominance plasticity (Videen et al., 1986).

Future experiments will test the model of plasticity regulation by intrinsic intracortical inhibition. If competitive mechanisms are indeed only reduced in the absence of GAD65, then perhaps much longer deprivations will distinguish even slight differences in afferent drive. Although intraventricular injections of the benzodiazepine agonist diazepam restored plasticity, similar results following local infusion directly into the binocular zone would greatly strengthen the specific involvement of the cortical GABA_A system. Direct demonstration of compromised rapid GABA release - by monitoring the rate of IPSC depression to trains of stimuli in cortical slices - is also crucial to our interpretation. In sum, such evidence would validate the use of GAD65 mice as a model system for exploring molecular candidates which might mechanistically underlie activity-dependent plasticity in the developing visual cortical.

Chapter 6

Intracortical Inhibition Regulates the Segregation of Ocular Dominance Columns

Summary

During development, geniculocortical afferents serving the two eyes segregate into discrete ocular dominance columns through an activity-dependent competition. In models of this Hebbian self-organizing process, intracortical interactions determine the final width of ocular dominance domains up to a possible limit set by arbor diameters. We examined directly the role of cortical circuitry in column formation by locally modulating postsynaptic inhibition during the segregation process. Chronic infusion of the benzodiazepine agonist diazepam did not affect visual responses *in vivo* severely, despite enhancing GABA_A-mediated synaptic currents *in vitro*. Surrounding and anterior to the diazepam infusion site, the spacing of proline-labelled columns in flattened visual cortex was found to be wider than in distant areas behind the cannula or in control hemispheres. The benzodiazepine inverse agonist DMCM produced the opposite effect. These results are consistent with a role for intracortical interactions in shaping the final layout of presynaptic afferent arbors to primary visual cortex, and provide strong evidence in favor of the activity-dependent development of cortical columnar architecture.

Introduction

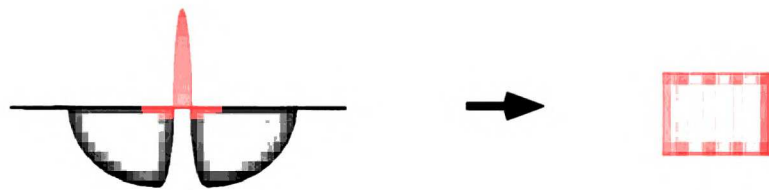
Manipulations of early visual experience have shown that the segregation of eye-specific columns is a competitive process between the two eyes' afferents to primary visual cortex (Wiesel, 1982). Patterns of input activity are detected by the target cell, which strengthens only those connections whose impulses coincide with its own (Hebb, 1949). Uncorrelated inputs are weakened (Stent, 1973). Gross disruptions of activity lend strong support to a Hebbian mechanism of refinement at the level of individual synapses (cf. Chapter 1). Yet, what determines the final dimensions of whole columns of input?

Computational models of activity-dependent development based on Hebbian rules may provide a clue (Miller et al., 1989; Miller and Stryker, 1990). In these schemes, local excitatory connections within cortex spread incoming afferent activity over a certain radius, which is ultimately limited by farther-reaching inhibition. Modulating the relative balance of excitation and inhibition alters the shape of this "mexican hat" interaction function, which is loosely based on experimental observations (cf. Chapter 1, (Berman and Martin, 1990; Peters and Yilmaz, 1993; White, 1989)). Hebbian mechanisms acting on these narrowed or widened central excitatory regions throughout development ultimately yield thin or fat columns, respectively (Fig. 6-1; (Miller and Stryker, 1990)).

Fig. 6-1. Intracortical interactions predict column spacing. In computational models, functional interactions within cortical circuits are often simulated as a central excitatory region (red) surrounded by more lateral inhibition (blue) - a 'mexican hat' function (Miller and Stryker, 1990). Levels of intracortical inhibition determine the spread of afferent activity through the cortical network. Hebbian rules of synaptic competition acting on these excitatory peaks during development define the final column periodicity in these models. Benzodiazepine modulators of GABA_A-mediated inhibition may allow experimental tests of this prediction *in vivo*.

Theoretical Predictions

Intracortical Interaction Function Columns



↑ **BDZ agonist ?**
(e.g. Diazepam)



↓ **BDZ inverse agonist ?**
(e.g. DMCM)



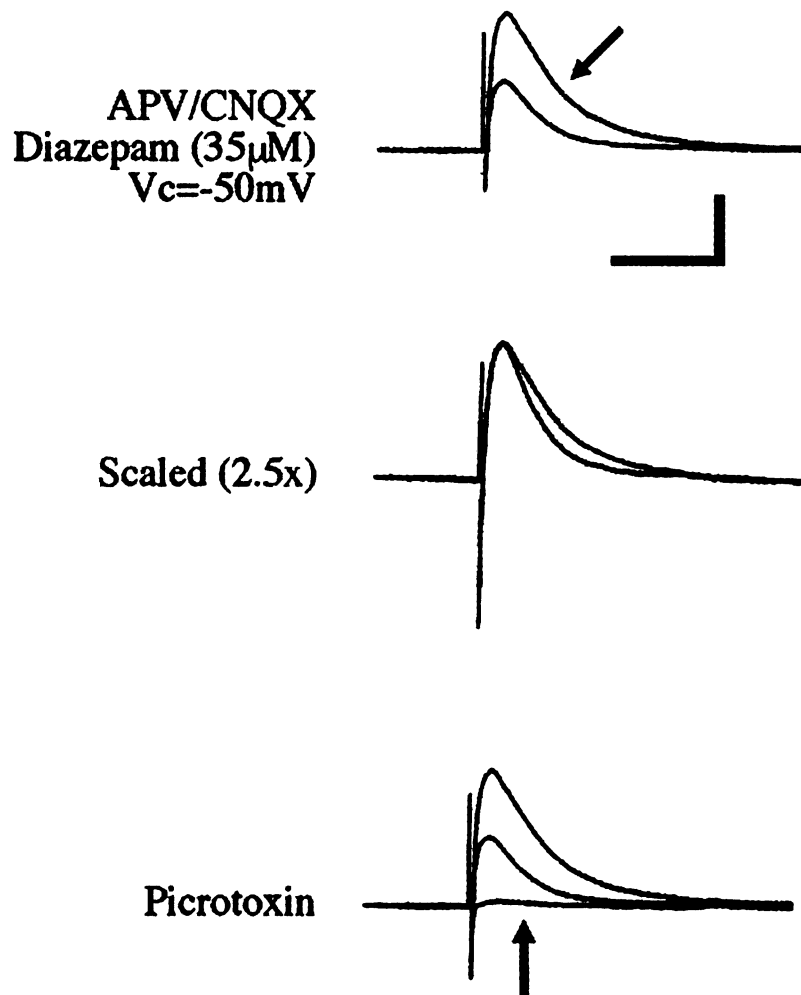
(cf. Miller and Stryker, 1990)

We sought to experimentally test the prediction that activation of intracortical circuits during the segregation process determines the final size of ocular dominance columns. Visual stimulation is known to release prominent amounts of the inhibitory neurotransmitter γ -amino-butyric acid (GABA) (Shirokawa and Ogawa, 1992). We, therefore, attempted to modulate the spread of afferent excitation by altering inhibitory interactions intrinsic to cortex. Since gross perturbations of activity interfere directly with coincidence detection mechanisms and disrupt column formation (cf. chapter 1), we focused on a subtle means by which to enhance intracortical inhibition without silencing the cortex completely.

Benzodiazepines are allosteric modulators of the GABA_A receptor, binding at the γ 2 subunit (Lueddens et al., 1995; Pritchett et al., 1989; Tallman and Galager, 1985). Benzodiazepine agonists, such as diazepam, reversibly potentiate the chloride flux through the GABA_A receptor ionopore (Fig. 6-2; (Haefely and Polc, 1986)); whereas, inverse agonists, such as β -carboline derivatives, decrease GABA_A currents (Capogna et al., 1994). Benzodiazepine binding sites are highest in the input layer IV of cats at all ages but are not altered by surgical undercutting of afferents (Shaw et al., 1987). This indicates that the receptors are associated with intrinsic cortical elements rather than thalamocortical axons or other subcortical inputs. Moreover, since benzodiazepines are inert in the absence of released GABA, they seemed like the

Fig. 6-2. Benzodiazepines specifically modulate postsynaptic GABA_A-mediated synaptic transmission in the visual cortex. IPSCs were evoked in supragranular pyramidal cells of mouse visual cortical slices by electrical stimulation in the presence of ionotropic glutamate receptor antagonists. The benzodiazepine agonist diazepam (35 μ M) approximately doubled GABA_A currents (top, arrow; middle, scaled trace). Picrotoxin (100mM) was bath applied to confirm the GABA receptor component of the IPSC.

Diazepam Enhances GABA_A Synaptic Currents



ideal tool for examining the role of intracortical interactions in the segregation of ocular dominance columns.

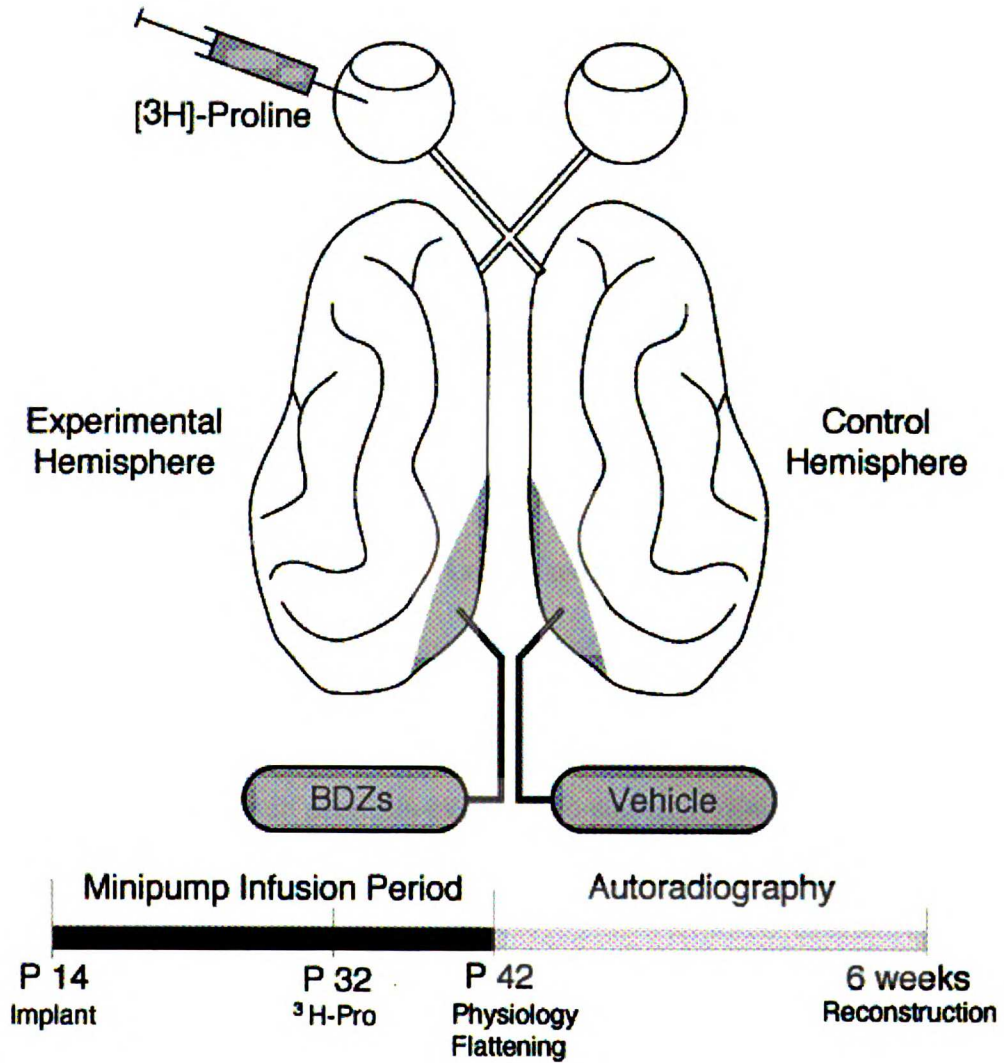
Materials and Methods

Benzodiazepine actions on GABAergic inhibitory postsynaptic currents (IPSCs) were first confirmed in slices of mouse visual cortex (adult C57Bl/6), prepared and maintained as described previously (cf. Chapters 3 and 4; (Hensch and Stryker, 1996)). IPSCs were evoked in supragranular pyramidal cells of mouse visual cortex by electrical stimulation of layer IV in the presence of ionotropic glutamate receptor antagonists (10 μ M CNQX and 50 μ M D-APV). Picrotoxin (100 μ M) was bath applied to confirm the GABA receptor component of the response (Fig. 6-2). Whole-cell patch pipette solutions contained (in mM): 122.5 KGluconate, 17.5 KCl, 10 HEPES buffer, 0.2 EGTA, 8 NaCl, 2.0 Mg-ATP, 0.3 Na₃-GTP (3-8M Ω , pH 7.2, 290-300mOsm).

Prior to the onset of ocular dominance column segregation (P14-17; (LeVay et al., 1978)), cannulae (31ga.) connected to osmotic minipumps (Alzet 2ML4 or paraffin-coated Alzet 2002, Alza) were implanted under sterile conditions into one hemisphere of kitten primary visual cortex (Area 17; Fig. 6-3). Pumps were previously filled with either a benzodiazepine agonist (3.5mM

Fig. 6-3. Protocol for local modulation of inhibitory circuits *in vivo*. Benzodiazepine agonists (Diazepam, 3.5 or 35mM), inverse agonists (DMCM, 50mM), or vehicle (propylene glycol, 20%) solutions were infused locally from osmotic minipumps (0.2 or 2.5 μ l/hr) into kitten striate cortex for 4 weeks beginning before ocular dominance columns normally segregate (P14). ³H-proline (2mCi) was injected into one eye to label columns 10 days prior to terminal electrophysiological recordings. Columns were revealed by autoradiographic techniques and measured in tangential sections through layer IV of unfolded and flattened visual cortex (Olavarria and Van Sluyters, 1985).

Methods

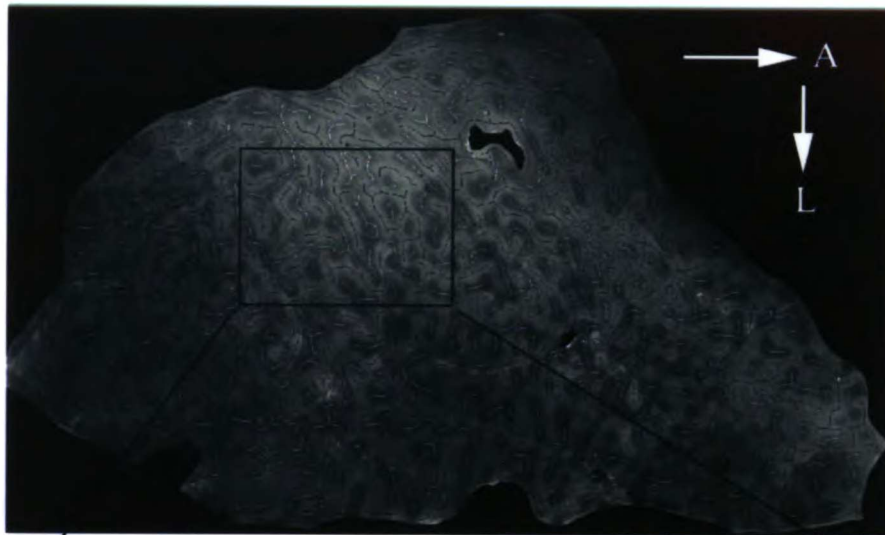


diazepam), inverse agonist (50 μ M DMCM), or vehicle (20% propylene glycol) solution. After four weeks of normal visual experience and local infusion, animals were prepared for single-unit recordings, and in some cases optical imaging of intrinsic signals, using standard techniques (cf. Chapter 2; (Bonhoeffer and Grinvald, 1993; Reiter et al., 1986)). Single-unit responses isolated with a window discriminator were assayed with a computer-based visual stimulation and data acquisition system pseudo-randomly presenting a moving high-contrast bar at 16 different orientations (or no stimulus for measurements of spontaneous activity).

Animals were perfused transcardially with saline and then with 4% paraformaldehyde in 0.1M phosphate buffer. The caudal part of the cortex, which includes the primary visual cortex, was unfolded and flattened between two glass slides and cut on a Leica freezing microtome (Olavarria and Van Sluyters, 1985). [3 H]-proline injections (2mCi in 20 μ l saline) into one eye 10 days prior to terminal physiology experiments revealed the pattern of ocular dominance columns in sections exposed to photoemulsion (Kodak) in the dark (6 weeks at 4 $^{\circ}$ C) (Anderson et al., 1988; LeVay et al., 1978). Photomontages of labeling in layer IV were made from several 40 μ m tangential sections using Photoshop 3.0 software (Adobe).

To quantify column spacing, images were further processed using two computer algorithms written in IDL (Research Systems). First, both labeled and unlabeled columns were reduced to their centers by a medial axis transform (Fig.

Fig. 6-4. Quantification of column spacing. Columns were first reduced to their centers using a medial-axis transform (top panel; magnified view, lower panel). A computer program (written in IDL) then measured the distance to the nearest column center representing each eye for every pixel in the image. A histogram of distances was automatically generated, and the mean values for particular regions of interest were calculated. Proline-labeled columns are dark in this negative image.



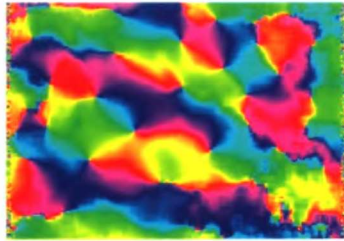
6-4). Second, the radial distance to the nearest column center was determined for each pixel in the skeletonized image. The nearest opposite eye column center was then located by searching in a user-specified range of angles emanating away from the initial column (cf. appendix). Column separation for each point was taken as the sum of the two distances, generating a distance map over the entire montage. To compare measurements with respect to the infusion site, mean spacing was determined for specific regions of interest outlined in template files.

Results

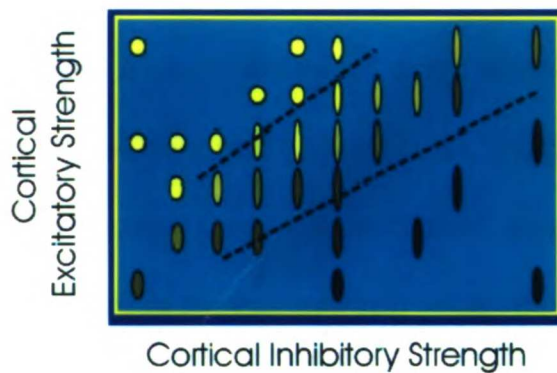
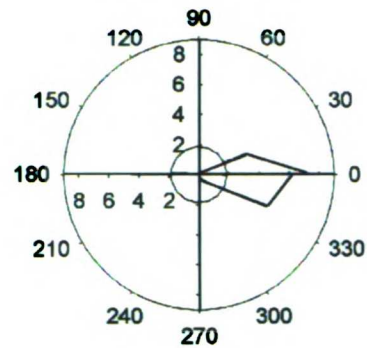
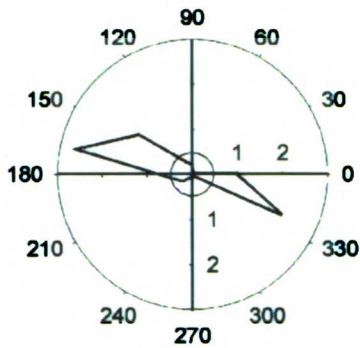
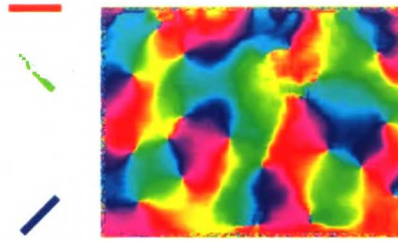
The benzodiazepine agonist diazepam enhanced fast GABA_A receptor-mediated IPSCs evoked in the supragranular layers of mouse visual cortical slices, as expected (Fig. 6-2; (Haefely and Polc, 1986; Rovira and Ben-Ari, 1993)). Since the effects were preserved even after four weeks at body temperature (37°C), we proceeded with *in vivo* infusion experiments. Remarkably, neither acute (data not shown) nor chronic administration of diazepam severely affected cortical processing (Fig. 6-5). Optical imaging of intrinsic signals revealed robust orientation maps, while single-units remained well-tuned for orientation.

Fig. 6-5. Physiological properties of cortical neurons under chronic benzodiazepine treatment. Top, Chronic benzodiazepine treatment did not disrupt the physiological properties of visual cortex. Diazepam-treated hemispheres exhibited robust orientation maps. Middle, Single units were well-tuned for orientation. However, overall response strength and spontaneous activity were lower than in control hemispheres recorded simultaneously. Response strength is indicated in arbitrary units of spikes/bin on the X and Y axes, while the inner circle represents the magnitude of spontaneous activity. Note the scale difference in the example shown. Bottom, These effects were consistent with enhanced intracortical inhibition in recent models of cortical circuitry (Somers et al, 1995). Visual cortical neurons normally operate in a regime of balanced excitation and inhibition in such models (dashed lines).

Diazepam



Vehicle



Orientation Tuning:



Response Strength:

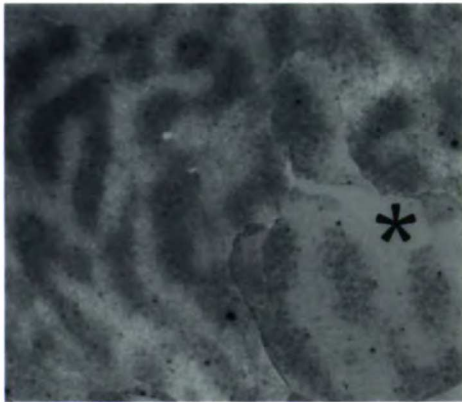


High Low
(adapted from Somers et al, 1995)

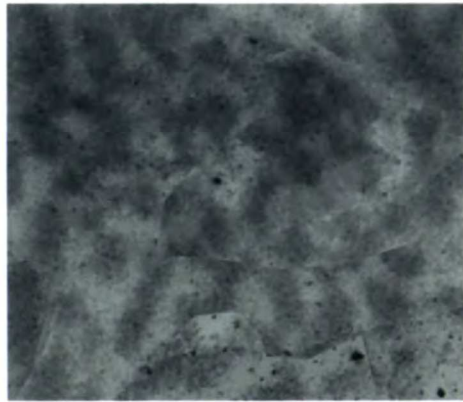
Both response strength and spontaneous activity were, however, consistently lower in the diazepam-treated hemispheres. This is best seen in simultaneous recordings of single units from drug and vehicle hemispheres to control for state-dependent effects of anesthesia (Fig. 6-5, middle). The preservation of orientation selectivity with reduced responsiveness is typical of enhanced inhibition in recent computational models of cortical processing (Fig. 6-5, bottom; Somers et al., 1995). Such models assume that cortical neurons normally operate in a regime of balanced excitation and inhibition. Our results support the notion that the visual cortex can rapidly recalibrate itself following alterations in neuronal activity.

With some assurance that intracortical inhibition was enhanced by diazepam *in vivo*, we examined the overall layout of [³H]proline-labeled ocular dominance columns in flattened visual cortex. Qualitatively, columns near the diazepam infusion site (3.5mM; 2.5 μ l/hr) appeared crisper and wider than in regions farther away behind the cannula (Fig. 6-6). Such a pattern would be expected from a point source of drug diffusing anterior to the cannula bevel. Pumping at a slower rate (0.2 μ l/hr) and higher dose (35mM) to create a wider radius of effective drug concentration produced a region of unsegregated columns in front of the cannula and wide columns behind (Fig. 6-7). Since direct activation of GABA_A receptors with muscimol desegregates columns (Hata and Stryker, 1994), we interpret our diazepam results as a continuum of GABA_A enhancing effects approaching saturation.

Fig. 6-6. Ocular dominance columns following chronic diazepam treatment. Local enhancement of GABA_A-mediated inhibition during development widened columns just anterior to the infusion site, as compared with areas farther away behind the cannula (lower panel). Scale bar, 5mm (top); 1mm (bottom). Proline-labeled columns are dark in this negative image.

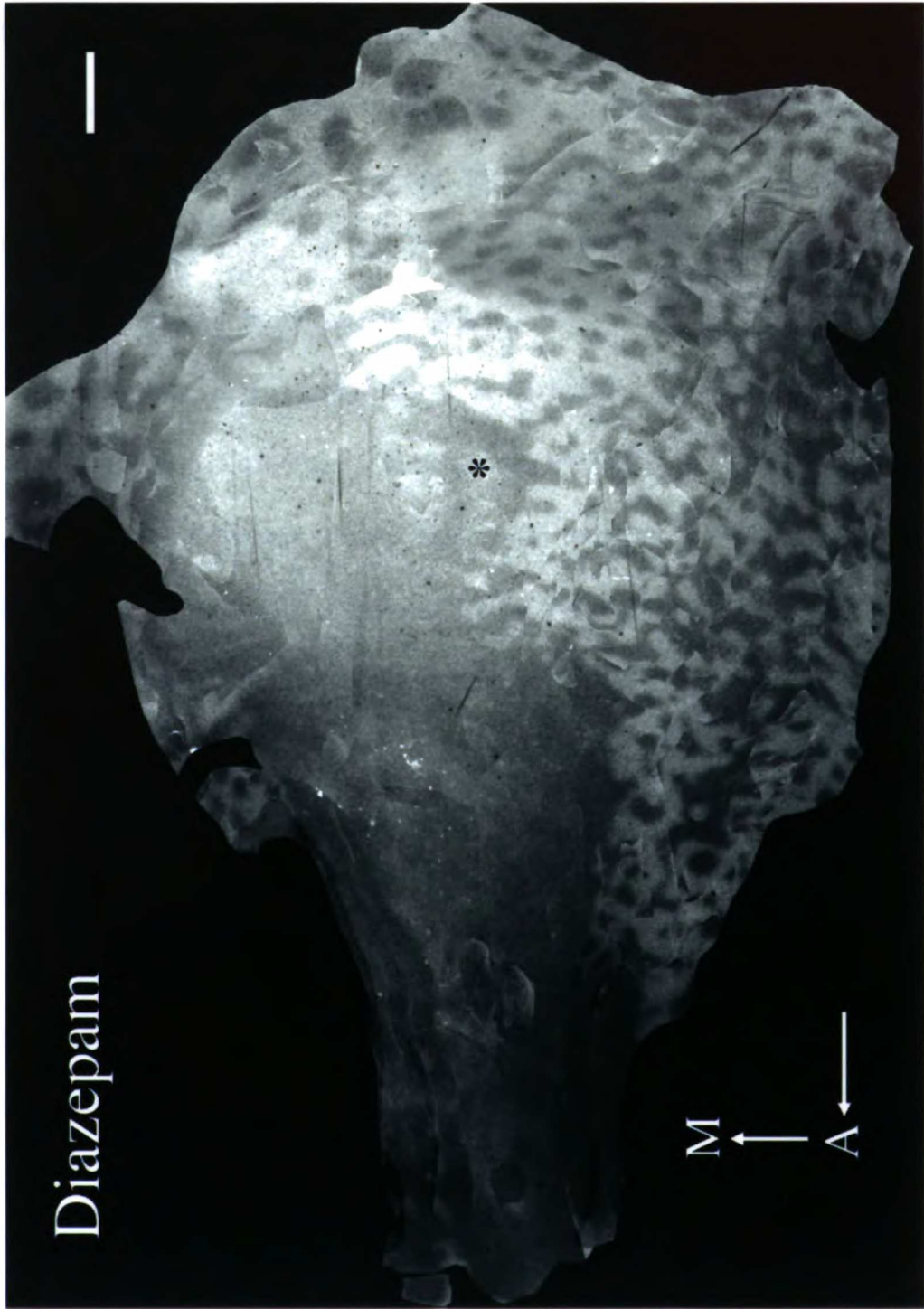


Near



Behind

Fig. 6-7. Graded effects of diazepam infusion. Consistent with a gradient of enhanced inhibition, effectively higher concentrations of diazepam yielded an area of column desegregation. This was reminiscent of the effects of full GABA_A receptor activation with muscimol (Hata and Stryker, 1994). Scale bar, 5mm. Proline-labeled areas are dark in this negative image.



In contrast to the graded changes in column widths seen with diazepam, control hemispheres exhibited a homogeneous spacing across the extent of area 17 (Fig. 6-8). This was true for brains infused with vehicle (propylene glycol), electrically inert substances (e.g. NGF), or nothing at all. We never observed the high degree of variability reported for cytochrome oxidase columns in monkey V1 (Horton and Hocking, 1996). It will be interesting to analyze more control data with our algorithm for comparison.

As a further control for the specificity of diazepam, we infused a benzodiazepine inverse agonist, methyl-6,7-dimethoxy-4-ethyl- β -carboline (DMCM) into 6 kittens. Since half of the animals died due to CNS metabolic toxicity at high doses, a concentration of 50 μ M appeared to be the threshold for safely enhancing cortical excitatory spread. The visual cortex from 2 animals which survived the 4-week infusion and flattening procedure, exhibited a reverse trend on column spacing from that of diazepam: near the cannula, columns were narrower than farther away or behind (Fig. 6-9).

In order to quantify the changes in periodicity, we used a computer-based algorithm to first skeletonize columns to their centers (Fig. 6-4) then compile a histogram of distance measurements between centers for every pixel in the image. Control columns ranged between 753 and 843 μ m with a mean of 791 \pm 18 μ m (n=5), in agreement with earlier published reports (Anderson et al., 1988; LeVay et al., 1978; Loewel and Singer, 1987). Near the diazepam infusion sites, columns were wider, ranging from 966 to 1104 μ m with a mean of

Fig. 6-8. Column layout in control hemispheres. Hemispheres treated with vehicle, no solution (shown), or electrophysiologically inert substances (e.g. NGF in saline) exhibited a homogeneous column spacing throughout the extent of area 17. Scale bar, 5mm. Proline-labeled columns are dark in this negative image courtesy of Dr. Y. Hata.

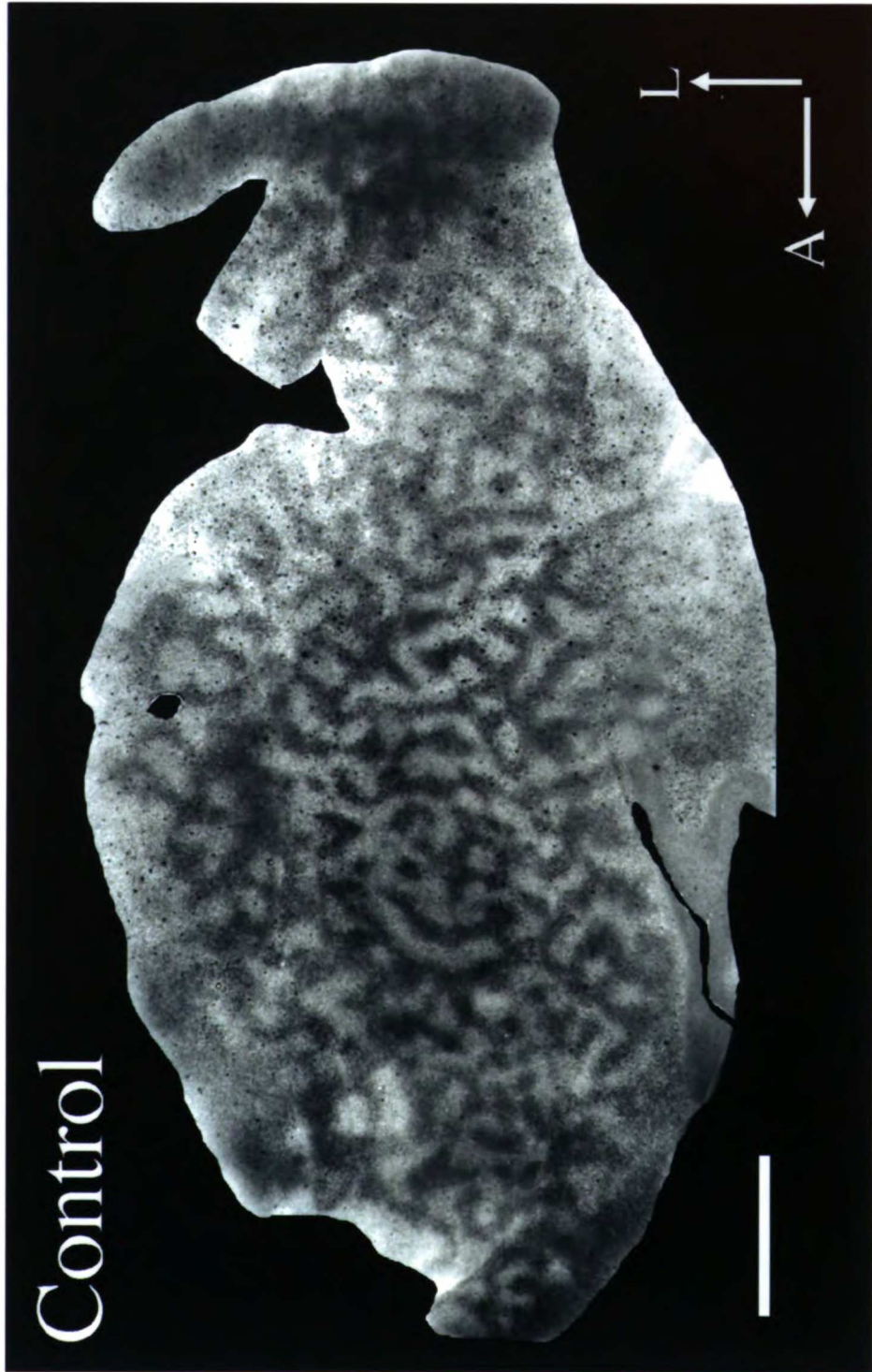
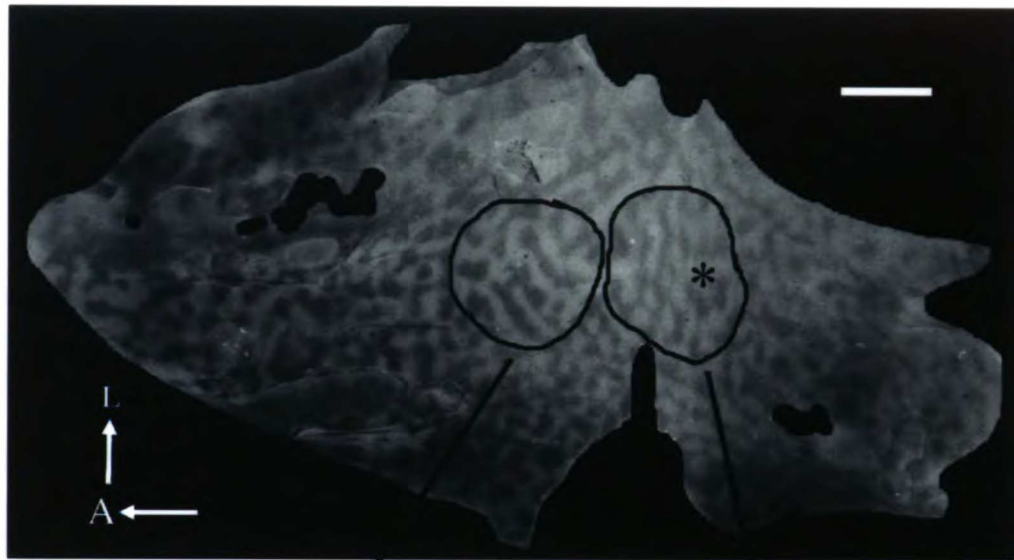
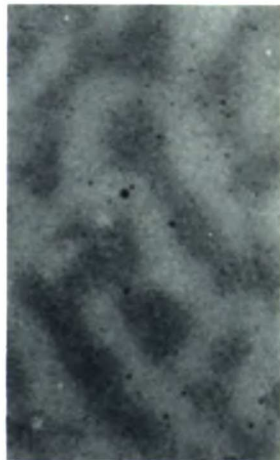


Fig. 6-9. Opposite effect of benzodiazepine inverse agonist on column size. Benzodiazepine inverse agonists (DMCM) exhibited a more complex effect on column spacing - **opposite** to that seen with diazepam. Columns near the infusion site were narrower than those farther away. Scale bars, 5mm (top); 1mm (bottom). Proline-labeled columns are dark in this negative image.

DMCM Effects on Column Spacing



Far



Near



1035±23µm (n=5). Plotting the percentage increase with respect to controls revealed a 31±2% increase which was highly significant (Fig. 6-10; $p < 0.001$, t-test). Behind the diazepam cannula, columns were also slightly larger than control but this difference was not significant ($p = 0.06$, t-test), consistent with a gradient of drug effects.

In the course of the present study, reports appeared of artificial strabismus changing the spacing of ocular dominance columns in cats (Loewel, 1994) and monkeys (Roe et al., 1995). Replotting Loewel's data normalized to her own controls showed a highly significant widening of similar magnitude to our results (34±2%, $p < 0.001$; Fig. 6-10). Curious that manipulations of either visual input or its target activity could produce the same effect on column segregation, we summarized the ocular dominance of cells recorded in all diazepam-treated hemispheres (Fig. 6-11). Indeed, there was a tendency toward reduced binocularity reminiscent of strabismic distributions (Hubel and Wiesel, 1965).

Discussion

We identified conditions for locally modulating intracortical inhibition throughout development of the kitten primary visual cortex. Chronic treatment

Fig. 6-10. Manipulations affecting column spacing in development. Column spacing was locally increased near diazepam infusion sites. Changes in column width were normalized to the mean of all control hemispheres (N=5 hemispheres each; t-test, ** $p < 0.001$). Column spacing was not significantly wider behind the diazepam infusion sites, consistent with a gradient of drug diffusion ($p = 0.06$). For comparison, changes in column spacing following divergent strabismus are plotted on the same graph, normalized to control animals (Loewel, 1994).

Manipulations Affecting Column Spacing

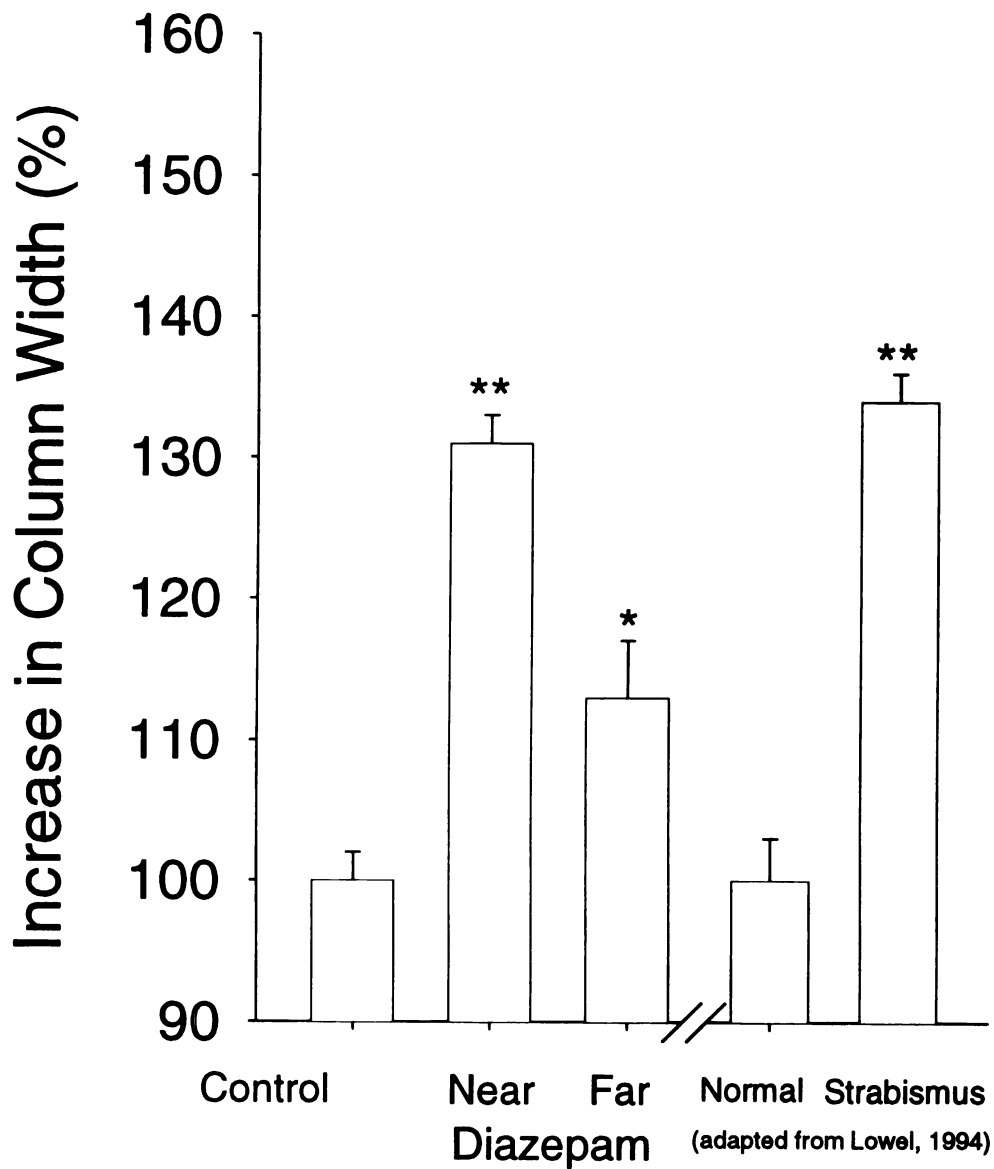
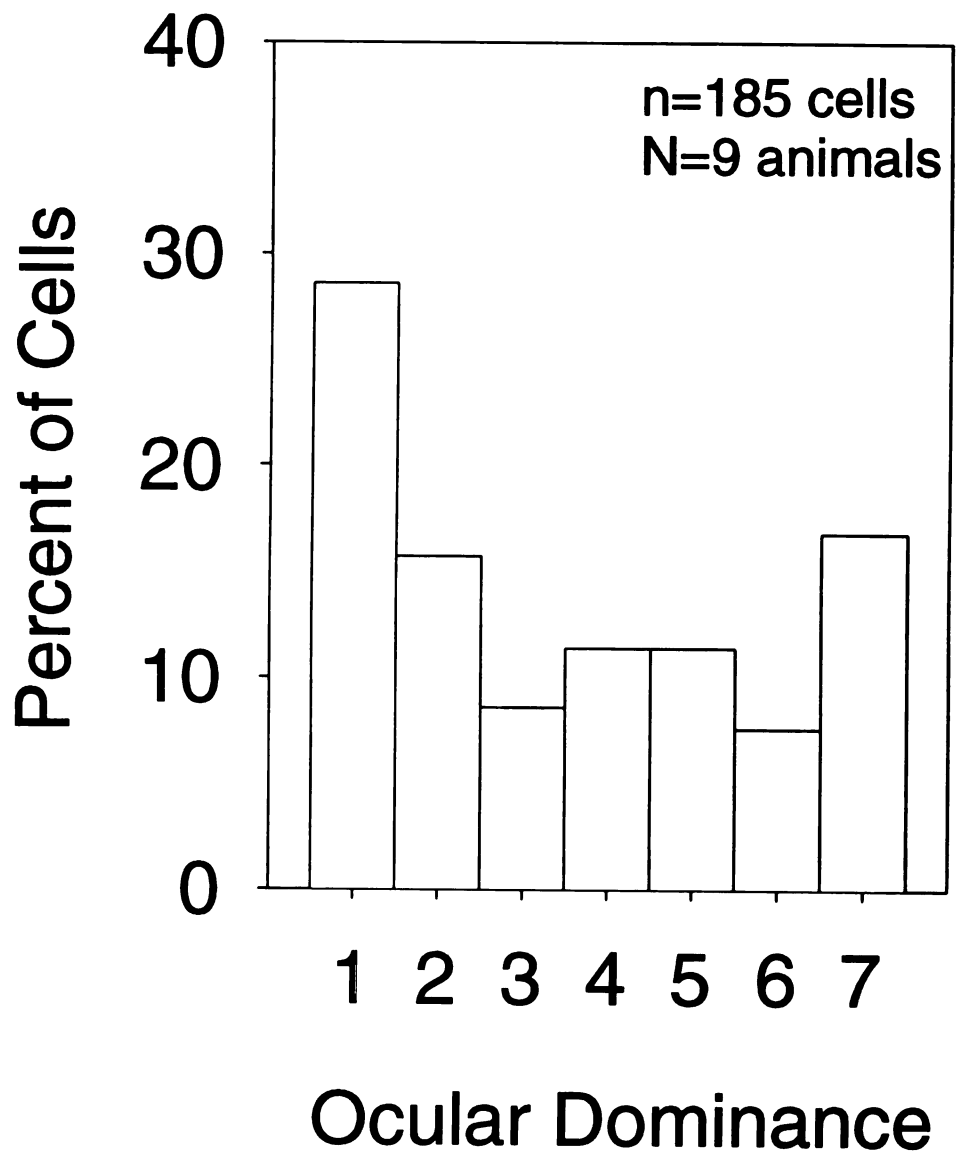


Fig. 6-11. 'Strabismic' ocular dominance distribution in diazepam-treated kittens. The ocular dominance of single units recorded in 9 diazepam-treated hemispheres tended toward a "strabismic" distribution with reduced binocularity.





with a benzodiazepine agonist had surprisingly little effect on the functional properties of cortical neurons *in vivo* (Fig. 6-5), although IPSCs were shown to be potently enhanced by the drug *in vitro* (Fig. 6-2). The maturation of intracortical networks, thus, appears to be quite robust to severe quantitative alterations of visual experience (Stryker et al., 1978) and neuronal activity. Nevertheless, we found pronounced effects on the columnar dimensions of afferents, which themselves are not responsive to benzodiazepines (Shaw et al., 1987). Taken together, these results demonstrate that intrinsic inhibition in target circuits of the cortex can regulate the segregation of presynaptic inputs -- strong evidence that column formation in cortical development is a self-organizing rather than genetically predetermined process.

The mechanism by which GABA_A receptor-mediated transmission alters column spacing appears complex. Benzodiazepine agonists and inverse agonists produced opposing effects on column size near the infusion site, as anticipated. However, these trends were precisely the reverse of predictions made by computational models of activity-dependent development (Fig. 6-1; (Miller and Stryker, 1990)). If anatomical connections in the cortex do indeed map out a "mexican hat" function (Fig. 6-1), then we must consider that diazepam exerts its actions via a disinhibition of cortical microcircuitry. Benzodiazepines are known to enhance depolarizing GABA_A-mediated responses (Staley, 1992). However, this feature of neonatal GABAergic transmission should have disappeared by the later critical period ages we studied (Cherubini et al., ; Staley et al., 1995).

Nevertheless, two pieces of evidence support the possibility of a functional disinhibition by benzodiazepines.

First, monoclonal antibodies directed against GABA_A receptor subunits reveal highest immunoreactive receptor density on somatic and dendritic plasma membranes of GABA-containing cells in layer IV (Somogyi, 1989). Among these are the large basket cells, which represent the primary lateral inhibitory connection in the cortex. Increased receptor expression may be accompanied by an increased sensitivity to GABAergic inhibition and modulation by benzodiazepines. Second, if receptor density differences rather than wiring may produce different physiological effects, then the possible regulation of GABA_A receptor expression on pyramidal cells following chronic benzodiazepine treatment may be important (Gallager et al., 1984; Marley et al., 1991; Shaw and Scarth, 1991). However, we found robust orientation selectivity with reduced responsiveness in diazepam-infused cortex (Fig. 6-4), consistent with enhanced inhibition in recurrent models of cortical processing (Somers et al., 1995).

An exciting alternative, then, is that our developmental results are informing us of an important organizing principle for inhibitory connectivity within the visual cortex. The discrepancy between theoretical predictions and experimental results regarding column spacing is likely due to our poor understanding of cortical circuitry. As reviewed in chapter 1, evidence for a "mexican hat"-like functional interaction is anecdotal at best. Moreover, there is no reason to expect intracortical interactions to remain the same throughout

development. Practical limitations are just now being overcome to resolve network properties on the scale of individual columns (cf. chapter 1).

Cortical inhibitory circuits in particular may operate (Kang et al., 1994; Van Brederode and Spain, 1995) and mature (Komatsu, 1983) in a laminar-specific fashion. Moreover, it has recently been suggested that fast GABA_A- and slow GABA_B-mediated synaptic inhibition originate from two distinct types of inhibitory interneurons (Benardo, 1994; Kawaguchi, 1993; Kawaguchi, 1992). Selective enhancement of only GABA_A pathways could, therefore, produce complex network effects. For example, benzodiazepine iontophoresis in somatosensory cortex suppresses pyramidal cell regular-spike firing in upper layers with relatively no effect on deep-layer bursting (Oka and Hicks, 1990; Oka et al., 1993). The spread of activity through cortical circuits needs to be examined further with *in vitro* resolution, under varying conditions of age and benzodiazepine treatment.

Lateral inhibition has been proposed to enhance the functional contrast between neighboring columns in normal animals (Sillito et al., 1980). This may explain the tendency toward reduced binocularity under our conditions of enhanced inhibition in the absence of any visual manipulation (Fig. 6-11). Conversely, strabismic kittens with reduced inhibition via bicuculline iontophoresis exhibit a similarly modest U-shaped ocular dominance distribution (Mower et al., 1984). In both cases, however, inhibition cannot solely account for the reduced binocularity. Exaggerated anatomical segregation of afferents into

wider patches may be the predominant determinant of the physiology (Fig. 6-6; (Loewel, 1994)). It will be interesting to determine whether other anatomical refinements produced by strabismus - such as targeting of horizontal connections to like ocular dominance columns (Loewel and Singer, 1992) or dendritic restructuring (Kossel et al., 1995) - are recapitulated by chronic benzodiazepine treatment.

Thus, local perturbation of intracortical interactions may manifest itself as a "cortical" strabismus, much like that following manipulations of visual input. The intriguingly similar changes in column spacing underscore the activity-dependent, self-organizing nature of ocular dominance column development and demonstrate for the first time the involvement of intrinsic cortical circuits in the segregation process.

Chapter 7

Conclusions and Future Directions

We have investigated the involvement of intracortical connections in the experience-dependent development of the primary visual cortex. Both in cats and rats at the peak of the critical period, responsiveness to an eye deprived of vision can be swiftly lost. This suggests a rapid underlying form of synapse modification which precedes gross anatomical rewiring of thalamocortical input by a few days, at least in the cat. These early stages of visual cortical refinement do not require PKC γ , which mediates the activity-dependent removal of other connections in the developing cerebellum (cf. Chapter 2).

Similarly, our results have shown that visual cortical plasticity is not necessarily the same as hippocampal LTP/LTD. Studying the *in vitro* plasticity of extracellular field potentials in the upper layers of cortex may mislead us about the principles guiding ocular dominance changes (cf. Chapters 3-5). This is not surprising given the relative complexity (and our ignorance) of the cortical network being stimulated in such experiments.

Indeed, circuits intrinsic to cortex do play an important role in both the development and plasticity of the visual cortex. Local inhibitory connections, in particular, appear to have profound regulatory effects (cf. Chapters 5, 6). Yet, again our models of developing intracortical interactions are inaccurate, providing us with predictions precisely the opposite of what we observe *in vivo*.

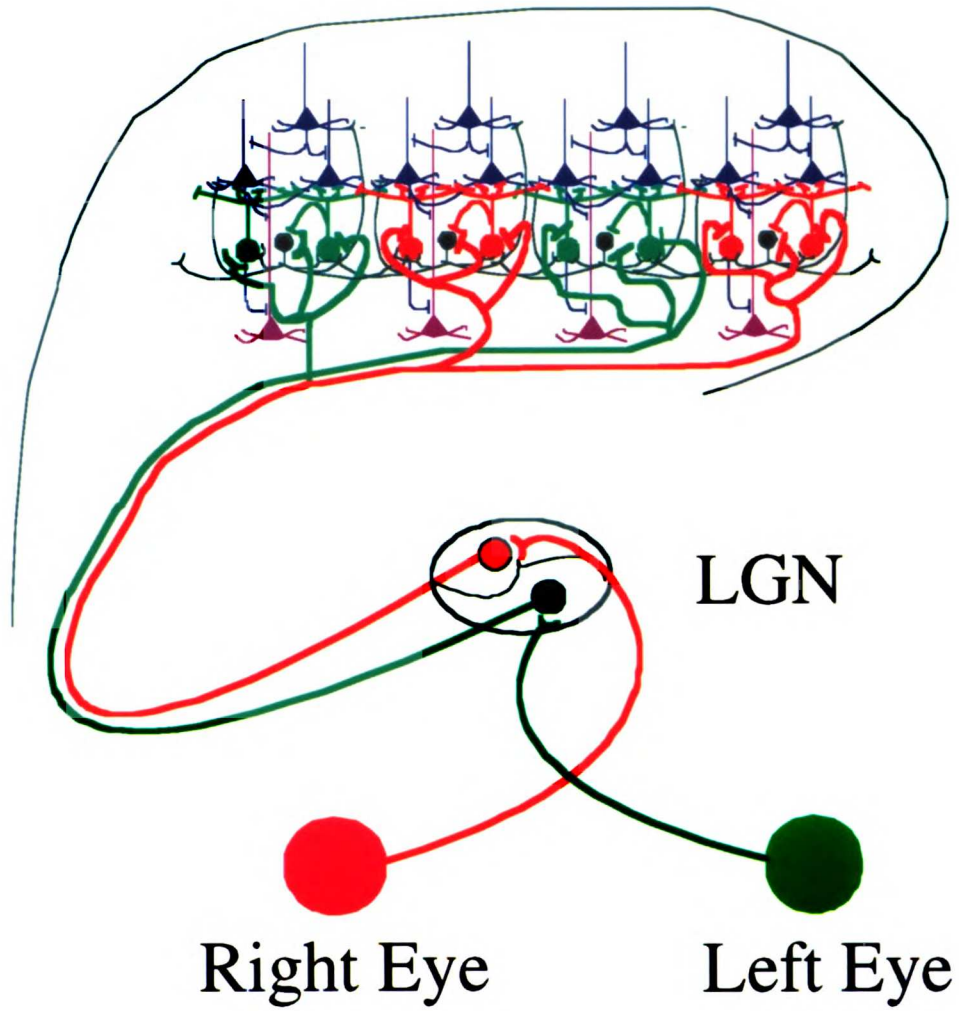
The agenda for the future seems clear. We must develop a deeper understanding of the connectivity within cortex and how it develops. Higher resolution analyses will require better stimulus control in reduced systems.

More creative preparations which preserve distinct thalamic inputs (Agmon and Connors, 1991), technically demanding recordings from pairs of synaptically-linked cells (Luebke et al., 1996; Stratford et al., 1996), or sophisticated innovations such as photostimulation (Callaway and Katz, 1993) or fast optical imaging (Nelson and Katz, 1995; Tanifuji et al., 1994) in slices of cortical tissue will offer new perspectives on thalamocortical interactions (Fig. 7-1).

The results presented in this thesis demonstrate a fruitful collaboration between *in vitro* and *in vivo* investigations of plasticity (Gordon et al., 1996). By taking advantage of the flexibility of both preparations and ease of comparison with other systems in the mouse, we have attempted to answer the request for “a well-defined rodent model” (Katz and Callaway, 1993). We hope that our approach will move us toward a molecular explanation of activity-dependent development, as well as refine our thinking about how networks of neurons interact to organize themselves in the brain.

Fig. 7-1. New perspectives on thalamocortical interactions.

Primary Visual Cortex



Appendix

```

pro DistMap, tiff_filename, ANGLERANGE = angrange, SAVEFLOAT=savefloat, $
  BATCHMODE=batchmode, CLOSEPAD=closepad, TOOCCLOSE=tooclose
; filename is a tiff of thinned column-centers, angrange is range of angles in radians
; requires a file of maximum distances and angles '/tmp/spiral.dat' produced by MakeSort
file (mkortff.pro)
; The input data file contains 3 regions, LINES (=2) which show the column centers, thin
; ARTIFACT (=0) where there is no VALID data, and VALID (=1) where there is the real spa
; ce between columns.
; Whenever the distance map from the first or second point encounters an ARTIFACT, zero
; is returned for dmap value.
;
; If we set things up as follows, then we can leave as a 0 in the dmap
; output (meaning no-VALID-data) anything that starts or ends in a
; zero value on the padded input picture.
; pad (outside border) = 0
; ARTIFACT regions = 0
; LINES = 2
; everywhere else (VALID) = 1
;
; Program now takes care of special cases of VERY short distances < TOOCCLOSE
; in a special way, by looking at the median of the points that are greater than TOOCCLOS
E
;
; in a neighborhood of square radius CLOSEPAD.
;
; Program now includes histogram plot of dmap values and means and medians and sdev of n
on-zero values.
LINES = byte(2)
VALID = byte(1)
ARTIFACT = byte(0)
if (keyword_set(tooclose)) then print, 'tooclose', tooclose else tooclose = float(4.0)
if (keyword_set(closepad)) then pad = closepad else pad = 8
if (keyword_set(angrange)) then ar=abs(angrange*IDTOR) else ar=PI/2.
; filename=spichfile(file=c:\tmp\spiral.dat', /write, title='Select Spiral Data Output fi
le )
maxi = fix(1) ; make sure it is an int
openr, unit, '/tmp/spiral.dat', /get_lun
readu, unit, maxi
dmap = float(2*maxi)
ds = fitarr(maxi)
xs = intarr(maxi)
ys = intarr(maxi)
readu, unit, xs, ys, ds, as
close, unit
TwoPI = float(2*PI)
as(when(as lt 0.)) = as(when(as lt 0.)) + TwoPI
iclose = 0
while (ds(iclose) le (tooclose + 0.5)) do iclose = iclose + 1 ; get the minimum search
distance for second hit
alist = fitarr(iclose)
print, 'maxi=', maxi, ' iclose=', iclose, '
ar(deg)=', ar/180./PI
; read in and pad the tiff input file
flush, -1
in = tiff_read(tiff_filename)

```

```

insize = size(in)
xlen = insize(1)
ylen = insize(2)
h=histogram(in)
print, 'opened', tiff_filename, ' xlen=', xlen, ' ylen=', ylen, ' histo=0.1.2.3
:255', h(0), h(1), h(2), total(h(3:255))
in = make_array(xlen*(2*maxi+1), ylen*(2*maxi+1), /byte, value = ARTIFACT)
in(maxi: maxi*xlen-1, maxi: maxi*ylen-1) = in
insize = size(in)
xlenn = insize(1)
ylen = insize(2)
wset, 0
tvsc1.byte(lnn), 0
dmax = float(maxi)
d1 = fitarr(xlen, ylen)
d2 = fitarr(xlen, ylen)
a1 = fitarr(xlen, ylen)
a2 = fitarr(xlen, ylen)
dmap = fitarr(xlen, ylen)
for y = maxi, maxi + ylen - 1 do begin
  print, y = ', strtrimly maxi.2), ' ', systime(0)
  flush, -1
  for x = maxi, maxi + xlen - 1 do begin
    ; print, ' ', format=('$,1A')
    if ( in(x, y) eq ARTIFACT ) then goto, nodata
    t initial point
    for i = 0L, maxi - 1 do begin
      z = inn(x + xs(i), y + ys(i))
      if (z eq LINES) then goto, firsthit
      if (z eq ARTIFACT) then goto, nodata
    here
    endfor
    nobit:
    d1(x-maxi, y-maxi) = 0 ; was dmax
    a1(x-maxi, y-maxi) = 0
    d2(x-maxi, y-maxi) = 0 ; was dmax
    a2(x-maxi, y-maxi) = 0
    goto, nextxy
  ; if (in(105,101) and (0 or 0)) then print, 'true' else print, 'false'
firsthit:
  d1(x-maxi, y-maxi) = ds(i)
  a1(x-maxi, y-maxi) = as(i)
goto, secondhit
; special cases
secondhit:
  at = a1(x-maxi, y-maxi) + PI
  if ( at gt TwoPI) then at = at - TwoPI
  att = at + TwoPI
  for j = 1+1, maxi - 1 do begin
    ; reject ARTIFACT ONLY if along petre
    ndicular line +- ar (i+1 now i) (main also now i)
    z = inn(x + xs(j), y + ys(j))
    if ( z ne VALID) and $
      ( (abs(at - as(j)) lt ar) or (abs(att - as(j)) lt ar) ) $
    then begin
      if (z eq ARTIFACT) then goto, nodata
      d2(x-maxi, y-maxi) = ds(j)

```

```

                a2(x-max1, y-max1) = a1(i)
            goto, nextxy
        end
    endfor
    d1(x-max1, y-max1) = 0
    a1(x-max1, y-max1) = 0
    a2(x-max1, y-max1) = 0
    goto, nextxy

nodata:
    d1(x-max1, y-max1) = 0
    a1(x-max1, y-max1) = 0
    d2(x-max1, y-max1) = 0
    a2(x-max1, y-max1) = 0
    goto, nextxy

nextxy:
    endfor
endfor

dmap = d1 + d2

; this first rather dumb fix for dmap tooclose values just replaces them with the median
; of the non-tooclose values
; that are located within a pd neighborhood
goto,nofix
dmf=dmap

dmp = fltar(xlen*2*pad,ylen*2*pad)
dmp(pad:pad*xlen-1,pad:pad*ylen-1) = dmap
for y = 0, ylen-1 do begin
    for x=0, xlen-1 do begin
        if ( (dmap(x,y) lt tooclose) AND (in(x,y) ne 0) ) then begin
            nabe1 = dmp(x:(2*pad)-1,y:(2*pad)-1)
            nabe2 = where(nabe1 gt tooclose ,count)
            if (count ne 0) then fv = median(nabe1(nabe2)) else fv = 0.
            dmf(x,y) = fv
        endif
    endfor
endfor

window, 6, xsize=xlen, ysize=ylen, title='dmf'
:window, 6, xsize=8*xlen, ysize=8*ylen, title='dmf'
wset, 6
tvsc1,dmf
:tvsc1,rebini(dmf,8*xlen,8*xlen)

dmapnottooclose = dmap( where (dmp ge tooclose) )
res = moment(dmapnottooclose)
print, 'Fixed Up Dmap: input file ', tiff_filename, ' mean = ', res(0), '
variance) = ', sqrt(res(1)), ' median = ', median(dmapnottooclose)

nofix:
dmapnottooclose = dmap( where (dmap ge tooclose) )
res = moment(dmapnottooclose)
print, 'Normal Dmap: input file ', tiff_filename, ' mean = ', res(0), '
variance) = ', sqrt(res(1)), ' median = ', median(dmapnottooclose)

window, 2, xsize=xlen, ysize=ylen, title='dmap'
:window, 2, xsize=8*xlen, ysize=8*ylen, title='dmap'
wset, 2
tvsc1,dmap

```

```

:tvsc1,rebini(dmap,8*xlen,8*xlen)
:goto, skipped
window, 1, xsize=xlen, ysize=ylen, title='dl'
wset, 1
tvsc1,dl
window, 3, xsize=xlen, ysize=ylen, title='d2'
wset, 3
tvsc1,d2
window, 4, xsize=xlen, ysize=ylen, title='a1'
wset, 4
tvsc1,a1
window, 5, xsize=xlen, ysize=ylen, title='a2'
wset, 5
tvsc1,a2

skipped:
window, 0, xsize=300, ysize=300, title='dmaphisto'
wset, 0
hmap-histogram(byte(dmap))
: hmf = histogram(byte(dmf))
plot,hmf(0:50),psym=10
: oplot,hmf(0:50)

:goto, nosave

st = str_sep(tiff_filename, '.')
if ( version.OS_FAMILY eq 'Windows' ) then begin
    ifilename=st(0)+'.t'
    if(strlen(ifilename) gt 8) then begin
        ifilename = st(0)
        strput.ifilename, 't'.0
    endif
    ifilename = ifilename+'.tif'
endif $
else ifilename = tiff_filename+'.dmt'.strtrim(string(ar*'RADEG,format='(F4.0)'.2)
if(keyword_set(batchcode)) then ifilename = ifilename $
else filename=pickfile(file=ifilename, /write, title='Select DistMap Tiff Output file')
tiff_write,filename, byte(dmap)
print, 'DistMap wrote tiff output file ', filename

goto, nosavedmf

st = str_sep(tiff_filename, '.')
if ( version.OS_FAMILY eq 'Windows' ) then begin
    ifilename=st(0)+'.f'
    if(strlen(ifilename) gt 8) then begin
        ifilename = st(0)
        strput.ifilename, 'f'.0
    endif
    ifilename = ifilename+'.tif'
endif $
else ifilename = tiff_filename+'.dmf'.strtrim(string(ar*'RADEG,format='(F4.0)'.2)
if(keyword_set(batchcode)) then ifilename = ifilename $
else filename=pickfile(file=ifilename, /write, title='Select DistMap fixed Tiff Output f
ile')
tiff_write,filename, byte(dmf)
print, 'DistMap wrote fixed tiff output file ', filename
nosavedmf:

if(keyword_set(savefloat)) then goto, savefloat else goto, nosave

savefloat:
ifilename = tiff_filename

```

```

if( !version.OS_FAMILY eq 'Windows' ) then begin
  filename=st(0)+'d'
  if(strlen(filename) gt 8) then begin
    filename = st(0)
    strput,filename,'d',0
  endif
  filename = filename+'.dm'
endif $
else filename = tiff_filename+'.dm'+strtrim(string(ar:'RADEG',format='(F4.0)'),2)
if(keyword_set(batchcode)) then filename = filename $
else filename=pickfile(file=filename, /write, title='Select DistMap Float Output file'
)
openw, unit, filename, /get_lun
writeu, unit, longixten, longiyten, float(ar), dmap, dl, d2, al, a2
close, unit
print, 'DistMap is done, wrote float output file ', filename
:stop

nosave:
return
end

```

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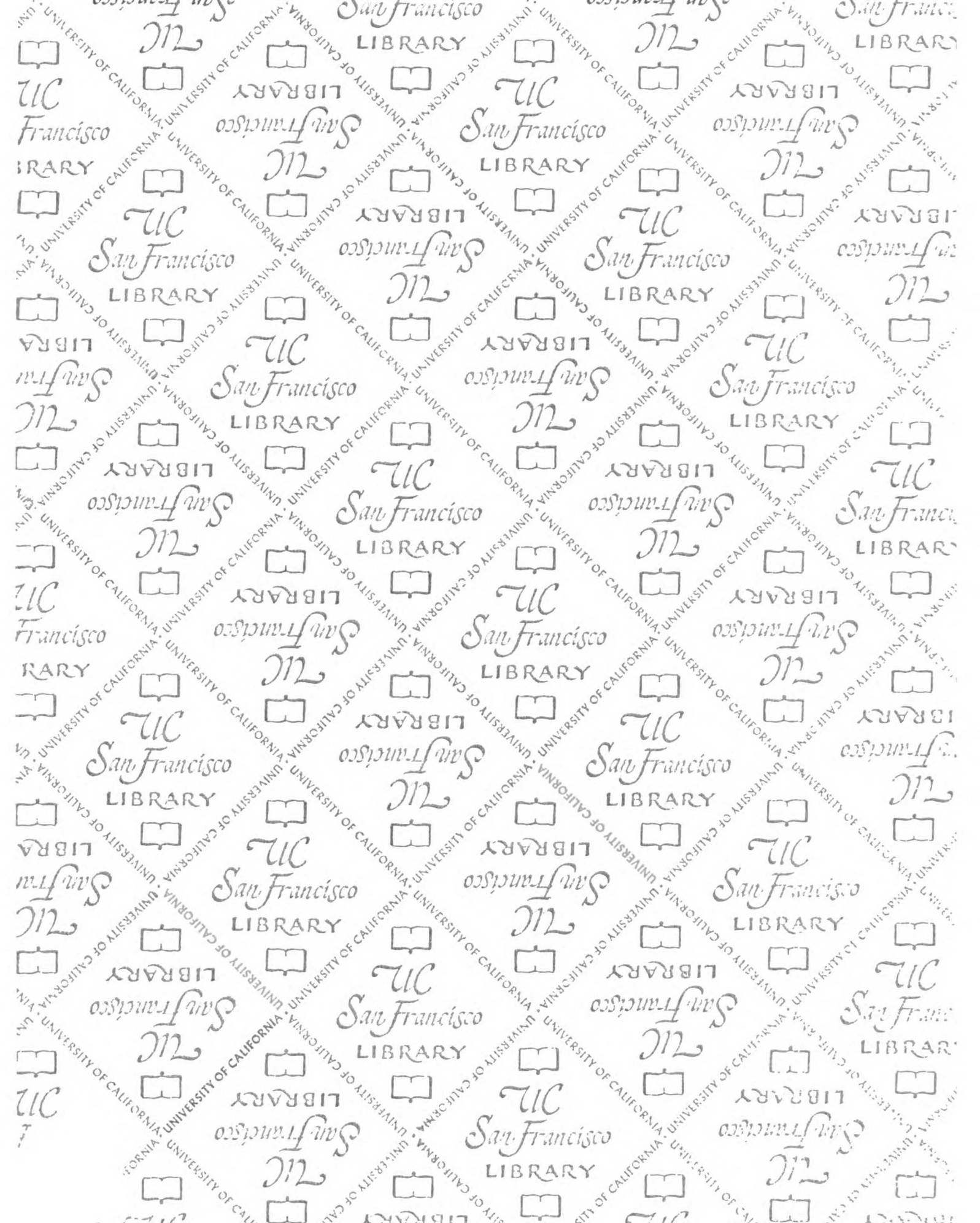
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