FUNCTIONAL ROLE *of* NMDA RECEPTOR SUBUNIT COMPOSITION *in* METAPLASTICITY

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ABSTRACT

Modification of synapses by neural activity has been proposed to be the substrate for experience-dependent brain development, learning, and recovery of function after brain damage. In the visual cortex, the strength of cortical synapses can be bidirectionally modified, where in response to a critical level of postsynaptic activation, synapses are strengthened (long-term potentiation; LTP) and below this level, synapses are weakened (long-term depression; LTD). Previous work in visual cortex has suggested that the threshold for synaptic modifications is dependent on the recent history of visual experience, a phenomenon called metaplasticity. Recent mechanistic studies have shown that experience-dependent adjustments of the modification threshold correlate with changes in the subunit composition and function of NMDA-type glutamate receptors (NMDARs). However, causality has not been conclusively established. Here we examined the mechanistic basis of metaplasticity, and specifically how this process is mediated by a switch in NMDAR subunit composition by focusing on the NR2A subunit of the NMDA receptor in visual cortex. We provide evidence for the functional significance of the NR2A subunit in metaplastic changes both in synaptic plasticity elicited *in vitro* and in naturally-occurring plasticity *in vivo*. We also performed a comparison of *in vitro* methods of inducing plasticity and those which subserve *in vivo* experience-dependent changes in synaptic strength. These findings represent an important step forward in understanding how plasticity thresholds are regulated in the brain.

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"isn't life quonderufl??"

-viren jain, 2006.

thank you,

my family
mark bear
robert crozier
benjamin philpot
lena khibnik
slice lab
jason coleman
arnold heynen
bear lab
mikhail frenkel
gordon smith
suzanne meagher
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CHAPTER I

Introduction and Background

For centuries researchers have sought to understand how one acquires, stores, and accesses information. As early as the fourth century, Hippocrates postulated that the brain is the seat of intelligence. Over a century ago, Ramon y Cajal made a conceptual breakthrough concerning the brain as the location of information storage (Bliss and Collingridge, 1993). By demonstrating that networks of neurons communicate with one another, it was further elucidated that such interactions participate in maintaining a representation that serves as a memory. Specialized junctions called synapses were found to house these dynamic interactions among neurons and be susceptible to modifications. Nonetheless, an understanding of how synaptic mechanisms may serve to underlie memory has remained elusive.

Sensory experiences help to refine synaptic connections throughout the life of the animal so that the world can be properly analyzed. For mammals, early postnatal development is a period of great use-dependent and experience-dependent modification in the brain, where cortical circuitry undergoes a considerable amount of maturation and refinement. However, the rate of such plasticity significantly decreases as a function of age. Although much research has been conducted on how neural connections are formed during development, of particular interest is how

ongoing sensory-driven activity can shape synaptic connectivity and underlie the basis of memory formation.

In 1949, Donald Hebb increased our understanding by proposing an innovative theory about how ensembles of neurons interact at the level of the synapse. Hebb postulated that "when an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased", suggesting activity-dependent changes in synaptic efficacy (Hebb, 1949). "Hebbian" modification thus arises from concurrent activation of both presynaptic and postsynaptic cells, resulting in an increase in synaptic strength. Bliss and Lømo in 1973 were the first to experimentally support Hebb's postulate (Bliss and Lømo, 1973). In an attempt to replicate robust sensory-driven activity, they administered high-frequency electrical stimulation (HFS) to monosynaptic excitatory pathways in the hippocampus, a structure known to be involved in some forms of learning and memory. The brief HFS caused a sustained increase in the efficacy of synaptic transmission. Thus, the long-lasting enhancement of synaptic transmission that was first described theoretically by Hebb (1949) and confirmed experimentally by Bliss and Lømo (1973) has been termed long-term potentiation (LTP). Alternatively, "anti-Hebbian" modifications occur when presynaptic activity fails to induce or synchronize with a strong postsynaptic response (Levy and Steward, 1979). For instance, weak postsynaptic activation induced by low-frequency stimulation (LFS) to monosynaptic excitatory pathways in the hippocampus result in long-term depression (LTD) of synaptic strength. Due to the stable, long-lasting synaptic modifications that characterize LTP and LTD, these phenomena have become popular models for the cellular mechanisms that may underlie information storage among neural systems.

The hippocampus and neocortex are both excellent model systems for studying changes in cell network connections. In hippocampus, plasticity can be evaluated by stimulating any of the main excitatory pathways (perforant, mossy fiber, and Schaffer collateral pathways). While LTP and LTD have been fully characterized in the CA1 region of the hippocampus, a number of studies have extended the principles of LTP and LTD to glutamatergic excitatory synapses throughout the mammalian brain (Kirkwood et al., 1993). For instance, the visual cortex provides an excellent model system for studying neocortical plasticity. In the visual cortex, sensory-driven activity can be easily modified in order to understand how experience modifies neural connections (Kirkwood et al., 1993). Intensive study of hippocampal and visual cortical plasticity promises to provide insights into the development of sensory systems and the acquisition and storage of memories.

PROPERTIES OF LONG-TERM POTENTIATION

There are a variety of ways to generate LTP, involving high-frequency stimulation to a pathway of choice. As stated earlier, LTP may be induced via brief (~1 sec) repetitive trains of high-frequency stimulation (~100 Hz) (Bliss and Lømo, 1973). Protocols such as theta burst (TBS) and primed-burst stimulation have also been used to induce LTP; these induction protocols are designed to mimic synchronous firing patterns similar to those that occur naturally in the hippocampus during learning (Otto et al., 1991). LTP can even be generated with low-frequency stimulation of presynaptic fibers, as long as the postsynaptic cell is still strongly depolarized. In this instance, injecting current into the postsynaptic cell to induce sufficient depolarization and giving 1 Hz stimulation to presynaptic fibers will also elicit LTP (Kirkwood and Bear, 1995). Therefore, strong postsynaptic activation will lead to the strengthening of synapses.

Although it can take only a few milliseconds to induce, LTP can last for hours *in vitro*, and even weeks and months *in vivo* (Bliss and Lømo, 1973; Abraham et al., 2002). In order to elicit LTP, pre- and postsynaptic activity must be paired: (1) presynaptic neurons must be activated (release neurotransmitter), and (2) postsynaptic neurons must depolarize (Hebb, 1949; Bear et al., 1987). There are three basic properties associated with LTP: (1) input-specificity, (2) associativity, and (3) cooperativity. LTP is input-specific, meaning that only those synapses that are activated are modified. Therefore, when a population of neurons receives two independent inputs, LTP induced at one set of inputs will not spread to synapses made by the second set of inactive afferent fibers on the same postsynaptic neurons (Kirkwood and Bear, 1994a). LTP also exhibits associativity; for example, weak non-LTP-inducing stimulation on one set of synapses is able to generate LTP when paired with strong LTP-inducing stimulation in an adjacent set of synapses to the same cell (Bliss and Collingridge, 1993). The third property, cooperativity, maintains the idea that while weak stimulation of one set of inputs might not lead to LTP induction, weak stimulation of many sets of converging inputs might be able to sufficiently depolarize postsynaptic neurons to produce LTP. There is also an intensity threshold for the induction of different forms of potentiation whereby the strength and pattern of tetanic stimulation can convey a difference in the time course of synaptic modification. For instance, weak tetanic stimulation will elicit a few minutes of post-tetanic potentiation, intermediate tetanic stimulation will elicit 15–30 minutes of short-term potentiation, and strong tetanic stimulation will elicit at least an hour of LTP (McNaughton et al., 1978; Malenka, 1991).

PROPERTIES OF LONG-TERM DEPRESSION

Whereas strong postsynaptic activation can induce long-term potentiation, weak postsynaptic activation can cause long-term depression. For instance, administering 900 pulses of stimulation of 0.5–3 Hz to the Schaffer collateral pathway, will generate LTD in the pyramidal cells of CA1 (Dudek and Bear, 1992). Like LTP, LTD has also been implicated as a mechanism underlying memory, and can be elicited in the visual, somatosensory, inferotemporal, and motor cortices (Bear, 1996). Thus, the strength of cortical synapses can be bidirectionally modified in both the hippocampus and the visual cortex, such that above a critical level of postsynaptic activation synapses are strengthened and below which synapses are weakened (Kirkwood et al., 1993).

There are three types of LTD: (1) heterosynaptic LTD, (2) associative LTD, and (3) homosynaptic LTD. LTD can be heterosynaptic, meaning that tetanic stimulation of one pathway can potentiate its target cells, but also depress the synaptic strength of target cells from converging untetanized or weak afferents (Lynch et al., 1977). LTD is associative when uncorrelated activation of a test pathway by LFS and tetanic stimulation of a converging pathway results in depression of synaptic strength of the test pathway (Stanton and Sejnowski, 1989). LTD is homosynaptic, or input-specific, when presynaptic activation of a pathway by LFS results in moderate postsynaptic activity, resulting in the depression of synaptic strength of the stimulated pathway (Dudek and Bear, 1992). In addition, the magnitude of homosynaptic LTD is frequency-dependent. The range of stimulation frequencies that elicit this synaptic depression varies between 0.1 Hz and 10 Hz (Dudek and Bear, 1992; Torii et al., 1995).

MOLECULAR MECHANISMS UNDERLYING SYNAPTIC PLASTICITY

THE ROLE OF THE NMDA RECEPTOR

A truly comprehensive description of the induction processes underlying LTP and LTD does not rest in the release of neurotransmitter and a subsequent depolarization of the postsynaptic neuron. What is needed is the existence of a molecular coincidence detector, a component that can appropriately tie together these phenomena and elicit a response.

The involvement of postsynaptic NMDA (N-methyl-D-aspartate) receptors (NMDARs), a pharmacologically distinguishable subtype of glutamate receptors, satisfies the requirements of a coincidence detector. By being both ligand- and voltage-dependent, the NMDAR channel differs in fundamental ways from non-NMDAR channels. When glutamate and co-agonist glycine binds to the NMDAR, the channel opens to allow cations, including calcium, to enter the cell (Benveniste and Mayer, 1991; Clements and Westbrook, 1991). However, the current of cations can only flow once the magnesium (Mg^{2+}) blockade within the ion channel is released. In order to relieve the Mg^{2+} block, the postsynaptic cell must be sufficiently depolarized. These ligand- and voltage-dependent properties, requiring both glutamate binding and membrane depolarization, make the NMDAR a suitable coincident detector of presynaptic and postsynaptic activity (Molinoff et al., 1994).

A wealth of data supports the idea that NMDAR activation is required for induction of the most commonly studied forms of LTP and LTD. By being both ligand- and voltage-dependent, the NMDAR allows calcium entry into the cell whereby large, transient elevations in intracellular calcium lead to LTP, while lower, sustained elevations in calcium lead to LTD (Molinoff et al., 1994). The role of the NMDAR as a molecular coincidence detector is thought to underlie the bidirectional regulation of synaptic strength by triggering signal transduction cascades (Mori and Mishina, 1995).

Indeed, the ability to block LTP and LTD *in vitro* and *in vivo* by NMDAR antagonists D-2-amino-5-phosphonopentanoic acid (APV) or (R, S)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) has been demonstrated in both hippocampus and visual cortex (Morris et al., 1986; Dudek and Bear, 1992; Kirkwood et al., 1993; Wong et al., 2007; Sato and Stryker, 2008). Because the experimental induction of LTP is typically sensitive to NMDAR antagonists, an approach to examine the importance of synaptic plasticity in learning and memory has been to block NMDARs in regions of the brain during specific learning tasks. For example, APV injected into the lateral ventricles of rats blocks LTP induction in the dentate gyrus and causes a deficit in spatial memory formation (Morris et al., 1986). What accounts for the induction of either LTP or LTD lies in the makeup of the NMDAR itself.

The cloning of cDNAs encoding the subunits of the NMDAR has led to a great increase in the knowledge and understanding of the structural and functional properties of this receptor. Expression studies have revealed three families of NMDAR subunits: the NR1, NR2, and NR3 families (Ishii et al., 1993; Sucher et al., 1995). By alternative splicing, NR1 subunits may be expressed in nine different variants from a single gene, of which eight isoforms form functional ion channels (Hollmann et al., 1993). Four genes give rise to NR2 subunits NR2A through NR2D, and three genes give rise to NR3A–1, NR3A–2, and NR3B (Monyer et al., 1994; Cavara and Hollmann, 2008). The different electrophysiological properties of the NR1, NR2, and NR3 subunit families suggest functional differences. The NR1 subunit is necessary to construct a functioning receptor, and combines with the NR2 and NR3 subunits in a variety of ways that determine its varied properties, including differences in single channel characteristics, sensitivity to Mg^{2+} blockade and coagonist stimulation (Cavara and Hollmann, 2008). Although NR1 expression in the brain is ubiquitous, there is specific regional and developmentally regulated expression of the NR2 and NR3 subunit families. While the functional contribution of NR3A and NR3B subunits are currently

being explored, peak expression of the NR3A subunits occurs as early as embryonic age E15, and reduces to hardly detectable levels after two weeks in most structures. NR3B subunit expression is restricted to motor neurons in the brain stem and spinal cord. However, of particular interest to developmental plasticity are the members of the NR2 subunit family. In the embryonic brain, the only subunits expressed are NR2B and NR2D, located in a wide distribution and contained in the diencephalon and brain stem, respectively. While NR2B is localized in the cortex, thalamus, spinal cord, hippocampus, superior colliculus, and hypothalamus, NR2D is mostly expressed in midbrain structures. By two weeks of postnatal life, a shift in expression occurs in favor of NR2A and NR2C, while NR2D expression is significantly lowered. While NR2A is upregulated in cortex and hippocampus, NR2C is restricted mainly to the cerebellum (Monyer et al., 1994). The main functional differences between the subunits lie in comparative differences regarding Mg^{2+} blockade, gating, and receptor kinetics. For example, receptors containing the NR2C subunit are less sensitive to blockade of Mg^{2+} and MK–801, an NMDAR antagonist, than are receptors containing NR2A or NR2B subunits (Molinoff et al., 1994).

Targeting NMDARs to the synapse is extremely important for efficient synaptic transmission. To understand how NMDARs localize at the synapse, biochemical studies have shown direct interactions of NMDARs to the PSD–95 family of proteins, a major component of the postsynaptic density (PSD) (O'Brien et al., 1998). The PSD, a cytoskeleton specialization beneath the postsynaptic membrane of neuronal synapses, contains organized signal transduction complexes that respond to the activation of postsynaptic receptors. Specifically, the C-terminal cytoplasmic domains of the NR2 subunits bind to the N-terminal PDZ domains of PSD–95 (Kornau et al., 1995). PSD–95 colocalizes with NMDARs at glutamatergic synapses, allowing for the clustering of NMDARs at the synapse (Niethammer et al., 1996).

The differences in expression patterns and receptor kinetics of NMDARs in the mammalian brain have implications regarding several features of synaptic plasticity. In postnatal visual cortex, the predominant NMDAR subtypes are NR2A- and NR2B-containing NMDARs (Monyer et al., 1994). NMDAR subunit composition in this region is developmentally regulated (Sheng et al., 1994). For example, juvenile rodent visual cortex shows a predominance of NR2B-containing NMDAR subunits. As the animals age, an increase in NR2A protein levels was observed while NR2B subunits remained constant. In addition, Carmignoto and Vicini observed longer NMDAR excitatory postsynaptic currents (EPSCs) in juvenile rats than in adults, which may contribute to why juveniles demonstrate greater susceptibility for plasticity than adults do (Carmignoto and Vicini, 1992). Consistent with this data, there was a concomitant increase in NR2A protein that was accompanied by shorter NMDAR-mediated EPSCs, indicating that the receptor subunits were indeed incorporated into full NMDAR complexes at the plasma membrane (Nase et al., 1999). Thus, the putative molecular basis for the duration of NMDAR-mediated EPSCs lies in the differences in NR2A and NR2B receptor kinetics. These differences run in parallel to the greater magnitude of LTP and LTD mechanisms in juveniles. For instance, Kirkwood and colleagues found a tight correlation between the duration of NMDAR EPSCs, the ability to elicit LTP and LTD, and age (Kirkwood and Bear, 1994b; Kirkwood et al., 1995). Taken together, the data demonstrate that a low NR2A/NR2B ratio results in the slower kinetics seen in young animals, whereas a higher NR2A/NR2B ratio is associated with faster currents seen in mature animals (Monyer et al., 1994). The fast kinetics of NR2A-containing NMDARs, which lend to reduced calcium entry into the cell, and the slow kinetics of NR2B-containing NMDARs, which increase calcium entry into the cell, can control the bidirectional regulation of synaptic strength in both hippocampus and visual cortex (Tang et al., 1999; Philpot et al., 2001a).

The influx of calcium ions through the NMDAR is essential to triggering the biochemical mechanisms underlying synaptic plasticity. For example, calcium-chelators, used to block the postsynaptic rise of Ca^{2+}, prevent the induction of LTP and LTD (Lynch et al., 1983; Mulkey and Malenka, 1992). The activation of NMDARs results in varied elevations in postsynaptic calcium: LTP of synapses is a result of a spike in calcium entry which leads to the activation of protein kinases, whereas LTD of synapses is a result of moderate and sustained calcium entry that favors the activation of protein phosphatases (Lisman, 1989). To induce NMDAR-dependent LTP and LTD, NR2A and NR2B proteins have been shown to bind to different sets of scaffolding and signaling proteins based on differences between their C-terminal cytoplasmic domains. Therefore, activation of either NR2A or NR2B can translate into changes in plasticity (Perez-Otano and Ehlers, 2005). Studies of the signaling cascades initiated following NMDAR-dependent calcium entry into the cell have led to attempts to find necessary and sufficient molecules, some of which include α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) trafficking, calcium/calmodulin-dependent kinase II (CaMKII), calcium/diacylglycerol-dependent protein kinase (PKC), cAMP-dependent protein kinase A (PKA), and calcineurin for regulating plasticity in the mammalian brain. The involvement of these mechanisms in synaptic plasticity is discussed below.

THE ROLE OF THE AMPA RECEPTOR

In addition to NMDARs, excitatory glutamatergic synapses also contain AMPARs that are subject to activity-dependent changes. Activation of the presynaptic neuron releases glutamate, which in turn activates AMPARs that can depolarize the postsynaptic cell sufficiently to relieve the voltage-dependent Mg^{2+} block of NMDARs. The opening of the NMDAR results in augmented calcium levels that can trigger protein expression and changes in phosphorylation states. AMPARs are heterotetramers that consist of a combination of GluR1–4 subunits that vary in their C-terminal tails, thus interacting with different sets of proteins (Wisden and Seeburg,

1993; Sheng and Sala, 2001). Unlike NMDARs, which are generally static (Benke et al., 1993), AMPARs are dynamic, i.e. they can be trafficked into and out of synaptic sites, in response to activation of AMPARs, NMDARs, metabotropic glutamate receptors (mGluRs), and insulin receptors (Malinow and Malenka, 2002). This trafficking to and away from the synapse has been recognized to play a major role in synaptic plasticity. The amount of AMPARs at the synapse is maintained by the phosphorylation of GluR2–GluR3 subunits that continuously cycle in and out of synapses, whereas the phosphorylation of GluR1–GluR2 subunits and GluR4-containing receptors are internalized from or added into synapses during periods of synaptic weakening or strengthening (Jiang et al., 2006). Blocking AMPAR endocytosis prevents LTD (Luscher et al., 1999; Crozier et al., 2007); blocking delivery of AMPARs to the synapse prevents LTP (Hayashi et al., 2000). Therefore, AMPAR trafficking actively mediates changes in synaptic strength.

THE ROLE OF CaMKII

CaMKII is a highly concentrated multi-subunit calcium/calmodulin-dependent serine-threonine kinase in the postsynaptic densities of hippocampus and neocortex (Kennedy et al., 1983); it is activated by calcium entry through NMDARs, which, in turn, leads to rapid autophosphorylation of CaMKII and renders it persistently active and calcium-independent (Miller and Kennedy, 1986). Upon autophosphorylation, CaMKII can phosphorylate multiple substrate proteins that mediate LTP until it is dephosphorylated by protein phosphatases. In particular, the phosphorylation by CaMKII at one site on the GluR1 subunit, Serine-831, will increase channel conductance and insertion of AMPARs to the synapse and allow for the expression of LTP (Barria et al., 1997a). By blocking phosphorylation of CaMKII, LTP cannot be induced in both the hippocampus and visual cortex (Funauchi et al., 1992; Barria et al., 1997b).

In particular, the role of one of the four isoforms of CaMKII, αCaMKII, has been implicated to be important for LTP expression in both the hippocampus and visual cortex (Funauchi et al., 1992; Silva et al., 1992b; Kirkwood et al., 1997).

Specifically, the interaction of CaMKII with NMDAR subunits may be important for eliciting a certain magnitude of plasticity. In this regard, the NR2B subunit of the NMDAR has a higher affinity to bind to CaMKII than does the NR2A subunit. Since the changes at synapses from NR2B-containing NMDARs to NR2A-containing NMDARs can result in reduced synaptic plasticity, Barria and Malinow performed mutations in the NR2A subunit that allowed for high-affinity binding of CaMKII and were able to restore plasticity. In addition, mutations in NR2B that resulted in lower association with CaMKII blocked synaptic plasticity (Barria and Malinow, 2005). Therefore, these studies show how NMDAR subunits can regulate synaptic plasticity by means of how they interact with downstream molecules such as CaMKII.

THE ROLE OF PKC AND PKA

PKC and PKA have also been implicated as important protein kinases that are capable of phosphorylating the GluR1 subunit of AMPARs, leading to the expression of LTP. Like CaMKII, PKC targets phosphorylation of Serine-831 (Roche et al., 1996). PKA, on the other hand, phosphorylates Serine-845 of the GluR1 subunit, modulating AMPAR function. The involvement of both PKC and PKA has been indicated mainly through the use of specific inhibitors of these kinases. The role of PKC has been established in the maintenance of LTP, such that inhibitor K-252b will not affect the induction of LTP, but will reduce LTP to baseline after an hour in the hippocampus (Reymann et al., 1988). However, other studies have shown that inhibition of PKC can prevent LTP induction, and postsynaptic infusion of PKC can produce potentiation (Hu et al., 1987; Hvalby et al., 1994). Support for a role of PKA arises from studies done in the hippocampus whereby the early and late phases of LTP are sensitive to PKA inhibitors, but postsynaptic infusion of PKA did not enhance synaptic transmission (Frey et al., 1993; Huang and Kandel, 1994; Blitzer et al., 1995). Notably, PKA has been shown to function as a gate for LTP by regulating protein phosphatase activity that can lead to LTD (Blitzer et al., 1995). High levels of PKA can enhance AMPAR function, whereas low levels of PKA can lead to LTD.

THE ROLE OF CALCINEURIN

With regard to homosynaptic long-term depression, a downstream effect of calcium entry through the NMDAR is the inhibition of PKA and the subsequent activation of calcineurin (protein phosphatase 2B), a serine/threonine protein phosphatase (Klee et al., 1979). Calcineurin can (in conjunction with protein phosphatase 1) dephosphorylate specific sites on NMDARs and AMPARs to contribute to the expression of synaptic plasticity (Lieberman and Mody, 1994; Ehlers, 2000). Okadaic acid, which inhibits both protein phosphatase 1 and calcineurin, inhibited LTD-induced dephosphorylation of Serine-845 with no effect on LTP in hippocampus or visual cortex (Mulkey et al., 1993; Kirkwood and Bear, 1994b; Lee et al., 2000). Mechanistically, PKA activators, which inhibit calcineurin, are found to induce enhancement of NMDAR-mediated currents (Cerne et al., 1993); on the other hand, application of calcineurin shortens the duration of NMDAR channel opening in rat dentate gyrus and superior colliculus (Shi et al., 2000; Arias et al., 2002). In addition to dephosphorylation of CaMKII (Barnes et al., 1995), the activiy-dependent induction of calcineurin activity has also been shown to dephosphorylate a site on NR2A subunits, which in turn, coincides with a shortening of NMDAR kinetics (Townsend et al., 2004). In particular, inhibition of protein phosphatase 1 and calcineurin by okadaic acid results in phosphorylation of NR2B subunits in rat hippocampus, which might explain the persistent broadening of NMDAR-mediated currents that are indicative of NR2B-containing NMDARs (Arias et al., 2002).

Collectively, the above discussion demonstrates that there are a number of molecules that can affect the induction of either LTP or LTD by altering their interactions with NMDAR subunits. In addition to these mechanisms, other non-NMDAR-dependent processes have been identified which also contribute to synaptic plasticity; these additional mechanisms are discussed in Chapter V.

THE BCM THEORY

In the brain, the strength of cortical synapses has been shown to be bidirectionally modifiable; in response to salient stimulation, synapses may be persistently enhanced (long-term potentiation; LTP) or weakened (long-term depression; LTD). These activity-dependent modifications of synaptic connections underlie refinement of cortical organization in visual cortex. Metaplasticity is a higher order feature of the brain: it is the plasticity of synaptic plasticity (Abraham and Bear, 1996). It is a process that describes how the activation history of synapses modifies their reactions to subsequent experience. Bienenstock, Cooper, and Munro incorporated bidirectional synaptic plasticity into a synaptic learning rule that accounts for experience-dependent acquisition of stimulus selectivity (Figure 1-1, p. 36). In this model, the point of crossover from weakening to strengthening of synapses is called the modification threshold (θ_m). The modification threshold is not fixed; rather, the direction the threshold slides depends on the history of postsynaptic activity. Essentially, it monitors the direction or magnitude of subsequent activity-dependent synaptic plasticity for maintaining homeostasis in the brain (Bienenstock et al., 1982; Bear, 2003). For instance, dark-rearing an animal provides an environment of low visual activity that will shift the threshold to the left, thus favoring subsequent LTP (Kirkwood et al., 1996). Conversely, activating synapses with sensory stimulation that does not lead to either LTP or LTD will shift the threshold to the right, thus favoring LTD (Huang et al., 1992). As a result, the properties of synaptic plasticity are "metaplastic", to keep synaptic strengths within a useful dynamic range (Bear, 2003).

VISUAL CORTICAL PLASTICITY

Numerous studies of how mechanisms of synaptic modification are incorporated in the developing mammalian brain have been performed in various sensory regions of neocortex. A well characterized sensory area is the visual cortex, where visual experience helps to refine synaptic connections throughout development (Gordon and Stryker, 1996). An excellent model system to study experience-dependent modifications is the rodent visual cortex as it is extremely sensitive to changes in visual experience, which are relatively simple to control. Furthermore, mice are particularly amenable to powerful genetic tools that are readily available for evaluating the molecular and cellular mechanisms underlying visual cortical plasticity.

PARADIGMS THAT INDUCE VISUAL CORTICAL PLASTICITY

Previous studies have suggested that the threshold for synaptic modifications can itself be modified by visual experience, and visual experience can be manipulated in a variety of ways. Environmental manipulations, such as dark-rearing or eyelid suture, demonstrate that the proper development of visual circuitry is governed by experience. In addition to dark-rearing that can delay developmental maturation of visual response properties (Carmignoto and Vicini, 1992), another way to understand the role of experience in development is by studying the properties of ocular dominance (OD) plasticity. Monocular deprivation (MD), achieved by suturing one eyelid shut, invokes rapid changes in visual cortex whereby synaptic connections subserving the deprived eye are weakened while synaptic connections subserving the non-deprived eye are maintained (Hubel and Wiesel, 1970). This manipulation invokes OD plasticity, which manifests anatomically as a decrease in the territory

occupied by inputs subserving the deprived eye, and as an increase in the area of inputs driven by the non-deprived eye (Antonini et al., 1999). Physiologically, there is a reduction in the magnitude of responses subserving the deprived eye, and an increase in the responses driven by the non-deprived eye (Frenkel and Bear, 2004). This OD shift has two functional phases: first, there is a rapid deprivation-induced depression of deprived-eye responses and, subsequently, a delayed potentiation of non-deprived eye responses (Frenkel and Bear, 2004). Importantly, the mechanisms of LTD appear to underlie the synaptic depression of deprived-eye inputs (Heynen et al., 2003).

The malleability of the visual system in response to various forms of visual experience is a great model to understand how experience modifies the brain. Dark-rearing or binocular deprivation can be used to reveal how visual experience shapes the receptive fields of visual cortical neurons. Extracellular recordings from the primary visual cortex in animals reared in complete darkness revealed an absence of orientation and stimulus selectivity that is normally present in animals that are light-reared (Fagiolini et al., 1994). When receptive field properties are measured in dark-reared rodents at postnatal day (P) 60, for example, visual acuity is immature and resembles that of light-reared animals before the onset of juvenile plasticity. However, light deprivation can extend the period of juvenile plasticity by several weeks. This process is reversible; exposure of as little as two hours of light can rapidly promote synaptic enhancements (Buisseret et al., 1978).

However, there are a number of visual cortical events that do not require visual experience such as the establishment of a cortical map of visual space (Ruthazer and Stryker, 1996), ocular dominance (Crair et al., 1998), and crude orientation selectivity (Stryker and Harris, 1986). Upon eye opening, rodent visual cortex undergoes a refinement of connections in response to visual stimulation; patterned visual experience provides refinement of orientation and direction selectivity

(Sengpiel et al., 1999; Li et al., 2006), modification of OD (Wiesel and Hubel, 1963), and alterations in receptive field properties (Smith and Trachtenberg, 2007). During this sensitive period in early postnatal life, the visual cortex is particularly susceptible to activity-driven modifications. This window of time in juveniles is known as the critical period, during which robust plasticity occurs (postnatal 3–5 weeks). During this time, many types of synaptic plasticity such as LTP are more robust than when the animal reaches the adult stage (Kirkwood and Bear, 1995). At this stage, experience-dependent plasticity takes longer to achieve and is of less magnitude (Katz, 1999). Therefore, since plasticity still occurs in adulthood, the critical period will be referred to as the period of juvenile plasticity (Sato and Stryker, 2008).

The influence of experience versus development is difficult to separate into two entities; the two phenomena are intertwined and edify one another. Thus, interpretations as to what mechanisms underlie either category have been challenging to assess (Crowley and Katz, 2000). In this discussion, mechanisms underlying experience-dependent plasticity have emerged based on the correlations of the spatial and temporal overlap of proteins and their subsequent changes as a consequence of disruptions in visual experience.

MOLECULAR MECHANISMS UNDERLYING METAPLASTICITY

THE ROLE OF THE NMDA RECEPTOR

Studies have established that normal, experience-dependent modifications of visual cortex receptive fields require activation of NMDARs. In particular, experience-dependent regulation of NMDAR composition appears to regulate the induction of synaptic plasticity. For example, upon eye opening, both biochemical and electrophysiological studies demonstrate a lateral switch of predominant NR2B-

containing NMDARs at the synapse to extrasynaptic sites while a steady increase of NR2A-containing NMDARs cluster at the synapse (Li et al., 1998; van Zundert et al., 2004). Similarly, PSD-95 is also redistributed to synapses in the mouse superior colliculus and visual cortex upon eye opening. This upregulation of PSD-95 has been found to facilitate the concomitant upregulation of NR2A subunits and the downregulation of NR2B subunits at the synapse (Yoshii et al., 2003). This process is also dynamic; if the eyelids are sutured shut after eye opening, the levels of PSD-95 decrease from the synapse along with a coincident decrease in NR2A subunits. Therefore, PSD-95 is important for increasing surface expression of NR2A-containing NMDARs, as well as decreasing the rate of their internalization (Lin et al,. 2004). In studies of animals with normal visual development, biochemical assays of primary cultures of juvenile cortical neurons have shown that NR2A-containing NMDARs tend to localize directly apposed to synaptic vesicle release sites, whereas NR2B-containing NMDARs are localized in perisynaptic or extrasynaptic locations (Li et al., 1998). Thus, the localization of NMDAR subunits at the synapse is altered by the introduction of visual experience.

Delaying the proper development of visual circuitry by dark-rearing from birth also delays the onset of shorter NMDAR EPSCs without affecting the amplitude or rise times of NMDAR responses in visual cortex (Carmignoto and Vicini, 1992). Indeed, biochemical studies demonstrated that dark-rearing mice delays the increase in NR2A levels (Chen and Bear, 2007). Therefore, a low NR2A/NR2B ratio results in the slower kinetics seen in dark-reared animals, whereas a high NR2A/NR2B ratio is associated with the faster currents seen in light-reared animals (Carmignoto and Vicini et al., 1992). In support of the BCM theory, previous studies have suggested that the threshold for synaptic modifications can be modified by visual experience; the induction of LTP is favored in the visual cortex of animals reared in the dark compared to that of light-reared controls (Kirkwood et al., 1996). Specifically,

dark-rearing animals will result in a shift in the modification threshold to the left, favoring the strengthening of synapses (Kirkwood et al., 1996; Philpot et al., 2003). Consequently, the two receptor subtypes contribute a difference in kinetics to the NMDAR as well as in synaptic plasticity.

The switch in subunits is also dynamic, i.e. metaplastic. For instance, while light deprivation promotes retention of high levels of NR2B-containing NMDARs and delays insertion of NR2A-containing NMDARs, light exposure for just 2 hours can lead to a robust increase in NR2A levels. This increase is absent following application of the translation inhibitor cycloheximide, indicating the importance of experience-induced NR2A protein synthesis (Quinlan et al., 1999b). Alternatively, dark-exposing light-reared animals or returning light-exposed dark-reared animals into the dark for 3–4 days can significantly decrease NR2A levels and increase the duration of NMDAR-mediated EPSCs, similar to those in age-matched dark-reared animals (Quinlan et al., 1999a). Therefore in juveniles, the change in the properties of NMDAR-mediated responses reflects the diversity in NMDAR subunit composition.

In the instance of MD, applying the NMDAR antagonist APV in kitten visual cortex prevents the consequence of shrinking lateral geniculate cells in response to the deprived eye. This study performed by Bear and Colman establishes the contribution of the NMDAR to cortical plasticity (Bear and Colman, 1990). Similarly, in the mouse, blocking NMDARs with competitive antagonist CPP showed that the temporal phases, depression of deprived-eye responses and the potentiation of non-deprived eye responses, comprising the OD shift are NMDAR-dependent in juveniles (Sato and Stryker, 2008; Cho et al., 2009). Furthermore, by using a CxNR1 mouse, a mutant mouse that loses functional NMDARs in adulthood, there was a loss of OD plasticity in the visual cortex by the age of P70, indicating that adult plasticity requires NMDARs (Sawtell et al., 2003).

To understand how OD plasticity affects NMDAR subunit composition, Chen and Bear also performed an assessment of NMDAR subunit composition following the two phases of MD in juvenile mouse visual cortex. After 3 days of MD when depression of deprived-eye responses takes place, NR2B protein is increased. After 7 days of MD when potentiation of non-deprived eye responses takes place, NR2A levels are decreased with no overall change in NR1 subunits (Chen and Bear, 2007). Therefore, there is a correlation of subunit change with the physiological changes induced by MD.

With regard to adult visual cortical plasticity, adult rats that received 10 days of dark exposure before brief MD (the time course can also include 1–3 days of light exposure in between) can exhibit a juvenile-like shift in OD plasticity (He et al., 2006). Quantitative immunoblot analysis of NMDAR proteins in these adults following MD showed a decrease in the NR2A/NR2B ratio due to an increase in NR2B protein in perisynaptic or extrasynaptic sites (Yashiro et al., 2005; He et al., 2006). Overall, these findings suggest that experience-dependent modifications in the properties of synaptic plasticity are correlated with, and possibly governed by, changes in NMDAR subunit composition. Although the biochemical and electrophysiological data demonstrate strong correlations that the NMDAR subunit composition and function underlie changes in the threshold for synaptic modifications, it has not been established by causality. With the use of a genetically modified mouse in which NR2A protein levels are not functionally expressed, it is possible to truly fix the NR2A/NR2B ratio and to observe its effects on the properties of synaptic plasticity and metaplasticity.

It has already been established that the function of NMDARs are unique; they serve as a gating mechanism for calcium to subsequently trigger second messenger cascades to establish a proper threshold for synaptic plasticity (Mori and Mishina, 1995). The importance of mechanisms downstream of NMDAR activation has also

been implicated in OD plasticity studies; the roles of activity-regulated cytoskeleton-associated protein (Arc), CaMKII, PKA, calcineurin, and tissue plasminogen activator (tPA) in metaplasticity are discussed below.

THE ROLE OF Arc

The immediate early gene protein Arc is observed at excitatory glutamatergic synapses in visual cortex and rapidly activates and accumulates in dendrites in response to visual stimulation (Lyford et al., 1995; Steward et al., 1998). Arc activation is necessary for activity-dependent refinement of visual projections to visual cortex (Wang et al., 2006), and its importance in synaptic plasticity was tested in hippocampus whereby Arc antisense oligonucleotides attenuate LTP (Guzowski et al., 2000). Its expression is also NMDAR-dependent, since blocking NMDARs prevents Arc expression (Lyford et al., 1995). In visual cortex, a main role for Arc is to regulate AMPAR internalization by interacting with the endocytic machinery (Chowdhury et al., 2006). Specifically, Arc KO mice display decreased endocytosis as well as an increased number of surface AMPARs (Chowdhury et al., 2006). This decrease in the ability for endocytosis will have an impact on the typical loss of AMPARs at the surface that occurs during LTD and MD (Heynen et al., 2003). Therefore, as expected, Arc KO mice exhibit a small OD shift due to reduced deprived-eye depression (McCurry et al., submitted). There is also a decrease in the amount of internalized AMPARs in these mutant mice. This versatile Arc molecule is now emerging as an active participant underlying synaptic plasticity.

THE ROLE OF CaMKII

With regard to CaMKII, Taha and Stryker used mutant mice that contained a variant of CaMKII that cannot be converted to the constitutively active, calcium-independent state. In visual cortical plasticity studies, they demonstrated that in the absence of constitutively active CaMKII, the induction of the OD shift took longer to acquire

(Taha and Stryker, 2005). Therefore, the conversion of CaMKII to the calcium-independent form is important for rapid expression of plasticity in the visual cortex.

THE ROLE OF CALCINEURIN AND PKA

In order to understand the role of calcineurin in metaplasticity, Yang et al. used a conditional calcineurin overexpressing mouse and discovered no OD shift compared to wild-types even if the deprivation was prolonged. The loss of the OD shift is due to the loss of depression of deprived-eye responses, suggesting that LTD is saturated in these animals. The loss of the OD shift is also particular to calcineurin overexpression, since suppression of calcineurin will restore OD plasticity (Yang et al., 2005). PKA inhibitors have also been shown to block the induction of OD shifts in kitten visual cortex (Beaver et al., 2001). Furthermore, PKA's four regulatory subunits (RIα, RIβ, RIIα, RIIβ) connote individual contributions to plasticity: deletion of RIβ leads to loss of both LTP and LTD without changing OD plasticity (Hensch et al., 1998b); deletion of RIIα decreases the magnitude of LTP, does not affect LTD but still allows for the depression of deprived-eye responses of the OD shift (Rao et al., 2004); and deletion of RIIβ does not affect LTP, but blocks LTD and the OD shift (Beaver et al., 2002). Although brief MD has been found to induce LTD in visual cortex (Heynen et al., 2003), the select effects of blocking LTD without affecting OD reveal different types of plasticity at play. Thus, the PKA signaling pathway is important for synaptic and OD plasticity. However, conferring their importance to particular subunits is complex and not fully determined.

THE ROLE OF tPA

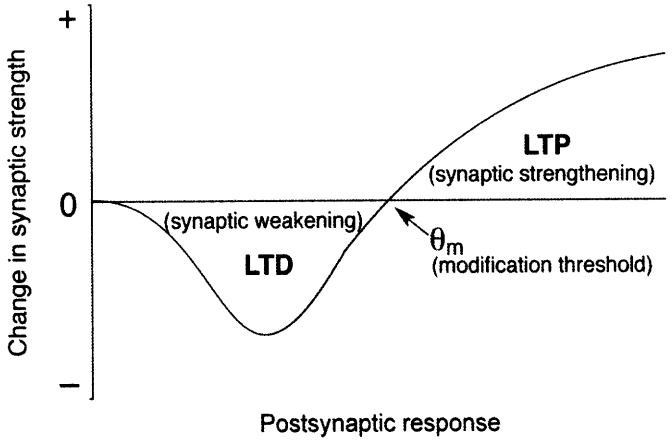
Another candidate molecular mechanism for metaplasticity is extracellular matrix molecule tPA. tPA forms a stable complex with the NR1 subunit of the NMDAR in mouse cortical cultures; its NMDAR-dependent release into the extracellular space via calcium-dependent secretion results in the cleavage of the amino-terminus of NR1.

The cleavage of NR1 enhances signaling mediated by NMDARs – too much cleavage can actually result in NMDAR-induced neuronal death (Nicole et al., 2001). In the hippocampus, tPA co-immunoprecipitates with NR2B and tPA expression stimulates NR2B expression; in tPA KO mice, the typical upregulation of NR2B is absent (Pawlak et al., 2005). Behaviorally, the tPA KO are deficient in hippocampus-dependent tasks whereby the animal takes longer to learn but eventually learns. Infusion of tPA reverses these deficits, and facilitates LTP and learning (Pawlak et al., 2002).

tPA is also expressed in primary rat visual cortex, particularly in layers II/III and IV. Its appearance correlates with the period of robust juvenile plasticity and declines thereafter (Zheng et al., 2008). Studies in visual cortex show a role for tPA in OD plasticity. It was already known that structural changes occur following long periods of MD whereby thalamocortical axons subserving the deprived eye retract whereas arbors subserving the non-deprived eye expand (Antonini and Stryker, 1993; Antonini et al., 1999). However following brief MD, rapid structural changes have also been found to occur at the level of dendritic spines. Dendritic spines are important for plasticity since they receive the majority of excitatory input, and their physical features are sensitive to activity (Hering and Sheng, 2001). For instance, spine motility decreases over development, and remains stable during the period for robust juvenile plasticity (Lendvai et al., 2000; Majewska and Sur, 2003). Brief MD causes spine motility to increase in the area contralateral to the deprived eye (Oray et al., 2004). This change in structural plasticity has been linked to tPA because tPA levels are upregulated after 2 days of MD. In addition, exogenous application of tPA has been shown to increase spine motility similar to that caused by brief MD (Mataga et al., 2004; Oray et al., 2004). tPA KO mice showed little or no OD shift, but the OD shift could be restored upon intracerebroventricular tPA infusion (Mataga et al., 2002). Therefore, the extracellular matrix can contribute to structural changes that ultimately affect its functional role in metaplasticity.

It is apparent that there are multiple mechanisms that may underlie experience-dependent modification of synaptic plasticity in visual cortex. To increase our understanding of cellular mechanisms in juvenile mouse visual cortex, we have utilized transgenic technologies to directly determine the functional significance of proteins that have been indicated to play a role in plasticity. Therefore, we took advantage of a genetically modified mouse in which the NR2A subunit is not functionally expressed and examined its effect on the properties of synaptic plasticity and metaplasticity. Speculations on other possible mechanisms underlying both synaptic plasticity and metaplasticity are discussed in Chapter V.

FIGURE 1-1



The Bienenstock, Cooper and Munro model of metaplasticity

The contribution of bidirectional synaptic modifications to ocular dominance (OD) plasticity in visual cortex can be modeled by a learning rule where low levels of postsynaptic activation induce long-term depression (LTD) and high levels induce long-term potentiation (LTP) (Bienenstock et. al., 1982). This model suggests that the magnitude of the postsynaptic response determines the modification of synaptic weight. The crossover from LTD to LTP is called the modification threshold (θ_m). Importantly, the value of θ_m is not fixed; rather, it "slides" as a function of the history of postsynaptic activation. The direction the modification threshold slides is determined by the history of postsynaptic activity.

CHAPTER II

Obligatory Role of NR2A for Metaplasticity in Visual Cortex

This chapter was published together with Dr. Benjamin Philpot and Dr. Mark Bear in Neuron (2007) Vol. 53, pp. 495-502.

ABSTRACT

Light deprivation lowers the threshold for long-term depression (LTD) and long-term potentiation (LTP) in visual cortex by a process termed metaplasticity, but the mechanism is unknown. The decreased LTD/LTP threshold correlates with a decrease in the ratio of NR2A to NR2B subunits of cortical NMDA receptors (NMDARs) and a slowing of NMDAR-mediated excitatory postsynaptic currents (EPSCs). However, whether and how changes in NR2 subunit expression contribute to LTD and LTP have been controversial. In the present study, we used an NR2A knockout (KO) mouse to examine the role of this subunit in the experience-dependent modulation of NMDAR properties, LTD, and LTP. We found that deletion of NR2A abrogates the effects of visual experience on NMDAR EPSCs and prevents metaplasticity of LTP and LTD. These data support the hypothesis that experience-dependent changes in NR2A/NR2B are functionally significant and yield a mechanism for an adjustable synaptic modification threshold in visual cortex.

INTRODUCTION

Metaplasticity – the plasticity of synaptic plasticity – is a change in the properties of synaptic modification as a function of the recent history of cellular or synaptic activation (Abraham and Bear, 1996). A robust demonstration of metaplasticity is the change in the properties of long-term potentiation (LTP) and long-term depression (LTD) in visual cortex of animals deprived of vision (Kirkwood et al., 1996; Philpot et al., 2003). After a period of reduced cortical activity caused by dark-rearing (Czepita et al., 1994; Maffei et al., 2006), LTP is enhanced and LTD is reduced over a range of stimulation frequencies compared to light-reared (LR) animals. These changes are rapidly reversed by light exposure of the dark-reared (DR) animals. Such findings are in accordance with the theoretical proposal of a synaptic modification threshold that "slides" as a function of the average activity of cortical neurons (Bienenstock et al., 1982). A sliding modification threshold enables the synaptic competition that yields stimulus-selective neuronal responses and contribute to homeostasis by keeping the network of modifiable synapses within a useful dynamic range (Bear, 2003).

The forms of LTP and LTD in visual cortex that are subject to metaplastic regulation are triggered by strong and weak activation of postsynaptic NMDA receptors (NMDARs), respectively. A mechanism for metaplasticity is suggested by the observations both *in vitro* and *in vivo* that NMDAR function is enhanced in visual cortex of DR animals and diminished after brief light exposure (Tsumoto et al., 1987; Fox et al., 1992; Philpot et al., 2003). Such changes in NMDAR function would be expected to lower and raise LTD/LTP thresholds, respectively.

While there are many ways to modulate NMDAR effectiveness, one appealing mechanism is the activity-dependent regulation of NMDAR subunit expression and assembly. NMDARs consist of the obligatory NR1 subunit in combination with NR2A–D and NR3A–B subunits (Perez-Otano et al., 2001). NR2A and NR2B subunits, which predominate in postnatal cortex (Watanabe et al., 1993; Monyer et al., 1994;

Sheng et al., 1994), exhibit several important differences that can influence NMDAR-mediated plasticity. First, NR2B-containing NMDARs have longer current duratinos than NR2A-containing receptors, due to a higher affinity for glutamate and slower rates of desensitization (Monyer et al., 1992; Laurie and Seeburg, 1994; Priestley et al., 1995; Vicini et al., 1998). Second, NR2B-containing NMDARs carry more calcium charge per unit current than NR2A subtypes (Sobczyk et al., 2005). Third, NR2A and NR2B subunits have distinct intracellular binding partners (Husi et al., 2000; Sans et al., 2000; Steigerwald et al., 2000; Gardoni et al., 2001; Vissel et al., 2002; Yoshii et al., 2003; Barria and Malinow, 2005).

Complementary biochemical, biophysical, and pharmacological studies have shown that dark-rearing leads to a decreased NR2A/NR2B ratio in synaptic NMDARs, which is rapidly reversed by light exposure (Quinlan et al., 1999a; Quinlan et al., 1999b; Philpot et al., 2001a; Chen and Bear, 2007). The time course of these bidirectional changes in NMDAR subunit composition correlates well with the changes in LTD/LTP thresholds in visual cortex. Thus, it has been hypothesized that inactivity caused by light deprivation gradually decreases the NR2A/NR2B ratio, which raises the LTD/LTP threshold. This model is attractive since a lowered NR2A/NR2B ratio has the effects of prolonging and increasing calcium curents through NMDARs and recruiting calcium/calmodulin-dependent protein kinase II – changes that would be expected to lower the threshold level of NMDAR activation required to trigger LTP.

Other than the correlations mentioned above, however, there is no direct evidence that NMDAR subunit composition contributes to metaplasticity in visual cortex. Resolving this question has taken on new importance with the publication of recent reports suggesting an entirely different view of how NR2A and NR2B subunits contribute to LTP and LTD, respectively (Liu et al., 2004; Massey et al., 2004). According to these studies, decreasing the NR2A/NR2B ratio should promote LTD over LTP. To distinguish among these very different hypotheses and to determine the

contribution of NR2 subunit expression to metaplasticity in visual cortex, we have studied the effects of visual experience and deprivation on the properties of NMDAR-mediated synaptic transmission, LTP, and LTD, in visual cortex of mice with a genetic ablation of NR2A.

RESULTS

NR1, NR2B, AND GluR1 LEVELS ARE UNCHANGED IN JUVENILE NR2A KO MICE

To test for possible compensatory alterations of glutamate receptor expression in NR2A-deficient mice, we first performed an immunoblot analysis of NMDA (NR1, NR2A, and NR2B) and AMPA (GluR1) receptor subunit expression in synaptoneurosomes prepared from visual cortex of +/+, +/−, −/− mice (Figure 2-1). To control for protein loading and the degree of synaptic enrichment, we normalized receptor subunit protein levels to the synaptic vesicle protein synapsin (Table 2-1). As expected, NR2A levels were significantly reduced in NR2A-deficient mice (*n*'s = 6, 5, and 7 animals for +/+, +/−, −/−, respectively; $F_{(2, 17)} = 17.83$, $p < 0.0002$). Consistent with previous observations, NR2A levels in +/− mice were approximately half that of wild-types (Sakimura et al., 1995; Kutsuwada et al., 1996). Despite reductions in NR2A levels in +/− and −/− mice, levels of the obligatory NR1 subunit were unchanged in NR2A-deficient mice ($F_{(2,17)} = 1.55$, $p = 0.24$). There was also no significant effect on NR2B levels ($F_{(1,5)} = 0.34$, $p = 0.59$) as observed previously (Morikawa et al., 1998). Levels of the GluR1 AMPA receptor subunit were also not changed in the mutant mice ($F_{(2,17)} = 0.88$, $p = 0.44$). These data indicate that there were no gross abnormalities in the expression of ionotropic glutamate receptors at the synapse, other than the expected loss of NR2A expression in +/− and −/− mice. Our observation that other ionotropic glutamate receptor subunit levels remain unchanged in the visual cortex

of NR2A-deficient mice is similar to previous findings demonstrating that NR2B, NR1, and GluR1 levels in the immature somatosensory cortex are unaltered by the loss of NR2A (Lu et al., 2001).

LOSS OF NR2A MIMICS AND OCCLUDES THE EFFECT OF DARK-REARING ON NMDAR EPSCs

To test for gross differences in NMDA receptor function in layer II/III of visual cortex, we generated input-output curves of pharmacologically-isolated NMDA receptor-mediated EPSCs in +/+, +/−, and −/− mice reared normally or in complete darkness (with sample sizes between 15 and 35 cells for each of the six groups, Figure 2-2; data from heterozygotes were excluded from this figure for clarity but were included in all statistics). In this and all subsequent comparisons, the experimenters were blind to genotype. Because ANOVA revealed a significant main effect ($F_{(5, 1590)} = 3.2$, $p < 0.008$), *post-hoc* analyses were used to test for group differences. This analysis revealed that dark-rearing significantly enhanced the input-output relationship of NMDAR responses in wild-type (WT) mice (Figure 2-2d; $p < 0.0007$). However, dark-rearing failed to alter the input-output curves of NMDAR responses in NR2A knockout (KO) mice (Figure 2-2e; $p = 0.79$). Consistent with our observation that NR1 levels were unchanged in NR2A-deficient mice, we detected no reduction in the evoked NMDAR currents in NR2A-deficient mice at this age. Our direct measurements of the relationship between stimulation intensity and NMDAR EPSC suggest that there is no net loss in the number of NMDARs functionally expressed at the synapse in NR2A-deficient mice. This finding is consistent with a report from Lu and colleagues who demonstrated that the ratio of NMDAR/AMPAR synaptic currents in the immature somatosensory cortex are not influenced by genetic deletion of NR2A (Lu et al., 2001). Coupled with our biochemical data demonstrating that there is no gross change in NR2B levels, our electrophysiological observations suggest that NR2B-containing NMDARs that would normally be targeted to perisynaptic sites are likely targeted to

synaptic sites in the absence of NR2A. Thus, at least at the ages examined and layer examined, NMDAR current amplitudes are not impaired in the absence of NR2A.

To test the contribution of NR2A subunits to experience-dependent modifications in current duration (Philpot et al., 2001a), we recorded NMDAR EPSCs in visually-identified layer II/III pyramidal cells. As expected, dark-rearing significantly increased NMDAR current duration in layer II/III pyramidal cells of normal mice (Figure 2-3a; $F_{(1,127)} = 13.31$, $p < 0.0005$). The NR2A deficiency also prolonged NMDAR current durations ($F_{(2,127)} = 13.72$, $p < 0.0001$), and there was a significant interaction between rearing condition and genotype on EPSC duration ($F_{(2,27)} = 4.40$, $p < 0.02$). *Post-hoc* analyses (Fisher's PLSD) revealed that LR, WT animals had significantly faster currents than that of DR animals and NR2A-deficient mice (+/–, –/–). Notably, dark-rearing had no effect in mice lacking functional NR2A subunits ($p = 0.89$). These data demonstrate that genetic ablation of functional NR2A subunits mimics and occludes the consequences of light deprivation.

We have previously demonstrated that the temporal summation of NMDAR-mediated EPSCs is extremely sensitive to small changes in NMDA receptor current durations (Philpot et al., 2001a). NMDAR currents in layer II/III pyramidal cells were evoked by extracellular stimulation of layer IV for 11 pulses at 40 Hz, and the total EPSC amplitude was measured after each pulse (Figure 2-3b). Total EPSC amplitude was described by a significant interaction between pulse number, genotype, and rearing condition ($F_{(20,1200)} = 2.4$, $p < 0.0007$). Consistent with the observation that NMDAR current durations from LR, WT mice differed significantly from all the other groups, *post-hoc* analyses revealed that the only significant differences in temporal summation arose between the LR, WT mice versus all the other groups. These data demonstrate that modest increases in NMDA receptor current duration, driven by a genetic or experience-dependent reduction in NR2A levels, are sufficient to maximize temporally summated NMDAR EPSCs generated by 40 Hz stimulation. Moreover, the genetic loss of NR2A occludes further changes by dark-rearing on NMDAR EPSC temporal summation.

DEPRIVATION-INDUCED METAPLASTICITY FAILS TO OCCUR IN NR2A KO MICE

To assess differences in cortical plasticity, layer II/III field potentials were evoked by layer IV stimulation in visual cortical slices of P21–P28 mice (Kirkwood et al., 1996; Philpot et al., 2003). There was no effect of deleting NR2A on LTP induced in layer II/III of slices prepared from LR animals. Remarkably, however, the metaplasticity of LTP and LTD was completely absent in the NR2A KO mice.

In WT mice, dark-rearing significantly enhanced LTP induced with a 40 Hz tetanus, confirming previous observations in rats (Kirkwood et al., 1996). LTP in LR mice measured 111.8 ± 4.2 % of baseline (n = 13) compared to 123.1 ± 1.9 % of baseline in DR mice (n = 8; $p < 0.05$; Figure 2-4a). In contrast, there was no effect of dark-rearing on the level of LTP induced by 40 Hz stimulation in NR2A KO mice compared with LR, NR2A KO mice (LR: 114.4 ± 2.9 % of baseline, n = 14; DR: 113.6 ± 3.8 % of baseline, n = 9; p = 0.88; Figure 2-4b). These data suggest that although NR2A is not required for LTP in visual cortex, it is essential for experience-dependent metaplasticity.

In WT mice, dark-rearing significantly reduced LTD induced with a 1 Hz tetanus, again confirming previous findings in rats (Kirkwood et al., 1996; Philpot et al., 2003). LTD in LR mice measured 82.0 ± 2.7 % of baseline (n = 6) compared to 92.7 ± 1.4 % of baseline in DR mice (n = 6; $p < 0.003$; Figure 2-4c). We were surprised to find that 1 Hz stimulation in LR, NR2A KO mice produced no LTD at all; instead, responses modestly potentiated (114.9 ± 1.8 % of baseline, n = 5; Figure 2-4d). Moreover, there was no longer any difference between LR and DR animals with respect to the plastic response to 1 Hz stimulation (DR: 111.3 ± 6.8 % of baseline, n = 8). These data suggest that the LTP threshold is greatly reduced in the absence of NR2A and no longer modified by the history of cortical experience.

To better determine how genetic deletion of NR2A altered the shape of the frequency-response function, we tested the consequences of 30 minutes of 0.5 Hz stimulation in WT and KO mice reared normally (Figure 2-4e-f). We found that this manipulation produced a modest but significant depression in both KO and WT mice (WT: 91.2 ± 8.2 % of baseline, n = 6; KO: 87.9 ± 1.6 % of baseline, n = 10). These data indicate that it is possible to weaken synapses in NR2A KO mice, and that the genetic deletion of NR2A produces an extreme leftward shift in the frequency-response function (see Figure 2-5).

DISCUSSION

To summarize the major findings of this study, our data show that visual experience regulates NMDAR EPSC kinetics and temporal summation, LTP, and LTD in layer II/III of mouse visual cortex as previously reported in rats (Carmignoto and Vicini, 1992; Kirkwood et al., 1996; Philpot et al., 2001a; Philpot et al., 2003), and that these effects of experience require the NMDAR NR2A subunit. In the absence of NR2A, the NMDAR EPSC kinetics and summation resemble those observed in DR, WT mice, and there is no additional effect of dark-rearing in the NR2A KO mice. Metaplasticity of LTP and LTD is completely lost in the absence of NR2A, and the loss of NR2A appears to greatly lower the threshold for inducing LTP with low-frequency stimulation. Taken together, the data support the hypothesis that experience-dependent changes in NR2A/NR2B, documented previously in visual cortex (Quinlan et al., 1999a; Quinlan et al., 1999b; Chen and Bear, 2007), are functionally significant and provide a mechanism for an adjustable synaptic modification threshold (Bear, 2003).

Our data provide direct evidence that experience-dependent modifications in NMDAR current duration are due to a change in NR2 subunit composition. Previous studies demonstrated a strong correlation between an increase in sensory

experience, an increase in the NR2A/NR2B ratio, and faster NMDAR current durations (Carmignoto and Vicini, 1992; Hestrin, 1992; Crair and Malenka, 1995; Flint et al., 1997; Stocca and Vicini, 1998; Nase et al., 1999; Quinlan et al., 1999b; Philpot et al., 2001a). The correlation between the expression of NR2A and NMDAR kinetics in hippocampus (Kirson and Yaari, 1996), visual cortex (Quinlan et al., 1999b; present study), and somatosensory cortex (Flint et al., 1997; Lu et al., 2001) suggested that changes in NMDAR composition underlie the developmental and experience-dependent modifications in NMDAR current duration (although see (Barth and Malenka, 2001), an idea supported by the absence of developmental shortening of NMDAR currents in neurons lacking NR2A (Fu et al., 2005). However, other mechanisms also contribute to the developmental shortening of NMDARs (Prybylowski et al., 2000; Rumbaugh et al., 2000; Shi et al., 2000). The present study provides direct evidence for the importance of NR2A for experience-dependent modifications in NMDAR currents, because the consequences of dark-rearing on NMDAR current duration are mimicked and occluded by the genetic loss of NR2A function.

While our data clearly show that the presence of NR2A is a molecular requirement for experience-dependent modifications in NMDAR current duration in visual cortex, it remains to be seen if NMDAR current duration is modified at the level of NR2A expression itself or through posttranslational modifications occurring through NR2A. For example, Shi and colleagues suggested that calcineurin activity can shorten NMDAR kinetics (Shi et al., 2000). Because the phosphatase can act through NR2A (e.g. (Krupp et al., 2002)), we cannot rule out the possibility that posttranslational modifications specific to NR2A are responsible for experience-dependent modifications in NMDAR current. Given evidence that NMDARs are largely unaffected by genetic deletion of calcineurin (Zeng et al., 2001), we favor the idea that the ratio of NR2A/NR2B is the major underlying factor for experience-dependent modifications in visual cortex. Regardless, our data indicate that experience-

dependent modifications in NMDAR kinetics within visual cortex require the presence of NR2A and are unlikely to occur through altered expression of NR1 splice variants or posttranslational modifications of NR1 or NR2B.

The importance of the NR2B to NR2A subunit switch for synaptic plasticity has remained a matter of controversy. Several influential papers suggest that NR2A and NR2B subunit-containing NMDARs have a separable role in synapse modification, with NR2A subtypes regulating LTP and NR2B subtypes regulating LTD (Liu et al., 2004; Massey et al., 2004). The validity of these findings is now being questioned because of suggestions that these studies were conducted using nonspecific concentrations of NR2A-selective antagonists and/or because of difficulty replicating the initial findings (Berberich et al., 2005; Neyton and Paoletti, 2006; Morishita et al., 2007). Recent data contradict the initial findings that NR2A and NR2B play distinct roles in regulating the polarity of synaptic plasticity, and instead suggest that either subunit is capable of inducing LTD and LTP (Berberich et al., 2005; Weitlauf et al., 2005; Zhao et al., 2005). While much of the confusion surrounding the role of NMDAR subtypes in synaptic plasticity may be the result of regional and developmental differences in NMDAR expression (see (Philpot et al., 2001b)), our data support an alternative interpretation of the function of the NMDAR subunit switch. We suggest that the NR2A/NR2B ratio reflects—and is set by—the amount of ongoing cortical activity, which normally increases during postnatal development. The NR2A/NR2B ratio establishes the threshold for subsequent activity-dependent synaptic modifications. We favor the idea that this threshold is set directly by the biophysical and biochemical properties of NMDARs at modifiable synapses, but additional sequelae, such as a change in inhibitory tone (Steele and Mauk, 1999; Fagiolini et al., 2003) may also contribute.

Although we observed an extreme reduction in the stimulation frequency required to induce LTP in the NR2A KO mice (see Figure 2-5), the absence of NR2A failed to enhance the magnitude of LTP that can be induced, consistent with previous

observations (Lu et al., 2001). This finding suggests that there may be separable mechanisms for regulating the threshold for inducing synaptic plasticity and the magnitude of the expressed plasticity. A parsimonious explanation for the lack of enhanced LTP in NR2A KO mice may be that there are limited resources available to make synapses stronger, and that these may be tightly and independently regulated in a homeostatic manner. Consistent with this idea, potentiated synapses have been observed to compete for a limited supply of "plasticity factors", which can limit the total synaptic expression of LTP (Fonseca et al., 2004).

Taken together, the data suggest that during visual cortical development the progressive increase in the NR2A/NR2B ratio in a normal visual environment raises the threshold for inducing LTP such that only the most strongly correlated inputs are maintained while less correlated inputs are more likely to be weakened. This experience-dependent adjustment in the plasticity threshold normally allows neurons to become progressively more tuned to select features of the environment (Bienenstock et al., 1982). Thus, the loss of orientation selectivity and incomplete expression of ocular dominance plasticity previously reported in the NR2A KO mouse (Fagiolini et al., 2003) is likely to be a manifestation of impaired metaplasticity *in vivo*.

MATERIALS AND METHODS

Subjects: Mice deficient in NR2A were generously supplied by S. Nakanishi. The mice were developed by replacing the region spanning the M2 transmembrane segment of NR2A subunits with a neomycin resistance gene as previously described (Kadotani et al., 1996). A pathogen-free line was rederived on a C57BL/6 background by Charles River Laboratories. Wild-type (+/+), heterozygote (+/−), and NR2A KO (−/−) mice were used between postnatal days (P) 21–28. Subjects were fed ad libitum and reared in normal lighting conditions (12/12 light/dark cycle) or in complete darkness.

Cortical Slice Preparation: Following an overdose of barbiturates (i.p.), mice were decapitated upon disappearance of corneal reflexes in compliance with the U.S. Department of Health and Human Services. The brain was rapidly removed and immersed in ice-cold dissection buffer (composition is as follows: 87 mM NaCl, 2.5 mM KCl, 125 mM NaH_2PO_4, 25 mM $NaHCO_3$, 75 mM sucrose, 10 mM dextrose, 1.3 mM ascorbic acid, 7 mM $MgCl_2$, and 0.5 mM $CaCl_2$) bubbled with 95% O_2 and 5% CO_2. The visual cortex was rapidly removed and 350 μm coronal slices were cut using a vibrating microtome (Leica VT100S). Slices recovered for 15 min in a submersion chamber at 32 °C filled with warmed artificial cerebral spinal fluid (ACSF; 124 mM NaCl, 5 mM KCl, 1.25 mM Na_2PO_4, 26 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM dextrose, saturated with 95% O_2 and 5% CO_2) and then cooled gradually to room temperature until use.

Extracellular Electrophysiology: Slices were transferred to an interface recording chamber maintained at 30 °C and perfused with ACSF at a rate of 2.5 mL/min. A stimulation electrode (concentric bipolar tungsten) was positioned in layer IV, and a glass recording electrode (~1 MΩ) filled with ACSF was positioned in layers II/III. The magnitude of responses evoked by a 200 μsec pulse was monitored by the amplitude of the field potential. Stimulation intensity was adjusted to elicit half the maximal response, and stable baseline responses were elicited every 30 sec. The resulting signals were filtered between 0.1 Hz and 3 kHz, amplified 1000 times, and captured at 10 kHz on an IBM-compatible computer using pCLAMP 9.2 software (Molecular Devices). After achieving a stable baseline (< 5% drift) for 15 minutes, slices were stimulated with either 40 Hz stimulation for 1 second, repeated three times with a 10 second interval, or 900 pulses at 1 Hz, or 900 pulses at 0.5 Hz. Field excitatory postsynaptic potential (FP) amplitudes were recorded every 30 seconds for 45 minutes following the cessation of the stimulation protocol. Control and experimental subjects

were run in an interleaved fashion. The data were normalized, averaged, and reported as means ± SEM. Changes in synaptic strength were measured by comparing the average response amplitude 35–40 minutes after conditioning stimulation to the pre-conditioning baseline response.

Voltage-Clamp Recordings: Slices were placed in a submersion chamber, maintained at 31 °C, and perfused at 2 mL/min with oxygenated ACSF containing 4 mM $MgCl_2$, 4 mM $CaCl_2$, 1 μM glycine, 50 μM picrotoxin (Fluka), and 20 μM CNQX. These conditions are sufficient to pharmacologically isolate NMDAR-mediated responses (Quinlan et al., 1999b; Philpot et al., 2001a; Philpot et al., 2001b). Layer II/III pyramidal cells were visually identified using a Nikon E600FN microscope equipped with IR-DIC optics. Patch pipettes were pulled from thick-walled borosilicate glass. Open tip resistances were 3–5 MΩ when pipettes were filled with the internal solution containing: 102 mM cesium gluconate, 5 mM TEA-chloride, 3.7 mM NaCl, 20 mM HEPES, 0.3 mM Na-GTP, 4 mM Mg-ATP, 0.2 mM EGTA, 10 mM BAPTA, and 5 mM QX-314 chloride (Alomone Labs, Jerusalem, Israel) with pH adjusted to 7.2 and osmolarity adjusted to ~300 mmol/kg with sucrose or ddH_2O. Cells were voltage-clamped at +40 mV in the whole cell configuration using a patch-clamp amplifier (Axoclamp 1D, Axon Instruments), and data were acquired and analyzed using a system from DataWave Technologies Inc. (Boulder, CO). Pipette seal resistances were > 1 GΩ, and pipette capacitive transients were minimized prior to breakthrough. Series resistance was monitored throughout the experiment by giving a test pulse and measuring the amplitude of the capacitive current filtered at 30 kHz. Only cells with series resistance < 30 MΩ were included in this study. No series resistance compensation was applied. Input resistance was monitored throughout the experiment by measuring the amplitude of the steady-state current, filtered at 2 kHz, evoked from a test pulse. Excitatory postsynaptic currents (EPSCs) were evoked

from a stimulating electrode (concentric bipolar stimulating; ~200 μm tip separation) placed in layer IV, and stimulation intensity was adjusted to evoke ~100 pA response. Stimulation was given for 200 μsec every 6 sec. To examine functional changes in the summation of NMDAR-mediated currents, 11 pulses of 40 Hz trains of stimulation were given every 6 sec for 3 min. In addition, input-output curves were generated by systematically adjusting the stimulation intensity from 0 to 80 μA. The deactivation kinetics of NMDAR-mediated EPSCs were described by averaging 30 evoked responses and fitting the current decay using the following formula:

$$I(t) = I_f \exp(-t/\tau_f) + I_s \exp(-t/\tau_s),$$

where "I" is the current amplitude, "t" is time, "I_f" and "I_s" are the peak amplitudes of the fast and slow components, respectively, and "τ_f" and "τ_s" are their respective time constants. A non-linear regression in GraphPad Prism software (San Diego, CA) was used to fit decay curves. For quantification purposes, we used the weighted time constant (τ_w), calculated as:

$$\tau_w = \tau_f \, (I_f/(I_f + I_s)) + \tau_s \, (I_s/(I_f + I_s))$$

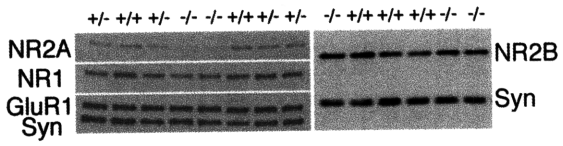
Biochemical Analysis: Synaptoneurosomes were prepared as previously described (Hollingsworth et al., 1985; Quinlan et al., 1999b). Briefly, animals were given a lethal dose of barbiturates (i.p.) and decapitated upon disappearance of corneal reflexes. The brain was quickly removed into ice cold dissection buffer (212.7 mM sucrose, 2.6 mM KCl, 1.23 mM NaH_2PO_4, 26 mM $NaHCO_3$, 10 mM dextrose, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.02 mM CNQX, 0.1 mM APV) saturated with 95% O_2 and 5% CO_2. Visual cortices were bilaterally removed and placed in homogenization buffer consisting of 10 mM HEPES, 2 mM EDTA, 2 mM EGTA, 0.5 mM DTT, 0.1 mM PMSF, 10 mg/L leupeptin, and 100 nM microcystin. The samples were homogenized using 2 mL glass tissue homogenizers (Kontes, Vineland, New Jersey), filtered through a double layer of 105 μm pore nylon mesh filter, and finally passed through 5 μm pore filter

paper. Homogenized tissue was centrifuged at 3,600 RPMs for 10 minutes at 4 °C. The sediment containing a high density of synaptic protein was then resuspended in 1% boiling SDS and stored at -20 °C. An optical densitometric assay (BCA Assay, Pierce) was used to measure the concentration of synaptic proteins. Protein concentrations across samples were normalized and preserved from decay with sample buffer. 10 μg of protein were loaded per lane and samples were run on 7.5% polyacrylamide gels using BioRad mini-protean II and III cells. Gels were then transferred to nitrocellulose membranes (BioRad) and probed against NR2A (Molecular Probes, 1:250), NR2B (Santa Cruz, 1:250), synapsin (Chemicon, 1:1500), GluR1 (Oncogene Research, 1:1000), or NR1 (Chemicon, 1:1000). Samples were next probed with the appropriate secondary antibody conjugated to horseradish peroxidase in Tris-buffered saline (pH 7.4) containing 0.1% Triton X-100. To visualize the immunoblots, enhanced chemiluminescence (Amersham ECL) was used with autoradiographic film (Amersham Hyper ECL). Developed autoradiographs were scanned using Alpha Imager software. Samples from +/+, +/–, and –/– were run simultaneously on each gel to control for antibody penetration and exposure time. Immunoblot bands were quantified by densitometric analysis using NIH Image software. Notably, long exposure times revealed a faint NR2A band in NR2A KO mice, suggesting some cross-reactivity of the NR2A polyclonal antibodies with NR2B. For quantifying NR1, NR2A, and GluR1 levels, densitometric intensities were expressed as a ratio of synapsin levels and normalized to the averaged protein levels in +/+ mice.

Statistics: Either multiple factor ANOVA's or mixed effect ANOVA's with a repeated measures factor were run with *post-hoc* analyses (Fisher's PLSD) to test for statistical significance between multiple groups. Data are expressed as means ± SEM, and significance was placed at $p < 0.05$.

Drugs: Unless otherwise noted, drugs were purchased from Sigma.

FIGURE 2-1



NR2A-deficient mice express normal levels of synaptic NR1, NR2B, and GluR1 subunits.

NR2A-deficient mice express normal levels of synaptic NR1, NR2B, and GluR1 subunits. The Western blot illustrates representative examples of NR2A, NR1, GluR1, NR2B, and synapsin (Syn) immunoreactive bands from the synaptoneurosome preparation in +/+, +/–, and –/– mice aged P21-P28. For quantification, receptor subunit levels were divided by synapsin levels to control for loading and efficiency of synaptic enrichment in the synaptoneurosome preparation, and the resulting ratio was normalized to the average value for +/+ mice (see Table 2-1). Note that NR2A levels were reduced to roughly half of control levels in +/– mice and absent in –/– animals, while NR1, NR2B, and GluR1 levels were not affected by the genetic manipulation.

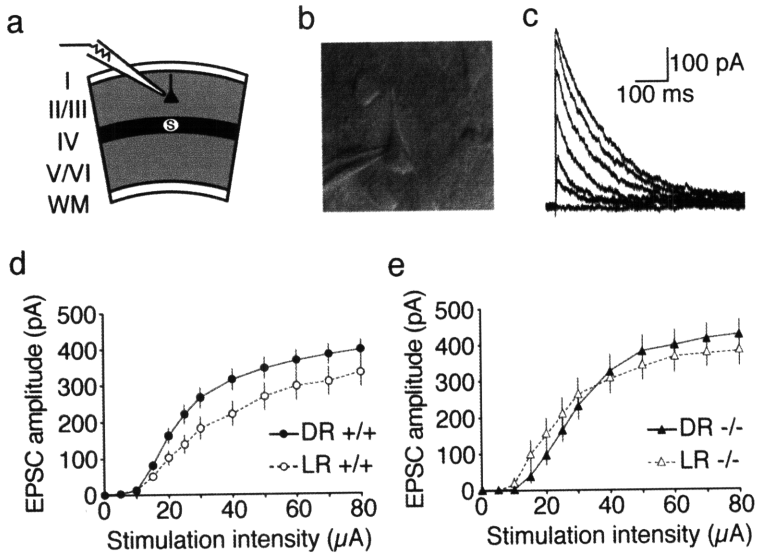
TABLE 2-1

NR2A genotype	NR2A protein	NR1 protein	GluR1 protein	NR2B protein
+/+	1.00 ± 0.05	1.00 ± 0.01	1.00 ± 0.06	1.00 ± 0.13
+/-	0.52 ± 0.25*	0.94 ± 0.11	1.08 ± 0.10	N.A.
-/-	0.03 ± 0.05*	0.83 ± 0.04	0.94 ± 0.06	1.16 ± 0.25

NR2A-deficient mice express normal levels of synaptic NR1, NR2B, and GluR1 subunits.

Normalized synaptic protein levels expressed as fraction of wild-type mean ± SEM.

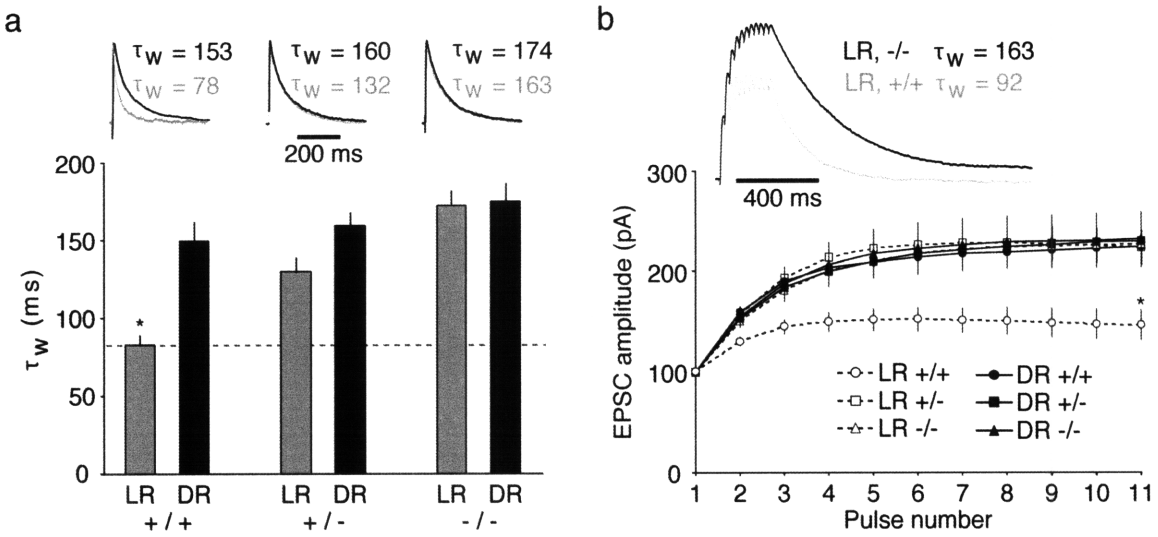
FIGURE 2-2



Experience-dependent enhancement of NMDAR currents evoked by layer IV stimulation of layer II/III pyramidal cells is absent in NR2A knockout mice.

(a) Schematic of recording configuration. (b) Example of IR-DIC image of a whole-cell recording from a layer II/III pyramidal cell. (c) Example demonstrating pharmacologically isolated NMDAR EPSCs evoked by increasing layer IV stimulation. (d) Dark-rearing enhanced the input-output relationship of NMDAR-mediated events compared to responses recorded from LR, WT mice (+/+). (e) NR2A-deficient mice maintain strong evoked NMDAR-mediated responses. Experience-dependent modifications of the input-output relationship of NMDAR EPSCs failed to occur in NR2A knockout mice. For graphs in (d) and (e), data are expressed as means ± SEM.

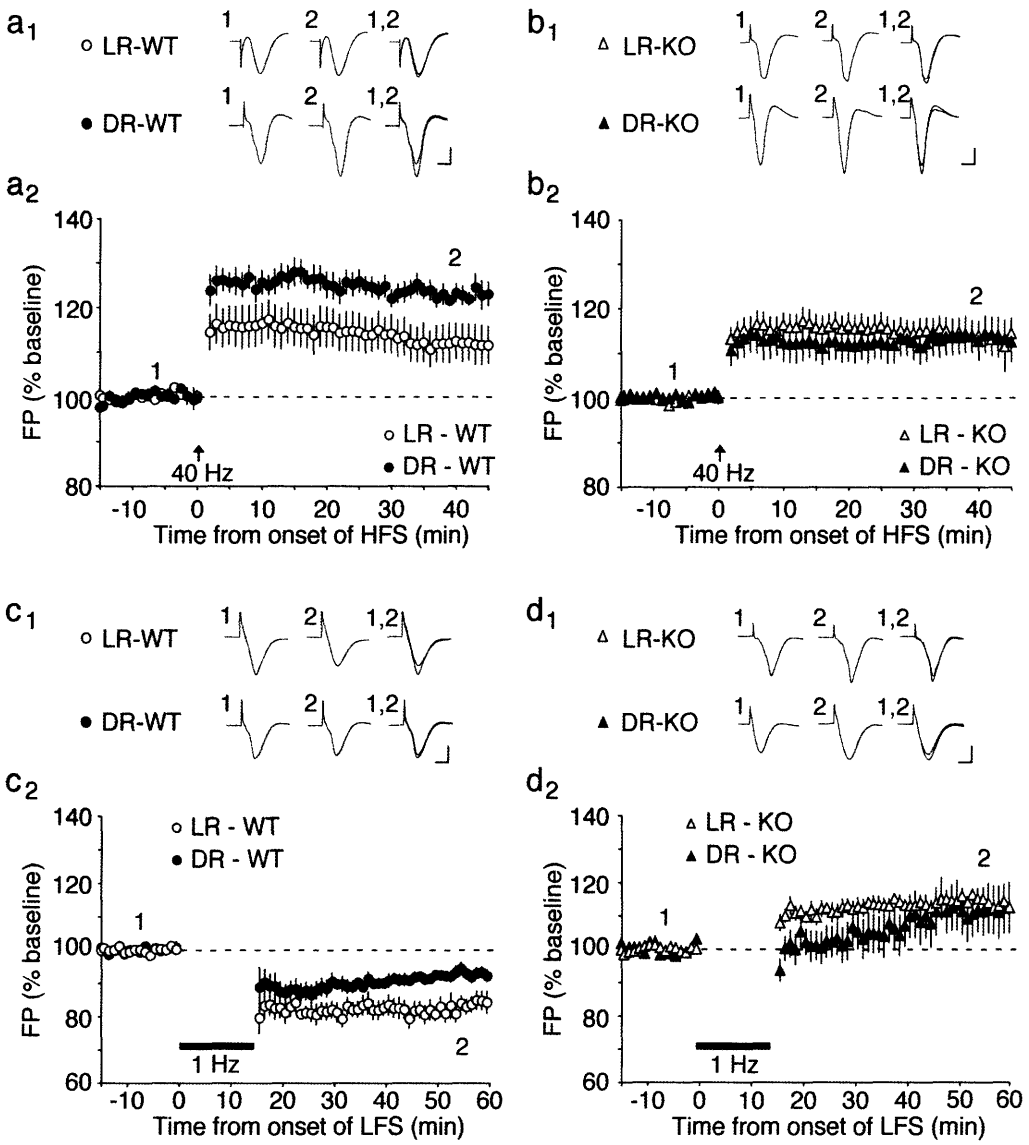
FIGURE 2-3



Loss of NR2A mimics and occludes the consequences of light deprivation on NMDAR EPSC duration in visual cortex layer II/III pyramidal cells.

(a) NMDAR EPSC decays were quantified by calculating a weighted time constant (τ_w) from the double-exponential fit (see Results). Bar graph depicts the averaged τ_w (± SEM) for NMDAR-mediated responses from LR or DR mice (+/+, +/−, and −/−). Traces are of normalized representative EPSCs, for the corresponding genotype in the bar graph below, recorded from DR (dark trace) or LR (light trace) animals. Note that NMDAR-mediated EPSCs are prolonged in DR cortex from +/+ mice, but loss of NR2A mimics and occludes the consequences of dark-rearing. Bar graph values from left to right are: 83 ± 6.4 ms, 150 ± 12.5 ms, 130 ± 8.6 ms, 159 ± 8.1 ms, 173 ± 9.2 ms, and 175 ± 11.5 ms. (b) Reducing NR2A expression genetically or by dark-rearing enhances the temporal summation of average (± SEM) NMDAR EPSCs evoked at 40 Hz (11 pulses). Traces represent normalized NMDAR EPSCs from WT (light trace) and NR2A KO (dark trace) mice. Stimulus artifacts were clipped for clarity.

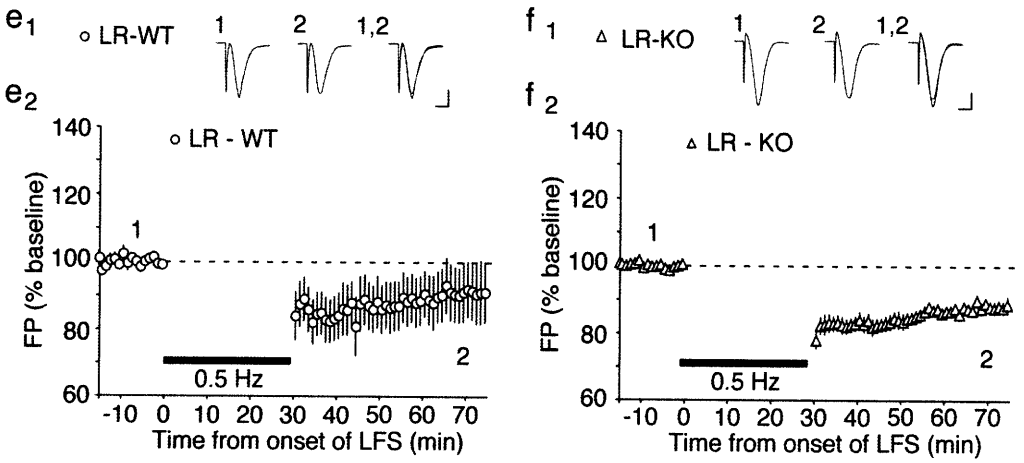
FIGURE 2-4



Loss of NR2A lowers the LTP threshold for synaptic potentiation and prevents experience-dependent modifications in LTP and LTD.

(a) Averaged data (± SEM) and representative waveforms demonstrating that dark-rearing enhances the induction of LTP at 40 Hz stimulation in wild-type mice. (b) Visual deprivation fails to modify the magnitude of LTP induced by 40 Hz stimulation in NR2A KO mice. (c) 1 Hz stimulation induces robust LTD in LR, WT mice. Dark-rearing reduces the magnitude of LTD. (d) 1 Hz stimulation induces LTP in NR2A KO mice, and dark-rearing fails to modify the magnitude of the plasticity. Scale bars: 5 ms, 500 μV.

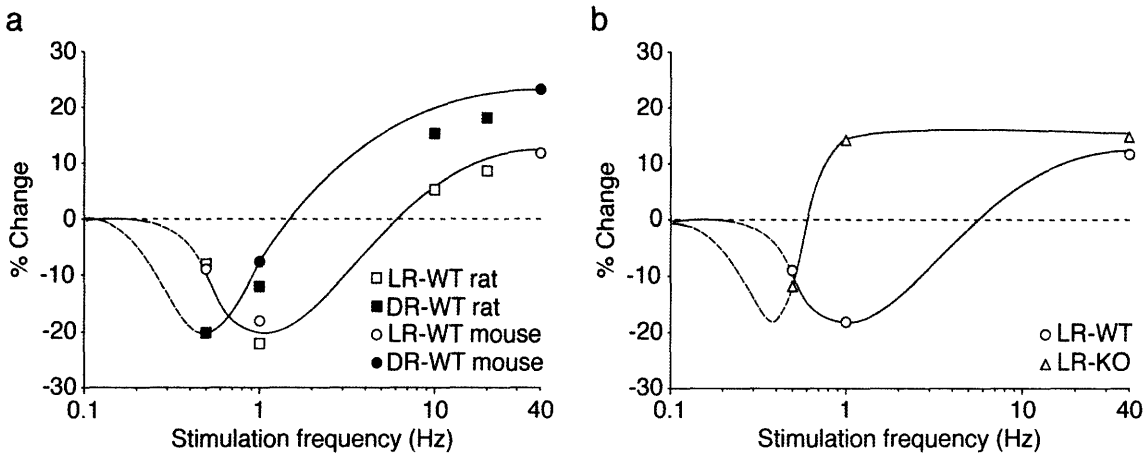
FIGURE 2-4 (cont'd)



Loss of NR2A lowers the LTP threshold for synaptic potentiation and prevents experience-dependent modifications in LTP and LTD.

(e) Averaged data (± SEM) and representative waveforms demonstrating that 0.5 Hz induces LTD in LR, WT mice as well as in LR, KO mice (f). Scale bars: 5 ms, 500 μV.

FIGURE 2-5



Comparison of the effect of dark-rearing and genetic deletion of NR2A on LTD/LTP frequency-response functions.

(a and b) Curves are semischematic, fit to data from the current study and, in (a), also to that previously reported for rats (Kirkwood et al., 1996; Phipot et al., 2003). Dashed regions of the curves are extrapolated from existing data and remain to be confirmed experimentally.

CHAPTER III

The ratio of NR2A/NR2B NMDA receptor subunits determines the qualities of ocular dominance plasticity in visual cortex

This chapter was published together with Lena Khibnik, Dr. Benjamin Philpot, and Dr. Mark Bear in Proceedings of the National Academy of Sciences (2009) Vol. 106, pp. 5377-5382.

ABSTRACT

Bidirectional synaptic plasticity during development ensures that appropriate synapses in the brain are strengthened and maintained, while inappropriate connections are weakened and eliminated. This plasticity is well illustrated in mouse visual cortex, where monocular deprivation during early postnatal development leads to a rapid depression of inputs from the deprived eye and a delayed strengthening of inputs from the non-deprived eye. The mechanisms that control these bidirectional synaptic modifications remain controversial. Here we demonstrate, both *in vitro* and *in vivo*, that genetic deletion or reduction of the NR2A NMDA receptor subunit impairs activity-dependent weakening of synapses and enhances the strengthening of synapses. While brief monocular deprivation in juvenile wild-type mice normally causes a profound depression of the deprived-eye response without a change in the non-deprived eye response, NR2A knockout mice fail to exhibit deprivation-induced depression and instead exhibit precocious potentiation of the non-deprived eye inputs. These data support the hypothesis that a reduction in the NR2A/NR2B ratio during monocular deprivation is permissive for the compensatory potentiation of non-deprived inputs.

INTRODUCTION

The circuitry of primary visual cortex is susceptible to changes in sensory experience during early postnatal development, as evidenced by the well studied paradigm of monocular deprivation (MD) (Hubel and Wiesel, 1970). MD and reverse suture studies demonstrate that the strength of synapses is bidirectionally modifiable (Blakemore and Van Sluyters, 1974; Movshon, 1976; Mioche and Singer, 1989). A detailed time course of the synaptic events following MD in mice shows that the initial consequence is a rapid depression of the deprived-eye inputs followed by a delayed strengthening of the non-deprived eye inputs (Frenkel and Bear, 2004). However, little is known about the molecular mechanisms that regulate the susceptibility of synapses to bidirectional modifications in their strength.

Bidirectional synaptic plasticity has been studied in slice recordings of visual cortex in the form of long-term potentiation (LTP) and long-term depression (LTD), whereby synapses strengthen and weaken in response to stimulation (Kirkwood et al., 1993). These activity-dependent modifications can be modeled by a learning rule where high levels of postsynaptic activation (evoked electrically by high-frequency stimulation) induce LTP and smaller levels of postsynaptic activation (evoked electrically by lower frequency stimulation) induce LTD (Bienenstock et al., 1982). The crossover point from synaptic weakening to strengthening is called the modification threshold (θ_m). An important feature of this model is that the value of θ_m is not fixed; rather, its value can "slide" as a function of the history of postsynaptic activation. According to the BCM theory, closing the dominant contralateral eye first leads to depression of the deprived synapses, followed by a leftward shift in θ_m due to the reduction in average cortical activity. This shift in θ_m is permissive for the subsequent increase in the responses to the non-deprived, ipsilateral eye (Frenkel and Bear, 2004).

A wealth of data now indicates that deprivation and experience during early postnatal development can indeed modify the plasticity threshold. For example, a

period of complete darkness lowers the plasticity threshold such that LTP is enhanced and LTD is attenuated across a range of stimulation frequencies (Kirkwood et al., 1996; Philpot et al., 2003; Philpot et al., 2007). These observations demonstrate that the susceptibility of synapses to plastic changes in visual cortex modifies in relation to their history of experience-driven activity.

Data suggest that the shift in the modification threshold is caused by a change in N-methyl-d-aspartate (NMDA) receptor function (Philpot et al., 2003), and regulation of the molecular composition of the NMDA receptor provides a powerful means to achieve this change. The NMDA receptor is a heteromer that contains the obligatory NR1 subunit and a mixture of NR2A-D subunits that alter receptor properties (McBain and Mayer, 1994; Monyer et al., 1994). At birth, most cortical NMDA receptors contain the NR2B subunit (Monyer et al., 1994). NR2A subunit levels gradually increase with development and reach a maximal expression between the peak and end of juvenile plasticity (Flint et al., 1997; Roberts and Ramoa, 1999). This switch from predominantly NR2B to NR2A subtypes is experience-dependent and reflects the recent history of visual experience (Quinlan et al., 1999a; Quinlan et al., 1999b; Philpot et al., 2001a). During MD, after the initial depression of deprived-eye responses, there is a transient reduction in the NR2A/NR2B ratio that slightly precedes open-eye response potentiation (Chen and Bear, 2007). Because lowering the NR2A/NR2B ratio reduces the threshold for inducing LTP in mouse visual cortex (Philpot et al., 2007), it has been proposed that activity-dependent regulation of NR2A and/or NR2B receptor expression is the molecular basis for the sliding modification threshold.

In the current study, we examined the connection between NMDA receptor subunit composition and the qualities of bidirectional synaptic plasticity in the visual cortex of NR2A knockout (KO), heterozygote (Het), and wild-type (WT) mice. We confirm in layer IV that reducing NR2A expression shifts to lower frequencies both the LTP threshold and the optimal stimulation for LTD. In response to MD, VEPs

evoked *in vivo* through the deprived eye fail to depress normally in NR2A mutants. Instead, an ocular dominance shift occurs by precocious potentiation of responses through the non-deprived eye. These data support the hypothesis that experience-dependent modifications in the NR2A/NR2B ratio at synapses provides a powerful *in vivo* mechanism for regulating subsequent induction of plasticity.

RESULTS

EFFECT OF NR2A GENE DOSAGE ON THE SYNAPTIC MODIFICATION THRESHOLD IN LAYER IV OF MOUSE VISUAL CORTEX

The goal of this study was to determine how decreasing the NR2A/NR2B ratio alters the LTD/LTP modification threshold *in vitro* and compare this with changes in the properties of naturally occurring plasticity in the visual cortex *in vivo* as a consequence of MD. We examined this question using mice with targeted disruption of one or both alleles of the NR2A gene (Kadotani et al., 1996). Because NR2A mutant mice do not display compensatory alterations in NR1 or NR2B subunit expression in visual cortex at the ages of interest, reducing NR2A expression effectively changes the NR2A/NR2B ratio (Philpot et al., 2007).

The bidirectional changes in visual responsiveness that occur after MD were established using visually evoked potentials (VEPs) recorded in layer IV of visual cortex (Frenkel and Bear, 2004). Current source-density (CSD) analysis *in vivo* has confirmed that changes in the amplitude of layer IV VEPs reflect changes in synaptic current sinks in this layer (Sawtell et al., 2003; Liu et al., 2008). However, previous studies of LTP and LTD in NR2A mutant mice were performed in layer II/III (Philpot et al., 2007), and it is now understood that there are significant laminar differences in the mechanisms of visual cortical plasticity in mice (Crozier et al., 2007; Liu et al., 2008). Therefore, our study began with an analysis of the effect of NR2A gene dosage

on synaptic plasticity in layer IV of slices of mouse visual cortex, the layer in which ocular dominance plasticity is also studied. We repeated in layer IV the experiments previously described in layer II/III (Philpot et al., 2007).

To test whether the plasticity threshold was altered by reducing NR2A gene dosage, we examined the consequences of a stimulation protocol (1 Hz for 15 min) in KO and Het mutants that typically results in LTD in normally-reared WT mice (aged between postnatal days (P) 21–28). Following collection of a baseline, 1 Hz stimulation produced reliable depression in WT mice (83.03 ± 2.56 % of baseline, n = 8 slices from 7 mice; Figure 3-1a$_1$). However, as previously shown in layer II/III of the KO mouse, we discovered that 1 Hz stimulation causes LTP of layer IV FP amplitudes in mice lacking NR2A (111.39 ± 2.33 % of baseline, n = 9 slices from 7 mice; Figure 3-1a$_1$). Moreover, in the NR2A Het mice, 1 Hz stimulation resulted in a modest depression of synapses (93.77 ± 5.33 % of baseline, n = 9 slices from 7 mice; Figure 3-1a$_1$) that was intermediate between the WT (p = 0.034) and NR2A KO (p = 0.001) values. Importantly, basal synaptic transmission was comparable between genotypes (Figure 3-1a$_2$) and there was no correlation between baseline FP amplitude and the percent change in synaptic transmission following 1 Hz stimulation (Figure 3-1a$_3$). These results are consistent with previous conclusions that that the LTD–LTP threshold is proportional to the level of NR2A expression in mouse visual cortex (Philpot et al., 2007).

To confirm that the plasticity observed in the NR2A mutants was still NMDA receptor-dependent, we repeated the experiment in the presence of the competitive NMDA receptor antagonist APV (D-2-amino-5-phosphonopentanoic acid; 50 μM). In addition to the expected effect of APV on LTD in WT mice (n = 5 slices from 3 mice) (Crozier et al., 2007), we found that blocking NMDA receptors prevented both the residual LTD in the Het mice (n = 5 slices from 3 mice) as well as the LTP induced by 1 Hz stimulation in the KO animals (n = 5 slices from 3 mice; Figure 3-1b).

Finally, to confirm that reducing NR2A caused a change in the induction requirements for LTD/LTP rather than a dose-dependent loss of LTD, we repeated the experiment using 0.5 Hz stimulation—a frequency that was shown previously to be optimal for LTD induction in visually-deprived animals (Philpot et al., 2003). We found that 900 pulses at 0.5 Hz elicited reliable and statistically significant depression in mice of all genotypes, with the greatest effect in the NR2A KO (NR2A KO: 70.56 ± 6.41 % of baseline, n = 10 slices from 5 mice; Het: 79.05 ± 6.86 %, n = 6 slices from 3 mice; WT: 84.43 ± 5.64 %, n = 6 slices from 3 mice; Figure 3-1c). Taken together, these data lead us to conclude that lowering the NR2A/NR2B ratio shifts the frequency-response curve to the left, and the degree of this shift is proportional to the amount of NR2A present in visual cortex.

EFFECT OF NR2A GENE DOSAGE ON THE OCULAR DOMINANCE SHIFT FOLLOWING MD IN LAYER IV OF MOUSE VISUAL CORTEX

We next examined the impact of altered NR2A and synaptic plasticity on ocular dominance plasticity in layer IV of mouse visual cortex. Electrodes were chronically implanted in layer IV of the binocular zone in primary visual cortex. Baseline VEPs were measured at P27–P29, and the eyelid of the eye contralateral to the experimental hemisphere was sutured closed. After three days of MD, the sutured eye was opened, the animal was allowed to recover from anesthesia, and VEPs were again recorded. We assessed ocular dominance plasticity by determining the ratio of contralateral to ipsilateral eye responses (C/I ratio), which is normally about 2:1 at baseline and decreases after MD to around 1:1. Our results show that NR2A KO and Het mice, as well as their WT littermates, exhibit a normal shift in the C/I ratio (day 0: 1.67 ± 0.21, day 3: 0.89 ± 0.13 in KO, n = 8, p = 0.01; day 0: 1.75 ± 0.09, day 3: 1.05 ± 0.14 in Het, n = 9, $p < 0.01$; day 0: 1.95 ± 0.22, day 3: 0.90 ± 0.13 in WT, n = 10, $p < 0.001$; Figure 3-2a), similar to what has been reported previously (Fagiolini et al., 2003). The degree of the shift is indistinguishable among the three genotypes (Kruskal-Wallis test, n = 27; p = 0.81).

However, upon closer examination of the deprived- and non-deprived eye responses, we discovered profound differences in the qualities of the ocular dominance shift between the genotypes (Figure 3-2b). As previously reported (Frenkel and Bear, 2004), we found that deprived-eye responses in WT mice were significantly depressed (day 0: 211.2 ± 17.1 μV; day 3: 127.6 ± 30.5 μV, n = 10, p = 0.002; Figure 3-2b), and non-deprived eye responses remained at baseline levels (day 0: 114.5 ± 13.2 μV, day 3: 151.9 ± 26.4 μV, n = 10, p = 0.07; Figure 3-2b). In stark contrast to WT mice, the deprived-eye responses in NR2A KO mice were unchanged (day 0: 188.2 ± 19.7 μV, day 3: 202.6 ± 21.4 μV, n = 8, p = 0.55; Figure 3-2b), whereas the non-deprived eye responses dramatically potentiated (day 0: 118.5 ± 11.6 μV; day 3: 247.9 ± 34.3 μV, n = 11, p = 0.005; Figure 3-2b). Results in the Het mice were intermediate: there was still a significant depression of the deprived eye (Figure 3-2b; day 0: 221.3 ± 14.7 μV, day 3: 180.0 ± 26.5 μV, n = 9, p = 0.04) and a slight but statistically significant potentiation of the non-deprived eye responses (day 0: 129.1 ± 10.4 μV; day 3: 175.2 ± 14.9 μV, n = 9, p = 0.03).

These findings are consistent with the idea that reducing the NR2A/NR2B ratio promotes the deprivation-induced adjustment of the BCM modification threshold, and thereby enhances open-eye response potentiation and reduces deprived-eye response depression *in vivo*, similar to what we observed in the slice experiments. However, an alternative explanation is that the shift occurs normally, but is superimposed on an exaggerated global upward scaling of responses caused by visual deprivation. To investigate the possibility of enhanced synaptic scaling in response to deprivation, we recorded VEPs before and after 3 days of binocular lid suture in NR2A KO and WT littermates. An increased homeostatic scaling response should lead to substantially increased visual responses after binocular deprivation (BD).

Our results show that the C/I ratios of both NR2A KO and WT do not change following this visual manipulation (day 0: 2.16 ± 0.19 μV, day 3: 1.92 ± 0.20 μV in KO,

n = 7; day 0: 2.34 ± 0.15 μV; day 3: 2.57 ± 0.43 μV in WT, n = 7; p = 0.31; Figure 3-3a). More importantly, BD did not affect the VEP amplitudes of contralaterally-projecting eyes (day 0: 228.4 ± 16.2 μV, day 3: 232.0 ± 35.5 μV in KO, n = 7; day 0: 236.5 ± 35.7 μV, day 3: 220.8 ± 35.1 μV in WT, n = 7; p = 0.72; Figure 3-3b) nor the VEP amplitudes of ipsilaterally-projecting eyes (day 0: 111.9 ± 11.5 μV, day 3: 121.6 ± 12.0 μV in KO, n = 7; day 0: 99.2 ± 12.2 μV, day 3: 87.0 ± 6.8 μV in WT, n = 7; p = 0.26; Figure 3-3b). These data indicate that reduction of NR2A does not promote synaptic scaling in response to 3 days of visual deprivation.

OPEN-EYE POTENTIATION IN WT MICE REQUIRES NMDAR ACTIVATION

In WT mice, MD for > 5 days causes potentiation of visual responses that we hypothesize is enabled by a deprivation-induced decrease in the NR2A/NR2B ratio (Chen and Bear, 2007). This hypothesis rests on the assumption that response potentiation is an NMDAR-dependent form of "Hebbian" synaptic plasticity (Bear, 2003; Smith et al., 2009). The alternative hypothesis is that responses increase by global upward scaling (Mrsic-Flogel et al., 2007), a process that has been shown to be independent of NMDAR activation (Turrigiano and Nelson, 2004). To distinguish among these hypotheses, we designed experiments in which NMDARs were blocked pharmacologically during the time span when response potentiation occurs (Figure 3-4a).

Following 3 days of MD, which allowed for deprived-eye depression, either CPP ((R, S)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid; 10 mg/kg) or saline was injected i.p. every 6 hours over the course of 4 additional days of MD. The saline controls showed a normal response to 7 days of MD. First, deprived-eye responses were significantly depressed relative to baseline (Figure 3-4b; day 0: 198.2 ± 16.6 μV; day 7: 155.4 ± 17.7 μV, n = 7, p = 0.007), but as described in previous studies (Frenkel and Bear, 2004), this depression was less than that observed after 3 days of MD (cf. Figure 3-2b, WT). Second, open-eye responses were significantly potentiated (Figure 3-4b; day 0: 104.7 ± 13.0 μV; day 7: 163.0 ± 21.0 μV, n = 7, p = 0.035). In contrast, the

mice that received CPP injections exhibited deprived-eye depression similar to what is obtained after 3 days of MD (Figure 3-4b; day 0: 200.4 ± 22.8 μV; day 7: 115.7 ± 31.8 μV, $n = 7$, $p = 0.004$), and the ipsilateral-eye responses remained unchanged (Figure 3-4b; day 0: 94.8 ± 6.1 μV; day 7: 106.3 ± 24.9 μV, $n = 7$, $p = 0.62$). The blockade of response potentiation with an NMDAR antagonist is not consistent with the scaling hypothesis.

DISCUSSION

Our data show that even a graded reduction of the NR2A subunit can dramatically alter the qualities of NMDAR-dependent bidirectional synaptic plasticity in layer IV of visual cortex. Reduced NR2A expression shifts the LTD–LTP threshold to the left; consequently, some stimulation frequencies that would normally lead to LTD cause LTP instead. *In vivo*, the patterns of synaptic activity that normally cause depression of responses from the deprived eye no longer have that effect, and the patterns of synaptic activity through the open eye that normally have no effect, cause precocious potentiation of responses instead. Our results are consistent with the hypothesis that the NR2A/NR2B ratio specifies the value of the synaptic modification threshold that choreographs the bidirectional cortical response to monocular deprivation.

A considerable body of work in the visual cortex has shown how the subunit composition of NMDARs varies during the course of early postnatal development and after periods of visual deprivation. As the cortex matures, the NR2A/NR2B ratio progressively increases, reaching an asymptote around the time of adolescence. This developmental profile is at least partially experience-dependent, as even brief episodes of visual deprivation can reversibly lower the NR2A/NR2B ratio. Changes in NR2A and NR2B expression also occur during the course of MD. In the hemisphere contralateral to the deprived eye, the NR2A/NR2B ratio is significantly reduced after 5 days of MD (Chen and Bear, 2007).

Three primary theories have been advanced in the literature regarding the possible significance of NMDAR subunit composition changes: (1) the subunit switch might bring the classically-defined critical period for ocular dominance plasticity to a close (Nase et al., 1999), (2) an increase in NR2A might favor the induction of LTP versus LTD (Liu et al., 2004; Massey et al., 2004), or (3) the activity-dependent increase in the NR2A/NR2B ratio adjusts the threshold for synaptic plasticity and facilitates the refinement of receptive field properties in juveniles (Katz, 1999; Quinlan et al., 1999a; Quinlan et al., 1999b; Philpot et al., 2001a).

The idea that the NMDAR subunit switch might bring the critical period to a close was attractive because the timing of the NMDAR subunit switch seemed to coincide with a reduction in NMDAR function and the end of the critical period. However, closer examination demonstrated that NR2A levels in layer IV are maximal during the period of maximal plasticity, not at the end, suggesting that the increase in NR2A is not the ultimate signal for terminating juvenile ocular dominance plasticity (Roberts and Ramoa, 1999; however, see Erisir and Harris, 2003). Moreover, NR2A KO mice continue to exhibit an age-dependent decline in ocular dominance plasticity (Fagiolini et al., 2003), corroborating findings in the somatosensory cortex (Lu et al., 2001).

The second putative role for NMDA receptor subunits was that NR2A-containing receptors were a requirement for the induction of LTP, while NR2B receptors were a requirement for the induction of LTD (Liu et al., 2004; Massey et al., 2004). This possibility was attractive because it provided a simple mechanism to describe the developmental loss of NMDAR-dependent LTD observed in many regions of the brain. However, the validity of these findings is now being questioned because these studies were conducted using nonspecific concentrations of NR2A-selective antagonists (Neyton and Paoletti, 2006). Moreover, recent data contradict the initial findings that NR2A and NR2B play distinct roles in regulating the polarity of synaptic plasticity (Berberich et al., 2005; Weitlauf et al., 2005; Zhao et al., 2005; Morishita et

al., 2007; de Marchena et al., 2008). Finally accumulating evidence (Sakimura et al., 1995; Lu et al., 2001; Frenkel and Bear, 2004; Weitlauf et al., 2005; Philpot et al., 2007), including findings from the present study, demonstrate that LTP can be induced in NR2A KO mice, suggesting that a synaptic requirement of NR2A for LTP is overly simplistic.

The current findings fit best the theory that NMDA receptor subunit composition regulates a sliding threshold for bidirectional synaptic plasticity (Bienenstock et al., 1982; Katz, 1999). As previously demonstrated in layer II/III (Philpot et al., 2007), we find in layer IV that reducing NR2A expression shifts the optimal LTD stimulation frequency leftward and enables LTP at low stimulation frequencies. It has been suggested previously that the drop in NR2A/NR2B protein that normally occurs between 3 and 5 days of MD enables the potentiation of the non-deprived eye by shifting the modification threshold to the left (Chen and Bear, 2007). Our finding of reduced deprived-eye depression and precocious open-eye potentiation after 3 days of MD in the Het and KO animals is consistent with this theory. However, rather than setting the threshold per se, reducing NR2A appears to remove a constraint on how fast it can adjust, so that 3 days of contralateral-eye MD is sufficient to cause potentiation of the ipsilateral-eye responses. Additional mechanisms for adjusting the threshold independently of NR2A could include regulation of NR2B (Chen and Bear, 2007) and/or the total number of NMDARs at the synapse (Philpot et al., 2007), among other possibilities (Abraham, 2008; Yoshimura et al., 2008).

The current data are relevant to the recent debate over whether the compensatory potentiation of the non-deprived eye after MD reflects a process analogous to input-specific LTP enabled by metaplastic adjustment of the modification threshold (Sawtell et al., 2003; Frenkel and Bear, 2004), or a cell-wide process of homeostatic synaptic scaling (Mrsic-Flogel et al., 2007; Kaneko et al.,

2008). Scaling is a phenomenon that does not require NMDAR activation (Turrigiano and Nelson, 2004), so the ocular dominance plasticity phenotype in the NR2A mutant mice is unlikely to result from altered scaling. Moreover, consistent with findings in adult mice (Sawtell et al., 2003; Sato and Stryker, 2008), we find that the response potentiation caused by 7 days of MD in juvenile mice requires NMDAR activation. Therefore, the current findings implicate metaplasticity rather than scaling as the mechanism for deprivation-induced response potentiation, at least in layer IV.

In conclusion, our data support the hypothesis that the experience-dependent regulation of the NR2A/NR2B ratio is critical for adjusting the threshold for synaptic modifications, both *in vitro* and *in vivo*. These data suggest that lowering the NR2A/NR2B ratio might provide a permissive milieu for strengthening weak cortical inputs. An exciting possibility is that manipulation of this ratio, either experientially or pharmacologically, could be exploited therapeutically to promote synapse rewiring after brain injury or disease.

MATERIALS AND METHODS

Subjects: Mice deficient in NR2A were generously supplied by S. Nakanishi. The mice were developed by replacing the region spanning the M2 transmembrane segment of NR2A subunits with a neomycin resistance gene as previously described (Kadotani et al., 1996; Sawtell et al., 2003; Sato and Stryker, 2008). A pathogen-free line was rederived on a C57BL/6 background by Charles River Laboratories. WT (+/+), heterozygote (+/−), and NR2A-KO (−/−) mice were used between postnatal days (P) 21–28 for *in vitro* experiments and P24–P36 for *in vivo* experiments. Subjects were fed ad libitum and reared in normal lighting conditions (12/12 light/dark cycle). All animals were handled according to the rules and guidelines set forth by the MIT Animal Care Committee. There was no significant difference in AMPAR-mediated

responses across genotypes, as evidenced by the facts that (1) the baseline VEP amplitudes were not different (Figures 3-2b, 3-3b), (2) the baseline FPs evoked in layer IV by white matter stimulation were not different (Figures 3-2a_2 and 3-2a_3), and (3) the stimulation intensities required to evoke a half-maximal FPs were not different. Whole-cell recordings of AMPA/NMDA receptor ratios in layer IV cells revealed no difference between KO and WT, suggesting a normal level of NMDA receptor expression at these ages (data not shown). As described previously, changes in NR2A gene dosage systematically alter NR2A protein and the properties of NMDA receptor-mediated synaptic currents in visual cortex (Philpot et al., 2007).

Cortical Slice Preparation: Following an overdose of barbiturates (i.p.), mice were decapitated upon disappearance of corneal reflexes in compliance with the U.S. Department of Health and Human Services. The brain was rapidly removed and immersed in ice-cold dissection buffer (composition: 87 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 75 mM sucrose, 10 mM dextrose, 1.3 mM ascorbic acid, 7 mM $MgCl_2$, 0.5 mM and $CaCl_2$) bubbled with 95% O_2 and 5% CO_2. The visual cortex was rapidly removed and 350 μm coronal slices were cut using a vibrating microtome (Leica VT100S). Slices recovered for 15 min in a submersion chamber at 32 °C filled with warmed artificial cerebral spinal fluid (ACSF; 124 mM NaCl, 5 mM KCl, 1.25 mM Na_2PO_4, 26 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM dextrose, saturated with 95% O_2 and 5% CO_2) and then cooled gradually to room temperature until use.

Extracellular Electrophysiology: Slices were transferred to an interface recording chamber maintained at 30 °C and perfused with ACSF at a rate of 2.5 mL/min. A stimulation electrode (concentric bipolar tungsten) was positioned in white matter, and a glass recording electrode (~1 MΩ) filled with ACSF was positioned in layer

IV. The magnitude of responses evoked by a 200 μsec pulse was monitored by the amplitude of the field potential. Stimulation intensity was adjusted to elicit half the maximal response, and stable baseline responses were elicited every 30 sec. The resulting signals were filtered between 0.1 Hz and 3 kHz, amplified 1000 times, and captured at 10 kHz on an IBM-compatible computer using pCLAMP 9.2 software (Molecular Devices). After achieving a stable baseline (< 5% drift) for 15 minutes, slices were stimulated with 900 pulses at 1 Hz or with 900 pulses at 0.5 Hz. Field excitatory postsynaptic potential (FP) amplitudes were recorded every 30 seconds for 45 minutes following the cessation of the stimulation protocol. The concentration used for bath application of D-APV was 50 μM. Control and experimental subjects were run in an interleaved fashion. Objective criteria (baseline drifts no greater than 5% and proper waveform alignment) were applied as inclusion criteria for further analysis. The data were normalized, averaged, and reported as means ± SEM. Changes in synaptic strength were measured by comparing the average response amplitude 35–45 minutes after conditioning stimulation to the pre-conditioning baseline response.

Miniature EPSC Recordings: Slices were maintained in ACSF containing 124 mM NaCl, 3 mM KCl, 1.25 mM Na_2PO_4, 26 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 20 mM D-glucose, saturated with 95% O_2 and 5% CO_2 with pH adjusted to 7.25 and osmolarity adjusted to 315 mOsm. Recording electrodes were filled with internal containing 20 mM KCl, 100 mM (K)gluconate, 10 mM HEPES, 4 mM Mg-ATP, 0.3 mM Na-GTP, and 10 mM Na-phosphocreatine with pH adjusted to 7.25 and osmolarity adjusted to 300 mOsm. AMPA receptor-mediated miniature EPSCs (mEPSCs) were recorded in the presence of blockers for voltage-gated sodium channels (tetrodotoxin; 200 nM), $GABA_A$ receptors (picrotoxin; 50 μM), and NMDA receptors (D,L–APV; 100 μM). To further block NMDA receptor currents the internal recording solution contained 1 μM MK801 and mEPSCs were recorded at negative

holding potentials (–80 mV). Events were first identified using an automatic template detection program (pCLAMP; Molecular Devices) and then manually verified so that only events with a monotonic rise time and exponential decay were included in the analysis. Over 100 events were analyzed for each data point for each cell. AMPA receptor-mediated mEPSC amplitudes in layer IV cells were not statistically significant between NR2A KO (18.58 ± 1.76 pA, n = 10 neurons from 3 mice) and WT (18.90 ± 1.30 pA, n = 13 neurons from 3 mice) mice.

In Vivo Electrophysiology: VEP recordings were conducted in awake mice as described previously (Frenkel and Bear, 2004). Mice were anesthetized with 50 mg/kg ketamine and 10 mg/kg xylazine i.p. Tungsten microelectrodes (FHC, Bowdoinham, ME) were chronically implanted into binocular visual cortex at P24. Reference electrodes were placed bilaterally into prefrontal cortex. All electrodes were secured in place with cyanoacrylate and the entire exposure was covered with dental cement.

For MD and BD, P27-P29 mice were anesthetized by inhalation of isoflurane (IsoFlo 2-3%). Lids were sutured using 6-0 vicryl. Animals were monitored daily to ensure a full seal. Mice whose eyelids did not remain fully shut for the entire duration of MD were excluded from the study. For CPP experiments, CPP (Tocris Bioscience) or saline was delivered intraperitoneally every 6 hours at 10 mg/kg (Heynen et al., 2003; Frenkel et al., 2006).

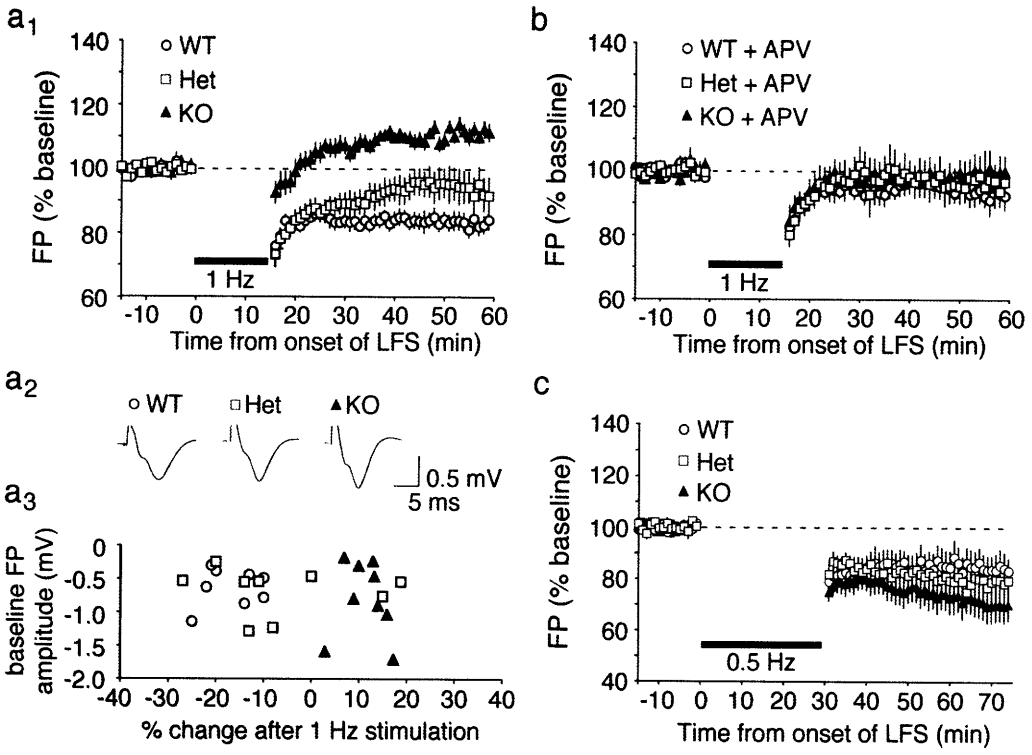
Visual stimuli consisted of full-field sine-wave gratings of 0% and 100% contrast, square reversing at 1 Hz, and presented at 0.05 cycles/degree. VEPs were evoked by either horizontal or vertical stimuli. As described previously, stimuli of orthogonal orientations were presented pre- and post-MD in order to avoid the phenomenon of stimulus-selective response potentiation (SRP) (Frenkel and Bear, 2004; Frenkel et al., 2006). Visual display occupied 92° x 66° of the animal's visual field. Visual stimuli were presented to left and right eyes randomly. A total of 100-

200 stimuli were presented per each condition. VEP amplitude was quantified by measuring trough-to-peak response amplitude, as described previously (Sawtell et al., 2003).

Statistics: Global ANOVA's with a repeated measures factor were run with *post-hoc* analyses (Fisher's PLSD) to test for statistical significance between multiple groups. Data expressed as means ± SEM, and significance was placed at $p < 0.05$.

Drugs: Unless otherwise noted, drugs were purchased from Sigma.

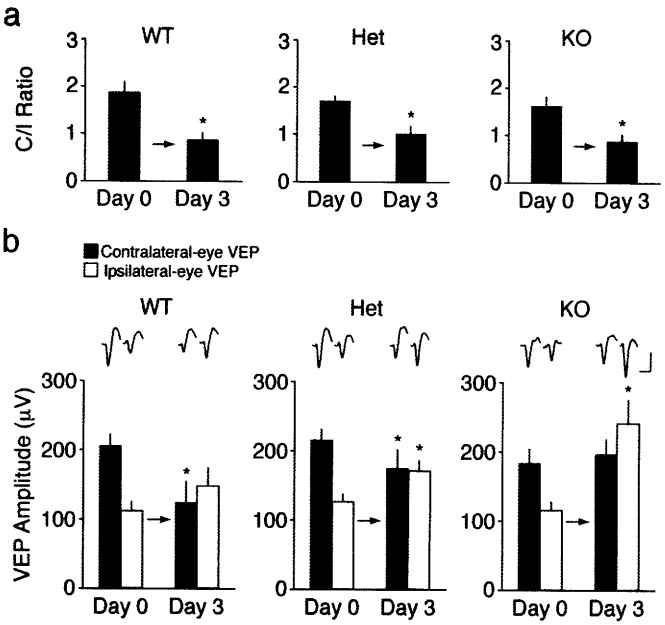
FIGURE 3-1



Loss of NR2A lowers the LTP threshold and the optimal LTD stimulation frequency.

(a_1) Averaged data (± SEM) demonstrating that 1 Hz stimulation (900 pulses) induces LTD in WT and Het mice and LTP in NR2A KO mice. (a_2) Baseline waveforms averaged across all the individual experiments summarized in a_1 (WT, n = 8; Het, n = 9; KO, n = 7). Note that averaged waveforms are of comparable size and shape in all 3 genotypes. (a_3) Scatter plot of individual baseline FP amplitudes of each genotype and percent change of synaptic transmission following 1 Hz stimulation. Note that the effect of 1 Hz stimulation is not correlated with initial response amplitude. (b) Averaged data demonstrating that bath-applied APV prevents the effects of 1 Hz stimulation in all genotypes. (c) Averaged data demonstrating that stimulation at 0.5 Hz (900 pulses) yields LTD in all genotypes, with maximal effect in NR2A KO mice.

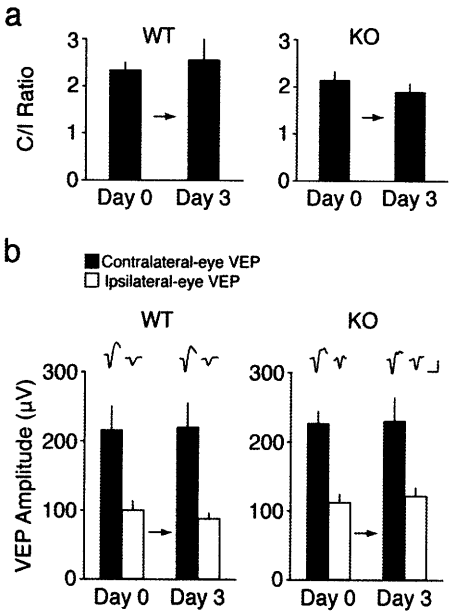
FIGURE 3-2



The ocular dominance shift following 3 days of MD is qualitatively different in NR2A KO, Het, and WT mice.

(a) There is a significant decrease in C/I ratios of WT (n = 10), Het (n = 9), KO (n = 8) mice following 3 days of MD. Average values of C/I ratios (± SEM) are plotted. Asterisks indicate $p <$ 0.01. (b) Day 0 and day 3 waveforms, averaged across all individual experiments (scale: 100 ms, 100 μV). Average trough-to-peak amplitude (± SEM, n = 10) of VEPs in WT mice in response to deprived-eye (filled bars) and non-deprived eye (open bars) stimulation during baseline (day 0) and after 3 days of MD. There is a significant decrease in the deprived-eye VEP amplitude and no change in the non-deprived eye VEP amplitude (left). Average amplitude (± SEM, n = 9) of VEPs in Het mice in response to deprived-eye (filled bars) and non-deprived eye (open bars) stimulation during baseline (day 0) and after 3 days of MD. There is a significant decrease in the deprived-eye VEP amplitude and significant increase in the non-deprived eye VEP amplitude (middle panel). Average amplitude (± SEM, n = 8) of VEPs in KO mice in response to deprived-eye and non-deprived eye stimulation during baseline (day 0) and after 3 days of MD. No change in deprived-eye VEP amplitude is observed, but the non-deprived eye VEP amplitude is significantly increased (right). Asterisks indicate $p <$ 0.05.

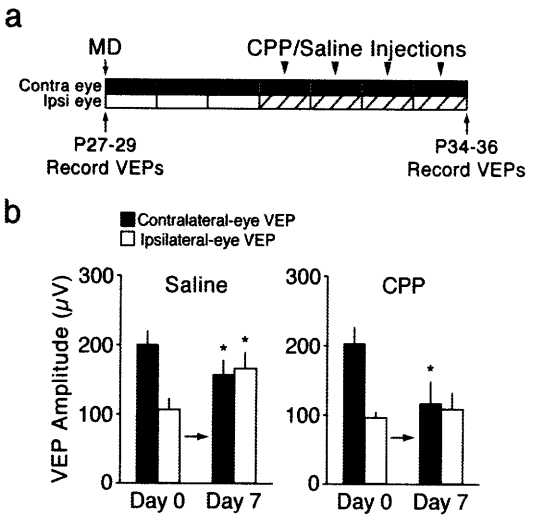
FIGURE 3-3



No evidence of synaptic scaling is seen following 3 days of BD in NR2A KO and WT mice.

(a) Three days of BD fail to modify the C/I ratio of VEPs in either WT or NR2A KO mice.
(b) Neither the ipsilateral nor contralateral eye VEP responses are modified by 3 days of BD in WT and NR2A KO mice. The scale for the averaged waveforms is 100 ms, 100 μV.

FIGURE 3-4



Ipsilateral-eye response potentiation following 7 days of monocular deprivation is NMDAR-dependent in juvenile, WT mice.

(a) Juvenile mice were treated with saline solution or CPP for the last 4 days of a 7 day MD.
(b) Ipsilateral-eye potentiation was blocked in mice treated with CPP.

CHAPTER IV

Comparisons of mouse visual cortical layer IV recordings in vitro *and* in vivo

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Among the data presented in this chapter, chronic VEP *recordings were done in collaboration with Lena Khibnik and Dr. Mikhail Frenkel.*

ABSTRACT

The circuitry in which visual information flows to the cortex has been well defined anatomically and electrophysiologically. These studies have provided a major basis for evaluating both LTD/LTP and naturally occurring synaptic plasticities in layer IV of visual cortex. However, the support for comparing *in vitro* white matter (WM) stimulation with *in vivo* retinal stimulation has not been characterized. *In vivo* plasticity studies have the advantage of an intact visual system, whereas visual cortical slices typically do not contain the presynaptic cell bodies of the thalamus that sends information to cortex. In this study, we evaluate the direct activation of thalamic afferents in layer IV of visual cortex by WM stimulation *in vitro*. WM stimulation activates thalamocortical axons, but it has not been demonstrated to be a valid substitute for an intact lateral geniculate nucleus (LGN) receiving patterned visual stimulation *in vivo*. By performing a comparison of current source-density analyses, using a novel pharmacological method to isolate thalamocortical input to cortex, and testing a functional assay, we demonstrate the validity in comparing mouse visual cortical layer IV recordings evoked by WM stimulation *in vitro* with patterned visual stimulation *in vivo*.

INTRODUCTION

Primary visual cortex is the first site of convergence of information from the two eyes. The input from retina to cortex defines the receptive field properties of postsynaptic cells in visual cortex (Sherman and Guillery, 1996; Sherman and Guillery, 1998). In the mouse, visual stimulation activates the retinal ganglion cells, which relay information along excitatory glutamatergic afferents to thalamic relay cells in the dorsal lateral geniculate nucleus (LGN) (Coleman et al., 2009). The LGN then delivers this retinal information with precision along excitatory axons that synapse primarily onto layer IV cells (Alonso and Swadlow, 2005; MacLean et al., 2006). Thalamic afferents in layer IV of visual cortex relays visual information to cells in layer IV, which transfers information to layers II/III, which continues to send feed-forward input to cells in layers V/VI (Liu et al., 2008). Deep layer cells then process modulatory input back to thalamus and non-thalamic subcortical structures for more refined processing of visual information (Brumberg et al., 2003).

Thus, layer IV cells constitute a first stage of cortical processing for sensory input. Therefore, many studies of the visual cortex aim to selectively activate and analyze responses of cells receiving thalamic input in layer IV of visual cortex. In monkey, cat, and mouse, ocular dominance plasticity has been observed by studying responses evoked in layer IV *in vivo* in response to patterned visual stimulation (Hubel et al., 1977; Shatz and Stryker, 1978; Frenkel and Bear, 2004). For that reason, many *in vitro* studies of the visual cortex aim to selectively activate thalamic afferents in layer IV of visual cortex by stimulating white matter (Daw et al., 2004; Crozier et al., 2007). However, there is sparse evidence that the two preparations are recruiting the same pathways, or if it is valid to correlate the findings from these two preparations.

With the use of the visual thalamocortical slice preparation, it is possible to maintain the anatomical connectivity between LGN and visual cortex (MacLean et al., 2006). Most importantly, MacLean et al. reported that layer IV cells in mouse primary

visual cortex receive direct LGN input. However, most *in vitro* studies prepare slices that do not preserve an intact LGN. In order to study responses in layer IV of visual cortex, a classical approach is to stimulate the thalamocortical axons that reside in white matter (WM). Although synaptic responses can be reliably evoked in layer IV, determining what portion of the response is functionally from LGN has not been characterized in the practice of stimulating WM to simulate activation of a detached LGN.

In the current study, we examined the relationship between WM stimulation *in vitro* and patterned visual stimulation *in vivo* for responses evoked in layer IV of mouse primary visual cortex. By performing current source-density (CSD) analyses, we confirm that activity evoked in layer IV by WM stimulation is comprised of direct LGN input. By utilizing a novel pharmacological method to suppress intracortical activity and preserve thalamocortical input, we also observe that a major portion of layer IV responses evoked by WM stimulation is monosynaptic and of thalamic origin. In this preparation, we show that the drug cocktail of muscimol + SCH50911 eliminates polysynaptic activity including intracortical inhibition and reveals thalamocortical activity when recording both field potentials and whole cell responses evoked by WM stimulation. Lastly, we show that experience-dependent changes in synaptic strength evoked *in vivo* occlude plasticity *ex vivo* in an *in vitro* preparation. Taken together, these data confirm that WM stimulation *in vitro* activates thalamocortical fibers sufficiently to represent LGN activation.

RESULTS

RETINOGENICULOCORTICAL PATHWAYS IN THE MOUSE VISUAL SYSTEM

To confirm the anatomical trajectory of axons from retina to cortex, we first injected 0.5% cholera toxin-B into the retina of postnatal day (P) 18 mice. Cholera toxin-B is a sensitive neuroanatomical tool used to label anterograde and retrograde connections

(Luppi et al., 1990). We allowed the tracer to diffuse for 24 hrs, resulting in the labeling of the retinogeniculate pathway (Figure 4-1a). Slices were then prepared and imaged using confocal microscopy. Figure 4-1b presents an example of the distribution of retinogeniculate cells labeled in mouse LGN; it is apparent that a substantial portion of the dorsal LGN (dLGN) receives eye-specific input from retina. To observe the anatomical connections between LGN and cortex, 0.5% cholera toxin-B was injected into the LGN in the same way described previously to label the thalamocortical and corticofugal pathway (Figure 4-1c). Figures 4-1d-e show an example of the anterograde and retrograde labeling of LGN afferents to and from visual cortex. The greatest density of the thalamocortical input is to layer IV of visual cortex; alternatively, considerable corticofugal input to LGN is from layer VI cells. Thus, neurons from LGN receive retinal afferents, and layer IV of primary visual cortex is the main entrance of sensory information from the LGN.

COMPARISON OF LAMINAR ACTIVATION PROFILES IN VIVO *AND* IN VITRO

CSD analyses have been used to determine the spatio-temporal pattern of current sinks and sources at different depths within cortex (Mitzdorf, 1985). Postsynaptic membrane currents are manifested as current sinks (inward currents) and sources (outward currents) and are evoked by the activation of presynaptic fibers. The CSD profile in visual cortex *in vivo* is derived from a laminar activation profile, which can be generated by tracking a recording electrode through the cortex while recording visually evoked field potentials (VEPs) (Sawtell et al., 2003). While historically it was used as a physiological method for the functional identification of specific lamina in cortex, it also reveals the properties of afferent activity that are responsible for generating laminar field potentials. In the *in vivo* preparation, the major source of input from LGN is to layer IV of visual cortex, as determined by its maximal current sink (Figure 4-2a).

As a result, layer IV was established to be the appropriate laminar position to record thalamorecipient cells. Recording VEP amplitudes in layer IV evoked by patterned visual stimulation has proven to be a useful technique to determine how visual experience can modify sensory processing. In addition to studying the properties of ocular dominance (OD) plasticity in this cortical layer, this method has been useful in understanding how repeated exposure to specific sensory stimuli can modify and improve perception of visual stimuli in mouse visual cortex (Sawtell et al., 2003; Frenkel and Bear, 2004; Frenkel et al., 2006). This phenomenon, called stimulus-selective response potentiation (SRP), is a selective enhancement of responses in layer IV of mouse visual cortex to repeated presentations of visual stimuli of a particular orientation. Accordingly, CSD analyses performed *in vivo* have confirmed that the changes in the amplitude of the layer IV VEPs following both the alterations in ocular dominance and the induction of SRP reflect changes in synaptic current sinks in this layer (Sawtell et al., 2003; Frenkel et al., 2006). Therefore, to understand and compare the *in vivo* and *in vitro* pattern of cortical activation in the mouse, there is a need to perform a CSD profile by recording extracellular field potential responses in layer IV evoked by WM stimulation in a slice.

In a visual cortical slice preparation, a recording electrode was tracked from pial surface to WM in 100 μm steps. At each recording depth, ten 200 μsec biphasic pulses were delivered to the WM, responses were averaged and a corresponding one-dimensional (depth) CSD profile was constructed (Figure 4-2b). WM stimulation produced a maximal current sink in layer IV of visual cortex. Therefore, the CSD analysis confirmed that the maximal negative-going extracellular field potential (FP) evoked in layer IV of mouse visual cortex by WM stimulation *in vitro* reflects a current sink qualitatively similar to the VEP *in vivo*. These data lead us to conclude that the greatest source of thalamic input in both *in vitro* and *in vivo* preparations is to layer IV cells.

CHARACTERIZATION OF THALAMIC INPUT TO VISUAL CORTEX BOTH IN VIVO *AND* IN VITRO

While it is known that visual processing consists of feed-forward inputs to cortex, intracortical processing, and feed-back inputs to LGN, we wanted to confirm that the field potentials we measure with VEPs and those following WM stimulation reflect feed-forward thalamacortical synaptic currents (Miller et al., 2001). Recently, Liu and colleagues developed a method for the functional dissection of thalamic and cortical circuits by silencing intracortical activity and isolating thalamic input to cortex (Liu et al., 2007). By infusing a cocktail of muscimol ($GABA_A$ receptor agonist to eliminate polysynaptic intracortical activity) and SCH50911 (specific $GABA_B$ receptor antagonist used to overcome the nonspecific effect of muscimol on presynaptic transmission), they were able to preserve the monosynaptic, thalamic input to auditory cortex *in vivo*.

We first sought to study the thalamocortical input to visual cortex *in vivo* by infusing the drug cocktail during patterned visual stimulation. Similar to what Liu and colleagues observed, application of the drug cocktail eliminated cortical spiking (Figure 4-3a-b) and preserved measurable thalamocortical responses (Figure 4-3c). The next step was to establish that the drug cocktail could eliminate polysynaptic activity *in vitro*. To do so, we recorded a series of stable responses in layer IV in response to WM stimulation, then we recorded responses following bath application of SCH50911 (70 μM), followed by muscimol (50 μM), and finally by CNQX (10 μM) to eliminate all synaptic activity. Field potentials (FPs) and postsynaptic potentials (PSPs) of single neurons were simultaneously elicited in layer IV by WM stimulation in visual cortical slices prepared from mice at P21–P28 (Figures 4-4a, 4-4b). Somatic whole-cell current-clamp recordings were obtained from pyramidal neurons and maintained at ≈ −90 mV, which is below the calculated reversal potential for chloride in our experiments (−82 mV) (Figure 4-4b). Thus, all polysynaptic activity, including depolarizing inhibitory postsynaptic potentials (IPSPs), is observed in the intracellular recordings following stimulation of WM. In order to show that intracortical inhibition

is eliminated with the use of the drug cocktail, we also examined both excitatory postsynaptic potentials (EPSPs) and IPSPs in pyramidal neurons of layer IV evoked by WM stimulation (Figure 4-4c). These somatic whole-cell current-clamp recordings were maintained at ≈ −40 mV, a relatively depolarizing steady-state potential, and a higher stimulation intensity was used to reveal both EPSP and IPSPs in the response.

Bath application of SCH50911 alone did not alter field potential responses (field potential responses: 100.22 ± 3.4 % of baseline, $n = 9$, $p = 0.96$, Figure 4-4a$_2$), however there was a significant reduction of whole-cell responses (85.15 ± 6.36 % of baseline, $n = 8$, $p = 0.003$, Figure 4-4b$_2$). The initial reduction of the whole-cell response by SCH50911 can be attributed to the loss of $GABA_B$ receptor-mediated inhibition of the depolarizing IPSPs. Bath application of both SCH50911 and muscimol significantly reduced the synaptic response as assayed in both field potential and PSP recordings (field potential responses: 47.1 ± 5.7 % of baseline, $n = 7$, $p < 0.0001$, Figure 4-4a$_2$; whole-cell responses: 24.67 ± 2.57 % of baseline, $n = 7$, $p < 0.0001$, Figure 4-4b$_2$). In the set of experiments detecting both EPSP and IPSPs, following bath application of SCH50911, the late onset inhibition incurred by the $GABA_B$ receptor activation is eliminated but does not change the overall amplitude of the response (EPSP: SCH50911: 103.53 ± 8.44 % of baseline, $p = 0.68$; IPSP: SCH50911: 100.32 ± 11.0 % of baseline, $p = 0.97$, Figure 4-4c$_1$). In addition, bath application of both SCH50911 and muscimol eliminated all polysynaptic activity, including inhibition, in the response (EPSP: muscimol + SCH50911: 71.42 ± 8.88 % of baseline, $n = 11$, $p = 0.002$; IPSP: muscimol + SCH50911: 1.65 ± 1.37 % of baseline, $n = 11$, $p < 0.0001$, Figure 4-4c$_2$). The reduction of all responses following SCH50911 + muscimol can be interpreted as the loss of intracortical activity while the thalamocortical input to cortex is largely preserved (Liu et al., 2007).

The kinetics of the cortical activation pattern in response to thalamic activation in the absence of the drug cocktail reflects a combination of both thalamocortical and intracortical components. For instance, the fast rising phase of

the response represents monosynaptic, thalamocortical input, whereas the slower phases represent integrated local and thalamic input. As expected, the latency to peak of the intracellular recordings decreased to one rapid phase following application of the drug cocktail, suggesting that the slower phase is due to intracortical activity (baseline: 10.09 ± 0.57 ms, $n = 9$; SCH50911: 9.55 ± 0.79 ms, $n = 8$, $p = 0.53$; muscimol + SCH50911: 5.32 ± 0.34 ms, $n = 7$, $p < 0.0001$, Figure 4-4b$_3$). Interestingly, the latency to peak of the field potential recordings did not change prior to and following the experiment irrespective of the presence of the drug cocktail (baseline: 5.48 ± 0.29 ms, $n = 9$; SCH50911: 5.30 ± 0.25 ms, $n = 9$, $p = 0.67$; muscimol + SCH50911: 5.31 ± 0.36 ms, $n = 7$, $p = 0.70$, Figure 4-4a$_3$). Therefore, most of the field potential response is of monosynaptic, thalamic origin. For the whole-cell recordings maintained at −40 mV, there are two peaks in the EPSP portion of the responses. The first peak represents thalamocortical activity, whereas the second peak represents intracortical activity. Following application of SCH50911 + muscimol, however, the multiple peaks in the EPSP are reduced to one monosynaptic peak. Since thalamocortical responses typically have a higher intensity threshold (Liu et al., 2007), the use of higher stimulation intensity explains the same latency to peak of the responses throughout the experiment (baseline: 4.73 ± 0.40 ms, SCH50911: 4.46 ± 0.35 ms, $p = 0.65$; muscimol + SCH50911: 4.85 ± 0.40 ms, $p = 0.98$, $n = 11$, Figure 4-4c$_3$).

Confirming that the remaining FPs and PSPs were a result of excitatory monosynaptic activity, the synaptic responses of both field and whole-cell recordings were eliminated following bath application of 10 μM CNQX (field potential responses: 12.5 ± 12.5 % of baseline, $n = 5$, $p < 0.0001$, Figure 4-4a$_2$; whole-cell responses: 5.35 ± 1.22 % of baseline, $n = 5$, $p < 0.0001$, Figure 4-4b$_2$; EPSP: 10.9 ± 1.84 % of baseline, $n = 11$, $p < 0.0001$; IPSP: 0 ± 0 % of baseline, $n = 11$, $p < 0.0001$, Figure 4-4c$_2$). Taken together, these data lead us to conclude that extracellular field potentials and intracellular recordings in layer IV evoked by WM stimulation are representative primarily of monosynaptic, thalamic input.

CHANGES IN SYNAPTIC STRENGTH IN VIVO *ALTER SUBSEQUENT PLASTICITY* IN VITRO

To show that the effects of *in vivo* plasticity can be studied *in vitro*, we tested the phenomenon of stimulus-selective response potentiation (SRP) *ex vivo*. SRP is a selective enhancement of responses in layer IV of mouse visual cortex to repeated presentations of stimuli of a particular orientation that are presented to the awake mouse over a number of days (Frenkel et al., 2006). It has been shown that the instructive effect of repeated presentations of a particular visual stimulus can be relayed and modified in mouse visual cortex, such that it will cause a stable increase in the magnitude of the cortical response to that particular stimulus. Preventing activity-dependent insertion of AMPA receptors into synapses, an event that has been shown to underlie LTP expression, blocked the increase in synaptic strength following SRP training. But can these plastic changes incorporated *in vivo* occlude plastic changes induced *in vitro*? It has already been demonstrated that changes in synaptic strength *in vivo* can alter subsequent plasticity *in vitro*. For instance, slices prepared from animals that have been reared in the dark demonstrate greater LTP than in animals reared in normal light conditions (Kirkwood et al., 1996; Philpot et al., 2007). Similarly, plastic changes incurred *in vivo* such as the depression of deprived-eye responses following monocular deprivation, occludes LTD in a slice preparation *ex vivo* (Heynen et al., 2003; Crozier et al., 2007). Thus, when Frenkel et al. demonstrated a novel form of perceptual learning in the visual cortex, it was important to determine if this phenomenon utilized the mechanisms of LTP in visual cortex. This can be tested *in vitro* by examining the magnitude of LTP following induction of SRP. If SRP utilized the same mechanisms that subserve LTP, then LTP should be occluded from slices from these animals, which can be tested by inducing LTP *ex vivo*. In these experiments, recording electrodes were implanted in layer IV of the binocular region of mouse primary visual cortex at P26. The mice were given a daily exposure to stimuli of several orientations over a period of 4 days to induce SRP and at P36,

visual cortical slices were prepared (Figure 4-5a). Extracellular FPs were recorded in layer IV evoked by WM stimulation. To test for gross differences in basal synaptic transmission, we generated input-output curves from both control (animals presented with a grey screen only) and trained animals undergoing SRP. In all comparisons, the experimenters were blind to training experience. This analysis revealed that SRP training enhanced the input-output curves of FP responses in layer IV (SRP: $n = 6$, control: $n = 8$, Figure 4-5b). To assess differences in cortical plasticity, a theta burst (TBS) protocol that typically induces LTP in control animals was used to induce plasticity in slices from both groups of animals. As expected in control animals, we found that TBS of WM resulted in a significant potentiation of responses in layer IV (108.57 ± 2.64 % of baseline, $n = 5$, $p < 0.05$, Figure 4-5c). Conversely, in those animals that underwent SRP, we found that the ability to induce LTP was occluded (97.96 ± 1.89 % of baseline, $n = 6$, $p = 0.15$, Figure 4-5c). Therefore, plastic changes in the visual cortex following patterned visual stimulation *in vivo* can occlude subsequent plasticity *in vitro*. Overall, these studies indicate a valid comparison between *in vitro* and *in vivo* recordings in layer IV evoked by either WM stimulation of patterned visual stimulation, respectively.

DISCUSSION

The visual cortex has become an excellent model system for understanding the mechanisms of experience-dependent plasticity. It is well supported that layer IV of visual cortex receives the bulk of visual information directly from LGN, which has led to a number of studies recording activity in this layer in response to visual stimulation *in vivo* (Sawtell et al., 2003; Frenkel and Bear, 2004; Frenkel et al., 2006; Crozier et al., 2007; Cho et al., 2009). Although electrical stimulation of white matter is believed to stimulate thalamocortical axons to layer IV *in vitro*, it has not been proven to be a good substitute for the LGN structure itself.

Our findings strongly support the notion that relating the plastic changes induced by WM stimulation and those induced by patterned visual stimulation *in vivo* is a valid comparison. We found that anatomical tracing of the retinogeniculocortical pathway leads to labeling of thalamic afferents primarily in layer IV of mouse visual cortex. To determine the visual cortical recipient of thalamic afferent activity, CSD analyses shows a maximal current sink in layer IV in both preparations (Sawtell et al., 2003). The use of a pharmacological method to isolate thalamic input to cortex demonstrates that a large portion of the extracellular FP and whole-cell responses in layer IV is monosynaptic and of thalamic origin. Lastly, the instructive effect of SRP training in layer IV *in vivo* occludes layer IV plasticity *in vitro*.

While the use of the muscimol and SCH50911 drug cocktail has been shown to largely preserve thalamic input to cortex *in vivo*, a few limitations arise when using WM stimulation to evoke responses in layer IV (Liu et al., 2007). In particular, this type of stimulation will activate all fibers that pass near the stimulating electrode, including axons of pyramidal cells located in supra- and infragranular layers. Some of these pyramidal cells make collateral synapses in layer IV. Therefore, even if polysynaptic activity is blocked via use of the drug cocktail, one cannot confirm whether the synaptic responses in layer IV are entirely due to thalamic input or collaterals of other pyramidal cells. While the possibility of stimulating nonthalamic fibers exists in this preparation, it is evident from the rapid kinetics of the response following application of the drug cocktail that the activity is monosynaptic and of thalamic origin. Thus, the most plausible interpretation to make from this experiment is that mostly axons originating from thalamus are being activated.

Overall, it is rather amazing that thalamocortical activity described here can be detected in an *in vivo* or an *in vitro* preparation. For example, in the cat visual system, it has been estimated that 5% of geniculate relay cells receive input from retina (Van Horn et al., 2000). From the 5% of LGN relay cells that convey visual information,

only 5–10% of cells in layer IV receive feed-forward transmission of visual information from thalamic afferents (Ahmed et al., 1994). The rest of the input to LGN is involved in modulatory function from cortex and various brainstem sites, thus illustrating a tightly coupled bidirectional interaction between thalamus and visual cortex (Sherman and Guillery, 2006). In the mouse visual system, neuroanatomical tracing and morphometric techniques have revealed that about 50–60% of retinal ganglion cells make synapses in LGN (Drager and Olsen, 1980; Coleman et al., 2009). Almost all of the relay cells in LGN send afferents to the visual cortex, 20% of which project to interneurons (Coleman et al., 2009). However, in mouse, the percentage of layer IV cells that receive thalamic input has not been quantified. Nevertheless, the ability to detect this synaptic transmission and the changes made in layer IV of visual cortex following manipulations of visual experience *in vivo* is fascinating. Even more so, the ability to stimulate a subset of thalamocortical axons that are functional after dissection from LGN and to record a measurable thalamic response *in vitro* is rather extraordinary.

MATERIALS AND METHODS

Subjects: Wild-type C57BL/6 mice (postnatal day 18–28) were obtained from Charles River Laboratories and housed at MIT. Subjects were fed ad libitum and reared in normal lighting conditions (12/12 light/dark cycle). All animals were handled according to the rules and guidelines set forth by the MIT Animal Care Committee.

Ocular Injections: For ocular injections, mice were anesthetized by inhalation of isoflurane (1%–2%) and placed under a surgical microscope. The temporal portion of the conjunctiva was exposed. To access the vitreal chamber, the temporal portion of the globe was reflected anteriorly by gently pulling a 7–0 silk suture that was threaded through the conjunctiva and secured to the operating surface. The conjunctiva was

then trimmed using fine angled scissors to expose the sclera. A small puncture was made into the vitreous chamber with a fine needle posterior to the corneoscleral junction. A glass micropipette with a tip diameter of 30 μm was attached to a manually driven microinjection apparatus (MMP, World Precision Instruments, Sarasota, FL, USA) and inserted into the vitreous chamber at a depth of approximately 2 mm. Both eyes were injected with approximately 1.0 μL of cholera toxin-B (CTB) subunit conjugated to Alexa-488 (green) and Alexa-555 (red) (Invitrogen, Carlsbad, CA, USA) (0.5% in 0.01 M phosphate-buffered saline and 2.0% DMSO). Following injection, the eye was rinsed with sterile eye drops and coated with an antibiotic ointment to prevent infection.

dLGN Injections: Mice were anesthetized with a mixture of ketamine and xylazine and prepared for stereotaxic injections as previously described (Frenkel et al., 2006). The following stereotaxic coordinates were used for each region: dLGN: −1.7 mm posterior to bregma and 3.3 mm lateral from the midline, 2.50 mm down from the dural surface. A small burr hole (1.0 mm in diameter) was made at each coordinate and a small bone flap was removed to expose the underlying dura and cortex. Dyes (prepared as described above) were injected using a Nanoject II injection system (Drummond Scientific, Broomall, PA, USA). Tip diameter for injection pipettes was approximately 20 μm and 10 × 13.2 nL (pausing 10–15 sec between injections) was injected into dLGN. The pipette was left in place for 2–5 min. Upon removal of the pipette, the exposed skull was covered with dental cement and the animals were allowed to recover for 2–3 days prior to perfusion.

Tissue Preparation and Immunohistochemistry: Animals were euthanized by an overdose of pentobarbital and transcardially perfused with 50 mL of 4% paraformaldehyde in 0.1 M phosphate buffer. Eyes and brains were removed and

postfixed for 1 day at room temperature or 2–5 days at 4 °C. For tracer-labeled brain tissue, 100 μm-thick sections were cut in the coronal plane using a vibratome. Serial sections were mounted onto glass slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA, USA), and allowed to adhere. The addition of DMSO to the CTB solutions facilitated the spread of label in retina and brain tissue. Samples were included for analysis if CTB tracer was present though the entire extent of medial dLGN or if the overlying optic tract was thoroughly labeled. All sections and flat-mounts were covered with an aqueous-based mounting medium (Biomeda GelMount, Foster City, CA, USA) and coverslipped, allowed to cure at room temperature for 2-18 hrs and then sealed with clear nail polish to prevent drying.

Confocal Imaging: All images (1024×1024 pixels unless otherwise noted) were acquired with an Olympus (Melville, NY, USA) FluoView 300 laser-scanning confocal microscope and FluoView 500 acquisition software using appropriate filter sets and excitation lasers. An Olympus 4× UPLanFl objective (NA=0.13) was used to acquire low-magnification images of visual cortex, with the confocal aperture (CA) set to 3 to maximize sensitivity. When acquiring images for morphometric analyses, we used Olympus 10× UPLanFl (NA=0.30) air objectives for the dLGN. For these images, the CA was set to reduce noise and minimize collection of signal outside the excitation plane (CA=1 for 10×; CA=2 for 20×). In order to obtain adequate signal for delineating dLGN regions containing contralateral- or ipsilateral-eye afferents, four to five optical sections (1.0 μm step size) were acquired by scanning ± 2-2.5 μm from the brightest focal plane.

Cortical Slice Preparation: Following an overdose of barbiturates (i.p.), mice were decapitated upon disappearance of corneal reflexes in compliance with the U.S. Department of Health and Human Services. The brain was rapidly removed and immersed in ice-cold dissection buffer (composition: 87 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 75 mM sucrose, 10 mM dextrose, 1.3 mM ascorbic

acid, 7 mM $MgCl_2$, 0.5 mM and $CaCl_2$) bubbled with 95% O_2 and 5% CO_2. The visual cortex was rapidly removed and 350 μm coronal slices were cut using a vibrating microtome (Leica VT100S). Slices recovered for 15 min in a submersion chamber at 32 °C filled with warmed artificial cerebral spinal fluid (ACSF; 124 mM NaCl, 5 mM KCl, 1.25 mM Na_2PO_4, 26 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM dextrose, saturated with 95% O_2 and 5% CO_2) and then cooled gradually to room temperature until use. At the completion of the recording session, the recording electrode was lifted along the z-plane and its tip immersed in FluoSpheres polystyrene microspheres and returned to its recording site to verify layer IV localization. The section was then mounted on gelatin-coated slides and fluorescently stained for Nissl substance (Neurotrace, Molecular Probes).

Visual Stimuli: For the drug cocktail infusion experiments, visual stimuli consisted of full-field sine wave gratings (0.05 cycles/deg) of 100% contrast, square-reversing at 1 Hz. For the SRP experiments, visual stimuli consisted of full-field sine wave gratings (0.05 cycles/deg) of varying contrast (0%–100%); VEPs were elicited by either horizontal, vertical of oblique (45° or 135°) bars. Stimuli were generated by a VSG2/2 card (Cambridge Research System, Cheshire, UK) and presented on a CRT computer monitor suitably linearized by gamma correction. The display was positioned 20 cm in front of the mouse and centered on the midline, thereby occupying 92° x 66° of the visual field. Mean luminance, determined by a photodiode placed in front of the computer screen, was 27 cd/m^2.

In Vivo Electrophysiology: VEP recordings were conducted in awake mice as described previously (Frenkel et al., 2006). Mice were anesthetized with 50 mg/kg ketamine and 10 mg/kg xylazine i.p. Tungsten microelectrodes (FHC, Bowdoinham, ME) were chronically implanted into binocular visual cortex at P26. Reference electrodes were placed bilaterally into prefrontal cortex. A small craniotomy (~1 mm) was made over binocular visual cortex (3 mm lateral to lambda), and tungsten

microelectrodes (FHC, Bowdoinham, ME) were inserted 450 μm below the cortical surface. For the drug cocktail infusion experiments, a second small craniotomy was made 0.5 mm lateral and 0.5 mm posterior to the electrode placement. A guide cannula (Plastics One, Roanoke, VA) was inserted 150 μm below the cortical surface at ~45° angle to the plane of electrode placement, thereby minimizing the distance between the tip of the electrode and the tip of the cannula. All electrodes were secured in place with cyanoacrylate and the entire exposure was covered with dental cement. Animals were monitored postoperatively and were allowed at least 24 hour recovery period before habituation to the restraint apparatus.

Infusion: On the day of infusion, the dummy cannula was removed and replaced with a 33 GA infusion cannula, attached with tubing to a 100 μL Hamilton syringe (VWR, West Chester, PA). The drug cocktail of muscimol (4 mM) and SCH50911 (6 mM), or artificial cerebrospinal fluid (ACSF), was infused with an infusion pump (VWR, West Chester, PA) over a 5 minute period at a rate of 6 μL/hr. VEPs were recorded throughout the infusion and after the infusion for an additional 1 hour or until the effect of the drug was observed.

Current Source-Density Analysis: *In vivo* CSD analysis was performed in order to determine the spatiotemporal pattern of current sinks and sources evoked in the visual cortex by pattern grating stimulation. In these experiments in addition to a reference recording electrode positioned at dural surface, a second roving electrode was tracked down through the visual cortex in 100 μm steps. At each recording depth > 300 VEPs were collected for contralateral, ipsilateral, and binocular viewing conditions. Roving electrode penetrations were performed perpendicular to the cortical surface to a depth of 1.4–1.6 mm from the dural surface. The recording electrode was then withdrawn in 100 μm steps and the recording and stimulating procedure repeated. Dorso-ventral tracking of the roving electrode had no observable

effect on the magnitude or location of current sinks and sources. At the completion of each recording session small electrolytic lesions were made at various depths along the recording track to allow for verification of the location and track of the roving electrode.

In vitro CSD analysis was performed to determine the spatiotemporal pattern of current sinks and sources evoked in layer IV by biphasic stimulation at the white matter/VI boundary of primary visual cortex. The glass recording electrode (~1 MΩ) filled with ACSF was tracked down through the layers in 100 μm steps. At each recording depth, ten 200 μsec pulses were delivered by biphasic stimulation (A-M Systems Isolated Pulse Stimulator Model 2100) and the responses were averaged.

From the FPs collected both *in vivo* and *in vitro*, the corresponding one-dimensional (depth) CSD profile was constructed according to the method described by Mitzdorf (Mitzdorf, 1985), using a spatial differentiation grid of 200 μm. A full account of the theoretical basis of CSD analysis has previously been presented (Freeman and Nicholson, 1975; Mitzdorf, 1985).

Extracellular Electrophysiology: Slices were transferred to an interface recording chamber maintained at 30 °C and perfused with ACSF at a rate of 2.5 mL/min. A stimulation electrode (concentric bipolar tungsten) was positioned in white matter, and a glass recording electrode (~1 MΩ) filled with ACSF was positioned in layer IV. The magnitude of responses evoked by a 200 μsec pulse was monitored by the amplitude of the field potential. Stimulation intensity was adjusted to elicit half the maximal response, and stable baseline responses were elicited every 30 sec. For the SRP *ex vivo* experiments, once a stable baseline of 15 minutes was collected, slices were stimulated with a theta burst stimulation protocol and field excitatory postsynaptic potential (FP) amplitudes were recorded every 30 seconds for 45 minutes following the cessation of the stimulation protocol. Control and experimental subjects were run in an interleaved fashion. Objective criteria (baseline drifts no greater than 5% and proper waveform alignment) were applied as inclusion criteria for further analysis. The data were normalized, averaged, and reported as means ± SEM. Changes in

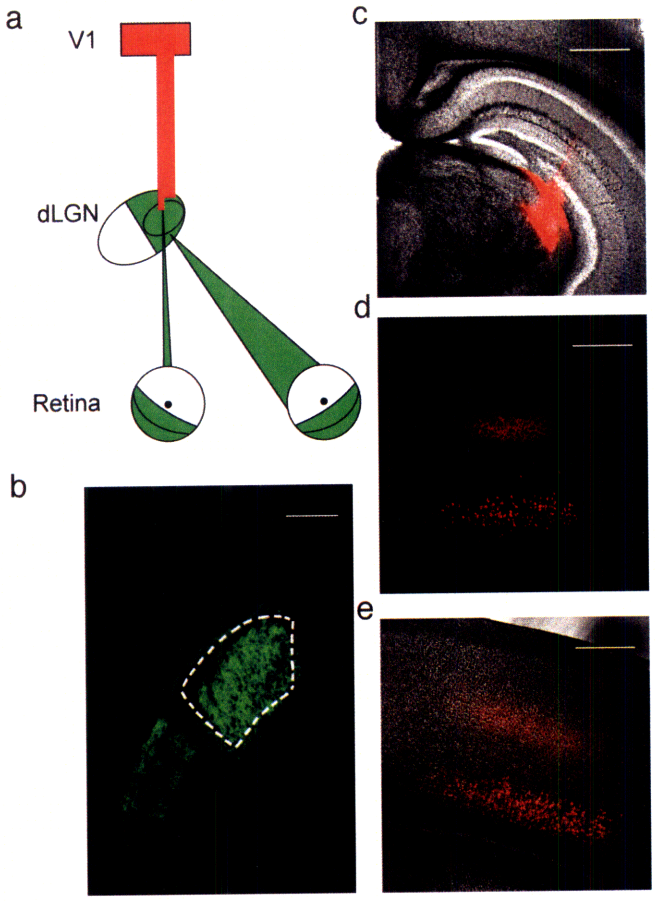
synaptic strength were measured by comparing the average response amplitude 35–45 minutes after conditioning stimulation to the pre-conditioning baseline response. For the drug cocktail experiments, responses were recorded every 20 sec. The resulting signals were filtered between 0.1 Hz and 3 kHz, amplified 1000 times, and captured at 10 kHz on an IBM-compatible computer using pCLAMP 9.2 software (Molecular Devices).

Current-Clamp Recordings: The internal solution consisted of: 130 mM K-gluconate, 4 mM KCl, 2 mM NaCl, 10 mM HEPES, 0.2 mM EGTA, 4 mM Mg–ATP, 0.3 mM Na-GTP, 14 mM phosphocreatine, 0.2% biocytin, with pH adjusted to 7.26, and osmolarity adjusted to 296 mOsm using ddH_2O. Pipette resistances were ≈6 MΩ when filled with internal solution. For current-clamp recordings, stimuli (0.2 msec) were delivered at 0.05 Hz. Recordings were considered acceptable if membrane potentials were maintained between −55 and −70 mV. At least 5 stable responses were collected before and after infusion of SCH50911, muscimol, and CNQX. EPSPs and IPSPs were acquired and analyzed via pClamp and Clampfit software. The reversal potential of chloride of these solutions is −82 mV.

Data Analysis: As a control, ACSF infusion does not significantly alter VEP amplitude (data not shown) in the *in vivo* drug cocktail experiments. In the muscimol + SCH50911 experiments, all responses over an hour of recording were summed and the mean was reported. All statistical analyses were performed using StatView 5.0.1 (Abacus Concepts, Berkeley, CA). A Student's paired t-test or global ANOVA was always performed where appropriate, and relevant *post-hoc* comparisons were made using Fisher's protected least square difference analysis. In all cases, significance was set at $p < 0.05$.

Drugs: Unless otherwise noted, drugs were purchased from Sigma (St. Louis, MO).

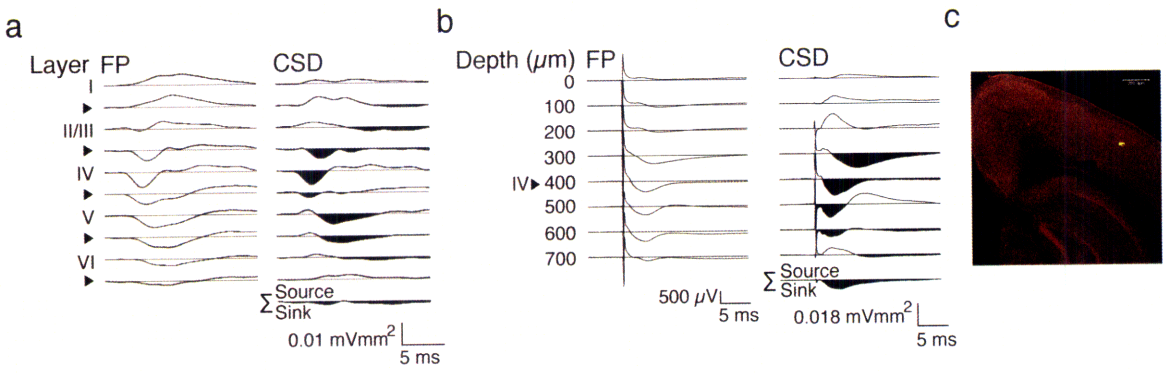
FIGURE 4-1



Distribution of anterograde and retrograde connections in the mouse visual system.

(a) Schematic of the visual pathway in mouse. The widths of the triangles and lines in the cartoon reflect the relative magnitude of the contralateral and ipsilateral (green) retinogeniculate and geniculocortical projections. The dLGN afferents serving each eye converge onto a subset of binocular cortical neurons in visual cortex (red). Adapted from Coleman et al., 2009.
(b) Example confocal image showing the distribution of retinal ganglion cell axon termination zones from both contralateral and ipsilateral retinas labeled by intraocular injection of 0.5% cholera toxin-B conjugated with Alexa-488 (green). An outline of dLGN is indicated in white.
(c) Example confocal image showing the central location of the injection site in the dLGN of 0.5% cholera toxin-B conjugated with Alexa-555 (red). (d-e) The panels contain example confocal images of retrograde and anterograde tracing experiments with cholera toxin-B that were used to anatomically define the location of LGN inputs to and from cortex. The distribution of thalamic afferents in visual cortex is mostly in layer IV; cell bodies in layer VI send feedback to the LGN. Scale bars: 200 μm.

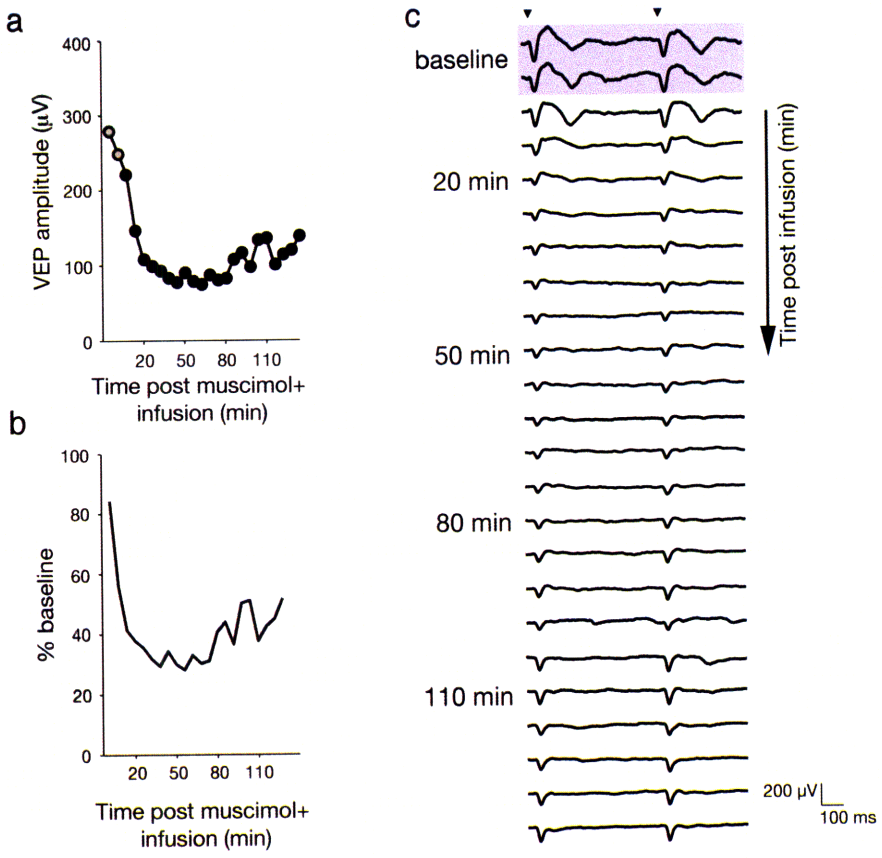
FIGURE 4-2



Comparison of *in vivo* and *in vitro* CSD analyses/Laminar activation profiles produced by patterned visual stimulation *in vivo* or WM stimulation *in vitro*

The left column in (a) and (b) shows field potentials (FPs) recorded at different cortical depths in response to patterned visual stimulation *in vivo* or WM stimulation *in vitro*, respectively. Cortical layers and depths are indicated at the left of each panel. The right column of (a) and (b) presents CSD profiles obtained from the FPs using a spatial differentiation grid of 200 μm. Current sinks are downward and shaded, and current sources are upward going. The bottom trace (Σ) is summation of all CSD traces across depth. In both preparations, the maximal current sink is in layer IV. (a) Adapted from Sawtell et al., 2003. (c) Nissl-stained coronal section showing fluorescent yellow beads in layer IV, indicating position of recording electrode tip at layer IV. Scale bar: 200 μm.

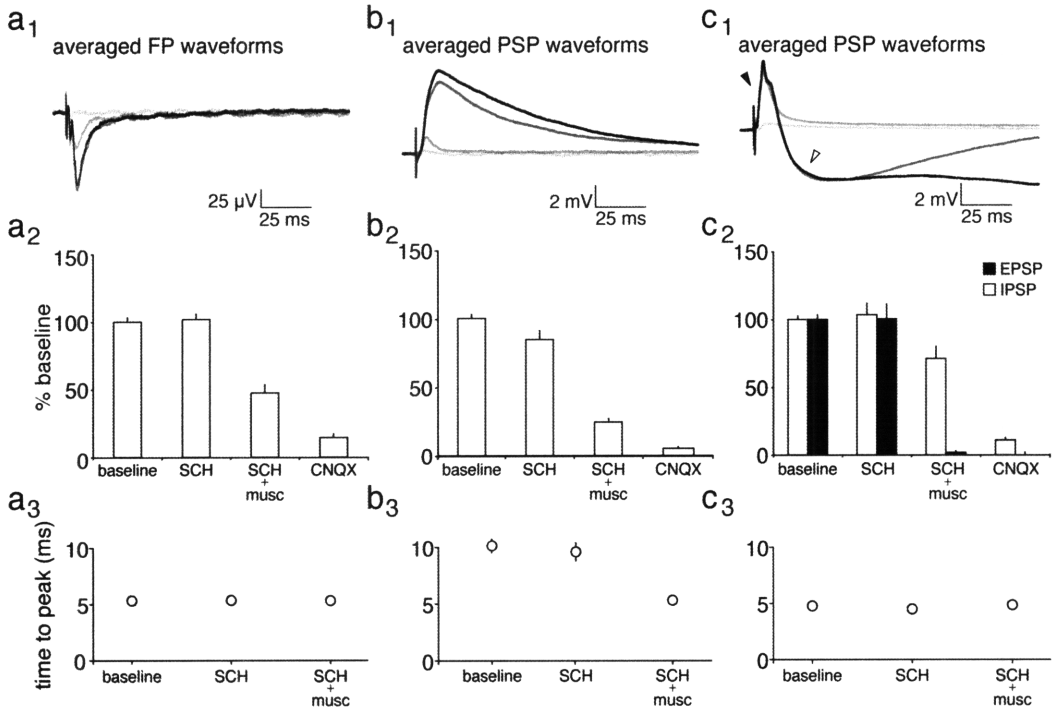
FIGURE 4-3



Isolation of the thalamocortical component of the VEP *in vivo*.

(a) VEP amplitude recorded before (gray circles) and immediately after (black circles) infusion of the muscimol + SCH50911 cocktail. The binocular VEPs were recorded continuously for 2 hours. (b) The amplitude of the responses decreased following drug infusion to about 30-40% of the baseline value, the same as was reported by Zhang and colleagues (Nature Neuroscience, 2007). (c) Following drug infusion the amplitude of the responses decreased, but remained stable and measurable (arrows indicate time of stimulus reversal). A negative component remained, reflecting a current sink in layer IV (the input layer for the thalamocortical axons) and representing the synaptic activation of layer IV neurons. Adapted from Khibnik et al., 2009 (in preparation).

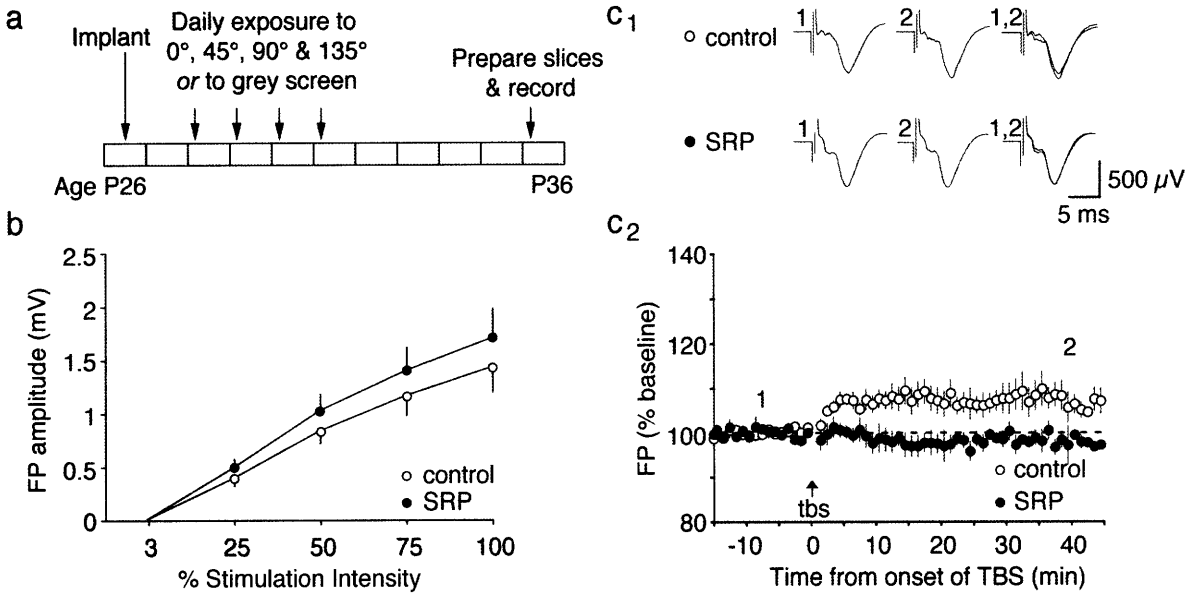
FIGURE 4-4



Isolation of the thalamocortical component to layer IV field potential and whole-cell responses evoked by WM stimulation *in vitro*.

(a_1) Overlay of averaged FP waveforms before (black) and after bath application of SCH50911 (dark grey), muscimol (grey), and CNQX (light grey). FPs were recorded simultaneously in the same slices as the whole-cell recordings maintained at -90 mV (n = 5-9 slices). Following SCH50911 + muscimol, the postsynaptic response becomes of thalamic origin and reduces in amplitude. (b_1 and c_1) Overlay of averaged PSP waveforms before (black) and after bath application of SCH50911 (dark grey), muscimol (grey), and CNQX (light grey). Following SCH50911 + muscimol, the postsynaptic response loses its polysynatic activity and reflects monosynaptic events which are reduced in amplitude. (b_1) Current-clamp recordings of postsynaptic potentials evoked by WM stimulation. Cells are recorded at -90 mV to reveal all polysynaptic activity, including depolarizing IPSPs (n = 5-9). (c_1) Whole-cell current-clamp recordings of excitatory (indicated by black arrowhead) and inhibitory (indicated by white arrowhead) postsynaptic potentials evoked by WM stimulation. Cells are recorded at -40 mV to reveal both EPSP and IPSP (n = 11). Following application of SCH50911, the late onset of GABAergic inhibition is blocked. (a_2, b_2, and c_2) Normalized amplitudes as a percentage of baseline (± SEM) before and after drug cocktail application. All experiments ended with CNQX infusion to block synaptic activity. (a_3, b_3, and c_3) Averaged time to peak (± SEM) latencies before and after cocktail application.

FIGURE 4-5



SRP occludes LTP *ex vivo*.

(a) Recording electrodes were implanted in the binocular region of mouse primary visual cortex at P26. They were either given a daily exposure to different orientation stimuli over a period of 4 days to induce SRP or were presented a grey screen (control animals) and at P36, slices were prepared. Recordings took place in layer IV evoked by WM stimulation. (b) SRP training enhanced the input-output relationship of responses compared to those recorded from control mice. (c) LTP of responses induced by thetaburst stimulation in layer IV control mouse visual cortex (n = 5) is occluded in layer IV of mice that have undergone SRP (n = 6).

CHAPTER V

Implications and Future Directions

Our findings provide experimental support that both experience and NMDAR subunit composition regulate the sliding modification threshold of synaptic plasticity as outlined by the BCM theory. The BCM theory is an influential model that proposes a mechanism by which neurons can acquire selectivity for such properties as ocularity and orientation preference (Bienenstock et al., 1982). The model suggests that stimulus selectivity and synaptic stability arise due to two neuronal properties: (1) synapses are bidirectionally modifiable, and (2) the ability to strengthen or weaken a synapse is a function of the activation history of the neuron, a process termed metaplasticity. That is, periods of low activity alter the regulation of synaptic plasticity such that the strengthening of synapses is favored, and periods of great activity will alter the modification threshold to favor the weakening of synapses. But how do these changes in synaptic strength vary as a function of the integrated postsynaptic activity of a neuron? The experiments described in this thesis were aimed at determining: (1) how the modification threshold shifts, and (2) a cellular mechanism by which visual experience modifies the modification threshold.

In support of the BCM theory, previous studies have demonstrated that the threshold for synaptic modifications can be modified by visual experience; the induction of LTP is favored in the visual cortex of animals reared in the dark as

compared to that of light-reared controls (Kirkwood et al., 1996; Philpot et al., 2007), suggesting that low activity in dark-reared animas shifts the modification threshold to the left (Figure 2-5a). Subsequent biochemical findings have shed further light on the synaptic mechanisms underlying experience-dependent modifications in the properties of synaptic plasticity. For instance, visual experience increases the levels of NR2A protein in visual cortex whereas the absence of visual experience maintains high levels of NR2B-containing NMDARs (Quinlan et al., 1999a). Thus, the effect of prior rearing shifts the modification threshold; molecularly, the modification threshold shifts to the left if the subunit composition favors NR2B expression, and shifts to the right when the NR2A subunit dominates. In addition, Philpot et al. demonstrated that visual experience increases the relative contribution of NR2A NMDAR-mediated kinetics to the response by shortening NMDA receptor current durations, while visual deprivation has an opposite effect (Philpot et al., 2001a). These data raise the exciting possibility that a change in NMDA receptor subunit composition might regulate experience-dependent modifications in synaptic plasticity.

While the biochemical and electrophysiological data cited above present a strong correlation between visual experience and the properties of synaptic plasticity, it has not established causality. To study the functional significance of either NMDAR subunit, it was important to truly fix the NR2A/NR2B ratio. Thus, transgenic and gene-targeting technologies have increased our understanding of cellular mechanisms governing plasticity in juvenile mouse visual cortex. As described in Chapter II, we took advantage of a genetically-modified mouse in which the NR2A subunit is not functionally expressed and examined its effect on NMDAR function and the properties of synaptic plasticity. By employing *in vitro* slice physiology, we demonstrated that visual deprivation from birth lengthens NMDAR EPSC duration and enhances temporal summation in visual cortical layers II/III pyramidal cells of juvenile wild-type mice. Remarkably, the consequences of dark-rearing on NMDAR

current duration and temporal summation were mimicked and occluded in mice deficient in the NR2A NMDAR subunit. While the data demonstrate that a loss of NR2A does not impair the expression of functional NMDARs at the synapse, the data also suggests that NR2A is required for experience-dependent modifications in NMDAR current duration during the period of juvenile receptive field development.

We performed a genetic test of the hypothesis that the regulation of the NR2A NMDA receptor subunits is required for experience-dependent modifications in the properties of synaptic plasticity by recording extracellular field potentials. Since previous studies have looked at the consequence of altering rearing conditions in rats, we first replicated this work in mouse visual cortex and demonstrated that there is an effect of rearing in layers II/III whereby dark-rearing allows for greater potentiation than in light-reared animals. Although NR2A KO mice do not have a deficit in LTP induction as previously suggested (Liu et al., 2004, Massey et al., 2004), there was no effect of dark-rearing on the level of LTP induced by 40 Hz stimulation compared with light-reared NR2A KO mice. These data suggest that NR2A is not obligatory for LTP, but is a requirement for metaplasticity. We then tested the consequences of low-frequency stimulation (1 Hz), and similar to observations in rats, LTD is attenuated in WT dark-reared mouse visual cortex compared with WT light-reared cortex. Surprisingly, we found that low-frequency stimulation produced potentiation of responses rather than depression in the light-reared NR2A KO mice. That is, a low NR2A/NR2B ratio (i.e. the absence of NR2A) reduces the synaptic modification threshold such that stimulation protocols that normally induce LTD now induce LTP (Figure 2-5b). Consistent with the hypothesis that a switch in NMDAR subunit composition is required for metaplasticity, we observed that the consequences of 1 Hz stimulation were similar in the cortex of light-reared and dark-reared NR2A KO mice. However, there is no deficit in LTD induction since a lower frequency stimulation such as 0.5 Hz produces LTD in NR2A KO mouse visual cortex. These data indicate

that the NR2A subunit does not only mediate experience-dependent modifications, but is also essential for modifications in the threshold of synaptic plasticity.

We were also interested in studying the properties of OD plasticity *in vivo*, and this was assayed by recording visually evoked potentials (VEPs) from the binocular region of primary visual cortex. According to the BCM theory, eyelid suture will first lead to a depression of deprived-eye responses, followed by a leftward shift in the modification threshold caused by the reduction in cortical activity. This shift in the modification threshold is permissive for the strengthening of non-deprived eye inputs (Frenkel and Bear, 2004). However, little is known about the molecular mechanism that underlies the bidirectional synaptic modification of ocular dominance. Our data in Chapter II demonstrated that the molecular subunit composition of the NMDA receptor provides a powerful means for regulating the receptor's function in metaplasticity. In addition, biochemical studies of the OD shift have shown that the rapid deprivation-induced depression of contralateral-eye responses after a period of 3 days of MD correlates with an increase in NR2B levels (Chen and Bear, 2007). Furthermore, the subsequent potentiation of ipsilateral-eye responses beginning after 5–7 days of MD correlates with a decrease in NR2A protein levels. Our *in vivo* OD studies employ recordings of local field potentials in layer IV of visual cortex where cells receive direct thalamic input as a result of patterned visual stimulation. Therefore, in Chapter III, we recorded field potentials both *in vitro* and *in vivo* in layer IV of binocular cortex of the NR2A KO mouse, in order to study how the reduction in the NR2A/NR2B ratio affects synaptic and OD plasticity

Similar to recordings performed in layers II/III, recording extracellular field potentials in layer IV *in vitro* also demonstrated that lower frequencies that typically elicit LTD in WT mice now elicit LTP in NR2A KO mice, demonstrating a shift in the threshold for synaptic plasticity. The low NR2A/NR2B ratio also appears to adjust the plasticity threshold *in vivo*, since in the NR2A KO mice, no deprived-eye

depression was observed following 3 days of MD, however a precocious potentiation of nondeprived-eye responses typically seen following 5–7 days of MD was (Frenkel and Bear, 2004). It has been proposed that synaptic scaling might contribute to this phenotype. Therefore, we investigated whether a global upward scaling of responses occurs following 3 days of binocular deprivation. The changes observed following 3 days of MD cannot be explained by synaptic scaling, since 3 days of binocular deprivation fail to modify the absolute amplitudes of contralateral- and ipsilateral-eye responses. These experiments also determined the effect of gene dosage by studying heterozygote subjects that have half the normal levels of NR2A protein typically present in WT control animals. The NR2A heterozygote mice exhibited a shift in the modification threshold that was intermediate between NR2A KO and WT mice both *in vitro* and *in vivo*. Thus, a graded reduction of the NR2A subunit expression can dramatically shift the modification threshold to the left, resulting in an alteration of subsequent plasticity *in vivo*. Taken together, these results indicate that the NR2A subunit is important for adjusting the modification threshold of metaplasticity following monocular deprivation.

Can the mechanisms of LTP and LTD account for key aspects of experience-dependent plasticity? Thus, it was important to determine the physiological relevance of linking these artificially induced forms of synaptic plasticity to naturally occurring plasticity. Many studies have suggested a role for LTP and LTD mechanisms in mediating naturally occurring synaptic plasticity in both hippocampus and visual cortex. For example, using a transgenic mouse approach, Silva et al. tested the relationship between the expression of LTP and LTD and the ability to perform spatial memory tasks. With the use of an αCaMKII KO mouse, Silva and colleagues demonstrated a deficiency in hippocampal LTP, in addition to an impairment in spatial learning memory tasks (Silva et al., 1992a; Silva et al., 1992b). These data strengthened the notion that tetanus-induced changes in synaptic strength *in vitro* can corroborate with memory formation *in vivo*.

Similarly, in mouse visual cortex, artificially induced synaptic plasticity in layer IV *in vitro* correlates with layer IV OD studies *in vivo*. For example, Chapter III demonstrates that the threshold for LTP induction is shifted to the left in the NR2A KO mice *in vitro*, parallel to that observed *in vivo*. *In vitro,* the resultant plasticity threshold for LTP induction is so low that low-frequency stimulation that normally elicits LTD now induces LTP *in vitro*. Likewise, a precocious potentiation of non-deprived eye responses occurs within a short 3 day period of MD in the NR2A KO mice *in vivo*. Therefore, there is a strong correlation of mechanisms at play between the properties of synaptic plasticity and OD plasticity.

The *in vitro* studies were performed by recording responses in layer IV in response to stimulation of WM, which contains axons originating from LGN. This preparation serves to simulate the activity of the LGN, which is intact in OD studies *in vivo*, receiving and relaying patterned visual stimulation to cortex. Surprisingly, however, the basis for stimulation of WM as a substitute for LGN has not been fully characterized. Chapter IV demonstrates the validity of comparing layer IV recordings *in vitro* and *in vivo* using a number of methods. The first method traces the retinogeniculocortical pathway in the mouse visual system and shows that most thalamic afferents innervate layer IV of visual cortex. The second method is a comparison of current source-density analyses that establishes that layer IV receives the greatest source of LGN input as assayed both *in vitro* and *in vivo*. The third method uses a pharmacological approach which preserves thalamocortical input to cortex, and confirms that both field potential and whole-cell responses in layer IV evoked by WM stimulation largely reflect monosynaptic, thalamic input. Therefore, both our anatomical and electrophysiological findings confirm that layer IV cells *in vitro* and *in vivo* are thalamorecipient and are activated by WM electrical stimulation or patterned visual stimulation via the LGN.

We were next interested to determine whether changes in naturally occurring plasticity *in vivo* can be observed *in vitro*. An example of this is described in Chapter II, which demonstrates that visual experience can result in differences in synaptic strength measured *in vitro*. Dark-rearing, a condition that produces periods of low visual activity *in vivo* (Czepita et al., 1994; Maffei et al., 2006), results in a greater magnitude of potentiation *in vitro*, compared to light-reared animals that receive the same frequency of stimulation. Conversely, low-frequency stimulation performed *in vitro* results in attenuated LTD in dark-reared animals compared to light-reared animals. Therefore, the effect of rearing conditions confers salient changes in synaptic plasticity *in vitro*.

In OD studies, it has already been established that a brief period of monocular deprivation induces LTD in visual cortex (Heynen et al., 2003). In the same vein, can certain forms of visual experience induce LTP in visual cortex? Chapter IV aims to characterize the mechanism(s) underlying a naturally occurring potentiation of responses (i.e. LTP) in mouse visual cortex that results *in vivo* following repeated exposure to a stimulus of a particular orientation (Frenkel et al., 2006). To test if plastic changes incorporated *in vivo* can occlude subsequent plastic changes induced *in vitro*, slices were prepared from animals undergoing stimulus-selective response potentiation (SRP) as well as control animals (presented with a grey screen only) and the consequences of LTP-inducing theta burst stimulation was tested. In the control animals, theta burst stimulation elicits a significant potentiation of responses in layer IV of visual cortex. Excitingly, this novel form of perceptual learning can occlude LTP *ex vivo* in a slice preparation. Therefore, not only does this phenomenon demonstrate the same electrophysiological characteristics as LTP, but it also suggests that WM stimulation recruits the same pathways which subserve patterned visual stimulation *in vitro*.

THE ROLE OF NMDARS IN SYNAPTIC PLASTICITY AND METAPLASTICITY

Chapters II and III describe numerous lines of evidence that explain how changes in NMDA receptor subunit composition are a molecular substrate for the sliding modification threshold. The NMDAR itself has shown to be very important in mediating the induction of synaptic plasticity. For instance, the NMDAR antagonist APV can prevent changes in synaptic strength in layer IV of mouse visual cortex (Chapter III). In studies of OD plasticity, Sato and Stryker demonstrated that the depression of deprived-eye responses is mediated by NMDARs in juvenile mice (Sato and Stryker, 2008) . In Chapter III, we also showed that the potentiation of nondeprived-eye responses following 7 days of MD is mediated by NMDARs. Therefore, both temporal phases of the juvenile OD shift are blocked following the application of NMDAR antagonist CPP. Thus, the NMDA receptor is essential for bidirectional, experience-dependent modifications in synaptic strength within the visual cortex.

SPECULATIONS ON POSSIBLE MECHANISMS MEDIATING SYNAPTIC PLASTICITY AND METAPLASTICITY

Although NMDAR-mediated signaling seems to be required for experience-dependent plasticity, it – as well as the molecules downstream of it – appears necessary for stable maintenance of the changes that occur (Mataga et al., 2002; Quinlan et al., 2004; Taha and Stryker, 2005). Invariably, it is the influx of calcium that is important to initiate changes, and other cellular mechanisms activated by calcium entry that run in parallel to NMDARs might underlie the maintenance phase of plasticity (Castellani et al., 2005). In rat visual cortex, LTP and LTD mechanisms have been shown to vary across layers (Wang and Daw, 2003; Rao and Daw, 2004). For instance, the conventional flow of information across the layers of neocortex can contribute to different types of plasticity with varying time courses. In the mouse, the direct activation of thalamic afferents in layer IV subsequently relays information to layers II/III and then to layers V/VI (Liu et al., 2008). Thus, it is possible that other cellular mechanisms running

in parallel to NMDAR activation can affect plasticity in distinct layers. For instance, while LTD underlies the depression of deprived-eye responses following monocular deprivation (Heynen et al., 2003), blocking the NMDAR-dependent endocytosis of AMPARs in layer IV during MD selectively occludes depression of deprived-eye responses without altering the potentiation of non-deprived eye inputs (Yoon et al., in press). Interestingly, the OD shift is not affected in layer II/III, indicating varied and perhaps distinct plasticity mechanisms across cortical layers. Furthermore, it has been demonstrated that the responses of cells in infra- and supragranular layers exhibit plasticity in response to sensory experience beyond juvenile development, even after layer IV cells are no longer plastic (Jiang et al., 2007). Other candidates that might regulate subsequent plasticity in supragranular layers include metabotropic glutamate receptors (mGluRs), cannabinoid receptors, and voltage-gated calcium channels (VGCCs). These mechanisms are discussed below, as well as illustrated in Figure 5-1, p. 125.

THE ROLE OF METABOTROPIC RECEPTORS

In hippocampus, mGluR activation can indirectly enhance NMDAR-mediated plasticity by depressing inhibitory transmission via GABAR-mediated inhibitory postsynaptic potentials (IPSPs) (Liu et al., 1993). However, Huemmeke and colleagues were able to parse out an NMDAR-independent form of LTP that required mGluR activation (Huemmeke et al., 2002). In addition, in GAD67–GFP mice where GABAergic neurons were labeled with GFP for identification, Sarihi et al. sought to understand layer II/III plasticity in visual cortex by recording excitatory responses from fast-spiking and non fast-spiking GABAergic neurons. By applying theta burst stimulation paired with postsynaptic depolarization, they found that the LTP that could be elicited at excitatory synapses onto fast-spiking GABAergic neurons was calcium-dependent. Calcium entry did not occur through NMDAR, L- or T-type channels, but rather was elevated intracellularly through mGluR5 receptor activation (Sarihi et al., 2008). Therefore, mGluR activation can work synergistically with NMDARs in experience-dependent plasticity.

THE ROLE OF CANNABINOID RECEPTORS

Cannabinoid receptors, in particular cannabinoid receptor type 1 (CB1R), have recently been identified as a mechanism that affects synaptic plasticity and OD plasticity. CB1Rs are located on either excitatory or inhibitory neurons (Azad et al., 2008). Endocannabinoids interact with cannabinoid receptors and are retrograde messengers that can modulate synaptic function. Although bath application of APV can block LTD in layers IV and II/III, it became evident that mouse layer IV plasticity was different than layer II/III plasticity. For example, loading cells in layer IV with PKA and inhibitors of AMPAR endocytosis blocked LTD in layer IV but not in layer II/III neurons. Only with the application of CB1R antagonist AM 251 were Crozier et al. able to significantly reduce LTD in layer II/III without affecting the plasticity in layer IV (Crozier et al., 2007). The most parsimonious explanation for these observations is the abundance of CB1Rs within layer II/III and its relative absence within layer IV (Deshmukh et al., 2007). The differences in plasticity between cortical layers as well as the time course of plasticity became even more evident following OD plasticity experiments performed by Liu and colleagues. A brief 1 day period of MD produces an OD shift that consists of depression of deprived-eye responses in both layers IV and II/III. Systemic administration of AM 251 left the OD shift intact in layer IV, but in layer II/III its expression was blocked. Therefore, plasticity in layer IV is not CB1R-dependent (Liu et al., 2008). These CB1R studies demonstrate that plasticity in layers II/III might bypass the requirement of NMDAR activation in layers II/III.

THE ROLE OF VOLTAGE-GATED CALCIUM CHANNELS

Another major source of calcium influx is through voltage-gated calcium channels. In juvenile rat visual cortex, Ohmura and colleagues were able to elicit LTP in the presence of APV using a stimulation protocol that was more intense than normally used. This form of LTP was blocked by nickel, which selectively blocks

T- and R-type calcium channels (Ohmura et al., 2003). The effect of dark-rearing the animals extended this type of plasticity into adulthood (Ohmura et al., 2003). Hence, metaplasticity can occur in the absence of NMDAR activation. Several types of voltage-gated calcium channels have been implicated in synaptic plasticity. For instance, in layer II/III of rat visual cortex, it was found that using P-type calcium channel blocker ω-agatoxin IVA (in low dose) following the induction of LTP returned responses to baseline. The same was observed following high doses of ω-agatoxin IVA that blocks Q-type calcium channels as well as for ω-conotoxin GVIA that blocks N-type calcium channels. However, this was not seen following the blockade of L-, T- or R-type calcium channels (Liu et al., 2004). To relate the necessity of T-type calcium channels from *in vitro* studies to *in vivo* studies, the Komatsu group tested the effects of T-type calcium channel blocker mibefradil during a 6 day monocular deprivation in juvenile rat. They showed that the depression of deprived-eye responses was unaffected, but the potentiation of non-deprived eye responses was blocked (Yoshimura et al., 2008). This result also shows the necessary but not sufficient requirement for both NMDARs (Cho et al., 2009) and VGCCs in OD plasticity.

THE ROLE OF THE EXCITATORY-INHIBITORY BALANCE

Over the course of postnatal development, inhibitory GABAergic transmission steadily increases (Morales et al., 2002). This increase in the strength of local inhibitory circuits also corresponds to the developmental decrease in plasticity (Luhmann and Prince, 1991; Guo et al., 1997). The balance of excitation and inhibition in OD plasticity was explored with the use of the GAD65 KO mouse, in which GABA release is significantly reduced. GAD65, localized to synaptic terminals, is an isoform of a GABA-producing enzyme (Hensch et al., 1998a). This disruption in inhibitory signaling in the GAD65 KO mouse did not affect spontaneous activity, habituation, retinotopic organization, orientation and direction selectivity, receptive field size, or LTP and LTD in visual cortex. However, following MD, there was no OD shift in the

GAD65 KO mice compared to their WT littermates. With infusion of diazepam to enhance inhibition, OD plasticity was restored (Hensch et al., 1998a). Thus, a causal role for inhibition in experience-dependent plasticity has been implicated. In effect, there is a loss of the closure of juvenile plasticity: OD plasticity could even be induced in adult GAD65 KO mice via brief MD and application of diazepam. Unlike the minimal OD shift observed after long-term MD in WT adult mice, in adult GAD65 KO mice, a period of 15 days of MD yielded a robust OD shift – leading to the hypothesis that the lack of inhibition plays a key role in extending juvenile-like forms of plasticity into adulthood (Steele and Mauk, 1999). It simply takes a longer period of deprivation for the changes to express themselves. With the infusion of diazepam earlier in life, however, this plasticity is not observed. Therefore, it seems that inhibition presented at a critical time point in the animal's life (i.e. during development) is enough to restore a normal time course of plasticity (Fagiolini and Hensch, 2000). To further pinpoint the locus of plasticity in mouse visual cortex, the use of knockins of various $GABA_AR$ subunits and their insensitivity to the application of diazepam revealed that the $\alpha 1$ subunit is important for OD plasticity (Fagiolini et al., 2004). The importance of inhibition in metaplasticity is that once it is mature, it gates changes in the magnitude of subsequent activity-dependent plasticity.

To investigate the effects of altering the excitatory-inhibitory balance in juvenile WT mice, Iwai and colleagues infused diazepam in P16 mice and then had the animals undergo MD at the peak of juvenile plasticity. They found that the OD shift was significantly attenuated; increasing the inhibitory to excitatory balance prematurely closed juvenile plasticity. In addition, dark-rearing animals until adulthood (> P50) prolonged juvenile plasticity and allowed for a robust OD shift, but a 2 day treatment of diazepam at P30 blocked the OD shift when these animals became adults (Huang et al., 1999; Iwai et al., 2003). Consequently, enhancing inhibition will close the period of juvenile plasticity even in the dark.

The results above lead one to ask: is GABAergic transmission bidirectionally modifiable (a la NR2A/NR2B protein levels) following experience? Morales et al. observed that the peak of GABAergic transmission overlapped with the peak of juvenile plasticity and, correspondingly, the decline of GABAergic transmission with the end of juvenile plasticity in rat visual cortex. By studying IPSCs in layer II/III in dark-reared and light-reared animals, they found that dark-rearing can decrease the maximal IPSC. Placing the animals in the light for 2 days is sufficient to reverse the IPSC to normal levels. Consistent with these findings, dark-rearing both juvenile and adult rat visual cortex resulted in a decrease in $GABA_AR$ expression (He et al., 2006). Thus, visual deprivation delays the maturation of inhibition. However, 3 weeks of normal rearing and subsequent dark exposure for 2 weeks does not alter the maximal IPSC, indicating that further sensory perturbations cannot alter the excitation/inhibition balance once it is in place. Therefore, this mechanism is likely not bidirectional or dynamic, which undermines the role of the excitatory-inhibitory balance as a mechanism underlying metaplasticity. These changes are manifested in the release probability of GABA onto synapses and the number of GABAergic synapses onto pyramidal neurons, not the strength of synapses (Morales et al., 2002).

Chattopadhyaya and colleagues examined the morphology of GABAergic synapses onto pyramidal neurons in visual cortex. The use of the bacterial artificial chromosome (BAC) mice that express GFP only in inhibitory parvalbumin-containing basket interneurons have shown that in the absence of visual experience, these cells are still able to target and surround pyramidal neurons in visual cortical slice cultures. Visual experience can also alter the extension and innervations of interneuron processes; for instance, dark-rearing will retard the maturation of perisomatic innervation of pyramidal neurons (Chattopadhyaya et al., 2004). As in the previously mentioned studies, once the maturation of inhibition occurs, the system is resistant to change. Moreover, the maturation of perisomatic innervation was disrupted only in

GAD67 heterozygote mice (GAD67 KO mice are lethal (Asada et al., 1997), but not in GAD65 KO mice, allocating the role of GAD67 for axonal and synaptic morphogenesis (Chattopadhyaya et al., 2007). Perisomatic innervation of pyramidal neurons is thus important for modulating excitatory firing, and the extent of innervation is experience-dependent.

To elucidate changes in inhibition following monocular deprivation, Gandhi et al. used two-photon microscopy with single-cell resolution to identify labeled inhibitory (GFP under GAD67 promoter) and excitatory (GFP-negative) neurons and observed responses by imaging calcium transients at different timepoints of a 4 day MD in mice. Initially, excitatory and inhibitory responses were equal, both showing the typical contralateral-eye bias to visual stimulation that is seen in rodents. However, following 2 days of MD, excitatory responses switch their favor from the contralateral- (deprived-eye) to the non-deprived eye. In contrast, the responses of the labeled inhibitory neurons are still driven by the contralateral, deprived eye. Given 2 more days of MD, the inhibitory responses finally switch their favor to the non-deprived eye and exhibit equal levels of strength for the non-deprived eye as do the excitatory cells (Gandhi et al., 2008). The delay in the modification of inhibitory neurons demonstrates how responses can reflect the absence of excitatory responses and show immediate depression of responses. Therefore, the metaplastic changes underlying MD can result from changes in the excitatory-inhibitory balance.

NEUROTROPHINS

A potential link between the developmental regulation of both excitatory glutamatergic and inhibitory GABAergic transmission in visual cortex are neurotrophins (Cotrufo et al., 2003; Gianfranceschi et al., 2003; Margottil and Domenici, 2003). Neurotrophins, in particular brain-derived neurotrophic factor (BDNF), are highly sensitive to activity-dependent manipulations. For example, nerve growth factor (NGF) infused into visual cortical slices of dark-reared animals

result in NR2A protein levels similar to those that were light-reared (Cotrufo et al., 2003). The expression of BDNF can modulate the activity and expression of NMDAR subunit composition specifically by increasing NR2A subunit levels in mouse visual cortex (Levine et al., 1998; Margotti et al., 2002). For instance, in patients with epileptic glioneuronal tumors and thus, increased excitability, there is co-localization of increased levels of BDNF and NMDARs (Aronica et al., 2001). In addition, BDNF can induce elevations of intracellular calcium, which are reduced by application of NMDAR and mGluR antagonists (Mizoguchi and Nabekura, 2003; Yang and Gu, 2005). Therefore, BDNF interacts with excitatory glutamatergic receptors in visual cortical plasticity. BDNF also shows a pattern for activity-dependent regulation (Castren et al., 1992): overexpression of BDNF leads to early closure of the period of juvenile plasticity (Hanover et al., 1999), whereas visual deprivation results in down-regulated levels of BDNF (Gianfranceschi et al., 2003). In terms of OD plasticity, decreased expression of BDNF follows monocular deprivation (Rossi et al., 1999). It was already understood that dark-rearing delays maturation of inhibition and the end of juvenile plasticity in visual cortex. The demonstration of the interaction between inhibition and BDNF results from studies comparing dark-reared and light-reared BDNF overexpressing mice. Despite rearing conditions that would, in normal mice, elicit opposite extensions of juvenile plasticity, the overexpression of BDNF in dark-reared mice showed similarly mature GABAergic inhibition as in light-reared mice (Gianfranceschi et al., 2003). Therefore, neutrophins interact with the maturation of NMDARs and inhibition in an experience-dependent manner, and show a link between metaplasticity mechanisms that might work in the absence of the other.

FUTURE EXPERIMENTS

UNDERSTANDING THE LINK BETWEEN MECHANISMS FOR SYNAPTIC PLASTICITY AND METAPLASTICITY

As discussed above, it appears that multiple mechanisms may underlie metaplasticity in rodent visual cortex. Fagiolini and colleagues looked at the NR2A KO mouse and tested the importance of the NR2A subunit in OD plasticity (Fagiolini et al., 2003). They observed that NR2A KO animals had longer NMDAR EPSCS, indicative of NR2B-containing NMDARs, which persisted into adulthood. They performed acute recordings in layer V of binocular cortex *in vivo* (as in Gordon and Stryker, 1996), before and after monocular deprivation, and found that the OD shift was significantly smaller than in WT littermates due to a loss of depression of deprived-eye inputs (Fagiolini et al., 2003). However, in this study of the NR2A KO mice, the infusion of diazepam, a $GABA_A$ receptor agonist, rescued the OD shift. Other molecules such as BDNF have been shown to increase GABA receptor-dependent inhibition, which has led to a premature close of juvenile plasticity (Hanover et al., 1999; Gianfranceschi et al., 2003). Is this maturation of inhibition caused by a modification of the NMDAR subunit composition to favor NR2A? An experiment to address these concerns may ask the following: (1) What is the level of basal inhibition in the NR2A KO mouse?

Experiment 1: Determining the link between the NR2A subunit and inhibition. It has been shown that inhibitory interneurons express more NR2A protein compared to excitatory pyramidal neurons (Kinney et al., 2006). Therefore, it is possible that the role of NR2A is to recruit the activity of inhibitory cells. In the absence of NR2A, inhibition may not increase. Thus, increasing inhibition via diazepam can rescue the smaller OD shift observed in the Fagiolini study of the NR2A KO mouse. To understand if plasticity observed in the NR2A KO mice is due to NR2A's role in increasing inhibition, it is necessary to determine the basal level of inhibition in these

mice. To do so, one can prepare visual cortical slices of the NR2A KO animals and compare layer IV input-output response curves of inhibitory postsynaptic potentials or currents to that of wild-type mice. If the animals do demonstrate less inhibition, it is possible that inhibition is recruited by the activation of NR2A. Furthermore, it is possible that overexpressing NR2A protein in the NR2A KO mouse during the period of juvenile plasticity will lead to normal levels of inhibition and amelioration of the plasticity phenotype, thus demonstrating a role of NR2A protein in the excitatory-inhibitory balance.

DETERMINING THE FUNCTIONAL SIGNIFICANCE OF THE NR2A SUBUNIT IN AN ACUTE PREPARATION

Studies using genetic manipulations have proven invaluable in determining possible mechanisms that underlie behavioral processes. Most of the studies described in this thesis have taken advantage of knockout technology that engineers a targeted gene mutation in mice resulting in inactivation of that gene's expression. Often, a targeted gene is vital for embryonic development and cannot be studied during the postnatal life of the mouse. Although the NR2A KO mouse is not lethal, it is possible that global elimination of this protein might result in related or unrelated developmental aberrations that can affect how ensuing plasticity studies are interpreted. We tested for the possibility of compensatory mechanisms in this knockout by confirming that the levels of NR1, NR2B and GluR1 protein were not affected, but one cannot be too careful to exclude underlying developmental defects. Therefore, conditional knockout or knockdown technology has provided a tool to overcome this restriction and has been used to study gene inactivation in specific regions, cell types, and time points in the life of the mouse. Therefore, the next set of experiments re-ask the questions of this thesis: (1) During the period of juvenile plasticity, will a conditional NR2A knockout or knockdown in visual cortex exhibit the same shift in the modification threshold as the NR2A KO? (2) Does overexpression of NR2A dictate the shift in the

modification threshold? and (3) Does introduction of NR2A in the NR2A KO mouse result in a rescue of the altered synaptic plasticity and OD plasticity observed?

Experiment 1: Conditional NR2A knockdown and re-expression of NR2A during the period of juvenile plasticity. The use of a conditional knockdown of NR2A would clearly show that the properties of synaptic plasticity as well as metaplasticity are affected acutely by the reduction in NR2A during the period of juvenile plasticity. Experiments described in Chapters II and III would be repeated under these conditions. Concurrently, if the resultant change in NMDAR subunit composition modulates the modification threshold, the re-expression of NR2A levels should be able to rescue the altered plasticity exhibited. Since there are different mechanisms responsible for plasticity according to layer, it would be advantageous if NR2A can be specifically absent in layer IV of visual cortex as this layer is thalamorecipient.

Experiment 2: Overexpression of the NR2A subunit – Do the levels of NR2A dictate/alter the modification threshold? Although Philpot et al. showed that overexpression of NR2B did not promote plastic changes as seen in the hippocampus, a valid concern from the paper is that while there was overexpression of NR2B mRNA, the protein itself was not translated in visual cortex (Philpot et al., 2001b). Since we have seen that exposing dark-reared animals to light can induce an elevation in NR2A protein levels, is it possible that in dark-reared animals, forcing an increase in NR2A protein levels alters changes in synaptic plasticity *in vitro*? To test this idea, overexpression of NR2A in the visual cortex would be conditional and region-specific. Wild-type mice would be raised in the dark until they are juveniles, thus delaying maturation of visual response properties (Carmignoto and Vicini, 1992), and conditional overexpression of NR2A would be induced in dark-reared visual cortex. Quantitative assessment of NR2A protein from synaptoneurosome or biotinylation preparations would confirm

overexpression of NR2A protein in visual cortex. Then, *in vitro* visual cortical slices would be prepared, and the effects of stimulation protocols such as 40 Hz or 1 Hz will be determined on responses collected in either layers II/III or IV. Even though it has been demonstrated that stimulation frequencies such as 10 Hz do not produce any synaptic change (Dudek and Bear, 1992), it is possible that overexpression of NR2A may result in the modification threshold to dramatically shift to the right, such that LTD may be induced. However, if the shift is not as dramatic, overexpression of NR2A levels (levels similar to those in light-reared WT mice) will result in a magnitude of potentiation or depression following 40 Hz or 1 Hz stimulation to be comparable to light-reared WT mice.

Experiment 3: Expression of the NR2A subunit in the NR2A KO mouse. Can expression of NR2A result in a rescue of synaptic and OD plasticity? This experiment tests the requirement of NR2A protein in obtaining WT plasticity. Using the electrophysiological assays described in Chapters II and III, an expression of the NR2A subunit (a level similar compared to that of light-reared WT mice) would be induced during the period of juvenile plasticity in either the dark-reared or light-reared NR2A KO mouse. If the NR2A subunit is sufficient, it should be able to correct the leftward shift in the modification threshold in both synaptic plasticity and OD plasticity studies.

FUNCTIONAL WM ASSAYS AND THE IMPACT OF THALAMOCORTICAL INPUT

Over the years, it is becoming increasingly apparent that the thalamus has a role in the ongoing moment-by-moment processing of sensory input. This occurs via the massive feedback pathway from the cortex and via nonspecific modulatory inputs from the brainstem and other areas (Sherman and Guillery, 2006). For instance, it has been shown in the cat visual system that corticofugal feedback is important for finely tuning receptive field properties (Andolina et al., 2007). While we discussed ways in which we validated the comparison between WM stimulation and patterned

visual stimulation in mouse visual cortex, it would be helpful to understand the impact of thalamocortical input alone in mouse visual processing. The use of channelrhodopsin-2 and the novel pharmacological method described in Chapter IV will help to address these questions: (1) Does specific activation of LGN afferents result in activation of cells in layer IV of mouse visual cortex? (2) How much of thalamocortical input underlies synaptic plasticity in layer IV *in vitro*? and (3) How much of thalamocortical input underlies visual processing *in vivo*?

Experiment 1: Demonstrating functional WM stimulation. In addition to the assays performed in Chapter IV, another way to validate functional thalamocortical connections *in vitro* is to use the visual thalamocortical slice preparation, where LGN is in the same plane as visual cortex (MacLean et al., 2006). It would be interesting to stimulate LGN and compare responses evoked in layer IV of visual cortex with the responses evoked in a slice that has been bath-applied with the muscimol + SCH50911 drug cocktail. Also, the advent of channelrhodopsin-2, a light-gated, cation-selecitive channel, can be used as a tool for selective noninvasive control of neuronal excitability along the thalamocortical pathway (Zhang and Oertner, 2007). Packaging channelrhodopsin-2 into an HSV vector will allow for anterograde expression of channelrhodopsin-2 along a particular pathway. Thus, injecting this virus into LGN will propagate expression of this receptor along thalamocortical axons. By shining light onto WM, it would be possible to selectively activate thalamocortical axons, and determine if responses can be evoked from layer IV cells from normally-prepared visual cortical slices. In addition, functional plasticity studies such as studying the properties of synaptic plasticity by providing noninvasive high- or low-frequency light stimulation to channelrhodopsin-2-positive afferents will also prove that WM stimulation is practical despite the absence of an intact LGN. These assays will contribute to our understanding of how much information is preserved following decussation of LGN in a visual cortical slice preparation.

Experiment 2: Plasticity Studies using the thalamocortical (TC) drug cocktail. Since we know *in vitro* that a large portion of the response evoked by WM stimulation is monosynaptic and of thalamic origin, it would be interesting to use the TC drug cocktail (muscimol + SCH50911) and then observe how much of the thalamic response following high- or low-frequency stimulation that would typically elicit LTP or LTD changes without activation of intracortical input. To test this, we would prepare mouse visual cortical slices and bath apply the TC drug cocktail, and collect a baseline of responses (either field potentials or PSPs) from cells in layer IV of visual cortex. In another group of animals, we would induce theta burst or 1 Hz stimulation to WM, bath apply the TC drug cocktail, and then observe the consequences of these protocols. These experiments would demonstrate the role of intracortical activity in synaptic plasticity mechanisms.

Experiment 3: Isolating thalamocortical input *in vivo* and testing alterations in plasticity *ex vivo*. By using the TC drug cocktail, it is possible to determine that the changes induced by SRP or OD plasticity is based on thalamocortical input. Furthermore, one can understand the impact of intracortical activity in visual cortical processing by testing if changes in thalamic input itself can occlude plasticity changes *in vitro* either in the presence or absence of the drug cocktail. This can be done by infusing the TC drug cocktail described in Chapter IV *in vivo* after SRP training sessions or after a brief 1 day of MD, which is sufficient to observe depression of deprived-eye responses (Liu et al., 2008). Measurable responses can be compared to responses of experimental animals that have not received TC drug cocktail infusion. Then, visual cortical slices will be prepared and theta burst stimulation or low-frequency stimulation can test the significance of thalamic input or the lack of intracortical activity in animals that underwent SRP or 1 day MD, respectively. Slices may be bath-applied with the TC drug cocktail before and after the stimulation

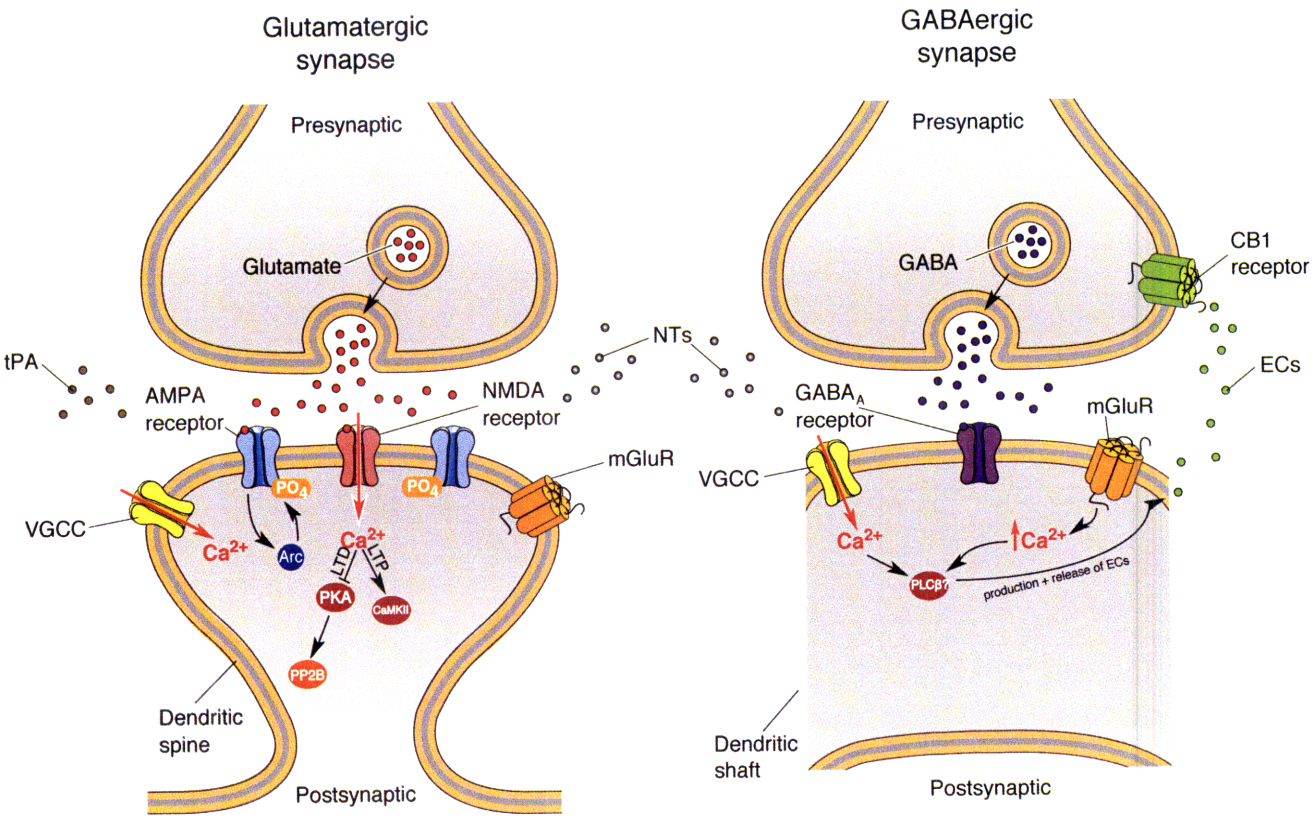
protocol to demonstrate how thalamic input changes *ex vivo*. Alternatively, slice preparations that allow the recruitment of intracortical activity may show different results, suggesting the importance of cortical activity *ex vivo*.

CONCLUDING REMARKS

Criteria to be considered as a mechanism for metaplasticity include correlative expression of molecules over the course of development, altered plasticity if the molecule's actions are disrupted, and amelioration of altered plasticity if the molecule is functionally reintroduced into the system. The introduction and discussion have mapped various mechanisms that mediate metaplastic changes in visual cortex that include NMDARs, their downstream effectors, as well as mGluRs, VGCCs, CB1 receptors, and the excitatory-inhibitory balance. However, controversy over interpretation of results is rooted in the difference of experimental protocols, paradigms, species and analysis. The clamor to support an overly simplistic view of an absolute mechanism underlying experience-dependent plasticity can cloud objectivity. Individual mechanisms do not have to be the only ones underlying plasticity, but may likely work together when intact and compensate when one is lacking. For example, it is not legitimate to rule out a mechanism underlying OD plasticity if the OD shift survives in spite of genetic deletion of that mechanism. While studying the mechanisms that might underlie plasticity in other regions of the brain and reassigning them to be important in visual cortex has proved beneficial, novel mechanisms can be overlooked. The use of DNA microarray analysis has also identified a number of candidate genes otherwise unsuspected that might regulate juvenile plasticity and metaplasticity in the visual cortex. Scientists in Mriganka Sur's lab are individually and combinatorially assessing these genes, including insulin-like growth factor binding protein-5 (IGFBP5), which has proven to be

important in OD plasticity (Tropea et al., 2006; Lyckman et al., 2008). The discovery of plasticity differences found in layers of cortex is also an interesting venture to pursue. Investigations into the time course at which rapid induction of plasticity takes places by a particular mechanism in a particular layer versus later and more sustained versions of plasticity in other layers is just starting to take form. There is much that needs to be resolved in terms of how the loss of one mechanism might start the compensatory activation of another mechanism. Most importantly, it would be of considerable value to determine the link between mechanisms instead of trying to discover one sole mechanism that is necessary and sufficient. The experiments described in this thesis focus on understanding one molecular mechanism and to this end, provide strong support that the NR2A subunit plays an important role in both synaptic plasticity and metaplasticity.

FIGURE 5-1



Proposed mechanisms underlying metaplasticity.

Schematic of glutamate- and GABA-receptor-mediated mechanisms of metaplasticity. A dendritic spine receives inputs from a glutamatergic presynaptic terminal, and a dendritic shaft receives inputs from a GABAergic presynaptic terminal. Postsynaptic glutamate and GABA receptors bidirectionally regulate synaptic strength and ocular dominance plasticity by triggering signaling molecules downstream such as CaMKII and calcineurin, Arc for AMPAR trafficking, and produce and release endocannabinoids to retrogradely activate presynaptic cannabinoid receptors. Other effectors include extracellular matrix molecule tPA and neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF).

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