Critical requirements for NMDA receptors in experience-dependent plasticity in the visual cortex

by

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Abstract

NMDA receptors play an essential role in many forms of experience-dependent plasticity in the visual system. NMDA receptors support the ocular dominance (OD) shift, development of receptive field properties in cortical neurons, and learning tasks associated with the visual cortex, as well as the cellular correlates of such plasticity measurements, ie long-term potentiation (LTP) and long-term depression (LTD). Complete removal of NMDA receptors abolishes plasticity, while selective removal of NMDA receptors from distinct circuits in the visual system can reveal a more specific requirement and identify circuits required for plasticity. Using this approach, I aimed to delineate the brain circuits involved in two forms of experience-dependent plasticity. First, I determined a layer-specific requirement for NMDA receptors in stimulus-specific response potentiation (SRP), which is a model for the plastic changes that may underlie perceptual learning. SRP acquisition requires NMDA receptors in the cortex, and it can be supported by expression of NMDA receptors in only deep layer 4 and layer 5. In contrast, this minimal expression of NMDA receptors did not support a paradigm for OD plasticity in adult mice. The contrasting requirements for NMDA receptors in these two forms of experience-dependent plasticity suggest that distinct forms of plasticity can be supported in separate cortical circuits.

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Chapter 1
General Introduction

The brain responds rapidly to changes in experience. In the visual system, this feature is essential for adapting to changes in sensory experience. This adaptability, also called plasticity, is apparent when the visual system has been deprived of visual experience or when the visual system has been exposed to new experiences in an instructive manner. Both phenomena have been observed in humans, and the mechanisms underlying experience-dependent plasticity have been extensively studied in animal models. The visual system is well-suited to these experiments, because experience can be controlled by paradigms such as rearing an animal in darkness (dark rearing), or suturing the eyelid of one eye (monocular deprivation, or MD), or by exposing the animal to new visual patterns or scenes. In addition, the basic circuitry of the visual system is straightforward; information flows directly from the retina to the lateral geniculate nucleus (LGN) in the thalamus to the visual cortex. Inputs to the visual cortex generally converge on layer 4, which projects to layers 2/3. In turn, layers 2/3 project to other cortical areas, or to layer 5. Experience-dependent plasticity is thought to occur in the neurons and synapses in the visual cortex.

Ocular dominance plasticity

In the 1960’s, David Hubel and Torsten Wiesel’s pioneering experiments established ocular dominance (OD) plasticity as a model for experience-dependent changes. OD plasticity was first described in the visual cortex of juvenile cats (Wiesel
and Hubel, 1963b) and is characterized by the change in neuronal responsiveness after a
period of monocular deprivation (MD). Under normal conditions, approximately 80% of
cortical neurons in the cat are driven binocularly, although most neurons show an ocular
dominance, and fire preferentially to input through one or the other eye (Hubel and
Wiesel, 1959, , 1962). After MD, the neuronal response shifts to favor the non-deprived
eye (Wiesel and Hubel, 1963b), even when the period of MD is as brief as 2 days (Olson
and Freeman, 1975; Mower, 1991). Accompanying the functional shift in ocular
dominance are anatomical changes, which include shrinkage of LGN neurons that receive
inputs from the deprived eye (Wiesel and Hubel, 1963a) and expansion of LGN afferents
representing the non-deprived eye (Hubel et al., 1977; Shatz and Stryker, 1978). In the
most simplistic model, these rearrangements at the LGN to layer 4 synapse are the basis
for the OD shift, and instruct plastic changes in subsequent layers in an “inside out”
sequence (Thompson, 2000).

Laminar analysis of cortical plasticity remains an active area of study. An
alternate model postulates that reorganization begins in superficial layers of the cortex,
and only later shapes the response in the input layer or subcortical structures
(Buonomano and Merzenich, 1998). Support for this model comes from several sources:
in the visual cortex, brief MD causes an OD shift in the neurons in superficial layers, with
no detectable change in layer 4 neurons (Trachtenberg et al., 2000). In addition,
functional changes in cortical neurons precede anatomical rearrangements of LGN axons,
which are marked only 4-7 days after MD (Antonini and Stryker, 1993, , 1996). Finally,
activity in horizontal connections between layer 2/3 neurons is thought to influence
formation of functional columns, independent of sensory experience (Ruthazer and Stryker, 1996; Crair et al., 1998).

OD plasticity is most robust during a brief period in early postnatal life (Hubel and Wiesel, 1970), after which the functional changes and anatomical rearrangements are not observed in layer 4. However, plasticity in superficial layers of the cortex is thought to mediate OD changes in adult animals. In older cats and monkeys, there are some reports that OD plasticity persists outside of layer 4 (Blakemore et al., 1978; Daw et al., 1992). Superficial layers also show dramatic changes after retinal lesions in adult animals, which are thought to be mediated by horizontal connections (Gilbert and Wiesel, 1992; Das and Gilbert, 1995) and not thalamocortical rearrangements (Darian-Smith and Gilbert, 1995). In addition, plasticity in the somatosensory cortex in adult animals seems to be mediated in superficial layers and not layer 4 (Fox, 2002).

Mechanisms of OD plasticity

What synaptic mechanisms underlie the OD shift? One possibility is that inputs from each eye compete for territory, and weak inputs from an eye deprived by MD lose the contest. A competitive mechanism could explain the effects of MD in juvenile animals, and is consistent with the observation that equal-sized eye-specific inputs are still formed after binocular deprivation (Hubel and Wiesel, 1965). On the other hand, a pure weak vs. strong competition would not explain how a deprived eye recovers during binocular visual experience (Mitchell and Gingras, 1998) or reverse suture, where the deprived eye is opened and the non-deprived eye is shut (Mitchell et al., 1977). Nor could competition between inputs explain why monocular inactivation, where the non-
deprived eye would be expected to win all, leads to a weaker shift than monocular deprivation (Rittenhouse et al., 1999).

Another possible mechanism invokes a homosynaptic learning rule, in which inputs from the 2 eyes are considered separately (Sengpiel and Kind, 2002; Bear, 2003). Here, the strengthening or weakening of inputs depends not on the relative strength of the two inputs, but rather on the activity and experience of the postsynaptic neuron. Thus, weak inputs from a deprived eye become depressed when the activity is too low, below a threshold of activity needed to strengthen the synapse. On the other hand, inputs from the non-deprived eye are active and correlated with the activity of the postsynaptic neuron, so these synapses are strengthened. The BCM theory expands the model further by postulating that the threshold of activity (or modification threshold, \( \theta_m \)) needed for synaptic strengthening changes with prior experience (Bienenstock et al., 1982). Thus, a paradigm like dark rearing or a period of binocular deprivation lowers \( \theta_m \) such that less activity is needed to strengthen the synapses. This is observed in long-term potentiation (LTP) and long-term depression (LTD) measurements, which are in vitro correlates for synaptic strengthening and weakening. Stimulation frequencies required to elicit LTP and LTD are different in dark-reared animals than in light-reared controls (Kirkwood et al., 1996). Finally, the BCM model can also explain the experimental observations that a deprived eye recovers after MD when the deprived eye is opened (Mitchell and Gingras, 1998) or when reverse suture is performed (Mitchell et al., 1977), although recovery depends on correlated inputs from the two eyes (Sengpiel and Kind, 2002).

LTP and LTD are correlates for the strengthening and weakening of eye inputs observed after MD. Thus, many of the cellular and molecular mechanisms are shared by
both systems. One mechanism is the requirement for NMDA receptors. NMDA receptors have a dual requirement of glutamate binding and sufficient depolarization to remove a Mg\(^2+\) ion from the channel pore, which means that pre and postsynaptic activity must be correlated. In the hippocampus, NMDA receptors were postulated to be coincident detectors for LTP, in a model for Hebbian plasticity. In the visual cortex, NMDA receptors are required for OD plasticity (Kleinschmidt et al., 1987; Bear et al., 1990), as well as LTP (Artola and Singer, 1987, 1990) and LTD (Kirkwood and Bear, 1994) in the visual cortex. Additional studies have monitored the expression of NMDA receptor subunits in different layers of the cortex across different ages (Fox et al., 1992; Catalano et al., 1997; Mower and Chen, 2003), in order to correlate NMDA receptors with juvenile and adult OD plasticity.

Insights from rodent models

Further understanding of the molecular mechanisms underlying OD plasticity has been enhanced by work in mice. The visual system in mice lacks some of the anatomical features found in the visual system of higher mammals, such as ocular dominance columns (Drager, 1975). Also, 97\% of projections are crossed, giving rise to a large monocular segment in the visual cortex, and a much smaller binocular region in which most neurons respond preferentially to the crossed, or contralateral, pathway. Nonetheless, a brief period of MD in rodents shifts the ocular dominance in favor of the non-deprived eye (Drager, 1978), and, as in cats and monkeys, the OD shift is most easily elicited during a critical period in early postnatal life (Gordon and Stryker, 1996). Using genetically-modified mice, researchers have identified many molecules which are
required for OD plasticity. In addition to NMDA receptors (Sawtell et al., 2003),
molecules associated with LTP and LTD have been shown to play a role (Daw et al,
2004). Other mechanisms implicated in juvenile OD plasticity include development of
inhibitory neurons (Hanover et al., 1999; Huang et al., 1999), maintenance of
excitatory/inhibitory balance (Fagiolini and Hensch, 2000), and changes in structural
plasticity that might correspond to synaptic rearrangements of deprived-eye inputs
(Mataga et al., 2002; Mataga et al., 2004; Oray et al., 2004). Another mechanism related
to structural rearrangements is the physical interactions of molecules in the extracellular
matrix (Pizzorusso et al., 2002) and the presence of myelin (McGee et al., 2005), both of
which appear to play a permissive role in OD plasticity. In particular, a juvenile-like OD
shift was uncovered in adult mice when these molecules were disrupted.

OD plasticity in adult rodents

OD plasticity in adult mice is an attractive model for experience-dependent
plasticity in mature animals, and could provide insight into adult learning, or recovery
from disuse or injury. A robust response to MD in adult mice has been demonstrated
using a variety of techniques, including single unit recordings (Fischer 2007), optical
imaging of intrinsic signals (Hofer et al., 2006), induction of immediate-early genes
(Pham et al., 2004; Tagawa et al., 2005) and visual evoked potentials (Sawtell et al.,
2003; Lickey et al., 2004; Pham et al., 2004; Fischer et al., 2007). So far, studies in adult
rodents have uncovered several mechanisms that restore juvenile-like plasticity to mature
animals (Pizzorusso et al., 2002; Pham et al., 2004; McGee et al., 2005; He et al., 2006;
Hofer et al., 2006). There are several differences between the adult and juvenile OD
shifts, indicating that adults require longer periods of MD to induce an OD shift (Frenkel et al., 2006; Hofer et al., 2006), that the shift is mediated by potentiation of non-deprived eye inputs rather than a loss of deprived eye inputs (Pham et al., 2004; Tagawa et al., 2005; Frenkel et al., 2006; Hofer et al., 2006), plasticity is sensitive to barbiturate anesthesia, persistence of MD effects are reduced in adults (Pham et al., 2004). Some of these requirements, like length of MD, may depend on the technique used, for example Lickey et al (2004) measured a change in VEP amplitudes after only 2d of MD, while Hofer et al (2006) required 6-7d of MD before observing a change in optical signal. The strengthening and weakening of deprived-eye and non-deprived eye inputs depends on the number of days of MD (Frenkel and Bear, 2004), which may explain some of the variation in dynamics observed by different groups. There is also the possibility that each technique might measure different characteristics of the OD shift.

Visual Evoked Potentials

Of the techniques used to measure the OD shift in adults, VEP recordings have been the most commonly used. We also use this method in this thesis. There is some criticism that VEPs measure subthreshold activity, rather than the response properties of neurons, and changes in VEP amplitudes after MD may not have relevance to visual function (Hensch 2005). However, several reports suggest that VEPs reflect visual capability. (Porciatti et al., 1999) used VEPs to measure spatial resolution, whose behavioral counterpart is visual acuity. In humans, VEPs are used to measure functional properties of the visual cortex (Tyler et al., 1979). Notably, the VEP response in humans changes after monocular deprivation, caused by unilateral cataracts (Sloper and Collins,
1995), suggesting that VEP measurements reflect functional changes associated with impaired vision.

Stimulus-specific response potentiation (SRP), a correlate of perceptual learning.

Another advantage of VEP recordings is the ability to implant chronic recording electrodes and take measurements in the same animal, before and after MD. The chronic recording technique led to the discovery of stimulus-specific response potentiation (SRP) (Frenkel et al., 2006), a novel plasticity paradigm in mice. SRP is characterized by an enhanced VEP response, upon repeated exposures to the same stimulus. This instruction-induced plasticity is a correlate for perceptual learning, which has been described in the visual system of higher primates (Fiorentini and Berardi, 1980; Karni and Sagi, 1993; Schoups et al., 2001; Furmanski et al., 2004). SRP may be a mechanism for perceptual learning, so it is important to characterize molecular requirements and layer-specific requirements for this form of plasticity.

Summary

Here, I explore molecular mechanisms of two forms of experience-dependent plasticity in adult mice: SRP and OD plasticity. In Chapter 2, I describe spatial requirements for NMDA receptors in acquisition of SRP. Using genetically-modified mice in which NR1 was deleted from specific cortical layers, I provide evidence that acquisition of SRP depends on NMDA receptors in the cortex, but SRP can still be acquired as long as NMDA receptors are expressed in deep layer 4 and layer 5. Chapters 3 and 4 address the molecular requirements of OD plasticity. In Chapter 3, I explore the
relationship between dendritic spine morphology and OD plasticity. Previous studies indicate that spine morphology and dynamics are affected by visual experience (Mataga et al., 2004; Oray et al., 2004; Wallace and Bear, 2004), but the functional significance of these changes, particularly in adult animals, is poorly understood. Here, I measured the OD shift in adult mice from a transgenic line in which the dominant-negative form of the p21-activated kinase (dnPAK) was expressed late in postnatal life. In these mice, cortical neurons in layers 2/3 show abnormal spine morphology, which correlates with deficits in LTD and enhanced LTP in the temporal cortex and corresponding memory deficits (Hayashi et al., 2004). The abnormal spine morphology did not, however, correlate with deficits in OD plasticity (see Chapter 3), suggesting that structural changes in spine morphology may have a more subtle effect on this form of plasticity. Another possibility is that the OD shift is mediated by neurons in cortical layers where dnPAK was not expressed. In Chapter 4, I explore this possibility by using NMDA receptor mutant mice in which the essential NR1 subunit has been removed from cortical layers 2/3, superficial layer 4, and layer 6. I present evidence that minimal expression of NMDA receptors in deep layer 4 and layer 5 is not sufficient to support OD plasticity, as measured by changes in induction of the immediate-early gene Arc. This finding indicates that deletion of NMDA receptors in superficial layers of the cortex has much more severe consequences for OD plasticity than does expression of dnPAK. In addition, this result highlights a major difference in spatial requirements for NMDA receptors in SRP and OD plasticity.
Abstract

The visual cortex in adult mice is highly responsive to changes in visual experience. One example of this experience-dependent plasticity is stimulus-selective response potentiation (SRP). SRP is an enhanced cortical response, measured by visual evoked potentials (VEPs), and observed only when awake, head-restrained mice are repeatedly exposed to the same visual stimulus. Here, we compare SRP measurements in 2 independent lines of conditional knockout mice to address critical requirements for SRP acquisition. In one knockout line, G35-3 NR1, the essential NR1 subunit of the NMDA receptor is deleted from all layers of the cortex. SRP was absent in G35-3 NR1 mutant mice, indicating that SRP relies on NMDA receptor-mediated plasticity in the cortex. In a second knockout line, CW2-NR1, the NR1 subunit is deleted in a layer-specific pattern that leaves NR1 signal only in deep layer 4 and layer 5 neurons. Interestingly, SRP is retained in CW2-NR1 mice, suggesting that NMDA receptors in deep layer 4 alone are sufficient for SRP acquisition. These findings provide evidence of a layer-specific requirement for NMDA receptors in SRP in adult mice.
Introduction

A novel form of experience-induced plasticity was recently identified in the visual cortex of mice (Frenkel et al., 2006). Called Stimulus-specific Response Potentiation (SRP), this plasticity shares several qualitative similarities with perceptual learning (De Weerd et al., 2006). Both require repetitive exposure to a particular stimulus and are characterized by an enhanced response to the stimulus. In addition, the enhanced responses reach a plateau after repeated exposures, and the responses are retained in the absence of further stimulus presentation. Finally, the potentiation is specific to the orientation of the stimulus. Based on these similarities, Frenkel et al (2006) postulated that SRP is a correlate and possible mechanism for perceptual learning. A dissection of the neuronal circuits and molecules involved in SRP could also reveal critical elements of perceptual learning.

One molecular mechanism essential for SRP is signaling through the NMDA receptors. This was demonstrated by systemic injection of CPP, an NMDA receptor antagonist, which blocked SRP acquisition (Frenkel et al, 2006). The finding is consistent with other reports in which NMDA receptors are required for induction of synaptic plasticity in cortical slices (Kirkwood et al., 1993; Wang and Daw, 2003), and for evoked plasticity in anesthetized rats (Heynen and Bear, 2001; Clapp et al., 2006). More generally, NMDA receptors act as coincidence detectors and mediate numerous forms of synaptic plasticity all over the brain (Lynch, 2004). Although Frenkel et al’s study (2006) confirmed that SRP is mediated by NMDA receptor signaling, it could not address which circuits in the visual system require them. In this study, we aimed to
identify the layers of the cortex where NMDA receptors play a crucial role in SRP. For this purpose, we adopted the strategy that has been used effectively in the dissection of mouse hippocampal functions (Tsien et al, 1996a; Nakazawa et al, 2003; McHugh et al, 2007). This spatially-restricted genetic deletion strategy resulted in a loss of plasticity at a specific set of hippocampal synapses and a behavioral impairment in specific aspects of learning and memory. Distinct memory processing could then be attributed to plasticity in specific regions of the hippocampus. Similarly, deletion of NMDA receptors in selected cortical layers was used here to identify the laminar site of the plasticity requirement for SRP.
Materials and Methods

Generation of Conditional NR1 Knockout Mice

The CW2-NR1 KO line was generated by crossing a forebrain-specific, αCaMKII promoter-driven Cre transgenic mouse line, referred to as CW2 (Zeng et al., 2001), with a second mouse line, the homozygous “floxed” NMDA receptor subunit-1 (fNR1) line, in which two loxP sites flank the transmembrane domain and C-terminal region (exons 11-21), (Tsien et al., 1996). Founder mice (Cre/+, fNR1/fNR1) were crossed to homozygous fNR1 mice (+/+, fNR1/fNR1) to produce CW2-NR1 KO mutant (Cre/+, fNR1/fNR1) and CW2-NR1 control (+/+, fNR1/fNR1) littermates. For this study, we used mutant mice at 4-5 months of age, in order to take advantage of the age-dependence of NR1 deletion in the cortex (Fukaya et al., 2003).

The G35-3 NR1 KO mouse line was generated as described (Sawtell et al., 2003). In short, a Cre transgene driven by a BAC containing the promoter and upstream regulatory sequence of the kainate receptor subunit KA1 was crossed to the homozygous fNR1 line. The progeny of this cross (Cre/+, fNR1/) were then crossed to a third mouse line in which one allele of the NR1 gene was deleted (Iwasato et al., 1997). These crosses produced G35-3-NR1 KO mutants (Cre/+, fNR1/NR1 null), and control littermates (+/+, fNR1/NR1 null). We used animals at 4-5 months old in order to match the age of the CW2-NR1 mutant line.
In situ hybridization experiments.

In situ hybridization experiments for NR1 mRNA and orphan nuclear receptor RORβ mRNA were performed on adjacent coronal sections from mice of all genotypes. Additional in situ hybridization experiments for Arc mRNA were also performed on neighboring coronal sections. Antisense oligonucleotide probes, corresponding to exon 11 of NR1, to the full-length cDNA of RORβ (IMAGE Consortium Clone ID5358124, Open Biosystems), or to the first 200 bp coding sequence of Arc (generated by PCR, using the primers 5'-atggagctggaccatatgacc-3' and 5'-taatacgactcactataggagacctgtgcaacctttcagc-3'), were generated by in vitro transcription (Riboprobe systems, Promega) and labeled with [33P]dUTP (Perkin Elmer). Probes were hybridized overnight to either cryostat sections (18 μm thick) or paraffin sections (8 μm thick). Specificity of the hybridization was confirmed by an absence of signal when the sense oligonucleotide was similarly labeled and hybridized. mRNA expression was verified by exposing the hybridized sections to a single x-ray film (Kodak BioMax) for 1 day. If the film indicated presence of hybridized probe, the sections were dipped in nuclear track emulsion (NTB-2, Kodak) and incubated at 4°C in a light-sealed box for up to 2 weeks. The sections were then developed in Kodak N19 developer, fixed, counterstained with Mayer’s hematoxylin and imaged under darkfield optics (Zeiss AxioVision).

Quantification of NR1 mRNA

NR1 expression was quantified in the darkfield images using Matlab (The MathWorks, Natick, MA). We selected 5-8 coronal sections from 2 mice of each
genotype, selecting sections that contained V1 at comparable rostral/caudal levels. A laminar expression profile was constructed by averaging pixel intensities in a 300 μm wide region of V1. To control for variability across slides, each profile was normalized to the average pixel intensity of the superior colliculus of the same section. Based on reporter gene analysis in CW2-Cre mice (data not shown) and G35-3-Cre mice (Sawtell et al, 2003), NR1 levels are not expected to change in the superior colliculus of either mutant line.

A similar procedure was followed to construct a laminar profile of RORβ expression in adjacent sections. These profiles were normalized to the peak in layer 4.

The boundaries of the cortical layers were identified on the basis of cell morphology and RORβ expression profile. As reported previously, the expression of RORβ was largely restricted to layer 4 and a few scattered neurons in layer 5 (Schaeren-Wiemers et al., 1997). We defined the boundaries of layer 4 as the width of the peak at half maximum in the laminar RORβ expression profile (see fig. 1B). Layers 2/3 and the cell sparse layer 1 are positioned superficial to layer 4, while layers 5 and 6 are positioned deep to layer 4. Additional subdivisions were approximated in layer 2/3, layer 4 for some of the analysis (see Results).

To facilitate comparison of expression profiles from individual sections, the average thickness of each layer was calculated for the whole dataset and each profile was scaled accordingly. To quantify the reduction of NR1 expression level in mutants, the averaged laminar profiles created for mutant slices was expressed as a percentage of the NR1 levels in control animals, after subtraction of background signal in layer 1. Unless
otherwise indicated, the values are presented as pixel intensity values or percentages ±
standard error of the mean.

Arc induction assay

Under isofluorane anesthesia, mice were subjected to eyelid suture. Briefly, eyelids of the left eye were trimmed, and the eye was washed with saline and covered with opthalmic ointment. The eyelids were sutured with Vicryl, and secured with Vetbond. When the animals had recovered from anesthesia, they were placed in the dark for 24 hours, then exposed to light for 30 minutes to induce Arc expression. The animals were sacrificed, and the brains were removed immediately, embedded in OCT freezing media (TissueTek), and flash frozen on dry ice. The brains were stored at -80°C, until cryostat sections of 18 μm were cut.

Electrode Implantation and Visual Evoked Potential Recordings.

We implanted adult mice (P120-P130) with tungsten microelectrodes (FHC, Bowdoinham, ME), as previously described (Sawtell et al., 2003). Mice were anesthetized with Avertin (2-2-2 Tribromoethanol, dissolved in tert-amyl alcohol), and a local anesthetic of 1% lidocaine hydrochloride was injected subcutaneously. After trimming the scalp to expose the skull, a head post was affixed slightly anterior to bregma, and small burr holes were drilled in the skull bilaterally at approximately 3mm lateral and 1 mm anterior to lambda (the intersection of the sagittal and lambdoid sutures), above the binocular visual cortex. Electrodes were inserted approximately 450-
500 microns from the surface of the exposed dura, and secured in place with
cyanoacrylate.

Mice were allowed to recover for 1 day, and were habituated to the head restraint
system for 15 minutes on the following day. Recording of visual evoked potentials
(VEPs) in awake, head-restrained mice were initiated 3 days post-surgery. Each
recording session consisted of the presentation of a visual stimulus, which was a full-field
sine wave grating of 100% contrast, 0.05 cycles/degree, and oriented at 0°. The stimulus
was presented in blocks of 100 presentations, in the following order: 1) 2 blocks
presented to both eyes of the mouse open, 2) 1 block presented to the right eye alone, 3)
one block presented to the left eye alone, 4) 1 block presented to the right eye alone, 5)
one block presented to the left eye alone. In a given training session, each eye was
exposed to 4 blocks of stimuli, of which 2 blocks were given to that eye alone. VEP
measurements were collected from the binocular zone of Area V1, quantified by
comparing the peak-trough amplitude (Sawtell et al., 2003), and averaged for the 2 blocks
of stimulus presentation given to each eye.
Results

Spatially-restricted deletion of NR1 in two independent mutant lines

In CW2-NR1 and G35-3 NR1 mutant mouse lines, the deletion of the NR1 gene was restricted to the postnatal life (Zeng et al., 2001; Sawtell et al., 2003). We selected animals at 4-5 months of ages, based on a mutant similar to the CW2-NR1 mouse (Fukaya et al., 2003), where NR1 deletion spread to distinct layers of the cortex after 4 months. To determine the exact pattern of deletion of NR1 in the visual cortex, we performed in situ hybridization in both mouse lines and quantified levels of NR1 mRNA in each cortical layer.

In the G35-3 control mice, NR1 mRNA was expressed in all layers of the visual cortex, except for layer 1 (see Fig. 1A, 1B). Quantification of the NR1 signal showed that expression levels were uniform across all cell layers, except for a small dip in layer 5, (Fig. 1C), which likely reflects the lower density of neurons in layer 5 (Peters and Kara, 1985).

Similar to controls, NR1 expression in the G35-3 mutant mice was present in all cortical layers, except for layer 1 (Fig. 1A, 1B). NR1 mRNA levels were comparable across layers 2-6, but appeared lower than in controls. Indeed, direct comparison of the average laminar profile in G35-3 controls and mutants revealed a uniform decrease of NR1 mRNA over all cortical layers (Fig. 1C). As shown in Figure 1D, the reduction amounted to approximately 50% in all cortical layers. (Pixel intensity values for each layer: superficial layer 2/3, control = 0.62 ± 0.06, mutant 0.28 ± 0.03; deep layer 2/3, control = 0.66 ± 0.07, mutant = 0.32 ± 0.03; superficial layer 4, control = 0.64 ± 0.06,
mutant = 0.38 ± 0.03; deep layer 4, control = 0.65 ± 0.06, mutant 0.40 ± 0.04; layer 5, control = 0.50 ± 0.05, mutant 0.29 ± 0.03; layer 6, control = 0.58 ± 0.04, mutant 0.31 ± 0.02. n=17 sections for control, n=18 for mutant. p<0.001 for all comparisons between cortical layers in controls versus mutants, t-test).

The NR1 expression in CW2-NRI control mice was qualitatively (Fig. 2A, 2B) and quantitatively (Fig. 2C) similar to that of G35-3 NR1 mice. In contrast, NR1 expression in CW2-NRI mutant mice was not uniform across all cortical layers (Fig. 2A, 2B). Comparison of laminar profiles from CW2-NRI controls and mutants showed a decrease in NR1 levels in superficial and deep cortical layers, but no change in intermediate layers (fig. 2C). These intermediate layers were identified as layer 5 and deep layer 4, based on relative position to the RORβ histogram, which encompasses all of layer 4. Other layers showed NR1 reduction by 50-80% of control levels, depending on the cortical layer (fig. 2D). (Pixel intensity values for each layer: superficial layer 2/3, control = 0.70 ± 0.09, mutant 0.17 ± 0.03; deep layer 2/3, control = 0.71 ± 0.10, mutant = 0.09 ± 0.03; superficial layer 4, control = 0.67 ± 0.09, mutant = 0.29 ± 0.04; deep layer 4, control = 0.61 ± 0.09, mutant 0.60 ± 0.07; layer 5, control = 0.46 ± 0.06, mutant 0.45 ± 0.07; layer 6, control = 0.48 ± 0.06, mutant 0.24 ± 0.03. n=10 sections for control, n=12 for mutant. p<0.001 for superficial layer 2/3, deep layer 2/3, superficial layer 4; p<0.05 for layer 6; p>0.05 for deep layer 4 and layer 5; paired t-test).

To determine the boundaries of layer 4, we used a histogram of RORβ signal (see methods). The RORβ histogram encompasses signal from both layer 4 and layer 5, and it is possible that the peak of the histogram, used to define the boundaries of layer 4, might include RORβ signal from layer 5. In that case, the width of layer 4 would be
overestimated, and the NR1 signal measured in the CW2-NR1 mutants could be attributed to NR1 in layer 5 alone. To confirm the presence of NR1 in deep layer 4 in CW2-NR1 mutants, we subjected the mutant animals to an NMDA receptor-dependent assay. In this assay, the immediate-early gene Arc is induced in the visual cortex by placing mice in the dark for 24 hours and then briefly exposing them to light (Tagawa et al., 2005). Because Arc expression depends on NMDA receptors (Wang et al., 2006), Arc can be induced in CW2-NR1 mutants only in the cortical layers where NMDA receptors remain. We probed Arc mRNA in cortical sections from control and mutant animals that were subjected to the Arc induction assay. Figure 2E shows that Arc levels are much diminished in CW2-NR1 mutants, but present in a middle band. As Arc expression is not induced in layer 5 (Tagawa et al., 2005), and see control section), we conclude that NR1 remains in deep layer 4 and supports Arc induction there.

Correlation of NR1 deletion and SRP acquisition

To correlate loss of NR1 mRNA in the cortex with function of the visual system, we measured SRP acquisition in both NR1 mutant lines. SRP is measured by the change in visual evoked potential (VEP) amplitudes after repeated exposures to a stimulus in the same orientation. In these experiments, we measured SRP acquisition through the contralateral and ipsilateral eyes separately. We used a stimulus oriented at 0° because the largest VEP amplitudes are observed at this stimulus orientation (Frenkel et al., 2006).

VEPs were measured in all genotypes, and no significant differences were observed between the VEP amplitudes on the first day of recording. (Figure 3a, CW2-
NR1 control, n=6, contra 167.38 ± 23.83 μV, ipsi 60.04 ± 4.51 μV; CW2-NRI mutant, n=11, contra 192.53 ± 20.69 μV, ipsi 63.08 ± 6.87 μV. G35-3 control, n=9, contra 163.49 ± 14.48 μV, ipsi 68.57 ± 10.80 μV; G35-3 NR1 mutant, n=4, contra 152.93 ± 28.43 μV, ipsi 71.51 ± 16.64 μV; ANOVA F(3,23), p = 0.53 for contra VEPs, p=0.74 for ipsi VEPs).

SRP acquisition was observed in G35-3 control mice (final VEP amplitudes: contra 230.59 ± 18.48 μV, ipsi 104.38 ± 13.70 μV; ANOVA F(5,8), p < 0.05 for contra and ipsi VEPs), indicating that both contra and ipsi VEPs reached a maximum amplitude that was 1.5x the initial VEP amplitude (Fig. 3B). In contrast, no SRP was observed in G35-3 mutant mice (final VEP amplitudes: contra 152.30 ± 28.43 μV; ipsi 71.51 ± 16.64 μV ; ANOVA F(5,3), p>0.05 for contra and ipsi VEPs) (Fig 3B). In G35-3 mutants, the VEP amplitudes remained constant across repeated stimulus presentations, suggesting that a lack of 50% of NR1 in the cortex could no longer support SRP.

To address whether NR1 in deep layer 4 and layer 5 alone would support SRP, we measured VEP amplitudes in CW2-NRI mutant mice. CW2-NRI control mice expressed SRP (final VEP amplitudes: contra 237.25 ± 40.52 μV; ipsi 94.60 ± 13.44 μV; ANOVA F(5,4), p <0.01 for contra and ipsi VEPs), reaching maximum VEP amplitudes that were 1.5X the initial amplitude (Fig 3C). Interestingly, CW2-NRI mutant mice showed SRP acquisition that was identical to that of control mice (final VEP amplitudes: contra 307.21 ± 33.20 μV; ipsi 107.10 ± 10.85 μV; ANOVA F(5,10), p <0.01 for contra and ipsi VEPs), suggesting that a NR1-mediated plasticity in a minimal circuit of deep layer 4 and layer 5 was sufficient to support SRP.
Discussion

Stimulus-selective response potentiation (SRP) bears qualitative similarities to perceptual learning, and we have used SRP as a model to study the mechanisms of NMDA receptor-mediated cortical plasticity that possibly underlie perceptual learning. Here we report that SRP was blocked in G35-3 mutant mice, where cortical NR1 is reduced by 50% throughout the cortical layers, except for layer 1. These data extend the observation that SRP is sensitive to systemic CPP administration (Frenkel et al., 2006), and show that NMDA receptors specifically in the cortex are required. Evidence for a cortical locus is supported by several features of SRP: first, there is no interocular transfer of SRP, suggesting that the modifications involve the LGN, where eye-specific inputs remain segregated. Second, the enhanced response in SRP is specific to stimulus-orientation, a feature that first appears in the primary visual cortex (Drager, 1975). Most significantly, SRP was blocked when cortical neurons were infected with a virus containing a peptide inhibitor of GluR1 insertion (Frenkel et al., 2006). Taken together, all the data suggest that geniculocortical synapses are involved in SRP, and NMDA receptors in the postsynaptic cortical neurons are required. In classic models of Hebbian plasticity, postsynaptic NMDA receptors function as coincidence detectors when both pre and postsynaptic sites are active (for review, see (Lynch, 2004). One example of this type of plasticity is long-term potentiation, or LTP, which has been demonstrated in the visual cortex in rodents (Heynen and Bear, 2001; Clapp et al., 2006). If NMDA receptors can function in this capacity in LTP, it is possible that they function similarly in SRP.
Data from G35-3 NR1 mutant mice address the role of cortical NMDA receptors, but SRP measurements could include potentiation of any synapses in the cortex, such as layer 4 to layer 2/3 or layer 2/3 to layer 5. However, data from CW2-NR1 mutant mice suggests that NMDA receptor-dependent plasticity of other synapses in the cortex may not be required for SRP. In CW2-NR1 mice, SRP acquisition was supported by NR1 in layer 4 and layer 5. These were the only layers to retain normal levels of NR1; all other layers had a 50% or greater reduction in NR1. As the reduction of 50% of NR1 was sufficient to block SRP in G35-3 mutants, we do not expect that any of the layers showing this level of reduction in CW2-NR1 mutants would support SRP. In particular, layer 2/3 shows an 80% reduction of NR1, which may down-regulate synaptic plasticity to the levels too low to be physiologically functional at the postsynaptic sites of LGN-layer 2/3 and layer 4 to layer 2/3 synapses. In CW2-NR1 mutants, NR1 expression was not uniform across layer 4. We compared the NR1 signal with a histogram of RORβ signal made from adjacent coronal sections. Because strong RORβ expression is confined to layer 4 neurons (Schaeren-Wiemers et al., 1997), we used the peak of the RORβ histogram as a marker for layer 4. In estimating the boundaries of layer 4, we used only the width of the RORβ peak where the signal was at half-maximum. This conservative estimate of layer 4 width is not expected to include contributions from other layers, particularly since RORβ is only weakly expressed in layer 5 (see Figs. 1B, 2B). In CW2-NR1 mutants, NR1 was reduced in the superficial area of the RORβ peak, and retained in the adjacent deep area. We confirmed that NR1 signal in the deep area corresponded to layer 4, by using an Arc induction assay. Surprisingly, the loss of NR1 in superficial layer 4 had no effect on SRP acquisition. One possible explanation would
be that total NR1 levels in layer 4 were high enough to support SRP, even if the distribution was skewed towards deep layer 4. In rodents, layer 4 is a largely homogeneous layer of which approximately 90% of the neurons are small “star” pyramids (Peters and Kara, 1985). There are no apparent anatomical subdivisions between deep and superficial parts of layer 4, and no evidence that deep layer 4 contains a unique population of neurons that support orientation-specific tasks like SRP.

Nonetheless, it is interesting to note that orientation-selectivity in the cortical lamina of higher mammals is diverse. In monkeys, physiological evidence was recently found for a highly orientation-selective region within layer 4C, a sublayer that otherwise contains neurons with low orientation-selectivity (Gur et al., 2005). While primates and rodents clearly differ in both neuronal types and laminar organization of the cortex, it is tempting to speculate that rodents too could have more diversity within layer 4 than previously reported. Subtle differences between neurons in deep and superficial layer 4 in rodents might confer different requirements for orientation-specific tasks like SRP.
Figure 1
NR1 deletion across all cortical layers in G35-3 NR1 mutant mice
Figure 2
NR1 deletion in specific cortical layers in CW2-NR1 mutant mice
Figure 3

A. VEP Amplitudes, Day 1

- CW2 control
- CW2 mutant
- G35-3 control
- G35-3 mutant

The graph shows the absolute amplitude (μV) across different genotypes (CW2 control, CW2 mutant, G35-3 control, G35-3 mutant) for both contra and ipsi conditions.
Figure 3
SRP Acquisition in CW2-NR1 mutant mice but not G35-3 NR1 mutant mice

B. G35-3 NR1 Mice

C. CW2-NR1 Mice
Figure Legend

Figure 1. NR1 deletion across all cortical layers in G35-3 NR1 KO mice.

A. Darkfield images, in situ hybridization on coronal sections from a 16-week old CW2-NR1 KO mouse and a control littermate, using a $^{33}$P-labeled NR1 cRNA probe. Scale bar = 1 mm.

B. Laminar analysis of NR1 signal. Cortical cross-sections, probed with $^{33}$P-labeled NR1 cRNA, were compared to adjacent sections probed with a ROR$\beta$, a layer 4 enriched gene. Scale bar = 100 $\mu$m. Densitometric analysis of ROR$\beta$ signal, used to define boundaries of cortical layer 4 (dashed vertical lines). All values have been normalized to the maximal ROR$\beta$ signal. Black histogram represents an average of ROR$\beta$ signal in all individual sections.

C. Quantification of NR1 reduction in G35-3 NR1 KO mice. Densitometric analysis of NR1 mRNA signal across the width of the cortex. The relative optical density of each section has been normalized to the signal level of the superior colliculus. Control (blue, n=17) and mutant (red, n=18) histograms represent an average of all individual sections. A histogram of ROR$\beta$ expression (black) was obtained from adjacent sections, and used to determine the boundaries of layer IV. Pixel intensity values for each layer: superficial layer 2/3, control = 0.62 ± 0.06, mutant = 0.28 ± 0.03; deep layer 2/3, control = 0.66 ± 0.07, mutant = 0.32 ± 0.03; superficial layer 4, control = 0.64 ± 0.06, mutant = 0.38 ± 0.03; deep layer 4, control = 0.65 ± 0.06, mutant = 0.40 ± 0.04; layer 5, control = 0.50 ± 0.05, mutant = 0.29 ± 0.03; layer 6, control = 0.58 ± 0.04, mutant = 0.31 ± 0.02. n=17 sections for
control, n=18 for mutant. p<0.001 for all comparisons between cortical layers in controls versus mutants, t-test.

D. Summary of NR1 quantification measurements. NR1 signal in G35-3 NR1 KO mice, expressed as a percentage of NR1 signal in control mice. The NR1 signal is significantly and uniformly reduced in all cortical layers.

Figure 2. NR1 deletion in specific cortical layers in CW2-NR1 KO mice.

A. Darkfield images of in situ hybridization on coronal sections from a 16-week old G35-3 NR1 KO mouse and a control littermate, using a $^{33}$P-labeled NR1 cRNA probe. Scale bar = 1 mm.

B. Laminar analysis of NR1 signal. Cortical cross-sections, probed with $^{33}$P-labeled NR1 cRNA, were compared to adjacent sections probed with a RORβ, a layer 4 enriched gene. Scale bar = 100 μm. Densitometric analysis of RORβ signal, used to define boundaries of cortical layer 4 (dashed vertical lines). All values have been normalized to the maximal RORβ signal. Black histogram represents an average of RORβ signal in all individual sections.

C. Quantification of NR1 reduction in CW2-NR1 KO mice. Densitometric analysis of NR1 mRNA signal across the width of the cortex. The relative optical density of each section has been normalized to the signal level of the superior colliculus. Control (blue, n=11) and mutant (red, n=13) histograms represent an average of all individual sections. A histogram of RORβ expression (black) was obtained from adjacent sections, and used to determine the boundaries of layer IV. Pixel intensity values for each layer: superficial layer 2/3, control = 0.70 ± 0.09, mutant
0.17 ± 0.03; deep layer 2/3, control = 0.71 ± 0.10, mutant = 0.09 ± 0.03; superficial layer 4, control = 0.67 ± 0.09, mutant = 0.29 ± 0.04; deep layer 4, control = 0.61 ± 0.09, mutant 0.60 ± 0.07; layer 5, control = 0.46 ± 0.06, mutant 0.45 ± 0.06; layer 6, control = 0.48 ± 0.06, mutant 0.24 ± 0.03. n=10 sections for control, n=12 for mutant. p<0.001 for superficial layer 2/3, deep layer 2/3, superficial layer 4; p<0.05 for layer 6; p>0.05 for deep layer 4 and layer 5; paired t-test.

D. Summary of NR1 quantification measurements. NR1 signal in CW2-NR1 KO mice, expressed as a percentage of NR1 signal in control mice. The NR1 signal is significantly reduced in cortical layers 2/3, superficial layer 4, and layer 6.

E. Darkfield images of in situ hybridization, the Arc induction assay, using a 33P-labeled Arc cRNA probe. Arc mRNA in the mutant (right) indicates the presence of NMDA receptors in layer 4.

Figure 3. SRP acquisition in CW2-NR1 KO mice but not G35-3 KO mice.

A. On the initial day of recording, VEP amplitudes from all genotypes are equal. Representative traces of contralateral and ipsilateral VEPs from each genotype, on initial day of recording. Scale bar, 50 μV, 500 ms.

B. SRP acquisition is observed in both CW2-NR1 control (n=5) and KO (n=11) mice. ANOVA, F(5,15), p<0.01 for both contralateral and ipsilateral VEPs in the control group, and ANOVA F(5,50), p<0.0001 for both contralateral and ipsilateral VEPs in the KO group.
C. SRP acquisition is not observed in G35-3 NR1 KO mice (n=4), while SRP acquisition is observed in G35-3 NR1 control mice (n=9). ANOVA, F(5,40), p < 0.01 for both contralateral and ipsilateral VEPs in the control group, and ANOVA, F(5,15), p > 0.05 for both contralateral and ipsilateral VEPs in the KO group.
Chapter 3

Spine morphology and ocular dominance: OD plasticity in dnPAK transgenic mice

Abstract

Dendritic spines are likely to be the structural site of experience-dependent plasticity in the sensory cortex. Dramatic changes in spine motility and morphology have been observed in the visual cortex during early postnatal development and beyond, particularly in response to deprivation. The functional significance of these changes is not well-understood. To provide a link between altered spine morphology and plasticity in the visual cortex, we measured ocular dominance (OD) plasticity in transgenic mice that express a dominant-negative form of p21-activated kinase (PAK). dnPAK transgenic mice were reported to have abnormal spine morphology, consisting of increased numbers of large spines with wide heads, which correlated with altered synaptic plasticity and deficits in memory consolidation. The spine phenotype emerges only in adult dnPAK mice in cortical layers 2/3 and layer 6, offering a good model system for studying plasticity in the adult visual system, while avoiding effects of altered spine morphology on early brain development. Here, we measured OD plasticity in adult mice (P60), after 7 days of MD. The dnPAK mice showed a normal OD shift, as compared to control mice, suggesting that OD plasticity is not sensitive to consequences of altered spine morphology in layers 2/3 and layer 6.
Introduction

When rodents have been deprived of visual experience in one eye (monocular deprivation, or MD), neurons in the visual cortex shift their responsiveness to favor the non-deprived eye. This so-called ocular dominance (OD) shift is manifested in several ways, likely beginning with changes in the electrophysiological properties of cortical neurons (Gordon and Stryker, 1996; Sawtell et al., 2003; Frenkel and Bear, 2004; Fischer et al., 2007) and eventually leading to structural rearrangements of the thalamocortical afferents from the non-deprived eye (Antonini et al., 1999). At the same time, dramatic changes also occur in the dendritic spines of cortical neurons after MD (Mataga et al., 2004; Oray et al., 2004). Spines are highly-motile protrusions located along the dendrites of neurons, where they serve as the main postsynaptic component of excitatory synapses (Tada and Sheng, 2006). As such, spines have a close relationship with synaptic plasticity; changes in spine size and shape often correlate with bidirectional synaptic changes such as LTP or LTD (Nagerl et al., 2004; Zhou et al., 2004), and with sensory experience in vivo (Trachtenberg et al., 2002; Majewska and Sur, 2003; Wallace and Bear, 2004; Holtmaat et al., 2006). Spine morphology may be the structural basis for synaptic plasticity (Alvarez and Sabitini, 2007). In the visual cortex, this model would link the increased spine motility (Oray et al., 2004) and spine pruning (Mataga et al., 2004) observed after MD with the electrophysiological changes that occur at the same time.
To date, no studies have provided definitive evidence that spine changes are involved in the OD shift in rodents. The closest evidence comes from mice that lack the tissue-type plasminogen activator (tPA) (Mataga et al., 2002; Mataga et al., 2004). A possible function of tPA is to prune spines in the apical dendrites of layer 5 neurons (Oray et al., 2004) or layer 2/3 neurons (Mataga et al., 2004), following MD. In tPA KO mice, the spine pruning after MD is gone (Mataga et al., 2004), as is the OD shift (Mataga et al., 2002). Both deficits were rescued by exogenously applied tPA, suggesting that tPA-mediated spine pruning might play a permissive role in the OD shift. While compelling, these results do not address the developmental consequences of early tPA deletion, which may be significant, considering that the endogenous tPA activity is highest just prior to the critical period (Mataga et al., 2004). If other functions of tPA (Melchor and Strickland, 2005) are impaired during development in the tPA KO mice, any of these deficits could also lead to the loss of the OD shift, independent of the spine pruning.

Stronger evidence that spine dynamics are a critical mediator of OD plasticity could be obtained from a mouse model in which spine characteristics were altered in adult life, after the critical period. To this end, we obtained transgenic mice that express a dominant-negative form of p21-activated kinase (PAK) in postnatal life. PAK mediates actin reorganization by functioning downstream from the small GTPases Rac and Cdc42, and inhibition of PAK by the transgene leads to altered spine morphology (Hayashi et al., 2004). In dnPAK mice, the transgene is expressed at low levels in early postnatal life so that PAK activity is not inhibited in mice at 3 weeks of age. By 8 weeks, however, increased levels of the transgene have led to preponderance of large spines in the cortex,
relative to control mice (Hayashi et al, 2004). We first aimed to confirm that OD plasticity was normal in juvenile mice (at 4 weeks of age). We then measured OD plasticity in adult mice (at 8 weeks of age) to determine how the adult-onset spine morphology phenotype would affect the OD shift.
Materials and Methods

Genetically-modified mice:

We obtained dnPAK transgenic mice, in which an 8.5 kb fragment of the αCaMKII promoter drives expression of the autoinhibitory domain of the p21-activated kinase (PAK) gene (Hayashi et al., 2004). This transgene inhibits all 3 isoforms of PAK found in mice. Expression of the transgene at maximal levels leads to approximately 50% reduction of PAK activity in the cortex. The transgene expression is first detected at P21, but the transgene levels remain low and no PAK inhibition is detected until P28. The striking spine morphology phenotype was observed in animals at P60 (Hayashi et al., 2004).

Electrode Implantation and Visual Evoked Potential Recordings.

We implanted adult mice with tungsten microelectrodes (FHC, Bowdoinham, ME), as previously described (Porciatti et al., 1999; Sawtell et al., 2003; Frenkel and Bear, 2004). Mice were anesthetized with Avertin (2-2-2 Tribromoethanol, dissolved in tert-amyl alcohol), and a local anesthetic of 1% lidocaine hydrochloride was injected subcutaneously. After trimming the scalp to expose the skull, a head post was affixed slightly anterior to bregma, and small burr holes were drilled in the skull bilaterally at approximately 3mm lateral and 1 mm anterior to lambda (the intersection of the sagittal and lambdoid sutures), above the binocular visual cortex. Electrodes were inserted approximately 450-500 microns from the surface of the exposed dura, and secured in place with cyanoacrylate.
Mice were allowed to recover for 1 day, and were habituated to the head restraint system for 15 minutes on the following day. Recording of visual evoked potentials (VEPs) in awake, head-restrained mice were initiated 3 days post-surgery. Each recording session consisted of the presentation of a visual stimulus, which was a full-field sine wave grating of 100% contrast, 0.05 cycles/degree, and oriented at $0^\circ$. The stimulus was presented in blocks of 100 presentations, in the following order: 1) 2 blocks presented to both eyes of the mouse open, 2) 1 block presented to the right eye alone, 3) one block presented to the left eye alone, 4) 1 block presented to the right eye alone, 5) one block presented to the left eye alone.

One hemisphere was selected for OD analysis, and the eye contralateral to this hemisphere was sutured. For eyelid suture, the mice were anesthetized under isofluorane, and the upper and lower eyelids were trimmed. The eye was rinsed with saline and antibacterial ophthalmic ointment was applied. The eyelids were then sewn shut with 3-4 mattress stitches, using Vicryl suture. Mice were allowed to recover from anesthesia, and returned to their home cage for 3d (mice at age P21) or 6-7 days (mice at age P60). Following the period of MD, the mice were again anesthetized under isofluorane and the sutures were removed. The eye was opened, cleaned, and the optics were checked for clarity. The mice were placed in the dark for 15-30 minutes to recover from anesthesia, and then exposed to 6 blocks of visual stimulus, in the same orientation as before MD. There was an additional recording session, in which the mice were exposed to 6 blocks of stimulus oriented at $90^\circ$. VEPs were collected from the post MD recording session, as before, and compared to the pre-MD VEP measurements. Values are presented as VEP amplitudes (mV) ± standard error of the mean.
Results

Ocular Dominance Plasticity

To assess the ocular dominance (OD) plasticity in dnPAK mice, we measured the change in visual evoked potentials (VEPs) that result from monocular deprivation (MD) (Porciatti et al., 1999; Sawtell et al., 2003; Frenkel and Bear, 2004). VEPs were recorded from the binocular zone of the visual cortex, in response to a sinusoidal grating stimulus. The peak to trough amplitudes of the VEP waveform was measured both before and after MD. We used two experimental paradigms which have been shown previously to give different results (Sawtell et al., 2003; Frenkel and Bear, 2004; Frenkel et al., 2006). In the first paradigm, the orientation of the sinusoidal grating stimulus was the same before and after MD (“same orientation”). In the second, the orientation after MD was rotated by 90° (“different orientation”) (Fig. 1). We used both experimental setups to probe for any differences between the plasticity in control and dnPAK mutant mice.

Normal OD plasticity in juvenile dnPAK mice

The dnPAK transgene is first detected in mutant mice one week after birth, although inhibition of the active form of PAK is not detected at 3 weeks of age. Expression of the transgene reaches a maximum only at 8 weeks of age, corresponding to a 50% of active PAK (Hayashi et al., 2004). Presumably, the inhibition of PAK increases between 3-8 weeks of age, a time window that includes the critical period for sensitivity to monocular deprivation in mice (3-5 weeks of age, (Gordon and Stryker, 1996). We
first tested whether low or moderate levels of dnPAK expression would have any deleterious effects on OD plasticity at this critical age.

One measure of the OD shift is a change in the ratio of contralateral eye VEP amplitude to the ipsilateral eye VEP amplitude (C/I ratio), following MD (Porciatti et al, 1999; Frenkel and Bear, 2004). In normal mice, prior to MD, the VEP amplitudes obtained by stimulating the contralateral eye are 2.5-3 times higher than those obtained from the ipsilateral eye (Porciatti et al, 1999). This ratio reflects the contralateral eye dominance in the binocular zone of the visual cortex (Drager et al, 1975; Porciatti et al, 1999). Following MD of the contralateral eye, the ocular dominance shifts to favor the ipsilateral (open) eye inputs, and the C/I ratio drops close to 1. The decline in the C/I ratio can reflect a depression of contralateral (deprived) eye inputs, or a potentiation of ipsilateral (open) eye inputs, or both.

In the “same orientation” paradigm, 3 days of MD in juvenile mice is reported to lead to a depression of contralateral eye VEPs (Sawtell et al., 2003). Starting with this paradigm, we measured the C/I ratio in juvenile control mice, using the same stimulus oriented at 0° for recordings performed before and after MD. After 3 days of MD, the C/I ratio was significantly reduced, indicating an OD shift (pre-MD, 2.68 ± 0.14; post-MD, 1.61 ± 0.10; n = 20, Student’s t-test, p <0.01) (Fig. 2A, left panel). Surprisingly, we found that the decline in C/I ratio was due to a potentiation of ipsilateral eye VEPs, while contralateral eye VEPs remained unchanged (VEP amplitudes: pre-MD, 191.21 ± 17.96 μV; ipsi 71.58 ± 4.84 μV; post MD, contra 187.48 ± 13.86 μV, ipsi 120.55 ± 8.67 μV, p = 0.84 for contra; p <0.01 for ipsi) (Fig. 2B, left panel).
Indeed, only when we used the “different orientation” paradigm (Frenkel and Bear, 2004) was the decline in the C/I ratio due to a depression of contralateral eye VEPs. Under these conditions, the shift in the C/I ratio after 3 days of MD was evident (C/I ratio: pre MD, 2.68 ± 0.13; post-MD, 1.67 ± 0.12; p << 0.01) (Fig. 3A, left panel). This time, the shift was due to a depression of contralateral eye VEPs, while ipsilateral eye VEPs remained unchanged (VEP amplitudes: pre-MD, contra 191.21 ± 17.96 μV and ipsi 71.58 ± 4.84 μV; post-MD, contra 106.54 ± 10.67 μV; ipsi 81.37 ± 7.54 μV; p < 0.01 for contra; p = 0.18 for ipsi) (Fig. 3B, left panel).

After determining the dynamics of the C/I ratio in control mice, we tested juvenile dnPAK mutant mice. In the “same orientation” paradigm, dnPAK mutants showed a shift in the C/I ratio that was slightly reduced, although this change was not significant (C/I ratio: pre-MD, 2.48 ± 0.10; post-MD, 1.88 ± 0.33; n=9; Student’s t-test, p = 0.10) (Fig. 2A, right panel). Here, the reduction in C/I ratio was due to the significant potentiation of ipsilateral eye VEPs, as in control animals (VEP amplitudes: pre-MD, contra 165.33 ± 14.56 μV; ipsi 67.97 ± 7.31 μV; post-MD, contra 188.87 ± 27.16 μV; ipsi 142.13 ± 35.67 μV; p = 0.277 for contra; p < 0.05 for ipsi) (Fig. 2B, right panel).

Juvenile dnPAK mutant mice also showed a robust shift in the C/I ratio, in response to the “different orientation” paradigm (C/I ratio: pre-MD, 2.48 ± 0.10; post-MD, 1.44 ± 0.18, p<0.01) (Fig. 3A, right panel). Similar to control mice, the shift was due to a depression of contralateral eye VEPs, (VEP amplitudes: pre-MD, contra 165.33 ± 14.56 μV; ipsi 67.97 ± 7.31 μV; post-MD, contra 90.27 ± 15.08 μV; ipsi 70.53 ± 12.60 μV, p < 0.05 for contra, p = 0.68 for ipsi) (Fig. 3B, right panel).
Taken together, these data indicate that dnPAK juvenile mice show normal cortical plasticity, and are not affected by low levels of the dnPAK transgene during the critical period.

Normal OD Plasticity in adult dnPAK mice

In adult mice, high levels of PAK inhibition led to abnormal spine morphology in cortical neurons. This morphology correlated with enhanced LTP and impaired LTD in the temporal cortex, as well as deficits in memory consolidation (Hayashi et al., 2004). We asked whether high levels of PAK inhibition and abnormal spine morphology would also affect OD plasticity. Accordingly, we repeated all the VEP measurements in adult mice, at age P60. We subjected the mice to 6-7 days of MD, as longer periods of MD are required to observe the change in C/I ratio in adult mice (M Frenkel, personal communication).

Using the “same orientation” paradigm, we observed a significant decline in the C/I ratio after 7 days of MD in adult control animals, as expected (C/I ratio: pre-MD, 2.81 ± 0.23; post-MD, 1.11 ± 0.17; n=5; Student’s t-test, p < 0.01) (Fig. 4A, left panel). Consistent with Sawtell et al (2003), this decline was due to a potentiation of ipsilateral eye VEPs (VEP amplitudes, pre-MD: contra, 289.01 ± 53.85 µV; ipsi 103.24 ± 16.94 µV; post-MD, contra 212.57 ± 33.29 µV, ipsi 202.03 ± 26.18 µV; p = 0.26 for contra; p < 0.05 for ipsi) (Fig. 4B, left panel). Using the “different orientation” paradigm, we also observed an OD shift (C/I ratio: pre-MD, 3.03 ± 0.25 ; post-MD, 1.44 ± 0.26; p <0.01) (Fig. 5A, left panel). Consistent with Frenkel et al (2006), this was caused by a depression of contralateral eye VEPs (VEP amplitudes: pre-MD, contra 262.07 ± 38.17
µV; ipsi 91.24 ± 14.18 µV; post-MD: contra 128.01 ± 15.19 µV; ipsi 105.64 ± 16.03 µV; p < 0.01 for contra; p = 0.511 for ipsi) (Fig. 5B, left panel).

We next measured the OD shift in dnPAK mice. Using the “same orientation” paradigm, we observed a normal OD shift in adult dnPAK mice (C/I ratio: pre-MD, 2.88 ± 0.33; post-MD, 1.40 ± 0.20; n = 6; Student’s t-test, p < 0.01) (Fig. 4A, right panel). As in adult control animals, this shift was due to a potentiation of ipsilateral eye VEPs (VEP amplitudes: pre-MD, contra, 218.86 ± 33.72 µV; ipsi 78.74 ± 12.03 µV; post-MD, contra 215.95 ± 39.11 µV, ipsi 151.32 ± 18.75 µV; p = 0.96 for contra; p < 0.01 for ipsi) (Fig. 4B, right panel).

In the “different orientation” paradigm, the change in the C/I ratio is due to a depression of contralateral eye VEPs (results above, and Frenkel et al, 2006). This depression observed after MD shares some common features with LTD (Heynen et al., 2003), and adult dnPAK mice show deficits in LTD (Hayashi et al, 2004). Therefore, we were curious to see if adult dnPAK mice would either lack the OD shift entirely, or would use a use potentiation, rather than depression, to shift the C/I ratio. However, adult dnPAK mice showed a significant reduction in the C/I ratio (C/I ratio: pre-MD, 2.86 ± 0.28; post-MD, 1.12 ± 0.10; p < 0.01) (Fig. 5A, right panel), and used the same depression of contralateral eye VEPs as control animals to achieve the shift (VEP amplitudes: pre-MD, 201.13 ± 19.13 µV; ipsi 74.56 ± 8.79 µV; post-MD, 119.97 ± 16.12 µV; ipsi 113.31 ± 19.72 µV; p < 0.01 for contra, p = 0.07 for ipsi) (Fig. 5B, right panel). These results indicate that high levels of dnPAK transgene expression and abnormal spine morphology did not lead to deficits in the OD shift.
Discussion

In these experiments, we aimed to study the relationship between spine morphology and OD plasticity, as measured by visual evoked potentials (VEPs). We used VEPs to compare the strength of evoked responses from each eye, before and after MD. Our results indicate both juvenile and adult dnPAK mice show an OD shift that is comparable to control mice. This finding holds true whether the same stimulus orientation is used for pre and post-MD recordings (Sawtell et al., 2003), or the stimulus orientation is changed (Frenkel and Bear, 2004).

In juvenile animals, we observed that in the case of the “same orientation” paradigm, the reduction of the C/I response ratio after 3 days of MD was due to a potentiation of the ipsilateral eye VEP. Previously, however, it was reported in a similar protocol that the reduced C/I ratio was caused by a decline in the contralateral eye VEP (Sawtell et al., 2003). This discrepancy might be explained by a difference in the way the recordings were performed. Sawtell et al (2003) performed several recordings prior to MD, in order to establish a baseline VEP amplitude. If this set of recordings caused the VEP amplitudes to reach a saturated maximum, a process later described as stimulus-selective response potentiation (SRP) (Frenkel et al., 2006), then the subsequent 3 days of MD could have led to the expected decline in contralateral eye VEP with no change in ipsilateral eye VEPs (Frenkel and Bear, 2004). Our result, which did not include any pre-MD baseline recordings, might reflect masking of a decline in contralateral eye VEPs by SRP, which would be induced by re-exposure to the same stimulus after MD. To circumvent the interfering effect of SRP, a better approach would be to establish
stimulation paradigms that do not lead to SRP, such as fewer stimulus presentations, lower contrast, longer intervals between presentations in a session or some combination of these. The same stimulus could then be used for recordings before and after MD, with no SRP effect, and no complications arising from a preferential response to a particular stimulus orientation (Frenkel et al., 2006). Nonetheless, our results suggest that all dnPAK mutant mice showed the same OD shift – or possibly OD shift plus SRP – as the control mice.

We found that juvenile dnPAK showed showed an OD plasticity that was intact and indistinguishable from control animals. This was expected at the age tested (P28), because the dnPAK transgene levels are low and PAK inhibition is barely detectable (Hayashi et al., 2004). Therefore, changes in either spine morphology or synaptic plasticity in mutants of this age are unlikely. However, the developing visual cortex is highly susceptible to manipulations of visual experience immediately after eye opening and a few weeks past (Gordon and Stryker, 1996; Tagawa et al., 2005). We show that developmental plasticity in dnPAK mice, as measured by VEPs, was not altered by even low levels of the transgene. By using a transgene that affects plasticity only in postnatal life, we could directly study the effects of the transgene on evoked, rather than developmental, plasticity. In the visual system, a similar approach was described by (Chakravarthy et al., 2006), to demonstrate that trkB is likely to be required for the maintenance of a mature mushroom spine morphology in the visual cortex.

We did not find any difference in OD plasticity between the control and dnPAK mutant mice at age P60. We can interpret this result in several ways. One possibility is that the phenotypes introduced by expression of dnPAK in cortical layers 2/3 and layer 6
do not affect OD plasticity. At P60, the expression level of the dnPAK transgene is maximal, and cortical neurons have abnormal spine morphology (Hayashi et al., 2004). In addition, LTP is enhanced and LTD is reduced in the cortex of these mice. These changes are correlated with an impairment of consolidation and/or retention of spatial and contextual fear memory (Hayashi et al., 2004). Requirements for memory consolidation may differ from those for OD plasticity, which would mean that subtle deficits in the balance between LTP and LTD have a significant impact on memory consolidation, whereas a larger change in LTP or LTD would be necessary to affect OD plasticity. We cannot rule out the possibility that altered spine morphology may have led to more subtle changes than our measure of potentiation or depression of VEP amplitudes can detect. In one model (Fagiolini et al., 2003), OD plasticity is controlled by molecules that balance the excitatory and inhibitory tone in the developing cortex, whereas another feature of visual function, orientation bias, is separately controlled by molecules such as NR2A and PSD95. We did not measure orientation bias or any properties of orientation specificity in dnPAK mice, but we observed different responses to stimuli oriented at 0° and 90°, suggesting that the dnPAK mice were, like controls, able to distinguish the two stimuli. A more thorough analysis of the functional properties of neurons in the dnPAK mice might have revealed other deficits in visual processing. Notably, the abnormal spine morphology in dnPAK mice, a larger percentage of short, fat spines, is qualitatively similar to the spine morphology observed in dark-reared rats (Wallace and Bear, 2004). Dark-rearing animals during the critical period is known to delay several features of visual system development, such as development of inhibitory neurons and closure of the critical period (Fagiolini et al., 1994; Kirkwood et al., 1995). It is possible that the short,
fat spines found in the adult dnPAK mice would indicate a return to immature-like
morphology, and consequently, the same OD plasticity that is observed in juvenile
animals. In this case, short periods of MD that would not affect control mice might be
sufficient to induce an OD shift in adult dnPAK mice.

A second interpretation of our results is that only layers 4 and 5, where dnPAK
expression is absent, are required for the observed OD shift measured by VEP changes.
The impaired LTD and enhanced LTP were observed when layer 4 was stimulated and
field potentials were recorded in layers 2/3 (Hayashi et al., 2004), which is mainly a
measurement of signaling between layer 4 to layer 2/3 synapses. In contrast, the VEP
measurements are field potentials that reflect the output from many different synapses,
including LGN to layer 4 synapses. If these synapses were not affected by the dnPAK
transgene, then the potentiation and depression of these synapses during the OD shift
would be normal. Changes in other synapses, such as layer 4-layer2/3, would be affected
by expression of dnPAK in layer 2/3 neurons, but these effects would might not be
reflected in our VEP measurement. Thus, it is possible that normal spine morphology in
layer 2/3 neurons is not required for normal OD plasticity, and the OD shift is mediated
layers 4 and 5 alone. In the next chapter, we address this possibility by testing the effect
of layer 2/3 NMDA receptor deletion on OD plasticity.
Figure 1
Experimental Set-up

“Same Orientation”

“Different Orientation”
Figure 2
3d MD in juvenile mice
“Same Orientation” paradigm

A. Decline in C/I ratio after 3d MD

B. OD shift due to potentiation of ipsi eye VEPs
Figure 3
3d MD in juvenile mice
"Different Orientation" paradigm

A. Decline in C/I ratio after 3d MD

WT control

dnPAK transgenic

B. OD shift due to a depression of contra eye VEPs

Contralateral eye VEPs
Ipsilateral eye VEPs

WT control

dnPAK transgenic
Figure 4
7d MD in adult mice
"Same Orientation" paradigm

A. Decline in C/I ratio after 7d MD

B. OD shift due to potentiation of ipsi eye VEPs
Figure 5
7d MD in adult mice
“Different Orientation” paradigm

A. Decline in C/I ratio after 7d MD

B. OD shift due to depression of contra eye VEPs
Figure Legend

Figure 1. Summary of experimental setup. Mice were implanted on Day 0, then allowed 1 day of recovery and habituation to the recording apparatus. On Day 3, mice were exposed to stimulus oriented at 0°, and VEPs were recorded. The contralateral eye was sutured for 3 days (juvenile mice) or 7 days (adult mice). The eye was re-opened, and VEPs were recorded when mice viewed a stimulus in the same orientation or a stimulus rotated 90°.

Figure 2. 3 days of MD in juvenile mice: the “same orientation” paradigm leads to an OD shift, mediated by potentiation of ipsi eye VEPs.

A. Decline in C/I ratio after 3 days of MD. Left, control mice (pre-MD, 2.68 ± 0.14; post-MD, 1.61 ± 0.10; n = 20, Student’s t-test, p < 0.01). Right, dnPAK mice (C/I ratio: pre-MD, 2.48 ± 0.10; post-MD, 1.88 ± 0.33; n=9; Student’s t-test, p = 0.10).

B. OD shift due to potentiation of ipsi eye VEPs. Left, control mice (VEP amplitudes: pre-MD, 191.21 ± 17.96 μV; ipsi 71.58 ± 4.84 μV; post MD, contra 187.48 ± 13.86 μV, ipsi 120.55 ± 8.67 μV, p = 0.84 for contra; p <0.01 for ipsi). Right, dnPAK mice (VEP amplitudes: pre-MD, contra 165.33 ± 14.56 μV; ipsi 67.97 ± 7.31 μV; post-MD, contra 188.87 ± 27.16 μV; ipsi 142.13 ± 35.67 μV; p = 0.277 for contra; p < 0.05 for ipsi) (Fig. 2B, right panel)

Figure 3. 3 days of MD in juvenile mice: the “different orientation” paradigm leads to an OD shift mediated by depression of contra eye VEPs.
A. Decline in C/I ratio after 3 days of MD. Left, control mice (C/I ratio: pre MD, 2.68 ± 0.13; post-MD, 1.67 ± 0.12; p << 0.01). Right, dnPAK mice (C/I ratio: pre-MD, 2.48 ± 0.10; post-MD, 1.44 ± 0.18, p<0.01)

B. OD shift due to a depression of contra eye VEPs. Left, control mice (VEP amplitudes: pre-MD, contra 191.21 ± 17.96 μV and ipsi 71.58 ± 4.84 μV; post-MD, contra 106.54 ± 10.67 μV; ipsi 81.37 ± 7.54 μV; p <0.01 for contra; p = 0.18 for ipsi). Right, dnPAK mice (VEP amplitudes: pre-MD, contra 165.33 ± 14.56 μV; ipsi 67.97 ± 7.31 μV; post-MD, contra 90.27 ± 15.08 μV; ipsi 70.53 ± 12.60 μV, p < 0.05 for contra, p = 0.68 for ipsi) (Fig. 3B, right panel).

Figure 4. 7 days of MD in adult mice: the “same orientation” paradigm leads to an OD shift mediated by potentiation of ipsi eye VEPs.

A. Decline in C/I ratio after 7 days of MD. Left, control mice (C/I ratio: pre-MD, 2.81 ± 0.23; post-MD, 1.11 ± 0.17; n=5; Student’s t-test, p < 0.01). Right, dnPAK mice (C/I ratio: pre-MD, 2.88 ± 0.33; post-MD, 1.40 ± 0.20; n = 6; Student’s t-test, p <0.01).

B. OD shift due to a potentiation of ipsi eye VEPs. Left, control mice (VEP amplitudes, pre-MD: contra, 289.01 ± 53.85 μV; ipsi 103.24 ± 16.94 μV; post-MD, contra 212.57 ± 33.29 μV, ipsi 202.03 ± 26.18 μV; p = 0.26 for contra; p < 0.05 for ipsi). Right, dnPAK mice (VEP amplitudes: pre-MD, contra, 218.86 ± 33.72 μV; ipsi 78.74 ± 12.03 μV; post-MD, contra 215.95 ± 39.11 μV, ipsi 151.32 ± 18.75 μV; p = 0.96 for contra; p < 0.01 for ipsi).
Figure 5. 7 days of MD in adult mice: the “different orientation” paradigm leads to an OD shift mediated by depression of contra eye VEPs.

A. Decline in C/I ratio after 7 days of MD. (C/I ratio: pre-MD, 3.03 ± 0.25; post-MD, 1.44 ± 0.26; p < 0.01) (Fig. 5A, left panel). Right, dnPAK mice (C/I ratio: pre-MD, 2.86 ± 0.28; post-MD, 1.12 ± 0.10; p < 0.01).

B. OD shift due to a depression of contra eye VEPs. Left, control mice (VEP amplitudes: pre-MD, contra 262.07 ± 38.17 μV; ipsi 91.24 ± 14.18 μV; post-MD: contra 128.01 ± 15.19 μV; ipsi 105.64 ± 16.03 μV; p < 0.01 for contra; p = 0.511 for ipsi). Right, dnPAK mice (VEP amplitudes: pre-MD, 201.13 ± 19.13 μV; ipsi 74.56 ± 8.79 μV; post-MD, 119.97 ± 16.12 μV; ipsi 113.31 ± 19.72 μV; p < 0.01 for contra, p = 0.07 for ipsi).
Chapter 4

Layer-specific requirement for NMDA receptors in one measure of ocular dominance plasticity

Abstract

After a period of monocular deprivation (MD), cortical responses to inputs from the non-deprived eye increase relative to inputs from the deprived eye. In rodents, the capacity for ocular dominance (OD) plasticity exists during the critical period and into adulthood. It is generally assumed that NMDA receptor-dependent mechanisms like LTP and LTD underlie the expression of OD plasticity. In this study, we utilized CW2-NR1 mice in which NMDA receptor expression is normal in deep layers 4 and 5, but greatly reduced in other layers. This allowed us to dissect the layer-specific requirements for NMDA receptors in OD plasticity in adult mice. Using a measure of OD plasticity that is based on induction of the immediate-early gene Arc, we report evidence that NMDA receptors in deep layer 4 and layer 5 are not sufficient to support the OD shift after 7 days of MD. This result suggests that the OD shift, as measured by changes in Arc induction, requires NMDA receptors in cortical layers 2/3 and superficial layer 4.
Introduction

In the previous chapter, we described ocular dominance (OD) plasticity in adult dnPAK mice, which have abnormal spine morphology and altered synaptic plasticity in cortical layers 2/3 (Hayashi et al., 2004). One possible explanation for this finding is that plasticity in the unaffected neurons in layers 4 and 5 may be sufficient to mediate the OD shift, such that synaptic changes in layers 2/3 are not required. This would agree with classical studies in which physiological and anatomical effects of monocular deprivation (MD) were mediated by geniculocortical afferents to layer 4 (Wiesel and Hubel, 1963b; Shatz and Stryker, 1978; Antonini and Stryker, 1993). A more recent model for OD plasticity, however, postulates that MD leads to rapid changes in extragranular layers, such as remodeling of horizontal connections (Trachtenberg and Stryker, 2001), in the absence of changes in layer 4 (Trachtenberg et al., 2000). Moreover, plasticity in older animals is thought to occur in extragranular layers, rather than layer 4 (Mower and Christen, 1985; Daw et al., 1992).

These models for OD plasticity were based on studies in young cats and monkeys, and it is unclear to what extent they can be extrapolated to mice. Unlike higher mammals, mice show an OD shift well into adulthood (Sawtell et al., 2003; Tagawa et al., 2005; Frenkel et al., 2006; Fischer et al., 2007) which is dependent on NMDA receptors. Some evidence suggests that this OD shift also occurs in layer 4 (Tagawa et al., 2005; Fischer et al., 2007). In addition, the OD shift in adult mice may not require the anatomical rearrangements described in cats and monkeys, as spines in the visual
cortex are very stable (Grutzendler et al., 2002) and a brief period of MD has no effect on spine motility or spine pruning (Mataga et al., 2004; Oray et al., 2004).

The exact mechanism and the cortical layers involved in the OD shift in adult mice have not been characterized. In this study, we used CW2-NR1 mutant mice to address a possible layer-specific requirement for NMDA receptors in OD plasticity in adult mice. CW2-NR1 mice have reduced levels of the essential NR1 subunit in cortical layers 2/3, superficial layer 4, and layer 5 (see Chapter 2). To measure the OD shift, we used an assay that detects changes in Arc induction after MD (Tagawa). This assay has the unique advantages of being sensitive to as little as 4 days of MD, layer-specific, and dependent on NMDA receptors. In addition, the changes in Arc induction are not observed in layers 5 or 6 (Tagawa et al., 2005), so we could focus our analysis on the OD shift in layer 4. Our study is the first attempt in adult mice to measure the OD shift in a distinct cortical layer, after NMDA receptor-dependent plasticity has been genetically removed from other layers.
Materials and Methods

Genetically-modified mice:

We generated CW2-NR1 mutant mice as described in Chapter 2. The CW2-NR1 KO line was generated by crossing a forebrain-specific, αCaMKII promoter-driven Cre transgenic mouse line, referred to as CW2 (Zeng et al., 2001), with a second mouse line, the homozygous “floxed” NMDA receptor subunit-1 (fNR1) line, in which two loxP sites flank the transmembrane domain and C-terminal region (exons 11-21), (Tsien et al., 1996). Founder mice (Cre/+, fNR1/fNR1) were crossed to homozygous fNR1 mice (+/+, fNR1/fNR1) to produce CW2-NR1 KO mutant (Cre/+, fNR1/fNR1) and CW2-NR1 control (+/+, fNR1/fNR1) littermates. For this study, we used mutant mice at 4-5 months of age, in order to take advantage of the age-dependence of NR1 deletion in the cortex (Fukaya et al., 2003).

Arc induction assay

Under isofluorane anesthesia, the left eye of each mouse was sutured, as described above. When the animals had recovered from anesthesia, they were placed in the dark for 24 hours, then exposed to light for 30 minutes to induce Arc expression. The animals were sacrificed, and the brains were removed immediately, embedded in OCT freezing media (TissueTek), and flash frozen on dry ice. The brains were stored at -80°C, until cryostat sections of 18 μm were cut.
In situ hybridization for Arc mRNA

In situ hybridization experiments for Arc mRNA were performed on coronal sections from CW2-NRI control and mutant mice. Antisense oligonucleotide probes, corresponding to the first 200 bp coding sequence of Arc (generated by PCR, using the primers 5'-atggagctggaccatatgacc-3' and 5'-taatacgactcactatagggagaacctgtgcaaccctttcagc-3'), were generated by in vitro transcription (Riboprobe systems, Promega) and labeled with $[^{33}\text{P}]$dUTP (Perkin Elmer). Probes were hybridized overnight to either cryostat sections (18 μm thick) or paraffin sections (8 μm thick). Specificity of the hybridization was confirmed by an absence of signal when the sense oligonucleotide was similarly labeled and hybridized. mRNA expression was verified by exposing the hybridized sections to a single x-ray film (Kodak BioMax) for 1 day. If the film indicated presence of hybridized probe, the sections were dipped in nuclear track emulsion (NTB-2, Kodak) and incubated at 4°C in a light-sealed box for up to 2 weeks. The sections were then developed in Kodak N19 developer, fixed, counterstained with Mayer’s hematoxylin and imaged under darkfield optics (Zeiss AxioVision).

Quantification of Arc mRNA signal

Arc mRNA was quantified in a manner similar to previous reports (Tagawa et al., 2005; Syken et al., 2006). All analysis was done blind to genotype and manipulation. For each genotype, darkfield images of 4-6 sections from 4-5 animals were scanned and imported into Matlab (The Mathworks, Natick MA). The border between layer 5, where Arc is not expressed, and deep layer 4, where Arc expression is apparent in the packed layer of cells, was clear in all images. A region encompassing all of layer 4 was defined.
by drawing a spline along the border of layer 5 and layer 4 and extending the spline
towards the pial surface by 200 \( \mu \text{m} \). All pixels within this region where projected onto
the spline and the average signal intensity for any point along layer 4 was computed by a
weighted average according to equation:

\[
I(x) = \frac{\sum_{p} y_p \cdot K\left(\frac{x_p - x}{h}\right)}{\sum_{p} K\left(\frac{x_p - x}{h}\right)}
\]

The variables were defined as follows: \( x_p \) is the position of pixel \( p \) along the
spline and \( y_p \) is the intensity of that pixel. \( K \) is a standard gaussian kernel and \( h \) is the
width of the kernel; here we used \( h=50 \ \mu \text{m} \).

The peak of the Arc induction zone in V1 was detected and the extent of
induction was defined as the width at half-maximum. To compile average Arc induction
zone measurements, curves were normalized and aligned to the peak. The signal
intensity was calculated as the average gray value along the width of the induction zone.
Values are presented as width of the Arc induction zone (in \( \mu \text{m} \)) or mean signal intensity
\( \pm \) standard error of the mean.
Results

To measure OD plasticity in adult mice, we used an assay of immediate-early gene expression (Pham et al., 2004; Tagawa et al., 2005). Baseline expression of Arc mRNA was induced by suturing one eyelid of a mouse and placing the animal in the dark for 24 hours. Subsequently, the animal was exposed to light for 30 minutes to induce Arc expression and then sacrificed for in situ hybridization (Figure 1A). Prior to the induction assay, mice experienced either normal binocular vision for the 7 days (non-MD group) or were subjected to 7 days of monocular deprivation (7d MD group). As described by Tagawa et al. (2005), the OD shift following MD is characterized by two major changes in the induction pattern of Arc. Both the intensity of the Arc signal and the width of the Arc induction zone are increased in the visual cortex of MD mice. This latter change is most apparent in the cortical hemisphere ipsilateral to the non-deprived (open) eye. In non-MD mice, Arc induction is restricted to the binocular zone. In MD mice, however, Arc induction is also observed in the monocular zone, implying a takeover of cortical territory by inputs subserving the non-deprived eye (Tagawa et al., 2005; see also schematic representation in Figure 1B). For this study, we compared the OD shift in CW2-NR1 mutant mice and littermate controls. We used mice at 16 weeks of age, in order to take advantage of the layer-specific deletion of NR1 in the cortex (Chapter 2).

Arc was induced in all non-MD mice from both genotypes (Figure 2). In non-MD control mice (Figure 2A, left panel), there is an intense band of Arc signal that corresponds to neurons in the binocular zone (yellow arrowhead). Arc is expressed in all
cortical layers except for layer 5 (Tagawa et al, 2005). Non-MD mutant mice also express Arc in the binocular zone (Fig 2B, left panel), but the signal is weak in layer 4 (immediately superficial to the Arc-negative signal in layer 5), likely due to the reduction of NR1 in superficial layer 4. In addition, Arc expression is absent from layers 2/3 and layer 6, consistent with a reduction of NR1 in these layers (Chapter 2) and the observation that Arc induction requires NMDA receptors (Steward and Worley, 2001; Wang et al., 2006).

Arc was also induced in 7d MD mice from both genotypes, but the consequences of NR1 deletion are profound. In 7d MD control mice, the Arc signal is stronger than non-MD controls, and has expanded medially into the monocular zone, indicating an OD shift (Fig. 2A, right panel). In contrast, mutant mice from the MD group show neither an increase in Arc signal intensity nor an expansion of expression (Fig 2B, right panel). We quantified these observations by measuring the height and the width of the Arc induction zones from all of the groups. In Figure 3, the mediolateral length of layer 4 was isolated along a curved line (spline), in order to generate a histogram plot of pixel intensity (mean gray value) as a function of distance along the spline. Histograms are presented for representative sections from control mice (Fig. 3A and B) and mutant mice (Fig. 3C and D).

From each histogram plot, the width of the Arc induction zone was conservatively defined as the width of the histogram at half-maximum. The signal intensity was calculated as average gray value along the width of the induction zone. All measurements are summarized in Fig 4. (For Arc signal intensity, non-MD control: 72.22 ± 4.33, n=12 sections; 7d MD control: 88.01 ± 5.33, n = 15 sections; non-MD
mutant: $56.32 \pm 5.80$, n=16 sections; 7d MD mutant: $42.90 \pm 4.79$, n=14 sections. For width of the Arc induction zone, non-MD control: $723.32 \pm 59.54 \, \mu m$, n=12 sections; 7d MD control, MD: $901 \pm 9.28 \, \mu m$, n = 15 sections; non-MD mutant: $615.83 \pm 52.55 \, \mu m$, n = 16 sections; 7d MD mutant: $735.77 \pm 52.92 \, \mu m$, n =14 sections). For both measures, there is a significant interaction between genotype and condition (p $< 0.01$, 2-way ANOVA; post-hoc comparison using the Bonferroni correction, p $<0.05$ for controls, p $>0.05$ for mutants), indicating that the Arc induction zone in CW2-NRI mice does not show a shift in response to MD. These data suggest that NR1 expression in deep layer 4 and layer 5 was not sufficient to mediate the OD shift, as measured by the Arc induction assay.
Discussion

We have observed a loss of the OD shift in CW2-NR1 mutant mice, as measured by Arc induction across the mediolateral extent of layer 4 in the primary visual cortex. Our data demonstrate that NR1-mediated synaptic plasticity in deep layer 4 and layer 5 is not sufficient to mediate an OD shift. Instead, these results suggest that NR1 receptors in other cortical layers, such as layers 2/3 or superficial layer 4, are required for the OD shift in adult mice. At first glance, these results are consistent with observations from cats and monkeys (Shatz and Stryker, 1978; LeVay et al., 1980), which show that OD plasticity in layer 4 is observed during the critical period, but only the superficial layers retain plasticity past the critical period (Daw et al., 1992), particularly if the animal has been dark reared (Mower and Christen, 1985). Similarly, cats show receptive field plasticity following retinal lesions, and the changes are thought to occur only in the horizontal connections between layer 2/3 neurons (Darian-Smith and Gilbert, 1995). The absence of an OD shift in CW2-NR1 mutant mice would also be consistent with a model for OD plasticity where superficial layers respond most rapidly to MD, with layer 4 following later (Trachtenberg et al., 2000).

On the other hand, there are two main differences between the studies in CW2-NR1 mice and the studies in cats or monkeys, which make comparison difficult. First, mice show OD plasticity well into adulthood, including layer 4 (Sawtell et al., 2003; Tagawa et al., 2005; Fischer et al., 2007). Because plasticity remains in layer 4 in adult mice, it is not clear if mice would share OD plasticity mechanisms with cats, where layer 4 plasticity is absent after closure of the critical period (Shatz and Stryker, 1978; Daw et
al., 1992). The other major difference is the technique used to measure the OD shift. OD plasticity in cats and monkeys is measured with single-unit recordings, whereas the OD shift in the CW2-NR1 mice was assessed by a change expression of the immediate early gene Arc. Arc is expressed in response to increased activity levels, but it is not clear what functional significance Arc expression has. There are some qualitative similarities between Arc expression and other measures of OD plasticity: Tagawa et al (2005) show that the expansion of Arc induction zone parallels the expansion of ocular dominance columns in young cats. In addition, the increased Arc expression following MD correlates with increased VEP amplitudes from ipsilateral (non-deprived) eye inputs after MD (Sawtell et al., 2003) and increased intensity of intrinsic optical signals following MD (Hofer et al., 2006). Finally, the lateral expansion of the Arc induction zone has an electrophysiological correlate; VEPs corresponding to ipsilateral (open) eye inputs appear after MD in the monocular zone (M Frenkel and M Bear, personal communication). In spite of all these correlations, it is not clear that expansion of Arc expression and electrophysiological recordings measure the same changes. Additional work in CW2-NR1 mice comparing single-unit recordings with VEP measurements and Arc induction changes are needed in order to definitively conclude that NMDA receptors are required in superficial layers for OD plasticity.
Figure 1
Arc induction assay

A. Experimental set-up

Non-MD Group

Normal binocular visual experience

7 days

24 hr dark

quiescence

30 minutes of light
SAC animal
In situ hybridization

Suture one eyelid

7d MD Group

Plasticity of neuronal circuits

Suture one eyelid

quiescence

30 minutes of light
SAC animal
In situ hybridization

B. Schematic representation of the OD shift
Figure 2
Arc induction in CW2-NR1 mutant and control mice

A. CONTROL

Non MD

7d MD

B. CW2-NR1 MUTANT

Non MD

7d MD
Figure 3
Histograms of Arc induction in layer 4

A. Selection of layer 4 in control mice

B. Sample histograms: pixel intensity along layer 4

C. Selection of layer 4 in CW2-NR1 mutant mice

D. Sample histograms: pixel intensity along length of layer 4
Figure 4
Summary of Arc measurements

A. Control mice

B. CW2-NR1 mutant mice
Figure Legend

Figure 1. The Arc induction assay.

A. Experimental set-up. The Arc induction assay was performed either in non-MD animals (non-MD Group, top) or in animals that were subjected to 7 days of MD (7d MD Group, bottom). In both cases, Arc was induced by placing the animals in the dark for 24 hours, then exposing them to light for 30 minutes. Expression of Arc mRNA is determined by in situ hybridization of coronal brain sections.

B. Schematic representation of the OD shift. Left, in the non-MD group, exposure to light induces Arc mRNA in the binocular zone of the cortical hemisphere ipsilateral to the open eye. Right, in the 7d MD group, exposure to light induces more intense levels and a wider expanse of Arc expression. Brain images from www.mbl.org (Rosen et al, 2000; Williams, 2000).

Figure 2. Arc induction in CW2-NR1 mutant and control mice.

A. Control mice. Left panel, Arc induction in a non-MD control mouse. The Arc expression in the visual cortex is restricted to the binocular zone (yellow arrowhead) and present in all cortical layers except for layer 5 (marked). Right panel, Arc induction in a 7d MD control mouse. As compared to the non-MD control mouse, Arc expression is more intense and more widespread in the 7d MD control mouse.

B. CW2-NR1 mutant mice. Left panel, Arc induction in a non-MD mutant mouse. Arc is induced in the binocular zone (yellow arrowhead) of layer 4, located
immediately superficial to the Arc-negative signal in layer 5 (marked). Overall levels of Arc are reduced, particularly in layers 2/3 and layer 6, because of the reduction in NR1 expression. Right panel, Arc induction in a 7d MD mutant mouse. Unlike the control mice, the Arc expression is neither more intense nor more widespread than the non-MD mutant mice.

Figure 3. Histograms of Arc induction in layer 4.

A,C. Layer 4 of the visual cortex in control mice (A) and CW2-NR1 mutant mice (C) was selected by drawing a spline along the border of layer 4 and layer 5, across the mediolateral extent of the visual cortex. The superficial boundary was defined at a fixed distance of 200 μm from the layer 4/5 border.

B,D. Histograms of pixel intensity along the mediolateral extent of layer 4 in control mice (B) and CW2-NR1 mutant mice (D). Data corresponds to images shown in A, C. Note that the histogram from the non-MD control mouse is narrower and flatter than the histogram from the 7d MD control mouse, reflecting differences in Arc induction in the control mice. In contrast, the histogram from the non-MD mutant mouse is very similar to the histogram from the 7d MD mutant mouse, indicating that the OD shift does not occur in CW2-NR1 mutant mice.

Figure 4. Summary of Arc measurements, a quantification of the Arc signal intensity and the width of the Arc induction zone in all mice.

A. Control mice. Left, an increase in mean signal intensity in 7d MD animals (non-MD control: 72.22 ± 4.33, n=12 sections; 7d MD control: 88.01 ± 5.33, n = 15
sections). Right, an increase in the width of the Arc induction zone in 7d MD animals (non-MD control: $723.32 \pm 59.54 \ \mu m$, $n=12$ sections; 7d MD control, MD: $901 \pm 9.28 \ \mu m$, $n = 15$ sections; Bonferroni post-test, $p < 0.05$).

B. CW2-NR1 mutant mice. Left, a slight decrease in mean signal intensity in 7d MD mutants (non-MD mutant: $56.32 \pm 5.80$, $n=16$ sections; 7d MD mutant: $42.90 \pm 4.79$, n=14 sections). Right, no change in width of the Arc induction zone in 7d MD mutants (non-MD mutant: $615.83 \pm 52.55 \ \mu m$, $n = 16$ sections; 7d MD mutant: $735.77 \pm 52.92 \ \mu m$, $n =14$ sections; Bonferroni post-test, $p > 0.05$).
Conclusions and Future Directions

Methodology

The removal of NR1 from selective neural circuits has been effectively used to dissect mechanisms of hippocampal plasticity, and we report here that this genetic lesion is also useful for studying circuitry in the visual cortex. The genetic lesion non-invasively targets a specific population of neurons in a specific region, and avoids problems associated with other methods used to disrupt synaptic function. In addition, the plasticity paradigms used in this study were specific to the primary visual cortex, so we could use the CW2-NR1 mice without concern that NR1 deletion in other brain areas, like the hippocampus, would confound our analysis.

The CW2-NR1 mice were a suitable model for dissecting plasticity mechanisms that depend on NR1 in deep layer 4 and layer 5. The story is not complete, however, until requirements for plasticity in other layers has been assessed. This will require new genetic models in which genes such as NR1 have been deleted or altered in other cortical layers. To this end, the promoter fragment from RORβ, which is enriched in layer 4, can be used to drive expression of other transgenes (or Cre recombinase) in layer 4. There are several other genes that show a layer-specific pattern of expression (Allen Brain Atlas) and their promoter fragments could be used in a similar manner to create a complement of layer-restricted mutant mice.

A potential problem with genetic models is that expression of the transgene or knockout may not be tightly restricted to a particular brain region. RORβ is enriched in layer 4 of the cortex and not in other cortical layers, but it is also expressed in other brain areas, including the retina and thalamus, as well as regions involved in regulation of
circadian rhythms. Any of these regions could non-specifically affect plasticity in the visual cortex, irrespective of the experimental paradigm. In addition, RORb is expressed early in postnatal life, and expression of a transgene at such an early age could have deleterious effects on brain formation and development. To circumvent this problem, a combination of technologies could be used. One possibility is to create a RORβ-Cre transgenic mouse and then infect neurons in the visual cortex with a virus containing a floxed transgene. Another option is to combine several mutant lines, in which interacting transgenes are driven by promoters whose expression overlaps. This approach can be used to create mice in which an inducibly-regulated gene can be restricted to a very specific brain region.

Layer-specific requirements for SRP

The requirement for NMDA receptors in deep layer 4 and layer 5 is compelling, because it suggests that SRP is supported by plasticity in the synapses in layer 4. Synapses present in layer 4 include those connecting LGN neurons to layer 4 neurons, as well as LGN neurons to apical dendrites from layer 5 neurons (White, 1979). Many neurons in layer 4 and layer 5 have symmetrical, non-oriented receptive fields (Drager, 1975; Mangini and Pearlman, 1980) so it is possible that SRP occurs only in a small population of neurons with oriented receptive fields. This could be explored by monitoring changes induced by SRP in this population of neurons. Changes occurring in apical dendrites of layer 5 during SRP could be explored in Thy-1 GFP mice, which express GFP in scattered neurons in layer 5. This technique has been used to monitor changes in spine motility and morphology in vivo (Majewska and Sur, 2003; Oray et al.,
CW2-NRI mice could be crossed with Thy-1 GFP mice and monitored by 2-photon microscopy for structural changes associated with potentiation, such as increased spine growth. Another way to assess the contributions of layer 4 and layer 5 would be to engineer genetically-modified mice that express NR1 in one or the other layer, but not both.

Layer-specific requirements for OD plasticity

Although NMDA receptors in deep layer 4 and layer 5 were sufficient to mediate SRP, this minimal complement of NMDA receptors was not sufficient to support OD plasticity, as measured by Arc induction. Unfortunately, this finding raises more questions than it answers. Are NMDA receptors in superficial layers necessary to mediate the OD shift? If so, would synaptic plasticity mechanisms like LTP and LTD in these layers underlie the OD shift? Would dnPAK mice still show an OD shift, as measured by VEPs, if LTP and LTD were required in superficial layers? Results from VEP measurements in dnPAK mice indicate a normal OD shift, even when spine morphology was abnormal and synaptic plasticity in the superficial layers was altered.

It is possible that the Arc induction assay does not reflect the same changes that VEPs measure. Unlike VEPs, induction assays of immediate-early genes have not been used to measure visual function, and the significance of their expression is unclear. Pham et al (2004) used a similar assay for OD plasticity, measuring changes in the induction of the immediate-early genes c-fos and zif-268 after MD. The authors reason that expression of immediate-early genes reflects the integration of many signals that depend on activity, which occurs over minutes or hours, unlike electrophysiological changes.

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which occur very quickly. This implies that requirement for induction of immediate-
early genes is stringent and would only occur under conditions where it had functional
significance. Notably, the changes in c-fos and zif-268 after MD mirrored the changes
measured by VEPs. On the other hand, Pham et al (2004) did not observe the expansion
of the induced genes into the monocular zone, as Tagawa et al (2005) observed with Arc
induction. In this study, we measured the expansion of the Arc induction zone, but a
correlating VEP measurement has not yet been made in adult mice. These findings could
be reconciled by measuring the OD plasticity in CW2-NR1 animals, using VEP
measurements. Further, recording electrodes implanted in the monocular zone could
record ipsilateral VEPs, in order to determine if emergence of this signal is a correlate of
Arc expansion into the monocular zone. If expansion of the Arc induction zone does not
correlate with the synaptic changes that are reflected by VEP measurements, then these
two paradigms may be measuring separate features of the OD shift. Indeed, any of the
variety of techniques used to measure OD plasticity might measure subtle but separate
components of the OD shift. Further comparative analysis will be necessary to answer
these questions. In the future, these analyses can be aided by the use of powerful genetic
technology like layer-specific deletion of NR1 or other genes.
References


He HY, Hodos W, Quinlan EM (2006) Visual deprivation reactivates rapid ocular
Heynen AJ, Bear MF (2001) Long-term potentiation of thalamocortical transmission in
mechanism for loss of visual cortical responsiveness following brief monocular
Hofer SB, Mrsic-Flogel TD, Bonhoeffer T, Hubener M (2006) Prior experience enhances
dependent and cell-type-specific spine growth in the neocortex. Nature 441:979-
983.
Huang ZJ, Kirkwood A, Pizzorusso T, Porciatti V, Morales B, Bear MF, Maffei L,
Tonegawa S (1999) BDNF regulates the maturation of inhibition and the critical
Hubel DH, Wiesel TN (1959) Receptive fields of single neurones in the cat's striate
Hubel DH, Wiesel TN (1962) Receptive fields, binocular interaction and functional
Hubel DH, Wiesel TN (1970) The period of susceptibility to the physiological effects of
Hubel DH, Wiesel TN, LeVay S (1977) Plasticity of ocular dominance columns in


White EL (1979) Thalamocortical synaptic relations: a review with emphasis on the projections of specific thalamic nuclei to the primary sensory areas of the neocortex. Brain Res 180:275-311.


