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Analysis of candidate plasticity genes in visual cortex.

Paul Joseph Kiser 1969-
University of Louisville

ANALYSIS OF CANDIDATE PLASTICITY GENES IN VISUAL CORTEX

By

Paul Joseph Kiser
B.S., The Florida State University, 1993
M.S., Univeristy of Louisville School of Medicine, 1997

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A Dissertation Approved on

April 22, 2010

by the following Dissertation Committee

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Dissertation Director

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DEDICATION

This dissertation is dedicated to my parents

Rev. and Mrs. Joseph W. Kiser,

and my wife,

Jennifer B. Kiser

for all of the love and support they have shown me;

and my daughters,

Kaitlyn and Kayleigh Kiser

who have patiently wondered where there father has been

as I worked to both support them and finish this research

over the past several years.

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Loving thanks is offered for my wife and family who have shown incredible patience while I disrupted our previously stable life through the pursuit of this degree in hopes that it will ultimately provide an even brighter future for us.

ABSTRACT
Analysis of Candidate Plasticity Genes in Visual Cortex

Paul J. Kiser

April 22, 2010

The goals of this dissertation is to further the understanding of the roles of two

genes identified by differential display polymerase chain reaction (ddPCR) of cat visual

cortex as candidate genes to play a role in visual cortical plasticity.   Analysis of normal

and dark rearing of cats at five and twenty weeks identified Dab-1, the mammalian

homologue of the *disabled-1* gene found in *drosophila* and Munc13-3, the mammalian

homologue of the *c. elegans* "uncoordinated" or *unc-13* gene. Dab-1 transcription

analysis showed a pattern of high levels during the peak (5 weeks) and low levels at the

nadir (20 weeks) of the critical period in normally reared animals with opposite

transcription levels in dark reared animals (the "plasticity" pattern). Munc13-3 showed

the opposite pattern with low levels of transcription at the peak of the critical period and

high levels at the nadir in normally reared and opposite pattern in dark reared animals

("anti-plasticity" patterm).

This project extended the mRNA findings on Western blot analysis to study

protein expression of both genes in the visual cortex during the critical period in both cats

and mice. This analysis confirmed both Dab-1 and Munc13-3 as candidate plasticity

genes showing "plasticity" and "anti-plasticity" patterns of protein expression, respectively, in visual cortex.

Immunohistochemical labeling of Dab-1 in cat visual cortex revealed a consistent developmental decline in density of immunopositive cells across all cortical lamina from birth through the critical period. Comparisons of Dab-1 immunohistochemistry in normal and dark reared cats at the peak and nadir of the critical period showed staining patterns consistent with the results of the total protein expression studies. There was a greater immunopositive cell density in normal vs. dark reared animals at the peak of the critical period and greater cell density in dark reared than normal at the nadir of the critical period.

Through the use of genetic mutants lacking the Dab-1 gene, additional Western blot analyses were performed to assess potential relationships between Dab-1 and expression of NMDA receptor subunits during the visual critical period. An inverse relationship was found between mutant and wild-type mice in the ratio of expression of NR2A and NR2B subunits between normal and dark-reared animals indicating a strong relationship between the proteins.

While future experiments need to be conducted to further elucidate more details on the specific functions of both genes, this research provides substantial evidence that Munc13-3 and Dab-1 are both genes with significant roles in the critical period of visual cortical development.

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INTRODUCTION

Visual cortex development is a complex series of anatomical and physiological processes that can be altered by visual experience. Through a combination of spontaneous activity and stimulus-driven responses, connections are fine-tuned in the cortex ultimately leading to the normal mature state. In the mid-1960s, Hubel and Wiesel discovered that there was a period of early postnatal development (a "critical period") during which the visual cortex has increased levels of malleability in response to visual experience. Experiences occurring within this developmental window yield permanent modifications to the anatomy and physiology of the circuitry of the cortex (Hubel et al., 1977). This phenomenon is the classic demonstration of the widely used term, neuronal plasticity. Critical period plasticity of the visual cortex results from an interaction of genetics and visual input.

The primary purpose of this study is to analyze two genes identified by our lab as possible candidate genes controlling neuronal plasticity during the critical period: Dab-1 and Munc13-3 (Yang et al., 2002; Yang et al., 2006). This introductory section will review important background information including: 1) the development of anatomical and physiological components of visual cortex; 2) the central tenets of the critical period

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and neuronal plasticity; 3) known roles and properties of Dab-1 and Munc13-3; and 4) experimental benefits of mouse versus cat studies in visual cortex.

Visual Pathway

In the cat visual system, stimuli are predominantly perceived through the primary visual pathway, also called the retinogeniculocortical pathway. Originating in the eye, retinal ganglion cells form fibers that are carried through the optic nerve (cranial nerve II). Those from the nasal retina of the contralateral eye decussate to merge with fibers from the temporal retina of the ipsilateral eye to form the optic tracts. This partial decussation of retinal fibers transmits the right visual hemi-field to the left side of the brain and the left visual hemi-field to the right side of the brain. The optic tracts each carry information from the contralateral visual hemi-field. These neurons synapse in the lateral geniculate nucleus (LGN) of the thalamus. Visual inputs from the two eyes are segregated in the six different layers of the dorsal LGN. The fibers from the ipsilateral eye synapse in layers 2, 3 and 5 and those from the contralateral eye synapse in layers 1, 4 and 6. Post-synaptic neurons from the LGN then travel through the optic radiations to the primary visual cortex in the occipital lobe. The projections from individual layers of the LGN remain segregated as alternating columns in layer IV of the primary visual cortex. The anatomically segregated pattern forms the basis for ocular dominance columns which will be discussed later in this chapter. Other brain regions receiving retinal afferent signals include the pretectal nucleus of the optic tract (for pupillary reflexes), the superior colliculus (for eye movements and tracking), and the

suprachiasmatic and ventrolateral preoptic nuclei (for circadian rhythms and sleep regulation) (Sherman and Spear, 1982).

Visual Cortex Development

Perinatally, there are five distinct layers present in the visual cortex. From pial surface to white matter, they are : layer I, a compact zone (CZ), a cortical plate (CP), layer V, and a combined layer VI/subplate (SP). The subplate is a transient embryonic layer that disappears within the first two to three postnatal weeks. The compact zone and the upper third of the cortical plate develop into layers II and III and the lower two-thirds of the cortical plate develops into the mature layer IV. During the first several postnatal weeks, neuronal migration and synaptogenesis result in the mature laminar pattern becoming evident by the third week of postnatal development (Luskin and Shatz, 1985a, b; Shatz and Luskin, 1986). The adult cat visual cortex consists of six different layers, numbered I-VI sequentially from the pial surface to the white matter (O'Leary, 1941) (Figure 1). These lamina are distinguished by the size, shape, packing density and receptive field properties of the cells, along with the afferent and efferent connections of these cells with other sites (Garey, 1971; Garey and Powell, 1971; Hubel and Wiesel, 1972; Hollander, 1974; Palmer and Rosenquist, 1974; Lund et al., 1975; LeVay and Gilbert, 1976; Gibson et al., 1978; Gilbert and Wiesel, 1979).

Figure 1. The layering of cat visual cortex as seen in a mature brain demonstrated by cresyl violet staining. There are six layers, numbered I – VI, from the pial surface to the white matter. WM: White matter. Calibration bar=100μm
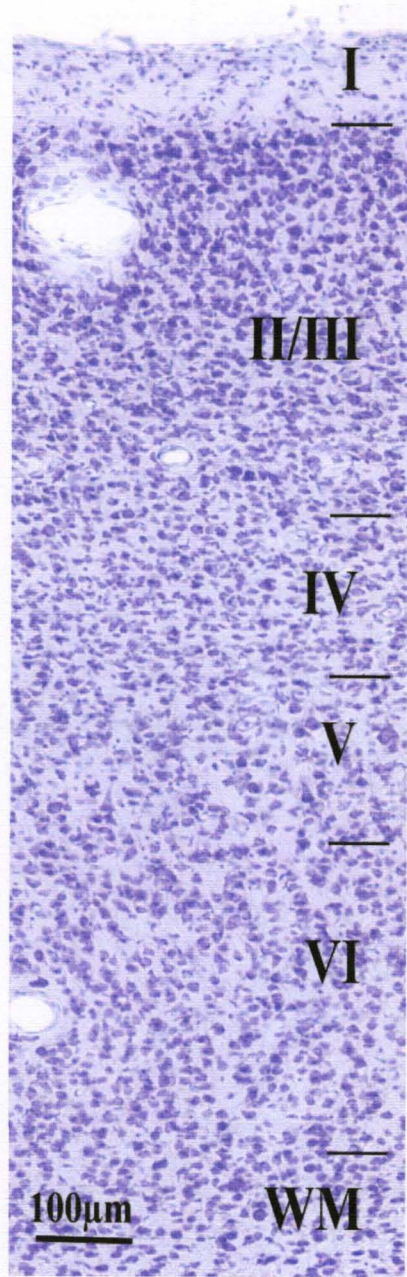
Figure 1

Developmental birth-dating studies by radioactive labeling of individual neurons show an "inside-to-outside" patterns of neurogenesis. In the cat cells in layer VI are generated early, between embryonic day 31 (E31) and E38, and cells in more superficial layers are generated progressively later (layer V, between E33 and E40; layer IV, between E37 and E44; layer II and III, between E40/41 and E57). Cumulatively, it takes approximately one week for cells in layers VI, V, and IV and two weeks for cells in layers II and III combined to develop ((Luskin and Shatz, 1985a, b)

As mentioned previously, in normal development afferent signals from the left and right eyes remain segregated, synapsing in different lamina of the LGN of the thalamus and ultimately entering layer IV of the visual cortex where they synapse on the cortical neurons. From layer IV these signals are carried primarily to nearby cells in layers II and III where they synapse and then relay to layers V and VI creating a columnar arrangement of the cells in cortex that respond to the same tiny region of the visual field. In primary visual cortex, area 17, there is a single representation of the cat's entire visual hemi-field. This retinotopic map in area 17 contains a first order transformation of the visual hemifield in which every adjacent point in that portion of the visual field is represented as an adjacent point in the cortex (Tusa et al., 1978). Within primary visual cortex there are individual neurons that respond to stimuli from either eye, the binocular segments. Columns in the binocular segments, although capable of being stimulated by either eye, do show distinct physiological preferences for stimulation by one eye or the other. There are also cortical regions in which neurons are specifically

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driven by stimuli from only one eye, the monocular segment, found in area 17 of the hemisphere contralateral to the stimulated eye (Lowel and Singer, 1987).

In normally reared cats and primates, the projections from the LGN to layer IV of the visual cortex terminate in anatomically distinct clusters representing axon terminals from geniculocortical afferents from each eye. These anatomical features, called ocular dominance columns, have been demonstrated by injecting radioactive labels into one eye producing, through transsynaptic neuronal transport, an alternating pattern of stained and unstained regions in layer IV (Shatz et al., 1977). Confirming the fact that this layer IV staining is representative of the projections from the injected eye through the LGN is the especially prominent and continuous staining pattern in the monocular segment of the contralateral eye and absence of staining in the ipsilateral monocular segment (Shatz et al., 1977).

Neuronal Composition of Visual Cortex

There are two principal types of cortical neurons. They are stellate cells and pyramidal cells which differ primarily in the shapes of cell bodies and lengths of axons. Pyramidal cells possess larger cell bodies and longer axons (Gilbert and Wiesel, 1979; Nicholls et al., 2001). Pyramidal cells are predominantly found in layers II, III, V, and VI, and stellate cells are mainly present in layer IV (also named the granular layer). Layer I has very few neuronal somas. Layers II and III receive input from layer IV and project to areas 18 and 19. There is no readily distinguishable border between these two

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layers; however, layer II is characterized by irregular clusters of superficial pyramidal cells. Layer IV receives the majority of the terminal input from the LGN and projects to layer II and III. Layer V receives input from layers II and III and projects back to layers II and III and superior colliculus and pontine and pulvinar nuclei. Layer VI also receives afferent information from the LGN and it projects back mainly to the LGN of the thalamus (Nicholls et al., 2001; Huppe-Gourgues et al., 2006).

Monocular Deprivation

Monocular Deprivation: Physiological effects

Hubel and Wiesel discovered that monocular visual deprivation, surgically closing or occluding one eye, during specific periods of development can lead to irreversible losses of binocularity of individual cells in the visual cortex (Hubel et al., 1977). Physiologically, effects are seen in response properties of the binocular cells in visual cortex that are typically driven by a preference for stimulation by one eye or the other. When one eye is closed by monocular deprivation through the critical period, these binocular cells lose their ability to respond to stimulation to that eye, instead responding only to the experienced eye (Hubel and Wiesel, 1977; Olson and Freeman, 1980). This is supported by the behavioral effects experienced by cats deprived of visual stimulation in one eye during this sensitive period of development showing permanent blindness in the closed eye when that the eye is opened and normal stimulus is restored (Mitchell, 1988).

Further experiments have demonstrated numerous other neuroanatomical and physiological effects of monocular deprivation. Some of these effects include decreases in stereoscopic depth perception (Timney, 1983), decreases in visual orienting skills (Sherman, 1973) and decreased visual acuity (Giffin and Mitchell, 1978).

Monocular Deprivation: Anatomical effects

The physiological effects of monocular deprivation were correlated by an anatomical basis for altered binocular receptivity. Monocular deprivation during the critical period produces alterations in both the LGN and visual cortex. In the LGN, monocular deprivation yields a reduction in cross-sectional area of cells in the layers that receive afferents from the deprived eye (Wiesel and Hubel, 1963; Guillery and Stelzner, 1970). In visual cortex, ocular dominance columns are affected such that the columns representing the deprived eye are reduced in area while those representing the non-deprived eye are expanded, implying a physical takeover of cortical region by the experienced eye (Shatz and Stryker, 1978; Mower et al., 1985). It has been shown that the physiological response to monocular deprivation is the result of rapid (within one week) activity-dependent remodeling via shortening of the terminal arborization of the geniculocortical afferents to layer IV. This fairly rapid reduction in connections from the deprived eye is contrasted by a slower increase in branching and rearrangement of geniculocortical terminals from the non-deprived eye. The evidence suggests that this reorganization of synaptic terminals results in projections from the non-deprived eye

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developing a broader arborization within layer IV and a decrease in the extent of the terminal arborization of the deprived afferents. This increased projection area yields a greater cortical representation of the non-deprived afferent neurons, thus providing a mechanism for the "takeover" of the cortex previously representing the deprived eye (Antonini and Stryker, 1996).

Critical Period

The critical period during which the developing cortex is most sensitive to experiential modification varies between species. Since Hubel and Wiesel's original cat experiments, that began the research into the developmental periods when the cortex was more or less modifiable by experience (Hubel and Wiesel, 1970), there have been nearly forty years of studies on visual plasticity. Research during this period has encompassed investigations into definitive time periods of greatest cortical plasticity as well as possible mechanisms controlling and influencing these processes. In spite of the volumes of research on the subject, and although there is consensus as to the time frames of greatest plasticity in specific systems of individual species, there remains surprisingly little unanimous agreement by experts in the field on ultimate determination of genetic and molecular mechanisms initiating and terminating the critical period. Recent transgenic studies have shed light on the role of inhibitory mechanisms in regulating both the timing of the critical period (Huang et al., 1999) as well as the entire susceptibility of visual cortex to modification by sensory experience during the critical period (Hensch et al., 1998). This, along with other significant recent findings, will be discussed in more detail

following an important review of the historical contributions to the research of the critical period and its traditional understanding.

Time Course of Critical Period

Cat visual cortex is a well documented, illustrative model for understanding neural development and plasticity at the cellular level. In the cat, the geniculocortical pathway is very immature at birth in terms of both its anatomy and physiology. By three weeks of age, cellular migration has completed formation of the mature lamination pattern in visual cortex, embryonic cells have disappeared, and geniculocortical afferents have reached their final position and begun the process of segregating into ocular dominance columns (Luskin and Shatz, 1985a; Shatz and Luskin, 1986). Correlated with these anatomical events is the appearance of adult-like physiological response properties, such as orientation selectivity, in visual cortex neurons by about 4 weeks of age and little change in response properties occurs thereafter (Pettigrew, 1974; Blakemore and Van Sluyters, 1975). Over the next several months these loosely established anatomical and physiological characteristics are stabilized under the guidance of visual experience. One major event during this period is synaptogenesis, which increases sharply from birth to a peak at about 5 weeks and then declines to adult levels at about 20 weeks of age (Cragg, 1975; Winfield, 1983). Maturation of both glial and neurotransmitter systems also occurs gradually over the first several postnatal months (Skangiel-Kramska, 1988; Muller, 1992).

It is now clear that ocular dominance columns in layer IV are already present in a state of incomplete segregation at birth or prior to the onset of the critical period in a number of species (Rakic, 1976; Horton and Hocking, 1996; Crowley and Katz, 2000; Crair et al., 2001). In cats, the process leading to incompletely segregated columns is complete by 2 weeks (Crair et al., 2001), activity dependent ocular dominance columns are clearly evident around 6 weeks of age (LeVay et al., 1978) and ocular dominance columns are no longer sensitive to monocular deprivation between 12 and 16 weeks in cats and macaque monkeys (Mower et al., 1985; Horton and Hocking, 1997).

It is during this stage, 6-16 weeks, of cortical development when plasticity, the capacity for anatomical and physiological development to be altered by visual experience, is at its greatest levels. Visual cortex is susceptible to monocular deprivation only during this critical period of postnatal life. In cats, physiological susceptibility to monocular deprivation is absent until about 3 weeks, peaks at 6 weeks, then gradually declines to low levels at 20 weeks and disappears completely between six months and one year of age (Daw et al., 1992).

Another marker for the onset and termination of the critical period that goes beyond a single cortical adaptation to loss of stimulus from monocular deprivation are studies that evaluate the ability of the cortex to recover from this sensory deprivation. These studies have proven significant in understanding critical period time limitations. Studies have been conducted utilizing reverse occlusion, where one eye is monocularly deprived for a set period of time, thus initiating the normal loss of function in the

deprived eye, then after several weeks, opened the original deprived eye and occluding

the other to force all stimulation to the previously closed eye. These studies permit

researchers to measure the amount of recovery of function in the originally sutured eye

(Blakemore and Van Sluyters, 1975). This allows for a more specific determination of

the actual period of true plasticity in visual cortex that includes not only a modification of

the neural pathways based on a one-time alteration of stimulus but an assessment of the

true period when ongoing cortical adaptations are possible as visual experience and

afferent signals are changed over time. These studies of recovery from monocular

deprivation with reverse occlusion confirmed the normal cat critical period as having

plasticity beginning around two weeks, peaking at 5-6 weeks and diminishing to very low

levels by week sixteen (Olson and Freeman, 1980; Fregnac and Imbert, 1984).

Dark Rearing

Dark rearing animals involves raising animals in a light-free environment from

birth, preventing any visual experience for the period of confinement. Although the exact

mechanisms remain undefined, it has been demonstrated repeatedly in the visual system

that the critical period in not simply a process of maturation based on the age of the

animal. There is a significant contribution of visual input during the critical period that

affects both the anatomical and physiological development of visual cortex. As

previously reviewed, in normally reared animals, approximately the first two weeks of

cortical development, prior to segregation of ocular dominance columns, is a process

independent of visual input, likely guided by predetermined genetic cues. Although these

early stages of cortical development in dark-reared animals appear concurrent with those raised in normal light conditions, as development continues anatomical and physiological differences quickly begin to become apparent between the two rearing conditions. Over subsequent developmental weeks, in normal light conditions, the competitive process guides the formation of mature ocular dominance columns. Through dark rearing experiments, this columnar maturation has been shown to be dependent on visually driven neuronal activity since the process does not occur in dark reared animals, where ocular dominance columns remain in a state of incomplete segregation (Swindale, 1981; Mower et al., 1985).

Dark Rearing: Effects on the Critical Period

Most importantly, rearing in total darkness extends the critical period and prolongs neuronal plasticity far beyond its normal age limits (Cynader and Mitchell, 1980; Mower et al., 1981). Monocular deprivation studies in dark-reared animals have shown that the length of time that effects of the procedure can be seen in visual cortex extend for a significantly longer period of time than the standard critical period (Cynader and Mitchell, 1980; Mower et al., 1981; Mower, 1991). As discussed, the critical period for susceptibility of normal cat visual cortex to monocular deprivation is low at two weeks, peaks at approximately 5 weeks and gradually declines over the next 4-5 months. Dark-reared cat visual cortex has low susceptibility to manipulation through the first five weeks, peaks at approximately 12 weeks of age and remains high in much older animals. As seen in Figure 2, it is important to acknowledge that this effect of extending the

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critical period through dark-rearing is not accomplished through prevention of either onset or decline of plasticity in the cortex, but instead slows down the entire time course and prolongs the entire process, both the rise and decline (Mower, 1991; Beaver et al., 2001). Dark rearing thus provides a means to dissociate changes associated with the state of visual cortical plasticity from changes associated with the general age-based maturation of the animal. This makes it is an ideal system for isolating changes in gene expression potentially involved in controlling neuronal plasticity.

Dark Rearing: Anatomical and Physiological Effects

Visual cortical neurons in kittens raised in normal light environments take on adult-like properties, specifically, orientation specificity, by 5-6 weeks of age (Fregnac and Imbert, 1978). Animals raised from birth in complete darkness show a significant reduction in the percentage of orientation selective cells (Blakemore and Van Sluyters, 1975; Buisseret and Imbert, 1976; Kratz and Spear, 1976; Fregnac and Imbert, 1978). This is not, however, due to an overall lack of receptiveness by visual cortical neurons because most of the neurons in the binocular segment of the dark-reared visual cortex remain receptive to stimuli from either eye (Cynader, 1983; Mower et al., 1985). In cat visual cortex, at two weeks of age, orientation-specific cell responsiveness is the same in both normal and dark-reared animals. However, beyond 17 days of dark-rearing, instead

Figure 2. The effects of dark rearing on the critical period. Cell recording studies show the effect of dark-rearing slows the entire time course of the critical period. The results of this are that near the peak of the critical period (5 weeks in cat visual cortex development) normal cats are more plastic than dark reared cats. Likewise, near the end of the critical period (20 weeks) dark reared cats are more plastic than normal. Deprivation Index (y-axis) is a measure of the percentage of cells that show responsiveness to the open after a period of two days of monocular deprivation. This graph is adapted from Mower (1991).

DARK REARING AND THE CRITICAL PERIOD
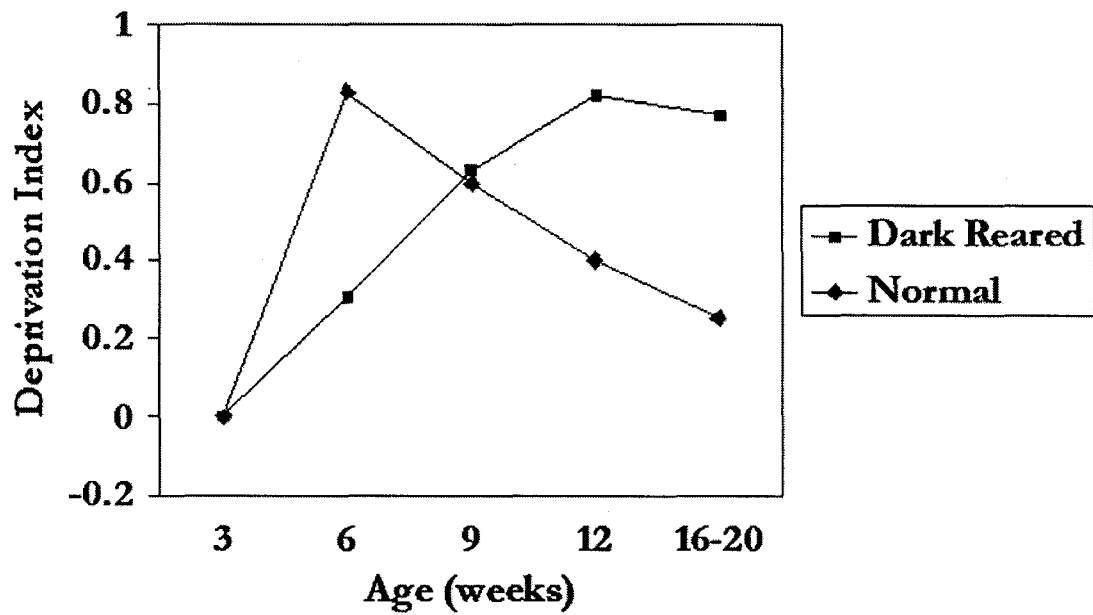
GRAPH ADAPTED FROM MOWER, 1991. DEV BRAIN RES 58:151-158.

Figure 2

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of continuing the normal maturation process of cortex involving increases in the ratio of orientation specific to non-specific and unresponsive cells (Fregnac and Imbert, 1978), there is a discontinuation of this process. Dark-rearing essentially freezes this maturation in an undeveloped physiological state leaving a higher ratio of non-selective to orientation selective cells in older dark-reared cortex.

Studies of ocular dominance columns and their susceptibility to monocular deprivation in animals that were dark-reared beyond the normal critical period revealed no anatomical changes in the distribution of the thalamocortical afferents to layer IV of area 17 and no alterations in the ocular dominance columns (Mower et al., 1985). This demonstrates that the anatomical plasticity of cortical area 17, unlike the physiological effects described above, is a product of age dependent processes and proceeds independent of visual experience.

Monocular Deprivation Effects: Revisited

As studies of plasticity in the visual cortical critical period continued to develop the research progressed from simple analyses of the thalamocortical effects in layer IV and began to assess impacts of monocular deprivation and the associated plasticity in other layers of cortex. There is a columnar arrangement of cerebral cortex perpendicular to the pial surface that extends beyond the ocular dominance columns previously described in layer IV (Hubel and Wiesel, 1962; Hubel et al., 1977; Shatz et al., 1977; Horton and Adams, 2005). The cells in the extragranular layers of these columns have

similar receptive fields and are linked through intracortical synapses. Although the effects of monocular deprivation were classically thought to result from anatomical reorganization of thalamocortical input to ocular dominance columns in layer IV, it is now clear that there is an extragranular mechanism for critical period plasticity. Numerous studies have revealed differences in critical periods in different cortical layers (LeVay et al., 1980; Daw et al., 1992). Layer IV has been shown to have a critical period that closes prior to that of the extragranular layers (LeVay et al., 1980) and is considerably less plastic than other layers (Mower et al., 1985; Daw et al., 1992). Specifically, monocular deprivation tests at week 14 in normal and dark-reared cats have shown layer IV to be least plastic and layers V and VI to be the most plastic, with layers II and III demonstrating intermediate plasticity (Beaver et al., 2001). However, like layer IV, the extragranular layers show similar effects of dark-rearing with an overall delay and prolonging of the effects of monocular deprivation in dark-reared animals (Mower et al., 1985; Beaver et al., 2001).

Perhaps the most significant finding of studies of the extragranular layers' roles in plasticity was that the immediate effect of brief monocular deprivation is outside layer IV. Extragranular layers show physiological responses to monocular deprivation prior to changes in layer IV (Trachtenberg et al., 2000). Since visual pathways primarily carry information from the LGN to layer IV, to layers II and III, and then to layers V and VI it is now certain that this extragranular mechanism, connected by intracortical synapses in the orientation column, is a primary factor in neonatal monocular deprivation, persisting

longer in the normal maturation of visual cortex, and is selectively maintained by dark rearing (Mower et al., 1985; Daw et al., 1992; Trachtenberg et al., 2000).

An intriguing implication of these results is that there is complex retrograde signaling from the extragranular layers back to cells in layer IV, which in turn feed back to geniculocortical afferents to guide relative expansion and contraction of eye specific ocular dominance patches in layer IV. Due to the differing states of cortical plasticity between normal and dark-reared developing animals of the same age, with associated differences in gene expression and physiological activity, dark rearing provides an ideal means to study this critical extragranular mechanism. Genes important to maintaining or preventing cortical plasticity will be differentially expressed in developing animals of the same age raised in the different rearing conditions. Those genes that promote plasticity would likely be expressed more highly in light-reared animals at the peak of the normal critical period and lower in dark-reared animals of the same age. These same genes would have their expression reversed in older animals, aged beyond the normal critical period, in which case the light-reared animals would have lower levels of expression while these genes in the dark-reared animals, with their prolonged critical period would, be more highly expressed. These opposing patterns of expression of these genes would contrast with genes that were simply developmentally regulated based on age which would be similarly expressed without regard to rearing condition.

Genes Regulating Neuroplasticity in Visual Cortex

Differential gene screening techniques (subtractive hybridization, differential display PCR [ddPCR], gene arrays) provide the means to identify genes whose expression is specifically elevated in one population compared to another. Numerous studies have manipulated neuronal activity levels to identify genes which may be important for neuronal plasticity, and this approach has proven fruitful in identifying candidate plasticity genes involved in the response of neurons to electrical (Yamagata et al., 1993) and chemical seizures (Nedivi et al., 1993; Qian et al., 1993) or blockade of neuronal activity in the visual system (Corriveau et al., 1998). These approaches have resulted in a great deal of important information on a number of genes which are responsive to visual input, regulated during the critical period and involved in structural and functional plastic responses (Corriveau et al., 1998; Lanahan and Worley, 1998; Corriveau et al., 1999; Huh et al., 2000). Differential screening techniques have been used to specifically identify genes involved in cat visual system plasticity. Shatz's group used ddPCR to screen for genes whose expression in the cat lateral geniculate nucleus is regulated by spontaneously generated action potentials present prior to vision (Corriveau et al., 1998). Results for one gene have been reported and they implicate a role in synaptic plasticity for a class of molecules classically thought to be involved only in the immune response. Cynader's group used subtractive hybridization and Arckens' group proteomics to identify candidates that are differentially expressed in cat visual cortex at the peak of the critical period versus adult animals (Prasad and Cynader, 1994; Van den

Bergh et al., 2003). While many genes can be identified by this approach they may simply be developmentally regulated and not related to visual cortical plasticity.

The following set of criteria had been proposed by Daw for any substance to be considered as a candidate for involvement in critical period neuroplasticity: 1) the substance should be present in young animals and absent in adults; 2) the substance should follow the critical period (high levels at the peak of the critical period and subsequently fall); 3) the substance should show changes in laminar expression that correspond to known variations with cortical layers and 4) the substance should change in accordance with procedures that change the time course of the critical period (dark rearing) (Daw, 1994).

Identification of Candidate Plasticity Genes

Based on the evidence that light reared cats have the greatest cortical plasticity at the peak of their critical period around 5 weeks and dark-reared animals do not reach the peak of their critical period until 12 weeks, the Mower lab established a stringent criterion to identify potential genes of importance for plasticity in visual cortex. Through the use of ddPCR one can identify expression levels of individual genes in animals of different ages raised in differing conditions. Genes that show different levels of expression indicate potential functional differences in the differing conditions. As it relates to plasticity and development, those genes most important to plasticity will show differences in expression between normal and dark reared cats that are of opposite

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direction in young versus older animals. The gene screening criterion of opposite direction differences, bidirectional gene expression screening, due to age and dark rearing offers a promising alternative to approaches based on seizures or blockade of neuronal activity for identifying genes important for neuronal plasticity. First, it provides a means to dissociate genes associated with the state of synaptic plasticity from genes associated with the general maturation of the animal. Second, it eliminates genes whose expression is tied directly to neuronal activity and focuses on genes whose expression is regulated according to synaptic plasticity. Dark rearing reduces the responsiveness of visual cortical neurons in both young and older cats (Fregnac and Imbert, 1984; Czepita et al., 1994) but does not affect spontaneous activity (Czepita et al., 1994). Therefore, the overall level of neuronal activity (including visual responses as well as spontaneous activity) in light reared animals is higher than in dark reared animals at all ages, inconsistent with the opposite direction criterion. Third, it is based on a physiologically salient manipulation of synaptic activity with effects restricted to the targeted (visual) pathway. Fourth, because the screen detects steady state levels of expression in the age/rearing condition combinations, it is less likely to detect transient genes such as transcription regulators and more likely to target long lasting effector genes.

In a recent series of studies, the Mower lab has utilized this criterion to identify candidate plasticity genes (Yang et al., 2001; Yang et al., 2002; Yang et al., 2006; Yang et al., 2007). Differential display PCR (ddPCR) screening of theoretically 96% (GenHunter Corp.) of the expressed genes in the visual cortex of normal and dark reared cats at 5 and 20 weeks was conducted to directly identify candidate plasticity genes that

show bi-directional gene expression differences. Two patterns of differential expression due to age and dark rearing were uncovered.

One pattern of differential expression is an elevation of gene product in normal cats at 5 weeks and an elevation in dark-reared cats at 20 weeks. This is the difference that the electrophysiology predicts, and it could indicate genes whose expression is required for neuronal plasticity ("plasticity" genes). The other pattern is the opposite: elevation in dark-reared cats at 5 weeks and elevation in normal cats at 20 weeks. This pattern could indicate genes whose expression is suppressed to allow for neuronal plasticity to occur ("anti-plasticity" genes). The finding of these complementary patterns of expression fits with current thinking that a balance between activator and repressor genes controls neural plasticity. The importance of a balance between activator and repressor genes has been documented in a number of systems such as tumorigenesis, axon guidance, synaptic growth and learning and memory (Abel et al., 1998). The visual cortical critical period also likely involves opposing processes with overlapping, but distinct, temporal profiles. One process maximizes adaptive responsiveness to external input while the other promotes crystallization of semi-immutable mature response properties. Identification and characterization of genes showing "plasticity" and "anti-plasticity" profiles is a promising new direction for insight into these interactive developmental mechanisms.

Visual cortical plasticity has historically been attributed to excitatory neuronal activation mechanisms, but recent evidence has indicated that repressor processes also

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play an important role. Chondroitin sulphate proteoglycans (CSPGs), a part of the extracellular matrix, have been implicated as inhibitory for visual cortical experience dependent plasticity. CSPGs organize at a time coincident with the end of the critical period, and more importantly, degradation of CSPGs in the adult restores ocular dominance plasticity (Pizzorusso et al., 2002). γ-Amino Butyric Acid (GABA) inhibition has also been implicated as a plasticity repressor. Mice engineered to overexpress BDNF show advanced maturation of glutamic acid decarboxylase, GAD65, a GABA synthetic enzyme, excessive cortical inhibition and a premature closure of the critical period for monocular deprivation (Huang et al., 1999). These results are consistent with the notion that inhibition acts as a plasticity "gate" whereby once a critical level of inhibition is exceeded, plasticity is lost (Kirkwood and Bear, 1994). Other evidence indicates a more complex role for GABA inhibition (Hensch et al., 1998; Fagiolini and Hensch, 2000; Iwai et al., 2003; Hensch and Fagiolini, 2005). GAD65 knockout mice show reduced plasticity during the rise of the critical period but pharmacological enhancement of GABA inhibition in young GAD65 mutants restores plasticity (Fagiolini and Hensch, 2000; Iwai et al., 2003). Pharmacological enhancement of inhibition in wild type animals prematurely closed the critical period (Hensch and Fagiolini, 2005). These pharmacological results suggest that a threshold of inhibition activates plasticity mechanisms. The combined results of these BNDF and GAD65 gene manipulation results suggest that inhibition plays a role both in activating plasticity mechanisms when they are low and in repressing plasticity mechanisms when they are high. Recent studies on normally raised adult rats deprived of light for 10 days reveal an adult susceptibility to

monocular deprivation that is also driven by significant decreases in inhibition to levels similar to those seen in juvenile animals (He et al., 2006).

This proposal will focus on two candidate plasticity genes Dab-1 and Munc13-3 , which, respectively, show "plasticity" and "anti-plasticity" bidirectional gene expression patterns for age and rearing condition.

Dab-1 and Neuronal Plasticity

Dab-1 has been shown to display the "plasticity" pattern of bidirectional gene expression in normal and dark-reared cats and mice: highly expressed in normally reared cats at five weeks and low in dark reared and highly expressed in dark reared cats at the 20 weeks and low in normally reared animals. (Yang et al., 2006). *Dab-1* is the mammalian homologue of the *disabled-1* gene in drosophila. It forms part of the Reelin/Dab-1 signaling pathway that controls neuronal positioning during brain development. Dab-1 acts downstream of Reelin in the signaling pathway as an intracellular, adaptor protein that is phosphorylated when the Reelin ligand binds to very low density lipoprotein receptor and Apolipoprotein E Receptor-2 (VLDLR and ApoER2) receptors on migrating neurons (Howell et al., 1997a; Rice et al., 2001). Recently research suggests that Dab-1 is important as more than just an adapter protein activating multiple kinases but also as structural component to facilitate downstream reactions in the Reelin pathway during development (Feng and Cooper, 2009).

In mice, *Dab-1* is found in both adult and developing brains. In the mature brain, it is expressed in extragranular cortical pyramidal neurons. In embryonic and post-natal mice, the gene has been shown to play a central role in directing the migration of cortical neurons (Rice et al., 2001). Mutants lacking the Dab-1 gene are viable, and appear behaviorally normal until post-natal day 10 at which time they develop a characteristic ataxia, however, they do survive into adulthood (Howell et al., 1997b). Morphologically these mutants show an abnormal cortical development that produces an inversion of cortical layers, as found in *reeler* mice (a mutation that does not produce Reelin) disrupting neuronal migration and altering lamination patterns in cortex, hippocampus and cerebellum (Howell et al., 1997b; Rice et al., 1998; Pesold et al., 1999; Deguchi et al., 2003).

5-Bromodeoxyuridine (BrdU) labeling of embryonic cells on different days of development demonstrates that neurons typically "born" on earlier dates (those that undergo their final S-phase and maintain a high concentration of the label) are normally found in deep layers of the cortex. In contrast, in the Dab-1 mutants they are found superficially. Cells born on later dates, which typically are found superficially in the cortex, are located in deep layers in the Dab-1 mutant mice. As would be expected with the removal of a downstream component in the Reelin/Dab-1 pathway, the Dab-1 mutation does not impact Reelin production or release and mutant mice do appropriately express Reelin in cortex (Sheldon et al., 1997; Rice et al., 1998). Surprisingly, the retinotopic map, electrophysiological membrane properties, single cell receptive field properties and afferent/efferent connections are largely unaffected in *reeler* mice

(Lemmon and Pearlman, 1981; Simmons et al., 1982; Silva et al., 1991); the same is likely true in Dab-1 mutants.

Several lines of recent functional evidence, in postnatal and adult hippocampus, directly implicate the Reelin/Dab-1 pathway in synaptic plasticity. Interference with Reelin signaling reduces long-term potentiation (LTP) and perfusion of Reelin enhances LTP in the hippocampus (Weeber et al., 2002; Beffert et al., 2005). Developmentally, changes in levels of exogenous Reelin affects normal developmental changes in NMDA receptor currents (Sinagra et al., 2005) and impairs dendritic maturation (Niu et al., 2004). Although it remains to be determined if the intracellular mechanisms of Reelin/ Dab-1 signaling in postnatal brain are similar to or different from those in embryogenesis, the pathway is a promising newly identified player in postnatal and adult synaptic plasticity. Gene screening results from our lab clearly indicate a likely role for Dab-1 in the postnatal critical period of both cat and rodent visual cortical development (Yang et al., 2006).

Munc13s and Neuronal Plasticity

In contrast to the "plasticity" pattern of bidirectional expression shown by Dab-1, recent studies show that Munc13-3 demonstrates the "anti-plasticity" pattern of gene expression in visual cortex (Yang et al., 2002; Yang et al., 2007). Munc13-3 is a member of a family of genes that also includes Munc13-1 and Munc13-2. The Munc13 gene family is the mammalian homologue of the *Caenorhabditis elegans*

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"uncoordinated" gene *unc-13*, which has been shown to express a protein that plays a significant role in exocytosis of synaptic vesicles and neurotransmitter release (Brose et al., 1995; Betz et al., 1997; Augustin et al., 1999a; Basu et al., 2007). The onset of expression of all three isoforms of the Munc13 gene corresponds to synaptogenesis, which includes no embryonic expression but a steady increase from birth through postnatal days 15-22. In mammalian brains, the expression patterns of the three genes are localized to different regions. In both cat and rats, mRNA for the predominant isoform Munc13-1 is expressed throughout the brain, but Munc13-2 and Munc13-3 mRNAs are primarily localized to rostral and caudal brain regions respectively (Brose et al., 1995; Yang et al., 2002), suggesting a primary function of Munc13-1 with complementary functions of Munc13-2 and Munc13-3 in differing brain regions. All three of these Munc13 proteins are found in differing subcellular compartments but they all are specifically targeted to presynaptic active zones and have all been shown to play an essential role in vesicle release at glutamatergic and GABAergic synapses (Brose et al., 1995; Betz et al., 1997; Augustin et al., 1999b). Munc13-3 mutants develop grossly normal brain anatomy but behaviorally express difficulty in learning motor tasks attributed to physiological impairments in synaptic transmission (Betz et al., 1998). Numerous studies indicate that Munc13-3 likely plays a role in short-term synaptic plasticity (Zucker, 1989; Augustin et al., 2001; Junge et al., 2004; Zikich et al., 2008; Shin et al., 2010).

Current published research indicates that Munc13-3 is normally an important up-regulator of neurotransmitter release, decreasing paired-pulse facilitation, at synapses supporting its potential role as a candidate gene impacting overall neuronal plasticity.

NMDA receptor

N-methyl-D-aspartate (NMDA) receptors are ionotropic glutamate receptors that likely play a role in neuronal plasticity of the visual cortex (Nakanishi, 1994; Sawtell et al., 2003; Philpot et al., 2007). Due to their relationship and subcellular colocalization with Dab-1 in the post-synaptic density, they are logical targets for study in Dab-1 mutant mice (Beffert et al., 2005; Chen et al., 2005).

NMDA receptors are multimeric proteins composed of NR1 and NR2 subunits. To date, seven subunits, NR1, NR2A-D and NR3A-B have been identified (Perez-Otano et al., 2001). NR1 is the mandatory subunit for all functional receptors and is necessary for the major characteristic properties. NR2A-2D contribute functional variability to the receptor (Nakanishi, 1992, 1994) and NR3A-B further that variability ((Perez-Otano et al., 2001; Rambhadran et al., 2010). NR1, NR2A and NR2B are the dominant subunits found in cerebral cortex (Monyer et al., 1994; Sheng et al., 1994).

In visual cortex, NMDA receptor activation has been implicated as critical for anatomical and physiological plasticity during early postnatal life. Inhibition of these receptors significantly reduces the shift in ocular dominance caused by monocular

deprivation in the critical period of visual cortex development (Bear et al., 1990; Roberts et al., 1998; Daw et al., 1999). Subunit composition of the NMDA receptor has been shown to change throughout development providing a mechanism for functional differences of the receptor in distinct anatomical regions at different periods of development (Perez-Otano et al., 2001; Chen et al., 2005; Chen and Bear, 2007). At birth NR2B and NR1 are the predominant subunits in rats and cats. NR2A increases in expression over the first 5 postnatal weeks. NMDA receptor subunit composition in visual cortex is bidirectionally regulated by visual experience and dark rearing reduces this increase in NR2A.(Quinlan et al., 1999; Chen et al., 2000) There are no significant effects of dark rearing on the levels of NR2B and NR1 (Chen et al., 2000). A recent study in mouse visual cortex found that translation of NR2B and the ratio of NR2A:NR2B expression is likely a major point of regulation. This implicates the differential translational regulation of NR2A and NR2B as a potential key contributor to experience-dependent modification in visual cortex plasticity.(Chen and Bear, 2007)

NMDA receptor activity has been shown to be enhanced by Reelin in wild-type cortical neurons but not in Dab-1 deficient cells (Chen et al., 2005). Recent research has shown that blocking exogenous Reelin can reversibly decrease the NR2A:NR2B ratio in hippocampal cultured neurons (Campo et al., 2009) further solidifying the justification for additional research into the relationship between Dab-1 and these two proteins.

Plasticity in Mouse vs. Cat Visual Cortex

The studies described here utilized both cat and mouse models; the bidirectional gene expression screening approach was extended to mice since mutant gene knockouts are possible.

Cat and primates have historically been studied as the definitive models for determining the chemical, anatomical and molecular markers that play a role in visual cortical plasticity. Studies in cats, where specific laminar changes are well documented (Shatz and Stryker, 1978; LeVay et al., 1980; Mower et al., 1985; Daw et al., 1992; Antonini and Stryker, 1993), and in rodents, show a number of parallels between the visual systems. The most prominent difference between them is that although rodents do have cortical regions, visible with optical imaging by transneuronal tracing, that are innervated by monocular or binocular afferents, they do not have highly organized ocular dominance columns as in higher mammals (Antonini et al., 1999). A primary factor in this differential cortical organization is that while LGN afferents in cats and primates terminate almost entirely in Layer IV, in rodents, these thalamocortical projections are not as localized, terminating not only in layer IV, but also in supragranular layers II and III and infragranular layers V and VI (Gordon and Stryker, 1996). The majority of the literature review in this dissertation comes from studies conducted in cats and primates implicating the importance of laminar differences in cortical plasticity. Since ocular dominance columns are not present in layer IV of rodents, but are well documented in cats, cats will be predominantly used for the histological analysis of Dab-1 expression.

Physiological and anatomical analyses of the critical period of the mouse visual corticex indicate other similarities and differences to cats (Gordon and Stryker, 1996; Antonini et al., 1999). Differences in the effects of monocular deprivation seen in rodents as compared to cats include the size and retinal location of the binocular visual field, quantitative differences in the magnitude of the physiological effect of monocular deprivation, and dramatic differences in the effects of monocular deprivation between hemispheres (Gordon and Stryker, 1996). In similarity with the cat, the mouse shows a measurable effect of monocular deprivation, a critical period for such effects, a prolongation of the critical period by dark rearing (Gordon and Stryker, 1996) and effects of monocular deprivation on terminal arbors of geniculocortical axons representing the open and closed eye (Antonini et al., 1999).

The normal critical period of cat visual cortex begins around 3 weeks, peaks around 5 weeks of age, diminishes and diminishes to low levels by week 20 (Daw et al., 1992) and ends around 30 weeks (Daw, 1994). Rodent visual cortical critical periods are shorter than those found in other mammals, with the classical definition of the mouse critical period beginning at eye opening, peaking around 3.5 weeks, diminishing thereafter and ending around 9.5 weeks (Guire et al., 1999; Fagiolini et al., 2003; Sawtell et al., 2003). Greater understanding of this time frame is being gained through finding that the balance of cortical excitatory/inhibitory signals and their rate of responsiveness to changes in visual experience (Gandhi et al., 2008) plays a significant role in its determination. New research indicates that molecular alterations can be made to

significantly extend or even "reopen" the critical period in adult animals through the introduction of inhibitory neurons to developed cortex (Southwell et al., 2010).

Benefits of Mice for Mechanistic and Mutation Studies

Although higher mammals have been the basis for the majority of the mechanistic studies of cortical development, murine models are becoming more popular for understanding the mechanisms responsible for activity dependent plasticity because they have been found to share many of the same mechanisms that are thought to guide this development. Beyond these similarities, however, mouse models have the distinct advantage of genetic manipulation which makes them more useful as a species to study the molecular and cellular mechanisms that contribute to cortical plasticity. Viable transgenic mice with specific exclusions ("knockout") or inclusions ("knockin") of genes thought to play roles in any system allow for detailed examination of those genes' contributions to the system. Several studies in this proposal will utilize Dab-1 knockout mice, in different rearing conditions, to specifically examine potential relationships between Dab-1 and other candidate genes identified through gene screenings and their roles in plasticity of the visual cortex.

Purposes of this Study

This project studied the potential role in critical period neuronal plasticity of visual cortex of two genes, Dab-1 and Munc13-3, which had been previously identified

as candidate plasticity genes (CPGs) through RNA analysis by our laboratory. This research was conducted in cats and mice to determine whether the results of the RNA screening can be extended to Dab-1 and Munc13-3 protein expression levels in the critical period, whether Dab-1 laminar expression in visual cortex correlates with known laminar differences in physiological plasticity during the visual cortical critical period, whether the laminar expression levels of Dab-1 are dependent upon visual input, and whether or not mutations in Dab-1 alter expression of other CPGs. [A secondary objective was to develop a reliable laboratory set-up and protocol to record mouse visual evoked potentials for future testing of physiological effects of Munc13-3 and Dab-1 mutations through the established plasticity model of monocular deprivation.]

To accomplish these objectives, four distinct aims were developed:

1. An important criterion for a molecule to be considered a candidate for critical period plasticity is that its expression should be changed by a manipulation that can alter the time course of the critical period, such as dark rearing. This predicts that if a molecule is critically involved in visual cortical plasticity there should be opposite directions of difference in the level of expression between normal and dark reared cats at 5 versus 20 weeks of age (peak and nadir of the critical period). Previous research by the Mower lab has identified numerous genes through ddPCR screening that were likely candidates to play a role in visual cortical plasticity. Published RNA analysis, by Northern blot, confirmed Dab-1 and Munc13-3 as two likely genes. This study expanded that analysis to

determine whether or not the RNA results could be extended to protein expression during the critical period in both cats and mice. To conduct this analysis protein from visual cortex of normal and dark-reared mice at 3.5 and 9.5 weeks of age were analyzed by quantification of Western blots.

2. Monocular deprivation studies have demonstrated that the critical period is different among lamina of cat visual cortex. Plasticity in layer IV ends earlier than the superficial (I, II and III) or deeper (V and VI) layers. If genes are involved in plasticity of the critical period the level of expression of the gene would likely be regulated during development in a manner that correlates with the known developmental laminar differences over the time course of the critical period. Dab-1 laminar protein expression was studied through immunohistochemical staining of cat visual cortex throughout development of the critical period (1wk, 5 wks, 10 wks, 20 wks and adult).

3. Numerous genes have been identified through the bidirectional paradigm as potentially playing a role in the critical period of visual cortex development. It is plausible that there exists a relationship between Dab-1 expression and the function or expression of other candidate plasticity genes. Through the use of normal and dark reared Dab-1 mutant mice (homozygous recessive mutants) at 3.5 and 9.5 weeks it was determined that other candidate plasticity gene proteins were impacted by the absence of Dab-1 during the critical period. Specifically, the proteins studied included NMDA receptor subunits that have been identified as

likely to play a role in visual cortical plasticity (NR2A and NR2B).

4. There is a well-documented physiological response to monocular deprivation during the critical period of mouse visual cortex in which binocular cells shift their response from primarily being driven by stimulation to the contralateral eye to a stronger ipsilateral response following suture of the contralateral eye. This project included establishment of a reliable laboratory set-up and experimental protocol to temporarily deprive mice of monocular visual experience and record visual evoked potentials (VEPs) in the Mower lab. Establishment of this protocol will permit future physiological experiments testing whether mice that are knockouts for Dab-1 and Munc13-3 show VEP responses to monocular deprivation that differ from normal.

MATERIALS AND METHODS

Animal Breeding and Rearing Conditions

Cats

Cats were purchased from Liberty Laboratory (New Jersey) and Snowhill

Breeding Kennels (West Harrison, IN). They were raised in the Research Resource

Center, University of Louisville. The procedures conform to the guidelines of the

National Institutes of Health and were approved by the Institutional Animal Care and Use

Committee. Our lab and this researcher have an extensive history of dark rearing animals

and records indicated no difference from the lighted colony in terms of survival rate and

body size.

Normal cats were raised in a 12-h light/dark cycle until 1, 5, 10, 20 weeks or adult

age. Dark reared cats were reared in complete darkness from birth and were sacrificed at

5 and 20 weeks without exposure to light.

Cats were sacrificed by an overdose of sodium pentobarbital (75mg/kg i.p. or

50mg/kg i.v.). For western and northern/slot blot studies, fresh brain tissue (all of Area 17

and possibly a small part of 18, frontal cortex and cerebellae) were dissected, immediately frozen in liquid nitrogen, and stored at -80°C until use. For immunohistochemistry studies, perfusion was performed intracardially through the left ventricle with 37°C PBS (0.91 NaCl in 0.1 phosphate buffer, pH=7.4) until fluid from right atrium ran clear indicating complete flushing of blood from systemic circuit. This was immediately followed by perfusion of 4% paraformaldehyde in PBS at 4°C for approximately 20 minutes. Brains were removed and blocked into cerebral hemispheres divided into anterior and posterior regions, cerebellar hemispheres and brainstem structures. The tissues were post-fixed in 4% paraformaldehyde in PBS overnight at 4°C. Finally, the tissues were then cryoprotected by immersion in 30% sucrose in PBS at 4°C until they sank. The blocked sections were frozen in crushed dry ice and stored at -80°C until use.

Mice

Mutant mice that have been genetically engineered as knockouts for Dab-1 were obtained from Jackson Labs (CBy.129S4-*Dab1^{tm1Cpr}*/J). They were raised in the Research Resource Center (RRC), University of Louisville. The procedures conform to the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee. Homozygous recessive mice for the Dab-1 knockout demonstrate a characteristic ataxia noticeable by postnatal day 14. Mice heterozygous for the knockout gene, as determined by multiplex PCR genotyping of the Dab-1, were identified and cross-bred to establish a breeding colony. Heterozygous breeding pairs

were kept in the barrier facility in the RRC barrier facility as back-up animals in the unlikely event that due to unforeseen circumstances the colony in the RRC needed to be depopulated. Every pup was ear-tagged and genotyped in order to maintain the most genetically diverse population possible.

Mice with a spontaneous mutation in Dab-1, "scrambler" (Jackson Labs, *A/A-Dab1^{scm}/J*) were also ordered as a supplemental colony to the knockout mice. While no genotyping protocol is available from Jackson Labs to determine wild-type homozygotes from heterozygotes, the homozygous recessive animals demonstrate the same characteristic ataxia and other known phenotypes of the knockout mice. It was the experience of this researcher that homozygous recessive scrambler pups have a much higher survival rate that those of the Dab-1 knockout, especially when dark reared.

Experimental mice were kept in 12-hour light/dark cycles or dark reared from birth. Dark-reared animals were housed from birth in standard mouse cages within a light tight enclosure. The cages were changed three times per week when the animals were fed. During cleaning and feeding, the investigator wore infrared amplifying goggles with an attached infrared light source filtered by a double-layer Wratten filter to reduce potential extraneous light. This allowed for visual inspection of the dark-reared animals on a regular basis without exposing them to visible light. The standard dark rearing periods for the mice in these experiments were from birth until 3.5 and 9.5 weeks.

While Dab-1 knockouts raised in normal 12-hour light/dark cycles had high survival rates, several months of dark-rearing of litters with Dab-1 knockout homozygous animals failed to produce survival of any mutants for more than two-weeks post-weaning (approximately 6-7 weeks of age). Their wild-type litter-mates survived normally. Subsequent to the onset of this rearing protocol, research was published indicating abnormal development of the rostral migratory stream and severe neuroanatomical defects in the olfactory bulb of Dab-1 mutant mice (Andrade, et.al. 2007). These olfactory deficits of the knockout mutants, combined with the ataxic phenotype, impair the mutants' abilities to smell and access food and water that they cannot see suspended above them in the standard mouse cage. Once the mothers had been removed post-weaning the homozygous mutants were apparently unable to find food or water in the cage. This situation did not occur in the light-reared cages. It is believed that the normally reared animals could see other mice consuming the food/water and thereby learn the behavior and survive. The post-weaning loss of mutant pups in dark-reared cages was somewhat alleviated through the introduction of "Napa Nectar" pouches (a gel-based water source) and food placed on the floor of the cage. Even with the added precautions, 9.5 week survival rates of dark-reared homozygous knockout pups were extremely low. The survival rate of dark-reared scrambler pups, a spontaneous Dab-1 mutant with identical phenotypes to Dab-1 knockout mutants, when given the same precautions, was markedly higher. The precise reasons for this are unknown but could be attributed to a hardier background mouse strain (agouti vs. white).

For western blot analysis, mice were sacrificed by an overdose of sodium pentobarbital prior to tissue dissection (75 mg/kg, i.p.). The animals were then decapitated and visual cortex (monocular and binocular regions), frontal cortex and cerebellum were immediately dissected, weighed and frozen in liquid nitrogen. The tissue was stored at -80°C until used for protein analysis.

Figure 3 demonstrates the region of the mouse brain representing both the monocular and binocular regions of mouse primary visual cortex. It is important to emphasize that both regions are included in this dissection meaning that any findings of altered gene expression in these experiments could be conservative underestimates of the effects in the binocular zone.

Tissue for immunohistochemistry was obtained from mice injected with an overdose of sodium pentobarbital (75 mg/kg, i.p.) and perfused intracardially through the left ventricle with 37°C PBS (0.91 NaCl in 0.1 phosphate buffer, pH=7.4) until fluid from right atrium ran clear indicating complete flushing of blood from systemic circuit. This was immediately followed by perfusion of 4% paraformaldehyde in PBS at 4°C for approximately 20 minutes. Brains were removed and blocked into cerebral hemispheres divided into anterior and posterior regions, cerebellar hemispheres and brainstem structures. The tissues were post-fixed in 4% paraformaldehyde in PBS overnight at 4°C. Finally, the tissues were then cryoprotected by immersion in 30% sucrose in PBS at 4°C until they sank. The blocked sections were frozen in crushed dry ice and stored at -80°C until use.

Figure 3. A. The primary visual cortex of the mouse, schematic diagram of the central visual pathway in the mouse. Retinal ganglion cell axons project to eye-specific regions of the LGN. Geniculocortical projections carrying information from the two eyes converge in the lateral one third of the primary visual cortex (V1), in the binocular zone. B. Image from Mouse Atlas of Gene Expression showing VI of mouse visual cortex that was removed for protein analysis.

A

Binocular Zone

V1

LGN

Retina

30°
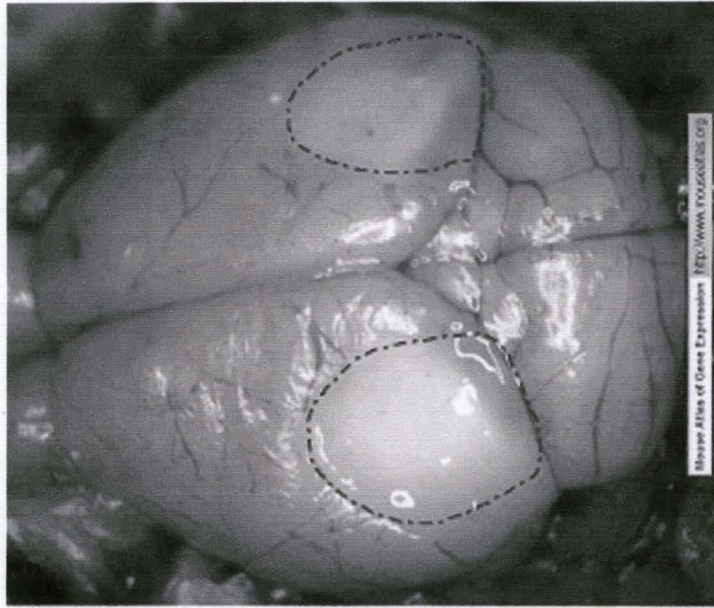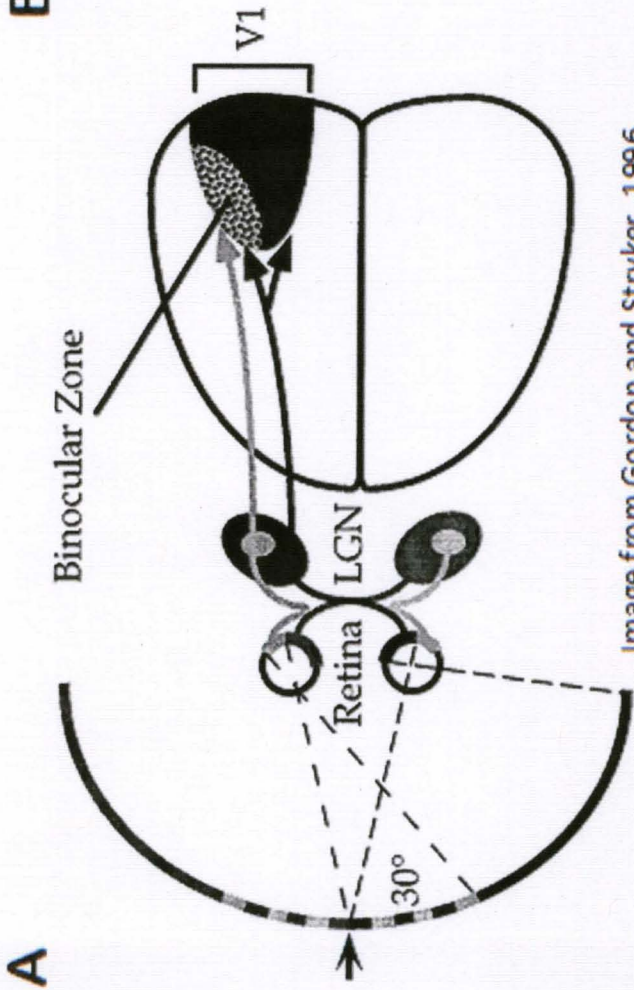
Image from Gordon and Stryker, 1996

B

Mouse Atlas of Gene Expression http://www.mouseatlas.org

Figure 3

The tissue was mounted with OCT and sectioned coronally (30 μm) on a cryostat (Cryocut 1800, Reichert-Jung, Austria). Sections were collected and stored in PBS (4°C) until processed. For each section processed for immunohistochemistry, an adjacent section was stained with cresyl violet (details below).

Western Blot Procedures

Sample Preparation

Homogenates of visual cortex were prepared on ice in the presence of a standard cocktail of homogenization buffer with protease inhibitor (PI) (aprotinin, 0.1 mg/ml; leupeptin, 0.1 mg/ml; antipain, 0.1 mg/ml pepstain, 0.01 mg/ml in Tris-HCl buffer [40 mM, pH 7.4] containing 1 mM EDTA-Na_2 and 0.2 mM PMSF). For mice, visual cortex from both hemispheres of a single animal were combined (approx. 12-17μg) for protein extraction. Crude synaptosomal fractions were obtained by the addition of 250μl of homogenization buffer with PI to each sample. The samples were homogenized with a 4.5mm Tissue Tearor™ rotor/stator tissue homogenizer. [50μl of homogenization buffer were then added to bring sample to 300μl.] Samples were centrifuged at 3000 RPM (900G) at 4°C for 10 min. The supernatant (S1) was removed and collected in a separate tube. An additional 250μl of homogenization buffer with PI was added to resuspend the pellet (P1) and was again centrifuged for 10 min at 3000RPM (900G) at 4°C. The supernatant (S1') was removed and combined with S1. The combined supernatants were centrifuged at 9900RPM (10000G) at 4°C for 20 min. The supernatant (S2) was removed

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(cytoskeleton, microsomes and soluble proteins) and stored separately. The remaining pellet was dissolved in 100μl of homogenization buffer with PI. This solution (crude synaptosome fraction) was divided into equal parts, quantified as described below, and stored at -80°C until used.

Total Protein Quantification

Protein quantification was performed by Bradford assay in a BioRad SmartSpec™ Plus spectrophotometer. 2.5μl of each sample was placed in a tube and brought to 50μl with 47.5μl of double-distilled H$_2$O (ddH$_2$O). 950μl of BioRad Protein Assay (diluted 1:5 with ddH$_2$O) was added for quantification. Protein standards using bovine serum albumin (BSA) were freshly made; a new standard curve was developed for each protein quantification. The standard curve was created with appropriately diluted aliquots of BSA concentrations ranging from 0 – 200μg/ml.

Protein Separation

Protein separation was performed by gel electrophoresis in a MiniPROTEAN II Cell apparatus (BioRad, Hercules, CA). Equal amounts of protein (20μg protein/sample) were denatured via incubation at 95°C with 2X Laemmli buffer (BioRad) for 4 min and cooled to room temperature before loading. Samples were loaded into individual wells of a 4-15% Tris-HCl polyacrylamide SDS gel (BioRad Ready Gel 161-1122), separated by electrophoresis (104V for approximately 1.5 hours). The protein was then

electrophoretically transferred to a nitrocellulose membrane using a Transblot SD semi-dry transfer apparatus (Biorad) at 25V for approximately 50 minutes. After transfer, the membrane was stained with Ponceau Stain in 1% acetic acid to assure transfer then destained by washing three times in TBS-T.

Protein Staining

Western Blot protein analysis protocol was performed as follows:

1. Nonspecific binding sites were blocked with 5% non-fat milk and 0.5% Tween 20 in TBS for 1 hour.

2. Rinse in TBS-T for five minutes

3. Incubation with the primary antibody in TBS-T at room temperature for 2 hours (Control blots were identically processed with the primary antibodies replaced by antibody diluents)

4. Wash in TBS-T for five minutes X 3.

5. Incubation with appropriate biotinylated secondary antibody in 5% milk in TBS-T.

6. Wash in TBS-T for five minutes X 3.

7. Protein visualization via ECL solution exposure (Amersham, Arlington, IL) for one minute.

8. Film exposure (0.5 to 3 minutes) to Hyblot CL autoradiography film

9. Film was developed in a Kodak X-OMAT 2000A automatic film processor.

10. Filters were then stripped with Restore™ western blot stripping buffer

(Thermo Scientific) for 15 min at 37ºC, rinsed completely with TBS-T (5 min

X 3) and reprobed with an antibody to β-actin to control for loading errors.

Quantification of Protein Expression Levels

The exposed films were quantified by computerized densitometric analysis

(Alpha Innotech). Protein expression levels for each individual antibody were calculated

as an average pixel density. Values were corrected by measured values of β-actin for each

sample and normalized to the mean of all four samples for each blot. The means of

animals in each age group/rearing condition from three independent samples were used to

express the relative expression levels.

Antibody Specificities

For Western blot analysis of Dab-1, commercial rabbit polyclonal antibody (Santa

Cruz) labeled a single band of appropriate size (approx. 80 kDa) (Figure 4A). For

immunohistochemical analysis of Dab-1, rabbit polyclonal antibody (Rockland

Immunochemicals, Inc.) was recommended by the laboratory of Dr. Jonathan Cooper.

Western blot analysis of this antibody labeled the appropriate band (approx. 80kDa,

Figure 4B). Immunohistochemical staining of the Rockland antibody in wild-type,

mutant, and negative controls (no primary antibody), compared to *in situ* Dab-1 mRNA

hybridization in normal 3.5 week mouse brain revealed appropriate staining levels and

similar results between wild-type immunohistochemical and *in situ* staining patterns further confirming specificity of the antibody (Figures 4C and 5). The Munc13–3 antibody, provided by Drs. Nils Brose and Frederique Varoqueaux, labeled an appropriate band at 206kDa (Figure 6A).Rabbit polyclonal antibodies specific to NMDA receptor subunits NR2A and NR2B were obtained commercially from Millipore, Inc. Each antibody labeled a single band of appropriate molecular weight (NR2A: 180kDa and NR2B: 180kDa) (Figure 6B-C).

Figure 4. Antibody specificity testing for the two different Dab-1 antibodies used for this project. A. The Santa Cruz antibody was utilized for the Western blot protein analysis and labeled an appropriately sized 80kDa band. B. The Rockland antibody was recommended by the laboratory of Dr. Jonathan Cooper for immunohistochemistry. It also labeled an appropriate band of approximately 80kDa in Western blot analysis. C. Comparisons of immunohistochemical tests of Rockland Dab-1 antibody to confirm its usage for analysis. Sections from four week old mice were stained for comparison including, from left to right, wild-type, Dab-1 knockout, no primary antibody (negative control) and an *in situ* stained section showing Dab-1 mRNA expression (unpublished results, Mower lab).
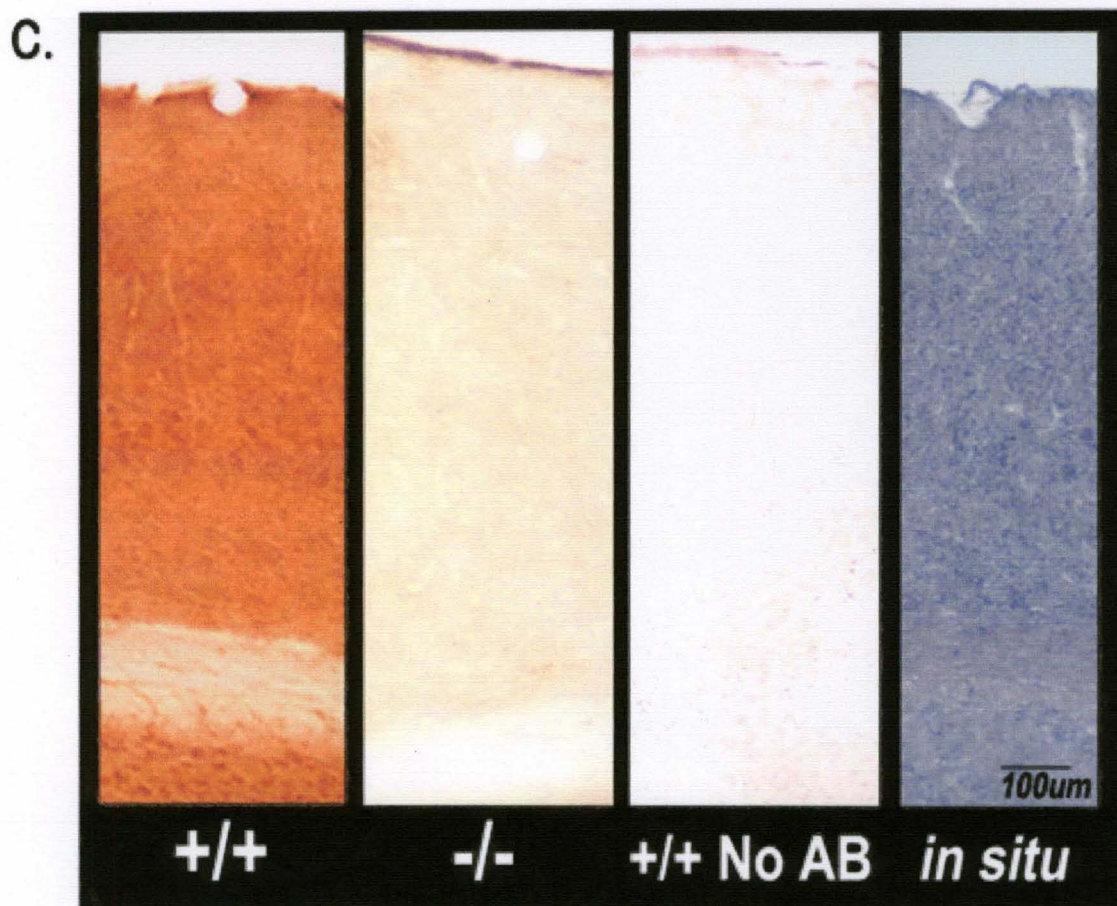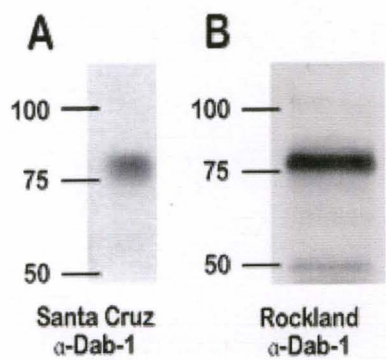
Figure 4

Figure 5. Further specificity testing of Rockland antibody through comparisons of staining patterns of immunohistochemistry of Rockland Dab-1 antibody and *in situ* labelling of Dab-1 mRNA in full sections of four week old wild-type mouse brain. Note similar staining in hippocampus both sections confirming antibody specificity.

Normal 28 day Mouse

Dab-1 Immunohistochemistry

Dab-1 *in-situ* hybridization

Figure 5

53
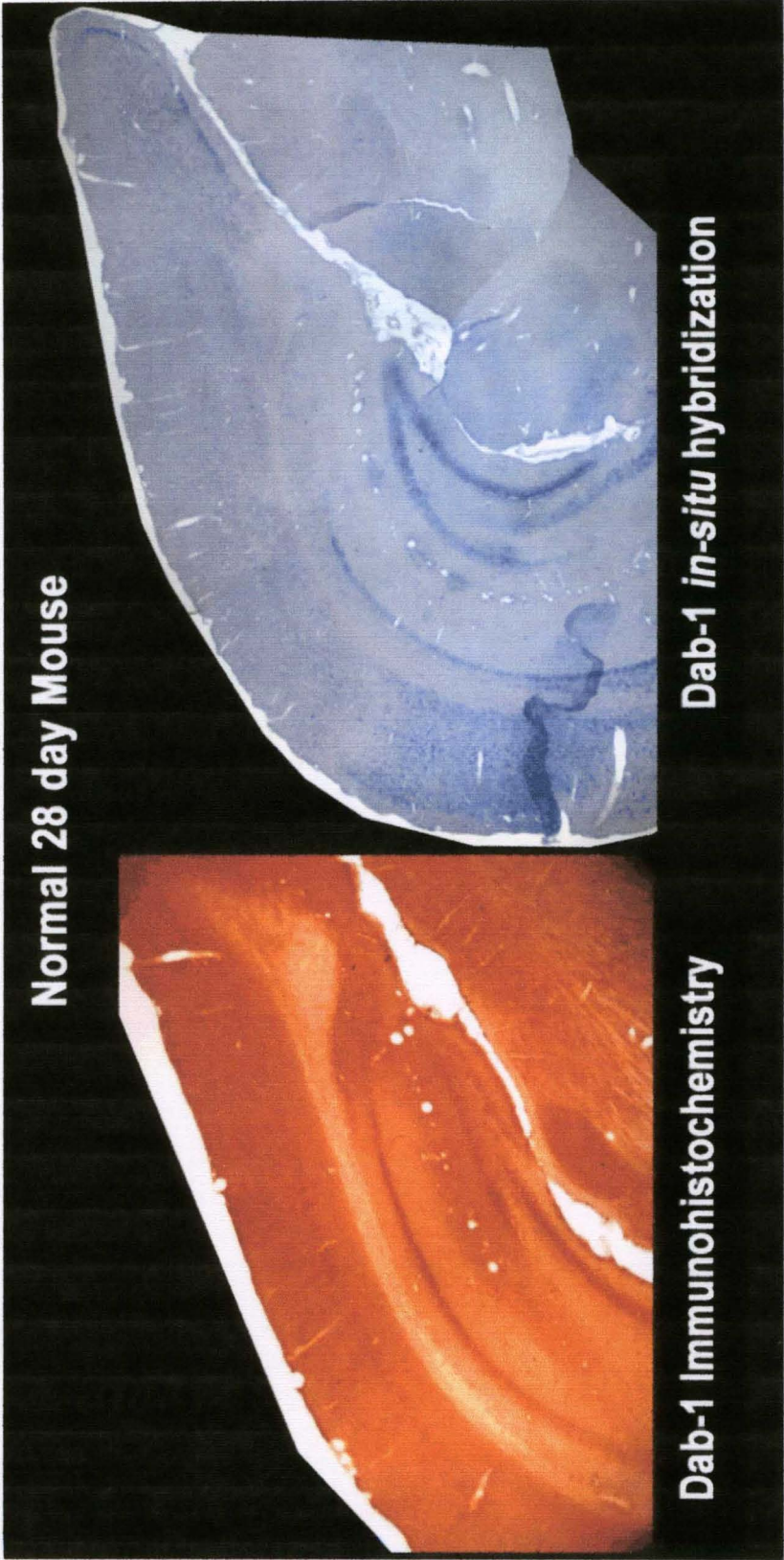
Figure 6. Western blotting showed the specificity of antibodies used for protein analysis in this dissertation. A. The antibody for Munc13–3 labeled an appropriate band at 206kDa Antibodies for NMDA receptor subunits NR2A (B) and NR2B (C) were obtained commercially from Millipore, Inc. Each antibody labeled a single band of appropriate molecular weight (approximately 180kDa).

A. α-Munc13-3 **B.** α-NR2A **C.** α-NR2B
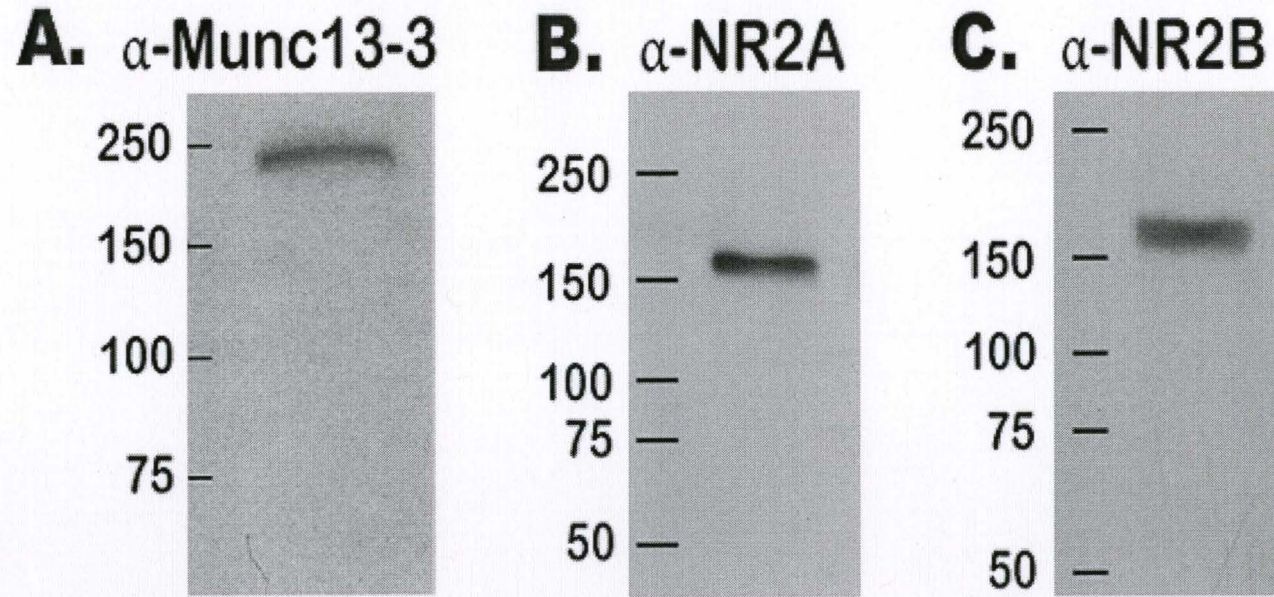
Figure 6

Immunohistochemistry Procedures

Dab-1 Antibody

Rabbit polyclonal anti-Dab-1 from Rockland Immunochemicals, Inc. (Gilbertsville, PA) was recommended by the laboratory of Dr. Jonathan Cooper for immunohistochemistry. Western blot analysis of this antibody labeled the appropriate band at 80kDa. Biotinylated secondary antibody was included in a Rabbit IgG ABC Kit from Vector Laboratories (Burlingame, California) for the avidin-biotin immunoperoxidase reaction staining. Comparisons of immune-staining in wild-type tissue, Dab-1 knockout mutant tissue, and wild-type tissue incubated without primary antibody showed immunopositive labeling solely in the wild-type tissue exposed to the Dab-1 antibody. Adjacent sections of each sample were stained at different concentrations of primary antibody (1:4000 and 1:8000) to verify staining patterns within individual sample runs.

Staining Procedure: Immunohistochemistry

Free floating 30μm sections were used for this study and all steps were performed on a shaker table at room temperature except for the overnight incubation in primary antibody.

The immunohistochemical protocol was performed as follows:

1. Wash/accomodate PBS 15 min X 3.

2. Blocking with 10% serum (eg. normal goat serum, NGS) in PBS with 0.1% Triton X-100 60 min (animal serum used depends on source of secondary antibody).

3. Primary antibody incubation (in PBS with 1% NGS and 0.1% Triton X-100 at 4°C) overnight.

4. 4% NGS in PBS 15 min X 3.

5. Biotinylated secondary antibody incubation (1:200 per Vector ABC Kit) 45 min.

6. Wash with PBS 15 min X 3.

7. ABC Reagent incubation (per Vector ABC Kit) 1 hr.

8. Wash with PBS 15 min X 3.

9. DAB reaction (0.05% diaminobenzedine-4 HCl with 0.01% H_2O_2 in 0.17M Tris-HCl) per DAB kit (Vector Laboratories, Burlingame, CA) for approximately 3-5 min or until clearly visible brown reaction product appears.

10. Wash with PBS, 15 min X 3.

The sections were mounted onto subbed Superfrost/Plus slides and air-dried overnight. The sections were then dehydrated through a sequential series alcohols for 3 minutes each, (50%, 70%, 95%, 100%), cleared in xylene (3 min X 2), and coverslipped with Permount (Fisher Scientific).

Staining Procedure: Cresyl Violet

The adjacent sections were stained with cresyl violet for laminar analysis and cellular quantification as follows:

1. Sections were mounted onto subbed Superfrost/Plus microscope slides and, air-dried overnight.

2. Sections were cleared in xylene for 3 min. and were then rehydrated through a sequential alcohol series for 3 min. each (100%, 95%, 70%)

3. Sections were rinsed with ddH$_2$O for 3 min

4. Sections were them immersed in warm Cresyl violet (1g Cresyl fast violet dissolved in 1000 ml ddH$_2$O with 10 ml 10% acetic acid, warmed to 45°C) for 30 sec.

5. Slides were briefly rinsed in ddH$_2$O.

6. Differentiation in 70% alcohol for 1 min (with a few drops of glacial acetic acid)

7. The sections were dehydrated through a sequential series alcohols for 3 minutes each (50%, 70%, 95%, 100%), and cleared in xylene (3 min X 2).

8. Sections were coverslipped with Permount (Fisher Scientific).

Staining Controls

Several control procedures were used. The positive controls were 1) an appropriate size single band on western blots and 2) staining seen in regions known to express the target protein (eg. hippocampus). Negative controls include staining of sections that are incubated as described above, except that the antibody diluent with no primary antibody is used to replace the primary antibody in step 3. This procedural control eliminates all specific staining. A second negative control, which also produced no specific staining was performed on sections of visual cortex from Dab-1 knockout mice that do not express the Dab-1 protein.

Immunohistochemistry Quantification and Analysis

The slides were photographed using a computerized system (SPOT software, v2.2, SPOT Diagnostic Instruments, Inc.) to obtain both high (400X) and low (40X) magnification images of the visual cortex. Qualitative analysis was based on visual observation of potential laminar staining differences within a given same and between samples of different ages and rearing conditions. Quantitative analysis was conducted through blind manual cell counts in different lamina using AlphaView software (version 2.0.1.1, Alpha Innotech Corporation) as described below.

In order to obtain unbiased results, digital images of immunostained sections and their adjacent cresyl violet stained sections were formatted for software analysis

(same file size and creation date) by this researcher and placed into folders on the computer desktop. A single copy of the coding key identifying each sample section was printed and, in the absence of the researcher, the project research advisor renamed each file with a random file name (numbers for immunolabeled and letters for cresyl violet sections) and recorded each on the code sheet. This sheet was kept secured in the advisor's office until quantification was complete.

Each unknown picture file was opened into the counting software. Two identical size counting area boxes were created for each layer examined (II/III, IV and V/VI) which were maintained across all samples analyzed. The boxes were placed within representative regions of the layers to be analyzed at a lower magnification to maintain accurate positioning within each layer. Once all boxes were positioned within an individual sample, magnification was increased in each box to accurately identify immunopositive cells. A cell was counted if it showed the avidin-biotin reaction product, had a definable soma and an evident nucleus. Cells intersecting the left and bottom margins of the counting box were not counted but those intersecting the top and right margins were included. Through the use of the software, a manual mouse-click on each cell marked it (with a green "X") as being counted, preventing it from being double-counted, and it was added to the running count maintained by the computer. Upon completion, the individual box cell count was recorded and reset each time for each box counted. Only after all samples had been counted and recorded were the codes revealed identifying which cell counts related to which ages and rearing conditions for data analysis. Immunohistochemical cell counts for both

boxes per layer were averaged and corrected against an average of counts of the two boxes from the same layer of the adjacent cresyl violet section giving an average cell density ratio (immunopositive cells:cresyl violet cells) for each layer in each section.

Electrophysiological Surgical and Recording Procedures

A protocol for recording of visual evoked potentials was received from the laboratory of Mark F. Bear, PhD (MIT, Cambridge, MA) but numerous specifications needed to be adapted for successful recording in our lab. The development of this protocol is included here as a part of the total research for this project. Specific issues requiring adaptations included: proper anesthesia determinations for surgical survival, modifications to the design of both the surgical and recording apparati, manufacture of apparati, surgical techniques for electrode implantation and monocular deprivation, visual stimulus and trigger pulse modifications/synchronization and circuitry set-up for proper recording of VEPs. All procedures in the test animals conform to the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee.

RESULTS

Western Blot Analysis of Dab-1 and Munc13-3 Protein Expression

Bidirectional Regulation of Dab-1 Protein in Mouse Visual Cortex

Western blots in mouse visual cortex were conducted to extend previous Dab-1 mRNA results to protein. Dab-1 was expressed at higher levels in visual and frontal cortex than cerebellum of normal 9.5-week-old mice (**Figure 7**). Dab-1 protein showed bidirectional regulation by age and dark rearing in the visual cortex of mice. When corrected against β-actin, densitometric analysis indicated Dab-1 was elevated 2.1-fold in normal visual cortex at 3.5 weeks and 2.3-fold in dark-reared visual cortex at 9.5 weeks (Figure 8). Analysis of variance of protein levels showed a significant interaction between variables (F[2,8], P < 0.002), indicating that the effect of dark rearing depended on age. In normal development, Dab-1 expression declined three-fold between the peak of the critical period (3.5 weeks) and its nadir (9.5 weeks).

To investigate the significance of Dab-1 for visual cortical plasticity we examined whether bidirectional regulation of its expression by age and dark rearing was specific to visual cortex or might also be seen in other cortical regions such as frontal cortex that

Figure 7. TOP: Western blot showing comparisons of Dab-1 protein expression in visual

cortex (V), cerebellum (C) and frontal cortex (F) of normal 9.5 week old mouse.

BOTTOM: Same blot reprobed with β-actin. Molecular weights shown in kDa.
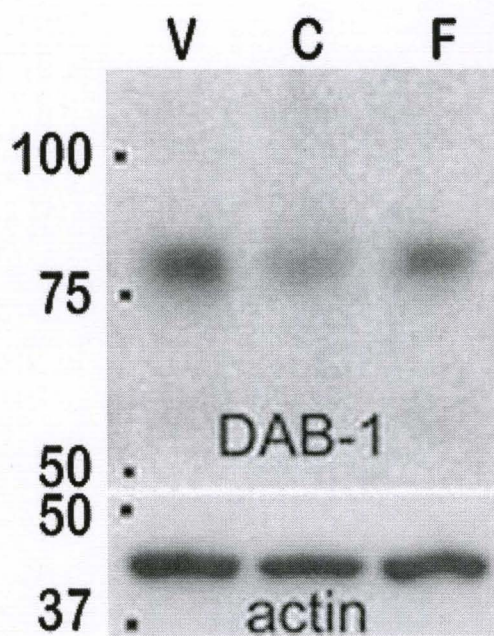
Figure 7

Figure 8. A. TOP: Western blot analysis of levels of Dab-1 protein expression in mouse visual cortex showing higher levels in normal (N3.5) than dark-reared (D3.5) mouse visual cortex at 3.5 weeks and higher levels in dark-reared (D9.5) than normal (N9.5) mouse visual cortex at 9.5 weeks. BOTTOM: The same blot stripped and reprobed with β-actin to control for loading errors. Size standards are indicated on Western blots (in kDa). B. Densitometric results for Dab-1 protein levels in mouse visual cortex. Mean levels of Dab-1 protein expression as determined by densitometry (corrected against β-actin) from three independent groups of mice. Means and standard errors are plotted. Data from each group were normalized against N3.5 animals.

Mouse
Visual Cortex

A N3.5 D3.5 N9.5 D9.5

100 •

75 •

DAB1

50 •

37 •

actin

B
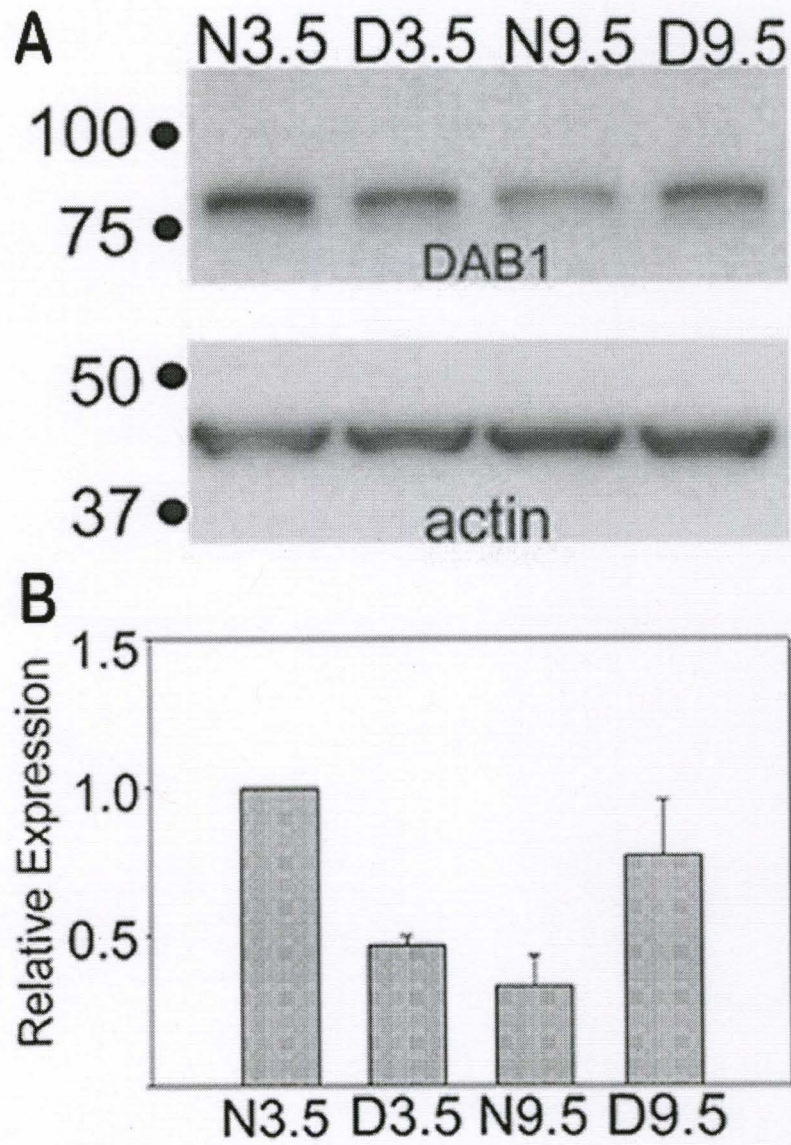
Figure 8

would not be influenced by visual experience (Figure 9). Levels of Dab-1 expression were similar in all rearing/age conditions in mouse frontal cortex, and analysis of variance of densitometric results indicated no statistically significant interaction due to age and dark rearing for Dab-1. The bidirectional regulation of Dab-1 protein expression was specific to visual cortex and was not present in mouse frontal cortex.

Bidirectional Regulation of Munc13-3 by Age and Dark Rearing in the Visual Cortex

Figure 10 shows Western blot analysis of visual cortex for three independent groups of three mice each. Each group contained the four age/rearing conditions (normal 3.5, dark reared 3.5, normal 9.5, dark reared 9.5). Standardized densitometric analysis indicated Munc13-3 was elevated 2.3-fold in dark-reared compared with normal mouse visual cortex at 3.5 weeks and two-fold in normal compared with dark-reared mouse visual cortex at 9.5 weeks. Analysis of variance showed a significant interaction between variables (F[2,8]=508.26, P=0.001), indicating that the effect of dark rearing was age dependent. In normal development, Munc13-3 expression increased twofold between the peak of the critical period (3.5 weeks) and its nadir (9.5 weeks). Munc13-3 protein showed opposite direction regulation by age and dark rearing in the visual cortex of mice.

To investigate the significance of Munc13-3 for visual cortical plasticity we examined whether bidirectional regulation of its expression by age and dark rearing was specific to visual cortex or might also be seen in other cortical regions. Levels of

Figure 9. A TOP: Western blot showing levels of Dab-1 protein expression in mouse frontal cortex in the four rearing and age conditions. BOTTOM: The same blot reprobed with β-actin. B. Mean level of Dab-1 protein expression in mouse frontal cortex as determined by densitometry (corrected against b-actin) from three independent groups of animals. Mean and standard errors are plotted.

Mouse
Frontal Cortex

A   N3.5 D3.5 N9.5 D9.5

Dab-1

actin

B

Figure 9

Figure 10. A. TOP: Western blot analysis of levels of Munc13-3 protein expression in mouse visual cortex showing higher levels in dark-reared (D3.5) than normal (N3.5) mouse visual cortex at 3.5 weeks and higher levels in normal (N9.5) than dark-reared (D9.5) mouse visual cortex at 9.5 weeks. BOTTOM: The same blot stripped and reprobed with β-actin to control for loading errors. Size standards are indicated on Western blots (in kDa). B. Densitometric results for Munc13-3 protein levels in mouse visual cortex. Mean levels of Munc13-3 protein expression as determined by densitometry (corrected against β-actin) from three independent groups of mice. For each of the three groups of mice, values for each age/rearing condition were normalized against the highest value in each group. Means and standard errors from the three groups for each age/rearing condition are plotted.

Mouse
Visual Cortex

A   **N3.5 D3.5 N9.5 D9.5**

250 -

150 -

50 -

37 -

B
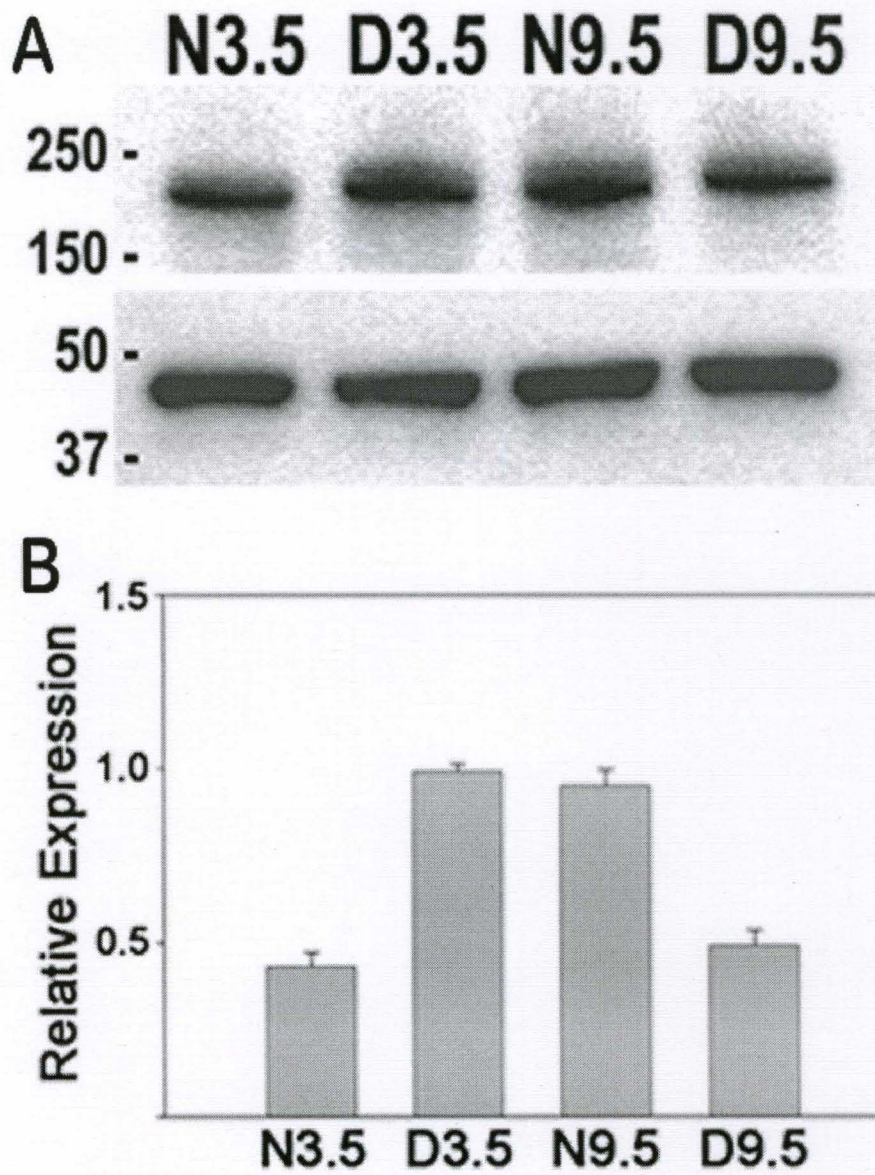
Figure 10

Munc13-3 expression were similar in all rearing/age conditions in frontal cortex, and analysis of variance of densitometric results (Figure 11) indicated no statistically significant interaction due to age and dark rearing for Munc13-3. The bidirectional regulation of Munc13-3 protein expression was specific to visual cortex and was not present in frontal cortex.

Immunohistochemical Analysis of Dab-1 Laminar Distribution in Visual Cortex

Figure 12 shows Dab-1 development in normally reared cat visual cortex. There is prominent Dab-1 immuno-staining across all layers at 1 week of age which diminished across all layers throughout the critical period. Cortical layers were identified and labeled following the designations of Luskin and Shatz ((Luskin and Shatz, 1985a, b) through cresyl violet staining of adjacent sections to those processed for Dab-1 immunohistochemistry (Figure 1). The observed developmental decline in Dab-1 cellular staining between each age was shown to be significant by analysis of variance of quantitative data obtained through cell counts which were corrected with total cell counts from the adjacent section stained with cresyl violet ($F[2,18]=41.319$, $P<0.001$, Tukey tests: 1wk vs. 5wk, $P<0.001$; 1wk vs 20wk, $P<0.001$; 5wk vs. 20wk, $P=0.007$) (Figure 13). Further analysis of laminar staining patterns throughout development revealed a significant difference between layers II/III and IV ($F[2,18]=8.143$, $p=0.003$, Tukey test: II/III vs.IV, $P=0.002$].

Figure 11. A TOP: Western blot showing levels of Munc13-3 protein expression in mouse frontal cortex in the four rearing and age conditions. BOTTOM: The same blot reprobed with β-actin. B. Mean level of Munc13-3 protein expression in mouse frontal cortex as determined by densitometry (corrected against b-actin) from three independent groups of animals. Mean and standard errors are plotted.
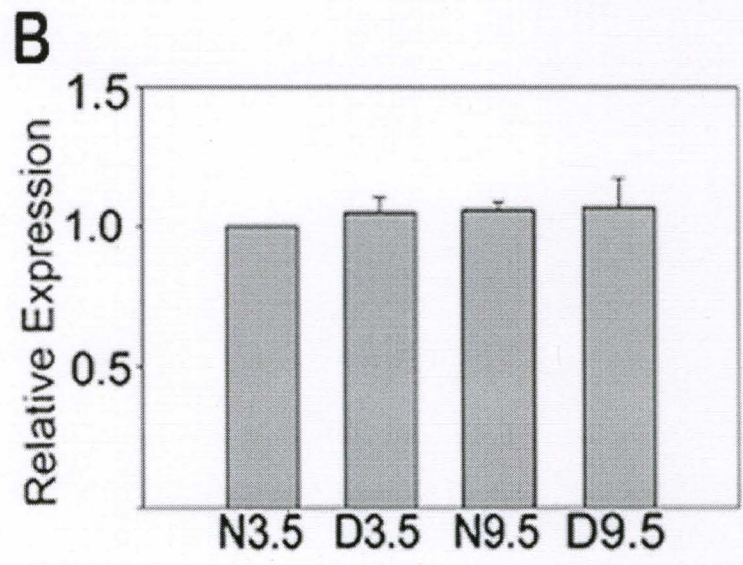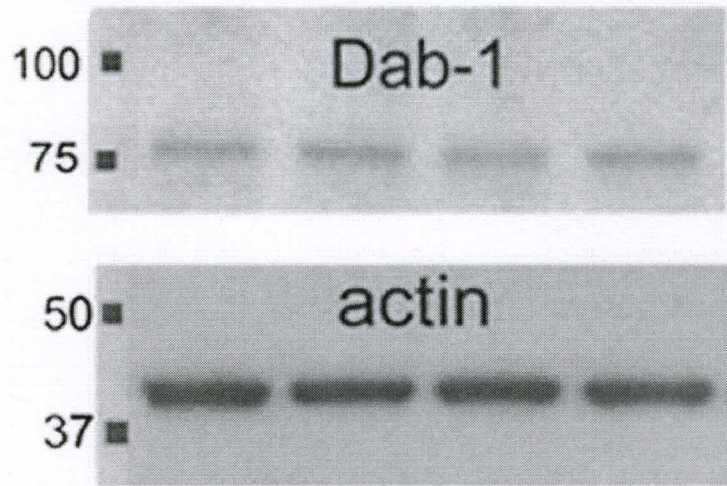
Mouse
Frontal Cortex

A   **N3.5 D3.5 N9.5 D9.5**

Munc
13-3

- 250

- 150

Actin

- 50

- 37

B
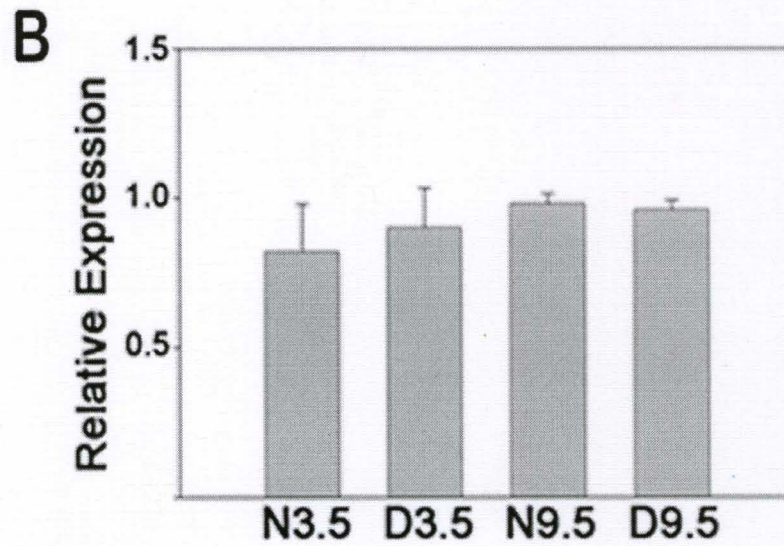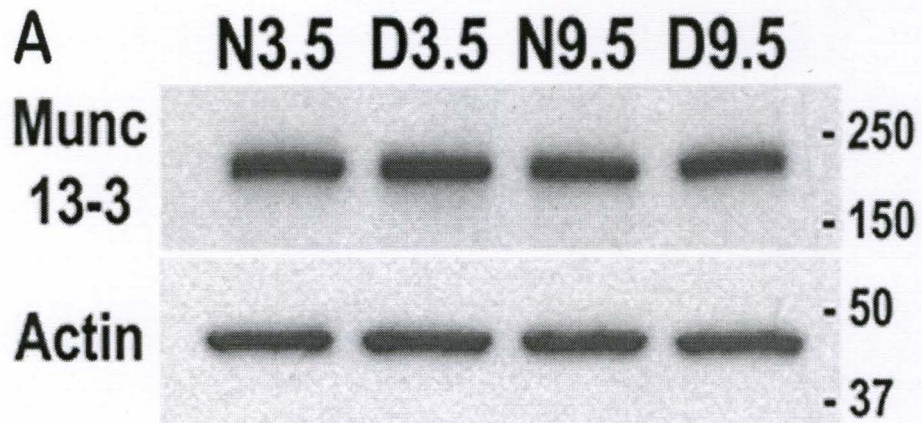
Figure 11

Figure 12. Low power (40X) photomicrographs showing Dab-1 immunoreactivity in cat visual cortex during postnatal development. The panel shows representative images of coronal sections of area 17 in 1, 5, 10, 20 week and adult cats. Cortical lamina are indicated and were determined by adjacent sections stained with cresyl violet. Rabbit polyclonal antibody to-Dab-1 (1:4000) was obtained from Rockland Immunochemicals, Inc. (Gilbertsville, PA) The slides were photographed using a computerized system (SPOT software, v2.2, SPOT Diagnostic Instruments, Inc.).

Dab1 Normal Development in Cat Visual Cortex
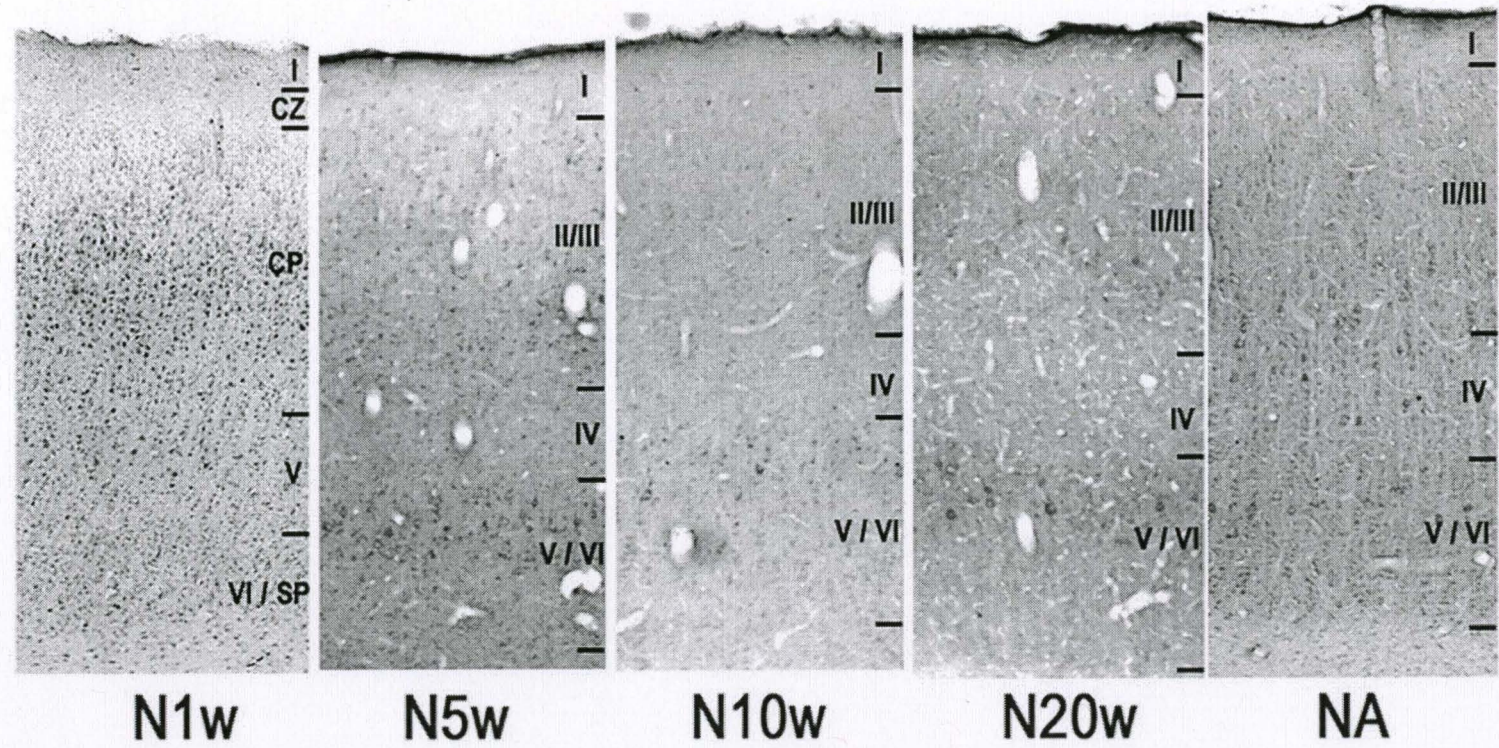
Figure 12

Figure 13. Line graphs showing quantification of Dab-1 immunohistochemisty in postnatal development. Top: Dab-1 expression in cortical lamina. . Immunopositive cell counts were an average of four unique counts in each lamina of three different animals at each age and corrected with cell counts from adjacent cresyl violet stained sections. Each data point represents an average of two independent immunohistochemical runs. Bottom: Combined results of all cortical lamina. Individual counts as described above from each layer in each animal at each age were averaged together to determine data points.
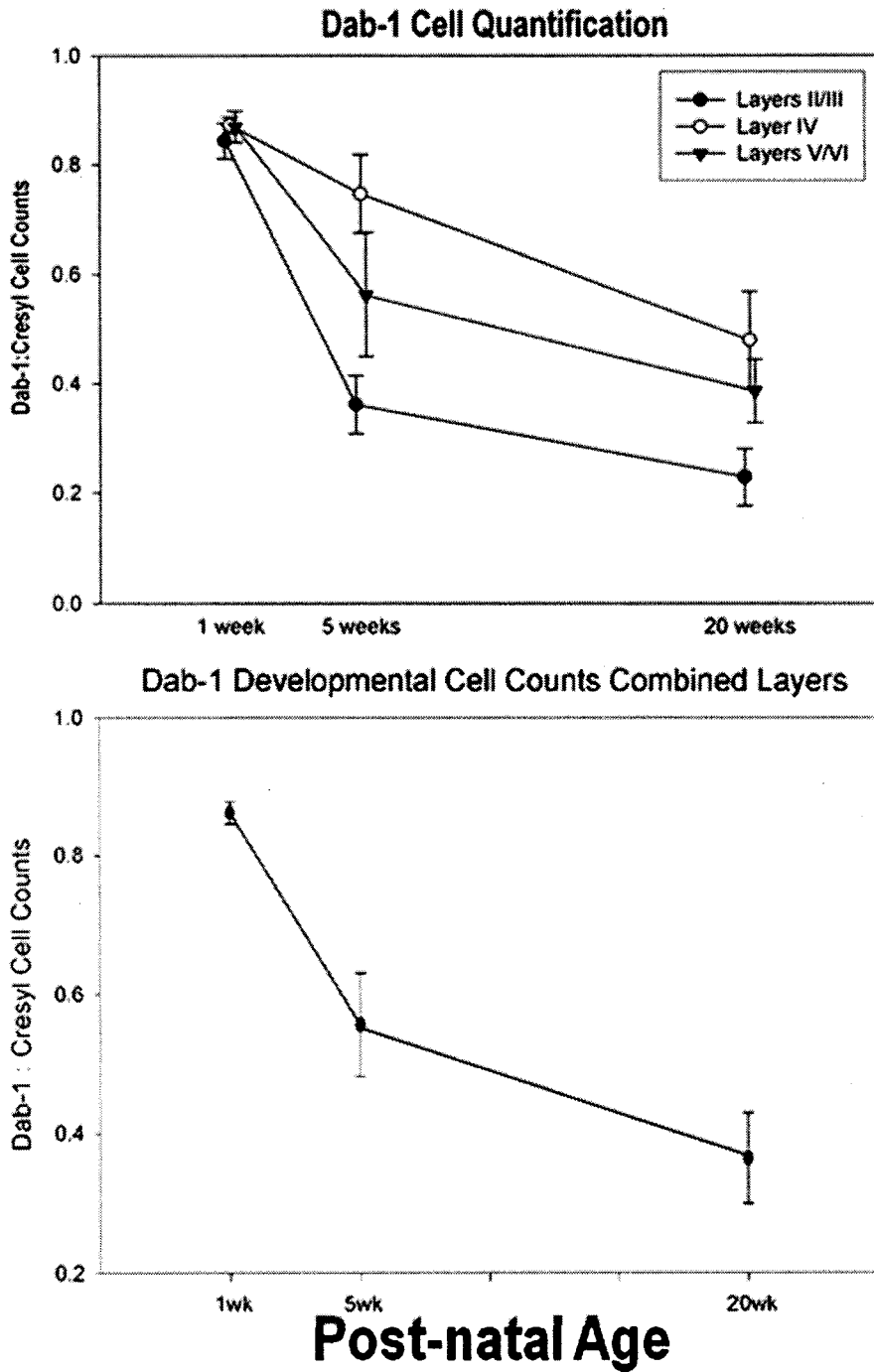
Figure 13

Figure 14 shows differences in Dab-1 immunohistochemical labeling between normal and dark reared cats at the peak (5 weeks) and nadir (20 weeks) of the critical period. Analysis of variance of these differences (Figure 15) revealed significant interaction between age and rearing condition ($F[1,24]=10.881$, $P=.003$) indicating that dark-rearing does have an effect on cellular expression of Dab-1 with significantly less Dab-1 labeled cells in the dark reared 5 week old cats compared to normal (Tukey test, N5wk vs. D5wk, $P=0.003$). This analysis also show significant reductions of Dab-1 immunopositive cells from normal 5 week to normal 20 week cats, as described previously (Tukey test: N5wk vs. N20wk, $p=0.005$). While the data trend in an opposite direction at 20 weeks, there was no significant difference found between normal and dark reared animals at the end of the critical period. Analysis of variance also revealed a significant difference found between layers ($F[2,24]=12.262$, $P,0.001$) with specific differences between layers II/III and IV (Tukey Test, $P<0.001$) and layers II/III and V/VI (Tukey test, $P=0.040$). No statistical differences were found between layers IV and V/VI. When considering these results, it is noteworthy to also refer back to the similar results pattern seen in the Western blot protein analysis of Dab-1 expression in mouse visual cortex (Yang et al., 2006) (Figure 8). The reduced immunopositive cell staining in dark-reared cats at five weeks of age when compared to normal animals and trending increases in dark-reared compared to normal at 20 weeks mirrors that seen in the total protein analysis of visual cortex.

Figure 14. Low power (40X) photomicrographs showing Dab-1 immunoreactivity in cat visual cortex. The panel shows representative images of coronal sections of area 17 in normal and dark reared cats at the peak (5 weeks) and nadir (20 weeks) of the critical period. Cortical lamina are indicated and were determined by adjacent sections stained with cresyl violet. Rabbit polyclonal antibody to-Dab-1 (1:4000) was obtained from Rockland Immunochemicals, Inc. (Gilbertsville, PA) The slides were photographed using a computerized system (SPOT software, v2.2, SPOT Diagnostic Instruments, Inc.).

Dab1 Normal vs Dark Reared in Cat Visual Cortex

N5w   D5w   N20w   D20w
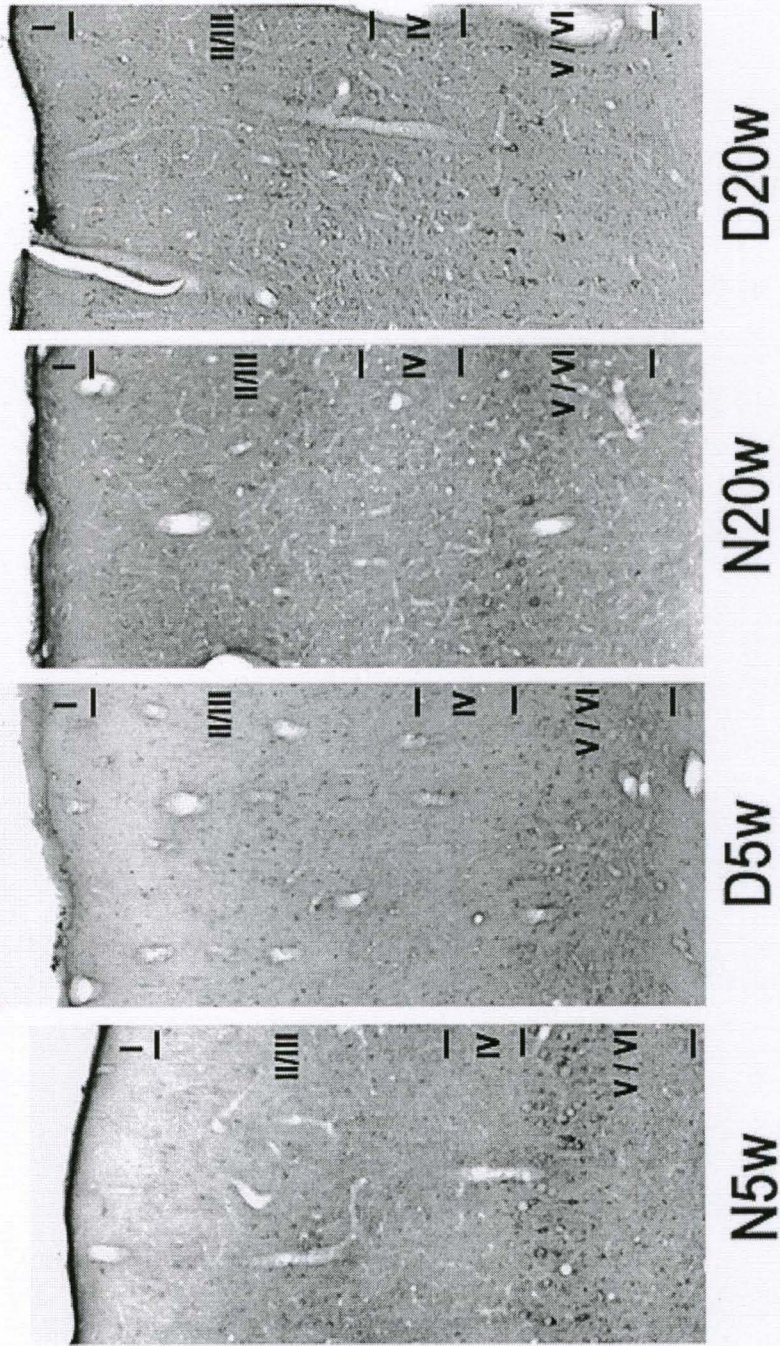
Figure 14

Figure 15 Line graphs showing quantification of Dab-1 immunohistochemisty in cortical lamina of cat visual cortex comparing normal and dark reared cats at the peak (5 weeks) and nadir (20 weeks) of the critical period. Top: Dab-1 expression in cortical lamina. Immunopositive cell counts were an average of four unique counts in each lamina of three different animals at each age and corrected with cell counts from adjacent cresyl violet stained sections. Each data point represents an average of two independent immunohistochemical runs. Bottom: Combined results of all cortical lamina. Individual counts as described above from each layer in each animal at each age were averaged together to determine data points.
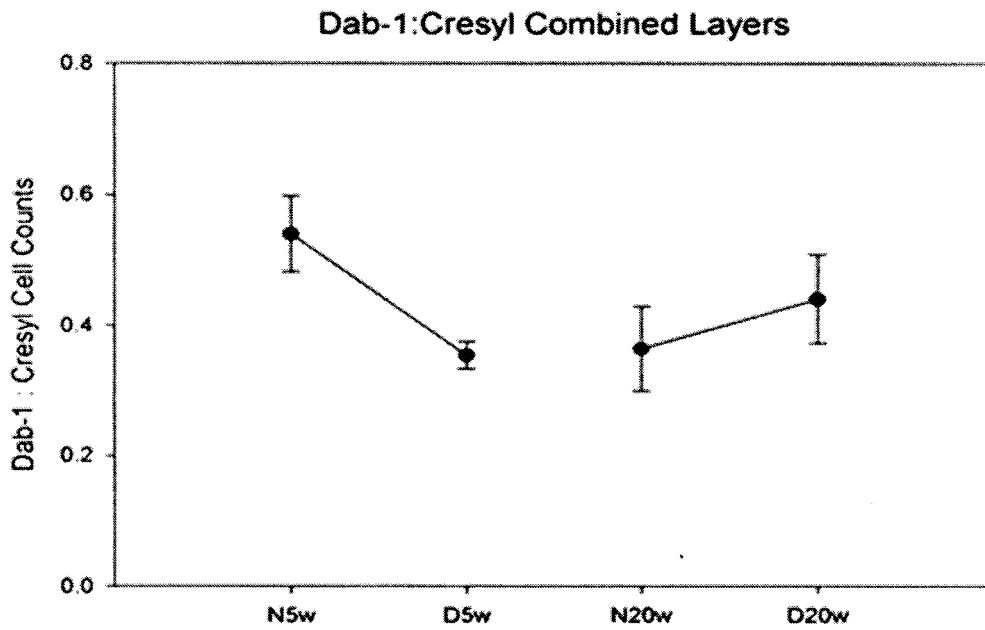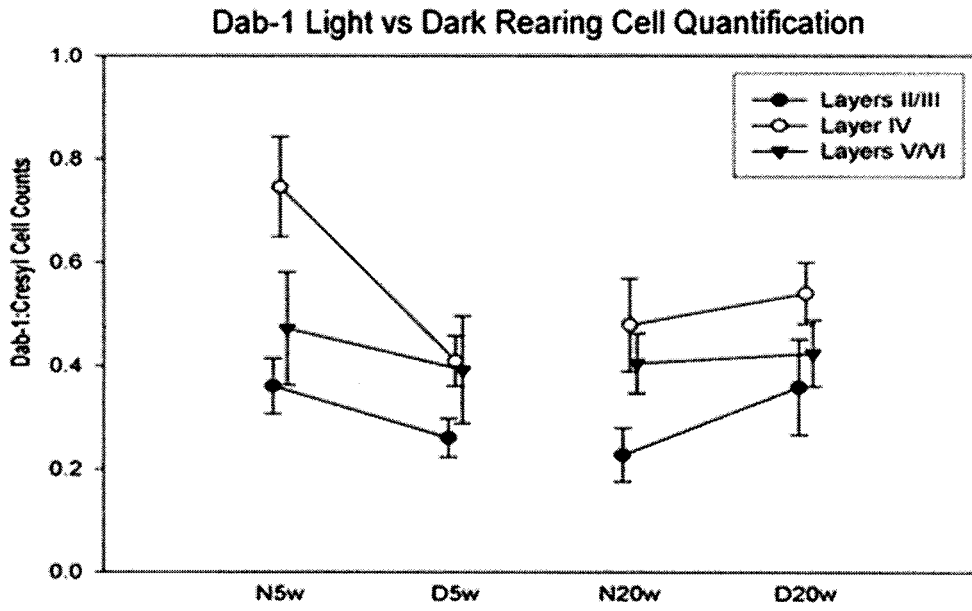
Figure 15

Dab-1 immunohistochemistry was also performed on normal and dark reared

mouse tissue (Figure 16) Dab-1 protein expression patterns were observed that support

those found in the cat however due to extreme laminar differences in afferent terminal

distribution between the species (described in the Introduction), quantitative analyses of

the staining patterns were not conducted.

Effect of Dab-1 Mutation on NMDAR Subunits

Figure 17 shows the effect of Dab-1 on NMDAR subunits NR2A and NR2B.

Analysis of variance revealed significant differences between Dab-1 mutants and wild-

type animals in the ratio of NR2A to NR2B subunits ($F[1, 8]$, $P<0.001$). In wild-type

animals, the NR2A:NR2B ratio shows a significant decrease from the peak to the nadir of

the critical period when expressed as a percentage of dark reared to normally reared

animals (DR/LR), (Tukey Test: Wild-type 3.5wk vs 9.5wk, $P=0.002$). This result is

significantly different from the opposite effect that is observed in Dab-1 mutant animals

where the percentage significantly increases from 5 weeks to 20 weeks (Tukey Test:

Mutant, 3.5wk vs 9.5wk, $P=0.015$). Comparisons of animal differences at both ages also

found significant effects. The DR/LR-NR2A:NR2B ratio is significantly lower in mutant

animals at 3.5 weeks (Tukey Test: 3.5wks, Wild-type vs Mutant, $P=0.003$) and

significantly higher in mutants at 9.5 weeks (Tukey Test: 9.5wks, Wild-type vs Mutant,

$P=0.009$).

Figure 16. Low power (40X) photomicrographs showing Dab-1 immunoreactivity in mouse visual cortex. The panel shows representative images of coronal sections of area 17 in normal and dark reared mice at the peak (3.5 weeks) and nadir (9.5 weeks) of the critical period. Rabbit polyclonal antibody to-Dab-1 (1:4000) was obtained from Rockland Immunochemicals, Inc. (Gilbertsville, PA) The slides were photographed using a computerized system (SPOT software, v2.2, SPOT Diagnostic Instruments, Inc.).

Dab-1 Light vs Dark Rearing in Mouse Visual Cortex

N3.5w   D3.5w   N9.5w   D9.5w
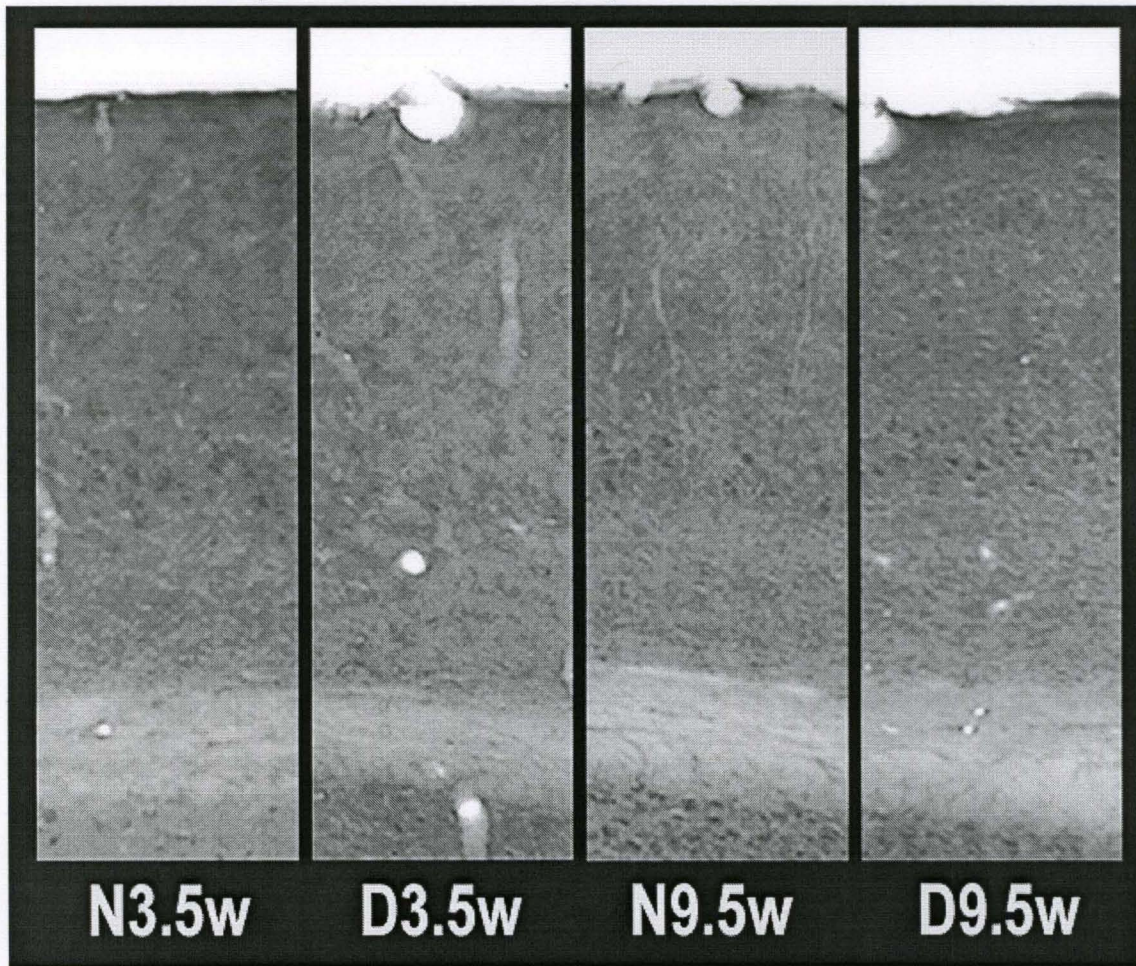
Figure 16

Figure 17. Histogram showing the effect of Dab-1 mutation on NMDAR subunits NR2A

and NR2B in mice at the peak (3.5 weeks) and nadir (9.5 weeks) of the critical period.

Each bar represents the ratio of dark-reared expression of the NR2A:NR2B ratio to the

normally reared expression of the same for each age in both wild-type and Dab-1

knockout animals.

Dab-1 Effect on NMDAR Subunits
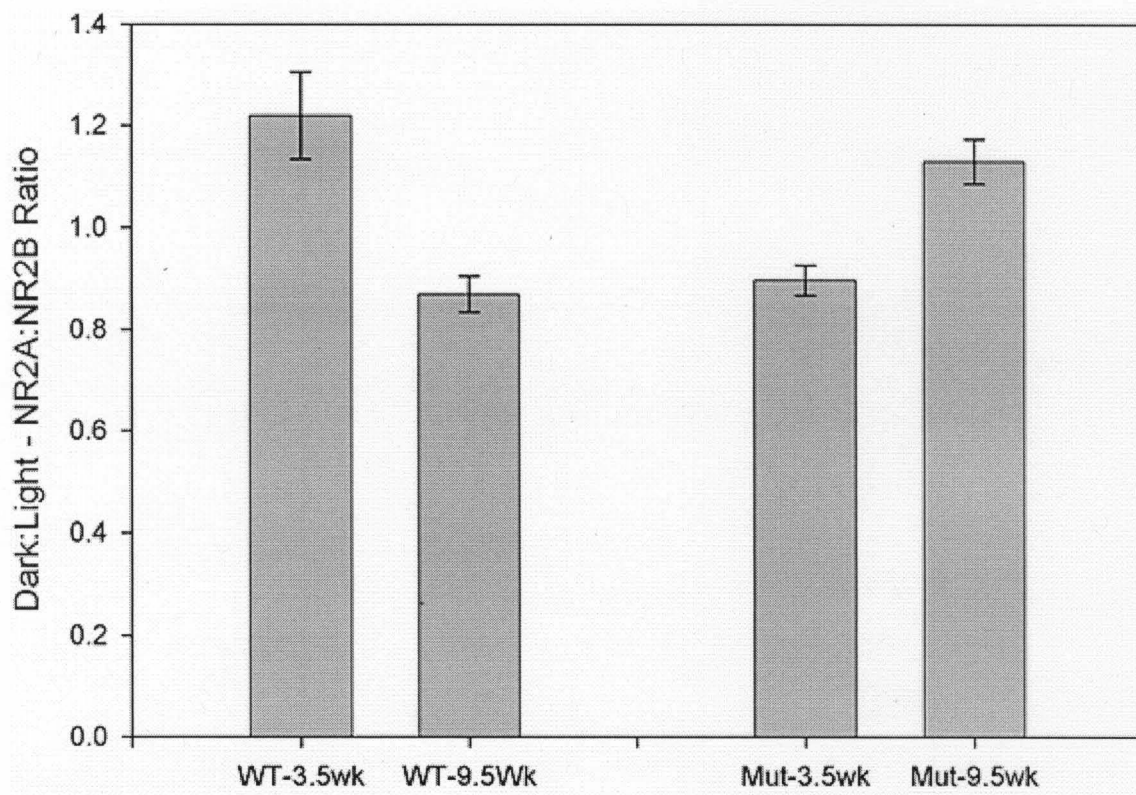
Figure 17

Establishing Procedures for Recording Evoked Potentials from Visual Cortex in Awake Mice

The following protocol for recording of visual evoked potentials from awake mice is the result of the developmental efforts of this project.

Electrode Implantation Procedure

The developed protocol was adapted from published work of the laboratory of Mark F. Bear, PhD (MIT, Cambridge, MA). All procedures in the test animals conform to the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee. Impedence of each tungsten recording microelectrode (#UEWSECBN1C, FHC, Bowdoinham, ME) was measured prior to implantation with an FHC NeuroCraft™ Impedence Conditioning Module (#55-70-0, FHC, Bowdoinham, ME).

Mice were anesthetized with a mixture of Ketamine and Xylazine (90mg/kg Ketamine/9mg/kg Xylazine). Levels of anesthesia were monitored throughout the entire procedure and supplemental "booster" injections of Ketamine only (90mg/ml) were administered as needed during extended procedures to maintain deep level of general anesthesia. Sterile ophthalmic ointment (Puralube, NDC17033-211-38, Dechra Veterinary Products, Overland Park, KS) was applied to each eye to prevent desiccation of the cornea during the surgery and recovery. This was reapplied as necessary during the

procedure. Prior to incision, approximately 0.1ml of 0.05% Lidocaine was also injected

under the scalp to supplement general anesthesia. A mid-saggital incision was made

exposing the skull. Approximately 3mm of tissue was removed from both sides of the

incision creating an oblong opening exposing the skull. The periosteal membranes above

the skull were scraped away with the broad edge of a scalpel blade, the skull was wiped

clean with sterile cotton swabs, swabbed with 70% EtOH and left to dry completely for

approximately 15 minutes.

A liquid cyanoacrylate adhesive (Loctite-495, Loctite Corp, Rocky Hill, CT) was

utilized to adhere a fixation post (#18 flathead nail cut to approximately 1 cm in length)

to the dried skull just anterior to bregma. Curing of the adhesive was hastened through a

single drop application of ZipKicker (Pacer Technology, Ranch Cucamonga, CA)

cyanoacrylate accelerator. The mouse was secured by the post into a custom designed and

fabricated, acrylic stereotaxic base (University of Louisville machine shop) for the

remaining procedure (Figure 18).

A small hole (approximately 1 mm) located 1 mm lateral to bregma for the

reference electrode (#19003-00, Fine Science Tools (USA), Inc, Foster City, CA with

1mm silver wire tip) was drilled in the skull using a high speed micro drill (#18000-17,

Fine Science Tools (USA), Inc, Foster City, CA) with a 0.7mm burr bit (#19008-07, Fine

Science Tools (USA), Inc, Foster City, CA) This electrode was inserted and secured with

a gel-based cyanoacrylate adhesive (Loctite-454, Loctite Corp, Rocky Hill, CT) directly

Figure 18. This acrylic surgical fixation apparatus was designed to screw into a stereotaxic stand for precise measurements for electrode placement. Once the fixation post was adhered to the skull, the mouse was secured into the apparatus by the skull post stabilizing the head for both drilling and implantation.
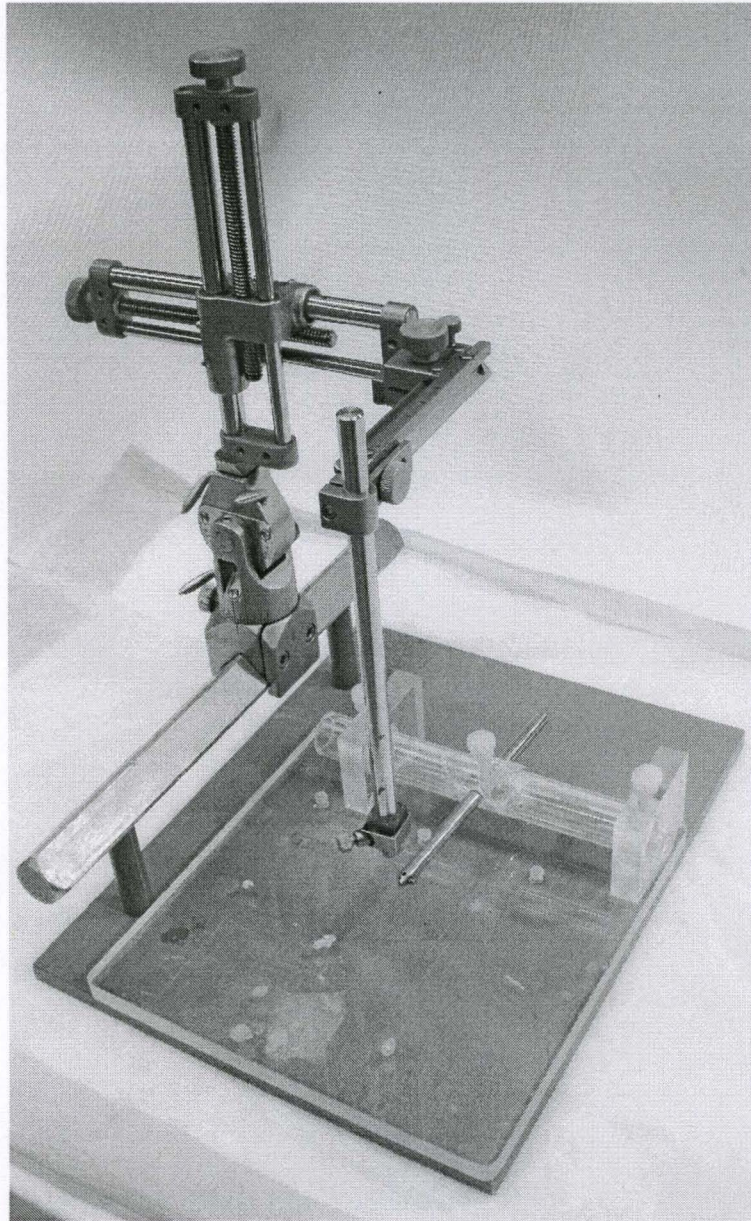
Figure 18

to the skull, again accelerated by ZipKicker. The hole for the tungsten recording

microelectrode placement was determined through stereotaxic positioning to allow for

insertion into the binocular region of mouse visual cortex. This location was ipsilateral to

the reference electrode, 3 mm lateral to lambda. The hole was made using the same rotary

tool and burr bit however it was not simply drilled directly through the skull as the one

made for the reference electrode. The recording electrode foramen was formed by the

creation of subsequently deeper circles approximately 2 mm in diameter centered on the

predetermined insertion point for the recording electrode. Once the depth of the ring had

nearly penetrated the skull, a sterile hypodermic needle and micro-forceps were utilized

to remove the remaining flap of bone (approximately 1mm in diameter) inside the circle

exposing the dura overlying the targeted visual cortex. The tungsten recording

microelectrode was lowered stereotaxically until the tip of the electrode contacted the

dura mater. A gentle tap was given to the entire apparatus to perforate the dura mater.

The electrode was then lowered approximately 0.45mm into the visual cortex. The gel-

based cyanoacrylate adhesive was used to secure the resin bead of the electrode to the

skull (and accelerated with ZipKicker) and the electrode guide wire was trimmed flush to

the resin bead. The lead wire of the recording electrode and recording pin were then

secured to the resin bead taking care to avoid direct contact between the wire and the

skull to reduce potential interference.

The mouse was removed from the stereotaxic apparatus. The entire exposed skull,

base of the fixation post and attachment points of both electrodes were then carefully

covered with a non-conductive two-part, rapid setting dental acrylic (OrthoJet Liquid

#1304, methyl methacrylate monomer, and OrthoJet Acrylic Resin Powder, #1300, Lang Dental Manufacturing Co., Inc., Wheeling, IL). This created an acrylic cap that not only protected and further secured the electrode placement but also completely sealed any exposed cut edges from the original scalp retraction/removal. Animals were given a 24 hour analgesic dosage of Ketoprofen (4mg/kg) and were allowed to recover on a heating pad and returned to their cages when alert. Animals were monitored postoperatively for signs of infection and/or discomfort and allowed at least 24 hours recovery prior to the first recording session.

Monocular Deprivation Procedure

Mice were anesthetized with a mixture of Ketamine and Xylazine (90mg/kg Ketamine/9mg/kg Xylazine). Sterile opthalmic ointment (Puralube, NDC17033-211-38, Dechra Veterinary Products, Overland Park, KS) was applied to both eyes to prevent corneal dessication while under anesthesia. The area immediately surrounding the eye to be sutured (contralateral to recording electrode) was wiped with 70% ethanol. Lid margins were trimmed (less than .5 mm from each eyelid), and the eye was flushed with saline and sterile ophthalmic ointment was applied to the eye. Two to three sutures were placed using 7-0 silk, opposing the full extent of the trimmed lids. The sutures and wound were sealed with VetBond cyanoacrylate tissue adhesive (#1469SB, 3M, St. Paul, MN). Animals were given a 24 hour analgesic dosage of Ketoprofen (4mg/kg) and were allowed to recover on a heating pad and returned to their cages when alert. Animals were checked daily to make sure that the eyes remained uninfected. After five days, the animal

was anesthetized again with the Ketamine/Xylazine mixture (90mg/kg Ketamine/9mg/kg Xylazine), recording and reference electrodes were placed in the contralateral visual and frontal hemispheres, as described previously, and sutures were removed to open the deprived eye. If holes developed in the sutured closure prior to eye-opening the animal was eliminated from the study. Eyes were then flushed with sterile saline and checked for clarity. Mice with corneal opacities or signs of infection were excluded from further study. All procedures in the test animals conform to the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee.

Visual-Evoked Potential Recording

A customized restraint system (Figure 19) was developed and manufactured in the University of Louisville machine shop for recording of visual evoked potentials (VEP). These recordings were then able to be conducted in awake, head-restrained mice through the use of a head-stage attached to a differential amplifier (A-M Systems Model 3000, high pass filter = 1Hz, low pass filter = 0.1KHz, Notch = "On" and gain = 100X) and digital storage oscilloscope (TekTronix TDS 1001B). Following the postoperative recovery period, animals were habituated to the head restraint system. Once habituated, animals sat quietly in tube/head restraint system with minimal movement throughout the duration of a recording session (approximately 30 minutes). Visual stimuli were presented to both eyes, alternately to right and left eyes, and with both eyes blocked (60 sweeps averaged/record). Each stimulus condition was recorded at least six times over

Figure 19. A custom restraint system was designed for awake recording of visual evoked potentials. The mouse was secured by the skull fixation post to a cross bar facing the visual stimulus while the body weight was supported by a plastic cylinder leaving the electrodes accessible for recording. A custom designed Faraday cage was built to reduce any noise that might mask the potential. Mice very quickly became accommodated to the set-up and would rest peacefully during the 20-30 minute recording sessions. A. Full view of recording set-up with mouse secured in recording apparatus in front of visual stimulus. B. Closer view of mouse resting in support tube while secured and facing the visual stimulus. C. Front view of mouse secured by fixation post and resting in support tube (Faraday cage removed). D. Top view of mouse secured in recording apparatus (Faraday cage removed).
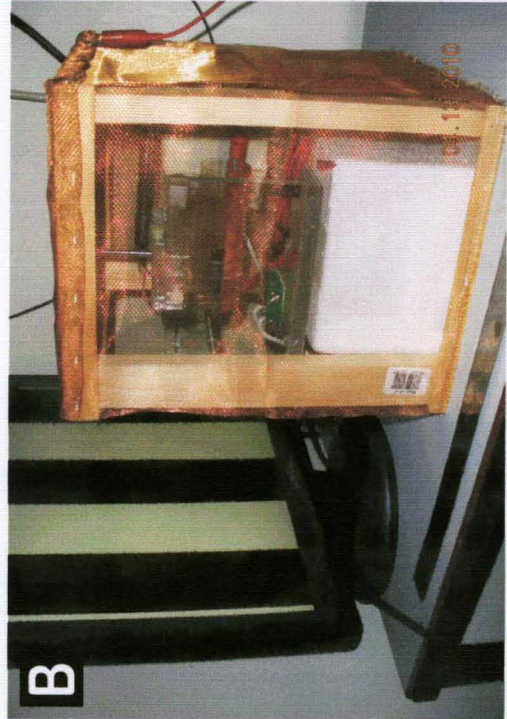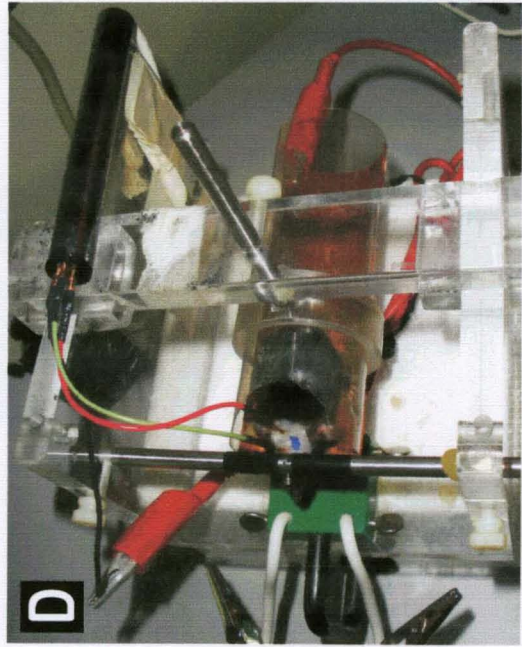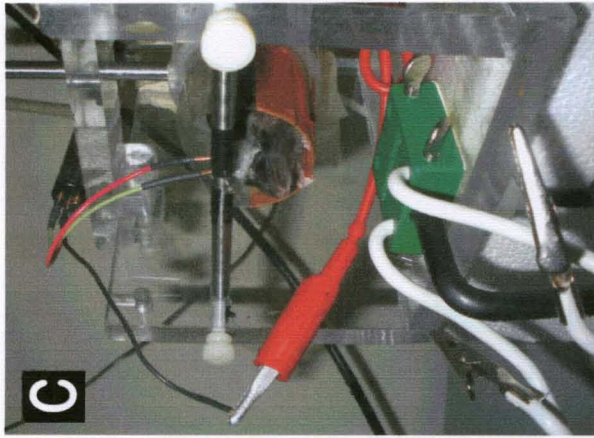
Figure 19

the course of a single session. Individual recording sweeps were initiated by a simultaneous 3mV trigger directly to the oscilloscope synchronized to the onset of each stimulus generated by StimulusMaker (version 2.1.35, Vision Research Graphics, Durham, NH).

Visual Stimulus

Visual stimuli consisted of full-field 1.98Hz counterphasing square-wave gratings of 100% contrast and 0.3 cycles/degree generated by an ATI Radeon graphics card and VisionWorks for Electrophysiology software (version 1.4.80, Vision Research Graphics, Durham, NH) presented on a Dell 19-inch flat-screen digital computer monitor. The display was positioned 20 cm in front of the mouse and centered on the midline.

Numerous challenges were encountered throughout the process as detailed in the Discussion section however protocols for monocular deprivation (MD) of animals and recording of VEPs were shown to be successful. Figure 20 shows VEPs recorded in the binocular region of visual cortex from stimulation to each eye in normal and MD mice in our lab. At 3.5 weeks, normal mice show a stronger response to stimulation to the contralateral over the ipsilateral eye. After five days of closure of the contralateral eye, followed by reopening of this eye, there is a marked depression in responsiveness of the deprived eye that significantly reduces the MD contralateral to ipsilateral ratio to about half of the normal ratio. This replicates published research on monocular deprivation in young mice (Frenkel and Bear, 2004) indicating successful protocol development.

Figure 20. Representative records of visual evoked potentials recorded in the binocular

zone of mouse visual cortex from stimulation to each eye in normal (top) and

monocularly deprived (bottom) 3.5 week old mice. Left side of each panel was recorded

from the eye contralateral to the hemispshere with the recording electrode. The right side

of each panel is a record from the ispsilateral eye. The contralateral eye was deprived for

a period of five days and reopened prior to recording for monocular deprivation

experiments. Each record is an average of sixty potentials from one recording session.

The visual stimulus was a 0.03 cycle/degree square-wave gradient at 100% contrast

counter-phasing at 1.98 Hz.

Normal 3.5 Week

Contralateral Eye

Ipsilateral Eye

2.0 mV

100 ms

MD 3.5 Week

Contralateral Eye

Ipsilateral Eye

2.0 mV

100 ms
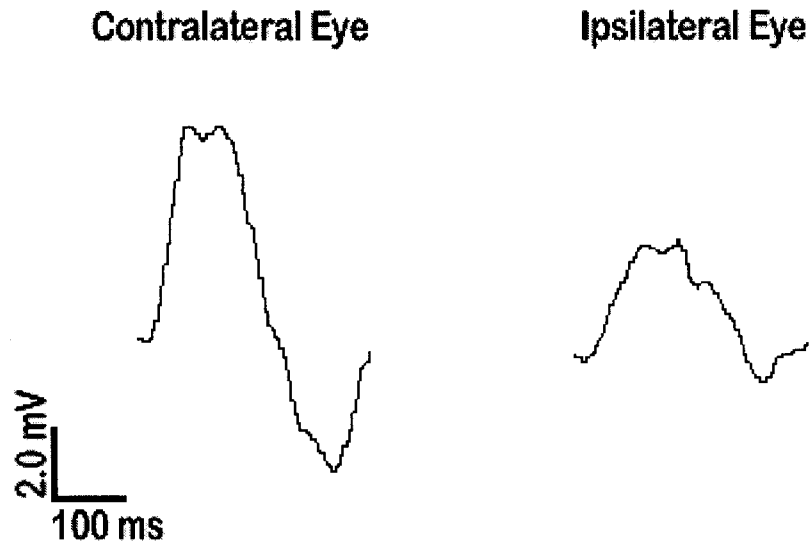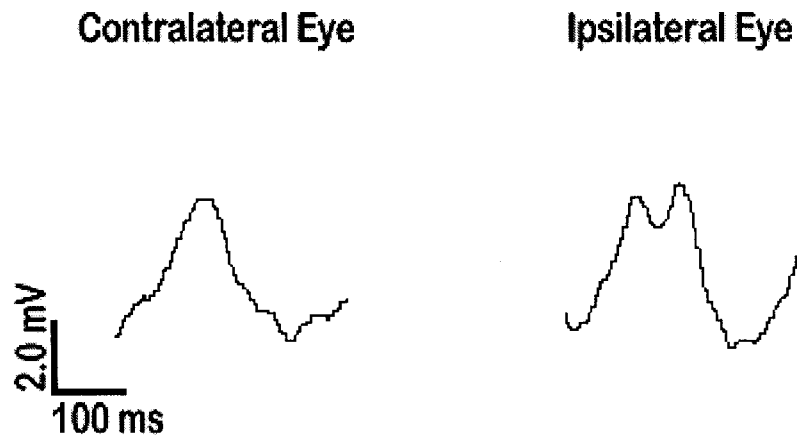
Figure 20

DISCUSSION

Summary of Results

1) Analysis of visual cortical protein expression of both Dab-1 and Munc13-3 genes agreed with mRNA gene screening data conducted previously in the Mower lab (Yang et al., 2001; Yang et al., 2002) and the results reported here are now published (Yang et al., 2006; Yang et al., 2007). Dab-1 protein showed opposite direction regulation by age and dark rearing in the visual cortex of cats and mice. Dab-1 protein expression was found to be greater in normal than dark-reared visual cortex at the peak of the critical period (5 weeks) and greater in dark-reared than normal visual cortex at the end of the critical period (20 weeks). Munc13–3 protein showed differences in expression due to age and dark rearing in mouse visual cortex in a pattern opposite to that seen in the Dab-1 expression. Munc13–3 protein expression was found to be greater in dark-reared than normal visual cortex at the peak of the critical period and greater in normal than dark-reared visual cortex at the end of the critical period. This bidirectional regulation of expression was specific to Munc13–3 and did not occur in other members of the Munc13 gene family. Bidirectional regulation of Dab-1 and Munc13-3 was also restricted to visual cortex, indicating that it was not a non-specific cortical effect of dark rearing. In

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normal development, Dab-1 decreased three fold between the peak and nadir of the critical period and Munc13-3 increased two-fold during the same time.

2) Analysis of the effect of Dab-1 on the protein expression of NMDAR subunits NR2A and NR2B showed a significant differences between Dab-1 knockout mutants and wild-type animals in the ratio of NR2A to NR2B subunits with age. When expressed as a percentage of dark reared to normally reared animals (DR/LR) the NR2A:NR2B ratio shows a significant decrease from the peak to the nadir of the critical period in wild-type animals. This result is significantly different from the opposite effect that is observed in Dab-1 knockout mutant animals where the percentage significantly increases between 5 and 20 weeks.

3) Quantification of immunohistochemical study of cat visual cortex showed a neonatal peak in Dab-1 cellular staining with a significant decline in staining density in all cortical layers throughout the critical period. A significant difference in cell labeling was found at one week of age with a higher number of immunopositive cells in the supragranular (developing layers II and III) than granular (layer IV) layers (no difference was found in infragranular (layers V and VI) layers). This difference abates, however and was found to be insignificant by 5 weeks of age with no significant laminar difference remaining throughout development. Comparisons of normal versus dark rearing conditions indicated a significant age/dark rearing interaction that affected all layers similarly. Thus, in agreement with the Western blot results, age and visual experience bidirectionally regulate Dab-1 expression in all visual cortical layers.

4) In support of laboratory efforts to conduct electrophysiological studies of the effects of Dab-1 and Munc13-3 on critical period cortical plasticity, appropriate methodology and procedures were developed as a part of this thesis to successfully implant electrodes into the binocular region of mouse visual cortex and reliably record visual evoked potentials with a square-wave counter-phasing gradient. Protocols were also developed to successfully monocularly deprive mice, open the deprived eye and repeat the results of published studies that show a shift in eye preference responses after monocular deprivation (Gordon and Stryker, 1996; Antonini et al., 1999). These methods and procedures will be used for future experiments to test physiological effects of mutations in the Dab-1 and Munc13-3 genes on cortical plasticity.

Candidate Plasticity Gene Protein Expression Analysis and Critical Period Neuronal Plasticity

Munc13-3

Munc13 isoforms, mammalian homologues of the *C. elegans uncoordinated* gene (*unc-13*), are phorbol ester receptors that form regulatory complexes, are specifically targeted to presynaptic active zones, and play an essential role in synaptic vesicle release (Brose et al., 1995; Betz et al., 1997; Augustin et al., 1999a; Basu et al., 2007; Breustedt et al., 2009). Munc13 proteins have been shown to be differentially expressed with isoforms present in different regions of the brain. Munc13-1 is expressed throughout all

brain regions, while Munc13-2 and Munc13-3 have been shown to be predominantly expressed in rostral and caudal brain regions respectively (Augustin et al., 1999b; Yang et al., 2002). This suggests a primary role for Munc13-1 in all synapses with a complementary role for either Munc13-2 or Munc13-3 depending on brain region. The results presented here further examine protein expression of Munc13-3 in cat and mouse visual cortex during the critical period of development.

Munc13–1 deletion mouse mutants do not feed and consequently die perinatally, but primary hippocampal cultures from these Munc13 mutant mice indicate an inability of docked vesicles to fuse in response to physiological stimuli in glutamatergic and GABAergic neurons (Augustin et al., 1999a; Varoqueaux et al., 2002). Study of Munc13–1, Munc13–2 and combined mutants in the hippocampus indicates that differential expression of Munc13 isoforms occurs within individual synapses and may be a mechanism that controls short- term plasticity by producing a mix of facilitating and depressing synapses (Rosenmund et al., 2002). This result has several potential implications. First, different types of synapses formed by the same axon may allow a given nerve cell to transmit different information to different target cells. Second, transforming depressing synapses into augmenting ones or vice versa by exchanging the Munc13 isoforms may represent a mechanism to induce lasting changes in synaptic efficacy that result from development and environmental input. Such mechanisms should be relevant to the ocular dominance shifts that define the visual cortical critical period.

Munc13 isoforms have also been implicated in short-term synaptic plasticity by establishing complexes with calmodulin to regulate synaptic vesicle priming (Junge et al., 2004; Zikich et al., 2008). Recent research suggests that the unique Ca^{2+}-binding site common to all Munc13 isoforms plays a significant role in short-term synaptic plasticity by offsetting synaptic depression induced by vesicle depletion caused by repeated action potentials (Shin et al., 2010).

Munc13–3 mutants have been studied in cerebellum. These mutants thrive and show grossly normal brain morphology, but there are impairments of synaptic transmission at cerebellar synapses and of motor learning (Augustin et al., 2001). Munc13–3 mutants show enhanced paired pulse facilitation at parallel fiber–Purkinje cell synapses which is indicative of decreased neurotransmitter release probability (Zucker, 1989). This suggests that Munc13–3 normally functions to increase release probability and decrease paired pulse facilitation. Conversely, mutation of BDNF, a molecule which promotes neuronal plasticity (Berardi and Maffei, 1999), results in decreased paired pulse facilitation at parallel fiber–Purkinje cell synapses (Carter et al., 2002). Thus, the data in cerebellum indicate that Munc13–3 acts to reduce synaptic plasticity, consistent with a potential repressor role in the visual cortical critical period. Firm conclusions must await determination of the function of Munc13–3 in visual cortex. The present results provide the basis for functional analysis of visual cortical critical period plasticity in Munc13–3 mutant mice. Overall, growing evidence indicates a role of Munc13 genes in presynaptic transmission and plasticity and Munc13–3 protein expression is a promising mechanism for regulating activity dependent critical period plasticity of visual cortex.

Dab-1

The results of this study indicate that Dab-1 likely plays a role in a pathway important for visual cortical critical period plasticity. It has already been shown that Dab-1 is an important component of the Reelin signaling pathway that controls cell positioning during early brain development and significant portions of the molecular apparatus of this pathway have been well described (Rice and Curran, 2001). Reelin is an extracellular protein secreted in developing cortex by Cajal-Retzius cells (Alcantara et al., 1998) and by GABAergic neurons in adult cortex and hippocampus (Pesold et al., 1998b). Reelin binds to its receptors, VLDLR and ApoER2, causing phosphorylation of intracellular Dab-1, which is a multifunction adaptor protein found in both phosphorylated and un-phosphorylated forms in both the cytoplasm and nucleus of labeled cells (Honda and Nakajima, 2006). Phosphorylated Dab-1 then recruits tyrosine kinases that are associated with the activation of a number of signaling pathways in migrating neurons and can function as a nonsynaptic mechanism to alter gene expression (Rice and Curran, 2001; Feng and Cooper, 2009). Dab-1 has also recently been identified as an intracellular scaffold for forming complex protein clusters with the intracellular Reelin receptor tails and numerous intracellular kinases and their target proteins. This suggests that Dab-1 is important as more than just an activator protein but also as structural component to facilitate downstream reactions in the Reelin pathway during development (Feng and Cooper, 2009).

Both Reelin (Alcantara et al., 1998; Pesold et al., 1998b; Pesold et al., 1998a; Pesold et al., 1999) and Dab-1 (Pesold et al., 1999; Deguchi et al., 2003) are shown to be expressed in the adult brain but the role of the Reelin/Dab-1 pathway in postnatal and mature brain is just beginning to be understood. Evolving evidence indicates that in postnatal brain the pathway plays a role in synaptic plasticity. Histologically, in neocortex Reelin has been found primarily in inhibitory interneurons (Alcantara et al., 1998; Pesold et al., 1998b; Pesold et al., 1998a) and Dab-1 predominately in pyramidal cells (Pesold et al., 1999). Thus, the Reelin/Dab-1 pathway is well positioned to mediate excitatory–inhibitory interactions believed to be critical for visual cortical plasticity (Hensch and Fagiolini, 2005). Ultrastructurally, in embryological development Reelin is strongly associated with the extracellular matrix, but in adults Reelin expression is also elevated in dendritic spines, the postsynaptic density and axon terminals, suggesting Reelin has a significant role at the synapse in the mature brain (Roberts et al., 2005).

In addition to the morphological evidence, recent functional evidence in postnatal hippocampus directly implicates the Reelin/Dab-1 pathway in synaptic plasticity. Interference with Reelin signaling reduces long-term potentiation (LTP) and perfusion of exogenous Reelin enhances LTP (Weeber et al., 2002). This enhancement of LTP is mediated by activity dependent alternative splicing of the ApoER2 receptor, which is required for Reelin-induced phosphorylation of NMDA receptors (Beffert et al., 2005). Developmentally, interruption of Reelin signaling slows and exogenous Reelin accelerates the normal developmental changes in NMDA receptor currents, which reflect changing NMDA receptor subunit composition (Sinagra et al., 2005). Reelin secreted by

adult GABAergic hippocampal neurons is implicated as an as in important trans-neuronal messenger required to maintain homeostasis of the subunit composition of NMDAR, suggesting that defects in the Reelin pathway could lead to significant disorders associated with lack of NMDAR regulation (Campo et al., 2009). Reelin has also been implicated in physiological enhancement of NMDA receptor activity by augmenting Ca^{2+} entry through the NMDA receptor. This effect is negated by the elimination of Dab-1 from the pathway (Chen et al., 2005) suggesting an important role for Dab-1 expression in NMDAR function.

Reelin mutation also impairs dendritic maturation and exogenous application of Reelin rescues this mutation deficit (Niu et al., 2004). Although it is still uncertain if the intracellular mechanisms of Reelin/Dab-1 signaling in postnatal brain are similar to or different from those found in embryogenesis, the pathway is a promising newly identified player in postnatal and adult synaptic plasticity. The protein analysis presented in this thesis further extends this evidence by directly correlating Dab-1 expression with the defined critical period of postnatal plasticity of cat and mouse visual cortex.

Effect of Dab-1 Mutation on NMDA Receptor Subunits during the Critical Period

Present results found significant differences between Dab-1 mutants and wild-type mice in the ratio of expression of NR2A to NR2B subunits of the NMDA receptor. In wild-type animals, the NR2A:NR2B ratio showed a significant decrease from the peak to the nadir of the critical period when expressed as a percentage of dark reared to

normally reared animals (DR/LR). This agrees with published research that also found significant temporal reductions in the NR2A:NR2B ratio in dark-reared mice over the same time period (Chen and Bear, 2007). Changes in the ratio of NR2A:NR2B have been implicated as a potential mechanism for experience-dependent modulation of synaptic plasticity in the visual cortex (Quinlan et al., 1999; Chen and Bear, 2007). This result is significantly different from the opposite effect that is observed in Dab-1 mutant animals where the ratio significantly increases from 5 weeks to 20 weeks. Likewise, differences between mutant and wild-type animals at both ages also found significant effects. The percentage of dark to light reared NR2A:NR2B ratios is significantly decreased in mutant animals at 3.5 weeks and significantly higher in mutants at 9.5 weeks.

While the physiological implications of these differences remain to be determined, a review of current literature shows numerous relationships between NMDAR and components of the Reelin/Dab-1 pathway that might contribute to the significant differences in the absence of Dab-1. There is a colocalization of NMDAR and Dab-1 in neuronal postsynaptic densities (Beffert et al., 2005; Chen et al., 2005) and Reelin has been shown to enhance glutamate stimulated Ca^{2+} influx through NMDAR. This effect is negated in the absence of Dab-1 (Chen et al., 2005) confirming a regulatory role for Dab-1 in NMDAR functions. Recent research has shown that blocking exogenous Reelin can reversibly decrease the NR2A:NR2B ratio in hippocampal cultured neurons (Campo et al., 2009) which, when considered with findings presented here implicates Dab-1 as a critical intermediary in a pathway regulating not only physiological – as an adapter protein required for phosphorylation and activation of numerous other

proteins – but also anatomical differences in the subunit composition of the NMDAR. As additional studies continue to examine the roles of the relationship of the NMDAR subunits to neuronal plasticity, future physiological studies of cortical plasticity in Dab-1 mutants will provide additional insight into the role of Dab-1 during the critical period and bring to light greater understanding of their interactions.

Immunohistochemical analysis of Dab-1 Cortical Expression

Developmental Expression of Dab-1

In cat visual cortex, ocular dominance column formation resulting from segregation of LGN axon terminals within layer IV occurs throughout the critical period extending from approximately three to six weeks of age (LeVay et al., 1978). Physiological cortical plasticity, as determined by monocular deprivation, varies in its time course among different layers of the visual cortex. Several studies have shown that plasticity in layer IV ends earlier than the deeper or superficial layers (LeVay et al., 1980; Mower et al., 1985; Daw et al., 1992). Results from this thesis of quantitative assessments of Dab-1 laminar development in cat visual cortex show some correlations between known anatomical or physiological critical period developmental events. There is a developmental decline in total immunopositive cell density across all layers that is significant through the critical period. The greatest decline in cell density is found between one week and the peak of the critical period. There is, however, also a

significant decline in total Dab-1 positive cell density from the peak (five weeks) to the nadir (20 weeks) of the critical period.

Prior to the critical period there is a significant difference in cell density between developing cortical lamina at 1 week of age with more immunopositive cells in the upper cortical plate (developing layers II/III) than the lower CP (developing layer IV). This disparity disappears by the peak of the critical period (five weeks) and remains insignificant throughout development.

A potential role for Dab-1 expression in facilitation of critical period events is furthered by the immunohistochemical analysis of visual cortex seen in this study. While the developmental changes are not specific to any laminar processes, quantitative analysis shows a uniform pattern of Dab-1 protein expression across all cortical layers that is higher at the peak of the critical period and declines significantly through the nadir.

Dab-1 Immunohistochemistry in the Critical Period in Normal and Dark Reared Cats

Immunohistochemical studies of Dab-1 cortical localization comparing normal and dark reared cats at the peak and nadir of the critical period found cellular staining patterns that also support the visual cortex total protein expression data presented. This provides further support for a potential role of Dab-1 in visual cortex critical period development.

There is a moderate conundrum found in the above immunohistochemical expression patterns that will require additional research. While protein and immunohistochemical analysis of Dab-1 demonstrate the lower levels of expression normally seen in candidate plasticity genes in dark-reared animals at the peak of the critical period, with Dab-1 this is apparently not merely of a delayed onset of expression as previously suggested (Mower, 1991). In normally reared animals, Dab-1 cellular staining was found at higher cell densities across all layers prior to the critical period and was already in decline by the peak. This would indicate that if dark rearing was to simply delay Dab-1 expression, it should be higher in dark-reared five week animals compared to normal. Likewise, at the nadir of the critical period, if Dab-1 were to simply be expressed on a delayed time course by dark rearing, it should be lower than the levels expressed in dark reared, five- week visual cortex. The results presented here showed the opposite effect. One possible explanation for this theoretical conflict is the proposed dual roles for Dab-1 in embryonic vs. postnatal development and function of the nervous system. Clearly, the Reelin/Dab-1 pathway is integral in neuronal migration during development of numerous parts of the nervous system (Bock et al., 2003; Stolt et al., 2005; Andrade et al., 2007; Yabut et al., 2007; Frotscher et al., 2009). It has also been shown to play an important role as not only a kinase switch but also a structural scaffold for numerous signaling complexes (Feng and Cooper, 2009). Likewise, Dab-1 has been demonstrated to play a role in the regulation of NMDA receptor subunit organization and facilitation (Beffert et al., 2005; Chen et al., 2005; Sinagra et al., 2005; Campo et al.,

2009). Perhaps visual experience upregulates some of these functions early in normal development causing the high concentrations of Dab-1 expression seen in normal one week animals but those components of Dab-1 function that operate regardless of experience, possibly playing a role in critical period plasticity, follow the typical "plasticity" pattern of bidirectional regulation seen in the protein analysis and immunohistochemical comparisons of normal and dark reared animals presented. Future studies that may elucidate some of these issues should include electrophysiology to determine any functional differences in cortical plasticity between normal and Dab-1 mutant animals.

This observation also causes one to question our proposed understanding of the "anti-plasticity" protein expression pattern, such as that of Munc13-3, seen in comparisons of normal and dark reared animals at the peak and nadir of the critical period. This pattern shows lower expression in normal than dark reared at the peak of the critical period and higher in normal than dark at the nadir. As in the Dab-1 analysis, there is a potential conflict if one interprets this data as a simple delay in the entire time course of plasticity caused by dark rearing. If this hypothesis is to be considered without other influences, it would indicate that Munc13-3 expression must be very high at, or even prior to, birth and quickly be down-regulated postnatally to reach the low levels seen in normal 5 week animals. As the critical period wanes, protein expression then increases back to the high levels seen at the nadir. Further developmental research both prior to and after the critical must be conducted to investigate this proposal.

Visual Evoked Potentials – establishment of an electrophysiological recording protocol

In order to continue research on the physiological effects of Dab-1 and Munc13-3 mutations on critical period plasticity a significant amount of time was devoted to the development of a successful laboratory set-up and protocol to monocularly deprive mice and reliably record visual evoked potentials (VEPs) from the binocular segment of visual cortex in awake mice. Although a basic protocol was generously shared with our laboratory by the laboratory of Mark F. Bear, PhD (MIT, Cambridge, MA), our facility was not originally equipped to perform these experiments and many structural and procedural adaptations needed to be developed to reliably record awake VEPs. These adaptations included: proper anesthesia determinations for surgical survival, modifications to the design of both the surgical and recording apparati, surgical techniques for electrode implantation and monocular deprivation, visual stimulus and trigger pulse modifications/synchronization and circuitry set-up for proper recording of VEPs. The finalized protocol is presented in the Results section of this dissertation but some of the more significant protocol issues are discussed below.

The initial issue arose with attempts to anesthetize mice with a survivable level dosage of general anesthesia for the extended surgical procedures (greater than 45 minutes) required to permanently implant recording electrodes in visual cortex. Ultimately a proper dosage of a Ketamine/Xylazine mixture was determined (90mg/kg Ketamine and 9mg/kg Xylazine) with a booster dosage of Ketamine only (9mg/kg) for

administration during prolonged surgeries where recovery of the mouse was evident prior to procedure completion as described in the protocol.

Surgical procedures for electrode implantation, although well described in the guidelines presented by the Bear lab, were also modified based primarily on apparatus differences and recording failures. These included alterations to ensure stable post fixation, accurate electrode placement and elimination of potential sources of circuitry conflicts (i.e. improper conduction by use of a conductive acrylic applied to protect surgical site and cement electrodes in position or through accidental skull contact with wire of recording electrode).

Although effective for recording purposes the procedure is currently limited in that electrode placement is only extremely reliable for a period of one to two weeks. Beyond this time period, especially in younger animals when the skull is growing more rapidly, the entire "skull cap" (acrylic with embedded electrodes and fixation post) frequently becomes dislodged and the animal is no longer useable for recording. Thus data analysis can only be conducted between groups of animals under the same treatment conditions.

Multiple visual stimuli were tested prior to development of final protocol. A specially designed flash stimulus was designed and manufactured by Alan Gibson (UNIVERSITY, Location). Testing of this stimulus revealed two concerns. The first was an intensity of response that was too strong to be useful for our research and the second

was that while the frequency of the stimulus was adjustable, it was not specifically measurable to be accurately set to a determined rate for repeat recordings. It was determined that a standard computer-generated gradient was the most effective stimulus for our research, ultimately it was determined that a 0.03 cycle/degree square-wave gradient alternating at a set rate (approximately 2Hz) was the optimal stimulus. Numerous rates were tested and it was determined that with the one-second recording sweep of the Tektronix oscilloscope being used a frequency of 1.98 Hz created the most reliable VEPs with minimal 60-cycle background noise.

Numerous issues arose in attempts to synchronize the trigger pulse (that initiates the recording sweep of the oscilloscope) with the visual stimulus. The flash stimulus was optimal in this regard because it generated an impulse with each flash, but the other issues discussed above precluded its use. Dr. Gibson created a trigger module with a photocell that, when secured to the computer screen, generated a 9-volt impulse coincident with each cycle of the stimulus gradient. This was an effective tool for recording reliable VEPs however there was an unexplainable delay in the latency of the response (although consistent within and between recording sessions) that caused us to seek other alternatives. The initial software utilized for the computer generated grating was described as having the capability of generating a simultaneous trigger pulse through the parallel port but multiple channel analysis of this trigger pulse compared to the one generated by the photocell trigger revealed that the computer-generated trigger was not synchronized to the visual stimulus and shifted significantly over the 60 sweeps (one minute recording) that were averaged for one record. This resulted in an overall decline

in VEP signal as each sweep was initiating a fraction of a second earlier or later than the previous essentially "averaging out," and eliminating the potential over the 60 sweeps. Through extensive efforts of Dr. Heywood Petry, and multiple software revisions, the program designer ultimately developed a computer-generated trigger pulse that was acceptably but still not perfectly synchronized with the onset of the visual stimulus. This system is currently in use and described in the Results section of this dissertation.

Successful development of this protocol allows for future monocular deprivation experiments. Both Munc13-3 and Dab-1 knockout animals will be tested to determine if either mutation causes any changes in VEP response indicating a physiological role in visual cortical plasticity. Modifications to the protocol are still being tested to obtain longer skull attachment times for electrodes in order to be able to implant electrodes, record from the mouse, monocularly deprive that animal for a period of time, reopen the eye and record again. This would allow for pre- and post-monocular deprivation comparisons within the same animal allowing it to be used as its own control for data analysis.

Future Directions

Based on the results presented here, the most logical immediate progression of this research will be to conduct the electrophysiological studies of the effects of monocular deprivation on visual cortical plasticity in the Munc13-3 and Dab-1 mutant animals. Now that a formal procedure and laboratory set-up have been established and

the animals are viable and housed under the care of the Mower lab this should be a fairly straightforward experiment to determine whether there is a physiological effect on cortical plasticity due to the absence of either of these genes. As additional genes that express the critical period bidirectional regulation indicative of candidate plasticity are identified the opportunity will be present for knockout mice lacking these genes that could be tested in a similar manner (if the mice are viable).

Another worthwhile experiment for consideration for future research would include a developmental study of protein expression levels of both Dab-1 and Munc13-3 in both normal and dark-reared animals from birth through the early critical period. While the results presented here address immunohistochemical changes in normal laminar and cellular expression of Dab-1 protein through this time period and protein expression levels in normal and dark reared animals at the peak and nadir of the critical period, they do not provide a complete quantification of total protein expression levels assessed through Western blot analysis. The results here showed a significant decline in immunopositive cells from one week that continued through the critical period into adulthood and the opposite direction of Dab-1 positive cell density seen in dark reared animals at the peak and nadir of the critical period. Perhaps a more extensive analysis of cortical protein expression throughout development would offer a greater understanding of whether lack of visual stimulus completely inverts protein expression levels of Dab-1 throughout visual cortex development or whether the rise in Dab-1 expression at the later time point in dark-reared animals is truly correlated with the delayed peak of the critical period and declines again subsequent to that developmental milestone.

Likewise, extending the time course of investigation of both pre- and post-critical period for Munc13-3 protein expression and immunohistochemical analysis would be an insightful investigation into the theory of an overall delay in the entire time course of plasticity caused by dark rearing. If the basic premise holds true, then based on the results of these experiments a proposed expression pattern can be predicted for Munc13-3 that will be high at birth (or perhaps even prenatally), diminish as observed through the peak of the critical period, return to high levels by the nadir and remain elevated throughout adulthood while cortical plasticity remains low. If this pattern is indeed revealed, immunohistological and electrophysiological evaluations would certainly be merited to determine if there are laminar differences in Munc13-3 expression that correlate with known developmental milestones in visual cortical plasticity. Further considerations of Munc13-3 in this research should also include the relatively low expression of Munc13-3 compared to the other Munc13 subunits that do not show any susceptibility to dark-rearing (Yang et al., 2007). While the changes in Munc13-3 expression are significant throughout the critical period in visual cortex the low overall expression levels would seem to indicate that very small changes in protein expression may have very significant physiological effects.

The knowledge gained from the Dab-1 and Munc13-3 electrophysiological studies and a complete developmental protein analysis, when considered with ongoing research in other labs identifying and clarifying the ever-increasing functions of the Dab-1 protein and Reelin/Dab-1 pathway, and the potential significance of relatively small

changes in Munc13-3 expression levels, may help further the understanding of which of these functions might be simply developmentally regulated and which are regulated based on visual experience.

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CURRICULUM VITAE

NAME: Paul Joseph Kiser

ADDRESS: 3429 Stony Farm Dr
               Louisville, KY 40299

DOB: Hammond, IN – September 10, 1969

EDUCATION
& TRAINING: B.S., Biology
               The Florida State University
               1987-1992

               M.S., Anatomical Sciences and Neurobiology
               University of Louisville School of Medicine
               1993-1997

CERTIFICATIONS: Internationally Certified Prevention Specialist, 2008

PROFESSIONAL
SOCIETIES: Kentucky Prevention Network, 2009

PUBLICATIONS: Yang CB*, Kiser PJ*, Zheng YT, Varoqueaux F, Mower GD
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                *Co-First Authors