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DEVELOPMENT AND PLASTICITY OF THE RETINOCOLLICULAR PROJECTION

by

MARIA MAGDALENA CARRASCO

Under the Direction of Sarah L. Pallas

ABSTRACT

Brain development and function depend on intrinsic and extrinsic factors. In particular, the proper functioning of sensory systems can be altered according to the quality of extrinsic sensory information received during life. In this context, questions concerning neuroplasticity take on special relevance when considering that sensory experience has a big impact on the degree of plasticity of the brain. In this thesis, we have sought to understand how visual deprivation affects the development and maintenance of visual centers in the brain and the role of visual deprivation on plasticity throughout life. We have addressed this question by studying the retinocollicular projection, which is the neuronal pathway that connects the retina with a visual input processing center, the superior colliculus (SC). Unexpectedly, we found that in Syrian hamsters (*Mesocricetus auratus*) the size of receptive fields (RFs) of neurons in the SC is plastic in adult animals if they have been deprived of a minimum of visual experience when juveniles. Specifically, dark-reared (DR) hamsters refine SC RFs as do their normally-reared counterparts, but they lose RF refinement if they remain in the dark after their RFs get refined. We found that a well defined period and duration of visual experience can stabilize RF size in

adulthood. Furthermore, we sought to investigate the mechanisms by which RF size is increased in adult DR hamsters. By testing the strength of intracollicular inhibition using electrophysiological and molecular techniques, we have found that visually-deprived animals have weaker inhibitory circuitry in their SC than normal animals. The quantity of GABA receptors and GABA containing neurons is decreased in the SC of adult DR animals. We propose that these results explain at least in part the RF enlargement we find in visually-deprived animals. Knowledge from this study provides general insight into sensory system plasticity in adulthood and new information about visual system development that is relevant for treatments of diseases.

INDEX WORDS: Neural plasticity, Superior colliculus, Visual deprivation, GABA, Inhibitory circuitry

DEVELOPMENT AND PLASTICITY OF THE RETINOCOLLICULAR PROJECTION

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MARIA MAGDALENA CARRASCO

A Dissertation Submitted in Partial Fulfillment of Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2008

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DEVELOPMENT AND PLASTICITY OF THE RETINOCOLLICULAR PROJECTION

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LIST OF ABBREVIATIONS

AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APV	2-amino-5-phosphonovalerate
BDNF	brain-derived neurotrophic factor
CaMKII	calcium/calmodulin-dependent protein kinase II
CREB	cAMP response element-binding
DR	dark-reared
GABA	γ -aminobutyric acid
GABA-ir	GABA immunoreactive
IEGs	immediate early genes
LGN	lateral geniculate nucleus
mEPSC	mini excitatory post synaptic current
mIPSC	mini inhibitory post synaptic current
NGF	nerve growth factor
NMDAR	<i>N</i> -methyl <i>D</i> -aspartate receptor
RFs	receptive fields
RGCs	retinal ganglion cells
SC	superior colliculus
SGS	stratum griseum superficiale
STDP	spike timing-dependent, Hebbian-type synaptic plasticity
TTX	tetrodotoxin

CHAPTER 1: **Introduction**

Although the basic plan of the vertebrate brain is highly conserved across species, its fine tuning depends in part on the actual conditions present during its development. Thus, the brain is a dynamic structure and the environment plays an important role in modifying it. Although brain plasticity has mostly been linked with early stages of the development of an organism, recent discoveries suggest that plasticity can be extended throughout life and that it is part of the normal aging process. Understanding how the brain changes according to the rearing environment and the relevant factors in that plasticity is a major challenge in neuroscience.

One of the major questions in the field of developmental neuroscience concerns the role of intrinsic and extrinsic factors in the development and plasticity of the nervous system. In the realm of sensory systems, it is clear now that both the quality and quantity of sensory experience have important effects on brain physiology and connectivity. Moreover, early sensory experience may have long-lasting consequences, altering brain function in adult life. What is not fully known, however, is how those changes take place and the mechanisms involved. The relevance of that knowledge becomes evident when examining cases of patients deprived of the normal functioning of a sensory system. It is only by understanding the mechanisms involved in brain plasticity that we can understand and learn how to alleviate the effects of anomalous sensory experience on the brain.

In this project, we have studied the role of visual experience in development and plasticity of the retinocollicular visual pathway that projects from the retina to the superior colliculus (SC) in the midbrain, using the Syrian hamster (*Mesocricetus auratus*). This model system allows us to test the effect of manipulating visual experience on a part of the brain that receives direct input from the eye, the SC. Research on the hamster's retinocollicular projection

has the advantage that neuronal properties of the SC are well described and several plasticity studies have been performed on this pathway. We investigated the mechanisms by which a lack of visual experience produces changes in the properties of neurons in the SC from different perspectives and levels of analysis. Our results provide interesting insights into the ways in which sensory experience influences the brain that can be extended to other sensory systems.

1. Activity-dependent and -independent factors in brain development and plasticity

The development and maturation of the brain is a fascinating process. In spite of the number of attempts directed toward obtaining a comprehensive understanding of how the brain develops and changes throughout an organism's life and experiences, numerous questions remain unanswered. Several events take place during brain maturation, such as refinement of neuronal morphology, synapse elimination, and modification of the quantity and composition of receptors present on neurons. Insights on how neural activity affects those processes would provide a better understanding of the extent of environmental influence on the brain.

a) Activity-independent factors: guidance molecules as an example

Topographic maps in the brain, which are projections that represent external space in an organized way, are a common feature of sensory systems. An ample set of data suggests that topographically organized projections in the brain are mainly guided by intrinsic molecular factors and not by neural activity. The mammalian retinocollicular projection and its homologue in non-mammalian vertebrates, the retinotectal projection, is a model system widely used to study molecular guidance cues in the central nervous system. The retinocollicular projection is organized in such a way that cells located close together in the retina project to neighboring

target cells in the SC. Furthermore, the SC contains an organized projection of the visual field, in which visual field axes are represented along the axes of the SC. Several kinds of evidence indicate that the gross topography in the SC is controlled by molecular gradients and that the fine-tuning process depends on neuronal activity (see Debski and Cline, 2002; and Ruthazer and Cline, 2004 for reviews).

One of the first studies of guidance molecules in the retinotectal system was performed by severing the optic nerve in frogs and allowing it to regenerate into the optic tectum (Sperry, 1944, 1963). In those experiments, the regenerating RGC (retinal ganglion cell) axons in the optic nerve maintained their original topographic position in the tectum even if the eye was surgically rotated prior to regeneration. Subsequent *in vitro* assays suggested that repulsive interactions between RGC axons and tectal neurons would explain the topographic preference of the retinotectal projection (Walter et al., 1987a; Walter et al., 1987b). Later studies identified ephrins and Eph receptors as molecules involved in guiding RGC axons in the tectum. These molecules are distributed in opposing gradients across the retina and tectum (Braisted et al., 1997; Davenport et al., 1998). Studies on transgenic mice in which A2 and A5 ephrins were knocked out showed that the map in the SC is profoundly disrupted in the absence of these ephrins (Frisén et al., 1998; Feldheim et al., 2000). Other studies have provided evidence that ephrins and Eph receptors are not only involved in axon guidance in the visual system, but are also involved in axon guidance in other brain areas (Knoll and Drescher, 2002; Quinn and Wadsworth, 2006).

b) Activity-dependent factors

Several studies have highlighted the necessity of neuronal activity for the normal development of neuronal properties in the visual system (Thompson and Holt, 1989; Shatz, 1990;

Cook et al., 1999; Zhang and Poo, 2001; McLaughlin et al., 2003), motor system (Kalb and Hockfield, 1992), auditory system (Sanes and Takacs, 1993; Kotak and Sanes, 1997), and somatosensory system (see Purves et al., 1994 for review; Wang et al., 2007). Neuronal activity can be spontaneous or driven by sensory inputs. While spontaneous activity has a relatively larger contribution early in development, particularly before sense organs mature, sensory input-driven activity is critical for the fine tuning of sensory systems. For example, in the auditory system, the acoustic environment plays a role in the formation of a tonotopic map in the auditory cortex (Recanzone et al., 1993; Weinberger, 1995; Chang and Merzenich, 2003; Nakahara et al., 2004; Yu et al., 2007). In the somatosensory system, sensory deprivation disrupts receptive field structure and responsiveness in the rat barrel cortex (Van der Loos and Woolsey, 1973; Stern et al., 2001; Shoykhet et al., 2005). These studies on auditory and somatosensory systems point out the role of sensory experience in the fine tuning of the cortical circuitry. Evidence in the visual system also shows that normal properties and maturation of visual cortex depend on normal patterns of visual input (Cynader, 1983; Mower and Christen, 1985). The current view is that both types of activity, spontaneous and sensory input-driven, contribute to brain development and plasticity at particular developmental stages.

i) Correlated spontaneous activity in development and plasticity of the visual system

The relationship between neural activity and brain development is still under debate. Some studies have argued that neural activity has an instructive role in visual system development (Stellwagen and Shatz, 2002; Torborg and Feller, 2005; Butts et al., 2007). Other studies have suggested that neural activity is instead only permissive, and thus that its pattern is not relevant in determining neuronal properties (Huberman et al., 2003). Both patterned and

spontaneous neural activity have been proposed to act through spike timing-dependent, Hebbian-type synaptic plasticity (STDP) to stabilize or destabilize synapses. Here, the central idea is that neurons firing together within a restricted time and space window strengthen their mutual synaptic contacts, and vice versa.

In the visual system of vertebrates, it is possible to find patterned waves of spontaneous activity sweeping across the retina before eye opening (Galli and Maffei, 1988; Meister et al., 1991). These waves of activity are first dependent on acetylcholine, and later, on glutamate, because they rely on the asynchronous development of cholinergic amacrine and glutamatergic bipolar cells in the retina. It has been proposed that the activation of adjacent neurons in the retina would facilitate the strengthening of their synapses with their postsynaptic target through coincidence detection by the postsynaptic neuron. The coordinate firing of presynaptic neurons could lead to refinement of retinotopy and segregation of eye-specific laminae in the lateral geniculate nucleus (LGN) (Eglen, 1999; Butts, 2002). There is an ongoing debate about whether the pattern or the amount of spontaneous activity is important for development of visual pathways. One study showed that mice lacking the $\beta 2$ subunit of the acetylcholine receptor exhibit an anomalous organization in the LGN (Grubb et al., 2003). In these animals, the absence of acetylcholine-dependent correlated activity in the retina disrupts fine grain retinotopy in the LGN, which suggests that this spontaneous activity is necessary for the normal development of this pathway (but see Sun et al., 2008). In addition, pharmacological blockade of acetylcholine receptors in the retina with epibatidine prevents eye segregation in the LGN of ferrets (Penn et al., 1998). On the other hand, a different study in ferrets showed that disrupting the patterned activity in the retina, without decreasing the absolute amount of activity, does not interfere with eye-specific lamina segregation in the LGN (Huberman et al., 2003). That study

argues for a permissive role of neural activity in brain development. In yet another study in the superior colliculus rather than LGN, the chronic pharmacological disruption of cholinergic spontaneous activity in the retina led to enlarged retinal ganglion cell (RGC) arbors (Chandrasekaran et al., 2005). That study also reports that spontaneous glutamatergic activity in the retina, which is present during the second postnatal week in mice, produces a partial refinement of RGC arbors, but is still deficient compared to that in wild-type mice. Blockade of the detection of retinal correlated activity by pharmacological blockade of NMDA receptors in the SC also argues in favor of the role of spontaneous waves of activity in the retina on the refinement of the retinocollicular projection. Chronic blockade of NMDA receptors in the SC of rats prior to visual experience leads to unrefined RGC projections in the SC (Simon et al., 1992). The same chronic treatment extended into adulthood and thus also reducing patterned sensory input, results in enlarged receptive fields (RFs) (Huang and Pallas, 2001). Although insightful, the studies are not necessarily conclusive due to possible compensations as the results of gene knockout or activity blockade treatments. Further studies would provide a more definite conclusion about the role of spontaneous correlated activity in the retina on visual pathway refinement.

ii) Experience-dependent activity in development and maintenance of the visual system

The involvement of visual experience in development of the visual system has been studied at different locations along the visual pathway. In the LGN, visually-driven activity has been shown to be necessary for maintenance of eye-specific lamina segregation (Chapman, 2000). In the same visual nucleus, visual deprivation disrupts the normal developmental increase of acuity (Mower and Christen, 1982; Blakemore and Vital-Durand, 1986), decreases cell size

(Prada, 1987; Lachica et al., 1990), and decreases the number of NMDAR (N-methyl-D-aspartate receptor)- immunoreactive cells (Fava et al., 1999). In visual cortex, the role of visual experience in defining neuronal properties has been extensively studied. Early observations showed that monocular deprivation leads to a marked decrease in the number of neurons responsive to the deprived eye (Wiesel and Hubel, 1963b). Visual experience is necessary for the normal development of orientation selectivity (Barlow and Levick, 1965) and receptive field refinement in the visual cortex. Rearing cats in a striped environment results in an increased proportion of neurons that are responsive to the experienced orientation (Hirsch and Spinelli, 1970; Sengpiel et al., 1999). Beyond the role attributed to visual experience in the development of neuronal properties, a handful of studies suggested that visually-driven activity is also necessary for their maintenance (Buisseret and Imbert, 1976; Fregnac and Imbert, 1978). Those studies argued that orientation selectivity develops in dark-reared kittens in a similar fashion as in light-reared kittens, but is lost afterwards.

In our model system, the SC, in which the effects of visual deprivation are comparatively less well known, visually-driven activity was shown to contribute to the development of orientation and speed selectivity in cats and rabbits (Hoffmann and Sherman, 1975; Fox et al., 1978). However, studies in hamster SC reported that visual experience has only a minimal role in the development and maintenance of direction selectivity and speed preference (Chalupa and Rhoades, 1978b). Also in hamsters, it was shown in behavioral experiments that dark-rearing does not modify visual orienting behavior, which depends on the SC (Rhoades and Chalupa, 1978a).

iii) The effect of visual experience on plasticity and maturation of the visual system

Brain plasticity is more extensive at relatively early stages of development. Wiesel and Hubel found that monocular deprivation early in life has profound effects on neuronal responsiveness in LGN (Wiesel and Hubel, 1963a) and visual cortex of cats (Wiesel and Hubel, 1963b). They found that kittens with one eye covered from birth to 2-3 months of age had almost no neurons in visual cortex that responded to visual stimulation of that eye, but the same results could not be reproduced if the animals were more mature at the time of visual deprivation. Thus, they described a 'critical period' for the effects of monocular deprivation in visual cortex. Since then, the monocular deprivation paradigm has been widely used to investigate the role of visual input in the development of physiological properties of neurons. Interestingly, it was found that binocular deprivation by dark-rearing prolongs the period of susceptibility to monocular deprivation in visual cortex far beyond the natural critical period (Cynader, 1983; Mower and Christen, 1985; Mower, 1991). Hence, visual input has the effect of promoting the end of plasticity to monocular deprivation in visual cortex. Whether that effect can be extended to physiological properties in other visual areas has not been extensively studied.

2. Mechanisms through which neural activity affects the development of the visual system

Normal brain development involves changes in receptor composition and distribution of excitatory and inhibitory neurotransmitters, among other factors. For example, NMDARs rich in NR2B subunits are predominant in the juvenile brain and cause the receptors to have a longer open time than receptors rich in NR2A subunits, which are predominant in the adult brain (Williams et al., 1993; Monyer et al., 1994; Sheng et al., 1994; Zhong et al., 1995). Several studies have shown that maturation of brain circuitry is modulated by neuronal activity and that

sensory deprivation results in immature molecular profiles of the affected sensory systems. In addition to neurotransmitter receptors, expression of neurotrophins, such as BDNF, NGF, and neurotrophin-3 (NT-3), which are involved in synapse maturation, modulation of synaptic efficacy and morphology, are also modulated by neuronal activity during development (Schuman, 1999; Poo, 2001). Alterations in the molecular characteristics of the brain due to a particular sensory experience could lead to profound modifications of neuronal properties.

a) NMDA system

The effects of visual deprivation on NMDARs have been studied at different levels of the visual system. In the LGN, visual deprivation causes a decrease in the proportion of neurons immunoreactive to NR1 (Fava et al., 1999), the obligatory subunit of functional NMDARs. In visual cortex of rats and cats, dark-rearing decreases the expression of the NR2A subunit of NMDARs during the critical period for ocular dominance shifts, and increases it afterwards without affecting NR2B or NR1 expression (Quinlan et al., 1999a; Chen et al., 2000b; Tongiorgi et al., 2003). In the superior colliculus, the molecular effects of visual deprivation are relatively less well studied. NMDAR-mediated currents assume a relatively greater importance for transmission of visual stimuli in the SC of dark-reared rats than in normal rats (Binns and Salt, 1998b). Because changes in NMDAR subunit composition result in alterations in the opening time of this type of glutamate receptor, visual deprivation could eventually lead to prolonged circuitry modification through STDP by increasing the time window for coincidence detection.

b) GABAergic system

In the visual system, decreased activity by means of visual deprivation or activity blockade has several effects on the inhibitory circuitry. In the retina, visual deprivation reduces the number of GABA-immunoreactive cells (Lee et al., 2006). In the cat LGN, visual deprivation decreases the expression of alpha 1, 2, 3, 4, and 5 subunits of GABA receptors (Huntsman et al., 1995; Huntsman and Jones, 1998), and in the monkey LGN GABA and GAD immunostaining are reduced after three weeks of bilateral enucleation (Hendry, 1991). Visual cortex also exhibits alterations in both the inhibitory and excitatory circuitry in visually-deprived animals. Thus, dark-rearing prevents the normal developmental increase of the GABAergic input in the rat visual cortex (Morales et al., 2002). Dark-rearing also alters the expression pattern of GABA receptor subunits, re-establishing immature levels of alpha1 and alpha3 GABA receptor subunits in cat visual cortex (Chen et al., 2001). Monocular deprivation reduces the number of GABA immunostained neurons within ocular dominance columns associated with the deprived eye (Hendry and Jones, 1986). Additional studies have shown that inhibition in the SC also seems to undergo changes under visual deprivation conditions. It was reported that dark-rearing reduces the benzodiazepine binding in the rat SC (Schliebs et al., 1986), although another study found no reduction in the number of GABA immunoreactive neurons in the SC of rhesus monkey (Mize and Luo, 1992). Taken together, these studies indicate that visual experience has a role in the development of the GABAergic circuit. Reduction in the strength of the GABAergic circuit as a result of visual deprivation could lead to significant changes of neuronal properties.

c) Neurotrophins

The expression of an important group of molecules, the neurotrophins, depends on visual experience. In the retina, visual deprivation decreases protein and mRNA levels of BDNF (Seki et al., 2003). In rat visual cortex, BDNF mRNA is significantly lower after three weeks of dark-rearing commencing at birth, whereas NGF is slightly increased after dark-rearing (Schoups et al., 1995). Alterations in the level of neurotrophin expression could lead to significant changes in physiological and synaptic properties of neurons due to involvement in synapse maturation, processes outgrowth and synapse modulation.

d) Signal transduction pathways underlying effects of neural activity on brain plasticity

The way in which neural activity affects cellular processes during refinement and development of the nervous system has been thoroughly investigated. One of the most remarkable effects of neuronal activity is the increase of presynaptic and postsynaptic intracellular Ca^{2+} , which leads to secretion of neurotransmitters and proteins, changes in synaptic efficacy, and changes in expression of neurotransmitter receptors. Hence, Ca^{2+} entering the cell through NMDARs or other ion channels can modulate gene expression either directly or through second messengers (Bliss and Collingridge, 1993; Bear and Malenka, 1994; Ghosh and Greenberg, 1995; Flavell and Greenberg, 2008). Several studies have shown that synaptic potentiation and depression depend on Ca^{2+} through molecular pathways involving CaMKII (Ca^{2+} /calmodulin-dependent protein kinase type II) (Malinow et al., 1989; Salin et al., 1996) and CREB (cAMP-responsive element binding protein) (Bourtchuladze et al., 1994). Activation of those pathways would increase neurotransmitter release probability and contribute to the insertion of AMPA-type glutamate receptors at the postsynaptic membrane, thus inducing

synaptic potentiation (Luscher et al., 1999; Malinow and Malenka, 2002). Thus, electrical activity regulates the function of these proteins and also of some 'candidate plasticity-related genes', such as *cpg15*, involved in synaptic maturation. Visual deprivation decreases *cpg15* expression in visual cortex (Lee and Nedivi, 2002) and brief visual experience causes the rapid expression of immediate early genes (IEGs) (Rosen et al., 1992). Hence, visually evoked neuronal activity has an important role in controlling the molecular profile of the visual pathway.

Visually-evoked neuronal activity, through Ca^{2+} influx, also upregulates expression of neurotrophins such as BDNF in cortical neurons (Ghosh et al., 1994) and NGF and BDNF in cultured hippocampal slices (Lu et al., 1991; Zafra et al., 1992). Moreover, BDNF can increase the levels of the NR1 and NR2A subunits of the NMDAR (Glazner and Mattson, 2000).

Implications from these studies include the possibility that neural activity can affect neuronal maturation and morphology and regulate synaptic efficacy through neurotrophic factors (Thoenen, 1995; Bonhoeffer, 1996; Schuman, 1999; Poo, 2001).

3. Why study the retinocollicular projection

The retinocollicular projection is the visual pathway that projects from the eye to the superior colliculus (SC) in the midbrain. The SC is a multisensory processing center involved in control of head and eye position, guiding of visuomotor behavior, attention, and generation of voluntary and involuntary eye movements, such as saccades (Schiller, 1972; Sprague, 1972; Dean and Redgrave, 1984; Wurtz and Optican, 1994). The SC has a layered organization. Deeper layers of the SC receive input from the auditory and somatosensory systems, which are in spatial register with the visual projection. The most superficial layer, the stratum griseum superficiale (SGS) (up to ~200 μm in depth), receives direct input from the retinal ganglion cells

(RGCs). This projection is organized in a topographic way, with a point-to-point representation of visual space given by the organized projection of RGCs. These features make the SC an appropriate model system for the study of development and refinement of topographic maps, a widespread characteristic of sensory systems.

Our model system, the retinocollicular projection of Syrian hamsters (*Mesocricetus auratus*), is well documented in terms of SC neuron properties and developmental plasticity. The fact that Syrian hamsters are altricial allows for manipulations and treatments to be performed postnatally, just as RGCs are reaching the SC. Because the development of the retinocollicular projection involves neuronal population matching and maturation of the intrinsic SC circuitry, it is an appropriate model system for the study of the mechanisms responsible for these processes. Questions regarding the role of neuronal activity-dependent and –independent development in the visual system can be better addressed by studying this direct projection from the RGCs to the midbrain than the indirect thalamocortical pathway.

4. Development of the retinocollicular projection

The SC serves as a sensory integration system involved in visually-guided behaviors. The superficial gray layer of the SC, the stratum griseum superficiale (SGS), contains a topographic representation of the visual field, provided by the ordered projection from the retina that forms a retinotopic map in the SGS. The retinotopic projection of RGCs in the SC is guided by activity-independent factors, such as molecular cues that are present in the retina and SC in concentration gradients (Ruthazer and Cline, 2004). Although the gross topography depends on molecular factors, development of the grain of the visual map in SC is an activity-dependent process. This refinement process involves cell death in the retina and SC as well as axon

collateral elimination and maturation of receptors in the SC (Finlay et al., 1982; O'Leary et al., 1986b; van Zundert et al., 2004). Experiments in which neuronal activity in the eye has been blocked by TTX injections have resulted in enlarged terminals in the developing and regenerating retinotectal projection, and enlarged tectal neuron receptive fields (Schmidt and Edwards, 1983; Kobayashi et al., 1990; Olson and Meyer, 1991). A different approach using β 2^{-/-} mice, which lack the β 2 subunit of nicotinic acetylcholine receptors, suggests that acetylcholine-dependent waves of spontaneous activity in the retina are needed for the anatomical refinement of RGC terminals in the SC (McLaughlin et al., 2003b; but see Sun et al., 2008) and RF refinement of SC neurons (Chandrasekaran et al., 2005). Moreover, chronic blockade of NMDA receptors by APV results in enlarged RGC terminals in the optic tectum (Cline and Constantine-Paton, 1989) and SC (Simon et al., 1992). The same treatment causes an enlargement of RFs of neurons in the SC of the hamster (Huang and Pallas, 2001), likely as a result of the increased RGC axon arbor size. Thus, the participation of NMDARs in the refinement of RGC terminal arbors suggests that neural activity has an instructive role that involves the coordinated firing of presynaptic and postsynaptic neurons. Due to the nature of the NMDARs, which are blocked by Mg^{2+} unless neurons are slightly depolarized, the coactivation of several inputs projecting to the same postsynaptic neuron enhances the probability of NMDAR activation and thus the molecular events triggered by Ca^{2+} influx. Support for the role of correlated activity in the refinement of this projection is also provided by experiments in which goldfish were reared with stroboscopic light, which artificially induces highly correlated activity within retinotectal projection and results in enlarged tectal RFs and RGC terminal arbors (Schmidt and Buzzard, 1993). Therefore, correlated activity coming from spontaneous synchronized firing appears to be required for the refinement of the retinocollicular and retinotectal projection.

Developmental plasticity studies have been performed using the retinocollicular projection as a model system. In hamsters, ablation of the caudal SC on the day of birth results in a compressed retinotopic map in the SC, whereas SC neurons conserve their properties, including normal RF size (Pallas and Finlay, 1989). Receptive field size, but not velocity or size tuning, is altered when APV is chronically applied in the normal or ablated SC, however (Huang and Pallas, 2001). Under APV treatment, RFs of individual SC neurons are enlarged, denoting the role of correlated activity in RF refinement in intact and partially ablated SC.

In sum, development of the retinocollicular projection depends on both activity-independent and –dependent processes that cooperate in different aspects. The role of visually-driven activity in these processes is the main topic of this study. We have investigated how visually-driven activity is involved in the refinement and maintenance of the retinocollicular projection and the SC cell response properties.

5. Receptive field properties of neurons in the superior colliculus

The SC is a layered midbrain structure, with its superficial layer, the stratum griseum superficiale (SGS), receiving almost exclusively direct input from RGCs. Properties of SC neurons depend on both their inputs and the intrinsic collicular circuitry. The input from the retina to the SC is mainly contralateral in rodents, although the far temporal portion of the ipsilateral eye projects to the rostral SC and thus it is possible to find binocularly responsive neurons there (Tiao and Blakemore, 1976). The intrinsic collicular circuitry, on the other hand, includes inhibitory neurons expressing GAD (glutamic acid decarboxylase) and GABA (Okada, 1974; Houser et al., 1983; Mize, 1988). More than one type of GABAergic neuron has been identified in the SC of cats and monkeys, but they do not seem to be organized in a layered

fashion (Mize, 1988; Mize et al., 1991; see Mize, 1992 for review). The importance of this inhibitory circuitry for neuronal response properties has not been extensively studied.

Neurons in the superficial SC of the golden hamster are exclusively visual, and respond preferentially to smaller, more slowly moving objects than neurons in visual cortex, with preferred velocities of less than 20 deg/sec (Tiao and Blakemore, 1976; Chalupa and Rhoades, 1977). Velocity tuning depends at least in part on lateral inhibition in the SC (Razak and Pallas, 2005). Most of the SC neurons also selectively respond to stimuli that are substantially smaller than their excitatory RF (Stein and Dixon, 1979). Size tuning depends on the strength of lateral inhibition within the RF (Razak and Pallas, 2006). Selectivity for direction of movement occurs in SC neurons (Tiao and Blakemore, 1976; Chalupa and Rhoades, 1977) and this property depends completely on ipsilateral input from visual cortex, although removing cortex does not alter other neuronal properties (Chalupa and Rhoades, 1977; Rhoades and Chalupa, 1978b).

6. Effects of visual experience on response properties of superior colliculus neurons

Several studies point out that the effects of alterations in visual experience on SC neurons are not as drastic as those seen in visual cortex (see Chalupa, 1981 for review). Complete and incomplete visual deprivation have been studied by dark-rearing animals and performing eyelid suturing. Eyelid suturing produces a loss of direction selectivity, reductions in general responsiveness, and reduced responsiveness specifically to rapidly moving stimuli in the SC of cats (Hoffmann and Sherman, 1975). In rabbits, visual deprivation does not change SC neuronal properties (Chow and Spear, 1974). In guinea pigs, dark-rearing or strobe rearing decrease the number of directionally selective cells in the SC (Thornton et al., 1996). In the SC of hamsters, dark-rearing produces longer latencies of “on” responses (Rhoades and Chalupa, 1978a), and

reduces stimulus size tuning (Razak and Pallas, 2006), but has no effect on direction or speed selectivity (Rhoades and Chalupa, 1978a). Strobe rearing, which allows the experience of visual pattern but not visual movement, seems to have more pronounced effects on SC than visual deprivation. Strobe rearing reduces the percentage of directionally selective neurons in the SC of hamsters (Chalupa and Rhoades, 1978a, b) and cats, in which it also produces a deficit of the Y-cell retinal input to the SC (Kennedy et al., 1980). These studies show that not only the occurrence of visual experience but also the quality of visual experience has consequences for SC neuronal properties.

Visual deprivation alters both glutamatergic and GABAergic circuitry in the SC. Dark-rearing of rats increases the contribution of NMDARs to visual responses in the SC, as shown by quantifying the effect of acute blockade of NMDARs by APV (Binns and Salt, 1998b, a). Dark-rearing decreases the effect of NR2A antagonists on SC neurons' responsiveness, suggesting that visual deprivation alters NMDAR subunit composition or receptor number (Binns et al., 1999). It also decreases binding to benzodiazepine receptors according to autoradiographic studies in the rat SC (Schliebs et al., 1986), although it has been reported that in the monkey SC, visual deprivation does not change GABA or calbindin immunoreactivity (Mize and Luo, 1992). These studies support the importance of visual experience in maintaining the excitatory/inhibitory balance in the SC circuitry and therefore its function in the proper working of the visual system. We have further investigated that issue by considering electrophysiological and molecular approaches to test the strength of intracollicular inhibition. We have found that visually-deprived animals have weaker inhibitory circuitry in their SC than normal animals and thus these results highlight the relevance of visual experience during development for the proper brain function.

7. Specific Aims of dissertation

Specific Aim 1 (Chapter 2): Determine the role of visual experience in refinement and maintenance of receptive fields of neurons in the superior colliculus. Previous studies have shown that sensory experience is necessary for the development of sensory systems. The role of visual experience in the development and maintenance of subcortical structures, such as the superior colliculus, is not fully known, however. This aim investigated the role of visual experience in the development of the retinocollicular pathway by studying receptive field (RF) refinement in dark-reared and normally reared hamsters.

Specific Aim 2 (Chapter 3): Determine how much and when visual experience is necessary to maintain the refinement of RFs of neurons in the superior colliculus. We have shown that visual experience is necessary for maintenance but not refinement of RFs in the superior colliculus. The aim of this chapter is to define the time window when visual experience can prevent RF enlargement produced by long term dark-rearing.

Specific Aim 3 (Chapter 4): Determine how visual experience contributes to maintenance of RFs in the superior colliculus by acting on the GABAergic circuitry. Our previous data have suggested that surround inhibition is decreased in the RFs of the SC of long term dark-reared hamsters, possibly accounting for the loss of RF refinement. This chapter elucidates the role of visual experience in maintaining RF refinement through its action on the GABAergic circuitry in the SC.

CHAPTER 2: **Visual experience is necessary for maintenance but not development of receptive fields in superior colliculus**

1. Abstract

Sensory deprivation is thought to have an adverse effect on visual development and to prolong the critical period for plasticity. Once the animal reaches adulthood, however, synaptic connectivity is understood to be largely stable. We reported previously that NMDA receptor blockade in the superior colliculus of the Syrian hamster prevents refinement of receptive fields (RFs) in normal or compressed retinotopic projections, resulting in target neurons with enlarged RFs but normal stimulus tuning. Here we asked whether visually driven activity is necessary for refinement or maintenance of retinotopic maps or if spontaneous activity is sufficient. Animals were deprived of light either in adulthood only or from birth until the time of recording. We found that dark rearing from birth to two months of age had no effect on the timing and extent of RF refinement as assessed with single unit extracellular recordings. Visual deprivation in adulthood also had no effect. Continuous dark rearing from birth into adulthood, however, resulted in a progressive loss of refinement, resulting in enlarged, asymmetric receptive fields and altered surround suppression in adulthood. Thus, unlike in visual cortex, early visually driven activity is not necessary for refinement of receptive fields during development, but is required to maintain refined visual projections in adulthood. Because the map can refine normally in the dark, these results argue against a deprivation-induced delay in critical period closure, and suggest instead that early visual deprivation leaves target neurons more vulnerable to deprivation that continues after refinement.

2. Introduction

Neural activity is thought to be essential for normal development in central visual structures such as the superior colliculus (SC), lateral geniculate nucleus (LGN), and visual cortex, but the specific contributions of spontaneous activity and visually driven activity remain under debate (Grubb et al., 2003; Huberman et al., 2003; McLaughlin et al., 2003b). Furthermore, little is known about how visual circuitry is maintained in adulthood, or how early deprivation might influence later maintenance and plasticity. This study addresses the unique contribution of vision itself to the development, refinement, and maintenance of visual receptive fields (RFs) in SC.

It has been argued based on studies in visual cortex that visual deprivation stabilizes an early, diffuse stage of connectivity (Blakemore and Van Sluyters, 1975; Emerson et al., 1982; Cynader, 1983; Derrington, 1984; Czepita et al., 1994; Chalupa, 1995; Daw, 1995), decreases GABAergic shaping of responses (Benevento et al., 1992; Benevento et al., 1995; Morales et al., 2002), and prolongs the critical period for plasticity (Lee and Nedivi, 2002). Consistent with the diffuse terminal arbors, dark rearing throughout postnatal development can also result in enlarged cortical receptive fields, as defined electrophysiologically (Fagiolini et al., 1994). An alternative explanation for these results, however, is that the enlarged receptive fields in deprived animals result not from preservation of an early, unrefined state, but from a failure to maintain visual projections that were previously refined by spontaneous activity alone. Thus, the extent to which spontaneous and visually driven activity contribute to the development and maintenance of stimulus specificity is unclear.

Examination of the factors contributing to the development and maintenance of response properties in a well-defined subcortical system such as the retinocollicular projection could help

to resolve the separate roles of vision and spontaneous activity. In rodents the superior colliculus plays a prominent role in visual perception. Moreover, unlike cortical ocular dominance column formation (Crowley and Katz, 1999; Crowley and Katz, 2000) or orientation tuning (Fregnac and Imbert, 1978; Crair et al., 1998; Chapman et al., 1999), RF refinement in the visual midbrain is known to require retinal activity (see Udin and Fawcett, 1988 for review), making it in some ways a better model system for studying the role of retinal activity in visual development.

There is substantial evidence suggesting that patterned, locally correlated retinal activity is required for development of subcortical retinotopic maps. In the superior colliculus (SC), after activity-independent establishment of gross retinotopy, the retinocollicular projection progressively refines, as measured by a reduction in the size of single unit receptive fields (Fortin et al., 1999; Huang and Pallas, 2001) and a corresponding reduction in retinal axon arbor size and extent (Simon and O'Leary, 1992; Yates et al., 2001). Blocking retinal activity with TTX prevents the normal refinement of retinal axon arbors in developing or regenerating retinotectal projections (Harris, 1980; Meyer, 1983; Schmidt and Eisele, 1985; O'Leary et al., 1986a; Schmidt and Buzzard, 1993), as does synchronizing activity across the retina with strobe rearing (Chalupa and Rhoades, 1978b; Schmidt and Eisele, 1985; Schmidt and Buzzard, 1990). Blocking NMDA receptor dependent activity from birth, which neither alters the level of activity nor blocks retinocollicular transmission, also prevents RF refinement (Huang and Pallas, 2001), and knockout of the $\beta 2$ acetylcholine receptor gene has the same effect (McLaughlin et al., 2003b). It has been reported in hamster SC that dark rearing has little effect (Chalupa and Rhoades, 1978b; Rhoades and Chalupa, 1978a). Thus, whether spontaneous activity is sufficient or whether vision is necessary for the developmental refinement and maintenance of

retinocollicular projections in mammals remains unclear. We have investigated this issue in the present study.

We tested the hypothesis that refinement of receptive field size in the SC would be delayed or prevented in the absence of visual experience. We also investigated whether vision would be necessary to maintain refined RFs, even if refinement was delayed by visual deprivation. Contrary to expectation, we found that the receptive fields in SC became fully refined in the dark, without any delay, yet they could not be maintained if animals remained in the dark as adults. These results are unexpected and important for understanding how early experience may influence the ability to recover from temporary vision loss late in life.

Some aspects of this study have been published previously in abstract form.

3. Methods

A total of 122 Syrian hamsters (*Mesocricetus auratus*) of different postnatal ages between P17 and P362 were used in this study. We chose Syrian hamsters as our model system because, although their visual system is much like that of rats and mice, they are born at an earlier stage of brain development, facilitating manipulations of early developmental events. All of the procedures used on animals met standards of humane care developed by the National Institutes of Health and the Society for Neuroscience and were approved by the Institutional Animal Care and Use Committee.

a) Rearing conditions

Syrian hamsters were obtained from Charles River Laboratories (Wilmington, MA) or bred in house. Normal hamsters were kept on a 14h/10h light/dark cycle. Dark-reared (DR)

hamsters were maintained in a light-tight, dark room from before birth and exposed only to a dim red light for husbandry purposes (not visible to Syrian hamsters (Huhman and Albers, 1994)). These conditions were maintained until the recording session. All were acute preparations.

b) Surgical procedures

Animals were prepared for terminal electrophysiological recordings as described previously (Pallas and Finlay, 1989; Huang and Pallas, 2001). Each animal was anesthetized with urethane (0.7 g/ml; 0.3ml/100g body weight in 4 i.p. aliquots at 20-30 min intervals), an anesthetic that has minimal effect on subcortical neurotransmission (Maggi and Meli, 1986). After surgical exposure of the SC, visual cortex was aspirated bilaterally in order to visualize the SC. Removal of cortex has no effect on SC neuron receptive field properties in hamsters, except for a loss of direction tuning (Rhoades and Chalupa, 1978b). The brain was kept covered with sterile saline solution, and the eye was protected by a custom designed, plano contact lens throughout the experiment. In some of the youngest animals, an endotracheal tube was placed in order to facilitate respiration. The animal was placed in a stereotaxic device and the conjunctivum was stabilized with 6-0 silk suture to prevent movement of the contralateral eye (Pallas and Finlay, 1989). Anesthesia level was periodically monitored during experiments by checking withdrawal reflexes, and supplemental doses of urethane were given if needed.

c) Electrophysiology

Teflon®-coated tungsten electrodes (1-2 M Ω , FHC, Bowdoinham, ME) were used for extracellular recording of single neurons within 200 μ m of the SC surface to ensure that all recorded units were contained in the stratum griseum superficiale (SGS, the retinorecipient layer)

in the right SC. Receptive field (RF) diameters of single neurons were plotted by hand or with a computerized method. For the manual method used in the first group of experiments, single units were electrically isolated by shape and amplitude of action potentials in response to stimulation with a penlight. Receptive field borders were plotted on a translucent dome fixed 30 cm from the eye, with the center of the dome aligned with the optic disk. A RF map was constructed by systematically recording along the rostrocaudal axis of the SC at 100 or 200 μ m intervals. Only neurons located in the rostral SC were considered for determining RF size (nasotemporal diameter), in order to be consistent with our previous studies and to provide a uniform population of cells across both experimental groups.

A computerized plotting method was used to gain greater resolution of RFs in some older animals. The data obtained by this method were analyzed separately because they necessarily yield different estimates of RF size. (The difference arises because the threshold for defining the RF edge is set differently and stimulus features are different, but the two methods are internally consistent (Pallas and Finlay, 1989). Stimuli were generated and data acquired as described previously (Huang and Pallas, 2001). Receptive field diameter of each neuron was determined by sweeping a spot of light (1 $^\circ$ diameter) from the top to the bottom of the computer monitor screen at successive nasotemporal locations. Successive sweeps started 2 $^\circ$ lateral to the previous sweep, allowing a determination of the naso-temporal extent of the RF. The light spot was swept at 5 $^\circ$ /sec for neurons that preferred slowly moving stimuli or at 30 $^\circ$ /sec for neurons that preferred rapidly moving stimuli (Pallas and Finlay, 1989). The estimated RF size did not change with the velocity of the stimulus used. Regardless of the method, we defined RF size as the naso-temporal diameter of the single unit RF. The zero position of the field was defined as the stimulus position that evoked the greatest response.

d) Analysis of RF refinement

In order to determine when RFs were refined during postnatal development, we grouped normal and DR animals into 5-day age intervals, and compared their RF diameters to those in the normal adult (>P80) using a Kruskal-Wallis One Way Analysis of Variance on Ranks, and Dunn post hoc pairwise comparisons. Within age groups, comparisons between normal and DR animals were made using a Mann-Whitney Rank Sum test.

e) Plotting of RF symmetry

We analyzed the symmetry of RFs by measuring the response level at progressive distances from the RF center. A ratio of the nasal location compared to the temporal location where response levels fell to 20% of maximum provided an estimate (Asymmetry Index) of how sharply the response declined on one side of the RF center (defined as 0) compared to the other. An Asymmetry Index exceeding one indicates that the decline in the response on the temporal side of the visual receptive field was sharper than on the nasal side, and vice versa.

f) Plotting receptive field substructure

To determine the extent of inhibition both within the RF and in the surround, two spots of light (diameter 1° each) were swept in parallel from the top to the bottom of the monitor. The second spot of light was swept at successive distances away from its previous location, while the first spot was always swept through the center of the RF. Each stimulus pair was repeated 3-7 times. The response to the two spots of light was normalized to the response elicited by the center spot presented alone. This allowed us to determine the spatial extent and strength of inhibition of the response to the first spot as caused by the second spot.

4. Results

In order to determine the effect of visual deprivation on retinocollicular map topography and refinement of receptive fields (RFs), we recorded extracellularly from single units in the superficial layers of the SC. Included in the study were 404 units from 68 normal hamsters and 409 units from 46 dark-reared (DR) hamsters. The ages of the animals ranged from postnatal day (P)17 to P362. Eye opening in Syrian hamsters occurs between P12 and P14, and sexual maturity occurs between P60 and P90. We also recorded 115 units from 8 hamsters whose dark rearing commenced at P60 and extended on into adulthood.

a) Dark rearing has no effect on gross retinocollicular map topography

The retinocollicular projection in rats is roughly retinotopic prior to eye opening (Frost et al., 1979; Yhip and Kirby, 1990; Simon and O'Leary, 1992). Our recordings revealed that the same is true in hamsters; an orderly map of visual space was present in all normal animals, at all ages (Fig. 2.1A). In the youngest normal animals examined (P22), an ordered retinotopic map was already present in the SC. We also found an orderly retinotopic map in animals dark reared from birth, at all ages (from P17 to P150) (Fig. 2.1B). There were no differences in the rate of change or in the linearity of RF position along the rostrocaudal axis of the SC between the normal and DR groups (Normal: 53.2 ± 4.15 deg/mm mean \pm SEM, $n = 77$ neurons from nine animals; DR: 48.2 ± 3.11 deg/mm, $n = 57$ neurons from eight animals; $p > 0.3$, t-test; $r \geq 0.90$ for all cases). These results are consistent with previous findings that patterned sensory input is not necessary for the development of gross retinocollicular topography (Harris, 1980; Thornton et al., 1996).

b) Receptive field refinement is complete by seven weeks of age

We assessed the refinement of the retinocollicular projection by measuring receptive field size (nasotemporal diameter) of single units in the SC. We observed that in normal animals, RF diameter decreased with age (Fig. 2.2) and RF borders became more sharply defined. In the SC of animals <P40, visual responses were robust in the RF center, but were less reliably elicited at the edges of the RF. As a result, measured variability in RF sizes was greater in young animals and became less variable with age. The extracellular recording methods used here cannot distinguish whether this variability might be caused by a rapid maturation of lateral inhibition or by a loss of weak excitatory inputs at the RF edges, but both processes likely contribute (Simon and O'Leary, 1992; Shi et al., 1997). In normal adults (>P80), RF diameter averaged $19.4 \pm 0.56^\circ$ (n=32 neurons). In order to quantify the time course of refinement, we divided postnatal development into 5-day age intervals and compared the mean RF size in these different age groups to that in normal adults (Fig. 2.2). By P46-51 in normal animals, RFs had refined to their adult size (mean RF diameter $21.3 \pm 0.84^\circ$, n=21 neurons). Prior to P46, RFs in normal animals were significantly larger than those measured in adults ($p < 0.05$, One Way ANOVA on Ranks). Within the group of normal adults, RF size did not vary significantly with increasing age beyond P46 ($p > 0.15$, ANOVA on Ranks), demonstrating that normal levels of visually driven activity are sufficient to maintain the refined map. The RFs in normal animals remained stable at least up to 12 months of age, the oldest age examined.

c) Dark rearing has no effect on refinement of the retinocollicular projection

We reasoned that if visually driven activity is needed for the refinement process, then RFs would fail to refine in DR animals and thus average RF size would be larger than normal.

Alternatively, if the correlated “waves” of spontaneous retinal activity that are present from birth to eye opening (see Wong, 1999 for review) or persistent spontaneous activity in the SC (Itaya et al., 1995) could compensate for a lack of visual input, then refinement might be delayed or only partially completed rather than prevented altogether. In the DR animals, contrary to our expectation, we found that dark rearing neither prevented nor delayed the refinement process. Receptive field diameters in the DR group, as in the normal group, attained normal adult size by P46-51 ($21.8 \pm 1.08^\circ$, $n=33$) (Fig. 2.2). Receptive fields in DR animals remained stable in size between P46 and approximately P80, and were not significantly different in size from those in normal animals within the same age groups ($p>0.05$, Mann-Whitney Rank Sum Test) up to P80. These findings demonstrate that refinement follows the same time course for both normal and DR animals. These results support the interpretation that visually driven activity is not necessary for the refinement of RF size in SC neurons and that visual deprivation does not alter the time course of this process.

d) Dark rearing causes a failure to maintain RF size

After learning that RFs could refine in the dark, we hypothesized that continued dark rearing might result in a failure to maintain a refined retinotopic map. Indeed, in DR animals older than P80, RFs were enlarged significantly beyond normal size (Fig. 2.2), suggesting a failure to maintain RF refinement (DR >P80: mean RF diameter $29.9 \pm 1.28^\circ$, $n=36$; $p<0.001$ compared to normal adults at $19.4 \pm 0.56^\circ$, $n = 32$). In order to examine the transition from developmental refinement to subsequent maintenance, we looked more closely at RF size between P70 and adulthood, using a more precise computerized plotting method (Fig. 2.3). We compared four DR age groups (P70, P80, P89 and P125-362) to the normal adults ($\geq P75$) using a

Kruskal-Wallis One Way Analysis of Variance on Ranks and Dunn post hoc comparisons. We found that despite the chronic lack of visual experience, RFs of SC neurons in DR hamsters had normal adult RF diameters by P70. There was no significant difference in RF size of SC neurons between normal adults ($\geq P75$) and P70 DR animals (Fig. 2.3A and B; normal adult: $9.7 \pm 0.30^\circ$, $n=71$; P70 DR: $9.6 \pm 0.42^\circ$, $n=14$; $p>0.05$, One Way ANOVA on Ranks). However, consistent with the hand-plotted data in Figure 2.2, we observed a progressive loss of refinement of the excitatory RF under prolonged dark rearing. DR animals $\geq P80$ had enlarged RFs compared to normal adult RFs (P80 DR: $13.6 \pm 0.56^\circ$, $n=23$; P89 DR: $14.1 \pm 1.16^\circ$, $n=14$; P125-P362 DR: $15.6 \pm 0.55^\circ$, $n=66$, $p<0.05$, One Way ANOVA on Ranks). A finer analysis of RF diameter in the normal adults confirms that receptive fields are stable in size throughout adulthood in normal animals (Fig. 2.3C) (life span for these animals is approximately one year). Together these data show that a loss of refinement in the RFs occurs in the continued absence of light beyond early adulthood. It appears that the visual receptive fields in DR adults may continue to degrade further into adulthood. The P125-P362 DR animals had slightly larger receptive fields than the P80-P89 DR hamsters (P80-P89 DR: 13.7 ± 0.55 , $n=37$; P125-P362: $15.6 \pm 0.55^\circ$, $n=66$; $p<0.02$, Rank Sum Test).

e) Dark exposure commencing after P60 does not affect RF size

To address the possibility that dark rearing can destabilize receptive fields regardless of when it occurs, we placed animals in the dark at P60, after the map would have reached its adult level of refinement in normal and DR animals, but prior to the time when animals that had been dark reared from birth would exhibit enlarged RFs. In these animals, RF size remained stable regardless of the amount of time spent in the dark. This was true up to 198 days, the last time

point tested (Fig. 2.4) (RF diameter: normal >P80: 19.4 ± 0.56 ; P122-P139 DR at P60: 19.4 ± 0.38 ; P177-P198 DR at P60: 17.9 ± 0.46 ; $p > 0.05$, One-way ANOVA on Ranks) (mean RF size of both age groups together = 18.9 ± 0.30 , $n=115$; $p > 0.4$, t-test, compared to normal adult). These results indicate that early visual experience is sufficient to protect against the detrimental effects of visual deprivation later in life.

f) Dark rearing alters the symmetry of receptive fields in SC

To examine how this loss of refinement might occur, we looked at the fine structure of receptive fields in SC using the computerized plotting method. We found that prolonged dark rearing altered not only the size but also the shape of the excitatory RFs. Although neurons in the P70-P89 DR group had a symmetric RF structure as in normal animals (see representative examples in Fig. 2.5A, B), DR animals older than P90 had asymmetric RFs that were expanded toward one side of the visual field (the nasal side in all cases so far examined) (Fig. 2.5C). In addition, dark rearing resulted in some neurons having more than one spatial peak in responsiveness (Fig. 2.5D). This was never observed in normal adult animals, either in this study or our several previous studies of adult hamster SC. Calculating the ratio of nasal to temporal RF extent (defined as the asymmetry index, AI) in neurons with a single response peak revealed that in the population of normal animals ($n=71$) and in the population of dark reared animals younger than P90 ($n=53$) the RFs were fairly symmetric (normal adults: $AI = 1.14 \pm 0.054$, $n=71$; <P90 DR adults: $AI = 1.12 \pm 0.05$, $n=53$) (Fig. 2.5E). However, in older adult DR hamsters (P125-P362), RFs were on average less symmetric than normal (DR P125-P362: $AI = 1.56 \pm 0.15$, $n=42$; $p < 0.05$ compared to >P90 normal adults, One Way ANOVA on Ranks) and were always biased in the nasal, not temporal, direction. This change in symmetry could result

from a loss of inhibition on one side of the RF, and thus we next examined the contribution of surround inhibition to the responsiveness of SC neurons in different parts of the RF, in DR adults compared to normal adults.

g) Dark rearing alters the strength of the inhibitory surround

In order to assess the spatial arrangement and strength of inhibition within the RF in normal and DR adults before and after 3 months of age, a second visual stimulus was placed at varying distances from the central stimulus as both were swept through the RF (Fig. 2.6). The center of the receptive field was defined as the location where the response to a single stimulus was highest. Response levels to the addition of the second stimulus at the other RF locations were normalized to the maximum response. This analysis revealed that inhibition was enhanced considerably beyond normal within the temporal part of the RF in 2-3 month old DR animals (n=53), but was reduced somewhat within the nasal part of the RF. In DR animals older than 3 months, inhibition was significantly reduced compared to normal on both sides of the RF, but the reduction was greater on the nasal side. These changes in the spatial arrangement of surround suppression, whether arising in SC or elsewhere, could account at least in part for the expanded and asymmetric receptive fields.

h) Dark rearing has no effect on responsiveness of SC neurons in adults

To address the possibility that differing levels of SC neuron responsiveness in normal compared to DR animals could bias RF measurements, we used a subset of the digitized recordings from the SC neurons in Figure 3 to compare peak response levels in a subpopulation of the SC neurons from the adult DR and normal animals (Fig. 2.7). Levels of spontaneous

activity are low in SC of normal animals under our recording conditions, and this was also the case for dark reared animals. We found no significant difference in peak response levels between the normal and DR animals (response in spikes/sec: normal: 15.8 ± 0.85 ; dark-reared: 16.6 ± 1.21 ; mean \pm SEM; $p > 0.25$ t-test), indicating that the enlarged receptive fields in the older DR animals are not an artifact of a general increase in responsiveness, and thus further supporting the hypothesis that dark rearing results in a failure to maintain refined receptive fields in the retinocollicular pathway of Syrian hamsters.

5. Discussion

The hypothesis tested in these experiments was suggested by results from sensory deprivation experiments in visual cortex. Those results led us to expect that visual experience would be necessary for refinement, but not maintenance, of visual receptive fields in SC neurons. We found the opposite. Animals dark reared from birth to adulthood, thus experiencing spontaneous activity but no visual stimulation (see Wong, 1999; Feller, 2002 for reviews), refined their receptive fields to the same extent, over the same period of time as in normal animals, with maximum refinement attained by two months of age. This seems appropriate for a fossorial rodent like the hamster that does not exit the burrow until later in development. Prolonged dark rearing into adulthood, however, led to a failure to maintain RF size commencing long after refinement was complete. Light deprivation in adulthood had no effect. These results show that spontaneous activity is sufficient and that visual experience is not necessary for developmental refinement of the retinocollicular projection. Further, they suggest that visual experience during development is necessary for long-term stabilization of synapses such that projections can be maintained in a refined state throughout adulthood.

a) Visual experience is not necessary for establishing gross retinotopy in SC

Using physiological methods, this study demonstrates that gross retinotopy in SC is present by the third postnatal week in hamsters, and possibly earlier. Furthermore, our recordings in this study and in a previous one (Huang and Pallas, 2001) never discovered any mistargeted projections, suggesting that retinal axon targeting is quite specific from the outset. This result is consistent with previous findings in lower vertebrates (see Udin and Fawcett, 1988 for review) and birds (see Mey and Thanos, 1992 for review), and supports previous anatomical evidence that initial topography is determined independently of visually driven activity (McLaughlin et al., 2003b). The preponderance of evidence to date thus argues strongly that formation of gross retinotopy is directed entirely by activity-independent molecular guidance cues (McLaughlin et al., 2003a; Mann et al., 2004).

b) Refinement of the retinocollicular projection occurs independently of visual experience

After establishment of gross retinotopy in SC, receptive fields are refined through retinal ganglion cell death and elimination of retinal axon collaterals. In normal hamsters, we found that complete refinement of SC receptive fields did not occur until P50 or later. This is surprising given previous reports of refinement by P30 or earlier (Simon and O'Leary, 1992; Binns and Salt, 1997a) and considering that hamsters are sexually mature by approximately P60. This may be explained by the finding that the number of synapses in SC is not stable until P80 (Warton and McCart, 1989). Syrian hamsters are altricial rather than precocial, and it is unlikely that development of their visual system occurs more slowly than in other rodents commonly employed in studies of the visual system (Clancy et al., 2001). This raises the possibility that a similarly protracted refinement period occurs in other rodents but has not been noted due to

differences in the timing of experiments. Regardless, this suggests that remodeling of synaptic connectivity can occur quite late in rodents, as it does in humans (Giedd et al., 1999).

Previous studies employing activity blockade via intraocular TTX (O'Leary et al., 1986a; Thompson and Holt, 1989) have shown that retinal activity in some form is necessary for complete RF refinement. Systemic knockout of the $\beta 2$ subunit of the nicotinic acetylcholine receptor (nAChR) results in unrefined retinotopic maps in LGN and SC (Grubb et al., 2003; McLaughlin et al., 2003b), consistent with the idea that the early cholinergic waves of correlated retinal activity (Wong, 1999; Feller, 2002) are necessary to achieve a mature pattern of connections. A side effect of $\beta 2$ nAChR gene knockout, however, is compensatory change in glutamatergic waves (Feller, 2002), complicating the interpretation. Blockade of NMDA receptors in SC, either throughout postnatal development, as shown physiologically (Huang and Pallas, 2001), or in the first two postnatal weeks when spontaneous waves are present and eyes are not open, as shown anatomically (Simon et al., 1992), also disrupts map refinement. These experiments suggest that both cholinergic and glutamatergic spontaneous retinal activity are important to retinocollicular development, although their separate contributions remain to be elucidated.

Prior to the present study, little was known about the specific role of visually driven activity in receptive field refinement. An early physiological study in hamsters found that dark rearing had little effect on SC response properties or on visual behavior (Chalupa and Rhoades, 1978b). In a study on rats, it was suggested that dark rearing causes a delay in RF refinement in SC (Binns and Salt, 1997a). Refinement was assessed in that study by measuring the size of the visual stimulus that evoked the best response. Cells in the SC are tuned to stimulus size, however, and prefer stimuli much smaller than the RF (Stein and Dixon, 1979; Razak et al.,

2003). Thus the apparent delay in refinement may have reflected an increase in the time taken for development of stimulus size tuning in DR animals. In this study we measured RF size directly to avoid this potential confound.

The conclusion that retinocollicular projection refinement occurs normally in the dark has important implications for understanding visual system development. Our finding that lack of visual experience did not prevent, delay, or prolong the development of a refined visual projection supports the conclusion that spontaneous activity is sufficient to refine the retinocollicular projection. This activity could arise from cholinergic or glutamatergic retinal input, from other inputs to the SC, or from the SC itself (Itaya et al., 1995). Our results contrast with previous studies in visual cortex, in which dark rearing prevented refinement of receptive fields, reduced acuity, increased RF size, and caused a delay in both the onset and the close of the critical period for ocular dominance plasticity (Sherman and Spear, 1982; Cynader, 1983; Mower and Christen, 1985; Swindale, 1988; Daw, 1995). Some reports suggest that the critical period for ocular dominance plasticity in visual cortex never closes in DR animals and that acuity never reaches normal values (Fagiolini et al., 1994). It has been suggested that a similar delay may also occur in SC (Fosse et al., 1989; Shi et al., 1997; Binns and Salt, 1998b). Indeed, our earlier work showed that chronic postnatal NMDA receptor blockade results in enlarged RFs (Huang and Pallas, 2001). Only adults were examined, however, and it is conceivable that RF enlargement occurred secondarily to initial refinement, or that postnatal NMDA receptor blockade resulted in RF enlargement through blocking detection of spontaneous retinal activity, and not by interfering with detection of coincidence in visually driven activity.

c) Visual experience is necessary for maintenance of refined retinocollicular projections

Despite the resistance of the refinement process to visual deprivation, we found that refined RFs could not be maintained in adulthood in the continued absence of light. It is surprising that deprivation led to a failure to maintain the refined projection already present in our adult DR animals. Late light deprivation had no such detrimental effect. The loss in refinement did not result from an increase in spontaneous activity or a differential increase in overall response levels in DR compared to normal animals, although this occurs in visual cortex (Benevento et al., 1992).

The signal leading to the loss of refinement in adult RFs could come directly from a reduction in overall activity independent of its source. In all vertebrate species studied to date, waves of correlated spontaneous activity in the retina cease when the eyes open (Wong, 1999; Feller, 2002). Dark reared mice do not present abnormal characteristics, or a different time course of loss of spontaneous retinal waves compared to normally reared animals (Demas et al., 2003). Thus correlated activity levels would drop at approximately P14 and not be replaced by visually driven activity in the DR animals. Spontaneous activity levels after eye opening are quite low in SC (Huang and Pallas, 2001) and thus may not be able to compensate for the lack of visual experience. We conclude from these results that spontaneous activity is not sufficient and visual experience is necessary for maintenance of refined retinotopy into late adulthood. Because the loss of refinement occurs well into adulthood, after the projection has been fully refined in the dark, we argue that it does not result from a delay in closure of a critical period as seen in visual cortex. Rather, retinocollicular synapses formed in the dark are apparently less stable over the long term. This finding is inconsistent with a period of retinotopic map plasticity that irreversibly closes in adulthood. The mechanism responsible for map maintenance may be

independent of the mechanism underlying initial map refinement (Sawtell et al., 2003; Frenkel and Bear, 2004) and may not be subject to a critical period.

d) Possible mechanisms underlying the failure to maintain refined RFs

What could be responsible for the latent instability in retinocollicular synapses in adulthood? If light exposure is required for maturation of NMDA receptors (Philpot et al., 2001; Yoshii et al., 2003), then the failure to maintain refined RFs may result from a late deprivation-induced loss of NMDA receptors containing NR2A at the postsynaptic density. This could affect the time course of NMDAR-dependent LTP and LTD (van Zundert et al., 2004). This scenario predicts that response levels in the center of the RF would decline, however, which does not occur.

An alternative explanation was pursued in the context of this study. The delayed enlargement of receptive fields in adult DR hamsters could result from a decrease in number or strength of inhibitory inputs. We found that visual responses were biased toward the nasal side of the RF in >3 month old DR animals, but not in normal adults or <3 month old DR animals. In many SC neurons surround suppression is greater on the nasal side of the RF (Razak and Pallas, unpublished data). A uniform loss of lateral inhibition would therefore result in a nasal expansion of the RFs in those neurons, biasing the population statistics. The results from our tests of surround suppression (see Fig. 2.6) show that lateral inhibition is undergoing dynamic changes in adult DR animals. Sensory deprivation leads to a shift in the balance between excitation and inhibition in visual cortex (Kilman et al., 2002; Morales et al., 2002) that can also occur in adulthood (Hendry et al., 1994), consistent with this suggestion. A failure in the maturation of inhibitory synapses could occur via failure to anchor mature GABA receptors

(Kneussel et al., 1999), as shown for activity-dependent NMDA receptor anchoring (Philpot et al., 2001; Yoshii et al., 2003), along with a delay in the maturation of GABA receptor composition. This would be an interesting avenue for further examination.

e) Conclusion

Our results suggest that a reinterpretation of some earlier studies of visual system plasticity may be warranted. If animals that are deprived of light during development are not examined until well into adulthood, then it may appear that projections have never refined, when instead they have refined and subsequently deteriorated. Because visual defects at birth are often not corrected until many months or years have passed, it is important to understand when intervention would be of most benefit, underscoring the clinical relevance of our findings. In conclusion, our results point out the relevance of early sensory experience to the maintenance of visual receptive field properties, and suggest that disuse of sensory organs during the early postnatal period could have severe consequences much later in life than might be expected.

6. Acknowledgements

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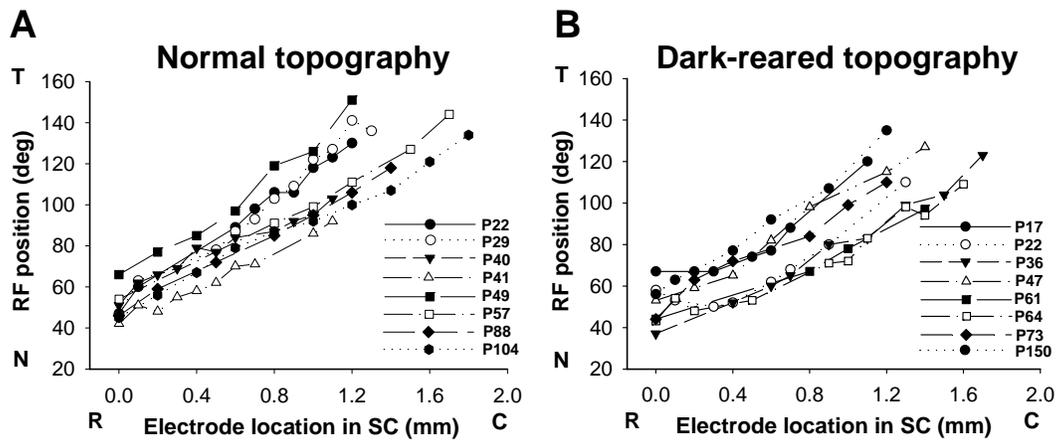


Figure 2.1. Visual deprivation does not affect map topography. Maps of visual field location in the SC for (A) normal and (B) dark reared hamsters. Symbols correspond to the center of each RF. Average values derived from linear regressions are comparable between normal (mean $53.2 \text{ deg/mm} \pm 4.15 \text{ SEM}$, $n=77$ neurons) and DR ($48.2 \pm 3.11 \text{ deg/mm}$, $n=57$ neurons) animals ($p=0.36$, t -test). N: nasal, T: temporal visual field; R: rostral, and C: caudal edge of SC.

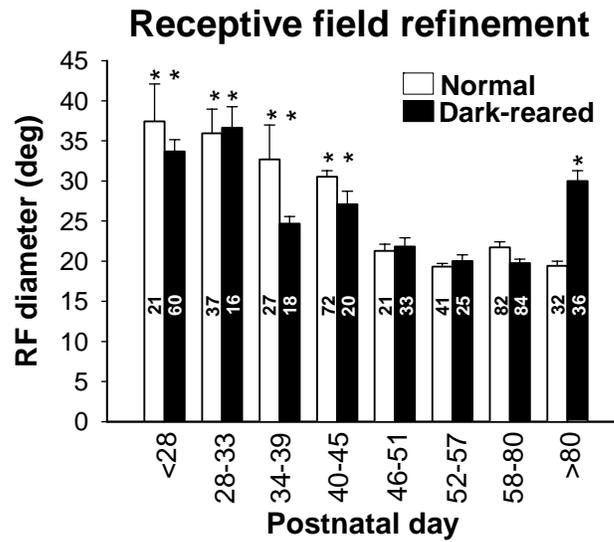


Figure 2.2. Visual experience is necessary for maintenance but not refinement of RF size. Data from single unit recordings in dark reared animals were grouped into 5-day age intervals and compared to that from the normal animals. There was no difference in RF size between normal and dark reared cases within any age group, with the exception of the >P80 group. * indicates statistical significance compared to normal adults ($p < 0.05$). Numbers inside bars represent number of single units recorded.

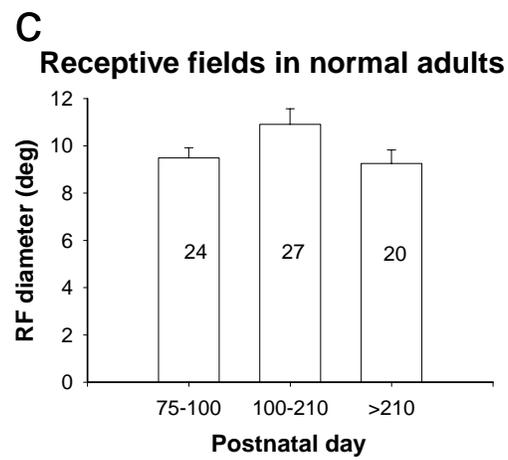
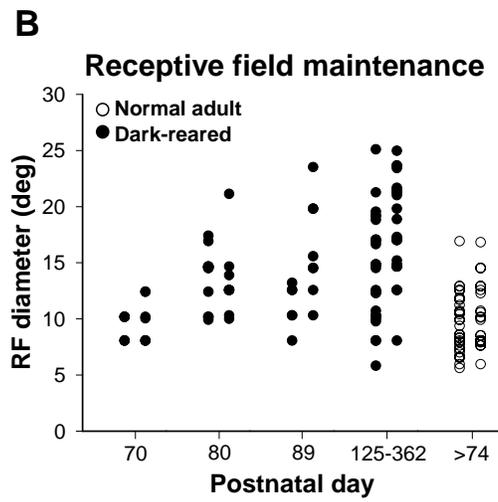
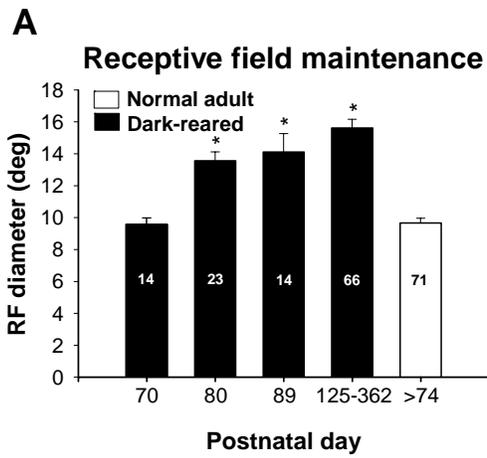


Figure 2.3. Long-term dark rearing leads to a loss of RF refinement. (A) Data from normal animals with RFs plotted by a higher resolution method showed that RF size at P70 in DR animals is similar to normal adult RF size (>P74). In animals dark reared for 80 days or more, RF diameters were significantly enlarged. (B) Data from (A) is plotted as a scatter plot (separated in two columns at each time point for visibility) to show that the increase in mean RF size arises from a gradual shift in the distribution of RF sizes with age. (C) Data from normally reared adults show that RFs maintain their refined size from P75 throughout adulthood. Conventions as in Figure 2.

Effect of late dark-rearing on receptive field maintenance

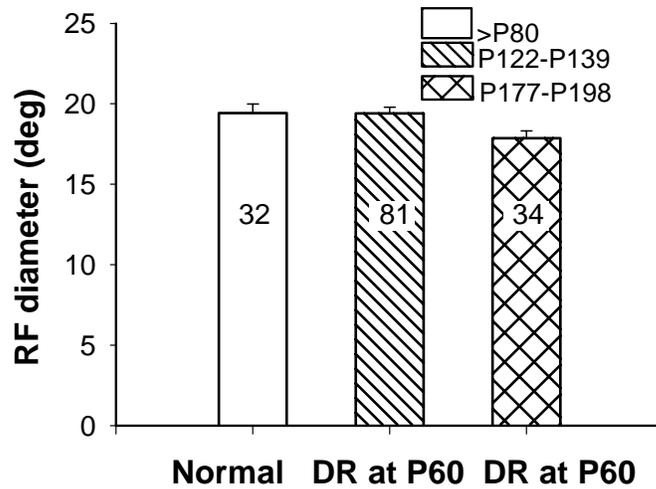


Figure 2.4. Late dark rearing does not interfere with maintenance of RF size. Animals were dark reared beginning at P60 and tested for RF size in two groups, one at P122-P139 and one at P177-P198. No significant differences in RF size were found when compared to normal adults.

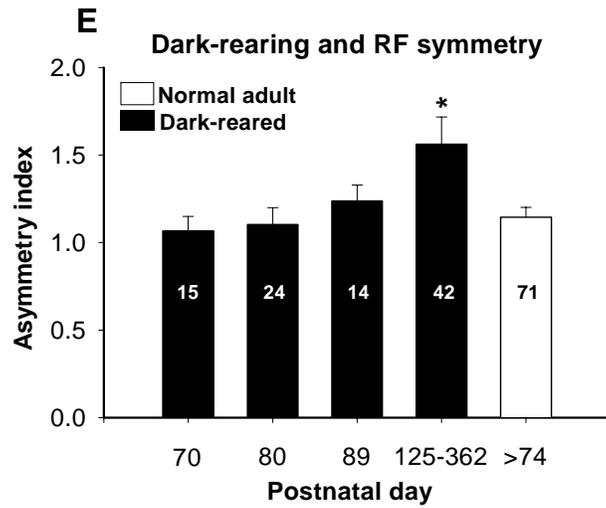
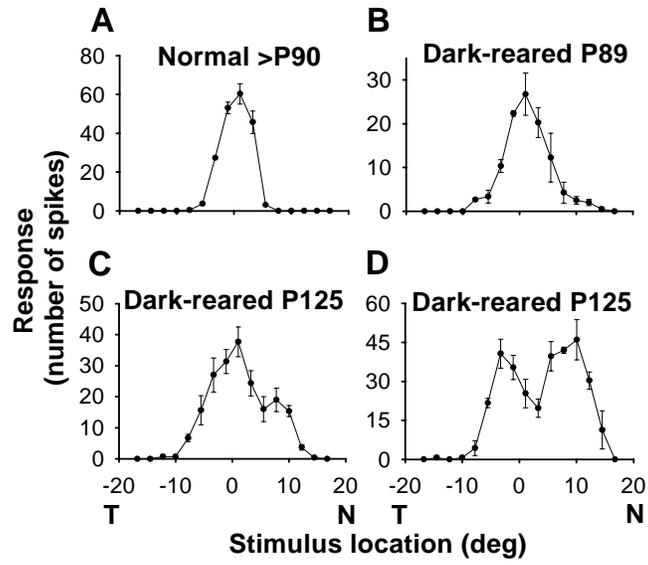


Figure 2.5. Long-term dark rearing alters RF structure of SC neurons. (A–D) Representative examples of RF structure. (A) The RFs of normal SC neurons were symmetric in the nasotemporal dimension, with a single response peak. (B) In DR animals < 3 months of age, RFs were symmetric. (C) After 3 months of dark rearing, asymmetric RFs extending further in the nasal than temporal direction were common. (D) In some neurons from DR animals at this age, multiple response peaks were seen. (E) Data from the entire population. An asymmetry index (AI) >1 indicates an asymmetric RF expansion toward nasal visual field. Asymmetries were seen only in the older DR animals (>P89). Numbers inside bars are number of single units. N: nasal, T: temporal.

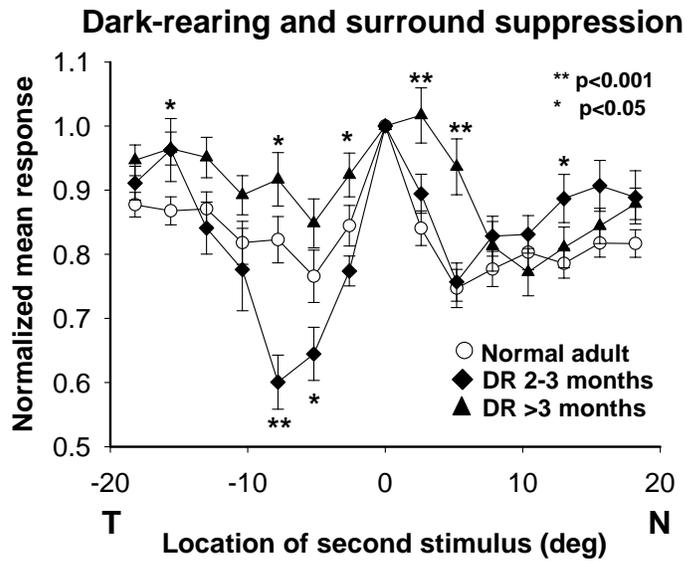


Figure 2.6. Long-term dark rearing alters the spatial arrangement of surround inhibition. Receptive field substructure was plotted using dual visual stimuli. One stimulus was swept through the RF center, and the other was swept at varying distances away along the x-axis, with nasal positive. In DR animals 2-3 months of age (n=30), inhibition was stronger than normal in near temporal visual field but slightly reduced in strength at one nasal location and one far temporal location (*). In DR animals >3 months old (n=52), inhibition was significantly reduced compared to normal animals in several locations, but highly significantly reduced in the nasal locations. Thus visual experience appears necessary to maintain the balance between excitation and inhibition in the RF.

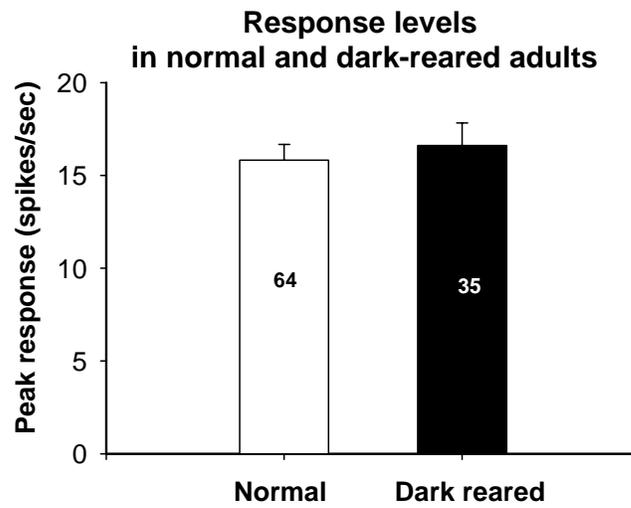


Figure 2.7. Dark rearing does not alter responsiveness to visual stimulation. The number of spikes per second to a visual stimulus in the center of the RF was measured, and was not significantly different between normal and dark reared adults.

CHAPTER 3: Early visual experience prevents but cannot reverse deprivation-induced loss of refinement in adult superior colliculus

1. Abstract

The role of sensory experience in the development and plasticity of the visual system has been widely studied. It has generally been reported that once animals reach adulthood, experience-dependent visual plasticity is reduced. We have found that visual experience is not needed for the refinement of receptive fields (RFs) in the superior colliculus (SC) but instead is necessary to maintain them in adulthood (Carrasco et al., 2005). Without light exposure, RFs in SC of hamsters refine by postnatal day 60 as usual but then enlarge, presumably reducing visual acuity. In this study we examined whether a brief period of light exposure during early postnatal development would be sufficient to prevent RF enlargement in adulthood, and whether prolonged light exposure in adulthood could reverse the deprivation-induced increase in RF size. We found that an early postnatal period of at least 30 days of visual experience was sufficient to maintain refined RFs in the adult SC. Prolonged visual experience in adulthood could not reverse the RF enlargement resulting from long term dark rearing, reflecting a loss of plasticity at this age. Our results suggest that, unlike in visual cortex, dark rearing does not indefinitely extend the critical period of plasticity in SC. Rather there is a limited time window when early experience can protect RFs from the detrimental effects of visual deprivation in adulthood. These results contribute to understanding adult brain plasticity and argue for the importance of early visual experience in protecting the adult visual system.

2. Introduction

The specific contribution of visually-driven activity to the development and plasticity of subcortical visual centers remains undefined despite considerable study. In the optic tectum

(superior colliculus, SC) of rodents, activity-independent cues establish gross retinotopy, but retinal activity, whether spontaneous (Meister et al., 1991) or visually-driven is required to refine receptive fields to their final small size (O'Leary et al., 1986a; Fortin et al., 1999). We have been exploring the mechanisms by which visual experience influences development and plasticity of retinotopic maps in the SC of Syrian hamsters (*Mesocricetus auratus*). We reported that visual stimulation is not needed for normal receptive field refinement in hamster SC, suggesting that spontaneous activity is sufficient (Carrasco et al., 2005). Vision was found to be necessary for maintaining refinement of the retinocollicular projection in adulthood, however. Thus, receptive fields (RFs) of SC neurons refine normally and without delay by 60 days after birth (P60) in dark-reared (DR) hamsters, but with continued light deprivation the refinement is lost by approximately P90, resulting in enlarged single unit RFs. Whether there is a critical time window within which visual experience must occur in order to prevent the enlargement of RF size in the SC of DR animals is unknown. Nor is it known whether visual experience after dark rearing can prevent or reverse the enlargement of RFs in the SC. Answers to these questions are relevant because understanding the role of visual experience in the maintenance of different areas within the visual system will inform strategies for treatment of patients that have been visually deprived. This issue is also important in considering risk factors for visual impairment.

Studies on visual cortex in rodents and carnivores have supported the idea that visual deprivation by dark rearing prevents maturation and extends the period during which the cortex is susceptible to visual experience. For example, dark rearing extends the critical period for ocular dominance shifts due to monocular deprivation in mice and cats (Mower et al., 1983; Mower, 1991; Fagiolini et al., 1994). In addition, dark rearing prolongs the period when LTP can be induced in the visual cortex of rats (Kirkwood et al., 1996) perhaps as a result of delaying

the normal developmental change in NMDA receptor subunit composition that reduces channel open time (Carmignoto and Vicini, 1992; also see Hensch, 2005). When light is provided, it has the effect of closing the critical period for ocular dominance plasticity in cat visual cortex (Mower et al., 1983; Mower and Christen, 1985). These studies show that dark rearing preserves visual cortex in an immature state, at least in some respects. In this study we address how dark rearing affects a subcortical visual center, the visual midbrain SC, with respect to refinement of its receptive fields.

In our previous study on hamster superior colliculus, we reported that dark rearing commencing at P60, when RFs have just refined, does not lead to a loss of RF refinement (Carrasco et al., 2005). This result is consistent with the concept of a critical period that closes when refinement is complete. An unexpected finding in this previous study, however, was that continued dark rearing into adulthood led to a loss of RF refinement. This adult plasticity cannot be explained by a deprivation-induced prolongation of the critical period, and suggests instead that although receptive fields refine normally in DR animals, their synaptic connections are less stable than in normal animals. This led to the hypothesis tested in the present study, that dark rearing until P60 (when RFs have refined in normal and DR animals) makes SC neurons more sensitive to subsequent visual experience than in normal adults, and thus that light exposure commencing at P60 might prevent the loss of RF refinement. We then tested whether visual experience after RFs have enlarged in adult DR animals could reverse the loss of refinement.

Previous studies have shown that brief visual experience has remarkable effects on visual cortex of DR animals, thus we also studied the effect of limited visual experience on the SC. In visual cortex of cats, two hours of daily binocular visual experience protects against the loss of visual acuity by monocular deprivation, as measured behaviorally (Mitchell et al., 2003, 2006).

Another study has shown, using physiological methods, that a period of visual experience as short as six hours during the critical period blocks the effects of monocular deprivation in kittens' visual cortex (Mower et al., 1983). Two hours of light exposure is sufficient to normalize the expression level of the NR2A subunit of NMDA receptor in the visual cortex of dark-reared rats (Quinlan et al., 1999a). Whether a similar time window of visual experience also prevents the effects of visual deprivation in subcortical visual areas has not been studied, to our knowledge. Given our previous result that visual experience until maturity at P60 maintains RF size in the SC even if animals are subsequently dark-reared, we hypothesized that there must be a limited time window before P60 when visual experience prevents the dark-induced failure to maintain refined RFs.

Our results suggest that the maintenance of refined RFs in adult SC neurons is highly dependent on a relatively long period of early visual experience. Interestingly, visual experience in adulthood could not reverse the effects of earlier deprivation, even though the loss of refinement did not occur until after the maturation of RF size. These results have relevant implications when considering cases in which patients have undergone periods of visual deprivation such as that produced by cataracts, retinopathy, or macular degeneration, and when considering plasticity of sensory systems in general.

3. Methods

A total of 60 Syrian hamsters (*Mesocricetus auratus*) of different postnatal ages between P61 and P360 were used in this study. We chose Syrian hamsters as our model system because although their visual system is much like that of rats and mice, they are born at an earlier stage of brain development, prior to the formation of retinocollicular synapses (Frost et al., 1979),

facilitating manipulations of early developmental events. Our long-term interest in superior colliculus stems from our and others' studies on the development and refinement of retinotopic maps (Chalupa and Rhoades, 1978b; Pallas and Finlay, 1989, 1991; Xiong et al., 1994; Huang and Pallas, 2001; Razak et al., 2003) and on the need for a relatively simple central visual structure with interesting, complex response properties that can be isolated from cortical influences (Rhoades and Chalupa, 1978b). All of the procedures used on animals met standards of humane care developed by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee.

a) Rearing conditions

Syrian hamsters were obtained from Charles River Laboratories (Wilmington, MA) or bred in house. Normal hamsters were kept on a 14h/10h light/dark cycle. Dark-reared (DR) hamsters were maintained in a light-tight, dark room from before birth and exposed only to a thin beam of dim red light [Philips 25W red A-type bulb #814546, not visible to Syrian hamsters (Huhman and Albers, 1994)] during brief, daily caretaker visits. Experimental groups used in this study are as follows (Figure 3.1): 1a) animals reared in a normal light/dark cycle, or b) reared in complete darkness from birth until the day of recording; 2) animals reared in light until either P60 or P80 and then moved to a darkroom to test whether late dark rearing can lead to enlarged RFs; 3) animals reared in darkness until P60, P93 or P130 and moved to light afterwards, to test whether late visual experience may prevent or reverse the loss of RF refinement in the SC of DR animals; and 4) animals that were exposed to light for 13, 22, or 32 days starting at P8 and then returned to the dark to test the existence of a time window when

visual experience is necessary to prevent the loss of RF refinement by dark rearing. All were acute preparations from animals that were P60 or older as indicated.

b) Surgical procedures

Animals were prepared for terminal electrophysiological recordings as described previously (Pallas and Finlay, 1989; Huang and Pallas, 2001). Each animal was anesthetized with urethane (0.7 g/ml; 0.3ml/100g body weight in 4 i.p. aliquots at 20-30 min intervals), an anesthetic that has minimal effect on subcortical neurotransmission (Maggi and Meli, 1986). The SC was surgically exposed by bilateral aspiration of the visual cortex. Removal of cortex has no effect on SC neuron receptive field properties in hamsters, except for a loss of direction tuning (Rhoades and Chalupa, 1978b). The brain was kept covered with sterile saline solution, and the eye was protected by a custom designed, plano contact lens during the experiment. In most of the animals, an endotracheal tube was placed in order to facilitate respiration. The animal was placed in a stereotaxic device and the conjunctivum was stabilized with 6-0 silk suture to prevent movement of the contralateral eye (Pallas and Finlay, 1989). Anesthesia level was periodically monitored throughout the experiments by checking withdrawal reflexes, and supplemental doses of urethane were given if needed.

c) Electrophysiology

Teflon®-coated tungsten electrodes (1-2 M Ω , FHC, Bowdoinham, ME) were used for extracellular recording of single neurons within 200 μ m of the right SC's surface to ensure that all recorded units were contained in the stratum griseum superficiale (SGS, the retinorecipient layer). Receptive field (RF) diameters (nasal to temporal) of single neurons were plotted by

hand on a translucent hemisphere fixed 30 cm from the eye, with the center of the hemisphere aligned with the optic disk. Single units were electrically isolated by shape and amplitude of action potentials in response to stimulation with a penlight. Only neurons located in the rostral SC, representing frontal visual fields, were considered for determining RF size, in order to be consistent with our previous studies and to provide a uniform population of cells across experimental groups.

4. Results

a) Dark rearing after RFs have refined does not affect RF size

We reported in our previous study that RF refinement in the SC occurs by two months of age (P60) regardless of whether visual stimulation occurs, and that this refinement is subsequently lost in animals that are continuously dark-reared from birth into adulthood (Carrasco et al., 2005). The goal of the current study was to determine whether and when exposure to light could prevent or reverse this loss of refinement. Our previous results suggest that maintenance of refined, small RFs is dependent on visual experience during adulthood. This led to the prediction that late dark rearing, after RFs have refined, would lead to a similar loss of refinement in adulthood as is produced by prolonged dark rearing. Here, we provide evidence contrary to this prediction; late dark rearing did not lead to enlarged RFs. We dark-reared P60 or P80 animals up to P198 and P360 days of age, respectively (Fig. 3.2). These animals were moved from a normal 14 light/10 dark environment to a darkroom and were maintained in the dark for four to nine months. We did not include a group dark-reared from P60 for 9 months because there was no difference in RF size at any age or treatment beyond P60. We found that RFs from single SC units were not significantly different in diameter between the late DR

hamsters and normal adult hamsters (normal adult: $19.4 \pm 0.31^\circ$ diameter, $n=92$; DR at P60: $18.9 \pm 0.31^\circ$, $n=115$; DR at P80: $19.5 \pm 0.34^\circ$, $n=72$; mean \pm SEM; $P=0.18$, One Way Analysis of Variance on Ranks). This result is surprising because the deprivation occurred at the same developmental stage when RF enlargement occurred in animals reared in the dark from birth. These results support the hypothesis that visual experience up to the age when RFs have refined in the SC prevents any later loss of RF maintenance that might result from subsequent visual deprivation.

b) Visual experience after RFs have refined preserves the refinement

We next asked whether late visual experience could prevent the RF enlargement that occurs in long-term dark-reared hamsters. We hypothesized that, although normal visual experience up to the age at which RFs have refined prevents subsequent dark-induced plasticity of RFs (see above), dark rearing could make RFs susceptible to late visual experience. To test this hypothesis, we dark-reared animals from birth up to P60, P93 or P130, and exposed them to normal visual experience thereafter (Fig. 3.3). Receptive fields of hamsters dark-reared from birth have a normal adult size by P60, start enlarging by P90, and have further enlarged by P130 (Carrasco et al., 2005). We found that when animals were dark-reared until P60 and then exposed to normal visual experience thereafter, RFs did not get any larger and were not significantly different in size from RFs of normal adult animals (Fig. 3.3, normal adult: $19.4 \pm 0.31^\circ$, $n=92$; DR until P60: 18.7 ± 0.33 , $n=58$, $P>0.05$, One Way ANOVA on Ranks). Thus, visual experience commencing at P60 in DR animals, before loss of RF refinement has occurred, did prevent dark-induced RF enlargement, but light exposure after P90, when RFs have enlarged,

did not. This result argues against the idea that the visual deprivation prolongs a critical period of susceptibility of the SC to the influences of light.

c) Visual experience after RFs have refined cannot reverse loss of refinement

In the next set of experiments, we addressed whether visual experience after loss of refinement could reverse the detrimental effects of dark rearing up to P90. Thus, animals were dark-reared until P93 or P130, after which they were exposed to a normal light cycle. We found that these animals presented enlarged RFs even after five months of normal visual experience. Their RFs were not significantly different from those of adult hamsters that were dark-reared from birth until the day of recording, but remained significantly larger than those of normal adults (Fig. 3.3, normal adult: $19.4 \pm 0.31^\circ$, $n=92$; DR until P93: $28.2 \pm 0.78^\circ$, $n=46$; DR until P130: $27.9 \pm 0.68^\circ$, $n=99$; DR >P80: $30.3 \pm 1.1^\circ$, $n=50$, $P<0.05$, One Way ANOVA on Ranks).

The experiments described above show that visual experience maintains RFs in their refined state, but cannot reverse the loss of RF refinement produced by long-term dark rearing. Additionally, visual deprivation starting at P60 does not lead to loss of RF refinement. These results, taken together, show that visual experience just after RFs have refined can prevent loss of refinement in hamster SC, but cannot reverse it. We next examined the time window when visual experience is necessary to prevent loss of RF refinement.

d) Thirty days of early visual experience protects against the effects of dark rearing

Because our results showed that visual experience up to P60 prevents any loss of RF refinement that might be induced by dark rearing after P60 (Fig. 3.2), we hypothesized that there is a distinct time window before P60 when visual experience must occur in order to protect the

retinocollicular projection from deprivation-induced failure to maintain RFs. In order to test this hypothesis, we recorded from the SC of adult hamsters that had either 13, 22, or 32 days of visual experience commencing at P8 (Fig. 3.4A). Because hamsters open their eyes at approximately P12, the actual period of visual experience was slightly shorter than the period of light exposure, although it is possible that light could activate the retina through the eyelids prior to eye opening (Akerman et al., 2002). After the period of visual experience and before P8, animals were maintained in the dark. Data were obtained from animals in the middle of adult life (P145-P205). Our results indicated that neither 13 nor 22 days of light exposure was sufficient to prevent loss of RF maintenance in light-deprived animals. The RFs from SC neurons in these groups were significantly larger than those in normal adults (normal adult: $19.4 \pm 0.31^\circ$, $n=92$; DR with 13d of light: $25.4 \pm 0.73^\circ$, $n=80$; DR with 22d of light: $27.1 \pm 0.75^\circ$, $n=53$, $P<0.05$, One Way ANOVA on Ranks). However, 32 days of light exposure commencing at P8 did prevent deprivation-induced loss of RF refinement. The mean RF size of SC neurons in the 32-day light exposure group was not significantly different from that of normal adults (normal adult: $19.4 \pm 0.31^\circ$, $n=92$; DR with 32d of light: $20.1 \pm 0.34^\circ$, $n=64$, $P>0.05$). Thus, dark rearing starting at P40 and maintained until late in adulthood did not lead to the loss of RF refinement seen in animals dark-reared from birth. Furthermore, animals with 32 days of light exposure had very stable RF sizes, even after approximately three months of visual deprivation (Fig. 3.4B). In contrast, the RFs of animals with either 13 or 22 days of light exposure were more variable during the analyzed time period. These results suggest that a period of approximately 30 days of visual experience commencing early in postnatal development has long-lasting effects on SC neuronal properties; in particular, it protects neurons from later visual deprivation-induced loss of RF refinement. Whether light exposure later in postnatal

development would have a similar effect would be an interesting question for investigation given the concept that plasticity decreases steadily with age after eye opening (see Hensch, 2004 for review).

5. Discussion

In a previous study we showed that long term-term dark rearing from birth, although it does not delay the developmental refinement of retino-SC projections, leads to a loss of refinement in adulthood, manifested as enlarged single unit RFs (Carrasco et al., 2005). In this study we have examined the time window during which visual experience is necessary to prevent this visual deprivation-induced loss of RF refinement. We have reported four main results (Fig. 3.5). First, we have shown that dark rearing commencing in adulthood does not interfere with the maintenance of previously refined RFs in the SC. Second, the loss of refinement incurred after early, long-term deprivation could not be reversed by many months of subsequent visual experience in adulthood. Third, enlarged RFs could be prevented in dark-reared animals if visual experience started at P60, when RFs have just reached their refined adult size. Additionally, we found that a >3 week period of early postnatal visual experience can prevent any subsequent loss of RF refinement caused by dark rearing late into adulthood. Briefer, early exposure to light (9 or 18 days beyond eye opening) was not protective. These results suggest that visual deprivation is promoting plasticity in the adult SC, but with certain limitations; visual experience during development can prevent the deleterious effects of deprivation, but visual experience during adulthood can only maintain the pre-existing state of refinement. As a result, loss of receptive field refinement in adult DR animals could not be reversed with visual experience.

a) Dark rearing promotes adult plasticity

Several previous studies in visual cortex have shown that plasticity is not only age-dependent, but also experience-dependent (see Hensch, 2004 for review). Visual deprivation in cats reportedly blocks the maturation of some aspects of cortical structure and function, and prolongs the critical period during which visual experience-dependent changes such as ocular dominance plasticity can take place in visual cortex (Cynader and Mitchell, 1980; Cynader, 1983; Mower et al., 1985). Something different is occurring in the SC, as seen here and in our previous study (Carrasco et al., 2005). Rather than causing the circuitry to remain suspended in a juvenile state, visual deprivation neither prevents nor delays normal maturation of RF size in the SC. A previous study in ferret lateral geniculate nucleus (LGN) has shown similarly that visually-driven activity is necessary to maintain LGN lamination (Chapman, 2000), suggesting that other subcortical visual areas also depend on vision for maintenance of at least some of their properties. In addition, a handful of studies on cat visual cortex have similarly suggested that visual deprivation causes a loss of mature properties rather than a failure to attain maturity in neurons of the visual cortex (Buisseret and Imbert, 1975; Fregnac and Imbert, 1978). More recent findings in ferrets, a species born earlier in development than cats, provide evidence for an activity-independent origin of ocular dominance columns (Crowley and Katz, 1999; Crowley and Katz, 2000; Crair et al., 2001), further supporting the idea that light exposure may promote maintenance rather than normal formation of visual cortical circuits. It would be interesting to reexamine the earlier work with more frequent sampling of postnatal time points and determine whether or not dark rearing has different effects in cortical versus subcortical visual areas or whether there may be species differences rather than or in addition to regional differences.

In our study the refined connections in the long-term DR animals were vulnerable to continued deprivation in adulthood, despite exposure to spontaneous retinal activity during development. Although our results do not reveal what the nature of the vulnerability is, it is apparent that non-visual retinal activity is insufficient to stabilize RFs in a fully mature state. Spontaneous activity is low in SC compared to visual cortex. Without light-driven activity then, there is substantially less activation of the SC. The quantity of activity is important in maturation of LGN (Stellwagen and Shatz, 2002; Huberman et al., 2003), and may be critical in other subcortical visual regions as well.

Our results demonstrate that adult brain plasticity can be influenced by the previous rearing conditions of the animal. Similar findings have been reported in visual cortex. Recovery of visual cortical responses in the deprived eye after monocular deprivation (MD) has been shown to occur after the critical period for MD in ferrets if the eye was exposed to light before the deprivation (Liao et al., 2004). In mice, a transient period of monocular deprivation renders the visual cortex susceptible to monocular deprivation in adulthood (He et al., 2006; Hofer et al., 2006). In this study we have shown that long-term dark rearing also elicits late brain plasticity in the SC, but the adult plasticity can be prevented by providing 30 days of normal sensory input during development. Thus, our results emphasize the role of early sensory experience in preventing detrimental adult brain plasticity that could lead to impaired visual acuity.

b) Light exposure in adulthood can stabilize pre-existing levels of RF refinement

Our finding that visual experience from P60 onward prevented enlargement of single SC neuron RFs, even if animals were previously reared in the dark, suggests that the early deprivation allows the SC to be modified by visual experience in adulthood. The opposite does

not occur, that is, dark rearing starting at P60 does not produce enlarged RFs. Thus, visual experience starting at P60 can prevent deprivation-induced loss of RF refinement, but dark rearing starting at P60 cannot reverse the effect of early visual experience on RFs. These results may indicate that dark rearing allows SC neurons to remain susceptible to changes in visual experience for a longer period of time than normal, despite the fact that the RFs refine on schedule. Alternatively, the deprivation may reopen a critical period for experience-dependent plasticity that had previously closed. Our results also showed that the loss of RF refinement could not be reversed, even with several months of visual experience, if animals were dark-reared until P90 or later. This suggests that there is a restricted period during which visual experience can protect RFs. Generally the term ‘critical period’ is reserved for plasticity that occurs during development, but in this case the loss of RF refinement does not occur until after sexual maturity. Exposure to light in adulthood, which can prevent loss of refinement if it commences at P60, may either be stabilizing the synapses or preventing them from being further destabilized. The stability of the synapses could be probed by dark rearing animals until P60, followed by light exposure, then retesting with another period of dark rearing. If synapses are stabilized by the light exposure, then the second deprivation should have no effect.

c) Early limited visual experience has long lasting protective effects on SC properties

We have stated above that visual deprivation starting at P60 did not reverse the effects of early visual experience on RFs; that is, RFs did not enlarge. Furthermore, our findings showed that a 32 day period of early visual experience has long lasting effects on RF size, preventing the future loss of RF refinement that would be caused by prolonged deprivation. Receptive fields in the hamster SC attain their normal adult size between P50 and P60 (Carrasco et al., 2005). Thus,

when dark rearing started at P40 for the animals that experienced 32 days of visual experience, RFs had not refined to the adult RF size, but nonetheless their refinement and maintenance occurred as usual. Our previous results show that visual experience is not necessary for the development of the retinocollicular projection but is necessary for its maintenance. Interestingly, we have found here that visual experience protects refined RFs, through unknown mechanisms, even if it takes place before RFs have been completely refined. However, 13 or 22 days of visual experience were not enough to protect RFs in adulthood. This result suggests that visual experience has to occur in a certain amount and/or at the time when RFs are close to being refined in order to maintain RF refinement.

Although the mechanism underlying the loss of RF refinement is not currently known, our previous results suggest that animals dark-reared from birth until late in adulthood have decreased surround inhibition in the SC (Carrasco et al., 2005). This finding is consistent with previous studies in developing SC of cats (Fosse et al., 1989) and in visual cortex, where it has been found in adult rats that dark rearing reduces benzodiazepine and muscimol binding and the number of GABA-immunopositive cells (Schliebs et al., 1986; Benevento et al., 1995; Gordon et al., 1997). It has been suggested that GABA has a preponderant role in triggering the closure of the plastic state of the visual cortex and that visual experience contributes to the maturation of the GABA circuit (see Hensch, 2005 for review). Thus, visual experience might prevent the depression of the inhibitory circuitry in the SC as it does in visual cortex. The effects of dark rearing on the SC could be similar to the effects of aging on visual cortex, in which it has been found that GABA function is depressed and that visual function improves with GABA administration (Leventhal et al., 2003). We are currently addressing this possibility and investigating the mechanism involved in RF maintenance in the SC.

Although decreased inhibition may account for at least part of the loss of RF refinement in DR animals, other possibilities also need to be taken into account. Previous studies in visual cortex and SC have shown that the subunit composition of NMDA receptors is experience-dependent (Carmignoto and Vicini, 1992; Binns and Salt, 1998b; Philpot et al., 2001). Visual deprivation in neonatal rats makes NMDA currents longer by increasing the NR2B/NR2A ratio (Quinlan et al., 1999a). Changes in NMDA receptor subunit composition can lead to changes in kinetics, which in turn might lead to changes in synaptic strength, in particular, to LTP, making previously silent synapses functional. Such a scenario if it occurred in SC could lead to a broader activation area and thus larger receptive fields.

d) Why might SC be different from visual cortex?

If it is the case that visual cortex is more susceptible than SC to visual experience during development, how could this be explained? One interesting difference in the molecular underpinnings of plasticity is that expression of *cpg15*, a gene involved in synaptic maturation, is affected by experience in V1 but not LGN or SC (Lee and Nedivi, 2002). Exploration of this and other activity-regulated genes may be fruitful. Results from studying the possible mechanisms underlying maintenance of visual circuitry and adult plasticity in different areas of the brain will provide knowledge that will contribute to a broader understanding and treatment of sensory impairment.

6. Acknowledgements

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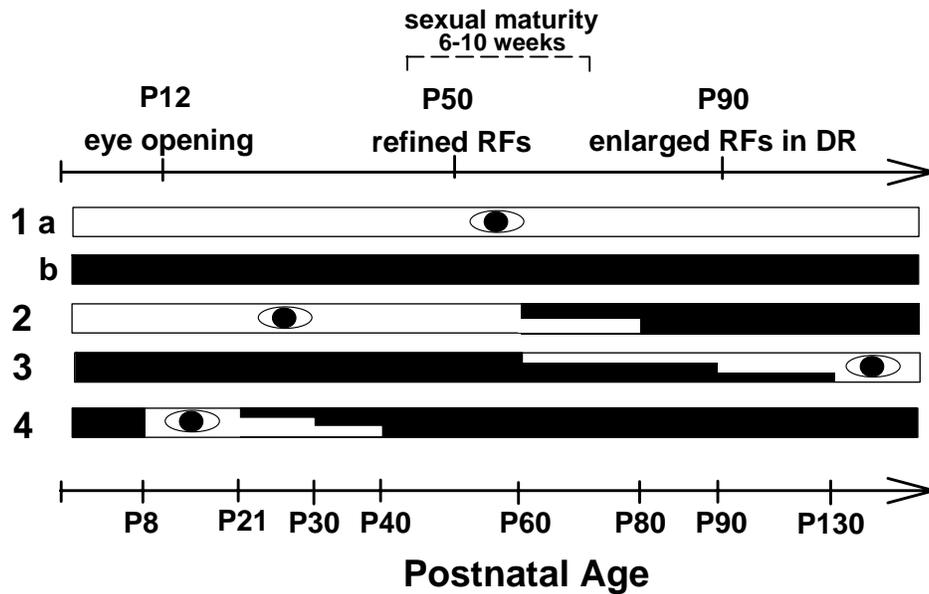


Figure 3.1. Experimental groups: 1) animals reared in a normal light/dark cycle a) or reared in complete darkness from birth until the day of recording b); 2) animals reared in light until either P60 or P80 and then moved to a darkroom to test whether late dark rearing can lead to enlarged RFs; 3) animals reared in dark until P60, P93 or P130 and moved to light afterwards, to test whether late visual experience may prevent or reverse the loss of RF refinement in the SC of DR animals; and 4) animals that were exposed to light for 13, 22, or 32 days starting at P8 and were reared in the dark before and after that period to test the existence of a time window when visual experience is necessary to prevent the loss of RF refinement by dark rearing.

Effect of late dark-rearing on receptive field maintenance

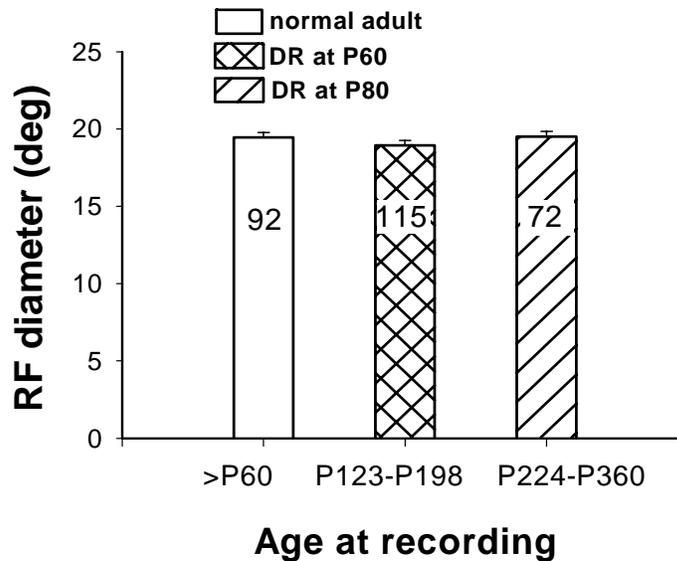


Figure 3.2. Late visual deprivation does not affect RF maintenance. Animals were dark-reared commencing at either P60 or P80. Receptive fields from animals dark-reared at P60 were obtained when animals reached P123-P198. Receptive fields from animals dark-reared at P80 were obtained at P224-P360. Neither of the experimental groups were different from the normal adult group (normal adult: $19.4 \pm 0.31^\circ$, $n=92$; DR at P60: $18.9 \pm 0.31^\circ$, $n=115$; DR at P80: $19.5 \pm 0.34^\circ$, $n=72$; mean \pm SEM; $P=0.18$ One Way ANOVA on Ranks). The label on the x-axis denotes the age of the animal in postnatal days on the day of recording. Numbers inside bars show the number of single units recorded in each group.

Receptive field size after visual experience in adulthood

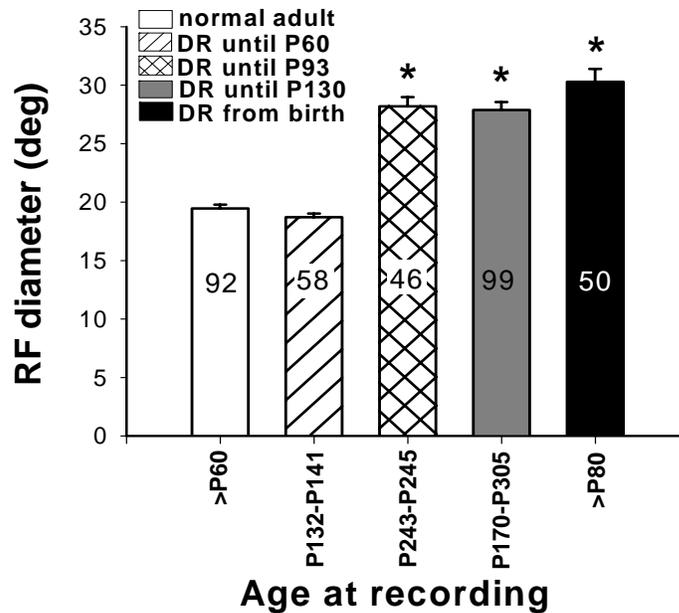
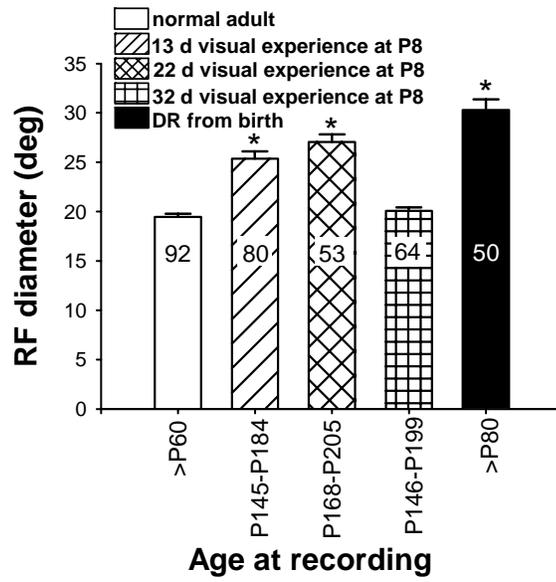


Figure 3.3. Late visual experience can prevent but cannot reverse the effects of long term dark rearing. Animals were dark-reared until P60, P93 or P130 and exposed to normal visual experience thereafter. Late visual experience prevented the loss of RF refinement in animals dark-reared until P60 (normal adult: $19.4 \pm 0.31^\circ$, $n=92$; DR until P60: 18.7 ± 0.33 , $n=58$, $P>0.05$, One Way ANOVA on Ranks), however it did not reverse loss of RF refinement in animals dark-reared until P93 or P130 (DR until P93: $28.2 \pm 0.78^\circ$, $n=46$; DR until P130: $27.9 \pm 0.68^\circ$, $n=99$; DR >P80: $30.3 \pm 1.1^\circ$, $n=50$, $P<0.05$, One Way ANOVA on Ranks). Labels on the x-axis denote the postnatal day on the day of recording. Numbers inside bars are number of single units. * denotes significant difference (<0.05) compared to the normal adult group.

A

Receptive field size after an early short period of visual experience



B

Receptive field size in animals with different visual experience

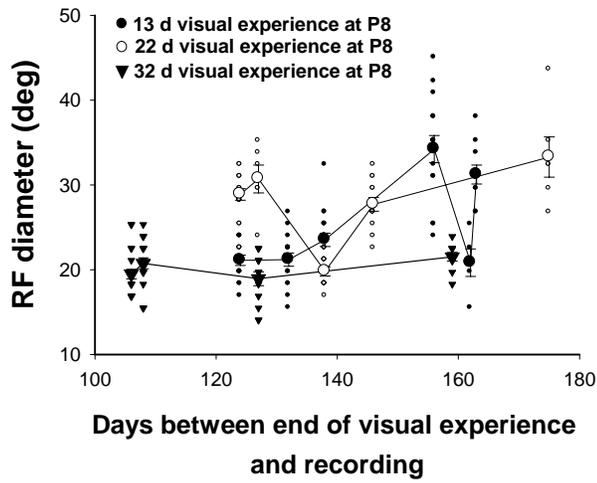


Figure 3.4. A 32 days period of visual experience in early life prevents the effects of long term dark rearing. Animals were exposed to light at P8 lasting 13, 22 or 32 days. A) Receptive field size from both the 13 and the 22 days of light exposure groups were significant different from those of normal adult animals (normal adult: $19.4 \pm 0.31^\circ$, $n=92$; DR with 13d of light: $25.4 \pm 0.73^\circ$, $n=80$; DR with 22d of light: $27.1 \pm 0.75^\circ$, $n=53$, $P<0.05$, One Way ANOVA on Ranks). Receptive field size of units in animals with 32 days of light exposure were not different from those of normal adults (DR with 32d of light: $20.1 \pm 0.34^\circ$, $n=64$, $P>0.05$). Label on the x-axis denote the postnatal day on the day of recording. Numbers inside the bars are number of single units. *indicates significant difference compared to normal adult group. B) Data from animals with 13, 22 or 32 days of visual experience are shown as RF diameter vs. days between the end of visual experience and the day of recording, that is, days in the dark after the visual experience period. Data points (mall circles) are represented as a scatter plot with mean values (large circles) \pm SEM.

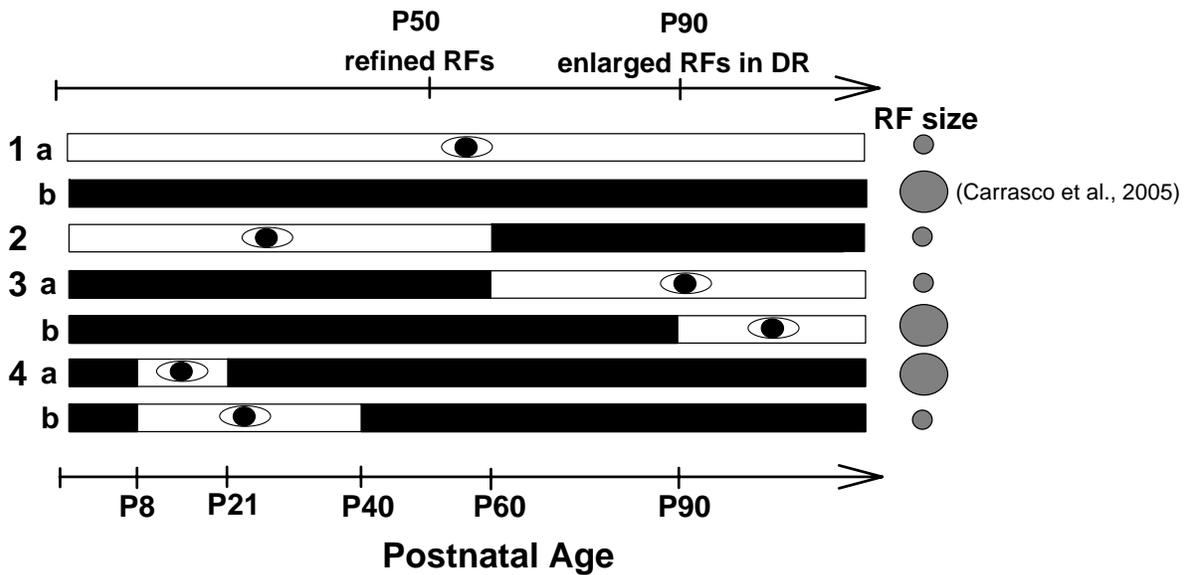


Figure 3.5. Summary of results. Different schedules of visual experience lead to either refined RFs or RFs that have lost their refinement, as indicated with the size of the circles on the right. 1) a) Visual experience throughout the lifespan leads to refined RFs by P60; b) Dark rearing from birth leads to refined RFs by P60, but RFs lose refinement by P90; 2) Visual experience until P60 protects against dark-induced loss of RF refinement; 3) a) Visual experience commencing at P60 after dark rearing from birth prevents the loss of RF refinement; b) Visual experience commencing at P90 does not reverse the loss of RF refinement; 4) a) 13 days of visual experience starting at P8 is not enough to prevent loss of RF refinement in adulthood; b) 32 days of visual experience starting at P8 is enough to prevent loss of RF refinement that would occur in adulthood as a result of dark rearing.

CHAPTER 4: **Inhibitory plasticity contributes to deprivation-induced loss of refinement in adult superior colliculus**

1. Abstract

The patterning of sensory pathways relies on activity-dependent and -independent factors. Increasing evidence shows that sensory experience is necessary for maintenance or plasticity of brain properties but not initial patterning. We have investigated the role of visual experience in development and plasticity of the retinocollicular pathway of an altricial rodent, the Syrian hamster. We reported previously that visual receptive field (RF) refinement in superior colliculus (SC) occurs with the same time course in dark-reared (DR) as in normally-reared hamsters, but RFs in DR animals become unrefined in adulthood. Here we provide support for the hypothesis that this failure to maintain refined RFs into adulthood is related to a decreased contribution of GABAergic inhibition in the SC of DR animals. Iontophoretic application of gabazine, a GABA_A receptor antagonist, or muscimol, a GABA_A receptor agonist, had less of an effect on excitability or RF size of SC neurons in adult DR animals with enlarged RFs than in normal animals or DR animals prior to loss of RF refinement. The percentage of GABA-immunoreactive neurons was significantly decreased in the SC of adult DR animals compared to normal animals. These results suggest that neurons in adult DR hamsters have a weaker inhibitory surround, which would contribute to the visual deprivation-induced enlargement of RFs in adult DR animals. Changes in inhibitory circuitry could occur through a homeostatic process that compensates for the lack of excitatory drive by a generalized depression of inhibition. Our results argue that visually-driven activity is necessary to maintain the inhibitory circuitry intrinsic to the SC and to protect against the consequences of visual deprivation. These

findings provide relevant insights into inhibitory plasticity and the role of sensory experience in the maintenance of neuronal properties.

2. Introduction

The way in which the brain responds to sensory experience during different stages of life is not fully understood. Specifically, how visually-driven activity contributes to the balance between excitatory and inhibitory inputs as animals age remains unclear, especially in subcortical visual centers (Hooks and Chen, 2007). Both spontaneous activity and visually-driven activity contributes to the development of retinal projections into the superior colliculus (SC) (Rhoades and Chalupa, 1978a; Thompson and Holt, 1989; Huang and Pallas, 2001; Torborg and Feller, 2005; Colonnese and Constantine-Paton, 2006; Chandrasekaran et al., 2007) and maintenance of the receptive field properties of LGN (Chapman, 2000; Hooks and Chen, 2008) and SC neurons (Carrasco et al., 2005). Our previous studies have shown that the receptive fields (RFs) of SC neurons refine normally in the absence of visual experience by postnatal day (P) 60, but they start losing their refinement and thus enlarging their RFs by P90 if deprivation continues (Carrasco et al., 2005). A period of about 30 days of visual experience early in life is sufficient to forestall the RF enlargement produced by long-term dark-rearing (Carrasco and Pallas, 2006). Our previous findings point out the importance of early visual experience for protecting neuronal circuits in the SC against the detrimental effects of sensory deprivation later in life, but did not address how this protection is conferred. We report here on our new findings regarding a possible mechanism for RF enlargement that suggest how early visual experience ensures the future maintenance of RFs in the adult SC.

a) The role of visual experience in the development of sensory systems

Visual experience plays a critical part in brain development and plasticity in several model systems. In the barn owl's midbrain, visual experience plays a fundamental role in normal development of the auditory map of space in the inferior colliculus and in its reconfiguration after prism rearing (Knudsen, 2002). In visual cortex, dark rearing delays the critical period for monocular deprivation and maintains the cortex in an apparently immature state (Blakemore et al., 1978; Mower et al., 1981; Mower et al., 1985). In addition, dark-rearing prolongs the critical period in visual cortex during which shifts in ocular dominance columns can be induced by monocular deprivation (Mower et al., 1985; Mower and Christen, 1985), as well as the critical period for LTP induction in visual cortex (Kirkwood et al., 1995). Understanding the mechanism by which visual experience alters neuronal circuits could shed light on general mechanisms involved in brain plasticity.

b) The role of visual experience in the modulation of glutamatergic and GABAergic circuitry

Much of the previous work on visual deprivation has focused on alterations of glutamatergic synapses, specifically the composition of NMDARs. In particular, in visual cortex and retina, visual deprivation alters the glutamatergic circuit by modifying the subunit composition of NMDA receptors and therefore channel open time (Quinlan et al., 1999b; Chen et al., 2000a; Philpot et al., 2001; Xue and Cooper, 2001; Tongiorgi et al., 2003). Visual deprivation decreases the number of NR2A subunits in all visual cortical layers, producing as a consequence a relatively higher proportion of NR2B subunits, which has the effect of prolonging currents through NMDARs. However, visual deprivation also alters the inhibitory GABAergic

circuitry. Dark-rearing weakens lateral inhibition (Kasamatsu et al., 1998), decreases GABA immunoreactivity (Hendry and Jones, 1986), and changes the subunit composition of GABA_A receptors (Chen et al., 2001) in visual cortex. It also prevents the normal developmental increase in GABAergic inputs converging on neurons in layer II/III (Choi et al., 2002). In the retina, visual deprivation decreases GABA immunoreactivity and the expression of GAD65 and GAD67 proteins (Lee et al., 2006). Because of these effects and because RF enlargement could result from a loss of lateral inhibition, we hypothesized that the loss of refinement in SC neurons in adult dark-reared hamsters results from a loss of inhibition.

c) Role of inhibition in visual receptive field properties

Visual deprivation-induced changes in inhibitory circuitry may provide particular insight into neural plasticity because of the important role of inhibition in regulating neuronal properties. For example, in visual cortex, GABAergic circuitry is involved in construction of spatiotemporal receptive field properties, including orientation selectivity and the substructure of receptive fields (Sillito, 1974, 1975; Wolf et al., 1986; Allison et al., 1996; Pernberg et al., 1998). Meanwhile, in the SC, a brain area with a relatively high number of GABAergic interneurons (Okada, 1974; Fosse et al., 1989; Mize, 1992; Okada, 1992), inhibitory inputs provide surround inhibition (Albus et al., 1991; Binns and Salt, 1997b), response habituation (Binns and Salt, 1997b), and stimulus size and velocity tuning (Razak and Pallas, 2005, 2006). Although the SC contains both metabotropic and ionotropic GABA receptors, visual response properties seem to depend mainly on ionotropic GABA_A receptors (Binns and Salt, 1997b). Given the role of GABA in the SC, we hypothesized that changes in the GABAergic intracollicular circuitry induced by dark-rearing would affect neuronal properties. Furthermore, we predicted that a weaker contribution of

GABA in the SC of DR hamsters could be a mechanism by which RFs lose refinement in long-term DR animals. In this study, we tested these predictions pharmacologically, using iontophoretic injection of gabazine, a GABA_A receptor antagonist, and muscimol, a GABA_A receptor agonist, in the SC while performing extracellular recordings. We compared the effect of gabazine and muscimol on RF size and responsiveness of neurons in normally reared and DR animals. In addition, we performed GABA immunohistochemistry to test the hypothesis that the loss of RF refinement results from differences in the number of GABA immunoreactive neurons between normal and DR animals. Our findings suggest that neurons of long-term DR hamsters have a weaker inhibitory surround, at least in part resulting from a decline in the number of GABAergic neurons in SC, and further suggest that a depression of the intracollicular inhibitory circuitry contributes to the failure to maintain refined RFs in adult DR animals. These results are relevant when considering environmental influences on brain plasticity and the role of sensory experience in maintaining the excitatory/inhibitory balance in the brain.

3. Methods

A total of 38 Syrian hamsters (*Mesocricetus auratus*) of different postnatal ages between P55 and P234 were used. All procedures used on animals met or exceeded standards of humane care developed by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee.

a) Experimental groups and electrophysiology preparation and procedure

i) Rearing conditions and experimental groups

Syrian hamsters were obtained from Charles River Laboratories (Wilmington, MA) or bred in house. Normal hamsters were kept on a 14h/10h light/dark cycle. Dark-reared (DR) hamsters were maintained in a light tight darkroom from before birth and exposed only to a thin beam of dim red light ((Philips 25W red A-type bulb #814546) not visible to Syrian hamsters (Huhman and Albers, 1994)) during brief, daily caretaker visits. Experimental groups included in this study were: 1) normal adult animals, aged P62-P217, reared in a light/dark cycle; 2) P55-P65 dark-reared (DR) animals, reared in the dark from birth until the day of the experiment; and 3) P138-P234 dark-reared animals, reared in the dark from birth until the day of the experiment. These age groups were chosen based on the timing of RF refinement and loss of refinement in DR animals. Receptive fields are refined at ~P60 in normally-reared and dark-reared animals and they lose refinement after P90 in DR animals (Carrasco et al., 2005).

ii) Surgery

Animals were prepared for terminal electrophysiological recordings in the superficial layers of the right SC as described previously (Carrasco et al., 2005). Each animal was anesthetized with urethane (0.7 g/ml; 0.3ml/100g body weight in 4 i.p. aliquots at 20-30 min intervals), an anesthetic that has minimal effect on subcortical neurotransmission and approximately equivalent effects on different neurotransmitter systems (Maggi and Meli, 1986; Hara and Harris, 2002; Sceniak and Maciver, 2006). The right SC was surgically exposed by bilateral aspiration of the visual cortex. Removal of cortex has no effect on SC neuron receptive field properties in hamsters, except for a loss of direction tuning (Chalupa et al., 1978; see also

Razak and Pallas, 2005). The brain was kept covered with sterile saline solution, and the left eye was protected by a custom designed, plano contact lens during the experiment (Conforma, Norfolk, VA). In most of the animals, an endotracheal tube was inserted in order to facilitate respiration. The animal was placed in a stereotaxic device and the conjunctivum of the left eye was stabilized with 6-0 silk suture to prevent movement (Pallas and Finlay, 1989). Anesthesia level was periodically monitored throughout the experiments by checking withdrawal reflexes, and supplemental $\frac{1}{4}$ doses of urethane were given if needed.

iii) Visual stimulation

Visual stimulation was delivered monocularly (usually to the left eye), because there is a strong contralateral dominance of visual inputs to the retino-recipient layers of the hamster SC (Tiao and Blakemore, 1976; Pallas and Finlay, 1989). A Sergeant Pepper graphics board (Number Nine, Cambridge, MA) was used in conjunction with “STIM” software (developed by K. Christian at Rockefeller University) to generate stationary and moving visual stimuli. Data were acquired by CED 1401 hardware and processed by Spike 2 software (Cambridge Electronic Design, Cambridge, UK).

Electrode penetrations were made perpendicular to the surface of the SC to locate visually responsive cells in the retino-recipient superficial gray layer (<200 μm depth). Edges and location of the excitatory receptive fields were determined with a penlight. A 14-inch computer display monitor was then placed 40 cm in front of the hamster’s eye such that the center of the neuron’s excitatory receptive field coincided with the center of the monitor. The stimulus consisted of a light spot of 1 degree diameter moving from the top to the bottom of the monitor screen, from temporal to nasal, with an interstimulus interval of 5s to prevent

habituation. The choice of stimulus velocity used, $10^{\circ}/\text{sec}$, was guided by previous results showing that the vast majority of hamster SC neurons in the superficial gray layer prefer for slowly moving spots of light (Tiao and Blakemore, 1976; Stein and Dixon, 1979; Pallas and Finlay, 1989; Razak et al., 2003). Each stimulus set was repeated 4 to 12 times.

iv) Electrodes, recording and iontophoresis

To determine the contribution of inhibition through GABA_A receptors in the different experimental groups, responses were quantified during control conditions, during gabazine or muscimol application, and during recovery from drug treatment. Multi-barreled micropipettes were used for the extracellular recording and iontophoretic drug application. The pipettes were broken under microscope control to a final outer tip diameter of about 4-10 μm (1-3 μm per barrel). The recording barrel contained a solution of 1 M NaCl. The remaining electrode barrels were filled with muscimol or gabazine (Sigma-Aldrich). The drug solutions were prepared at 10 mM for gabazine and 5 mM for muscimol (Celada et al., 1999; Waroux et al., 2005; Windels and Kiyatkin, 2006). All drug solutions were adjusted to pH 3.7 with 0.1 M HCl and thus were positively charged. An iontophoresis device (Cygnus Technology, Inc, Delaware Water Gap, PA) was used for drug administration. Negative retaining currents of 10 nA were applied to drug barrels not in use. Muscimol and gabazine were ejected using positive currents at 5 nA for muscimol and 15-20 nA for 10 mM gabazine. In each penetration, only the first neuron encountered was isolated, which in combination with the monitoring of electrode depth ensured that recorded neurons were from the retinorecipient, superficial gray layer.

Application of both muscimol and gabazine was maintained throughout the period when their effects were being tested. Typically this lasted 20-30 min for each drug. The changes in

neuronal excitability due to maintained drug application took considerable time to reverse. It was possible, however, to obtain data concerning recovery from the effects of the drugs in most neurons.

v) *Data analysis*

We carried out an off-line data analysis by using Spike2 software and we isolated single units in according to their waveform. We determined the effect of the drugs by quantifying the change in RF size and the number of spikes of each single unit under control and drug application conditions. Receptive field center was obtained by determining the spatial location producing the highest number of spikes (peak response) obtained for a single stimulus within each trial. Stimulus locations generating spike numbers less than 20% of the peak response were defined as no response and were considered to be outside of the RF.

b) Immunohistochemistry

i) *Rearing conditions and experimental groups*

Two experimental groups were used in this part of the study: 1) P91-218 normal adult hamsters (n = 5), reared in a light/dark cycle, and 2) P141-153 long-term DR hamsters (n = 5), reared in the dark since birth. These age groups were chosen because RFs in the SC become refined at approximately P60 and long-term DR animals lose that refinement after P90.

ii) *Tissue preparation*

Animals were euthanized with a lethal overdose of sodium pentobarbital (150 mg/kg) and were perfused through the heart with 0.1 M phosphate buffered saline (PBS) adjusted to pH 7.4

with NaOH, followed by 4% paraformaldehyde in PBS containing 0.2% of glutaraldehyde, at pH 7.4. Brains were removed and stored at 4°C in the same fixative for 48-72 h and then transferred to 30% sucrose in 0.1M phosphate buffer for cryoprotection. Brains were sectioned frozen in the coronal plane at 50 µm for Nissl staining and at 30 µm for GABA immunohistochemistry.

iii) Immunohistochemistry procedure

Sections were processed free-floating using the avidin-biotin method for localization of antigens with peroxidase (Vector, Burlingame, CA). They were first rinsed in 0.1 M PBS at pH 7.4 with 0.02% sodium azide (PBS/A) and then treated for 1h in 0.34% L-lysine and 0.05% sodium periodate (NaIO₄) to reduce free aldehydes. Blocking of nonspecific staining was achieved by incubating the sections in 3% normal goat serum (NGS) in PBS/A for 1h at room temperature. Incubation with the primary antibody (mouse anti-GABA from MP Biomedicals, Solon, OH, diluted with PBS/A plus 3% NGS at 1:1,000) in NGS was done for 48 h at 4°C under constant agitation. After rinsing in PBS/A, sections were incubated in the secondary antibody solution for 2h (biotinylated goat anti-mouse in PBS/A plus 3% NGS, Vector Labs, Burlingame, CA at a dilution of 1:200), washed in PBS, and then incubated in ABC solution according to package directions (Vectastain Elite ABC kit, Vector, Burlingame, CA) for 1h. Sodium azide was left out of the buffer after incubation in the secondary antibody. The peroxidase reaction was performed with 0.01% diaminobenzidine and 0.004% hydrogen peroxide and intensified by adding 1% nickel ammonium sulfate and 0.34% imidazole. Sections were mounted from saline, dehydrated, and coverslipped with Permount.

iv) Quantitative analysis

We utilized NeuroLucida (MicroBrightfield, Burlington, VT) to perform quantitative analysis of immunolabeled neurons. Three 30 μm coronal sections per animal located at approximately 25, 50 and 75% of the rostrocaudal extent of the SC were selected for counting. A 300 μm wide rectangular boundary was drawn in the mediolateral center of the right SC to define the area within which neurons would be counted. We counted only GABAergic neurons located in the superficial gray layer of the SC, defined according to adjacent Nissl-stained sections. All of the GABA immunopositive neurons found within the defined area were counted. We obtained the total number of neurons from 50 μm Nissl sections by counting neurons within every 4th bin of a 25 μm x 25 μm grid and multiplying by 4 (Pallas and Finlay, 1991; Gao et al., 1999). We did not account for differences in section thickness between the tissue treated for immunohistochemistry (30 μm) and Nissl substance (50 μm) because the antibody does not penetrate the sections completely (see Gao et al., 1999). To determine whether there were any differences in soma size between normal and experimental groups that might bias the counts, we measured soma diameter (average of the widest and narrowest diameters) of 100 GABA-ir SC neurons in each experimental group and compared them using a Student's t-test.

4. Results

We investigated the mechanism underlying the loss of refinement of RFs in the SC of long-term DR hamsters by examining the influence of GABAergic inputs, using electrophysiological and immunohistochemical methods. We used three experimental groups: 1) normal adult animals reared in a light/dark cycle; 2) P55-P65 DR animals, which have refined RFs; 3) >P90 (P100-P250) DR animals, which have enlarged RFs. We hypothesized that a

weaker GABAergic circuit in the SC exists in >P90 DR animals, possibly contributing to the loss of RF refinement.

a) The effect of GABA_A receptor blockade on visual responsiveness is reduced after prolonged dark-rearing

In order to test the hypothesis that GABAergic influence is decreased in the SC of long-term DR hamsters, we tested the effect of gabazine, a competitive GABA_A receptor antagonist, on the responsiveness of neurons in the SC of our different experimental groups. Previous studies suggest that GABA_A receptors are selectively involved in surround inhibition of RFs in the SC (Binns and Salt, 1997b), visual cortex, and retina (Sato et al., 1996; Pernberg et al., 1998; Flores-Herr et al., 2001), as compared to GABA_B or GABA_C receptors. As expected, gabazine increased the number of spikes recorded in response to visual stimulation from single units in the normal group. The mean number of spikes per visual stimulation series in the normal adult group increased from 48.83 ± 6.18 to 112.1 ± 21.4 (mean \pm S.E.M., $n = 32$) under iontophoretic application of gabazine. Those values were significantly higher than normal ($p = 0.016$, Rank Sum Test) and are in accordance with a previous study on somatosensory cortex that examined the effect of similar concentrations of gabazine on neuronal responsiveness (Foeller et al., 2005). The ratio between the number of spikes under the influence of gabazine and the number of spikes without any drug application was 2.10 ± 0.15 for the same group of neurons from normal adults (Fig. 4.1). For the P55-65 DR group, the number of spikes of single units in response to visual stimulation was significantly increased by gabazine application from 36.1 ± 3.83 to 69.3 ± 6.51 , $n = 30$ ($p < 0.001$, Rank Sum Test). The ratio between the number of spikes with gabazine and without was 2.27 ± 0.26 for this group, which was not significantly different from the normal

group. In sharp contrast, there was no significant difference in single unit responsiveness to visual stimulation before and after gabazine application in the >P90 DR group. The values obtained for that group were 30.98 ± 3.80 spikes without gabazine and 40.12 ± 9.01 spikes with gabazine ($n = 27$, $p = 0.71$, Rank Sum Test), and the ratio was 1.24 ± 0.10 . There were significant differences between the >P90 DR group and both the normal and the P55-65 DR group in the effect of gabazine on the visual responses ($p < 0.05$, ANOVA on Ranks, post hoc Dunn's Method).

b) The effect of GABA_A receptor blockade on RF size is reduced after prolonged dark-rearing

In order to test the hypothesis that GABAergic influence is decreased in the SC of long-term DR hamsters, we examined the contribution of surround inhibition to RF size in animals with refined RFs and in animals whose RFs were enlarged as a consequence of long-term dark-rearing. We found that gabazine application enlarged RFs of single units by approximately 50% in normal adult animals, which is close to the magnitude of RF enlargement that we previously reported in long-term DR animals (Carrasco et al., 2005). The ratio of RF size under gabazine application to that under control conditions in the normal group was 1.533 ± 0.0503 ($n = 32$) (Fig 4.2). In the P55-65 DR animals, whose RFs were not yet significantly different from those of normal adults, RF size increased on average by 41% for a gabazine/no gabazine RF size ratio of 1.465 ± 0.0882 ($n = 30$), which was not significantly different from the normal group. The long-term DR group on the other hand, whose RFs were significantly larger than those of the other two experimental groups, presented only a 6% average increase in RF size after gabazine treatment, which was not significantly different from the RF size obtained from the same

neurons before gabazine application ($p = 0.28$, Rank Sum Test), with a ratio of 1.088 ± 0.0365 ($n = 27$). The increase in RF size under gabazine application was significantly different between the long-term DR group and both the normal and P55-65 DR groups (ANOVA on Ranks, $p < 0.001$, post hoc Dunn's Method). In summary, these results show that blockade of GABA_A receptors increased RF size in long-term DR animals to a lesser extent than in normal animals and suggest that surround inhibition is decreased as a consequence of chronic visual deprivation.

c) The effect of muscimol on visual responsiveness is reduced after prolonged dark-rearing

As an additional test of the hypothesis that the increase in RF size in SC of the >P90 DR animals was due to a loss of inhibition, we tested the effect of muscimol, a GABA_A receptor agonist, on neuronal responsiveness in the SC of the normally reared and >P90 DR groups. The expectation was that application of the agonist muscimol would produce opposite effects from the antagonist gabazine. Our results with muscimol were consistent with this expectation, and thus provide further support for the hypothesis. Muscimol application resulted in a decreased responsiveness of neurons to visual stimuli in normal animals from 14.19 ± 2.35 to 5.52 ± 1.18 ($n = 18$), which represents a 61% decrease (Fig 4.3). The ratio between the number of spikes with and without muscimol obtained during a visual stimulation trial was 0.4497 ± 0.0675 . In the long-term DR group, on the other hand, muscimol decreased neuronal responsiveness only by 4%, from 28.52 ± 5.54 to 27.21 ± 6.02 ($n = 16$), which did not represent a significant change (Rank Sum Test, $p = 0.624$). The ratio between the number of spikes with and without muscimol was 0.8995 ± 0.146 for this group. These data show that the effect of muscimol on neuronal responsiveness was significantly reduced in the long-term dark-reared animals in comparison to normal animals ($p = 0.011$, Rank Sum Test).

d) The effect of muscimol on RF size is reduced after prolonged dark-rearing

We also quantified the effect of muscimol on RF size. As expected from the above results, muscimol application significantly decreased RF size in the normal group, but not in the long-term DR group. The amount of reduction in RF size was 45% and 14% in the normal and long-term DR group, respectively. The ratio of the RF sizes obtained with and without muscimol was 0.5555 ± 0.0533 ($n = 17$) for the normally-reared group and 0.8800 ± 0.0726 ($n = 16$) for the DR group (Fig 4.4). The effect of muscimol on these two groups was significantly different ($p < 0.001$, t-test). Taken together, the data from these pharmacological manipulations suggest that alterations in the number/effectiveness of GABA_A receptors occur in the SC as a consequence of chronic dark-rearing.

e) The proportion and density of GABA immunopositive neurons in the SC of >P90 DR animals is significantly lower than that in normal animals

To determine whether dark-rearing also affects intracollicular GABAergic circuitry presynaptically, we quantified the number and density of GABA-containing neurons in the SC of normal adult (P91-218) and long-term DR (P141-153) hamsters using an antibody to GABA visualized with a biotinylated secondary antibody in an ABC reaction (see Methods). The superficial layers of the SC in long-term DR hamsters have a significantly lower density of GABA-ir neurons than in normal hamsters, by over 50% (Fig 4.5, normal: 2113 ± 301 neurons/mm²; DR: 904 ± 130 neurons/mm², mean \pm SEM, $p = 0.006$, t-test). In addition, the proportion of GABA-immunoreactive (-ir) neurons compared to total neurons was reduced in the long-term DR group compared to that in normal hamsters (Fig 4.6, $p = 0.003$, t-test). GABA-ir neurons comprised 10% of total neurons in normal animals, but only 4% in DR animals. To test

the alternative hypothesis that the reduction in the proportion and density of GABA-ir neurons in long-term DR animals was a result of an overall decrease in neuronal density or number, or a smaller soma size in that group, we also quantified those parameters. We found that visual deprivation did not affect the neuronal density in the superficial layers of the SC (Fig. 4.7 A, normal: $20,066 \pm 524$ total neurons/mm²; DR: $20,380 \pm 898$ total neurons/mm²; $p = 0.771$, t-test). Size of GABA-ir neuronal somata was not significantly different between the two groups (Fig. 4.7 B, normal: 6.041 ± 0.11 μm , $n = 100$; DR: 5.846 ± 0.093 μm , $n = 100$, $p = 0.201$, t-test), and furthermore, the total number of neurons was not significantly different between the two experimental groups (normal adult: 742 ± 24.8 ; >P90 DR: 680 ± 21.3 , $p = 0.097$, t-test). Taken together, these results support the hypothesis that long-term dark-rearing produces an increase in RF size in the SC as a consequence, at least in part, of a reduction in the intrinsic collicular inhibition.

5. Discussion

We investigated mechanisms underlying deprivation-induced plasticity in the SC of long-term DR animals (Carrasco et al., 2005; Carrasco and Pallas, 2006). In particular, we tested whether a reduction in inhibition can explain all or part of the DR-induced RF enlargement. We examined the strength of the intracollicular GABAergic circuit by using electrophysiological and immunohistochemical methods. We found that the effects of activating or blocking GABA_A receptors in the SC are reduced in the long-term DR group compared to normal and that DR animals have a lower proportion of GABA-ir neurons compared to the normal group.

The SC has one of the highest concentrations of GABAergic neurons in the brain (Mize, 1992). Intracollicular inhibition plays an important role in some receptive field properties of

neurons in the SC, including velocity and size tuning (Razak and Pallas, 2005, 2006), surround inhibition, and habituation (Binns and Salt, 1997b). In this study, we tested the strength of lateral inhibition in the SC itself by local application of the GABA antagonist gabazine and the GABA agonist muscimol, and showed that surround inhibition in the SC is weaker in long-term DR animals but not in short-term DR animals that still have refined RFs. Moreover, our results suggest that the loss of RF refinement is due in large part to a decreased number of GABA_A receptors and GABA in the SC.

Results from a recent study raise the possibility that changes at the retinal level are also involved in the loss of RF refinement with chronic visual deprivation (Lee et al., 2006). That study reported that GABA immunoreactivity is decreased in the retina of P30 DR mice, raising the possibility that decreased lateral inhibition in the retina could also contribute to the RF enlargement in the SC. Decreased inhibition in the retina could increase RF size of RGCs, which would in turn increase RF size of SC neurons. Although interesting, those results do not explain the RF expansion after P60, however, because they observed decreased retinal inhibition much earlier, at P30. Our results with gabazine and muscimol iontophoresis in SC directly showed that both drugs change RF size of SC neurons by about 50% up or down, respectively, in normally-reared animals. In contrast, gabazine and muscimol only changed RF size by +6% and -14%, respectively, in long-term DR animals. Interestingly, the average increase in RF size that we observed in the SC of long-term DR animals corresponds to approximately a 40% expansion beyond normal (Carrasco et al., 2005). Our results thus suggest that a large part of the RF enlargement in long-term DR animals can be explained by the changes in the GABAergic SC circuitry that we have reported in this study.

The loss of inhibition in long-term DR animals could occur through different mechanisms and could manifest itself at the presynaptic level, the postsynaptic level, or both. The weak effect of gabazine and muscimol on neurons in the SC of long-term DR hamsters could be due to a reduction in the number of GABA_A receptors. Alternatively or in addition, changes in the subunit composition of GABA receptors could be responsible. It has been shown that age and visual experience alter GABA_A receptor composition in the SC and visual cortex (Chen et al., 2001; Clark et al., 2001). More than twenty different subunits can contribute to the pentameric GABA_A receptor, in addition to the obligatory one, γ_2 (Sieghart, 1995). Certain changes in receptor composition affect the receptor affinity for gabazine and muscimol (see Hevers and Luddens, 1998 for review; Stell and Mody, 2002). Modulation of plasticity by changes in the strength of the inhibitory circuitry has been reported in visual cortex (Fagiolini and Hensch, 2000; Fagiolini et al., 2004). GABA_A receptors containing α_1 are necessary for the expression of ocular dominance plasticity (Fagiolini et al., 2004) and, furthermore, the level of GABA_A receptor-mediated inhibition is proposed to control the critical period for ocular dominance plasticity in visual cortex (Fagiolini and Hensch, 2000). Our study points out a similar kind of plasticity involving GABA_A receptors. In our study, however, we found that visual experience may affect GABA_A receptor number, which we suggest accounts for the plasticity observed. Further examination of GABA receptor composition in the SC of DR hamsters might provide more detailed information regarding the role of inhibition in plasticity.

Several previous studies have shown that neuronal networks are capable of compensating for alterations in their own activity (see Turrigiano, 1999 for review). This homeostatic plasticity occurs in both sensory and motor systems (see Rich and Wenner, 2007 for review). A handful of studies have addressed plasticity at inhibitory and excitatory synapses after treatments

that alter input activity (He et al., 2004; Froemke et al., 2007). In the somatosensory system, where removal of sensory afferents reorganizes representation of the body surface in the somatosensory cortex (see Kaas, 2000 for review), this reorganization involves modulation of AMPA and GABA_A receptors (Wellman et al., 2002; He et al., 2004). The expression level of these receptors stabilizes several weeks after deafferentation, at which point RFs in the somatosensory cortex return to their refined size. Their and our studies demonstrate that inhibition can be modulated by sensory afferents and that a balance between excitation and inhibition is necessary to maintain or regain a refined RF size. In the auditory cortex of rats, increasing excitatory input by stimulation of subcortical afferents produces a rapid reduction of inhibition in the cortex, followed by increased excitation and a slow increase in inhibition (Froemke et al., 2007). This latter increase in inhibition may balance the changes in excitation. Our results suggest that decreased neuronal activity due to the lack of visual input in DR animals has been balanced with decreased inhibition in the SC.

In the visual system, alterations in sensory input activity also regulate both excitatory and inhibitory synaptic strength. Visual deprivation may cause an increase in the effectiveness of glutamatergic synapses in visual cortex through a decrease in NR2A subunit expression, without affecting NR1 or NR2B levels (Quinlan et al., 1999a; Quinlan et al., 1999b; Tongiorgi et al., 2003). Binding of flunitrazepam, a GABA_A receptor antagonist, is decreased in the SC and LGN of rats reared in the dark until P25 (Schliebs et al., 1986). The effect of dark-rearing on GABA_A receptors observed by Schliebs et al. (1986) occurred at a much earlier age than in our study, thus it is possible that alterations in GABA_A receptor composition occur earlier than P90 but that they have no effect on RF size and were not detected by measuring the effect of gabazine in our study. Nevertheless, our results are in concordance with the conceptual framework of

homeostatic plasticity and suggest that a lack of impulse activity by visual deprivation triggers changes in inhibition that preserve the inhibitory/excitatory balance in the subcortical visual system. The reduction in the GABA immunostaining that we found in the SC of long-term DR hamsters is consistent with that idea. We do not know, however, whether visual experience maintains refined RFs in the SC through the pattern of activity (instructive effect), the amount of activity derived from sensory experience (permissive effect), or both.

Whether the regulation of synaptic strength by neuronal activity occurs presynaptically or postsynaptically has been debated. In our study, we investigated deprivation-induced changes at the presynaptic and postsynaptic levels in the strength of the inhibitory inputs to SC neurons. Our results show that GABAergic inputs are weaker in long-term DR hamsters at both levels. Whether a decrease in the amount of GABA present in neurons decreased or change composition of GABA_A receptors in postsynaptic neurons, or vice versa, was not tested in our study, but would be interesting to examine. Blockade of spiking activity in hippocampal cultures of neonatal rats decreases the density of GABAergic terminals (Hartman et al., 2006) and GABA transporters can be regulated by neuronal activity (Erickson et al., 2006). On the other hand, many studies have demonstrated changes in both pre- and postsynaptic loci as a result of altering activity levels (see Rich and Wenner, 2007; and Turrigiano, 2007 for reviews). More studies are needed to resolve where the sensors for altered network activity are located and how the compensatory synaptic changes are manifested.

In summary, our results offer a mechanism by which lack of visual experience can affect maintenance of neuronal properties in rodent visual midbrain. Our results point to the importance of intrinsic collicular circuitry for construction and maintenance of neuronal response properties. This study offers insight into plasticity of a subcortical visual system that could be

extended to other sensory systems. Moreover, it contributes to the knowledge about adult plasticity occurring through modulation of network inhibition.

6. Acknowledgements

We thank the Pallas laboratory for its contribution to the development of this project.

**Reduced effectiveness of gabazine
on neuronal response level in long-term DR animals**

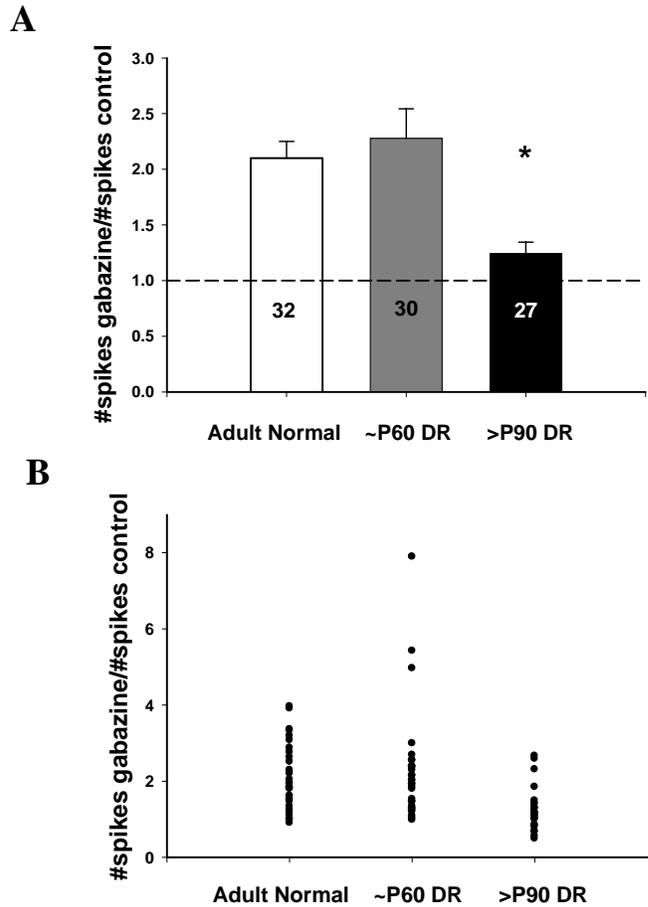
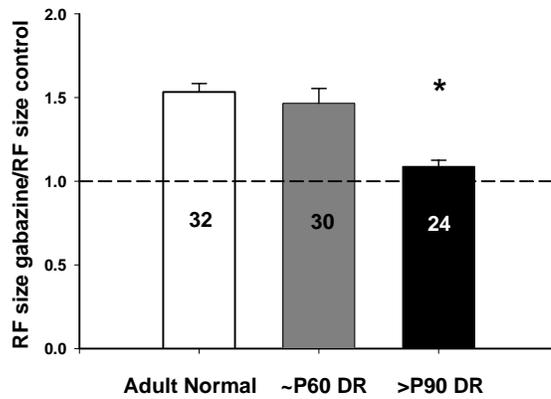


Figure 4.1. Gabazine reduces neuronal responsiveness to a greater extent in SC of normal adult animals or P60 DR animals than in long-term (>P90) DR animals. (A) mean \pm SEM and (B) raw data represent the ratio between the number of spikes per single unit, averaged over all visual stimulation trials under control conditions and under gabazine (20 mM, 20 nA) iontophoresis. The increase in neuronal responsiveness under gabazine is significantly lower in the >P90 DR group than in the other two experimental groups ($p < 0.001$, ANOVA on Ranks, post hoc Dunn's Method), suggesting that deprivation reduces GABA_A receptor number or effectiveness.

**Reduced effectiveness of gabazine
on receptive field size in long-term DR animals**

A



B

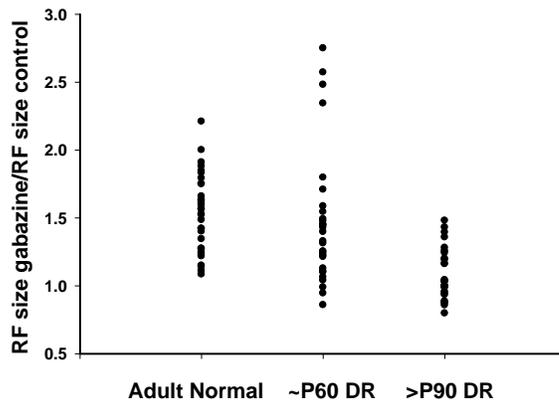


Figure 4.2. Gabazine enlarges RF size to a greater extent in normal or P60 DR animals than in >P90 DR animals. (A) mean \pm SEM and (B) raw data represent the ratio between the RF size of single units under control conditions and gabazine iontophoresis. The effect of gabazine is significantly less in the >P90 DR group than in the other two experimental groups ($p < 0.001$, ANOVA on Ranks, post hoc Dunn's Method), suggesting that long-term dark-rearing reduces GABA_A receptor number or effectiveness.

Reduced effectiveness of muscimol on neuronal response level in long-term DR animals

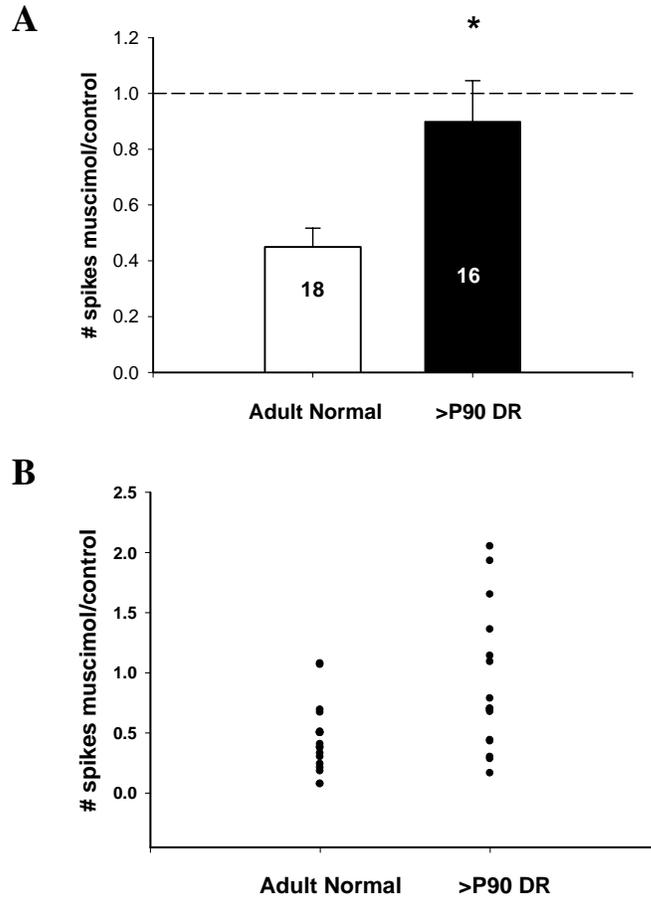


Figure 4.3. Muscimol decreases neuronal responsiveness to a lesser extent in the >P90 DR group than in the adult normal group. (A) mean \pm SEM and (B) raw data represent the ratio of the number of spikes from single SC neurons per trial of visual stimulation with iontophoretic injection of muscimol to that without muscimol (5 mM, 5 nA). The effect of muscimol was significantly reduced in the DR group compared to the normal group ($p = 0.013$, Rank Sum Test), supporting the interpretation that long-term visual deprivation reduces the number or effectiveness of GABA_A receptors.

Reduced effectiveness of muscimol on receptive field size in long-term DR animals

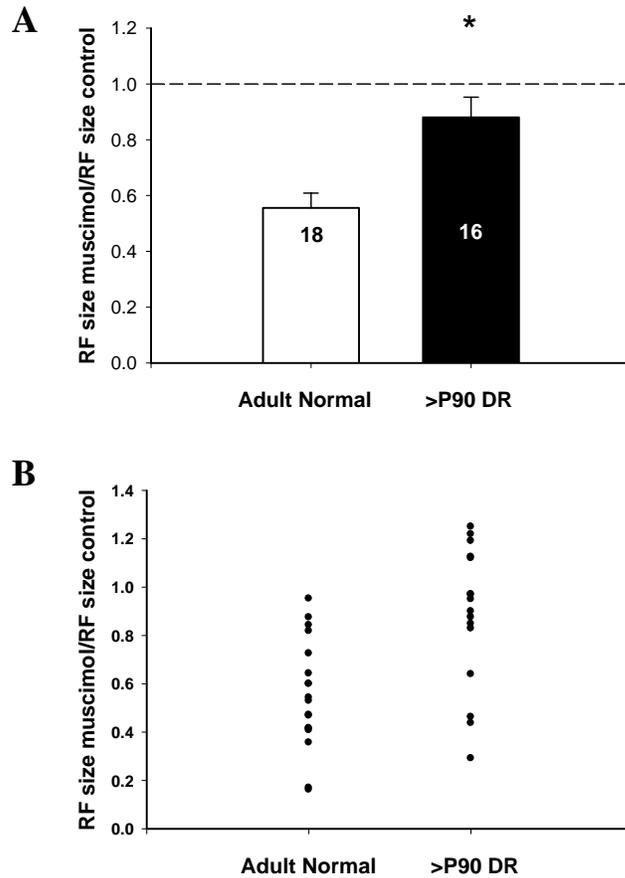
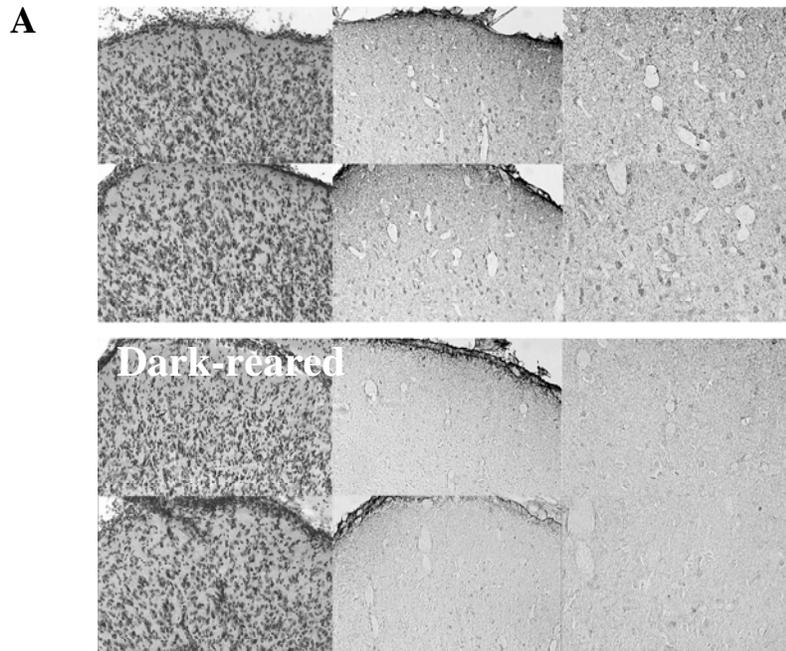


Figure 4.4. Muscimol decreases single unit RF size to a lesser extent in the >P90 DR group than in the normal adult group. (A) mean \pm SEM and (B) raw data represent the ratio of the RF size from single units with iontophoretic injection of muscimol to that without muscimol (5 mM, 5 nA). The effect of muscimol was significantly reduced in the DR group compared to the normal group ($P = 0.003$, Rank Sum Test), in agreement with the interpretation that long-term visual deprivation decreases the number or effectiveness of GABA_A receptors in the SC.



B **Dark-rearing reduces the density of GABA-ir neurons in the SC**

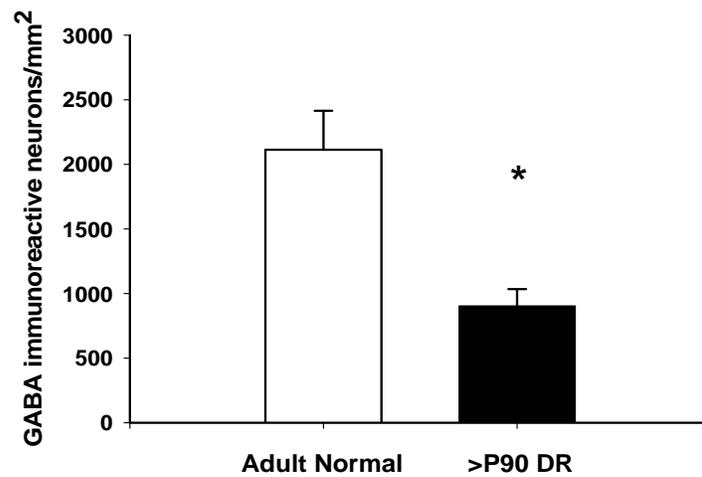


Figure 4.5. The density of GABA immunoreactive neurons is significantly lower in the SC of >P90 dark-reared animals compared to normal animals. (A) Examples of Nissl staining (10x) and GABA immunohistochemistry (10x and 20x) from the SC of normal adults and >P90 DR animals B) Density of GABA-ir neurons in both experimental groups (normal: 2113 ± 301 , DR: 904.7 ± 130 , $p = 0.006$, t-test). Thus there is a loss of inhibitory influence under chronic DR.

Dark-rearing decreases the proportion of GABA-ir neurons in the SC

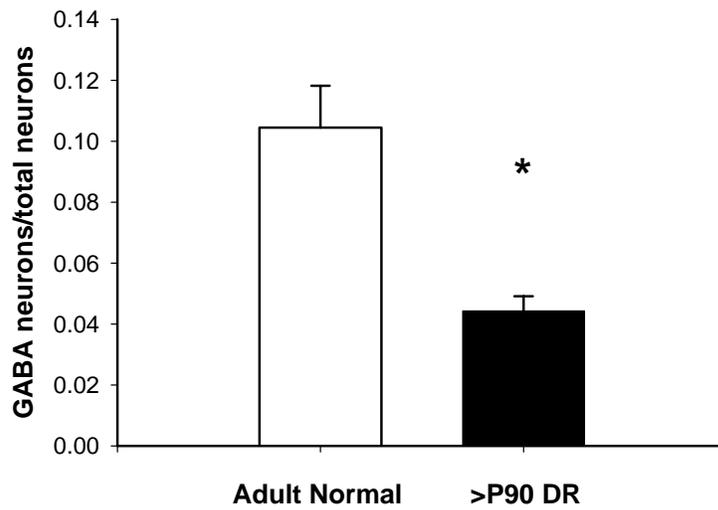


Figure 4.6. Adult DR animals have a significantly lowered proportion of GABA immunoreactive neurons in the SC compared to normal adults. Proportions values were: normal: 0.104 ± 0.013 , DR: 0.0443 ± 0.0047 , mean \pm SEM, $p = 0.003$, t-test, suggesting that a reduction in the number of GABA-containing neurons is also partially responsible for the RF enlargement seen in that group. Refer to methods section to see how data were obtained.

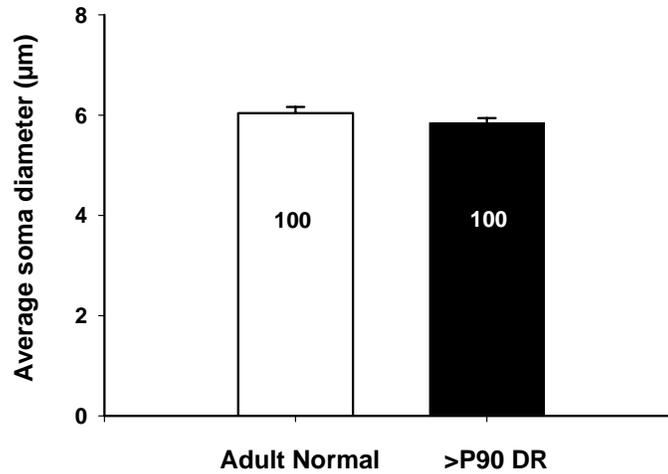
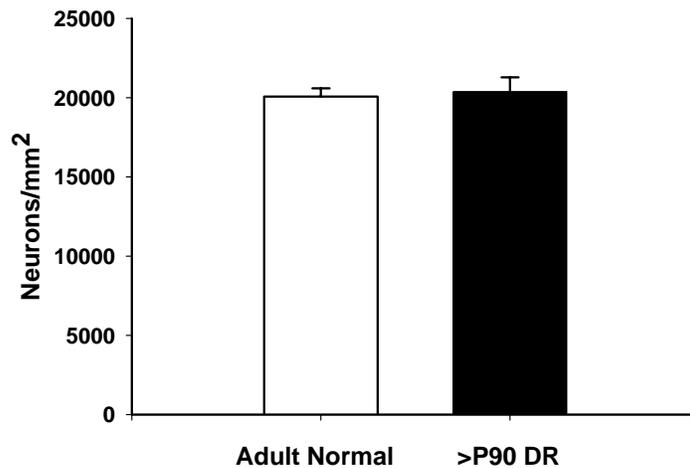
A**Size comparison of GABA-ir neurons****B****Neuronal density in the SC**

Figure 4.7. The size (A) and neuronal density (B) of immunopositive neurons in the SC of >P90 DR animals do not differ from that in normal animals ($p > 0.05$, t-test). These results refute the alternative hypothesis that a generalized decreased in neuron density or size explains the differences found in proportion (Fig 4.5) and density (Fig 4.6) of GABA-ir neurons in the SC of >P90 DR animals compared to normal adults. Data represented as mean \pm SEM.

CHAPTER 5: **Discussion**

Our results concerning the effects of dark-rearing on development and maintenance of the retinocollicular projection of the hamster point out the relationship between early sensory experience and brain plasticity later in life. Our results provide the first example of adult plasticity in a subcortical visual structure. They provide knowledge necessary to understand the effects of anomalous visual experience, and should be taken into account when considering treatments for patients with visual abnormalities.

1. Visual experience is necessary for maintenance but not refinement of receptive fields in the superior colliculus

Refinement of RFs is a necessary process that occurs during maturation of sensory systems. In the visual system, refinement of receptive fields results in higher visual acuity (Prusky et al., 2004). Our results show that visual experience is necessary for the maintenance of refinement in adulthood but not for the development of refined RFs (Carrasco et al., 2005). This conclusion derives from our finding that RFs of SC neurons become refined in DR hamsters at the same rate and age as in light/dark reared hamsters, but they lose refinement and thus enlarge if the animals remain in the dark. Unexpectedly, this loss of refinement occurs around P90, when hamsters are sexually mature and considered adults. Although a previous study showed that neuronal activity is necessary for maintenance of neuronal properties (Chapman, 2000), our results were unexpected because the loss of RF refinement occurred in animals that were further into adulthood and after RFs had attained their normal size.

a) Adult plasticity in sensory systems

While the notion of brain plasticity in sensory systems has been studied primarily in juvenile systems, several studies argue that the adult and aging brain are also susceptible to modifications in response to experience or damage, although in a more limited fashion (see Chen et al., 2002; and Mahncke et al., 2006 for reviews). A very well known example occurs in the somatosensory system. Whisker trimming in adult rats leads to alterations in inhibition in the barrel cortex (Akhtar and Land, 1991; Fuchs and Salazar, 1998). That brain plasticity can occur in adults has also been shown in the auditory and visual systems. Adult barn owls can shift their midbrain sound localization map in response to distortion of visual cues by prism-rearing if they have had previous prism experience as juveniles (Linkenhoker and Knudsen, 2002). In the deep layers of the SC of guinea pigs, the auditory map is disrupted after a period of dark-rearing in adulthood (Withington et al., 1994). Thus, brain plasticity in sensory systems is not limited to the juvenile brain.

Additional examples of adult brain plasticity have been reported in the visual system. In the visual cortex of adult rodents, ocular dominance can be shifted beyond the previously defined juvenile 'critical period' (Guire et al., 1999; Sawtell et al., 2003; Liao et al., 2004; Pham et al., 2004). Plasticity of the visual cortex occurs in adult cats within a few hours of retinal lesion (Chino et al., 1992). Interestingly, reorganization of cortical receptive fields only occurs if the intact eye is removed, suggesting that the intact eye would compete on an activity-dependent basis with the lesioned eye. Another example of adult visual system plasticity comes from a study on adult humans that have attained a substantial improvement of visual acuity with their amblyopic eyes after practicing a visual acuity task (Levi and Polat, 1996). Although there are no examples of adult plasticity in subcortical visual structures, one anatomical study showed that

lesions to visual cortex in adult cats produce synaptic rearrangements of the retinal afferents in the LGN (Kalil and Behan, 1987). Ours is the first report of plasticity in the adult superficial SC, therefore offering novel insight into the effects of sensory experience later in life on a subcortical visual structure.

b) The role of neuronal activity in maintaining receptive field properties in the visual system

Although numerous studies showed the importance of neural activity in the development and plasticity of neural connections and neuronal properties in the visual system, few studies have addressed their maintenance. In ferrets, blockade of glutamatergic activity in the retinae after segregation of eye-specific laminae in the LGN and before eye-opening produces desegregation (Chapman, 2000). In visual cortex, in addition to studies suggesting that visual experience is necessary for development of direction and orientation selectivity (Mower et al., 1981; Fagiolini et al., 1994), some earlier studies suggested that it is also necessary for their maintenance. Recordings from cat visual cortex have shown that direction and orientation selectivity are recognizable as soon as visual responses can be obtained in both light and dark-reared animals, but visual experience is necessary for their maintenance after the first few weeks of postnatal life (Buisseret and Imbert, 1975, 1976; Fregnac and Imbert, 1978). Similarly, our study showed that visual experience has a stabilizing effect on RF size in the SC. Furthermore, dark-rearing after RFs are refined does not affect RF size. Spontaneous activity might have a preponderant role relatively early in life, but later, when levels of spontaneous activity decrease (Itaya et al., 1995), visual experience becomes necessary to maintain the circuitry. Our data suggest that certain levels of neuronal activity are necessary even in adulthood to preserve

neuronal properties in the SC, although we do not know whether the pattern or the amount of activity is the relevant factor in maintaining the SC circuitry (see Crair, 1999; and Chalupa, 2007 for reviews).

c) Spontaneous and visually-evoked activity during map formation

We show, as reported previously (Thornton et al., 1996), that development of gross map topography in the SC is independent of visual experience. Gross retinotopy was present at the earliest age recorded in both DR and normal animals. Several studies on non-mammalian vertebrates suggest that initial establishment of an organized representation of the visual field in the optic tectum, the non-mammalian homologue of the SC, depends on molecular cues (see Flanagan, 2006 for review) but spontaneous, correlated retinal activity is required for refinement. Spontaneous waves of correlated activity that depend first on acetylcholine and later on glutamate have been described in the retinae of different vertebrate groups during the first postnatal weeks (Galli and Maffei, 1988; Meister et al., 1991; see Wong, 1999 for review). Studies on the role of spontaneous correlated retinal activity on the retinothalamic projection show that topography in the LGN is disrupted in mice lacking the $\beta 2$ acetylcholine receptor subunit (Feller, 2002; Grubb et al., 2003; but see Sun et al., 2008). Other studies on the role of glutamatergic waves of activity point out the importance of NMDARs as coincidence detectors during map refinement. NMDAR blockade in the SC during the first two postnatal weeks disrupts the anatomical and physiological refinement of RGC axon arbors (Simon et al., 1992; Huang and Pallas, 2001) presumably by interfering with the detection of the spontaneous correlated activity that takes place in the retina during that period. In our study, we did not disrupt spontaneous activity and thus as expected map formation proceeded normally. The

relative roles of acetylcholine and glutamate-dependent spontaneous activity in the retina on the development of the retinocollicular projection remain undefined.

2. Early visual experience prevents but cannot reverse deprivation-induced loss of refinement in adult superior colliculus

Our findings point out the role of early visual experience on plasticity later in adulthood. We show that a 30-day period of visual experience early in the life of hamsters is necessary to prevent RF enlargement in adult SC neurons induced by subsequent dark-rearing. We also show that visual experience after dark-rearing can prevent but not reverse the loss of RF refinement. Our results may offer insight into experience-dependent plasticity in other sensory systems.

a) Plasticity and sensory experience

That sensory experience modulates the degree of plasticity in sensory systems has been reported by several studies. In the visual system, dark-rearing extends the period when cortex is more susceptible to the effects of monocular deprivation (Mower et al., 1985; Mower and Christen, 1985; Mower, 1991), which normally occurs only in juvenile animals (Daw et al., 1992; Fagiolini et al., 1994). Ocular dominance plasticity is also achievable in adult animals (Liao et al., 2004; Pham et al., 2004; Fischer et al., 2007; Goel and Lee, 2007). We show that dark-rearing after RFs have been refined in the SC, at P60, does not produce a loss of RF refinement, suggesting that early visual experience leaves the visual system in a state less likely to undergo plasticity. Furthermore, dark-rearing from birth until P60 allows the SC to remain capable of being affected by late visual experience, because light exposure after that age prevents loss of RF refinement. Hence, early deprivation leaves the brain susceptible to later sensory experience. Our results also suggest that there is a defined period early in life when visual experience must

occur (see Appendix 1, Fig. 1) to forestall detrimental effects of light deprivation in adulthood. The amount of visual experience needed to protect against loss of RF refinement, ~30 days, seems relatively extensive compared to the 6-h period of visual experience needed to prevent the effects of monocular deprivation in cat visual cortex (Mower et al., 1983). Differences in the requirements for plasticity in these two visual areas may originate from distinct mechanisms of plasticity and/or their intrinsic malleability. Visual deprivation has severe effects on visual cortex, but is ineffective in changing receptive field properties other than RF size in the superior colliculus (this thesis; (Rhoades and Chalupa, 1978a; Chalupa, 1981)).

b) Early experience and adult plasticity

Our findings suggest that visual experience has a stabilizing effect in the retinocollicular projection and that its effects extend well into adulthood. Another example of the role of previous experience in achieving plasticity in adulthood has been reported in visual cortex. Although ocular dominance plasticity is achievable in adults as well as in juvenile animals, this type of plasticity can be enhanced by previous experience with monocular deprivation (Hofer et al., 2006), even though adult and juvenile plasticity involve different cellular mechanisms (Sawtell et al., 2003; Frenkel and Bear, 2004). Similarly, in the auditory system, early training promotes adult plasticity. Adult barn owls can learn sound localization based on an abnormal association of visual and auditory cues as adults only if they have experienced the association as juveniles (Linkenhoker and Knudsen, 2002). Anatomical remnants of this early experience found in the inferior colliculus of adults barn owls support the idea that adult learning is based on anatomical traces left in the brain from a previous learning process (Linkenhoker et al., 2005).

Our results from Chapter 4 suggest that a decrease in the strength of the inhibitory intracollicular circuit mediates the loss of RF refinement seen in the SC of long-term DR animals. Thus, a 30-day period of early visual experience may act by stabilizing the inhibitory circuit in the SC. Although we do not currently know the mechanism involved, several studies show that neuronal activity regulates the strength of inhibition in neuronal networks (Memo et al., 1991; Seil and Drake-Baumann, 1994). NMDAR activation increases the strength of the inhibitory circuitry in the rat SC (Aamodt et al., 2000). Those results together with our study suggest that a certain level of NMDAR activation early in life could have a long-term effect on inhibitory circuitry in the SC. Further investigation of the molecular nature of the long-term stabilization of the inhibitory circuitry by visual experience could provide important information about the mechanisms involved.

3. Adult plasticity in the superior colliculus results from the loss of surround inhibition

We have investigated the mechanism underlying the loss of RF refinement in the SC of long-term DR animals. Our results show that iontophoresis of GABA agonists and antagonists had a significantly reduced effect on both responsiveness and RF size in neurons of long-term DR animals with enlarged SC receptive fields than in normal animals (Chapter 4). We have also found that the density and number of GABA immunoreactive neurons is reduced in the SC of long-term DR animals compared to normal ones. Our results strongly suggest that the loss of RF refinement in long-term DR animals is due in large part to a partial loss of intracollicular inhibition. This study argues that modulation of the inhibitory circuitry is an important factor, and may be entirely responsible for adult plasticity in SC.

a) Visual deprivation and inhibitory circuitry

That visual deprivation leads to changes in inhibitory circuitry has been previously reported. In the retina, dark-rearing mice from birth until P30 decreases the levels of GAD65 and GABA, and 15 days of visual experience commencing at P30 recovers normal levels of GAD65 (Lee et al., 2006). In the LGN of monkeys, activity blockade by TTX eye injections decreases the number of GABA and GAD immunoreactive neurons and the expression of alpha1 and beta2/3 GABA receptor subunits in the deprived-eye laminae of the LGN (Hendry, 1991; Hendry and Miller, 1996). Presynaptically, neither dark-rearing nor monocular deprivation affect the level of GAD65 or GAD 67 in the visual cortex of cats (Benson et al., 1989; Mower and Guo, 2001). In monkeys, on the other hand, eyelid suture decreases the number of GABA immunoreactive neurons in the deprived-eye dominance columns of area 17 (Hendry and Jones, 1986). Interestingly, that study was done in adult monkeys, which shows a previously unrecognized level of plasticity in adulthood. At the postsynaptic level, in the cat visual cortex, the number of alpha1 and alpha3 GABAR subunits is elevated after dark-rearing, giving the cortex a juvenile-like molecular profile for these subunits (Chen et al., 2001). Our results suggest that long-term dark-rearing affects the GABAergic collicular circuitry both pre- and postsynaptically in adult animals: presynaptically, by decreasing the number of GABA immunoreactive neurons, and postsynaptically by decreasing the response mediated by GABA_A receptors.

b) Homeostatic plasticity of the inhibitory circuit

Homeostatic plasticity in the nervous system refers to the phenomenon of regulation of activity levels within certain limits in a given network. Homeostatic plasticity occurs in several

systems including cortical and hippocampal cell cultures (see Turrigiano and Nelson, 2000 for review). In cortical cultures, for example, blocking activity with TTX increases mEPSC amplitudes. Conversely, GABA_A receptor blockade decreases mEPSC amplitudes (Turrigiano et al., 1998). Our results show that neither stimulus-induced nor spontaneous spiking levels differ between the visually-deprived group and the normal group (Chapter 2) and that GABA_A receptor antagonists and agonists have a reduced effect on neurons of long-term DR hamsters compared to normal hamsters. We show a reduction of the number and density of GABA immunoreactive neurons in the SC of long-term DR hamsters. Interestingly, in visual cortex cultures, activity blockade with TTX decreases the amplitude of mIPSCs and this occurs through a reduction of the probability of channel opening accompanied by a reduction of GABA_A immunoreactivity (Kilman et al., 2002). One of the mechanisms responsible for plasticity of GABA_A receptors is their phosphorylation, which modulates insertion of many receptors, including GABA_A receptors, into the membrane (Wan et al., 1997; Wang et al., 2003). Presynaptically, a reduction in the level of GABA in visual cortex has been reported after intraocular TTX injections (Hendry and Jones, 1988). mRNA and protein expression of GABA and glutamate vesicle transporters are bidirectionally regulated by changes in activity levels (De Gois et al., 2005). These mechanisms involved in the regulation of activity seem to be common in the nervous system and therefore, it is possible that what is seen in the SC also occurs in other sensory systems.

4. Clinical implications

a) Visual impairments and plasticity

Studies that confirm the possibility of achieving brain plasticity in adulthood offer hope for possible treatments of anomalous conditions of sensory systems that are present beyond

infancy. We have focused on the role of visual experience in maintenance of neuronal properties in a subcortical visual structure, the superior colliculus. We found that visual deprivation can have negative effects during adulthood and that early visual experience is crucial to prevent future consequences of visual deprivation. Visual deprivation of the active eye as a treatment for amblyopia has been one of the most common methods used to treat that condition (Daw, 1998; Wu and Hunter, 2006). In light of our results, it is valid to consider that deprivation of the sound eye early in life could potentially cause irreversible damage to central circuitry in the long term. Our research, by addressing the long term effect of visual treatments, should shed light into the capability of the system to respond to visual experience beyond youth.

b) Plasticity of GABAergic circuitry and psychiatric disorders

One of the highlights of this work is related to plasticity of the inhibitory circuitry as a result of visual deprivation. Plasticity of inhibitory circuitry has become a relevant topic due to the role of inhibition in modulating the excitability of neural circuits. Several disorders, such as epilepsy, anxiety, insomnia, and substance abuse have been attributed to the anomalous functioning of inhibition at different locations in the central nervous system (see Mohler, 2006 for review).

Anxiety disorders have been related to a decreased clustering of GABA_A receptors at the synapse (Crestani et al., 1999). Decreased benzodiazepine binding in the orbitofrontal and temporal cortices and reduced GABA levels in occipital cortex have been found in patients suffering from anxiety (Tiihonen et al., 1997; Goddard et al., 2001). Epilepsy is another condition that has been suggested to arise from a dysregulation of GABA_A receptors (see Coulter, 2001 for review). A complex pattern of changes in the functionality, receptor subunit

composition, and distribution of GABA_A receptors has been found in models of epilepsy (Loup et al., 2000; Coulter, 2001; Mohler, 2006). Thus, the dynamic control of the different factors affecting the functionality of GABA_A receptors seems to be key in regulating the excitability of neuronal systems. These findings point out the relevance of learning about the nature of and mechanisms underlying plasticity of the inhibitory system.

c) GABA circuitry and aging

Functional decline is a normal process in the aging brain. Much of the functional degradation may be due to alterations of the inhibitory circuitry in addition to changes in the morphology of neurons and tissue density (see Mora et al., 2007; and Rissman et al., 2007 for reviews). Therefore, understanding the factors that modulate inhibition in the central nervous system will offer insights into the process of brain aging.

One of the most interesting examples of plasticity of the inhibitory system in normal aging occurs in the visual cortex of macaque monkeys, where data suggest a decreased level of intracortical inhibition in old age (Leventhal et al., 2003). In that study, an improvement in direction and orientation tuning was reported in the visual cortex of senescent monkeys with acute GABA or muscimol application. In addition, bicuculline, a GABA_A receptor antagonist, had a much weaker effect on cortical neurons from aged monkeys than on neurons from juveniles. Our results on the effects of gabazine and muscimol iontophoresis show a decreased effect of these drugs in the long-term DR group compared to the other experimental groups. Thus, our study also suggests inhibitory plasticity in the adult brain, but in this case as a consequence of early visual deprivation. Gaining knowledge of the physiological consequences

of a depressed inhibitory circuit may help to understand changes occurring during the normal aging process.

Our results on plasticity of the GABA circuitry in the SC add to the understanding of the relationship between circuit properties and neuronal response properties. Knowledge about the nature of the plasticity in inhibitory synapses gives insight into possible mechanisms of plasticity involved in sensory systems in particular and in the brain in general.

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Appendix A

1. Visual experience must occur early in life to prevent the effects of dark-rearing in the

SC

In Chapter 3, we reported that a 32-day period of visual experience starting at P8 is necessary to avoid the loss of RF refinement by dark-rearing (Carrasco and Pallas, 2006). Because we did not test whether visual experience was necessary at a particular developmental stage, we added an additional experimental group, as reported in this section. We tested whether 30 days of visual experience starting at P37, instead of P8, is sufficient to maintain RF refinement. We chose P37 because we wanted to test whether visual experience starting at an age far from eye opening but still before RFs would refine would prevent from the effects of long-term dark-rearing. We found that this experimental group also lost RF refinement when tested as adults. Our results suggest that visual experience needs to occur early in life in order to prevent the effects of dark-rearing in the SC.

Receptive field size after a short period of visual experience

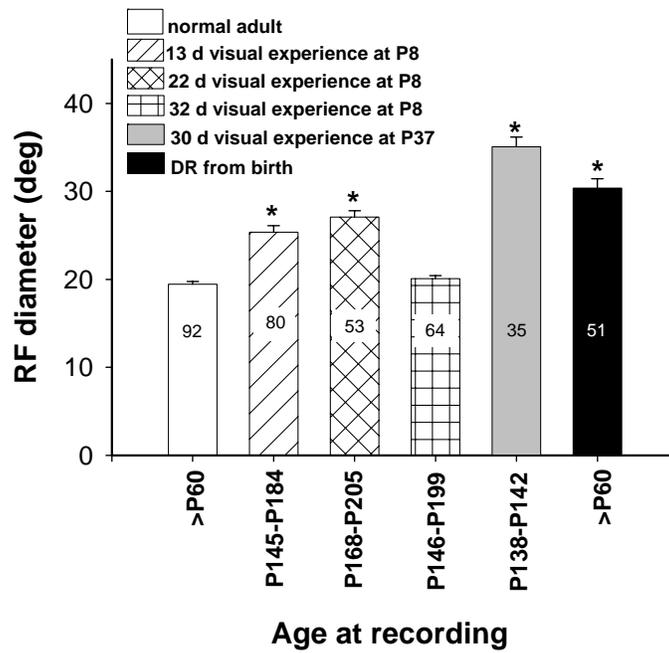


Figure A.1. Visual experience starting at P8 but not P37 prevents loss of RF refinement by dark-rearing. Animals exposed to 30 days of visual experience at P37 have significantly larger RFs in the SC than normally-reared animals (normal: 19.4 ± 0.31 , $n = 92$; DR with 30 days of visual experience: 35.0 ± 1.0 , $n = 35$; $P < 0.001$, Mann-Whitney Rank Sum Test).

Appendix B

1. Introduction

Development and maintenance of the properties of sensory systems depend on sensory input. In the visual cortex and LGN, the development of normal response properties of neurons requires visual experience (see Chapman et al., 1999; and Crowley and Katz, 2002 for reviews). Our previous studies showed that in the Syrian hamster (*Mesocricetus auratus*) superior colliculus (SC), visual experience is necessary to maintain rather than drive receptive field (RF) refinement (Carrasco et al., 2005; Carrasco and Pallas, 2006). The question that remains unanswered in several systems concerns the mechanism by which visual experience acts on modifying neuronal properties.

In the visual cortex, the level of expression of several molecules depends on visual experience. Specifically, and very importantly due to its role in coincidence detection, the subunit composition of NMDA receptors (NMDARs) depends on developmental stage and sensory experience (Carmignoto and Vicini, 1992; Philpot et al., 2001; Tongiorgi et al., 2003). Normally the NR2B subunit, with its longer channel open time, is replaced by the NR2A subunit during development, accounting at least in part for the shortening of NMDA currents with age. It has been suggested that NMDARs containing a relatively high proportion of NR2B may facilitate induction of long-term potentiation (LTP), as seen in young animals (Yoshimura et al., 2003). On the other hand, visual deprivation has been shown to decrease levels of NR2A in visual cortex, and visual experience rapidly increases its levels (Quinlan et al., 1999a; Quinlan et al., 1999b; Tongiorgi et al., 2003). Thus, changes in the relative amount of NR2A and NR2B may be involved in plasticity of physiological responses, which depends on age and sensory experience.

In the SC, which receives direct input from the retina, hamsters reared in complete darkness since birth or with less than 30 days of early visual experience show refinement of RFs of retinorecipient neurons, but RF refinement is lost in adulthood (Carrasco et al., 2005). Consequently, RFs of SC neurons become enlarged in hamsters that have not received enough visual input at the appropriate time. Some studies have suggested that dark-rearing increases the percentage of cells sensitive to NMDAR antagonists and that NMDA currents have a greater importance in visual transmission in the SC compared to light reared rats (Binns and Salt, 1998a, b). Those results support more recent evidence that visual experience and age modulate the subunit composition or number of NMDARs in the SC.

NMDARs are involved in synapse strengthening in several model systems. Long-term potentiation elicited in the retinotectal projection, the non-mammalian homologue of the retinocollicular projection, is NMDAR-dependent and may be involved in the refinement of retinal projections (Schmidt, 1990; Zhang et al., 1998). Therefore, we propose that abnormalities in the amount of NMDARs and/or in the ratio between NR2A and NR2B subunits underlie the loss of refinement in DR animals. In particular, we hypothesize that if the NR2B subunit is present in the long-term DR groups in a relatively higher proportion than in the normal group, and/or in NR2A is unchanged or decreased, then previously silent synapses could become potentiated and thus make RFs larger. To investigate this possibility, we performed Western Blotting to analyze the relative amounts of NR2A and NR2B protein in the SC of normally reared and DR hamsters.

2. Materials and methods

a) Experimental groups

Syrian hamsters (*Mesocricetus auratus*) were used in this study. We compared the quantity of NR2A and NR2B proteins in membrane fractions of four experimental groups (n=14 animals in each group): 1) Adult animals at postnatal day 136 (P136)-P163 adult animals reared in a 14/10 h light/dark environment, 2) P54-57 14 light/10 dark, 3) P135-149 animals reared in an internal dark room since birth (dark-reared -DR), and 4) P57-58 DR. We chose to compare these age groups because hamsters have developed refined RFs in the SC by P60, and DR hamsters begin to lose RF refinement after P90. All of the procedures used on animals met or exceeded standards of humane care developed by the National Institutes of Health and the Society for Neuroscience, and were approved in advance by our Institutional Animal Care and Use Committee.

b) Protein extraction

For protein extraction, brains were extracted after animals were deeply anesthetized with sodium pentobarbital (15 mg/100g). With the brain on dry ice, rapid dissection of the superficial layers of the superior colliculus (SC) was performed. We tried different lysis buffers (50 mM HEPES only and 20 mM HEPES to which was added 150 NaCl, 2mM EDTA, 10 % glycerol, and 0.5% Nonidet P-40) and centrifugation at ~10,000xg for at least 30 min to extract NMDARs from the tissue. Because one-step centrifugation at that speed in the different lysis buffers did not yield the expected band at 180 kDa, the size of the NMDARs subunits, we sought more specific methods to isolate synaptosomes, which should be enriched in NRs. Thus, we followed the protocol of a previous study (Shi et al., 1997) and the SC tissue of each animal group was

homogenized in 500 μ l of lysis buffer (10 mM phosphate buffer, pH 7.0, to which was added 5 mM EGTA, 5 mM EDTA, 1 mM DTT, and Complete Mini protease inhibitor (Roche Diagnostics, Indianapolis, IN)). Homogenates were fractionated by centrifugation at 4°C for 10 min at 16,000 \times g. The supernatants (crude soluble fraction) were collected and placed on ice. The pellets were resuspended in 150 μ l of 2 mM HEPES, pH 7.2, and centrifuged at 4°C for 30-45 min at 200,000 \times g. The supernatants were discarded and the pellets resuspended in 140 μ l of 0.5 mM HEPES, pH 7.3, 0.32 M sucrose and centrifuged at 4°C for 8 min at 450 \times g. The supernatants obtained correspond to the membrane fraction, in which synaptic proteins including the NMDAR subunits were found. All of the preceding procedures were performed on ice or at 4°C. Protein concentration was calculated using a *DC* (detergent compatible) protein assay, which is a modified Lowry assay (*DC* protein assay reagents, Bio-Rad, Hercules, CA). Samples were mixed in equal amounts of Laemmli sample buffer (Bio-Rad, Hercules, CA) and 5% mercaptoethanol and heated to 90°C for 5 min. Samples that were not immediately used were stored at -80°C.

c) Western Blotting

Samples and standards (Precision plus protein standard, Bio-Rad) were run on 7% SDS-Polyacrylamide minigels at 110 V for ~1 h, taking care to load the same amount of protein in each well (20-24 μ l of sample per well). Proteins were transferred to nitrocellulose membranes by electroblotting (Bio-Rad) at 400 mA for 130 min at 4°C on ice. Blots were washed in TTBS (0.1% Tween in TBS) and blocked with 5% nonfat dry milk in TTBS for 5 h at room temperature. Membranes were washed in TTBS three times for 5 min each. Blots were incubated in the primary antibody solution made in 2% nonfat dry milk in TTBS (rabbit anti-

NR2A 1:500; rabbit anti-NR2B 1:500, Chemicon, Temecula, CA) for 17 h at 4°C. Blots were washed three times for 5 min each in TTBS and incubated in secondary antibody solution made in 2% nonfat dry milk in TTBS for 1 h (horseradish peroxidase-conjugated goat anti-rabbit, 1:10,000, Bio-Rad). Blots were then washed three times for 5 min each in TTBS and three times for 5 min in TBS, and reacted with a chemiluminescent substrate kit (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL). Films (X-OMAT LS, Kodak, Rochester, NY) were exposed to the membrane for 15-30 s and processed in a Kodak processor (X-OMAT 2000A processor).

d) Data analysis

We measured optical density of bands in the films using ImageJ software. All experimental groups were compared to the optical density of bands from the adult normal group and were represented as a percentage of normal. Only bands from the same blot were compared in this way, but results from different samples and blottings were pooled together.

3. Results

We performed Western Blotting for NR2A and NR2B proteins from membrane fractions from the four experimental groups: 1) P54-57 normal animals 2) P136-163 normal animals, 3) P57-58 DR animals, and 4) P135-149 DR animals. Bands for both proteins, NR2A and NR2B, appeared at the expected location in accordance with their molecular weight, 180 kDa.

We hypothesized that NR2B levels would be comparatively increased in the long-term DR group and NR2A would be the same or decrease compared to the normal adult group. Figure 1 shows examples of NR2A bands obtained from the four different experimental groups. We

found that the levels of NR2A in the older DR group were significantly different from those found in normal adult animals (Fig. 2). P135 DR animals had decreased levels of this protein compared to the P136 normal group ($p < 0.05$, ANOVA on Ranks, post hoc Tukey test). There were no significant differences found between the normal and DR group earlier in development (<P57-58).

We expected to see an increase in the amount of NR2B in the DR animals. Contrary to this expectation, we found no significant differences in the levels of NR2B protein between any of the experimental groups (Fig. 3). However, there was a trend for this protein to decrease in quantity with age and to be diminished in the P149 DR group.

4. Discussion

We demonstrated that adult DR animals have less NR2A protein in the SC compared to normal adult animals. Taken together with our previous studies showing that DR hamsters >P90 have enlarged RFs in the SC (Carrasco et al., 2005; Carrasco and Pallas, 2006), this result raises further questions about causation. Previous studies showed that dark-rearing decreases NR2A in the visual cortex of rats (Quinlan et al., 1999a; Quinlan et al., 1999b; Tongiorgi et al., 2003) and that visual experience has the opposite effect. Our results, although in a different brain area, are consistent with those studies.

Visual deprivation facilitates LTP induction in rat visual cortex (Kirkwood et al., 1996), and this could be due in part to changes in NMDAR subunit composition (Yoshimura et al., 2003). A relatively higher proportion of NR2B in relation to NR2A would increase the duration of glutamatergic currents and thus make spike-time dependent plasticity (STDP) more likely. We found no difference in the level of NR2B in DR animals compared to normal, although there was

a tendency for this protein to be decreased in the older DR group compared to the other groups. It has been shown that NMDARs are involved in the appearance of LTP in the SC (Okada and Miyamoto, 1989). Consistent with that idea, we expected to find a larger proportion of NR2B in old DR animals, which would facilitate the potentiation of synapses that were previously silent and thus contribute to the enlargement of the excitatory RF in the SC. We think that a more detailed electrophysiological study that measures changes in the contribution of NR2A and NR2B dependent currents would help to draw a more solid conclusion. Thus, although interesting, our results are not conclusive.

Nevertheless, our finding that visual experience regulates NMDAR composition in the SC is still of interest. Further studies are necessary to describe the physiological effects of that regulation. Regulation of the amount or composition of NMDARs modulates Ca^{2+} conductance through these receptors and therefore several cellular processes that involve Ca^{2+} binding proteins and gene regulation related to synaptic plasticity (Rauschecker, 1991; Malenka and Nicoll, 1993; Barria and Malinow, 2005; Citri and Malenka, 2008; Huang et al., 2008). Whether these changes in NMDAR composition are related to the RF enlargement in the SC of DR hamsters is still uncertain.

Results presented in Chapter 4 suggest that there are fewer GABA immunoreactive neurons in the SC of long-term DR hamsters than in normal animals. In addition, our electrophysiological results show that SC neurons of long-term DR animals are less responsive to antagonists and agonists of GABA_A receptors, suggesting that they have fewer of these receptors or that they are altered in composition. Several previous studies have shown that inhibitory and excitatory inputs can regulate each other through their postsynaptic neurons and therefore maintain excitation in the system within certain levels (Liu, 2004; see Turrigiano and

Nelson, 2004 for review; Echegoyen et al., 2007). The downregulation of the GABAergic system of DR animals may also downregulate the glutamatergic system, a hypothesis that would coincide with the decreased amounts of NR2A and NR2B subunits. On the other hand, the opposite situation may be true: regulation of NMDARs may have regulated the GABAergic circuit in the SC. Distinguishing between these possibilities requires further study.

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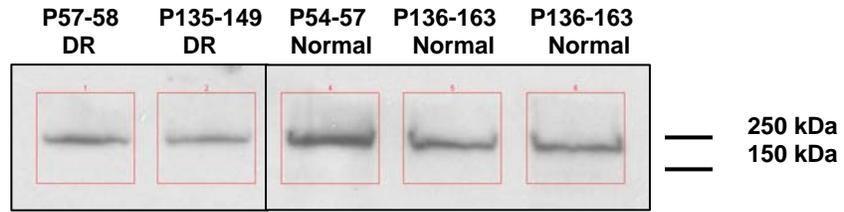


Figure B.1. Examples of NR2A bands from dark-reared (DR) and Normal animals at the postnatal (P) ages indicated. The band located between 150 and 250 kDa corresponds to NR2A, a 180 kDa protein.

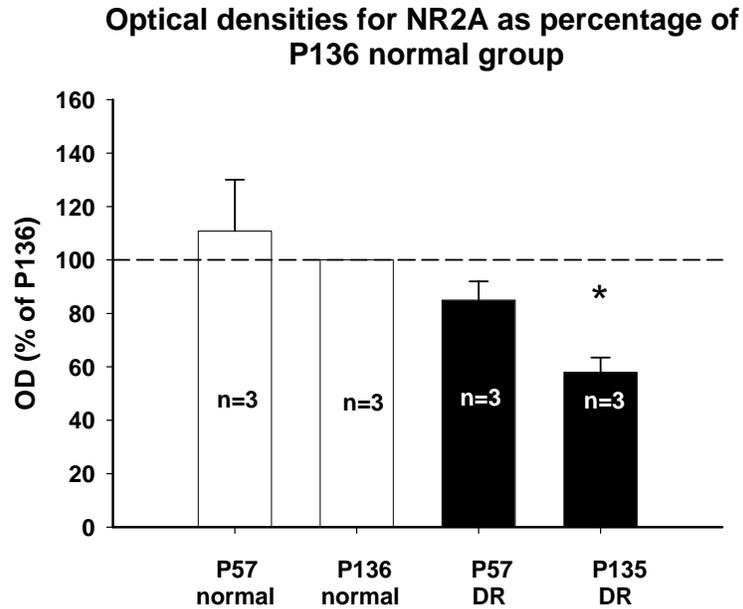


Figure B.2. NR2A is decreased in old DR animals. Optical densities were expressed as a percentage of the density of the band in the P136 normal group run in the same gel and blot. Each sample was run three times for each of the groups and their percentage values combined and expressed as mean \pm SEM. The amount of NR2A in the P135 DR group was significantly less than that in the P136 normal group (ANOVA on Ranks, $P = 0.036$, post hoc Tukey test). Data represented as mean \pm SEM.

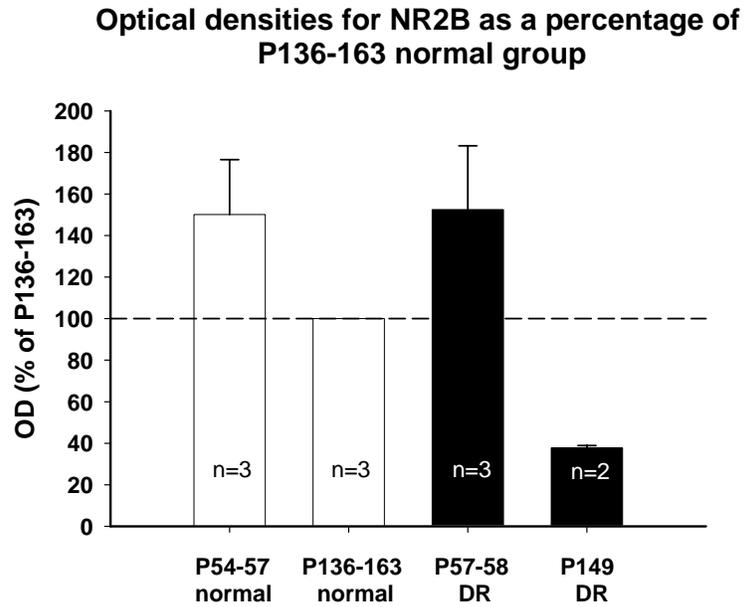


Figure B.3. No significant differences were found for NR2B. Optical densities were expressed as a percentage of those from the P136-163 normal group run in the same gel and blot. Samples were run at least twice for this protein for each of the groups. No significant differences were found between the groups, although the P149 DR group tended to have a decreased amount of this protein compared to the other groups (ANOVA on Ranks, $P = 0.171$). Data represented as mean \pm SEM.