SUSCEPTIBILITY TO MONOCULAR DEPRIVATION FOLLOWING IMMERSION IN DARKNESS JUST PRIOR TO THE CRITICAL PERIOD PEAK

by

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To the MacBook that just wouldn't quit.

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ABSTRACT

Immersion of animals in complete darkness highlights that the maturation and plastic capacity of visual circuitry is regulated by visual experience. Recent investigations have shown that imposing 10-days of darkness exposure during juvenile life instates a neurobiological context wherein an animal's susceptibility to subsequent modification of visual experience is augmented. For instance, immersion of 12-week old cats in complete darkness for 10-days promotes neurobiological changes that render the effects of monocular deprivation (MD) more severe than those observed in age-matched controls. This result indicates that darkness increases an animal's capacity for plasticity and recapitulates a developmental state akin to a much younger animal. The current study sought to explore whether short-term darkness immersion could similarly promote visual plasticity when applied just prior to the peak of the critical period), an age when sensitivity to visual perturbation is at its biological maximum. Although 7-days of MD at this age produced potent alterations of neuronal soma size in the dorsal lateral geniculate nucleus (dLGN), 10-days of preceding darkness immersion did not increase the subsequent MD effect. Similarly, MD imposed following darkness induced changes in dLGN neurofilament immunoreactivity equivalent, but not greater than those observed in animals subjected to MD alone. These results reveal that the propensity of darkness to promote susceptibility to MD depends on the animal's initial capacity for visual plasticity. Further, it appears that the ceiling for critical period plasticity may be constrained by a neural environment that is resistant to modification by darkness.

LIST OF ABBREVIATIONS USED

αCaMKII: α-Ca2+/calmodulin-dependent protein kinase II

BDNF: brain-derived neurotrophic factor

chABC: chondroitinase-ABC

CNS: central nervous system

CREB: cAMP response element-binding protein

CSPG: chondroitin sulfate proteoglycan

dLGN: dorsal lateral geniculate nucleus

DNA: deoxyribonucleic acid

DR: darkness

DR+MD: darkness + monocular deprivation

E-I: excitation-inhibition

ECM: extracellular matrix

ERK: extracellular-signal-regulated kinase

GABA: gamma-aminobutyric acid

GAD65: glutamic acid decarboxylase 65

HDAC: histone deacetylase complex

LGN: lateral geniculate nucleus

LTD: long-term depression

LTP: long-term potentiation

Lynx1: Ly6/neurotoxin 1

MAG: myelin-associated glycoprotein

MD: monocular deprivation

MeCP2: methyl–CpG-binding protein 2

nAchR: nicotinic acetylcholine receptor

NF-H: neurofilament - heavy

NF-L: neurofilament - light

NF-M: neurofilament - medium

NGF: nerve growth factor

NMDA: N-metyhl-D-aspartate

NR2A: NMDA receptor subunit 2-A

NR2B: NMDA receptor subunit 2-B

Narp: neuronal activity-regulated pentraxin

NCAM: neuronal cell adhesion molecule

NgR: nogo receptor

Otx2: orthodenticle homeobox 2

OMgp: oligodendrocyte-myelin glycoprotein

PBS: phosphate buffered saline

PirB: paired immunoglobulin-like receptor B complex

PKA: cAMP-dependent protein kinase

PNN: perineuronal net

PSA: polysialic acid

PV+: parvalbumin-expressing

tPA: tissue plasminogen activator

TTX: tetrodotoxin

V1: primary visual cortex

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CHAPTER 1: INTRODUCTION

Modern research reveals that an animal's phenotype is necessarily predicated on the influence of both genes and experience. Genes dictate a molecular road map that instructs initial developmental processes. Experience, however, can both modify and make additions to this structurally- and functionally-serving map, allowing for vast experience-dependent phenotypic differences. This two-factor conceptualization of biological outcomes is readily apparent in the study of monozygous twins, who despite sharing a common genotype, gradually incur distinguishing experience-dependent changes in their gene-expression profile (Fraga et al., 2005). That experiential variation produces phenotypic discordances, even between twins of the same genotype, alludes to the vast variability in population phenotypes that can be induced by differential living experiences.

Neural plasticity encompasses the diverse structural and functional changes undertaken by the brain in response to experience. Plastic processes allow for the modification of existing connections between brain cells and regions or even the formation of completely new connections. This flexibility of neural circuitry underlies many important functions, including one's capacity to learn (Galván, 2010), to form memories (Neves et al., 2008), or to recover from brain damage (Kleim and Jones, 2008). One particularly notable and general feature of neuronal plasticity is its relationship with age. Diverse fields of research have converged upon the notion that the brain's sensitivity to experience is maximal

during early life. For instance, the acquisition of language (Lenneberg, 1967), learning of motor skills (Watanabe et al., 2007), and also the development of sensory systems (Kral, 2013; Hooks and Chen, 2007; Erzurumlu and Gasper, 2012) each appear governed by mechanisms whose sensitivity to experience is maximal during early life and gradually dissipates with age thereafter. During these periods of elevated plasticity, so-called “critical” or “sensitive” periods, neuronal circuitry is readily shaped by intrinsically- (spontaneous) and extrinsically-evoked neural activity. It is thought that this period of heightened flexibility allows one to manage the novelty and instability of early experiences and to develop adaptive neural functioning while a later-occurring decline in plasticity ensures trained responses persist throughout life (Fusco and Minelli, 2010).

However, when early development is perturbed in some way that precludes the formation of functional neural circuitry, the recession of elevated critical period plasticity can cause intractable functional deficits. Sometimes it may therefore be desirable to restore a state of heightened plasticity beyond the critical period such that aberrant neural circuitry may be altered to salvage normal function. Further, while biology permits an extraordinary degree of plasticity during critical periods, it remains unknown whether this sensitivity to experience may be further elevated from its maximum by experimental means. Here, I discuss my investigation of critical period plasticity in the visual system and means by which it may be augmented to facilitate experience-dependent anatomical malleability.

1.1 - Plasticity of the visual system

The developing visual system has become a well-established model for the study of critical period plasticity. Alterations of visual experience during early life provoke profound changes to anatomical and physiological components of the visual system as well as to visual behaviour. For instance, conditions that preclude a balanced visual experience between the two eyes during early life are pre-disposing of a collection of visual deficits characteristic of a condition called amblyopia. In humans, natural imbalances in visual input can derive from deviations in ocular positioning (strabismus), in opacity of the lenses or corneas (congenital cataract), or in the relative refractive strength of the two eyes (anisometropia). These visual abnormalities are thought to presage changes in the visual system that subsequently impair visual functioning, particularly of the initially perturbed eye (Webber and Wood, 2005).

Amblyopia prevalence is estimated to range from 0.25% to 5.8%, usually centering around 2-3%, depending on the specific population and diagnostic criteria used (Thompson et al., 1991; Vinding et al., 1991; Attebo et al., 1998; Brown et al., 2000; Rosman et al., 2005; MEPEDS, 2008; Drover et al., 2008; Friedman et al., 2009; Xiao et al., 2015). This high occurrence makes amblyopia the leading cause of monocular visual loss in adults, ahead of age-related macular degeneration and diabetic retinopathy (Buch et al., 2001). Although unilateral vision loss is considered a relatively mild impairment compared to conditions that cause binocular or complete blindness, amblyopes have worsened career prospects (Adams and Karas, 1999), decreased quality of life

(Brown et al., 2001), and impaired psychosocial development (Koklanis et al., 2006). Further, unilateral vision loss caused by amblyopia is thought to elevate one's risk of accident-induced vision loss in the healthy eye (Tomila and Tarkkanen, 1981; Rahi et al., 2002; Leeuwen et al., 2007), possibly as a result of impaired depth perception (Thompson and Nawrot, 1999) or reduced field of vision. It is therefore evident that the prevention and/or treatment of amblyopia plays an important role in preserving not only vision but also a variety of characteristics that facilitate employment, quality of life, and mental wellness.

Consistent with critical period regulation, amblyopia is unlikely to manifest in adults, and childhood amblyopia becomes near intractable when traditional interventions are imposed beyond about 7 years of age (Holmes et al., 2011). Even prior to this age, the efficacy of treatment declines dramatically with progressively later intervention (Epelbaum et al., 1993). Preventing amblyopia relies on robust screening procedures that either assess for conditions pre-disposing of ocular imbalances, including strabismus, congenital cataracts, and anisometropia, or for the presence of amblyopia itself during childhood. Screening of this type is associated with reductions in the prevalence of amblyopia by 45-62% (Schmucker et al., 2009), presumably by facilitating the earlier prescription of treatment designed to prevent or reverse the impairment. Initial treatment aims to resolve any ocular imbalances, perhaps by re-aligning a strabismic eye, by removing a unilateral cataract, or by prescribing glasses that nullify eye-specific refractive differences. Once ocular balance has been restored, the goal of treatment shifts towards reversing any existing neural bias that has

induced the visual impairment. Most commonly this is achieved by imposing the use of an eye patch over the “good” eye, forcing usage of the amblyopic or “bad” eye (Webber and Wood, 2005).

However, even when compliance is good, the efficacy of conventional patching in reversing the effects of amblyopia is relatively weak. While the forced disadvantaging of the initially strong eye may strengthen the initially weak eye, patching fails to allow binocular visual experience. Therefore, while visual balance may be restored between the acuity of the two eyes by patching, the brain gains little experience in using the two eyes together, culminating in impaired stereopsis or depth perception. Further, that patching is only effective when employed prior to about 7 years of age (Holmes and Lazar, 2011) precludes effective treatment of amblyopes whose condition has circumvented diagnosis during early life. An ideal treatment for amblyopia is possibly one that re-instates a neural context similar to that at the peak of the critical period, wherein an ocular imbalance is rapidly equilibrated and subsequent binocular experience facilitates the formation of precise, binocularly-tuned neural circuitry. While such a treatment does not yet exist in humans, investigation using animal models has allowed great progress in understanding the cellular and molecular mechanisms underlying plasticity and in designing interventions that enhance plasticity to reverse developmental abnormalities like amblyopia.

1.2 - Modelling of amblyopia in non-human animals

Monocular deprivation (MD), a unilateral deprivation of vision, has long been used to study the sensitivity of the visual system to visual experience and has provided a means of modelling amblyopia-like changes in animal models. MD can be employed via a variety of experimental techniques. MD similar to that of conditions presaging amblyopic changes in humans might be modelled by inducing a unilateral cataract, or by imposing the unilateral use of a distorted lens. More severe MD effects can be produced by occluding a single eye with an eye-patch or eye-lid suture that occlude both light and patterned visual stimulation. Occlusion via eye-lid suture is often preferred to that employed with patches as it is easier to maintain and imposes a more substantial difference in visual input between the two eyes. Binocular asymmetry can also be established by preventing the propagation of retinally-driven activity such as would occur following monocular enucleation or unilateral intraocular tetrodotoxin (TTX) injection. For the purposes of this paper, the abbreviation “MD” will be used to refer to monocular deprivation imposed via lid suture closure, as this technique is most commonly employed in the field. When MD of another form is conducted, this will be indicated. Despite the plethora of means by which MD can be induced, each technique is related in reducing the amount or quality of retinal activity, in one eye only, that is propagated through the central visual pathway.

The seminal work of Hubel and Wiesel (1963) first described the effects of MD in kittens. They famously showed that a unilateral deprivation of light and patterned visual input during early postnatal development rendered neurons in

the primary visual cortex insensitive to stimulation of the deprived eye, and the visual performance of the deprived eye severely impaired. It is largely upon the basis of this work that fields of sensory plasticity are founded. Since Hubel and Wiesel's initial contribution, a vast and diverse range of research has investigated the effects of visual experience on development (Daw, 1998).

1.3 - Visual development in animal models

Understanding the influence of abnormal visual experience on neural development requires an understanding of visual system components as well as an understanding of how the visual system develops under normal rearing conditions. Our knowledge of the mammalian visual system and its sensitivity to experience has been aided particularly by the study of non-human primates, cats, and rodents amongst other species. The visual system of each of these species shares important characteristics with that of human. Each possesses two eyes with corneal optic characteristics and a posterior layer of specialized retinal cells. Upon exposure to light, the cells of the retinae initiate processes that transform light signals into the chemical/electrical signals requisite for information transfer within the brain. This information is first relayed to a thalamic area, the lateral geniculate nucleus (LGN), and thereon to a variety of regions including cortical areas, namely the primary visual cortex (V1) whose output is subsequently propagated to progressively more specialized extrastriate visual regions. It is by

way of information transmission along these neural pathways that our environment becomes visually accessible (Figure 1).

Despite these similarities, there are a variety of inter-species differences in visual system structure and function that emerge from species-specific developmental differences. For instance, the retinae of sub-human primates and cats are characterized by a central retinal specialization – a fovea, or area centralis – that permits high acuity vision and is absent in rodents (Rapaport and Stone, 1984; Provis et al., 2013). The degree to which optic nerve axons decussate (Dräger and Olson, 1980), as well as the structure and organization of visual structures like the LGN and V1 (Discenza and Reinagel, 2012) are also important sources of species-specific differences in the visual system. As a result of these differences, it is paramount that experimental results be interpreted with acknowledgement of the species-specific contexts within which observations are made.

In each of these species, however, the development of the visual system, involves both activity-independent processes as well as those that require either spontaneous or evoked neural activation (Katz and Shatz, 1996). Each of these types of processes are important in facilitating the development of the visual system and are differentially influenced by manipulations of visual experience before, during, or after the critical period for ocular dominance.

1.3.1 - Pre-critical period

A considerable degree of visual system development occurs prenatally. As with other cells of the brain, cells of the visual system are not generated in visual structures but rather migrate to them following their birth whereupon they form dendrites to receive input from other cells and/or form axons to relay their neural output (Rakic, 1977; Daw, 2014). Initially, axons emerging from retinal ganglion cells exit the posterior surface of the eye, guided by molecular cues to the optic chiasm, a region characterized by the crossing of the optic nerves. While afferents from the nasal retinae tend to decussate, projecting to the contralateral dLGN, afferents from the temporal retinae tend to forego decussation, projecting to the ipsilateral dLGN, such that visual information from each field of view is processed in the contralateral hemisphere of the brain (Reese, 2012). This partial decussation is true of the retinogeniculate projection in higher order mammals including humans, non-human primates, and cats, enabling binocular processing within each hemisphere (Larsson, 2015). In rodents, however, there is a much lower proportion of retinal afferents projecting to the ipsilateral dLGN (Dräger and Olsen, 1980). The optic chiasm of rodents is therefore characterized by a greater proportion of contralateral retinogeniculate projections than in higher mammals, meaning that most of the input from a single eye is processed in the contralateral hemisphere.

Upon reaching the dLGN, retinal afferents from both eyes initially innervate an intermingled distribution of geniculate cells. In humans, non-human primates, and cats this jumbled collection of terminating arbors is gradually refined, partially

through spontaneous retinal activity (Wong, 1999; Penn et al., 1998) to produce eye-specific dLGN layers. In rhesus monkeys, this lamination of the dLGN occurs prior to birth (Rakic, 1977) whereas in cats dLGN laminae are only distinguishable upon the first day of postnatal life (Kalil, 1978; Shatz, 1983). The LGN of humans and non-human primates is characterized by six laminae, each exclusively receiving monocular input. In cats, three laminae are distinguishable, two of which are monocular layers (A and A1 lamina), receiving input from exclusively the contralateral (A) or ipsilateral (A1) eye. The third lamina, layer C, receives input that is not segregated by eye of origin. Nocturnal rodents, such as rats and mice have lateral geniculate nuclei that show no apparent lamination in Nissl preparation but that contain multiple eye-specific spatially segregated subdomain regions (Discenza and Reinagel, 2012). However, due to a high proportion of contralateral retinogeniculate projections in rodents, the area of the dLGN devoted to cells receiving input from the ipsilateral eye is relatively small (Dräger and Olsen, 1980). Regardless, the segregation of LGN regions by eye of origin appears a conserved organizing feature of the mammalian dLGN.

In humans, non-human primates, and cats, thalamo-cortical afferents of left- and right-eye serving dLGN layers initially synapse with layer IV cells of the primary visual cortex in an overlapping fashion. That is, the nascent visual cortex lacks distinct segregation of eye-specific input. Over time, this intermingled distribution of monocularly driven cells is progressively segregated into a tiled pattern of eye-specific “ocular dominance columns”. The segregation of monocular input occurs in the absence of spontaneous or visually-evoked retinal

activity (Crowley and Katz, 1999; Crair et al., 2001), suggesting that this initial pattern of development occurs via guidance from genetically encoded molecular cues. In normally developing animals the width of cortical columns receiving input of left eye origin is roughly equal to those receiving input of right eye origin (Levay and Gilbert, 1976). Subsequent projections of monocularly driven layer IV cells converge upon neurons of the extragranular layers (I, II, III, V, and VI), conferring a sensitivity to activity originating from either eye. The degree to which these extragranular neurons are binocularly responsive varies with some cells sensitive mostly to stimulation of a single eye, and others about equally sensitive to stimulation of either eye. This pattern of cortical architecture differs greatly from that observed in rodents whose ocular dominance appears to be organized at the level of intermingled, individual cells in a so-called “salt and pepper” distribution (Ohki and Reid, 2007; Kaschube, 2014).

That the majority of developmental changes described here occur in utero or very early in postnatal life indicates that visual development is initially independent of visually-evoked activity. Consequently, manipulations of visual experience appear to have no effect very early in life. For instance, the segregation of eye-specific inputs in layer IV of the developing visual cortex is unaltered by monocular enucleation at the time of eye opening (Crowley and Katz, 1999). Binocular enucleation is similarly ineffective in altering the development of ocular dominance columns when imposed prior to the critical period (Crowley and Katz, 2000). This of course, does not exclude the fact that spontaneous activity originating in the retina, dLGN, or other sources, could

provide development-guiding input prior to the advent of experience sensitivity (Wong et al., 1993).

1.3.2 - During Critical Period

While the formation of thalamic and cortical visual processing areas occurs initially in the absence of visually-evoked stimulation, weeks later visual architecture becomes highly dependent on visually-evoked activity and is readily altered by modulation of normal visual experience. In cats and rodents, a sensitivity to visual experience is suddenly established at about 20-days of age (Fagiolini et al., 1994; Gordon and Stryker, 1996; Hubel and Wiesel, 1970; Issa et al., 1999; Wiesel and Hubel, 1963). When imposed during the critical period, MD causes a constellation of changes that can permeate throughout the visual system. While early deprivation of patterned visual input induces slight retinal alterations, including reduced dopamine synthesis and metabolism (Iuvone et al., 2009), reduced retinal ganglion cell density (Mwachaka et al., 2015a), and reduced retinal thickness (Mwachaka et al., 2015b) in deprived retinae, it is MD-induced alterations in higher level components of the visual stream that are thought to more likely precipitate amblyopia-type deficits in animals. Of course, this does not exclude the fact that MD-induced retinal changes may contribute to more impactful alterations in downstream visual structures.

Downstream of the retina, dLGN and V1 have also proven sensitive to the effects of MD during early life. In kittens, reduced input from retinally-driven

activity causes a shrinkage of soma size and narrowing of neuronal dendritic field width in layers of the dLGN driven by activity of the deprived eye (Wiesel and Hubel, 1963a; Kupfer and Palmer, 1964; Guillery and Stelzner, 1970; Guillery, 1970; Friedlander et al., 1982). In layer IV of V1, MD alters the typical ocular dominance column architecture such that columns of neurons driven by stimulation of the deprived eye shrink while those driven by the non-deprived eye expand (Shatz and Stryker, 1978; Antonini and Stryker, 1996). In extragranular layers of V1, MD causes a reduction in the number of cells that respond to stimulation of the deprived eye (Wiesel and Hubel, 1963b; Blakemore and Van Sluyters, 1974). Following experimental strabismus (misalignment of the eyes is imposed), rather than an occlusion MD, there is a reduction in the number of binocularly responsive cortical neurons, sparing cells that are exclusively driven by one of the two eyes (Hubel and Wiesel, 1965; Smith et al., 1979).

Binocular deprivation, or the closure of both eyes during early life induces far less severe reductions in the number of binocularly responsive V1 cells than does MD (Wiesel and Hubel, 1965). It therefore seems that experience dependent refinement of cortical circuitry is governed not only by the absolute level of retinal input but also by the correlation of retinal input between the two eyes. It is the competition of the two eyes for limited processing resources in visual areas of the brain that is thought to precipitate MD-induced deficits in deprived-eye function.

While it may seem intuitive that MD-induced effects would manifest in a serial progression paralleling that of information processing (from retina, to dLGN,

to granular cortex and then to extragranular cells), some evidence suggests this is not true. In particular, it appears that the extragranular layers of the visual cortex are responsive to MD-induced reorganization before cells of layer 4 or of the geniculocortical afferents (Trachtenberg et al., 2000). This finding supports the hypothesis that MD-induced changes are initially manifested at higher levels of visual processing and that these higher level changes guide or induce the reorganization that occurs in lower level structures (Buonomano and Merzenich, 1998). That the dLGN receives an enormous amount of feedback input from the visual cortex (Sherman and Guillery, 1996; Sillito and Jones, 2002) lends credence to the notion that MD-induced dLGN changes may reflect alterations that occur in higher level structures.

Behaviourally, these MD-induced anatomical and physiological perturbations manifest as deficits in visual performance using the deprived eye. Monocularly deprived animals have reduced visual acuity of their deprived eye (Dews and Wiesel, 1970; Giffin and Mitchell, 1978), deficient contrast sensitivity (Kratz and Lehmkuhle, 1983), as well as impaired stereopsis and depth perception (Timney, 1983), the severity of which correlates with the duration of MD.

1.3.3 - Post-Critical Period

The response of an adult animal to MD is profoundly different from that of a young animal. MD imposed during adulthood fails to induce the anatomical

(Wiesel and Hubel, 1963), electrophysiological (Wiesel and Hubel, 1963), and behavioural (Dews and Wiesel, 1970) alterations that younger animals are susceptible to. It appears that in cats, susceptibility to MD effects peaks at around 4 weeks of age and thereafter declines to very low levels by about 12 weeks of age (Hubel and Wiesel, 1970; Olson and Freeman, 1980) though some electrophysiological measures of ocular dominance shifts have detected MD-induced changes when 3-month long MD is imposed at up to one year of age (Daw et al., 1992). The efficacy of "reverse occlusion", the technique wherein an initially deprived eye is opened and an initially non-deprived eye is occluded so as to reverse MD-induced changes, has a temporal profile similar but slightly faster than that of one's susceptibility to MD. Following an MD, reverse occlusion at about 5 weeks of age facilitates an inversion of the previously established cortical state from favouring the initially non-deprived eye to favouring the initially deprived eye (in ocular dominance column width and stimulation sensitivity) (Blakemore and Van Sluyters, 1974). However, the same intervention imposed beyond 12 weeks of age causes little to no reversal of the previously induced MD state (Blakemore and Van Sluyters, 1974).

1.4 - Mechanisms of critical period plasticity

The functional consequences of visual perturbation during the critical period are precipitated by a variety of biological processes that provoke structural changes to neural circuitry. In response to MD, the visual cortex undergoes

anatomical changes wherein monocular ocular dominance columns corresponding to the deprived and non-deprived eye shrink and expand respectively (Shatz and Stryker, 1978), and functional changes as evidenced by reduced response to deprived eye stimulation (Wiesel and Hubel, 1963b; Blakemore and Van Sluyters, 1974). Necessarily, a significant degree of thalamocortical synaptic rewiring is requisite to instate these MD-induced effects. However, it would appear that such changes are also governed by dynamic pruning and regrowth of dendritic spines, outgrowths of the dendrite specialized for reception of excitatory input (Lai and Ip, 2013). Indeed, dendritic spines of the hippocampus have proven highly dynamic both during development and as a response to changes in environmentally-evoked synaptic activity. While potentiating neuronal activity stimulates an increase in spine size and number (Matsuzaki et al., 2004), reduced synaptic activity or blockade of glutamatergic transmission is associated with reduced spine size and number (Zhou et al., 2004; McKinney et al., 1999). Notably, this motility of spine morphology dissipates with age (Lendvai et al., 2000), reaching a stable state near the peak of the critical period for ocular dominance (Konur and Yuste, 2004; Majewska and Sur, 2003). Spine dynamics are similarly modulated by visual experience as both binocular and monocular deprivation destabilize the morphology of spine outgrowths in binocular regions of V1 (Majewska and Sur, 2003; Oray et al., 2004). It is thought that changes to the motility of spine morphology underlie a capacity for activity-dependent neuronal rewiring such as that required to induce shifts in ocular dominance (Mataga et al., 2004; Yuste and Bonhoeffer, 2001).

Molecules which engage or inhibit axonal rewiring or spine motility are therefore direct modulators of experience-dependent plasticity. Of particular interest has been the role of tissue plasminogen activator (tPA) in executing plasticity processes. Upon its activation at the start of the critical period, the secretion of tPA induces the conversion of extracellular plasminogen into plasmin that subsequently engages a proteolysis of extracellular material, facilitating a capacity for axonal rewiring and spine motility (Oray et al., 2004; Mataga et al., 2004). By this means, cortical spines or circuits receiving input from a deprived eye may be lost or converted, while those receiving input from a non-deprived eye are conserved (Oray et al., 2004). In accordance with this, MD promotes the proteolytic activity of tPA in the visual cortex (Mataga et al., 2002) and the pharmacological inhibition of tPA is associated with attenuated ocular dominance plasticity following MD or reverse occlusion during the normal critical period (Mataga et al., 1996; Müller and Griesinger, 1998) that is rescued by exogenously applied tPA (Mataga et al., 2002).

The engagement of the tPA pathway is thought to be preceded by a cascade of signalling mechanisms that set the stage for experience-dependent neuronal rewiring. A variety of kinases, enzymes that phosphorylate their substrates, have been found to have obligatory roles in MD-induced shifts of ocular dominance. These include cAMP-dependent protein kinase (PKA; Beaver et al., 2001), extracellular-signal-regulated kinase (ERK; Cristo et al., 2001), and α-Ca2+/calmodulin-dependent protein kinase II (αCaMKII; Taha et al., 2002). Visually-driven activation of the mentioned kinases is thought to trigger their rapid

and local phosphorylation of a variety of substrates that mediate features of synaptic transmission and neuronal excitability and morphological stabilization (Berardi et al., 2003).

Though these initial changes to synaptic efficacy are independent of new protein synthesis, long-lasting changes in neuronal circuitry such as changes to ocular dominance induced by visual perturbations are reliant on gene expression and the production of proteins (Mower et al., 2002; Taha and Stryker, 2002). Achieving protein synthesis requires that the mentioned kinases activate transcription factors, whose actions engage the production of gene transcripts. cAMP response element-binding protein (CREB) is one such transcription factor that may be activated by PKA and ERK (Impey et al., 1996; Mayr and Montminy, 2001) and whose presence is requisite for ocular dominance plasticity (Mower et al., 2002; Liao et al., 2002; Pham et al., 1999). The kinase-mediated activation of CREB permits it to facilitate the production of gene transcripts that orchestrate a set of plastic processes implemented by downstream effectors (Silva et al., 1998) such as tPA-induced changes to axonal wiring and spine motility (Oray et al., 2004).

The described plasticity-engaging mechanisms are not active or are unable to induce maximal plasticity before and after the critical period. This window of elevated plasticity is therefore mediated by plasticity triggers and brakes whose actions modulate the capacity for plasticity with age.

1.5 - Regulation of critical period plasticity

An animal's susceptibility to monocular deprivation and responsivity to reverse occlusion jointly outline the temporal features of a critical period for sensitivity to visual experience during early life. Prior to the critical period, developmental processes appear to occur independently of visually-evoked activity and are uninfluenced by manipulations of an animal's visual rearing environment. Upon the onset of the critical period, animals become susceptible to the effects of MD and are responsive to interventions that reverse these MD-induced changes. However, with increased age an animal becomes progressively less susceptible to MD and the efficacy of reverse occlusion attenuates to the point of being absent. Unlike song birds that experience an annual critical period, during which they become sensitive to auditory stimuli for the purposes of song learning (Nottebohm and Nottebohm, 1978), the critical period for sensitivity to visual deprivation appears to occur only once during an animal's lifetime (Fagiolini and Hensch, 2000). The initial spark in sensitivity to visual experience, the maintenance of this sensitivity during the critical period, and the subsequent age-related decline in vulnerability to alterations in visual experience are collectively indicative of underlying neurobiological processes that modify the capacity for plasticity with age.

The use of rodent models has proven particularly useful in investigating the molecular underpinnings of the critical period for ocular dominance plasticity. Despite their nocturnal lifestyle and developmental differences from higher mammals, rodents display a vulnerability to changes in ocular dominance

mirroring many of the features observed in cats. During the critical period, MD in rodents reduces the sensitivity of cortical neurons to stimulation of the deprived eye (Fagiolini et al., 1994), ceases the growth of geniculocortical afferents serving the deprived eye (Antonini et al., 1999), and produces deficits in visual acuity of the deprived eye (Gordon and Stryker, 1996). Although the cortex of rodents is not characterized by ocular dominance columns, the likes of which observed in higher mammals, they still exhibit ocular dominance at the level of each cell. This cell-by-cell organization of ocular dominance allows an accessible means of assessing the physiological effects of MD in V1. The susceptibility of rodents to these changes is maximal when MD is imposed at about 4-5 weeks of age (Fagiolini et al., 1994).

The sudden increase and subsequent decline in sensitivity of neural circuitry to variations in visual experience has been linked with the emergence of a number of age-related cellular and molecular factors. In particular, it is now widely thought that a shift in the degree of balance between excitatory and inhibitory cortical circuitry most prominently influences the initial capacity of the cortex for experience-dependent change (Hensch, 2005). Consequently, factors that influence the maturation of inhibitory or excitatory circuitry, including NMDA receptors, neurotrophins, and a variety of other molecular factors also contribute to the regulation of critical period plasticity. Lagging behind these changes to cortical excitation and inhibition is the accumulation of extracellular and intracellular scaffolding proteins as well as transcriptional regulators that progressively restrain plasticity beyond the critical period. The contribution of

each of these factors to experience-dependent plasticity of the visual system will be discussed below. Where possible, the relationships between these accepted and putative modulators of visual plasticity will be acknowledged. However, the manifestation of cortical plasticity is certainly governed by a constellation of factors whose complex interactions are as yet only partially understood.

1.5.1 - Maturation of inhibitory circuitry

In altering the balance of intracortical excitation and inhibition, the maturation of inhibitory circuitry, particularly gamma-Aminobutyric acid (GABA)ergic circuitry, appears prerequisite for the induction of experience-dependent plasticity. The targeted genetic deletion of glutamic acid decarboxylase 65 (GAD65), one of two GABA-synthesizing enzymes, produces transgenic mice that appear insensitive to the effects of early MD (Hensch et al., 1998). The subsequent infusion of benzodiazepines, GABA agonists, restores a vulnerability to MD regardless of the age at which the intervention occurs (Hensch et al., 1998). It therefore seems that the initial expression of GABAergic tone opens a gate that enables a sensitivity to visual experience during early life. Furthermore, though mice deficient in GABAergic circuitry initially lack the capacity to undergo plastic change, they retain a potential for such changes that is harnessed following the eventual simulation of GABA tone to rapidly induct the critical period. This notion is substantiated by work that has demonstrated a precocious induction of critical period plasticity in 19 day-old mice following early

cortical infusion of diazepam, a benzodiazepine (Fagiolini and Hensch, 2000). Infusion of diazepam after the critical period has occurred does not appear to induce the same plasticity promoting effect (Fagiolini and Hensch, 2000). Rather, this result suggests either that critical period plasticity is only possible once during life or that plastic opportunities beyond the first critical period are not accessible by means of elevated inhibitory tone. Indeed, restoring ocular dominance plasticity after the natural critical period requires reducing inhibition to immature levels (Harauzov et al., 2010) or transplanting immature inhibitory neurons into the postnatal visual cortex (Southwell et al., 2010; Davis et al., 2015).

One neural substrate of inhibitory maturation appears to be a developmental progression in the composition of $GABA_A$ receptors, one of two receptors for GABA transmission. Immature GABAergic circuitry, such as that observed prior to the critical period, is characterized by a dominant expression of the α3 $GABA_A$ receptor subunit (Chen, 2001). The maturation of GABAergic circuitry appears to drive a shift in dominant receptor subunit expression from α3 to α1 near the peak of the critical period (Chen, 2001). Rearing animals in complete darkness both prevents this switch in GABA receptor subunit expression and also prolongs critical period plasticity (Chen 2001; Cynader and Mitchell, 1980; Mower, 1991). Further, genetic manipulations of GABA receptor subunit composition have revealed that the presence of the GABA receptor α1 subunit is requisite for the precocious induction of experience-dependent plasticity induced by infusion of GABA agonists (Fagiolini et al., 2004).

Cumulatively these results suggest that the shift in GABAergic receptor expression during early life, from predominantly α3 to α1 subunit composition, may be one factor underlying the induction of critical period plasticity during early life.

That the α1 subunit is the target of contacts from GABAergic parvalbumin-expressing (PV+) basket cells (Klausberger et al., 2002), implicates these cells in an especial role in inducing critical period plasticity. PV+ neurons also mature with a timeline correlating with that of critical period expression (Huang et al., 1999) and genetic manipulations that disrupt their function impair the capacity for ocular dominance plasticity (Matsuda et al., unpublished observations). Indeed, the deletion of methyl–CpG-binding protein 2 (MeCP2), a transcriptional modulator involved in brain development, in PV+ cells confines them to an immature cellular state and abolishes the normal sensitivity of V1 to MD (He et al., 2014). Deletion of MeCP2 from other cortical cell types does not similarly disrupt experience dependent plasticity (He et al., 2014), substantiating an obligatory and exclusive role of PV+ cells in inducting the critical period.

The maturation of GABAergic PV+ cells is initially restrained by interactions with specific molecules, restricting plasticity before and immediately after eye opening. In particular, the attachment of polysialic acid (PSA) to neuronal cell adhesion molecule (NCAM), forms an aggregate molecule (PSA-NCAM) whose joint expression appears to inhibit the maturation of PV+ basket cells, delaying a capacity for experience-dependent refinement until PSA-NCAM levels decline after eye opening (Cristo et al., 2007). On these bases, it would

seem that the maturational state of PV+ neurons is the foremost substrate of inhibitory maturation that ultimately triggers the induction of critical period plasticity.

The exact reason underlying why increased inhibition triggers the onset of critical period plasticity is yet to be uncovered. One theory supposes that the maturation of GABAergic PV+ cells might facilitate spatial and temporal filtering of visual responses, allowing the detection of activity differences between activity driven by the two eyes (Feldman, 2000). It is this detection of disparate input between the two eyes, either in magnitude or temporal features, that permits a competition for resources between neural units driven by each eye (Stryker and Strickland, 1984). Alternatively, it could be that the maturation of inhibition selectively suppresses spontaneous input that is equal between the two eyes, sparing the imbalanced visually-evoked activity that occurs following MD (Toyoizumi et al., 2013). This shift in sensitivity to different activity sources, from internal to external cues, is thought to permit a competition between the two eyes that manifests as changes to ocular dominance when MD is imposed (Toyoizumi et al., 2013).

Paradoxically, it also appears that the maturation of inhibitory circuitry triggers the decline of plasticity at the end of the critical period. When inhibitory tone is enhanced, the entire plasticity profile, including its onset and its closure, is accelerated (Fagiolini and Hensch, 2000; Huang et al., 1999). It could be that as it does in V1 in vitro, inhibitory circuitry prevents induction of long term potentiation (LTP) (Kirkwood and Bear, 1994), prompting the age-related decline

in visual plasticity. However, this explanation would seem to be discordant with the obligatory role of inhibitory circuitry in initially triggering the induction of the critical period. More likely is the speculation that while inhibitory tone initiates plasticity, its elevation to some threshold level may engage downstream biological processes that act to dissipate an animal's sensitivity to visual experience.

1.5.2 - Neurotrophins

Neurotrophins constitute a family of proteins that regulate survival, development, and function in the nervous system (Huang and Reichardt, 2001). Several lines of investigation now suggest that one of the diverse roles neurotrophins play is in the regulation of experience-dependent plasticity. Many of these links between neurotrophins and plasticity are mediated by effects on the balance of cortical excitation and inhibition. For instance, brain-derived neurotropic factor (BDNF) is known to promote the differentiation of GABAergic neurons (Mizuno et al., 1994) and its overexpression prior to the critical period is associated with accelerated GABAergic maturation and experience-dependent plasticity in V1 (Huang et al., 1999). The expression of BDNF is itself dependent on light-responsive visual activity as levels of BDNF expression are reduced after monocular deprivation and after an animal is dark-reared, or placed in darkness for a period of time (Bozzi et al., 1995; Castrén et al., 1992). In addition to, and likely as a result of reducing BDNF expression, dark-rearing has been shown to

retard GABAergic circuit maturation (Benevento et al., 1992; Benevento et al., 1995; Lee at al., 2006) and prolong ocular dominance plasticity in cats (Cynader and Mitchell, 1980; Mower, 1991) and in rats (Fagiolini et al., 1994). Studies on cats that have employed longer durations of dark-rearing demonstrate ocular dominance plasticity even at 2 years of age, a time at which virtually no ocular dominance plasticity would be expected to occur in normally reared animals (Cynader, 1983). Furthermore, overexpression of BDNF or infusion of benzodiazepines in dark-reared animals eliminates the darkness-induced critical period delay (Gianfranceschi et al., 2003; Iwai, 2003). Jointly, these results suggest that BDNF expression is crucially involved in inhibitory GABAergic maturation and thus also in the initiation of the critical period for ocular dominance plasticity.

Neurotrophins also appear to mediate plasticity through pathways not obviously involving modulation of the cortical excitation/inhibition balance. Key to these distinct pathways is the fact that the expression of neurotrophins is dependent on neural activity (McAllister et al., 1999; Berardi and Maffei, 1999). That mRNA levels of neurotrophins are rapidly elevated in cells following neural activity (Gall and Isackson, 1989; Zafra et al., 1990), permits the hypothesis that neurotrophins may be involved in the selective strengthening of active connections such as would occur in afferents originating from a non-deprived eye following MD. This notion is supported by the fact that the expression of neurotrophins, NGF and BDNF, increase with postnatal age, plateauing just prior to the peak of the critical period in rodents (Schoups et al., 1995).

1.5.3 - NMDA receptors

In conjunction with amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainic acid (KA) receptors, N-methyl-D-aspartate (NMDA) receptors facilitate the transmission of glutamate, the main excitatory neurotransmitter in the central nervous system (CNS). Of the family of glutamate receptors, NMDA receptors have been most prominently linked to roles in regulating experience-dependent plasticity. For instance, the antagonism of NDMA receptors during early development reduces the severity of MD-induced effects during the critical period (Bear et al., 1990; Daw et al., 1999). These findings suggest that glutamatergic transmission via NMDA receptors is an important component in conferring an experience-sensitivity to visual circuitry.

NMDA receptors also appear to be developmentally regulated in a manner paralleling that of GABA receptors. As GABA receptors progressively mature from predominantly α3 to α1 subunit composition throughout the critical period so too are changes in NMDA receptors observed. Immature NMDA receptors, such as those at the peak of the critical period, are defined by a dominant expression of the NR2A subunit type (Roberts and Ramoa, 1999; Chen et al., 2000). As the critical period progresses, the relative dominance of NR2A subunit expression over that of the other NR2 subunits is significantly attenuated (Chen et al., 2000). That dark-reared animals have prolonged ocular dominance plasticity (Cynader and Mitchell, 1980; Mower, 1991) and immature-type NMDA receptors (NR2A subunit dominant) (Quinlan et al., 1999a; Chen et al., 2000) jointly suggests that expression of the NR2A subunit may be promoting of experience-dependent

synaptic plasticity. Further, the genetic deletion of the NMDA-receptor NR2A subunit appears to cause a reduced sensitivity to MD during the natural critical period (Fagiolini et al., 2003).

One putative mechanism by which NMDA receptors may facilitate synaptic plasticity is in modifying synaptic efficacy in accordance with Hebbian rules. Hebbian plasticity dictates that the frequent activation of a given pathway causes changes that improve the efficacy of transmission in that pathway. Conversely, disuse of a pathway causes changes that decrease the efficacy of the pathway. These processes describe long-term potentiation (LTP) and long-term depression (LTD) respectively. Specific features of NMDA receptors are thought to allow them to mediate activity-dependent changes in synaptic efficacy. Namely, NDMA receptors are distinguished in being both transmitter- and voltage-dependent. At resting potential, NMDA receptors are blocked with magnesium ions, which are only removed following depolarization of the membrane (Nowak et al., 1984). Following removal of the magnesium blockade, an ionic channel permits the entry of calcium upon the binding of glutamate. By this means, the passage of calcium ions signals concurrent activation of pre- and postsynaptic elements. Several studies suggest that this calcium conductance elicits biochemical changes that alter the efficacy of a synapse (Lynch et al., 1983; Malenka et al., 1988). In accordance with this hypothesis, LTP induction in the somatosensory cortex is regulated by a critical period that matches temporally with developmental changes in NMDA receptor composition and parallels that of the critical period for sensory deprivation (Fox, 1995). However, LTP inducibility and MD-induced

plasticity do not always correlate (Hensch et al., 1998; Gordon et al., 1996), indicating that visual plasticity is not entirely mediated by Hebbian change to synaptic efficacy. Undoubtedly the regulation of experience-dependent plasticity is contributed to by a wide spectrum of biological processes, one of which is likely to be changes in synaptic efficacy afforded by NMDA receptors during specific periods of development.

1.5.4 - Extracellular Matrix: Lynx1, and PNNs

Subsequent to changes in the balance of cortical excitation and inhibition is the formation of a variety of late-emerging molecular proteins at the closure of the critical period (Pizzorusso et al., 2002). The association of these proteins with a decline in critical period plasticity has lead to their identification as “molecular brakes”, so called because they appear to inhibit plastic processes (Hensch, 2005). One such molecule whose expression appears to restrain plasticity beyond the critical period is Ly6/neurotoxin1 (Lynx1). Lynx1 is an endogenous prototoxin that binds to the nicotinic acetylcholine receptor (nAChR; Miwa et al., 1999) and is expressed in the dLGN and in GABAergic PV+ neurons of the visual cortex following the critical period (Morishita et al., 2010). Mice engineered to lack Lynx1 retain a susceptibility to MD and capacity for MD recovery that extends into adulthood (Morishita et al., 2010), perhaps because the presence of Lynx1 inhibits the motility of spine motility (Sajo et al., 2016) or because the transmission of acetylcholine somehow facilitates experience dependent

plasticity. Jointly, these findings indicate a possible role for Lynx1 and cholinergic signaling in constraining and promoting ocular dominance plasticity, respectively.

Also occurring at the closure of the critical period is the accumulation of chondroitin sulfate proteoglycans (CSPGs) that form aggregates of extracellular matrix (ECM), perineuronal nets (PNNs), specifically surrounding PV+ GABAergic interneurons (Celio and Blümcke, 1994; Härtig et al., 1994; Härtig et al., 1999). The aggregation of PNN constituents in the visual cortex occurs with a temporal profile mirroring that of the decline in ocular dominance plasticity during the critical period (Sur et al., 1988; Lander et al., 1997; Kind et al., 2013). That PNNs specifically envelope cells known to be mediating of ocular dominance plasticity, the PV+ GABAergic interneurons, and that their accumulation appears coincident with the closure of the critical period has prompted the hypothesis that components of the ECM may be involved in the maturation and stabilization of experience-refined neuronal circuitry (Hockfield et al., 1990). Supporting this notion, dark rearing, which prolongs ocular dominance plasticity, reduces the expression of PNN constituents (Sur et al., 1988; Guimaraes et al., 1990; Lander et al., 1997; Kind et al., 2013).

Components of PNN structure are degraded in vivo by chrondroitinase-ABC (chABC). When this enzyme is injected in the visual cortex of adult rats, a sensitivity to MD-induced shifts in ocular dominance is restored (Pizzorusso et al., 2002). Indeed, in compliment with reverse occlusion in adulthood, the same treatment can be used to promote functional recovery from a long term MD (Pizzorusso et al., 2006). It therefore seems that the accumulation of PNNs plays

an important role in the stabilization of mature neural circuitry in rodents, inhibiting plastic changes beyond the critical period. However, digestion of PNNs with chABC fails to enhance experience-dependent malleability in the visual cortex of adult cats (Vorobyov et al., 2013). Structural and functional barriers constraining plasticity would therefore seem to be more complex or intricate in higher level mammals than those observed in rodents.

The mechanism by which components of the ECM may impair experience-dependent plasticity could be derived either from PNN mediated control of the extracellular ionic milieu or by interactions with plasticity-regulating molecular factors (Hensch, 2005). In particular, interactions of PNNs with cell adhesion molecules inhibit a cell's capacity for axonal extension and cell migration (Grumet et al., 1996) and CSPG's inhibit the motility of dendritic spines (Vivo et al., 2013). In this manner, the accumulation of CSPG's directly restrains a capacity for experience-dependent structural remodeling. This stabilizing effect of PNNs may also be precipitated by interactions with two molecules, Otx2 and Narp, that will be discussed below.

1.5.5 - Interactions with the Extracellular Matrix: Otx2 and Narp

A variety of molecules appear to be involved in regulating critical period plasticity, as a function of their interactions with components of the extracellular matrix. Orthodenticle homeobox 2 (Otx2) is a transcription factor whose involvement in regulating experience-dependent plasticity has been increasingly

suggested (Huang and Cristo, 2008). Prior to eye opening, Otx2 is expressed in the retina and then, in response to a threshold level of visual activity, transported to the visual cortex during the critical period, where it is taken up by PV+ GABAergic interneurons (Sugiyama et al., 2008). In the cortex, Otx2 appears to play a role in advancing the maturation of GABAergic circuitry, therefore promoting the induction of critical period plasticity (Sugiyama et al., 2008). Direct Otx2 gain- or loss-of-function genetic manipulations respectively enhance and prevent ocular dominance plasticity (Sugiyama et al., 2008). Further, cortical infusion of either benzodiazepines or Otx2 restore critical period plasticity induced by an initial absence of Otx2 (Sugiyama et al., 2008). Collectively these effects of Otx2 appear consistent with a role in inducing critical period plasticity.

As might be expected, the effect of Otx2 on neural circuitry is developmentally-regulated, changing as a function of the diverse and complex molecular interactions that vary throughout the critical period. In particular, the interaction of Otx2 with late-emerging extracellular proteins (PNNs) has been associated with the maintenance of mature neural circuitry, thus contributing to waning experience-sensitivity at the end of the critical period (Beurdeley et al., 2012). In mature animals, the blockade of Otx2 entry to PV+ neurons (Beurdeley et al., 2012) or knockdown of Otx2 synthesis (Spatazza et al., 2013) rejuvenates ocular dominance plasticity, permitting recovery from MD-induced changes. Collectively, it therefore appears that though the initial entry of Otx2 into PV+ neurons triggers the critical period (Sugiyama et al., 2008), subsequent

accumulation of Otx2 restricts the capacity for experience-dependent plasticity beyond the critical period (Beurdeley et al., 2012).

The formation of PNNs by constituent CSPGs also facilitates the accumulation of an immediate early gene product, neuronal activity-regulated pentraxin (Narp), whose secretion appears to enhance excitatory input to PV+ neurons of the visual cortex, facilitating their maturation and plasticity-inducing function (Chang et al., 2010). Importantly, Narp knockout mice fail to demonstrate ocular dominance plasticity throughout life, suggesting an obligatory role of Narp in inducting the critical period (Gu et al., 2013). While the proportion of Narp that accumulates on CSPGs surrounding PV+ interneurons is small compared to its secretion in other regions (Chang et al., 2010), this result nonetheless indicates an indirect role of perineuronal nets in promoting experience dependent plasticity. This would seem to be discordant with the typical plasticity-inhibiting functions of the ECM, driven by its physical stabilization of dendritic spines or axonal wiring (Vivo et al., 2013) and through interactions with Otx2 (Beurdeley et al., 2012). It might be that the influence of the ECM on an animal's capacity for plasticity is dynamically balanced by these plasticity-promoting and stabilizing functions or interactions. For instance, any stabilizing effect of the ECM on neuronal circuitry might be nullified shortly following their emergence by the attraction of Narp and of Otx2, whose entry to PV+ interneurons is initially plasticity-inducing (Sugiyama et al., 2008). Over time however, the accumulation of CSPGs and Otx2 likely contributes to the formation of a stabilized neural environment less responsive to the plasticity-promoting effects of Narp. This hypothetical shift in the plasticity-

regulating function of the ECM is merely a speculation, but could provide some insight into the complex and dynamic roles of CSPGs and PNNs in modulating neural responses to experience.

1.5.6 - Myelin and Myelin-associated proteins

Paralleling the typical effect of the ECM, myelin and myelin-associated proteins have been increasingly linked with a role in limiting the capacity or structural flexibility beyond the critical period. In particular, the interaction of Nogo-A/B, myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp) with the Nogo receptor (NgR) and paired immunoglobulin-like receptor B complex (PirB) limits ocular dominance plasticity (Atwal et al., 2008). In accordance, the genetic disruption of NgR or PirB permits abnormally prolonged sensitivity to MD (McGee et al., 2005; Syken et al., 2006). The absence of NgR has been associated with augmented dendritic spine motility (Akbik et al., 2013), suggesting a role of myelin-associated proteins in limiting structural changes to neuronal circuitry.

1.5.7 - Epigenetic Changes

Ocular dominance plasticity also appears to be limited beyond the critical period by the accumulation of experience-dependent chromatin modifications. These “epigenetic” changes alter gene expression without disrupting the sequence of deoxyribonucleic acid (DNA). Accumulating evidence suggests that

a variety of these experience-dependent chromatin modifications may mediate the waxing and/or waning of ocular dominance plasticity across the lifespan (Fagiolini et al., 2009). Of particular relevance has been the role of histone acetylation and deacetylation in modulating experience dependent plasticity. Histones are proteins, produced by chromatin, that DNA wraps itself around so as to facilitate its storage in the restricted nuclear space. Histone acetylation and deacetylation are the process by which an acetyl group is added or removed from the histone protein, respectively. The addition and subtraction of acetyl groups is thought to provide a means of transcriptional regulation, wherein acetylated histones are associated with facilitated transcription and deacetylated histones with restricted transcription. Interestingly, enzymes that remove acetyl groups from histones, histone deacetylases (HDACs), have been associated with a role in inhibiting plasticity. The age-related deacetylation of histones correlates with reduced plasticity (Vierci et al., 2016) and interventions that promote histone acetylation by inhibiting HDACs rejuvenate a sensitivity to MD or reverse occlusion beyond the critical period (Putignano et al. 2007; Silingardi et al., 2010).

1.5.8 - Intracellular Components: Intermediate filaments

While a great deal of evidence supports a role of the extracellular matrix, myelin-related proteins, and HDACs in stabilizing neural circuitry, it would also seem likely that the regulation of intracellular stability may be an important

mediator of plasticity. For instance, changes in dLGN neuronal soma size associated with MD presumably reflect changes in the internal cellular scaffolding. This notion is corroborated by evidence that changes to the intracellular cytoskeleton may presage anatomical changes induced by MD (Kutcher and Duffy, 2007). The mammalian cytoskeleton is comprised of three major filament families: microfilaments, intermediate filaments, and microtubules (Morris and Lasek, 1982). Of particular relevance is the role of intermediate filaments, a family of proteins that contribute predominantly to cell shape and stability (Goldman et al., 2012). The specific intermediate filaments types that are expressed is developmentally regulated. Nestin and vimentin are expressed prenatally (Tapscott et al., 1981) but in postnatal life the expression of intermediate filaments shifts towards α-internexin and neurofilament in mature neurons (Kaplan et al., 1990). Neurofilament is the most commonly expressed intermediate filament and is formed from arrangements of 3 distinct subunits: neurofilament heavy (NF-H), medium (NF-M), and light (NF-L) (Morris and Lasek, 1982). Neurofilament is initially expressed at a time corresponding with the decline of the critical period and its accumulation continues into adulthood at which point levels plateau (Song et al., 2015). This temporal relation of neurofilament accumulation and the decline of ocular dominance plasticity prompts the inclusion of neurofilament as a putative molecular brake (Liu et al., 1994; Duffy and Slusar, 2009; Song et al., 2015). Indeed, neurofilament expression in the dLGN is responsive to monocular deprivation, following which levels decline in deprived-eye layers in parallel with reductions in soma size

(Bickford et al., 1998; Kutcher and Duffy, 2007; Duffy and Slusar, 2009), and to dark-rearing, following which levels decline in both deprived- and non-deprived-eye layers (O'Leary et al., 2012; Duffy and Mitchell, 2013). Presumably these changes in neurofilament expression reflect activity-dependent adjustments of the activity of proteases involved in the catabolism of neurofilament protein. Despite the identification of proteases degrading of neurofilament (Paggi and Lasek, 1984; Zimmerman and Schlaepfer, 1982; Tanii et al., 1988), these have not yet been studied in relation to visual deprivation nor other forms of plasticity.

It is the directness with which molecular brakes, such as neurofilament, appear to influence experience-dependent anatomical changes that makes them an interesting research target. Though cytoskeletal alterations are not likely the central mechanism underlying cortical shifts in ocular dominance, they provide a tool with which to analyze specific anatomical changes that could have widespread functional consequences. Interventions that alter the expression of molecular brakes have proven invaluable in identifying the relationship of a cell's anatomy to its internal and external environment.

1.6 - Darkness immersion

Immersion of animals in complete darkness presents a non-invasive means of simultaneously modulating a broad spectrum of factors regulating ocular dominance plasticity. Dark rearing inhibits the normal maturation of GABAergic (Chen, 2001) and glutamatergic neuronal circuitry (Quinlan et al.,

1999a), decreases BDNF expression (Castrén et al., 1992), and reduces the accumulation of molecular brakes including PNNs (Sur et al., 1988; Guimarãs et al., 1990; Lander et al., 1997; Kind et al., 2013) and neurofilament (O'Leary et al., 2012; Duffy and Mitchell, 2013). Likely due to these interactions with known plasticity-modulating factors, rearing animals in complete darkness results in prolonged sensitivity to visual alteration (Cynader and Mitchell, 1980; Mower, 1991).

The manner by which darkness is suggested to prolong sensitivity to visual experience differs depending on the manner in which darkness is imposed. When long periods of darkness are imposed from birth, researchers have tended to suggest that the enhanced plasticity observed upon re-entry to light is prompted by a slowing of the progression of the critical period, or mere inhibition of age-dependent maturation (Mower and Christen, 1985; Mower, 1991). In contrast, when darkness is imposed for brief durations (~10 days) after visual experience has already occurred, it appears that an elevated sensitivity to visual experience is brought about by an active reversal of the maturational processes restricting plasticity (He et al., 2007; Montey and Quinlan, 2011; Freeman and Olson, 1982; O'Leary et al., 2012; Duffy and Mitchell, 2013).

A previous publication from our lab investigated the age-related limits associated with the plasticity-promoting effects of brief darkness immersion (Duffy et al., 2016). We found that immersing kittens in darkness for 10 days at 12 weeks of age restored the dLGNs anatomical sensitivity to MD akin to that which would be expected of a much younger animal (Figure 4A). This enhanced

anatomical MD-effect was accompanied by changes in neurofilament accumulation (Figure 4B), suggesting a recapitulation of immature-type neural circuitry. However, when the same duration of darkness was imposed in adult cats, susceptibility to MD was not elevated beyond that of normal, age-matched animals, nor were levels of neurofilament altered (Duffy et al., 2016).

The current study sought to explore whether short-term darkness immersion could similarly promote visual plasticity at the peak of the critical period (p30), when an animal's sensitivity to visual perturbation is at its biological maximum. We immersed young kittens in darkness from 19- to 29-days of age and then subject them to 7 days of monocular deprivation. Their consequent susceptibility to MD was compared relative to animals undergoing the same period of MD in the absence of prior darkness immersion. In conducting this study, we questioned whether biology bestows an optimized sensitivity to visual experience during life or whether an experimental manipulation of visual experience can render the visual system supra-responsive to its environment.

Susceptibility to MD was assessed by a stereological quantification of neuronal soma size and neurofilament immunoreactivity in right- and left-eye receiving layers of the dLGN. Although often misperceived as a "relay" structure from retina to visual cortex, the dLGN appears to play more complex roles in visual processing than originally thought (Sherman and Guillery, 2001). In particular, the dLGN receives a substantial degree of feedback from layer VI of the visual cortex. Because MD-induced reorganization appears first in the visual cortex (Trachtenberg, 2000), it would seem that this feedback pathway from V1

instructs downstream changes in the dLGN. It is likely that changes in the dLGN reflect changes occurring in higher level visual structures. This, in conjunction with the dLGN's distinct monocular segregation in cats makes it a convenient and well-controlled region for the study of deprivation-induced effects. This anatomical selection also allowed for comparison of my findings to previous studies of similar phenomena in the same region (Duffy et al., 2016).

The employed measures were similarly selected to facilitate this inter-study comparison. Quantification of soma area and neurofilament expression have previously provided a precise and robust means of assessing the severity of MD effects in cats and rodents (Duffy et al., 2016; Kutcher and Duffy, 2007; Duffy and Mitchell, 2013). Changes to soma area likely reflect functional changes in ocular dominance and cellular size is thought to correlate with dendritic branching and other functionally-serving parameters. Such anatomical changes have long been observed in concert with functional measures of MD-induced changes to ocular dominance (Wiesel and Hubel, 1963; Hubel and Wiesel, 1963). Quantifying neurofilament expression, on the other hand, provides a means of exploring the potential mechanisms by which darkness immersion could enhance plasticity.

The results of the current study indicated that 10-days of darkness immersion just prior to the critical period peak does not confer an enhanced susceptibility to subsequent MD. This incapability of darkness to promote changes in cell size and neurofilament accumulation in the current study stands in contrast to the effects of darkness when imposed at 12 weeks of age (Duffy et

al., 2016). The findings of this study are informative of the limits of critical period plasticity as well as of the mechanisms and temporal factors associated with darkness-induced plasticity.

CHAPTER 2: EXPERIMENTAL DESIGN AND HYPOTHESES

2.1 - Animal Selection

While non-human primates provide a visual system model most similar to that of humans, there are many ethical, political, and financial issues associated with their experimental use. An alternative and long-established animal model for the study of the visual system has been the cat. While their acuity and colour vision are different from primates (Hall and Mitchell, 1991; Jacobs, 1981), cats have central visual pathways similar to humans in morphology and in their response to visual deprivation (Stone, 1983; Hess et al., 1981). Of particular relevance to the current study is the fact that the cat dLGN is segregated into laminae of exclusively monocular input, just as occurs in the human LGN. This laminar segregation of input is not true of the dLGN in rodents where monocular input is separated but not in a manner that may be visualized by Nissl stain. Anatomical investigation of the rodent dLGN is therefore limited as it is very difficult or impossible to discern whether cells receive input from the left- or right-eye. For this reason and to allow for comparison with previous related studies (Duffy et al., 2016), the cat model was regarded as being well suited for the current study.

2.2 - Cat Colony and Housing

Fourteen kittens were used to conduct the experiments involved in this collection of studies. Prior to any manipulation of visual experience, the kittens were housed with their mothers in colony rooms with a 12:12 hour light/dark schedule. While the animals were confined to large interconnected cages during the night, they were allowed to roam their colony room freely during the day. Food and water were provided ad libitum and a litter box was emptied and cleaned daily.

2.3 - Design

The fourteen kittens were divided into four experimental groups: 1) Normal (Norm): Animals were normally reared until approximately 30 days of age (n=3); 2) Dark-rear (DR): Animals were immersed in darkness for approximately 10-days at approximately 20-30 days of age (n=4); 3) Monocularly-Deprived (MD): Animals were monocularly-deprived for 7-days at 30-days of age (n=3); 4) Darkness Immersion + Monocular Deprivation (DR+MD): Animals were immersed in darkness at 19-days of age for 10-days and then immediately monocularly-deprived for 7-days (n=4). Normal control animal tissue had been collected for previous studies and kept in antigen preservative (50% ethylene glycol, 1% polyvinylpyrrolidone in PBS) to allow for processing in future research, including the current study. The division of animals by experimental condition is outlined in Figure 2.

2.4 - Ethical Approval

All experimental protocols, including breeding, surgery, and rearing were approved by the University Committee on Laboratory Animals in accordance with policies established by the Canadian Council on Animal Care.

2.5 - Experimental Procedures

2.5.1 - Monocular Deprivation

In kittens subjected to monocular deprivation, the upper and lower palpebral conjunctivae of the left eye were sutured with vicryl suture material, followed by closure of the eyelids with silk suture (Murphy and Mitchell, 1987). This procedure produced a two-layer occlusion of the eye, depriving the animal of patterned visual stimulation. Monocular deprivation was performed under general gaseous anesthesia (3-4% isofluorane in oxygen) and a heating pad (37 degrees Celsius) was used to maintain the animals' body temperatures. Anesthetized animals received a subcutaneous injection of Anafen for post-procedure analgesia, Alcaine (proparacaine hydrochloride) sterile ophthalmic solution as a local anesthetic, and a broad-spectrum topical antibiotic (1% Chloromycetin). Following the MD procedure, animals were monitored daily to ensure their overall health and to assess for any gaps emerging in the eye-lid suture. No such gaps were observed in any of the animals used in the current study. As a result, no

animals required additional surgical procedures beyond those expected from the project design.

The decision to impose a 7-days of MD was made because the effects associated with this duration of MD have been well documented in kittens of this postnatal age. Additionally, employing this duration of MD allows for comparison with previous studies (Kutcher and Duffy, 2007; Duffy and Slusar, 2009; Duffy et al., 2016) so as to observe the effect of darkness at different developmental stages.

2.5.2 - Darkness Immersion

Kittens in conditions requiring darkness immersion were housed for 10-days in a darkroom facility, which has previously been described in detail (Mitchell, 2013). The darkroom facility is shown to scale in figure 2. The facility consists of six rooms including two core darkrooms (Figure 3, C1 and C2), three dark anterooms (Figure 3, A1, A2, A3) and one illuminated area with a sink to clean the animals' cages. Kittens of each litter were housed, with their mother, in a large playpen (1.5m x 0.7m x 0.9m) in the primary core darkroom (Figure 3, C1), which is accessible by two dark anterooms (Figure 3, A1 and A2). The playpen contained raised shelves on each side, a large cardboard box lined with blankets, a litter box, and ad libitum food and water. Once daily, animals were moved from the primary core darkroom (Figure 3, C1) to the secondary core darkroom (Figure 3, C2) in cat carriers. One carrier was used to carry up to 4

kittens or 1 adult cat. Once the animals were relocated to the secondary darkroom, the primary core dark room (Figure 3, C1) could be illuminated allowing for cleaning of the cage(s), cleaning of the room, emptying of litter, and supply of food and water. Once these procedures were complete, lights within in the primary dark room would be turned off. In darkness, the carriers containing the animals were retrieved from the secondary darkroom and returned to their playpen in the primary darkroom.

Animals were cared for by technicians experienced with the blueprint and protocols of the darkroom facility. Extensive care was taken to ensure animals were not exposed to any source of light. For instance, cellular phones, watches, and key chains were all deposited outside the darkroom facility prior to feeding or monitoring the animals. When the experimental design required MD to be imposed following immersion in darkness, animals were transported to a surgical facility in opaque, light impermeable chambers. The same chambers were designed with a port for the administration of gaseous anesthetic without any risk of light exposure. The importance of these procedures is highlighted by evidence that brief pulses of light nullify the effect of darkness immersion (Mitchell et al., 2016).

The overall health of animals housed in the darkroom facility was monitored with a charged couple device (CCD) camera and infrared illumination system (>820nm), which remains off when not in use. The darkroom facility contained a radio that was set to turn on (low volume setting) and off at times coincident with the light cycle of regular animal rooms. This procedure was

intended to provide some comfort to the cats housed in the darkroom facility as well as to entrain a circadian rhythm similar to that of animal care personnel.

The decision to immerse animals in darkness for specifically 10 days was made for two reasons. First, employing the same period of darkness as that in previous studies of darkness immersion at later ages (Duffy et al., 2016) allowed for comparison of the effects of darkness at different developmental stages. Second, previous research has identified that shorter periods of darkness immersion (5-days) are ineffective in eliciting the same plasticity-enhancing effects of darkness at 5- and 12-weeks of age (Mitchell et al., 2016).

2.5.3 - Histology

Kittens were anesthetized with isofluorane (5% in oxygen) and euthanized with an intraperitoneal lethal dose of sodium pentobarbital (Euthanyl; 150mg/kg). Animals were transcardially perfused with 150mL of phosphate buffered saline (PBS) followed by 150mL of 4% dissolved paraformaldehyde in PBS.

The brain of each animal was extracted immediately following perfusion and the thalamus, containing the dLGN, was dissected with a razor blade. The dLGN containing tissue was placed in a cryoprotectant solution (30% sucrose in PBS). Following cryoprotection, the dLGN containing block of tissue was sliced into coronal sections of 50µm thickness using a freezing microtome (Leica SM2000R; Germany). Tissue sections to be stained for Nissl substance were mounted on glass slides and allowed to dry over night. The tissue from each

individual animal was mounted on a single slide with 3-8 coronal sections mounted per slide. Sections were immersed in a graded series of ethanol concentrations, placed in a solution of 0.1% cresyl violet acetate dye in distilled water, and then immersed in the graded series of ethanol concentrations a second time for differentiation. Sections were cleared using Histo-clear (DiaMed Lab Supplies Inc.; Mississauga, ON, CAN). A mounting medium, permount (Fisher Scientific; Canada) was be used to coverslip the sections.

Sections to be labeled for neurofilament protein were immersed in PBS containing the mouse monoclonal antibody targetting NF-H (1:1000 dilution; SMI-32; BioLegend, San Diego, CA; table 2). Sections were left in the primary antibody solution overnight, washed with PBS the following day, and then immersed in the secondary antibody (1:500 dilution; goat anti-mouse; Jackson ImmunoResearch, West Grove, PA) for one hour. After being washed with PBS a second time, the tissue was immersed in an avidin and peroxidase-conjugated biotin solution for one hour (PK6100; Vector Labs, Burlingame, CA). Following a third wash with PBS, neurofilament labeling was made visible by reaction of the conjugated tissue with 3,3' – diaminobenzadine. Labelled sections were washed with PBS once more and then mounted onto glass slides and allowed to dry overnight. The tissue from each individual animal was mounted on a single slide with 3-8 coronal sections mounted per slide. Sections were dehydrated by immersion in a graded series of ethanol concentrations, cleared with Histo-clear, and cover-slipped with permount.

The specificity of the neurofilament antibody, SMI-32, which targets specifically non-phosphorylated NF-H, was verified with an immunoblot of homogenized normal cat primary visual cortex. The labelling blots revealed bands corresponding with the expected mass of NF-H (Goldstein et al., 1987), validating the antibody for labeling of NF-H in the current study (Table 1).

2.5.4 - Quantification & Analysis

Quantification of neuronal soma size and neurofilament immunoreactivity in the dLGN was performed blind to the condition of the animal. Quantification was performed using a BX-51 compound microscope (Olympus; Markham, Ottawa, Canada) fitted with a DP-70 digital camera (Olympus; Markham, Ottawa, Canada), which allows for transmission of the microscope image to a computerized stereology software suite (newCast; VisioPharm, Denmark). Two coronal sections containing the dLGN were selected from each animal to be quantified, for both Nissl stained slides and slides labelled for NF-H. Caution was taken between and within animals to select sections with similar dLGN morphologies, indicating similar locations on the anterior-posterior axis of the brain. The cross-sectional area of neuronal somata was manually quantified in both the A and A1 layers of the right and left dLGN of Nissl-stained sections at 600x magnification using the “nucleator” probe on the stereology software. The cross-sectional area of approximately 1,000 neurons was manually quantified for each animal (500 per section). The number of neurofilament immunoreactive

cells was quantified in the A and A1 layers of the right and left dLGN of sections labeled for NF-H at 400x magnification using the “optical dissector” probe on the computerized stereology software. The absolute number of neurofilament immunoreactive neurons was divided by the area of the relevant dLGN area to calculate a density of neurofilament immunoreactive cells in each dLGN layer. Neurons in both Nissl stained and neurofilament stained sections were selected for quantification on the basis of a pre-defined selection criteria. Cells were selected if they exhibited a uniformly dark cytoplasm and a weakly stained nucleus, characteristics of cells cut through the somatic midline.

A deprivation index was calculated to assess the within-animal percent difference in neuronal somata size and density of neurofilament immunoreactivity between deprived- and non-deprived-eye, A and A1, layers of the dLGN (1). Positive deprivation index values indicate that neuronal soma size or density neurofilament immunoreactivity is less in deprived-eye layers than in non-deprived layers, while negative values indicate that the measured values are less in non-deprived layers than in deprived-eye layers.

Deprivation Index:

$$= \frac{(\text{Non-deprived A} + \text{Non-deprived A1}) - (\text{Deprived A} + \text{Deprived A1})}{(\text{Non-deprived A} + \text{Non-deprived A1})} \; x100 \quad (1)$$

Statistical analyses were performed to assess the significance of differences in neuron somata size and density of neurofilament immunoreactivity

deprivation between experimental conditions. One-way ANOVA's were used to assess for differences between groups and a Tukey's honestly significant difference (HSD) post hoc test was used to assess between which groups the differences occurred.

All statistical analyses and data visualizations were conducted using RStudio (RStudio Team, 2016), an integrated development environment for R (R Development Core Team, 2017). In addition to the base software, the Tidyverse package (Wickham, 2016) was used.

CHAPTER 3: RESULTS

3.1 - Anatomical susceptibility of the dLGN to MD in isolation or following darkness immersion

Gross examination of the Nissl-stained dLGN revealed an apparent shrinkage of cell size in deprived-eye layers, relative to non-deprived layers, following 7-days of MD imposed at 30 days of age (Figure 6A; MD). This distinction in soma area between deprived and non-deprived layers appeared about equal to that observed when the same period of MD was applied following 10-days of darkness immersion (Figure 6A; DR+MD). Examination of Nissl staining in normal animals and in animals immersed in darkness without subsequent MD (DR) revealed no apparent difference in cell size between left- and right-eye dLGN layers (Figure 6A). However, overall soma size appeared slightly smaller in all dLGN layers of animals immersed in darkness (Figure 6A; DR).

These gross observations paralleled results from stereological quantification of the cross-sectional area of neuron somata in right- and left-eye layers of the dLGN. Initial analyses showed that while soma size appeared to be balanced between right- and left-eye layers in normal and dark-immersed animals, there was a disparity between the smaller cells of deprived layers and larger cells of non-deprived layers in MD and DR+MD animals (Figure 5). These results were further probed by calculating the percent difference in soma size

between right- and left-eye layers of the dLGN (Figure 6B; Table 2). Following 7 days of MD at p30, neuronal soma area in deprived layers was reduced by an average of 21% relative to non-deprived layers. Similarly, when the same period of MD was imposed following immersion in darkness for 10-days, neuronal soma area in deprived layers was reduced by an average of 22% relative to the same measure in non-deprived layers. In normal animals and in animals immersed in darkness without subsequent MD, no apparent difference between the mean cell area in deprived vs. non-deprived dLGN layers was observed (0% and 2%, respectively). A one-way ANOVA performed on the measurement of soma size difference between deprived and non-deprived dLGN layers indicated a significant effect of condition ($F(3,10) = 54.58$, $p = 1.67e-06$). A post-hoc Tukey's test confirmed that while MD, in isolation and following darkness, produced deprivation effects significantly different from those of normal and dark-immersed animals, there was no statistically significant difference in the deprivation effects driven by MD and MD following darkness, respectively ($p=0.62$). This result indicates that the ability of MD to produce alterations in dLGN soma size is not enhanced by prior immersion in darkness at 19-days of age.

3.2 - Sensitivity of dLGN neurofilament expression to MD in isolation or following darkness immersion

Following 7 days of MD imposed at p30, gross examination revealed an apparent reduction in immunolabelling for neurofilament in deprived, relative to

non-deprived dLGN layers (Figure 7A; MD). A roughly equivalent difference in immunolabelling between deprived and non-deprived layers was observed when the same period of MD was applied following 10-days of darkness immersion (Figure 7A; DR+MD). Gross examination of neurofilament immunolabelling in normal animals and in animals immersed in darkness without subsequent MD (DR) revealed no apparent distinction between deprived and non-deprived dLGN layers (Figure 7A). However, immunolabelling did appear sparser in both right- and left-eye dLGN layers of animals immersed in darkness without a following period of MD (Figure 7A; DR).

Stereological quantification of the density of immunolabelled neurofilament-positive neurons in deprived and non-deprived layers of the dLGN was performed to investigate a putative mechanism of MD-induced anatomical effects (Figure 7B; Table 3). This measure revealed an apparent reduction of neurofilament expression in deprived relative to non-deprived layers of the dLGN following MD in isolation and following darkness immersion (50.42% and 53.15%, respectively). In contrast, the density of neurofilament labelled neurons appeared about equal across deprived and non-deprived dLGN layers in normal animals and in animals immersed in darkness with no subsequent MD (1.13 and 3.85 percent difference between layers, respectively). A one-way ANOVA performed on the difference in density of neurofilament labelled neurons between deprived and non-deprived dLGN layers indicated a significant effect of condition ($F(3,9) = 107.7$, $p = 2.3e-07$). A post-hoc Tukey's test confirmed no significant difference in the alteration of neurofilament expression produced by MD and MD following

darkness, respectively. There was however, a significant difference in the neurofilament deprivation effect produced in normal animals and following darkness immersion as compared to following MD and MD following darkness. This result suggests that the inability of darkness to enhance anatomical MD effects is accompanied by an inability to enhance modulations of neurofilament expression in the dLGN.

3.3 - Effect of darkness on absolute dLGN neuron soma size

In addition to analyses of the relative difference in neuron size between right- and left-eye dLGN layers, further analysis was conducted to make inferences on the absolute nature of this measure. Of particular interest was analysis of the absolute mean neuron area across both right- and left-eye dLGN layers in each of the four conditions. A mean value was calculated from the set of neuron area measurements for each animal (including both right- and left-eye dLGN layers) and for each condition (Figure 8; Table 4). The mean neuron area in right- and left-eye layers of the dLGN appeared similar in normal animals (147.79μm^2), following 7-days of MD (151.48μm^2), and following 7-days of MD that was preceded by 10-days of darkness (153.59μm^2). However, animals that were immersed in darkness for 10-days appeared to have dLGN neurons with reduced soma areas relative to the other conditions (110.58μm^2). A one-way ANOVA performed on the mean dLGN neuron area (both right- and left-eye layers) indicated a significant effect of condition ($F(3,24) = 7.6$, $p = 0.001$). A

post-hoc Tukey's test confirmed that 10 days of darkness immersion at p19 produced neurons smaller than those observed in the MD ($p=0.006$), DR+MD ($p=0.002$), and normal ($p=0.01$) conditions.

CHAPTER 4: DISCUSSION

Following demonstration that 10-days of darkness immersion facilitates an enhanced sensitivity to visual experience in juvenile kittens (Duffy et al., 2016; Duffy and Mitchell, 2013) and in adult rats (He et al., 2006; He et al., 2007), the current study sought to explore whether the same 10-day period of complete darkness immersion could similarly promote plasticity when imposed at about the peak of the critical period. By imposing 10-days of darkness immersion at 19-days of age, the current study questioned whether the visual system provides a maximum sensitivity to experience during early life or whether a brief removal of visual experience could render the visual system supra-responsive to its environment. Following stereological quantification of soma area and neurofilament immunolabelling in the dLGN of young kittens, monocular deprivation imposed immediately after 10-days of darkness immersion at 19-days of age was observed to produce alterations of cell size and neurofilament immunoreactivity that were no more severe than those observed following an equivalent MD not preceded by darkness. In contrast to observations of enhanced plasticity following darkness immersion at 12 weeks of age (Duffy et al., 2016; Duffy and Mitchell, 2013), the current study suggests that brief darkness immersion is incapable of conferring a plastic advantage at the peak of the critical period. These findings and their related implications are discussed below.

4.1 - Effects of darkness throughout the lifespan

Evidently, the influence of darkness upon mechanisms that mediate vulnerability to MD is contingent on the age at which darkness is applied. While brief periods of darkness elevate an anatomical sensitivity to visual experience during juvenile life (Duffy et al., 2016; Duffy and Mitchell, 2013), the same treatment is ineffective when imposed on adult cats (Duffy et al., 2016) or when imposed prior to the peak of the critical period (current study). Rearing animals in darkness from birth to 30-days of age is similarly futile in facilitating enhanced MD effects at the peak of the critical period (Olson and Freeman, 1975). Jointly these results would seem to indicate a critical period associated with the ability of darkness to promote experience-dependent visual plasticity. While in early development dark immersion appears to not influence the effect of subsequent alterations of visual experience (current study), weeks later the same treatment exerts plasticity enhancing effects (Duffy et al., 2016), the potency of which dissipates to zero by the time an animal reaches adulthood (Duffy et al., 2016). The foremost factor seemingly linked to this parabolic progression of darkness efficacy is the age-related change in an animal's capacity for plasticity. When plasticity is at its maximum, at about 4 weeks of age (Olson and Freeman, 1980), darkness is unable to enhance experience-dependent neural alterations. When plasticity has waned to some threshold level, the capacity of darkness to promote anatomical changes is elevated (Duffy et al., 2016; Duffy and Mitchell, 2013), but when plasticity has declined to adulthood levels, darkness is again ineffective in promoting plastic changes (Duffy et al., 2016). The critical period for the effect of

darkness therefore lags slightly behind the critical period for vulnerability to MD, which peaks at 4 weeks of age and thereafter declines progressively to completely undetectable adult levels (Olson and Freeman, 1980).

One interesting point of discussion involves the pattern by which the ability of darkness to promote experience-dependent plasticity emerges. It could either be that darkness-enhanced plasticity becomes rapidly feasible at or just beyond the critical period peak, or that its efficacy builds gradually as an animal ages. These contrasting temporal profiles would seem to be indicative of distinct underlying neurobiological processes. For instance, a gradual increase in the ability of darkness to promote plasticity would parallel the progressive accumulation of molecular brakes and accession of maturational changes to neuronal circuitry. This pattern of functioning would suggest darkness's ability to enhance plasticity relies on the presence of plasticity-inhibiting changes that it may act to reverse. In contrast, an immediate activation of darkness-induced effects would seem to suggest the flipping of a hypothetical biological "switch" that rapidly permits darkness-induced plasticity, perhaps by way of sudden changes in gene expression. Regardless, that the ability of darkness to influence subsequent exposure to visual experience changes throughout development is indicative of changes to the underlying neurobiological processes that darkness acts upon.

4.2 - Implications for mechanisms of darkness-induced plasticity

The ability of darkness to promote plasticity has been linked to a wide spectrum of darkness-induced neurobiological changes. Rearing animals in complete darkness from birth appears to disturb the maturation of GABAergic inhibitory circuitry (Chen, 2001; Benevento et al., 1992), likely as a result of reducing the expression of BDNF (Bozzi et al., 1995), a neurotrophin known to promote the differentiation of GABAergic neurons (Mizuno et al., 1994). Dark-rearing also appears to delay the normal developmental progression of NDMA receptor types (Quinlan et al., 1999a; Chen et al., 2000) and inhibits the aggregation of extracellular materials into PNNs (Sur et al., 1988; Guimarãs et al., 1990; Lander et al., 1997; Kind et al., 2013). These neurobiological alterations are thought to underlie the prolonged sensitivity to visual experience observed in dark-reared animals (Cynader and Mitchell, 1980; Mower, 1991). The emergence of dark-reared animals into a lit environment is associated with rapid changes to NMDA receptor expression (Quinlan et al., 1999b) and immediate early gene expression, whose protein products are thought to mediate subsequent responses to visually elicited activity (Rosen et al., 1992). These light-induced changes rapidly induct delayed critical period plasticity in dark-reared animals.

In contrast, darkness immersion imposed on initially normally-reared animals appears to involve a set of neurobiological changes differing from those induced by chronic dark-rearing. Rather than the delay or prevention of maturation induced by dark-rearing from birth, the enhancement of ocular dominance plasticity in animals already exposed to visual experience appears to

be mediated by an active reversal of accrued maturational changes. In adult rats, brief darkness immersion restores a juvenile-like sensitivity to MD, seemingly by reducing the ratio of GABAA receptors relative to AMPA receptors and by causing an increase in NR2b NMDA receptor expression (He et al., 2006; He et al., 2007). In juvenile cats, brief darkness immersion both enhances anatomical sensitivity to visual experience and causes an absolute reduction in the expression of neurofilament, a putative molecular brake (Duffy et al., 2016; Duffy and Mitchell, 2013). It would therefore seem that brief darkness immersion following some period of normal visual experience can rejuvenate a sensitivity to visual experience, re-instating a neural state similar to that of younger animals.

While the current study parallels these previous examples in imposing brief darkness immersion following a period of normal visual experience, it is unique in doing so before plasticity has waned from its natural peak. Specifically, darkness was imposed for 10-days at 19-days of age. This imposition of darkness coincides with the advent of critical period plasticity (Hubel and Wiesel, 1970; Olson and Freeman, 1980), approximately 10-13 days after a kitten's eyes open and just 10 days prior to the peak of the critical period for ocular dominance plasticity. My experimental design therefore imposes darkness immersion during the entirety of the upstroke in a kitten's temporal profile for susceptibility to MD. As a result, this study provides an important and previously unobserved perspective on the susceptibility of plasticity-inducing mechanisms to a brief absence of visual stimulation.

At around 20 days of age in kittens, a cascade of biological processes either precipitated by the maturation of inhibitory cortical circuitry or contributing to this maturation of inhibition is thought to induce an inaugural sensitivity of neural architecture to visual experience. Unlike dark-rearing from birth, darkness immersion imposed at this age is incapable of preventing critical period induction because it has already occurred. The effect of darkness at this time must also differ from when it is imposed during juvenile life in kittens (Duffy et al., 2016; Duffy and Mitchell, 2013) or adult life in rodents (He et al., 2006; He et al., 2007). At such ages, dark immersion causes a reversal of plasticity-inhibiting maturational changes. At 19-days of age, however, plasticity is rising and it is unlikely that many plasticity-resistant factors have yet emerged. Any augmentation of plasticity induced by darkness at this time would seemingly rely upon the facilitation of plasticity-inducing mechanisms, such as the activation of pathways involving PKA, ERK, and CREB, rather than inhibition of those inhibiting plasticity. That the current study did not find any enhanced MD effect following immersion in darkness from p19-p29 would seem to be indicative that darkness does not enhance plasticity by means of directly promoting plasticity-inducing mechanisms. Rather, the current evidence appears to support the notion that darkness may facilitate experience-dependent plasticity only by engaging mechanisms that reverse or prevent the effects of plasticity-inhibiting factors. The relative scarcity of these stabilizing factors in early life presumably renders darkness immersion unavailing in boosting plasticity at the peak of the critical period.

This issue is made more complex by the observation in the current study that darkness immersion, without subsequent MD, significantly reduces the size of dLGN neurons. Changes to neuronal cell size have been repeatedly linked to alterations in the expression of neurofilament (Bickford et al., 1998; Kutcher and Duffy, 2007; Duffy et al., 2007; Duffy and Slusar, 2009), a putative molecular brake whose accumulation occurs in compliment to the decline in ocular dominance plasticity (Song et al., 2015). While the stereological methods employed in the current study did not allow for quantification of absolute neurofilament expression, it would seem that darkness-induced soma shrinkage requires some degree of neurofilament degradation. That a reduction of neurofilament expression may have permitted the observed dark-induced shrinkage of cell size and that darkness had no plasticity-enhancing effect is seemingly problematic for the hypothesis that neurofilament acts as a molecular brake. Were neurofilament to actively inhibit plasticity, it would be expected that its reduction in darkness would elicit a state of heightened plasticity, as evidenced by an elevated susceptibility to MD, unobserved in the current study. Alternatively, it could be that neurofilament only acts as a brake on plasticity when it has accumulated to a threshold not yet reached at the ages studied in the current experiment or that the degree to which it is post-translationally modified dictates its effect on plasticity. Indeed, neurofilament appears to be made more stable by its age-related phosphorylation (Pant, 1988; Gong et al., 2003). It could therefore be that the phosphorylation of neurofilament, rather than its accumulation more strongly facilitates an inhibition of plasticity. That markers for

phosphorylated neurofilament are low before and during the critical period (Song et al., 2015) may partially explain the inability of darkness to promote plasticity in the current study.

However, it could also be that neurofilament's plasticity-inhibiting function relies upon interactions with later-emerging molecular factors. This suggestion is not unprecedented as it appears stabilizing factors in the extracellular matrix exert some of their plasticity inhibiting effects via interactions with cell adhesion molecules or transcription factors (Grumet et al., 1996; Beurdeley et al., 2012). Similar stabilizing interactions with neurofilament proteins might not become accessible until ages beyond those observed in the current study, perhaps explaining the absence of augmented plasticity following a presumed decline in neurofilament expression. Future studies that examine ocular dominance plasticity in response to genetic manipulation of cytoskeletal elements may prove fruitful in further unravelling the role of neurofilament in modulating neural plasticity. For instance, one might dampen or augment the expression of neurofilament by genetic means to potentially uncover its causal role in constraining experience dependent plasticity.

4.3 - Potential for saturation of measures

While the current study appears to show that darkness immersion before the peak of the critical period fails to exacerbate the effect of following MD, one might speculate that our measures were insufficiently sensitive to detect an

effect. One such concern could be the possibility that our measures of dLGN cell size and neurofilament immunoreactivity were at or near saturation following MD at 30-days of age. Were this to be true, any plasticity-promoting effect of darkness would not have been detected in the current study. It could be that monocular deprivation can only shrink neurons and decrease the expression of neurofilament in deprived dLGN layers by a certain amount. However, previous studies conducted by our lab have demonstrated deprivation effects much greater than those observed in the current study either following a longer period of MD (Kutcher and Duffy, 2007) or following monocular TTX injection (Fong et al., 2016). For instance, following 7-months of MD imposed at the peak of the critical period, cells of deprived dLGN layers were more than 50% smaller than their non-deprived counterparts (Kutcher and Duffy, 2007), while deprivation effects in the current study peaked much lower at about 25% (Figure 6B). The same 7-month period of MD was associated with an 80% loss of neurofilament labelling in deprived relative to non-deprived dLGN layers (Kutcher and Duffy, 2007), easily surpassing the most severe neurofilament deprivation effect observed in the current study (60%; Figure 7B). With acknowledgement of the difficulty in comparing stereological quantification values between studies, it does not appear that our measures were saturated in the current study. To more definitely address this issue, the current study might have been conducted with a shorter period of monocular deprivation. For instance, because 2-days of MD produces deprivation effects smaller than those produced by 7-days of MD, the

effect would certainly not be saturated and the ability of darkness to promote any MD-induced changes might be more clearly observable.

One might also speculate as to whether darkness could have enhanced a functional sensitivity to MD in the absence of anatomical changes. This hypothetical scenario would require that the electrophysiological responsiveness of cortical cells to stimulation of a deprived eye be altered without any corresponding anatomical changes in the dLGN or visual cortex. While the current study was not equipped to assess for this possibility, previous studies have long documented the coincident manifestation of MD-induced anatomical and functional changes to ocular dominance (Wiesel and Hubel, 1963; Hubel and Wiesel, 1963). Supporting this speculation, however, is the case of minute MD effects that are only detectable by electrophysiological measures and not by anatomical measures such as that of cell size in the dLGN (Cynader, 1983). It is therefore plausible that darkness immersion in the current study could have promoted a small enhancement of MD susceptibility that was undetected by our anatomical measures. Regardless of this possibility, it is obvious from the current data that the ability of darkness to promote experience dependent plasticity varies dramatically on the basis of the age at which it is imposed.

4.4 - Other means of enhancing peak plasticity

That 10 days of darkness immersion appears unable to facilitate an enhanced susceptibility to MD at the peak of the critical period does not preclude

the notion that more severe forms of binocular deprivation might prove efficacious. While immersion in darkness prevents patterned visual stimulation, it does not impede the propagation of spontaneous retinally-driven activity throughout the central visual stream. In contrast, binocular intravitreal injection with tetrodotoxin (TTX), a potent sodium channel blocker, temporarily ceases the transmission of any retinally-driven activity. Binocular TTX injection has been previously employed to facilitate more rapid recovery from previous MD than would be afforded by treatment with darkness (Fong et al., 2016). On this basis, it would seem plausible that substituting 10-days of darkness for 10-days of retinal inactivation in the current study may have permitted an enhanced susceptibility to MD at the peak of the critical period. However, if plasticity afford by retinal inactivation is similarly dependent on an inhibition of plasticity-resistant factors, which are evidently limited prior to the critical period peak, it is unlikely to provide an advantage over darkness immersion.

Relatedly, it might be speculated that intervention with multiple plasticity-promoting treatments could provide a heightened susceptibility to MD at the peak of the critical period. The cortical infusion of enzymes degrading of molecular brakes (Pizzorusso et al., 2002) or of NGF (Gu et al., 1994), as well as environmental enrichment (Tognini et al., 2012) have each been demonstrated to enhance ocular dominance plasticity in older animals. On this basis, it might seem that the conjunctive application of these treatments with darkness immersion could potentially prove more efficacious in promoting plasticity than treatment with darkness immersion alone. This speculation, however, does not

account for the apparent differences in the regulation of plasticity before and after the critical period peak respectively. While the enzymatic degradation of PNN constituents promotes plasticity in adulthood (Pizzorusso et al., 2002), the same treatment is likely less efficacious early in life when PNNs are yet to form. Similarly, while the infusion of NGF during adulthood induces an enhanced sensitivity to MD (Togini et al., 2012), its up-regulation during the critical period actually prevents MD-induced alterations of ocular dominance (Yan et al., 1996). The use of exogenously applied molecules to enhance plasticity would therefore require particular attention to the timing of the introduction of these molecules so that impact can be optimized. Finally, environmental enrichment is relatively incompatible with concurrent dark immersion and is therefore not likely to facilitate any effect of darkness on experience-dependent plasticity. Furthermore, the ability of environmental enrichment to promote plasticity in adulthood is linked to similar mechanisms as darkness-induced plasticity including modulation of GABAergic inhibition (Baroncelli et al. 2010), BDNF expression (Cancedda et al. 2004; Sale et al., 2004) and stability of the extracellular matrix (Sale et al., 2007), and is therefore unlikely to promote changes beyond those that darkness already triggers. Though there is a possibility that interactions between multiple interventions such as those described might produce effects distinct from the summation of each individual treatment, the current evidence does not support that peak plasticity can be enhanced.

4.5 - Optimization of the visual system

The inability of darkness immersion to exacerbate the effect of MD at the peak of the critical period seems to suggest that the visual system is optimized during early life to provide a maximal and biologically ideal sensitivity to visual experience. An evolutionary perspective would suggest that this period of heightened responsiveness allows the visual system to develop neural circuitry capable of processing a wide variety of novel experiences during early life. The subsequent decline in neural plasticity presumably facilitates a stabilization of early-refined circuits perhaps to increase the efficiency with which visual stimuli is processed throughout life. Elucidating the mechanisms involved both in the induction and subsequent decline of heightened ocular dominance plasticity will be invaluable in continuing to improve therapies for conditions caused by sensory disturbances during early life, such as amblyopia. The results of the current studies suggest that enhancement of visual system plasticity beyond its natural maximum will be challenging, and appears to require an intervention more potent than 10 days of darkness. This observation yields valuable insight regarding the viability of darkness for translation to the treatment of human amblyopes.

4.6 - Darkness as an adjunct therapy for amblyopia

Previous observations that 10-days of darkness promotes recovery from MD in juvenile kittens (Duffy and Mitchell, 2013) and in adult rats (Montey and Quinlan, 2011) have prompted the notion that darkness immersion could be used

to improve therapies for amblyopia in humans. This hypothesis is predicated on the basis that darkness appears to re-instate a neurobiological context similar to that which would be expected of younger and more plastic animals. In humans, latent or absent screening for amblyopia is one primary risk factor for the persistent manifestation of the condition in adulthood (Eibschitz-Tsimhoni et al., 2000). In such cases, therapeutic intervention is necessarily prescribed beyond ages associated with optimal plasticity, subsequently impairing the therapeutic outcome. Should darkness immersion promote plasticity in humans as it does in animal models, it could be used to reverse those maturational changes that limit plasticity so as to improve the efficacy of subsequent treatments.

My findings, however, reveal that the timing with which darkness immersion is imposed is crucially important to the ultimate outcome. Evidently, immersion in darkness prior to the natural peak in plasticity does not render the visual system more responsive to experience, suggesting a similar treatment in humans would not facilitate amblyopic recovery. This finding is complimented by the observation that darkness immersion is unable to rejuvenate recovery-inducing plasticity when it is imposed in adulthood (Duffy et al., 2016). The temporal features governing the effects of darkness immersion are further complicated by the fact that brief darkness immersion is more rapid in facilitating recovery from prior MD when imposed following 5-8 weeks of binocular vision as compared to when it is imposed immediately following MD (Duffy and Mitchell, 2013). Furthermore, darkness immersion actually impairs vision, causing blindness, when it is imposed before 10-weeks of age in kittens (Mitchell et al.,

2015). Relatedly, while 10-days of darkness is sufficient to induce plasticity in animal models, these changes in humans may require longer periods of light deprivation, as evidenced by humans having a slower emerging and prolonged susceptibility to amblyopia (Vaegan and Taylor, 1979; Birch et al., 1998). It therefore seems that both the age at and duration with which darkness immersion is imposed plays a crucial role in predicting its biological or behavioural outcome. Determining the human correlates of these temporal parameters for efficacy of darkness treatment will be important for if a translation to human therapy is desirable. Comparing the developmental profile of molecular brakes in the visual cortex of cats and humans could provide one such means of temporal scaling between species (Song et al., 2015).

These temporal intricacies are unlikely to be any more restrictive than those associated with comparable pharmacological treatments, over which darkness holds many advantages. Perhaps the most significant of these advantages is the relative non-invasiveness of darkness immersion. In contrast to the administration of exogenous agents, darkness is unlikely to directly induce undesired side effects. Furthermore, darkness is holistic in the manner by which its effects are exerted. Rather than targeting a single biological process or component, darkness modulates a constellation of neural processes and molecules all within appropriate visually-serving brain regions (Mitchell and Duffy, 2014). A pharmacologically-based manipulation of plasticity is unlikely to be sufficiently specific to target relevant biological processes in relevant brain regions. Certainly there is a risk with such treatments to induce wide spread

changes in plasticity, which although promoting of amblyopic recovery, may induce severe negative consequences in other modalities.

Darkness-based amblyopia therapy is also compelling as a result of its binocular nature. While patching, the occlusion of one's non-amblyopic eye, can promote visual recovery of an amblyopic eye, it only permits monocular visual experience, precluding any coordination of activity between the two eyes. For this reason, deficits in depth perception tend to persist following patching treatment despite restoration of visual acuity in the amblyopic eye (Levi et al., 2015). Following dark immersion in juvenile kittens, binocular visual experience alone facilitates a recovery of visual acuity and of stereoscopic depth perception (Duffy and Mitchell, 2013). It would seem that the balancing of circuitry induced by darkness facilitates a symmetrical refinement of visual functioning between the two eyes following binocular experience, perhaps circumventing a need for patching.

There are, however, aspects of darkness immersion that make its translation to human benefit problematic. The first of which relates to the temporal factors governing the effects of darkness immersion. The requirement to impose darkness immersion during childhood or juvenile years for a relatively long length of time (likely >10 days) could make it a difficult sell to individuals and parents. Exacerbating this is that brief periods of light exposure appear to rapidly nullify the plasticity-promoting effects of darkness (Mitchell et al., 2016). Darkness therapy therefore requires a strict compliance to voluntary sensory deprivation. That patient compliance to conventional patching therapies is already

a major concern (Al-Yahya et al., 2012) perhaps foreshadows the difficulties that might be expected in promoting adherence to darkness therapy in the future.

At least some of these issues may be resolved via the substitution of darkness immersion with retinal inactivation such as that afforded by binocular intravitreal TTX injection. In particular, complete retinal silencing induced by binocular TTX injection permits the same enhanced plasticity as darkness immersion with a much shorter period of sensory deprivation (Fong et al., 2016). Furthermore, because intraocular TTX confers a complete insensitivity to light, this treatment does not require avoidance of lit environments, subsequently circumventing a potential issue of compliance. These benefits, however, are accompanied by increased risks. Namely, in rare cases intravitreal injections can cause endophthalmitis, a retinal irritation that cause infection (Scott and Flynn, 2007), and tetrodotoxin, although apparently harmless in small amounts, does pose a lethal risk at higher doses (Hwang and Noguchi, 2007). As with any medical treatment, TTX-based amblyopia therapy therefore requires a careful assessment of benefit and risk.

Jointly, the emergence of darkness- and TTX-based therapies appears a promising avenue in the treatment of a condition whose range of clinical therapies has not ventured far from the likes of basic patching procedures. Research that furthers our understanding of the temporal and molecular factors governing these forms of light deprivation will be of critical importance in assessing their viability for translation to the treatment of human amblyopes.

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APPENDIX A Figures

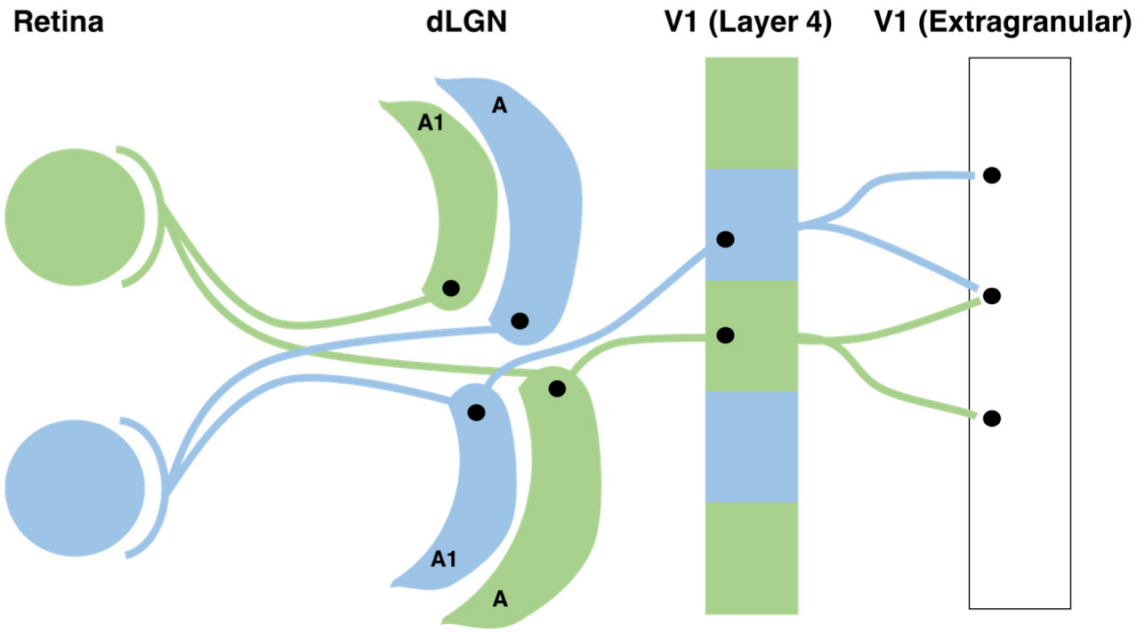

Figure 1. Schematic of the visual system in cat

Figure 1. Schematic of the visual system in cat.

Retinal input is fed through the optic chiasm to the dorsal lateral geniculate nuclei (dLGN) of the thalamus. The colours of projections and regions are indicative of the eye from which the input originates. The LGN is segregated into eye-specific layers, A and A1. A and A1 laminae are innervated by the contralateral and ipsilateral eye, respectively. Afferents from the dLGN project to the granular layer, layer IV, of the primary visual cortex (V1), that is arranged in an alternating pattern of monocular regions, so-called “ocular dominance columns”. Visual information is relayed from the granular layer to extragranular layers of the visual cortex, layers I, II, III, V, and VI. Extragranular layers are the first point at which input from the two eyes converge. While some cortical neurons in these layers only receive monocular input, the majority are targeted by afferents originating from both eyes. The degree to which this population of neurons are driven by each eye varies neuron-by-neuron and can be visualized via ocular dominance histograms such as those employed by Wiesel and Hubel (1963).

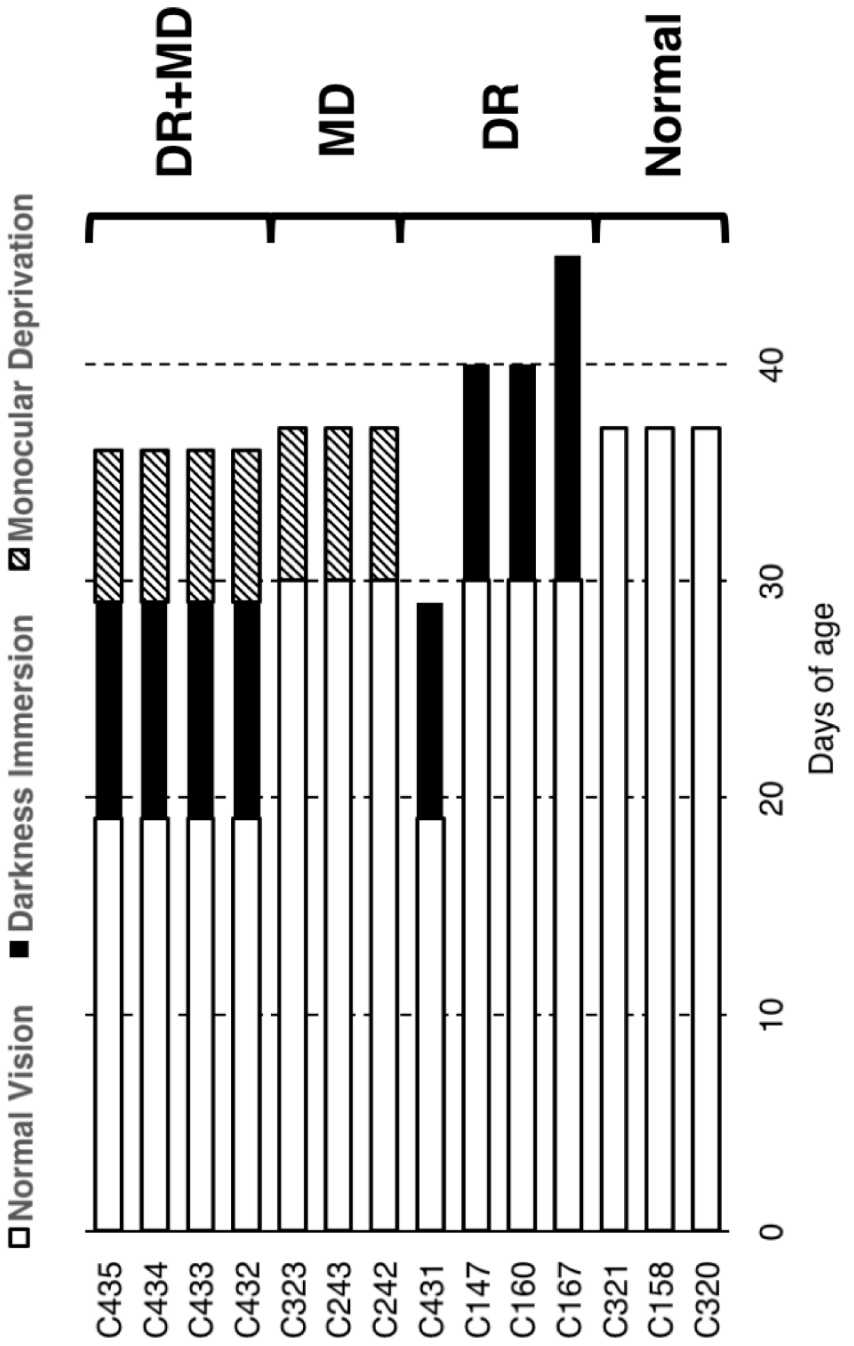

Figure 2. Animal Rearing Conditions

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Fourteen kittens were used in the current study. Each was assigned a unique cat identification number (C###), as listed on the left side of the figure. The animals were subdivided into four distinct experimental groups, as indicated on the right side of the figure. Animals of the DR+MD (Darkness + Monocular Deprivation) group, C432, C433, C434, and C435, were normally reared until p19, then immersed in darkness for 10-days. Immediately following, at p29, the animals were subject to 7-days of MD and then sacrificed at p37. Animals of the MD (Monocular Deprivation) group, C323, C243, and C242, were normally reared until p30, subject to 7-days of MD, and then sacrificed. Animals of the DR (Darkness) Group were initially normally reared and then immersed in darkness for a brief duration of time. While, C147 and C160 were immersed in darkness from p30-p40, C431 was immersed from p19 to p29, and C167 was immersed from p30-p45. Normal animals, C321, C158, and C320, were normally reared until p37 and then sacrificed. Unfilled bars indicate periods of normal vision. Filled bars indicate a period of sensory deprivation via darkness immersion. Hatched bars indicate periods of MD.

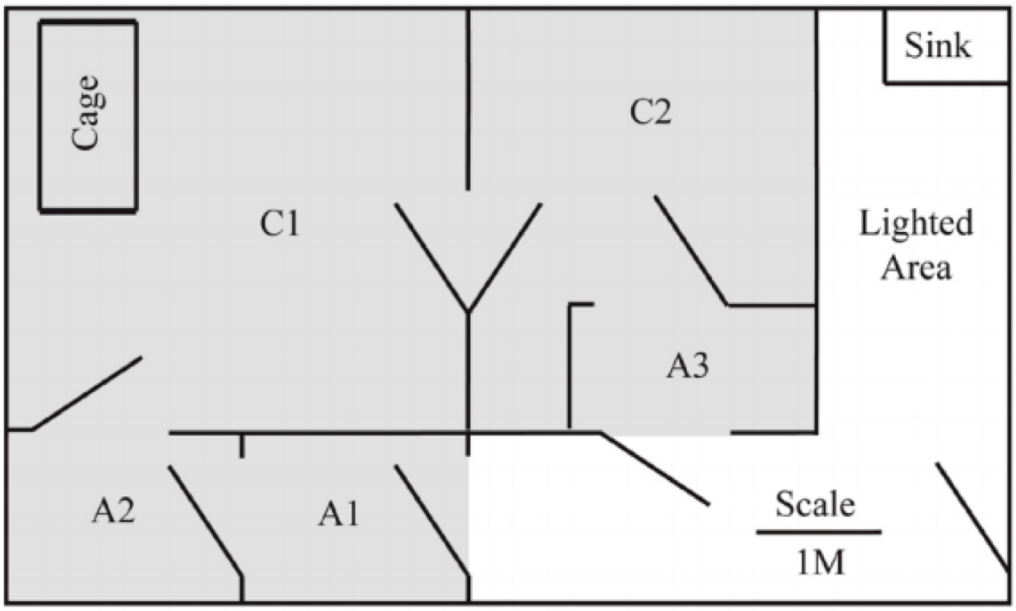

Figure 3. Dark-room facility depicted to scale

Figure 3. Dark-room facility depicted to scale

The darkroom facility, shown to scale, consists of two core darkrooms (C1 and C2), three dark anterooms (A1, A2, A3) and a single illuminated area with a sink to clean the animals' cages. Animals are communally housed in a cage in the primary core darkroom (C1). Animals are moved from the primary core darkroom to the secondary core darkroom (C2) to allow for cleaning of their cage(s), cleaning of the room, emptying of litter, and supply of food and water in the primary core darkroom.

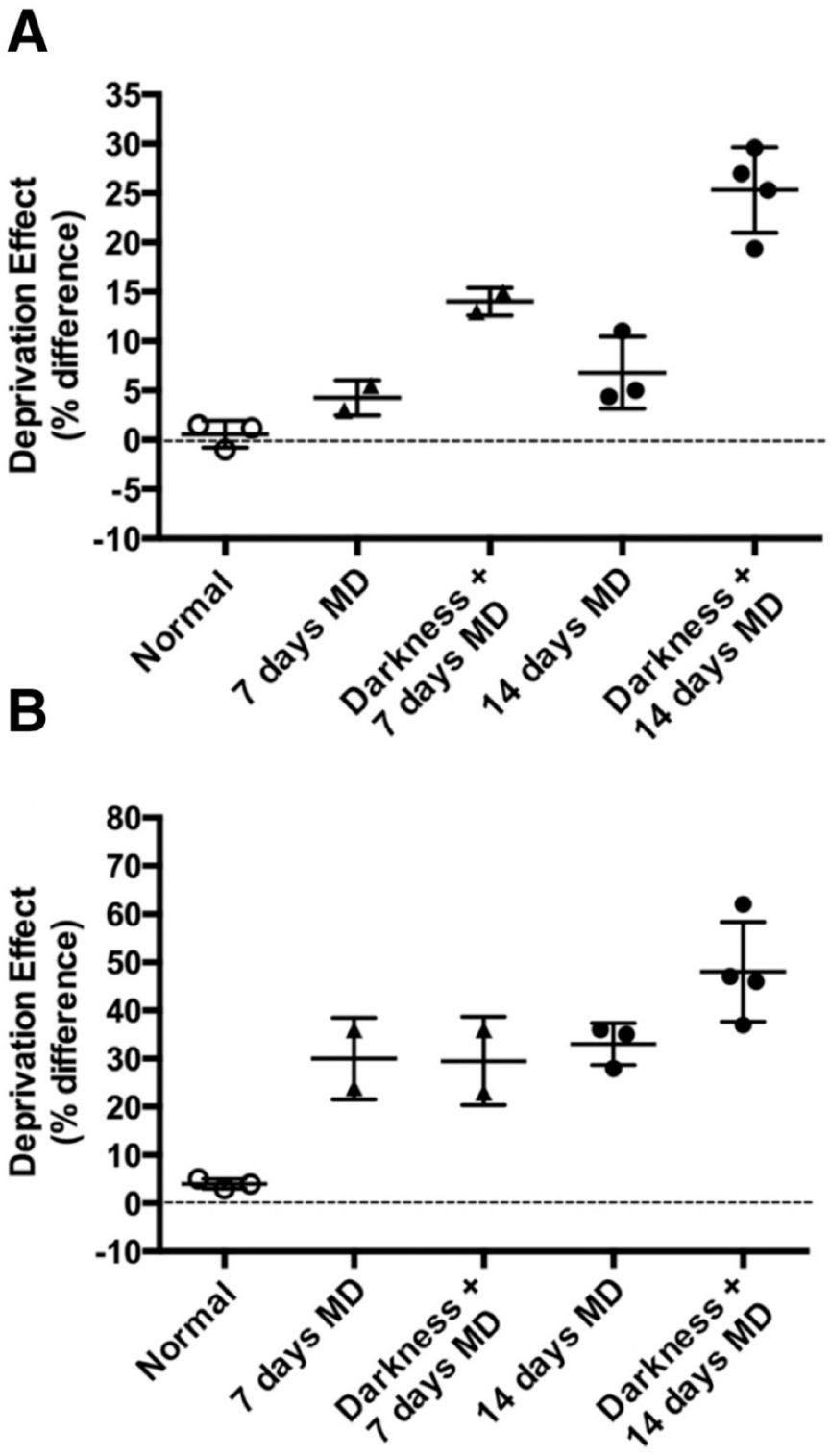

Figure 4. Effects of 10-days of darkness on the capacity of MD to provoke changes to dLGN soma size and neurofilament expression in juvenile kittens (Duffy et al., 2016).

Figure 4. Effects of 10-days of darkness on the capacity of MD to provoke changes to dLGN soma size and neurofilament expression in juvenile kittens (Duffy et al., 2016).

The results from this paper indicated that 10-days of darkness immersion at 12-weeks of age restores a sensitivity to monocular deprivation akin to that of a younger animal. (A) Stereological quantification of cross-sectional soma area revealed a significant shrinkage of deprived dLGN neurons, relative to non-deprived neurons when darkness preceded 7- or 14-days of MD. Neither 7- or 14-days of MD without preceding darkness provoked deficits in deprived layer neuron area different from that of normal. (B) Stereological quantification of neurofilament labelling density revealed a more significant loss in deprived layers when 14-days of MD followed 10-days of darkness as compared to when 14-days of MD occurred in isolation. For (A) and (B), a dashed line indicates zero difference between left and right-eye dLGN layers.

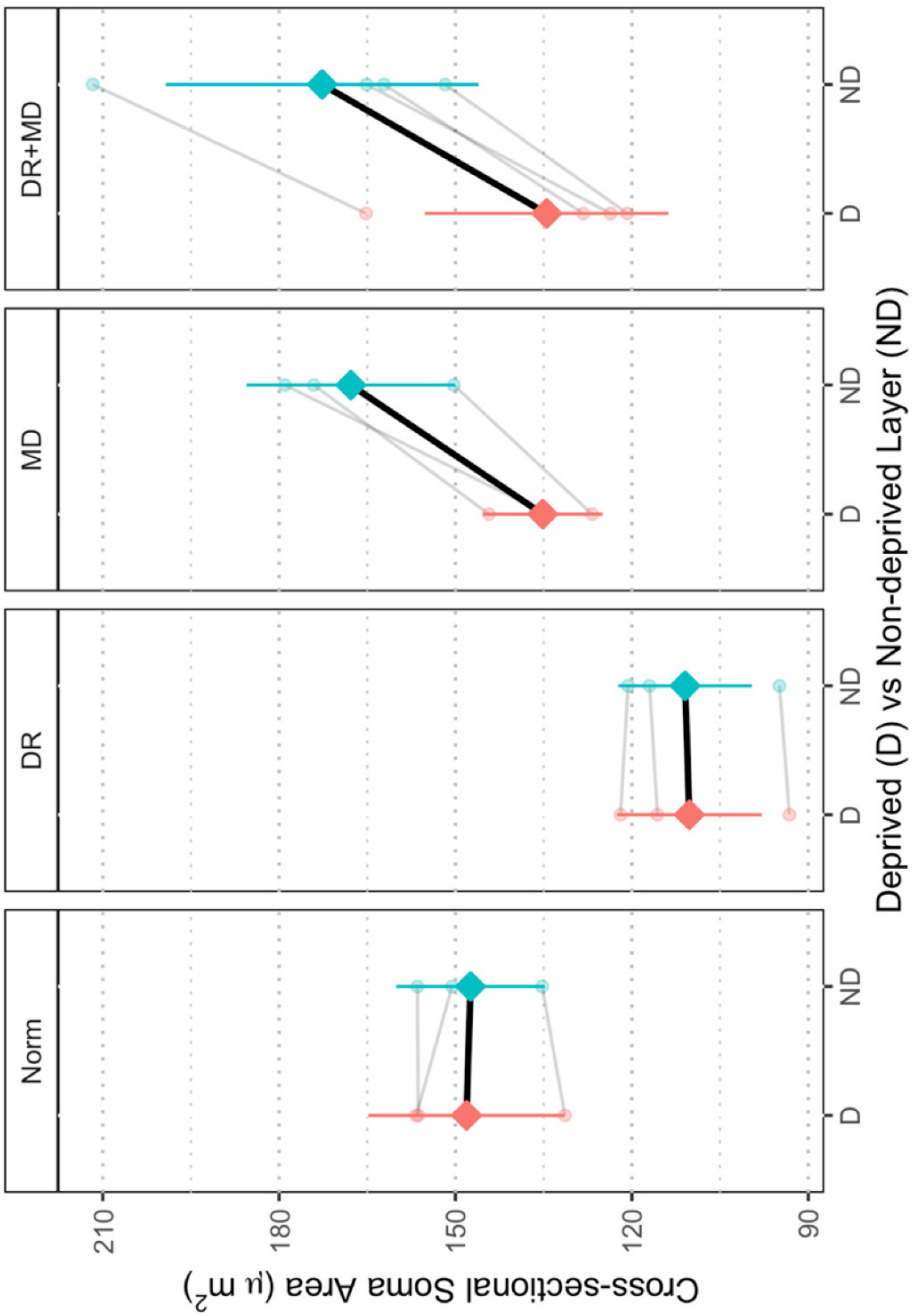

Figure 5. Cross-sectional soma area in deprived and non-deprived dLGN layers in each experimental condition

Figure 5. Cross-sectional soma area in deprived and non-deprived dLGN layers of each experimental condition

While measures of cross-sectional soma area in deprived and non-deprived dLGN layers are balanced in normal (Norm) and dark-immersed (DR) animals, they are imbalanced following monocular deprivation (MD) and following monocular deprivation that is preceded by darkness immersion (DR+MD). Specifically, in MD and DR+MD animals, cross-sectional soma area was found to be smaller in deprived- relative to non-deprived layers of the dLGN. While cross-sectional soma area appears to be balanced between deprived and non-deprived dLGN layers in DR animals, there is an overall reduction in neuron area compared to animals of other conditions. Large red and large blue diamond symbols indicate the group mean neuron area in deprived and non-deprived dLGN layers, respectively. The error bars extending from these large diamond points indicates the standard error of the group mean for each measure. The bolded black line connecting these large diamond symbols indicates the degree of balance between neuron area in deprived and non-deprived layers for each group. The smaller, faded, red and blue circle symbols indicate the mean neuron for each animal in deprived and non-deprived dLGN layers, respectively. The faded black lines connecting these symbols indicate the degree of balance between neuron area in deprived and non-deprived dLGN layers for each animal.

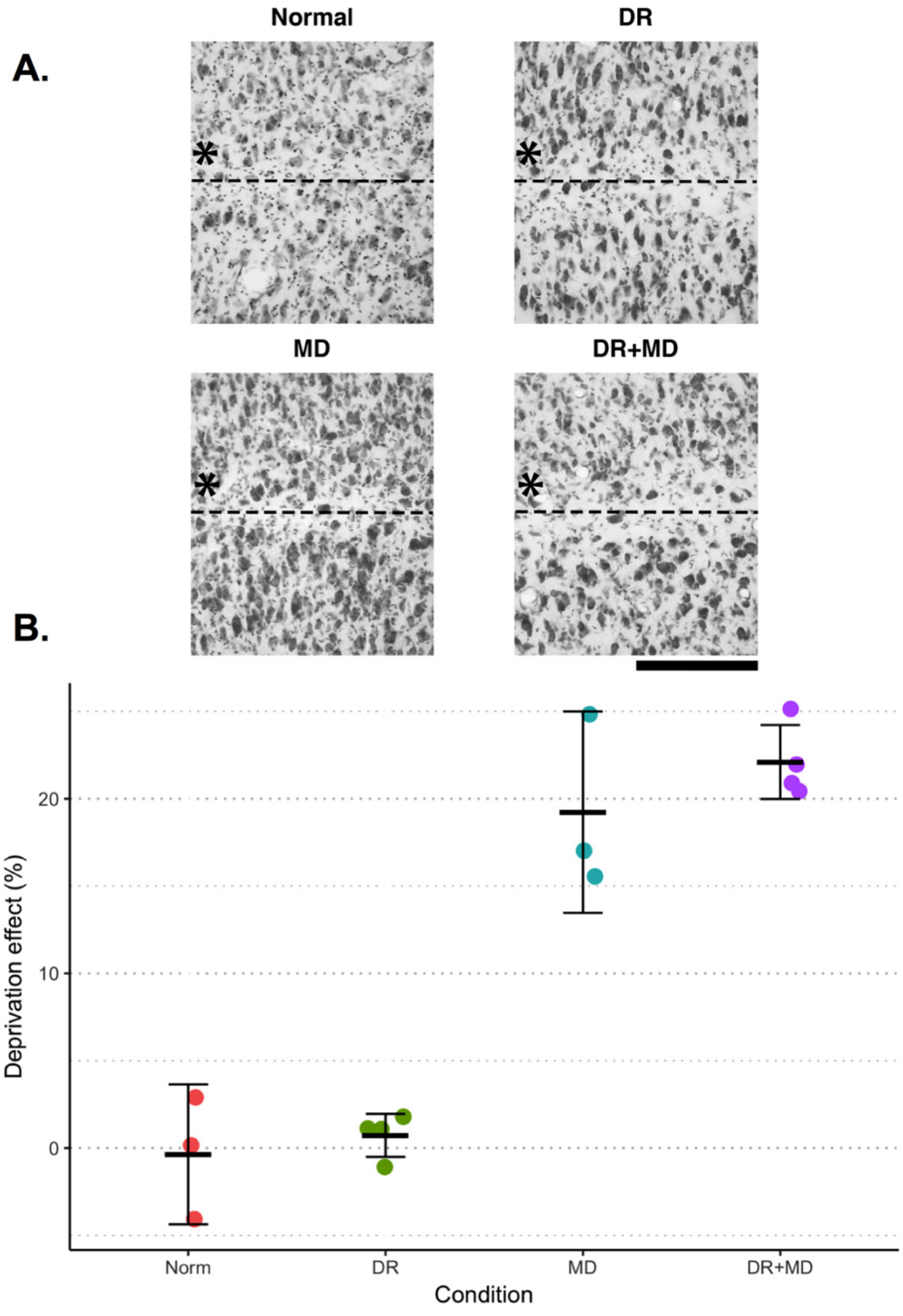

Figure 6. Effect of darkness and MD on dLGN neuron size in young kittens

Figure 6. Effect of darkness and MD on dLGN neuron size in young kittens.

The effect of MD on the cross-sectional area of dLGN neurons following 10-days of darkness in young kittens prior to the peak of the critical period. (A) Deprivation-induced structural changes in the dLGN were revealed by Nissl staining. 7-days of monocular deprivation, either in isolation (MD) or following darkness immersion (DR+MD) appears to cause a shrinkage of neuron area in deprived (asterisk) relative to non-deprived layers of the dLGN. This difference in soma size between deprived and non-deprived dLGN layers did not manifest in normal or dark-immersed (DR) animals. Scale bar = 100µm. (B) Stereological quantification of cross-sectional soma area in deprived and non-deprived layers of the dLGN across conditions. The percent difference between soma size in deprived and non-deprived layers (deprivation effect) was calculated for each animal (circular symbols), as described in the methods section. The mean deprivation effect (bold, central horizontal bar) and associated standard error (error bars) were calculated for each group. No difference was observed between the deprivation effect provoked by monocular deprivation alone (MD) or following darkness (DR+MD).

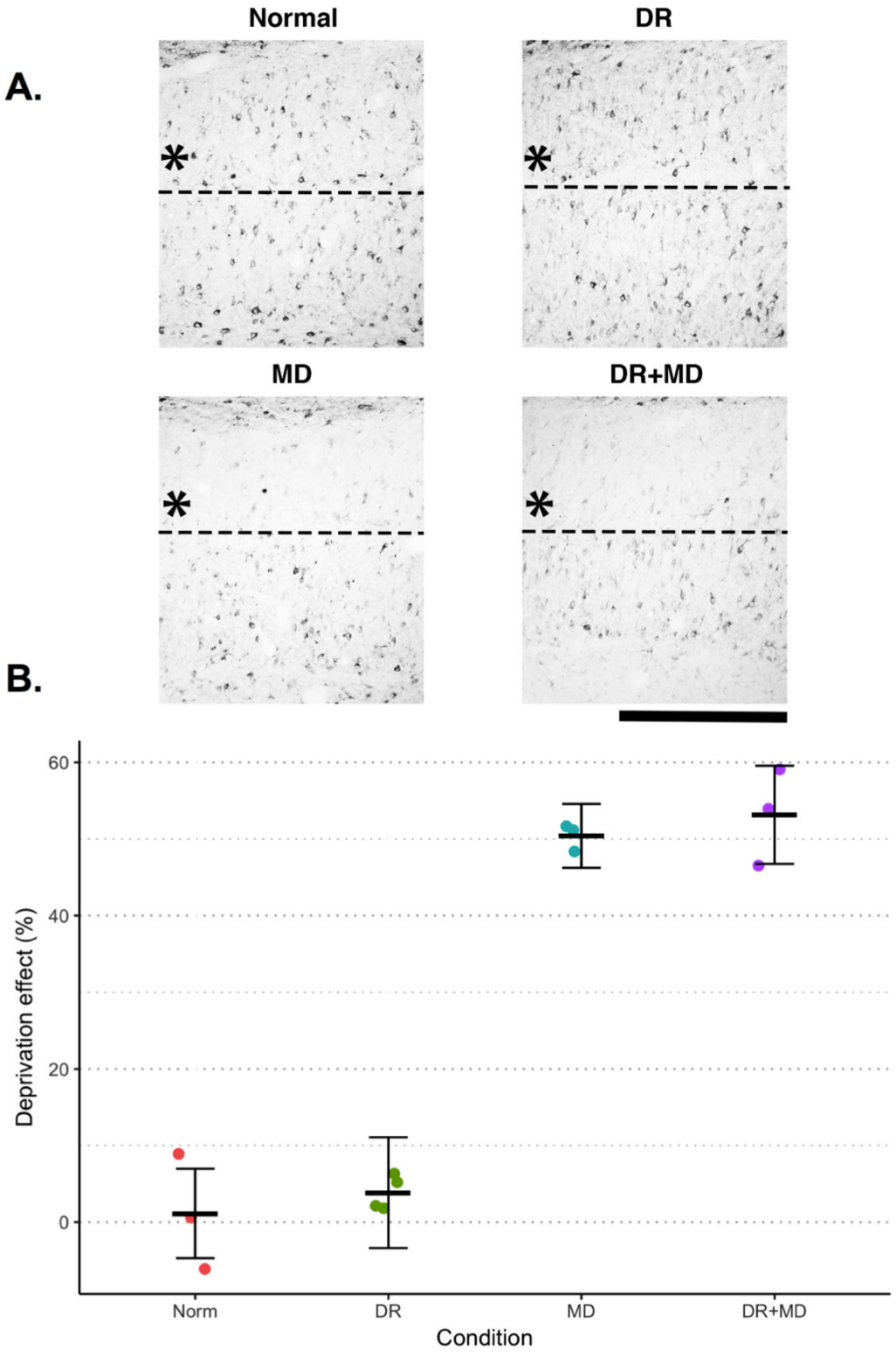

Figure 7. Effect of darkness and MD on neurofilament expression in the dLGN of young kittens

Figure 7. Effect of darkness and MD on neurofilament expression in the dLGN of young kittens.

The effect of MD on neurofilament expression in the dLGN following 10-days of darkness in young kittens prior to the peak of the critical period. (A) Deprivation-induced changes in neurofilament expression were revealed by immunolabelling tissue with SMI-32, an antibody specified for NF-H. 7-days of monocular deprivation, either in isolation (MD) or following darkness immersion (DR+MD) appears to cause a reduction of neurofilament labelling in deprived (asterisk) relative to non-deprived layers of the dLGN. This difference in labelling intensity between deprived and non-deprived dLGN layers did not manifest in normal or dark-immersed (DR) animals. Scale bar = 500µm. (B) Stereological quantification of neurofilament labelling density in deprived and non-deprived layers of the dLGN across conditions. The percent difference between neurofilament labelling density in deprived and non-deprived layers (deprivation effect) was calculated for each animal (circular symbols), as described in the methods section. The mean deprivation effect (bold, central horizontal bar) and associated standard error (error bars) were calculated for each group. No difference was observed between the deprivation effect provoked by monocular deprivation alone (MD) or following darkness (DR+MD).

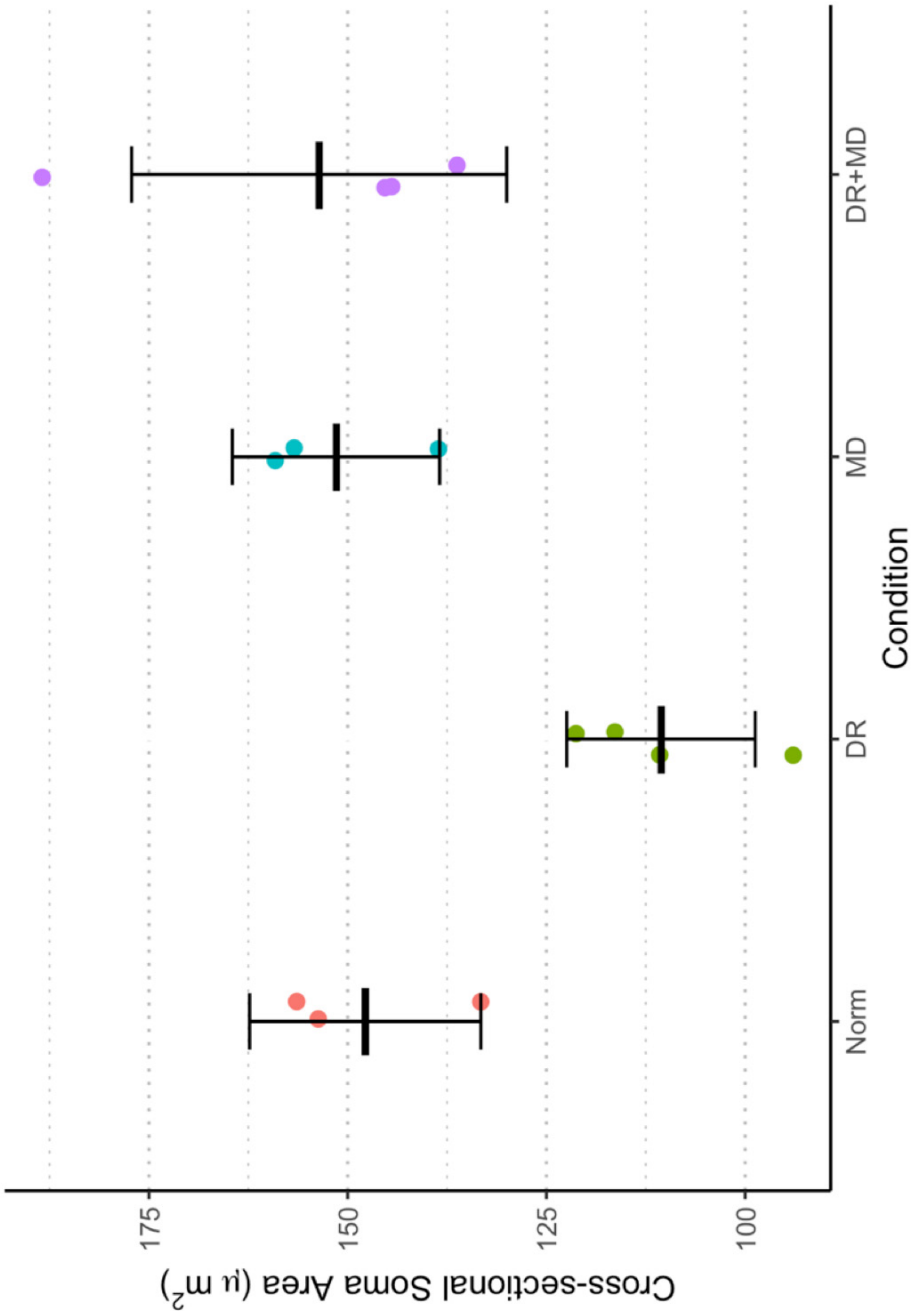

Figure 8. Absolute dLGN cross-sectional neuron area in young kittens of each group

Figure 8. Absolute dLGN cross-sectional neuron area in young kittens of each experimental group.

Stereological quantification of cross-sectional soma area across both deprived and non-deprived layers of the dLGN in each animal. The mean cross-sectional soma area quantified for each animal (circular points), for each group (bold, central horizontal lines), and the standard error associated with the group mean (error bars) are presented. No difference in mean dLGN soma area was found between normal animals, following monocular deprivation (MD), or following MD preceded by darkness (DR+MD). Animals that were immersed in darkness for 10-days (DR) had overall smaller dLGN neurons than animals in the other groups.

APPENDIX B Tables

Table 1. Table of primary antibodies used

Antigen	Immunogen	Source	Dilution
Neurofilament H	Homogenized rat hypothalamus	Covance (Princeton, NJ), mouse monoclonal, clone SMI-32, No. SMI-32. AB_509998	1:1000

Table 2. Mean deprivation effect (DE) for the difference in cross-sectional soma area between deprived and non-deprived layers of the dLGN in young kittens of each experimental group

Values were recorded from animals following normal-rearing, 10-days of dark immersion (DR), 7-days of monocular deprivation (MD), and 7-days of monocular deprivation preceded by 10-days of darkness (DR+MD). A positive DE indicates that deprived layer dLGN neurons were smaller than those of the non-deprived layer.

Animal	Condition	Individual Animal DE	Mean Condition DE
C158	*Normal*	0.10%	-0.36%
C320	*Normal*	2.86%	
C321	*Normal*	-4.03%	
C147	*DR*	-1.05%	0.73%
C160	*DR*	1.04%	
C167	*DR*	1.77%	
C431	*DR*	1.15%	
C242	*MD*	17.11%	19.23%
C243	*MD*	24.93%	
C323	*MD*	15.65%	
C432	*DR+MD*	20.90%	22.10%
C433	*DR+MD*	21.95%	
C434	*DR+MD*	25.12%	
C435	*DR+MD*	20.43%	

Table 3. Mean deprivation effect (DE) for the difference in neurofilament labelling between deprived and non-deprived layers of the dLGN in young kittens

Values were recorded from animals following normal-rearing, 10-days of dark immersion (DR), 7-days of monocular deprivation (MD), and 7-days of monocular deprivation preceded by 10-days of darkness (DR+MD). A positive DE indicates that deprived layer dLGN neurofilament labelling was reduced compared to that of the non-deprived layer.

Animal	Condition	Individual Animal DE	Mean Condition DE
C158	*Normal*	0.60%	1.13%
C320	*Normal*	-6.20%	
C321	*Normal*	8.99%	
C147	*DR*	5.20%	3.85%
C160	*DR*	2.17%	
C167	*DR*	1.71%	
C431	*DR*	6.30%	
C242	*MD*	48.42%	50.42%
C243	*MD*	51.25%	
C323	*MD*	51.58%	
C432	*DR+MD*	46.48%	53.15%
C433	*DR+MD*	53.85%	
C434	*DR+MD*	59.13%	
C435	*DR+MD*	N/A	

Table 4. Mean cross-sectional neuron area in deprived and non-deprived dLGN layers of young kittens

Values were recorded from animals following normal-rearing, 10-days of dark immersion (DR), 7-days of monocular deprivation (MD), and 7-days of monocular deprivation preceded by 10-days of darkness (DR+MD). Units are μm^2.

Animal	Condition	Deprived Area	Non-Deprived Area	Mean Deprived Area	Mean Non-Deprived Area	Mean Overall Area
C158	*Normal*	156.32	156.47			
C320	*Normal*	131.40	135.26	148.13	147.44	147.79
C321	*Normal*	156.66	150.60			
C147	*DR*	121.93	120.66			
C160	*DR*	110.10	111.25	110.22	110.95	110.58
C167	*DR*	93.20	94.88			
C431	*DR*	115.65	116.99			
C242	*MD*	144.31	174.10			
C243	*MD*	134.39	179.02	135.16	167.80	151.48
C323	*MD*	126.77	150.28			
C432	*DR+MD*	128.27	162.17			
C433	*DR+MD*	165.24	211.70	134.48	172.69	153.59
C434	*DR+MD*	123.64	165.12			
C435	*DR+MD*	120.78	151.78			