10 DAYS OF DARKNESS DOES NOT RESTORE VISUAL OR NEURAL PLASTICITY IN ADULT CATS

by

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DEDICATION PAGE

I dedicate this thesis to my brother, Josh Holman, whose unwaivering ability to exceed the expectations of every neurosurgeon and neurologist assigned to his case has inspired my study of neuroscience and has continued to provide a constant and marvelous reminder that perceptual limitations do not have to be absolute.

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ABSTRACT

Recently it was demonstrated that 10-days of darkness could restore visual plasticity and promote fast and complete recovery from amblyopia in juvenile kittens (Duffy and Mitchell, 2013). To test whether 10-days of darkness could restore plasticity and promote recovery from amblyopia in adult cats (≥1year), two sets of studies were conducted. First, the effect of darkness on promoting recovery from monocular deprivation was tested behaviourally by examining the visual acuity of amblyopic adult cats placed in darkness, and examined histologically by measurement of soma size and neurofilament-H (NF-H) label density in the dorsal lateral geniculate nucleus (dLGN). Second, the capacity of darkness to induce plasticity in normal adult cats was tested through examining the effect of a subsequent period of monocular deprivation on visual acuity and on soma size and NF-H label density in the dLGN. In all conditions examined, darkness was wholly unable to promote visual or anatomical plasticity.

LIST OF ABBREVIATIONS USED

Binoc: binocular

BDNF: brain-derived neurotrophic factor

BV: binocular vision

CSPG: chondroitan sulfate proteoglycan

DE: deprived-eye

dLGN: dorsal lateral geniculate nucleus

DR: dark rear

ECM: extracellular matrix

GABA: gamma-aminobutyric acid

LTD: long-term depression

LTMD: long-term monocular deprivation

LTP: long-term potentiation

Lynx1: Ly6/neurotoxin 1

MD: monocular deprivation

nAChR: nicotinic acetylcholine receptor

NDE: non-deprived eye

NF-H: neurofilament – heavy

NF-L: neurofilament – light

NF-M: neurofilament – medium

NMDA: N-methyl-D-aspartate

Npas4: neuronal per arnt sim domain protein 4

NR2A: NMDA receptor subunit 2-A

NR2B: NMDA receptor subunit 2-B

NT-4/5: neurotrophic factor 4; neurotrophic factor 5

Otx2: orthodenticle homeobox 2

PBS: phosphate buffered saline

PD: post-natal day

PEDIG: pediatric eye disease investigator group

PNN: perineuronal net

VEP: visually evoked potential

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CHAPTER 1: INTRODUCTION

The term “amblyopia” is derived from two Greek words that translate as ‘blunt sight’, and is used to describe a decrease in visual acuity (typically in one eye) that cannot be attributed to disease, and which persists despite correction of any optical perturbation. First described as a decrease of vision in healthy eyes by Hippocrates in 480BC (Loudon and Somonsz, 2005), amblyopia currently affects ~3% of children (Thompson et al, 1991). Amblyopia has long been known to arise in early life and has been linked to various peripheral conditions, many of which are optical in nature, that preclude similar imagery in the two eyes. Common optical perturbations thought to lead to amblyopia are unequal refractive errors in the two eyes (ansiometropia), or medial opacities of either the lens (congenital cataract) or the cornea. In addition to such purely optical conditions, an early disturbance of the optical alignment of the two eyes as in various types of strabismus has long been suspected to lead to amblyopia because of their common joint occurence.

Historically, many methods have been attempted to treat amblyopia. In conjunction with optical correction of any anisometropia or surgical interventions to straighten binocular eye alignment in the case of strabismus , or surgical removal of congenital cataract, the most effective treatment regimes have included the use of occlusion therapy early in life, introduced in modern times in 1743 (Loudon and Somonsz, 2005). Occlusion therapy entails occlusion by patching of the “good” eye for periods of time in an attempt to promote an improvement of vision in the amblyopic eye through forcing its mandatory use. Many variations of occlusion therapies have been attempted in an effort to improve outcomes. These include manipulations of the age of onset of therapy and its duration as well as the length of occlusion each day. Although a biological explanation for amblyopia

remained elusive until comparatively recently, it was generally believed that amblyopia was a disorder of visual development and that improvement of vision was possible only if treatment was attempted sufficiently early in life. However, the pivotal discoveries of Hubel and Wiesel in the 1960's provided for the first time a glimpse into possible anatomical and physiological explanations for amblyopia and as well introduced an animal model of amblyopia which could also be used for study of the way sensory nervous systems develop under the influence of the environment.

Hubel and Wiesel began by mapping the receptive field properties of neurons in the visual thalamus (Hubel and Wiesel, 1961) and the primary visual cortex (Hubel and Wiesel, 1959) of normal adult cats, thereby laying a foundation for an understanding of the functional architecture of the neural networks which serve visual perception (Hubel and Wiesel, 1962). Shortly thereafter they published results from a series of studies on the development of receptive field properties of cells in the visual cortex. Arguably, the two key studies from this series were directed toward exploration of the role played by the animal's early visual experience. To this end, they used electrophysiological recordings to document the properties of cells in very young kittens deprived of all visual experience from birth until 1 – 2 weeks old (Hubel & Wiesel, 1963). As many of the features that define the receptive field characteristics of adult cortical cells, such as ocular dominance and orientation selectivity were evident in reduced form in the kitten cortex they concluded that the neural connections that underlie these specific responses were present near birth and that this early development could occur in the absence of patterned visual experience. In an influential companion study, they examined the visual system of juvenile cats that had the eyelids of one eye sutured closed from shortly after birth until the time of

the experiments 2-3 months later (Wiesel and Hubel, 1963a,b) . The receptive field properties of neurons in the striate cortex of these kittens were very abnormal in comparison to those observed in normally reared juvenile kittens and in adult cats (Hubel and Wiesel, 1962; 1963b). In normal adult and juvenile cats, ~4/5 of cortical cells exhibited binocular response properties and were excited to some degree by stimulation of either eye (Wiesel and Hubel, 1962; Wiesel and Hubel, 1963b), whereas in the monocularly deprived kittens, most cells were responsive only to stimulation of the non-deprived eye, with very few cells exhibiting responses to stimulation of the deprived eye and almost no cells exhibiting binocular response properties (Wiesel and Hubel, 1963b). In contrast to the profound effects observed in the visual cortex, the functional characteristics of retinal ganglion cells in the deprived eye appeared normal as assessed from either electroretinograms (Wiesel and Hubel, 1963b) or by electrophysiological recordings (Cleland et al., 1980). Although minimal differences in the receptive field properties of neurons in the visual thalamus (the dorsal lateral geniculate nucleus - dLGN) were found, significant structural changes were evident histologically in the form of atrophied cell bodies in layers of the dLGN that receive afferents from the deprived eye (Wiesel and Hubel, 1963a). The atrophy noted in the deprived-eye recipient layers of the visual thalamus, is now thought to reflect the reduced cortico-thalamic feedback from deprived-eye circuitry in the visual cortex. Wiesel and Hubel (1963c) suggested that both the anatomical changes observed in the dLGN as well as the dramatic functional changes observed in the visual cortex following monocular deprivation may together represent the anatomical and physiologcial basis for amblyopia.

Later, Hubel and Wiesel (1970) found that there was a sensitive period for the effects of monocular deprivation on ocular dominance in the visual cortex, where the effects of monocular deprivation were particularly potent. This period peaked early in postnatal life at 4-5 weeks of age, and beyond this age the effects of monocular deprivation (MD) declined moving into the second and third postnatal months. Periods of monocular deprivation imposed in adulthood, even those lasting as long as 5 years, produced no shifts in the ocular dominance distribution. However, during the peak of what is now known as the critical period at 4-5 weeks of age, monocular deprivation imposed for as little as one day was sufficient to produce a marked shift in ocular dominance toward the open eye, and a period of deprivation lasting 6 days produced a shift in ocular dominance and changes in dLGN cell sizes which resembled those seen after periods of deprivation lasting several months (Hubel and Wiesel, 1970; Olson and Freeman, 1975). Later experiments have refined this work and extended the estimates of critical period length. By use of a 10-day period of monocular deprivation imposed on kittens of sequentially older ages, Olson and Freeman (1980) mapped the susceptibility of ocular dominance to monocular deprivation, effectively defining the profile of the critical period for ocular dominance plasticity in the cat. Although abnormal distibutions of ocular dominance were evident in kittens deprived as early as 2 weeks of age, the largest effects on ocular dominance were observed at between 4 and 5 weeks of age followed by a gradual decline in the effects that were still evident in the oldest kittens in the study that were deprived at 4 months of age (Olson and Freeman, 1980). Subsequent work that employed longer periods of MD lasting 1-3 months indicated that ocular dominance changes can be observed following MD to between 6 and 8 months of age, which suggests

that the critical period for ocular dominance plasticity in cats lasts until 8 months of age (Cynader et al, 1980; Jones et al 1984; Daw et al, 1992).

A large body of research has employed monocular deprivation to model amblyopia in a variety of species in order to investigate various developmental issues ranging from studies of the molecular mechanisms that underly neuronal plasticity and the timing of critical periods (Rauschecker, 1991; Hensch, 2005) to studies of experiential influences on the development of neural networks in the visual cortex and the computations they perform (Cooper and Bear, 2012). Studies of animal models of amblyopia have also contributed to an understanding of the development of other sensory systems which undergo similar environmentally regulated sensitive periods, but which may otherwise be difficult to study in isolation.

In parallel to the use of animal models of amblyopia to inform approaches to treatment (e.g. Mitchell, 1991; Tang et al., 2014), over 200 eye-care professionals from the United States, Canada and the UK coalesced in 1997 to form the collaborative research group PEDIG (Pediatric Eye Disease Investigator Group), whose aim is to conduct and report on multi-centre clinical trials that work towards the optimization of clinical therapies for several pediatric vision disorders, including amblyopia. In a series of such trials the various therapies that have been suggested for amblyopia have been examined in a systematic fashion on large groups of patients

One particularly noteworthy set of clinical clinical trials examined the efficacy of different regimens of patching as well as the issue of compliance with the prescribed dose. In this trial, one fourth of patients aged 7-12 demonstrated an improvement in amblyopia (of >2 lines on a logMAR eye chart) with only optical correction and the addition of a

patching regimine of 2-6 hrs/day nearly doubled the number of patients who demonstrated this level of visual improvement (Scheiman et al., 2005). However, at home compliance for regimes as prescribed remains notoriously difficult to verify, thereby complicating the interpretations of treatment efficacy (Bloom, 2004).

To set the stage for the studies described in this thesis it is necessary to provide an overview of visual development, critical period plasticity and the effects of monocular deprivation in the cat. Next, there will be an introduction to promising new research which employs a period of total binocular visual deprivation by immersion in complete darkness to promote recovery of vision in amblyopic kittens

Mammalian visual development begins *in utero*. The formation of the retina, its retinotopically ordered projections to the thalamus and subsequent thalamocortical projections are guided *in utero* by intrinsic molecular signalling (Sperry, 1963; Chalupa and Williams, 1984; Triplett and Feldheim, 2012) and co-ordinated retino-thalamic waves of activity (Catalano and Shatz, 1998). In cats, ~50% of retinal afferents from the left and right eye decussate at the optic chiasm before reaching their thalamic targets in the dLGN. Each dLGN has 2 monocular layers that receive exclusive input from either the left or right eye, and are visible in Nissl-stained sections, separated by a zone of lower cell density (interlaminar zone). The dorsal layer is referred to as the A lamina and receives input from the contralateral eye. Immediately ventral to this layer lies A1, which receives input from the ipsilateral eye. There is a 3rd layer ventral to A1, at one time referred to as the B layer but which is now referred to as the C laminae because it consists of multiple eye-specific layers visible only through use of monocular injection of anatomical tracers. Cells in the dLGN exhibit response properties that reflect those of the retinal ganglion

cells which project to them (Hubel and Wiesel, 1961; Hoffman et al., 1972) which lead earlier to the belief that the dLGN was simply a relay structure sending retinal information to the cortex. However, subsequent work has shown that the activity of dLGN cells can be modulated by many other inputs (Sherman and Guillery, 2000). Among them include cholinergic input from the brainstem (de Lima and Singer, 1987; Fjeld et al., 2002), GABAergic input from the pretectum (Cucchiaro et al, 1991; Cucchiaro et al., 1993), and feedback pathways from layer 6 of the the visual cortex (Sherman and Guillery 1996; Sillito and Jones, 2002; Andolina et al., 2013).

Afferents from the dLGN to the primary visual cortex synapse with cells in layer IV such that right eye and left eye afferents innervate neighboring regions of cortical space in a tiled pattern referred to as ocular dominance columns. In cat, these ocular dominance columns are clearly demarcated in layer IV and can be made visible by monocular injection of intraocular anterograde tracer. Monocular regions of cortex corresponding to the eye injected with tracer will then appear as distict bands of label interlaced with unlabeled regions serving afferents from the unlabeled eye (Lowel and Singer, 1987; Anderson et al. 1988), such that each eye innervates a roughly equal amount of cortical area (LeVay and Gilbert, 1976). These monocularly driven cells of layer IV form synapses with cells in the extragranular cortical layers (I, II, III, V and VI), resulting in the majority of cells in primary visual cortex receiving binocular input but with varying degrees of responsiveness to stimulation of each eye. However, the anatomical pattern of ocular dominance is significantly altered by monocular deprivation experienced early in life (Hubel and Wiesel, 1963b). In conjunction with the predominent emergence of cells responding exclusively to the non-deprived eye in the primary visual cortex, and the

atrophy observed in deprived eye recipient layers of the dLGN, the anatomical effect of monocular deprivation in cortex is reflected in the structure of ocular dominance columns in layer IV; the regions of cortex serving the non-deprived eye expand to occupy the majority of cortical space, while those regions serving the deprived eye shrink to thin strips (Shatz and Stryker, 1978; LeVay et al., 1980; Kossut et al., 1993). While the initial stages of visual development that occur *in utero* are governed by molecular cues and instrinsically generated activity, the emergence of distinct ocular dominance domains occurs during the first two weeks of post-natal life at a time early in that period of development referred to as the critical period for ocular dominance plasticity.

The term 'critical period' has become widely used to describe any window of suceptibility of a neural response property (i.e. orientation or direction selectivity) to permanent alteration by extreme environmental manipulation. The term has been used variously to refer either to the time during which a particular functional response property or anatomical feature develops (the critical period for development), the period in which development is susceptible to disruption (the critical period for disruption), or the period during which some recovery can occur in response to experiential therapeutic interventions (the critical period for recovery). Additionally, critical periods vary in both duration and onset depending on particular species, cortical layer, and response property (Daw, 2006). However, it is generally the case that the critical period for the disruption of ocular dominance encompasses those specific to cortical layer and other selective response properties such that it has become most commonly referrred to as simply 'the critical period'. As such, unless further clarification is provided, 'the critical period' will refer to the critical period for the disruption of ocular dominance.

Apparently, the cascade of molecular and physiological events leading up to the onset of critical period plasticity in the cat occur irrespective of prior visual experience. Dark-rearing from birth to 3 weeks of age, followed by a short period of monocular deprivation (from 1-10 days long) results in ocular dominance shifts that are indistinguishable from those seen in animals which were reared in normal lighting conditions prior to the period of monocular deprivation (Olson and Freeman, 1975). However, if the period of dark-rearing is extended to between 4 and 10 months of age prior to light experience, physiological susceptibility to monocular deprivation is apparent at ages well beyond the conventional critical period observed in normal animals (Cynader and Mitchell, 1980). Additional testing demonstrated that dark-rearing could preserve ocular dominance plasticity in cats up to 2 years of age, implying that complete darkness could prolong the activation of critical period plasticity irrespective of the age of the animal (Cynader, 1983). Later experiments showed that visual experience as brief as one day is sufficient to trigger the cascade of events leading to the opening of the critical period, the maturation of response properties and the subsequent closing of the critical period (Mower and Christen, 1985).

Since the development of transgenic models, rodents have become the most widely used species for study of the molecular events that underly coritical plasticity. Like cats, rodents have a well-defined critical period for ocular dominance plasticity early in life, and shifts in ocular dominance towards the non-deprived eye can be recorded after periods of monocular deprivation during the critical period (Fagiolini et al. 1994). Unlike cats (and monkeys), rodents do not have anatomically segregated ocular dominance columns in their visual cortex, but instead have cells of varying ocular dominances distributed throughout

the cortex with the majority of cells preferring stimulus by the contralateral eye (Wiesenfeld and Kornel, 1975). Shifts in ocular dominance and deficits to visual acuity as a result of monocular deprivation in rodents are much smaller in magnitude than those observed in cats and monkeys, but nonetheless provide an accessible model for the study of the underlying molecular mechanisms governing the emergence and maintenance of ocular dominance in the cortex.

A hallmark component of critical period plasticity and the development of ocular dominance columns is the balance between the maturation of inhibitory and excitatory neural circuitry in visual cortex (Hensch, 2005). The developmental emergence of widespread GABAergic inhibitory neural circuitry has been causally linked to the initiation of critical period plasticity (Fagiolini and Hensch, 2000), an assertion supported by experiments that examined ocular dominance formation in GABA knockout mice (Hensch et al., 1998) as well as the effects of pharmaceutical manipulation including local application of GABAergic agonists and antagonists (Hensch and Stryker, 2004). Similarly, the closure of critical period plasticity has been linked to the BDNF driven maturation of GABAergic parvalbumin-positive interneurons (Huang et al., 1999). Immature GABA receptors express elevated levels of the subunit $\alpha 3$ component compared to the $\alpha 1$ component, but by the end of the critical period the levels of expression of these two components are reversed and this expression profile is maintained into adulthood (Chen et al., 2001). Dark-rearing prevents the switch of the predominante subunit from $\alpha 3$ to $\alpha 1$, so that GABA receptors maintain putative immaturity through persistance of the neonatal isoform of the receptor (Chen et al., 2001). Excitatory NMDA receptors undergo similar

developmental changes in subunit composition, expressing elevated levels of NR2A at the peak of the critical period and two-fold less expression at the end of the critical period and into adulthood (Roberts and Ramoa, 1999; Chen et al., 2000). This reduction in expression is prevented by dark-rearing, and high levels of NR2A are maintained for the duration of dark-rearing (Quinlan et al. 1999a; Quinl et al., 1999b; Chen et al., 2000), suggesting that their developmental decline may be a factor in delineating the end of critical period plasticity. The presence of NMDA receptors expressing NR2A is known to promote synaptic plasticity by enabling long-term potentiation (LTP) at synapses in the hippocampus, although its role in visual system critical period plasticity appears to be more correlational than causal (Fagiolini et al., 2003). NR2A knock-out mice have a weakened response to monocular deprivation during the critical period, but their susceptibility remains within the critical period and is extended by dark-rearing from birth to the same extent seen in normal animals (Fagiolini et al., 2003). Importantly, a full expression of critical period plasticity can be rescued in these animals by targeted pharmocological enhancement of inhibition (Fagiolini et al., 2003), further indicating that threshold levels of inhibition, not excitation, govern critical period plasticity. Interestingly, the post-natal infusion of BDNF or NT-4/5 onto kitten visual cortex prevents the formation of ocular dominance columns entirely, highlighting the crucial role of neurotrophic factors in mediating the activity-dependent segregation of dLGN afferents into ocular dominance columns (Cabelli et al., 1995).

At the end of the critical period, inhibitory GABAergic interneurons become enveloped by extracellular matrix (ECM) proteoglycan molecules (CSPGs) which form perineuronal nets (PNNs) thought to mediate adult levels of inhibitory GABAergic

potency by exerting control of the extracellular ionic milieu (Hartig et al., 1999). Axon growth and cell migration become stifled by the formation of PNNs with constituent CSPGs, phosphacan and neurocan, specifically implicated in this inhibition (Grumet et al., 1996). Recently, another CSPG, aggrecan, has been shown to acculumate in the cortex of kittens in a manner that correlates with the decline of critical period plasticity (Kind et al., 2013), further implicating the ECM in its active restriction of plasticity in maturity. The BDNF driven maturation of GABAergic interneurons, and the co-incident development of enveloping PNNs and their constituent CSPGs are activity dependent processes which are delayed or otherwise disrupted by dark-rearing early in life (Castren et al., 1992; Lein and Shatz, 2000; Chattopadhyaya et al. 2004; Kind et al., 2013; Ye and Miao, 2013).

A number of molecules are involved in the active maintenance of restricted plasticity in post-critical period visual cortex, including Lynx1 (Ly6/neurotoxin1) and Otx2 (Orthodenticle homeobox 2) (Morishita et al. 2010; Beurdeley et al, 2012). Lynx1 is an endogenous prototoxin which becomes stably expressed in the visual cortex and the LGN of mice at the end of the critical period. Lynx1 binds to nicotinic acetylcholine receptors (nAChR) and reduces sensitivity to excitatory acetylcholine transmission. Transgenic mice lacking Lynx1 exhibit normal development of ocular dominance throughout the critical period, and exhibit normal ocular dominance shifts in response to periods of monocular deprivation during the critical period; however, unlike normal animals, these mice maintain suceptibility to ocular dominance in adulthood, and are also able to recover fully from periods of monocular deprivation that extend into adulthood (Morishita et al, 2010). That Lynx1 is selectively expressed in parvalbumin-positive GABAergic interneurons, provides yet another piece of evidence that regulatory changes

to the inhibitory-excitatory balance affect critical period plasticity (Hensch, 2005; Morishita et al, 2010).

Because the large majority of critical period plasticity-related molecules studied are, by definition, examined in an activity-dependent manner, the recent discovery of Otx2's involvement in regulating critical period plasticity has been particularly compelling (Huang and Cristo, 2008). Otx2 is a homeoprotein expressed in the retina throughout life, but is only found in the visual cortex during the critical period. Interestingly, cortical Otx2 is derived from retina, arriving in the cortex during the critical period through intercellular anterograde transfer in response to a threshold level of visual activity (Sugiyama et al., 2008). Otx2 accumulates preferentially in GABAergic parvalbumin positive interneurons during the critical period and plays a distinct role in nuturing the maturation of GABAergic circuitry, and with the initiation and closure of the critical period (Sugiyama et al., 2008). Dark-rearing has no effect on the expression of retinal Otx2, but substantially decreases its cortical presence (Sugiyama et al, 2008), consistent with the now well-established observations that dark-rearing prolongs the timecourse of critical period plasticity (Cynader and Mitchell, 1980; Timney et al., 1980; Mower and Christen, 1985; Mower, 1991). Further, the PNNs which surround mature parvalbumin GABAergic interneurons have specific binding sites for Otx2 and their interaction is crucial for the maintenance of mature neural circuitry (Beurdeley et al., 2012). Disruption of Otx2 accumulation in pavalbumin interneurons and on PNNs through exogenous pharamcological intervention is sufficient to restore critical period plasticity in adulthood such that amblyopic mice recover visually-evoked potentials in deprived-eye recipient cortex, and normal adult mice once again show susceptibility to monocular deprivation

(Beurdeley et al., 2012). Similarly, disruption to the PNN through the enzymatic digestion of its constituent CSPGs has been shown to restore critical period plasticity in adult mice (Pizzorusso et al., 2002).

That the maturation of the visual system apparently entails an accumulation of molecules which coincide with a decline in plasticity, and that their disruption can reintroduce periods of plasticity in adulthood, has given rise to the idea that restricted plasticity in adulthood is governed by a collection of putative "molecular brakes" on plasticity (Hensch, 2005). Otx2, the constituent CPSGs of PNNs and Lynx1 are all thought of as acting like molecular brakes. Thus far, the molecular brakes on plasticity have involved the extracellular stabilization of neural circuitry, though it seems logical that the gross structural changes in neurons that can be evoked during periods of plasticity (namely, changes in cell size as a result of monocular deprivation) are likely to also be under some form of developmentally regulated intracellular control. Recently, an intracellular scaffolding protein, neurofilament, has been put forth as another putative molecular brake on plasticity following demonstrations of an environmental manipulation that perturbs its expression and coincides with a period of heightened plasticity (O'Leary et al., 2012; Duffy and Mitchell, 2013).

Three kinds of proteins make up the neuronal cytoskeleton: actin microfilaments, intermediate filaments and microtubules (Siegel et al, 1999). Neurofilament, known to contribute stability to the mature neuronal cytoskeleton (Morris and Lasek, 1982) is the most plentiful intermediate filament in mature neurons. Neurofilaments provide structural support to the neuronal cytoskeleton through the ordered assembly of 3 subunits, each

named after their relative molecular masses: neurofilament – light (NF-L), neurofilament – medium (NF-M) and neurofilament – heavy (NF-H). NF-L is the obligatory subunit and its interaction with either NF-M or NF-H is required to generate filament assembly that provides functional cytoskeletal support (Lee et. al, 1993). Studies of *in vivo* neurofilament assembly have revealed the presence of NF-L and NF-M prior to NF-H (Shaw and Weber, 1982; Carden et al., 1987), which has led to the presence of NF-H being used as an indicator of neuron maturity (Liu et al., 1994; Lavenex et al., 2004; Borne and Rosa, 2006). NF-H is known to specifically contribue to axon calibre, conduction velocity (Marszalek et al., 1996; Kriz et al., 2000) and axonal outgrowth (Lee and Shea, 2014), and is posttranslationally modified in both phosphorylated and non-phosphorylated forms (Foster et al., 1987). Developmentally, the expression of non-phosphorylated NF-H precedes expression of phosphorylated NF-H, with both forms co-expressed in maturity (Foster et al., 1987). An increased expression of both NF-H isoforms with age has been linked to decreased levels of plasticity in the cat visual cortex (Liu et al., 1994), with the non-phosphorylated isoform localized mostly to the somata and dendrites, while the phosphorylated isoform is localized mostly to axons in adult animals (Sternberger and Sternberger, 1983; Foster et al., 1987).

The role of neurofilament as a molecular brake on plasticity can be demonstrated through examination of changes to its expression profile in the context of monocular deprivation and dark-rearing. Monocular deprivation imposed during the peak of the critical period leads to a loss of neurofilament label in the dLGN which can be detected as early as 4 days after monocular deprivation and which becomes asymptotic at 8 days, demonstrating levels of loss comparable with that observed after long-term (7 months)

monocular deprivations (Kutcher and Duffy, 2007). This loss of neurofilament label as a result of monocular deprivation has been demonstrated with all neurofilament subunits (Duffy and Slusar, 2009) and coincides closely in time with the reduction of cell size in deprived-eye recipient layers of the dLGN (Kutcher and Duffy, 2007) and to the loss of vision (Duffy and Mitchell, 2013), indicating that alteration to the cytoskeleton may somehow be involved in mediating the changes to gross structure (Kutcher and Duffy, 2007) and the loss of functional vision (Duffy and Mitchell, 2013). A loss of neurofilament labeling has been observed not only in the deprived eye recipient layers in the dLGN of the cat (Bickford et al., 1998; Kutcher and Duffy, 2007; Duffy and Slusar, 2009; O'Leary et al, 2012) and the primate (Sesma et al., 1984; Sengpiel et al., 1996) but also in ocular dominance columns serving the deprived eye in the cortex of primates (Duffy and Livingstone, 2005) and humans (Duffy et al, 2007). Further, changes in the sizes of deprived-eye receeipient cells have been noted in the dLGN of cats (Kutcher and Duffy, 2007; Duffy and Slusar, 2009; O'Leary et al, 2012) and primates (Hubel et al., 1977). Taken in aggregate, these results extend the idea that neurofilament contributes to the normal functioning and processing of neurons, with its loss from deprived-eye recipient circuitry across species paralleling the losses in function and cell size known to accompany monocular deprivation imposed during the critical period.

The deficits induced by monocular deprivation on vision, dLGN soma size and NF-H label are closely linked in time However, the recoveries from the deficits induced by monocular deprivation on vision, dLGN soma size and NF-H expression, are not similarly linked in time. Regarding visual function, monocular deprivation relieved early during the critical period allows for minor recovery of vision in the deprived eye simply upon

restoration of vision to the deprived eye (binocular recovery), though slightly more recovery can be promoted by employing periods of reverse occlusion (Dews and Wiesel, 1970; Giffin and Mitchell, 1978). Unlike the limited recovery of vision, full recovery of deprived eye dLGN cell size is observed following either binocular recovery, reverse occlusion, or dark-rearing early on in the critical period (O'Leary et al., 2012). Additionally, the full recovery of dLGN neurofilament labeling occurs either by providing binocular vision or reverse occlusion when either is imposed early in the critical period (O'Leary et al., 2012). However, no recovery of cell size (Cragg et al., 1976) is observed after long-term deprivation extending well beyond the peak of the critical period under any recovery condition and even in the extreme case of enucleation of the non-deprived eye, only minimal improvements in vision are observed (Smith, 1981).

Interestingly, 8-days of darkness imposed immediately following relief of a 7-day period of monocular deprivation during the peak of the critical period promotes a near complete recovery of deprived-eye recipient cell size in the dLGN of the cat while simultaneously causing a widespread loss of neurofilament that extends to the non-deprived layers, leaving the dLGN nearly void of neurofilament labeling and thus resembling an earlier developmental state (O'Leary et al., 2012). This result provided the basis for a set of experiments conducted by Duffy and Mitchell (2013) which hypothesized that 10-days of darkness could potentially provide functional therapeutic benefit for animals that had experienced an amblyogenic event by reinstating a period of plasticity which could allow for the recovery of normal visual function.

The first part of their experiment was a behavioral assessment of animals that had experienced a 7-day period of monocular deprivation imposed during the peak of the critical period (PD30-37) followed by a 10-day period of darkness immediately upon relief of monocular deprivation. These animals were tested by use of a jumping stand (Figure 2; see Chaper 2, section 2.1.3) and initially appeared completely blind in both eyes but steadily recovered acuity in both eyes to normal levels over ~2 months (Duffy and Mitchell, 2013). Notably, these animals did not develop amblyopia in the originally deprived eye.

A second group of normal animals were placed into darkness from PD30-40, and the level of the NF-L present in their visual cortexes was assessed at PD40. When compared to normal animals of the same age, the animals reared in darkness expressed 50-60% less NF-L label throughout all layers of the cortex (Duffy and Mitchell, 2013), extending previous results which demonstrated that darkness could catalyze a widespread loss of NF label in the entire dLGN (O'Leary et al., 2012). That dark experience early in the critical period has the propensity to catalyze a widespread loss of neurofilament in the visual system (O'Leary et al., 2012; Duffy and Mitchell 2013) and that this loss of neurofilament is coincident with a loss of vision that becomes fully recovered in both eyes with time, provides evidence that darkness experienced early on provokes a heightened period of plasticity that can ameliorate both anatomical and functional effects of an amblyogenic event, possibly through the provokation of instability to a maturing neuronal cytoskeleton (Duffy and Mitchell, 2013).

A third group of animals experienced a 7-day period of monocular deprivation, after which point were provided with either 5 weeks or 8 weeks of binocular vision prior to being exposed to 10-days of darkness at 10-13 weeks of age. In these delayed-dark animals, the development of vision in both eyes was measured daily by use of a jumping stand and all animals developed a clear and stable amblyopia prior to the period of darkness. Upon emergence from the darkroom, all animals demonstrated deprived and non-deprived eye acuities identical to those measured immediately prior to the period of darkness; however, in the days that followed, the deprived eye acuties of all animals showed remarkable and rapid improvement each day until achieving normal levels after only one week of binocular vision (Figure 3; Duffy and Mitchell, 2013).

That the acuities of the two eyes upon emergence from the darkroom were identical to those measured prior to entry is consistent with previously published research which suggested that darkness alone does not promote recovery from the effects of monocular deprivation in cats (Yinon and Goshen, 1984) or rats (He et al., 2006). Instead, the results of Duffy and Mitchell (2013) highlight the crucial role that binocular vision plays in darkness-promoted recovery. Darkness imposed well beyond the peak of the critical period appears to promote a period of heightened plasticity that permits the deprived eye to develop functional connections to the visual cortex in only a few days at no cost to connections of the non-deprived eye to allow full recovery of the visual acuity of the deprived eye (Duffy and Mitchell, 2013). This remarkable improvement of visual acuity promoted by darkness in 3-4 month old kittens begs the question as to whether or not 10-days of darkness may provide similar benefit to amblyopic adult cats.

Converging evidence from a series of rodent studies supports the hypothesis that darkness may be capable of promoting heightened periods of plasticity in adulthood. Ten days of darkness preceeding a short period (≥3d) of monocular deprivation in adult rats appears to reinstate juvenile-like ocular dominance plasticity such that significant shifts in ocular dominance towards the non-deprived eye are evident in measures of visually-evoked potentials (VEP) (He et al., 2006). Significant recovery of VEP amplitude in the case of long-term monocular deprivation extending into adulthood (from eye-opening to between PD70-100) was observed in rats following 10 days of darkness imposed prior to a period of either binocular vision or reverse occlusion (He et al., 2007). Further, 10-days of darkness imposed in adult rodents restores juvenile levels of NR2A expression and GABAergic activation (He et al., 2006), and when combined with either binocular vision or reverse occlusion, increases the density of dendritic spines in all layers of deprived eye recipient cortex (Montey and Quinlan, 2011).

If a 10-day period of darkness was able to promote functional recovery of vision in amblyopic adult cats and/or provoke instability of the neuronal cytoskeleton, it would help build a case for the use of darkness as an adjunct to, or as a potential treatment for adult human amblyopes. In recent years, a growing body of evidence from both animal models of amblyopia and from psychophysical studies of human amblyopes have provided evidence for the view that therapies for amblyopia which promote binocular cooperation may supersede in both efficacy and long-term stability the outcomes from traditional monocular treatments that employ patching to improve the vision of the amblyopic eye (for review see Mitchell and Duffy, 2014). In addition to being easier to deliver than the often difficult to enforce compliance of prescribed patching regimes (Bloom, 2004), the

use of darkness as a possible treatment for amblyopia in adulthood could introduce new therapeutic options for patients whose amblyopia was discovered too late in life to benefit from conventional therapies, or even for whom previous attempts at convential treatment failed to provide benefit (Scheiman et al., 2005).

Therefore, to address whether or not a 10-day period of darkness has the propensity to provoke heightened periods of visual plasticity and/or the instability of the neuronal cytoskeleton in adult cats, two set of experiments were designed as outlined in detail in Chapter 2.

CHAPTER 2: EXPERIMENTAL DESIGNS AND HYPOTHESES

Two sets of experiments were designed that employed both anatomical and behavioural approaches to test for the possible introduction of plasticity in adulthood in response to brief periods of darkness. The first class of experimental explorations represented an extension to adult cats of earlier studies conducted on amblyopic kittens (Duffy & Mitchell, 2013) that documented the recovery induced by a 10-day period of darkness. The ability of short periods of darkness to induce anatomical and/or behavioural recovery in adult cats from a prior amblyogenic event would provide evidence that plasticity could be evoked at any age. In contrast, the second class of experiment investigated the possibility of darkness-induced adult plasticity in normal adult cats as demonstrated by susceptibility to monocular deprivation at an age well beyond the accepted end of the critical period of sensitivity to such deprivation. Both sets of experiments were conducted on animals at about one year of age at a time well beyond the accepted end of the critical period for ocular dominance plasticity in the cat primary visual cortex (Daw, 2006). The specific methodological details for the two sets of experiments, that investigated the possibility of darkness-induced plasticity in adulthood in terms of either recovery from, or induction by, monocular deprivation are described separately in respectively, sections 2.2 and 2.3. The methodological procedures that were common to both sets of experiments will be described first.

2.1 – Materials and Methods

A total of 17 male and female cats reared and bred in a closed colony at Dalhousie University were used in this study. All procedures were approved by the Dalhousie University Committee for Laboratory Animals and followed the guidelines outlined by the Canadian Council on Animal Care. In common for all animals in both the anatomical and behavioural experiments were either long or short periods of monocular deprivation and 10 days spent in total darkness. Separate groups of animals were used for the behavioural and anatomical investigations. Procedures common to all animals, and those unique to the animals in either the anatomical or behavioral investigations, are described below in sections 2.1.1 – 2.1.5.

2.1.1 - Monocular deprivation

Animals were anaesthetized for surgery on post-natal day (PD) 30 with gaseous isofluorine (1-3% in oxygen) and body temperature maintained with a heating pad kept at 37°C. An analgesic (Ketoprofen, 0.2ml/kg) was injected subcutaneously prior to the beginning of surgery. Monocular deprivation was achieved by suturing the upper and lower conjunctiva together with Ethicon 6-0 vicryl, applying an ophthalmic broad spectrum antibiotic ointment, and then suturing together opposing inner edges of the upper and lower outer lid with Ethicon 5-0 silk, just behind the lid margins. This provided two occlusion layers. For short-term deprivation (7 or 14 days), sutures were surgically removed under anesthesia at the end of the deprivation period, the conjuctiva separated if

fused and any scar tissue excised to alleviate potential corneal irritation. The left-eye was deprived in all cases.

For long-term (11 months) monocular deprivation, initial procedures were identical to those described above, with the added step of creating small, crisscrossing wounds with a scalpel on the inner surfaces of both the upper and lower lids prior to suturing. This facilitated their fusion through the healing process. Outer sutures were surgically removed ~2 weeks after the onset of deprivation and the extent of fusion was assessed. The inner conjuctiva and outer palpebral tissues healed together, forming two occlusion layers. This allowed for a long-term deprivation without further surgical intervention. Animals were monitored daily for pinholes throughout the extent of the deprivation period. No pinholes were found in any of the animals and no surgical intervention post-suture removal was required for any of the animals used in this study.

2.1.2 Darkroom facility

Animals were placed into darkness for 10 full days at around one year of age. Darkness was achieved through use of a light-tight dark room facility, depicted to scale in Figure 1. Cats were housed in a large playpen (1.5m long x 0.7m wide x 0.9m high) within the darkroom containing raised shelves at both ends (0.2m wide, 0.4m off of the floor), a litter box, ad libitum food and water, cardboard boxes and toys. When timelines were co-incident, cats were housed 2 per playpen and in no cases were cats in the darkroom alone. Cats were removed from their playpens daily and put into carriers that were placed in a secondary dark room (Figure 1, C2). The primary dark room (Figure 1, C1) and

playpen(s) were then cleaned under illumination, litter trays changed, and food and water replaced. Lights were turned off once cleaning was finished and cats returned to their playpens.

The dark room was equipped with a radio programmed on a 12h:12h L:D cycle coincident with the light cycles in their home colony room in order to keep animals entrained to a circadian rhythm.

2.1.3 Behavioural testing

Behavioural measures of acuity were made by use of a jumping stand (Figure 2) (Murphy & Mitchell, 1987; Mitchell & Duffy, 2014). The jumping stand consisted of an open-ended rectangular starting wooden box (Figure 2, A:for kittens- 13.5 cm W x 19 cm H x 21 cm L; for adults- 18 cm W x 22.5 cm H x 31 cm L), attached to a wooden platform (Figure 2, B: 20.5 cm W x 44.5 cm L), that was supported by 2 yoked laboratory jacks (Figure 2, C) to enable continuous changes in height. The starting boxes could be placed on the platform to provide an overhang of 6-10 cm so that the animals were directly above the front of the stimuli beneath them. The stimuli were placed on top of a rectangular box (Figure 2, D: 42 cm H x 65 cm W x 38 cm L), with two hinged doors, (Figure 2, E: 31 cm W x 35 cm L), on top. The platform and starting box could be lowered flush with the stimulus, or gently raised incrementally using the jacks to a maximum height of 72 cms above two adjacent stimuli. Stimuli consisted of identical two square-wave gratings (19 x 19 cm) surrounded by a gray tape border (3.5 cm wide) placed on the two hinged doors separated vertically by a wooden divider (Figure 2, F: 1.5 cm W x 4.5 cm H x 35 cm L). The two doors were nearly always closed but one was opened during training or when it

was suspected that the vision was so poor that the animal possessed only rudimentary vision. With one door open the animal faced a choice between a grating and an open door and a 40 cm drop to the floor below. Stimuli were generated by a laser printer, coated with a matte protective finish, illuminated by a 60 watt incandescent lamp located in front of the laboratory jacks and had a luminance of 100 cd/m^2.

Reliable testing of adult cats by use of this method requires that cats learn the task at an early age through daily training sessions and that visual salience be maintained by regular testing into adulthood. Two-choice discrimination training began for each animal at between PD36 and PD52 with a vertically oriented grating used as the positive stimulus. Initial training began with the starting box lowered to the height of the stimuli. Kittens were placed into the starting box and encouraged with gentle pushes and guidance to step off the platform onto a vertically oriented grating having the highest period (32mm), placed adjacent to an open door. Kittens were positively reinforced with a food reward (wet cat food mixed with raw chicken liver) and patting for walking onto the vertical grating. The side of the grating was switched randomly for about 10 trials or until the kitten walked onto the grating with little hesitation. This simple task was repeated the next day followed by 10 trials in which both trapdoors were closed so that the animal now had to chose between a vertical and a horizontal grating. In most cases kittens continued to walk onto the vertical grating. Vertical gratings were presented on the left or right side in a pseudorandom order according to a Gellerman series (Gellerman, 1933) that avoided multiple (>2) successive presentations on the same side that could precipitate a side preference. When animals made 10 correct choices consecutively without guidance, a new trial block was started using gratings of a slightly higher spatial frequency. If the kitten

made a mistake, it was immediately placed back on the jumping stand and made to repeat the trial. Animals required between 7 and 14 training sessions before demonstrating reliable performance of the task. The height of the jumping stand was gradually raised above the gratings throughout the entire training period commensurate with their motor skills so that kittens learned to jump to the stimulus at the maximum testing height of 72 cm. After initial training, animals were required to make 5 correct jumps in a row to the vertical grating before moving to the next trial block which used a grating of higher spatial frequency. If an error was made in any trial block, animals were then required to correctly jump to the vertical grating for a minimum of 7 out of 10 trials before moving on to the next block. Between trial blocks, gratings were increased in spatial frequency in small steps that were equated on a logarithmic scale, with as many as 12 steps per octave (a factor of 2). Tests of acuity by use of logarithmic steps in spatial frequency closely mimics clinical testing of human visual acuity with a logMAR eye chart, albeit on a much finer scale of 12 versus 3 steps/octave (Mitchell, 2014).

Performance of trained cats was typically at 100% correct until spatial frequencies very close to threshold. Here, the animal's behaviour changed dramatically in a manner consistent with them having reached the limit of their visual resolution. In addition to their performance falling to chance (50% correct), animals exhibit uncharacteristic behaviours such as mewing, panting, head-bobbing, and a reluctance to jump. The spatial frequency of the last trial passed with ≥70% accuracy before performance fell to chance was the conservative measure used as the visual acuity (cycles/degree). The small changes in spatial frequency between trial blocks ensured that cats continued to respond to the visual cues and did not switch to other potential solutions such as alternation or responses to one

side. As well, the small steps in spatial frequency that were barely visible to a human allowed for a precise titration of visual threshold.

Measurements of monocular acuities were achieved by placing an opaque contact lens in the eye opposite to the one being tested. An analgesic ophthalmic solution (Alcaine, 1%) was applied to the eye prior to application of the contact lens to alleviate potential discomfort. Monocular testing of the non-deprived eye was not conducted for all animals as it has been observed that binocular acuity accurately reflects the monocular acuity of the non-deprived eye. Additionally, when binocular and monocular acuity measurements were collected on the same day, binocular measures preceeded monocular measures. This ensured that any corneal distortion due to the contact lens would not affect subsequent acuity measurments.

Training was maintained into adulthood using bi-weekly, weekly or bi-monthly training sessions and varied based on the individual animal's behaviour from session to session. The total number of testing sessions (after initial training) each animal underwent before the 10-day period of dark exposure was as follows: C187 – 62; C188 – 73; C173 – 48; C199 – 45; C200 – 72. While the animals received different numbers of testing sessions leading up to experimental manipulation, all animals reliably performed the task at the time of experimental manipulation. One week before the animals were placed in darkness, the frequency of testing was increased to ensure that the established baseline acuity being measured throughout the maintenance period was accurate and to rule out the possible interference of behavioural inconsistencies that could arise with a sudden change in testing frequency post-experimental manipulation. After removal from the darkroom

measurements of acuity were made every 24 hours for a minimum of 5 days to ensure any rapid changes in acuity would be detected.

2.1.4 - Histology

Cats were deeply anesthetized with isofluorane (1-3% in oxygen) prior to receiving a lethal dose of euthanol (150 ml/kg) delivered intraperitoneally. Cats were perfused transcardially to exsanguinate brain tissue with 250-350 ml phosphate buffered saline (PBS, 0.1M, 4° C, pH 7.4) followed by 250–350 ml of 4% paraformaldehyde.

Brains of each animal were removed from the cranium and dissected with a razor blade to reveal the thalamus. The occipital, frontal and temporal lobes were removed from each hemisphere. The resulting block of tissue contained both left and right dLGNs on the temporal portion of the thalamus and was approximately 3cmL x 4cmW x 2cmH. The dissected tissue was placed in 30% sucrose in 0.1M PBS at 4° C to establish cryoprotection. Each sample was embedded in OCT Tissue Freeze (Triangle Biomedical; Durham, NC, USA) and mounted on a freezing microtome (Leica SM2000R; Germany) where 50μm thick coronal sections were cut through the entire lateral geniculate nucleus. Sections used for Nissl were wet-mounted onto glass slides, allowed to dry overnight, dehydrated using a graded dilution series of ethanol and then placed in cresyl violet for 5 minutes. Sections were then differentiated using the ethanol dilution series to optimize staining of perikaryon, cleared in Histo-clear (DiaMed Lab Supplies Inc.; Mississauga, ON, CAN) and coverslipped with permount (Fisher Scientific; Canada). Sections adjacent to the Nissl stained sections were used for immunohistochemistry and were left free-

floating in PBS and probed for the presence of Neurofilament-H (NF-H) using the monoclonal SMI-32 antibody (Covance, 1:1000) that targets the non-phosphorylated epitope of the obligatory subunit of the neurofilament protein. Antibody specificity was verified using immunoblots of cat visual cortex that gave rise to a single band of immunoreactivity at 130kDa, congruent with previously published data on NF-H (Julien and Mushynski, 1982; Kaufmann et al. 1984; Georges and Mushynski, 1987). Sections were incubated overnight at 4° C in SMI-32, rinsed with PBS and incubated in a biotinylated secondary antibody (Jackson Immunolabs: 115-065-146, 1:1000), then conjugated with an avidin-biotin complex using a Vectastain ABC kit (Vector Laboratories; Burlingame, CA, USA). Antibodies were then made visible using the 3’3-diaminobenzadine tetrahydrochloride as chromogen. Reacted sections were mounted onto glass sides, dried overnight, dehydrated with ethanol, cleared in Histo-clear and cover-slipped using permount.

2.1.5 Quantification

All quantifications were performed by the author (KDH) using slides labelled by Dr. Kevin Duffy so that KDH was completely blind to the experimental condition throughout quantification. The density of NF-H label was quantified separately in layers A and A1 of the right and left dLGN by stereologically counting positively labeled neurons within the defined regions of interest. Positively labeled neurons exhibited a uniformly dark cytoplasm around a weakly labelled nucleus. Neurons were made visible at 600X magnification using a BX51 compound microscope fitted with a high-resolution DP-70

digital camera (Olympus, Markham, Canada). Counts were performed using an optical dissector probe from a computerized stereology program (newCAST; VisioPharm, Denmark), which generated a random 50% surface area sample of each A and A1 layer of each section examined of the right and left dLGN.

Measurements of cell size were performed under the same conditions as the density counts, except for use of the nucleator stereology probe (newCAST; VisioPharm, Denmark) in place of the optical dissector probe. Only those cells exhibiting darkly stained cytoplasm and a weakly stained nucleus were measured, ensuring measurements were limited to neurons that had been cut through the presumed somatic midline and avoided the inclusion of measurements from glial cells. Measurements were taken from 2-3 sections per animal yielding a minimum of 1458 cells measured per animal.

Within animal comparisons were made between measurements taken from the A and A1 layers of the dLGN both for cell size and density using a deprivation metric (1).

Deprivation Metric:

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

A value of this metric near zero indicates no difference between values measured in deprived and non-deprived layers, as observed in normal animals (Kutcher and Duffy, 2007; Duffy and Slusar, 2009). A positive value indicates that measures taken from deprived layers are less than those in non-deprived layers; a negative value indicates deprived measures are greater than non-deprived.

2.2 – Recovery Experiments: a search for darkness-induced recovery from Monocular deprivation in adult cats

2.2.1 Behavioural investigation

Three cats received an early period of monocular deprivation from PD30 to either PD37 or PD43 and in adulthood were exposed to a 10-day period of darkness beginning at ages between PD383 (C199) and PD412 (C200). If 10-days of darkness imposed in adulthood was able to restore plasticity in the adult visual system, it would be anticipated that some recovery of vision in the deprived eye would be possible, the extent and speed of which would provide a measure of the extent of plasticity that remained.

2.2.2 Anatomical investigation

The effect of darkness on dLGN soma size and neurofilament levels was studied on 7 adult cats reared to one year of age with long-term monocular deprivation (LTMD) that began at PD30. At one year of age, 3 cats had the eyelids of their deprived eye surgically opened to allow either for a 30-day binocular recovery period while the remaining 4 cats experienced an immediate 10-day period of darkness followed by 20 days of binocular recovery in their illuminated colony room.

If 10 days of darkness imposed in adulthood were able to restore plasticity in the visual system, it would be expected that animals which experienced 10 days of darkness prior to 20 days of binocular vision would show recovery of both cell size and neurofilament labeling in deprived layers. In contrast, the animals that experienced just 30

days of binocular vision would not be expected to show recovery of cell size or neurofilament labeling.

2.3 – Inducing visual plasticity using darkness

2.3.1 Behavioural investigation

Two littermates (C187, C188) were reared normally into adulthood and then had a 14-day period of monocular deprivation imposed beginning at PD482. In the case of C188, the period of monocular deprivation was immediately preceded by a 10-day period of darkness beginning at PD472.

If the 10 days of darkness imposed in adulthood was able to restore plasticity in the visual system, it would be anticipated that C188 would exhibit deficits in the visual acuity of the deprived eye immediately following the period of monocular deprivation. The extent of the visual loss and the speed of any subsequent improvement would provide a measure of the plasticity promoted by darkness. On the other hand, the acuity of the deprived eye of C187 that was not placed in darkness prior to the period of monocular deprivation would expected to be unchanged, consistent with imposition of MD well beyond the documented end of known critical periods in the visual cortex.

2.3.2 Anatomical investigation

This investigation was conducted on 5 normally reared adult cats that received a 7-day period of monocular deprivation beginning at PD375. For 3 of these animals, the period of monocular deprivation was immediately preceded by a 10-day period of

darkness beginning at PD365. If 10 days of darkness imposed in adulthood were able to restore plasticity in the visual system, it would be expected that those animals placed in darkness would show a reduction in cell size and neurofilament labeling in deprived layers following monocular deprivation, while those animals that just experienced a period of monocular deprivation without a prior period of darkness would not.

CHAPTER 3: RESULTS

3.1 Recovery Experiments: a search for darkness-induced recovery from monocular deprivation in adult cats

3.1.1 Behavioural Investigation

The 3 cats raised into adulthood following an early period of monocular deprivation (from P30 – 37 or 43) all developed severe amblyopia in the deprived eye that remained stable into adulthood. As exemplified by the results for C199 displayed in Figure 4B, the emergence of amblyopia in each of the 3 animals followed a profile consistent with those documented previously (Duffy & Mitchell, 2013) and appeared to stabilize about 50 days following the end of the period of MD.

Prior to entering the darkroom, the acuities of the deprived eye of C199, C200 and C173 were respectively, 1.12, 0.88 and 2.22 cycles/deg. In relation to the acuities of their individual fellow eyes (as reflected by the measurements of binocular visual acuities as indicated in Table 1) the deprived eye acuities were reduced by factors of respectively, 6.6, 8.1 and 2.9, or 2.7, 3.0 and 1.54 octaves.

Binocular and monocular acuity measurements were made immediately before each animal entered the darkroom, and within 30 minutes of the animal's emergence back into light 10 days later. After darkness, the initial measures of binocular and monocular acuities of C199 and C200 were identical to those measured before the period of darkness and remained unchanged in the days that followed. Upon emergence from the darkroom, C173 adopted non-visual strategies on the jumping stand including extreme sensitivities to movements by the experimenters so that the immediate measures of visual acuity were

deemed unreliable. These behavioural issues were resolved satisfactorily after about a month, at which time the acuities of the two eyes had changed only by a very small amount from those measured prior to the period of darkness so that the deficit remained little changed as a factor of 3.8 or 1.9 octaves.

In stark contrast to the fast recovery of deprived eye acuity following a 10-day period of darkness observed in juvenile kittens (Duffy & Mitchell, 2013), the deprived-eye acuities of the 3 adult cats of this study were unchanged by the period of darkness (Figure 4; Figure 5; Table 1).

3.1.2 Anatomical Investigation

The anatomical investigation included measurement of the recovery of neurofilament labeling and recovery of soma size in deprived layers of the dLGN following relief of LTMD in the context of two different recovery conditions. In the first recovery condition, C201, C204 and C207 were provided with a 30-day period of binocular vision following opening of the eye that was deprived from PD30 to PD365. Upon gross examination of Nissl stained dLGN sections, perikarya were well defined against a light background, and a substantial deprivation effect was still evident in the deprived-eye recipient layers of the dLGN with cells in these layers appearing smaller than cells in non-deprived counterpart layers. For each animal, ~ 1500 cell measurements were taken from 50% of the area of the medial region of the A and A1 layers of both the left and right dLGN in 4 sections from about midway through the anterior-posterior axis of the dLGN. Mean cell size for C201: deprived = 140μm^2, non-deprived = 188μm^2 . Mean cell size for C204: deprived= 120μm^2, non-deprived = 178μm^2. Mean cell size for C207:

deprived = $142\mu m^2$, non-deprived = $224\mu m^2$. Expressed in terms of the deprivation metric (1), cells in deprived layers were, on average, smaller than those in counterpart non-deprived layers for C201, C204 and C207 by, respectively, 34%, 33% and 37%, (Table 2; Figure 6A-C). Statistical comparison using an independent unpaired samples t-test found the average soma areas between deprived and non-deprived layers of all animals to be significant: $t(8)=-5.130$, $p<0.001$.

Similar to our observations from Nissl-stained sections, gross observations of sections immunolabeled for NF-H revealed a substantial reduction of labeling in deprived eye recipient layers. Quantifications of NF-H positive neuron density in each layer of the dLGN revealed densities for each animal as follows: C201: deprived = 115 neurons/mm^2 , non-deprived = 235 neurons/mm^2; C204: deprived = 134 neurons/mm^2, non-deprived = 240 neurons/mm^2; C207: deprived = 120 neurons/mm^2 , non-deprived = 219 neurons/mm^2. Subsequent analysis using the deprivation metric (1) revealed a reduction in the density of positively labeled neurons in deprived layers for C201, C204 and C207 of 51%, 44% and 45%, respectively (Figure 7A-C; Table 3). Differences in the densities measured for all animals between deprived and non-deprived layers compared with an independent unpaired samples t test revealed a significant difference between groups: $t(7) = -8.8775$, $p < 0.00007$.

In the second recovery condition, 4 cats received a 10-day period of darkness immediately upon relief of LTMD, followed by a 20-day period of binocular vision. Gross observations of Nissl stained sections from these animals again showed darkly stained perikarya against a lighter background and notably smaller cells were evident in deprived layers of the dLGN. Cell measurements revealed mean cross-sectional soma areas as

follows: C170: deprived = 194μm^2, non-deprived = 245μm^2 ; C171: deprived = 137μm^2, non-deprived = 194μm^2; C203: deprived = 152μm^2, non-deprived = 215μm^2; C206: deprived = 154μm^2, non-deprived = 222μm^2. Expressed in terms of the deprivation metric (1), cells in deprived layers were smaller than their non-deprived counterparts for C170, C171, C203 and C206 by 21%, 29%, 29% and 31%, respectively (Figure 6D-G, Table 2). Differences in the average soma sizes between deprived and non-deprived layers for all animals were compared statistically with an independent unpaired samples t test which revealed a significant difference between groups: $t(14)=-5.149$, $p<0.0002$.

Similarly, gross observations of dLGN sections immunolabeled for NF-H appeared to have a substantial loss of labeling in deprived layers. Quantification of NF-H positive neurons revealed densities for each animal as follows: C170: deprived = 83 neurons/mm^2 , non-deprived = 213 neurons/mm^2; C171: deprived = 123 neurons/mm^2 , non-deprived = 271 neurons/mm^2; C203: deprived = 84 neurons/mm^2, non-deprived = 206 neurons/mm^2; C206: deprived = 118 neurons/mm^2, non-deprived = 206 neurons/mm^2. Subsequent analysis in terms of the deprivation metric (1) revealed a loss of label in deprived layers as compared to non-deprived layers for C170, C171, C203 and C206 of respectively, 61%, 55%, 59% and 43% (Figure 7D-F; Table 3). Differences in the densities measured for all animals between deprived and non-deprived layers were statistically compared with an independent unpaired samples t test which revealed a significant difference between groups: $t(11)=-8.506$, $p< 0.000002$.

To compare the effect of the two recovery conditions on soma area and NF-H label, the raw data used to compute the deprivation indexes for each group of animals in

each recovery condition was statistically compared using an independent unpaired samples t test. For the recovery of soma area, no significant difference was revealed between the two recovery conditions: $t(10)=1.164$, $p < 0.271$ (Figure 6G). For the recovery of NF-H label, no significant difference was found between the two recovery conditions: $t(4)=-1.724$, $p<0.158$ (Figure 7G). Therefore, 10-day of darkness did not facilitate the recovery of NF-H label, or soma area when imposed following a period of monocular deprivation which extends into adulthood.

3.2 Inducing visual plasticity using darkness

3.2.1 Behavioral Investigation

Two cats from the same litter were reared normally until they were >1yr old, at which point they each received a 14-day period of monocular deprivation. For one cat (C187), the period of MD was immediately preceded by a 10-day period of darkness. Both animals possessed normal visual acuities binocularly and monocularly prior to experimental manipulation: C188 acuity = 6.49-7.1c/deg, C187 acuity = 7.1c/deg. As expected, upon termination of monocular deprivation, the acuity of C188's deprived eye was unaffected, remaining at between 6.49-7.1c/deg. For C187, the acuity of the deprived eye remained unchanged at 7.1c/deg following the period of MD so that it appeared that the preceding 10-day period of darkness was unable to reinstate ocular dominance plasticity (Figure 8; Table 4).

This result can be reinforced through detailed comparisons of each animal's behavioural reliability across trials before and after experimental manipulation. Given the

multiplicity of jumps required to pass each trial leading up to visual threshold, and the minute changes in spatial frequencies between trials, even a small change in the visual saliency of the stimuli would manifest themselves in discernable behavioural changes. As demonstrated by Figure 9, the performance of both C188 and C187 prior to and following the period of monocular deprivation were virtually identical.

3.2.2 Anatomical Investigation

Five cats from the same litter were used to investigate whether a 10-day period of darkness could enhance anatomical susceptibility to the effects of monocular deprivation on soma area and NF-H label density in the dLGN. Animals were raised normally until one year of age. Three animals (C194, C195, C197) experienced a 10-day period of darkness beginning at PD365, that was immediately followed by a 7-day period of monocular deprivation beginning at PD375. The two remaining animals (C193, C196) experienced only a 7-day period of monocular deprivation beginning at PD375.

Gross examination of Nissl stained sections for C193 and C196 revealed no apparent difference in cell size between deprived and non-deprived-eye recipient layers, an impression that was confirmed by formal measurements of cross-sectional soma area. The mean cell areas in the deprived and non-deprived eye recipient layers for each animal were as follows: C193: deprived = 231μm^2, non-deprived = 232μm^2 ; C196: deprived = 179μm^2, non-deprived = 181μm^2 . Subsequent analysis using the deprivation metric (1) revealed differences of cell size between deprived and non-deprived layers that were small and comparable to normal animals: 0% and 1% for C193 and C196, respectively (Table 5;

Figure 10A-C). Differences between the average soma areas in deprived and non-deprived layers for both animals were compared using an independent unpaired samples t test and revealed to be non-significant: $t(6)=-0.079$, $p<0.940$.

Similarly, no difference in NF-H label density between deprived and non-deprived layers was evident upon gross examination. Quantification of NF-H positive neurons revealed densities as follows: C193: deprived = 168 neurons/mm^2, non-deprived = 164 neurons/mm^2; C196: deprived = 231 neurons/mm^2, non-deprived = 240 neurons/mm^2. Subsequent analysis using the deprivation metric (1) revealed a difference in density between deprived and non-deprived layers that was comparable to normal animals: -2% and 0% for C193 and C196, respectively (Table 6; Figure 11A-C). Differences between the NF-H label density in deprived and non-deprived layers for both animals were compared using an independent unpaired samples t test and were revealed to be insignificant: $t(6)=-0.074$, $p<0.943$.

In the second condition, three animals experienced a 10-day period of darkness prior to the 7-day period of monocular deprivation. Gross examination of Nissl stained sections for C194, C195 and C196 revealed no apparent difference in cell size between deprived and non-deprived layers. Formal cross-sectional soma area measurements revealed mean soma areas for each animal as follows; C194: deprived = 190µm^2, non-deprived = 195µm^2 ; C195: deprived = 197µm^2, non-deprived = 200µm^2 ; C197: deprived = 220µm^2, non-deprived = 214µm^2. Subsequent analysis using the deprivation metric (1) revealed differences in cell size between deprived and non-deprived eye recipient layers for C194, C195 and C196 of 3%, 2% and -3% respectively (Table 5; Figure 10D-F).

Differences between the average soma areas in deprived and non-deprived layers for all animals was compared using an independent unpaired samples t test and was revealed to be non-significant: $t(10)=0.016$, $p<0.988$.

Similarly, gross observations of NF-H labeled sections for C194, C195 and C196 revealed no difference in labeling density. Quantification of NF-H positive neurons revealed densities as follows: C194: deprived = 188 neurons/mm^2, non-deprived = 181 neurons/mm^2; C195: deprived = 190 neurons/mm^2, non-deprived = 199 neurons/mm^2 ; C197: deprived = 169 neurons/mm^2, non-deprived = 171 neurons/mm^2. Subsequent analysis using the deprivation metric (1) revealed differences in labeling density between deprived and non-deprived eye recipient layers for C194, C195 and C197 of –4%, 5% and 1%, respectively (Table 6; Figure 11D-F). Differences between the NF-H label density in deprived and non-deprived layers for all animals was compared using an independent unpaired samples t test and was revealed to be non-significant: $t(7)=-0.42$, $p<0.968$.

To assess the effect on deprived-eye layer soma size and NF-H label density of 10-days of darkness preceeding a period of monocular deprivation, the raw data used to compute the deprivation indexes for each group of animals in each recovery condition was assessed statistically using an independent unpaired samples t test. For the effect on soma area, no significant difference was revealed between the two experimental conditions: $t(8)=-1.059$, $p<0.322$ (Figure 10G). For the effect on NF-H label, no significant difference was found between the two experimental conditions: $t(8)=-0.806$, $p<0.444$ (Figure 11G). Ten days of darkness imposed in adulthood has no ability to induce anatomical susceptibility to subsequent monocular deprivation as measured by soma area and NF-H label density.

CHAPTER 4: DISCUSSION

On the basis of reports that 10 days of darkness can induce plasticity in the central visual pathways and of vision in both 3-month old kittens (Duffy and Mitchell, 2013) and in adult rats (He et al., 2006; He et al, 2007; Montey and Quinlan, 2011) my study explored whether or not a similar period of complete darkness could re-introduce plasticity in adult cats. The major findings of the current study were very clear, and together point to the conclusion that darkness-induced plasticity is either completely absent or severely reduced in cats at ≥1 year of age. The lack of darkness-induced plasticity in adulthood was demonstrated through both behavioral examinations of the effects of darkness on the vision of amblyopic and normally-reared cats and through anatomical examinations of the effects of darkness on cell size and NF-H label density in deprived-eye recipient layers of the dLGN of long-term monocularly deprived and normally-reared cats. The findings from each portion of the study will be discussed in detail within sections 4.1 and 4.2.

4.1 – Dark-induced visual plasticity appears absent in adult cats.

In the first behavioral investigation, the effects of 10 days of darkness on the vision of amblyopic adult cats was examined, and in stark contrast to the profound improvement of the vision of the deprived eye of kittens as old as 13 weeks (Duffy and Mitchell, 2013), the acuity of the 3 adults studied was unaffected by the same period of darkness. This result indicates that a critical period for the effects of darkness must exist, and that it ends sometime between the ages of 13 weeks and 1 year of age. This result parallels the original observation of Hubel and Wiesel (1963) that demonstrated that monocular deprivation

could induce profound changes in the distribution of cortical ocular dominance when experienced early in life, but not when long periods of such deprivation were imposed in adulthood, which led them to conclude that cortical ocular dominance was susceptible to change in response to monocular deprivation only when imposed within a critical period.

In the second behavioral investigation, the ability of darkness to induce plasticity in adult cats was measured by comparison of the effects of 2 weeks of monocular deprivation on the visual acuity of the deprived eye of normal 1 year old cats with that of animals that had been placed in darkness for 10 days prior to the period of MD. In neither case did the period of MD influence the visual acuity of the deprived eye, indicating that darkness was unable to reinstate visual plasticity. That 10-days of darkness in adulthood appears unable to improve the acuity of amblyopic cats, or induce susceptibility to monocular deprivation in normal cats, provides evidence that darkness-induced plasticity is absent or greatly diminished in adulthood. A logical question to ask is whether or not 10-days of darkness is of sufficient length to induce plasticity in adulthood. While it cannot be ruled out that a longer period of time spent in darkness may provoke some limited plasticity in adulthood, the following argument based on consideration of the likely mechanisms governing the initiation of plasticity by darkness in juvenile cats suggests that this is unlikely to be so. When amblyopic juvenile kittens between 10 and 13 weeks of age experienced a 10-day period of darkness, the vision in their deprived-eye recovered to normal levels in approximately one week following reintroduction to the light (Duffy and Mitchell, 2013). That the functional recovery of vision occurs entirely in the light suggests that the complete removal of visually driven activity for 10 days is able to prime the visual system so that the deprived eye is once again able to form functional connections with

cells in the visual cortex upon reemergence into the light. That this reinstatement of ocular dominance plasticity occurs upon emergence into the light suggests one mechanism by which darkness may produce plasticity early in life is related to the relative changes in the levels of spontaneous retinally-driven activity versus visually driven activity. In turn, this change in activity could alter the expression patterns of the various molecular brakes on plasticity such that a period of heightened plasticity occurs. Interestingly, a theoretical model has been recently proposed that describes the initiation of critical period plasticity in similar terms (Toyoizumi et al., 2013). This model proposes that the switch from spontaneous, retinally-generated activity to visually driven activity after birth initiates thalamo-cortical plasticity through the selective inhibitory silencing of spontaneous activity, and that this plasticity can arise independently from those mechanisms that govern smaller-scale synaptic plasticity, namely, LTD and LTP (Toyoizumi et al., 2013).

Preliminary data (Mitchell, MacNeill, Holman & Duffy, in preparation) suggest that 5 days of darkness is insufficient to produce any change in the vision of the amblyopic eye of kittens, a result congruent with the observation that 5 days of darkness is insufficient to produce changes in the expression of NF-H label in the visual cortex (Duffy & Mitchell, 2013). That 5-days seems insufficient for manifestation of functional and anatomical changes resulting from darkness indicates that these changes require between 5 and 10 days of darkness. This time frame rules out the possibility that these changes occur as a direct result of the expression of immediate early genes, that typically manifest their protein products in a matter of hours (Curan and Morgan, 1995) and are instead more closely linked in time with the translated products of late response genes. Recently, in young and juvenile mice, an early-response transcription factor, Npas4, was shown to be

involved the induction of different transcriptional programs in inhibitory and excitatory neurons in response to changes of neuronal activity level (Spiegel et al., 2014). These induced transcriptional programs produce differential late-response protein products that promote the generation of new inhibitory synapses on excitatory neurons, and new excitatory synapses on inhibitory neurons (Spiegel et al., 2014). That the widespread activity-dependent induction of a transcription factor has been shown to induce cell-specific transcriptional programs, which then directly affect the homeostatic stability of the inhibitory/excitory cortical balance, highlights the potential role that age-dependent genetic regulation may have in the ineffectiveness of darkness to induce plasticity in adulthood. It may be the case that transcription factors are necessary for inducing the molecular cascades which lead to widespread homeostatic plasticity as a result of darkness experienced early in life, and that these transcription factors are incapable of being similarly activated by darkness experienced in adulthood.

Because the changes in visual function which accompany darkness imposed early in life are likely the result of threshold changes in network activity, which mediate changes in the expression profiles of an array of molecular species, it is likely that the ineffectiveness of darkness in adult cats is not due to the length of darkness experienced, but rather the inability of darkness to sufficiently relieve molecular brakes on plasticity in adulthood.

To understand why this happens, it will first be necessary to map the critical period for darkness-induced plasticity in its entirety. Previous results (Duffy and Mitchell, 2013) combined with the results presented in the current study reveal that the critical period for darkness-induced plasticity in cats ends at some point between 13 weeks and one year of

age. There are two possibilities for how the critical period for darkness could end: first, it could end gradually, resulting in effects of progressively less magnitude until finally the effects of darkness extinguish entirely, or second, it could end abruptly consistent with the lack of any decline in the robustness of the beneficial effects on vision of darkness imposed at either 71 or 93 days of age (Duffy and Mitchell, 2013. The former possibility would follow the known profile for ocular dominance plasticity in cats which ends gradually at between 6 and 8 months of age (Olson and Freeman, 1980; Cynader et al, 1980; Jones et al 1984; Daw et al, 1992), while the latter would be indicative of a particular molecular brake, or constellation of molecular brakes, simultaneously reaching a threshold which hinders the capacity of darkness to be effective. In either case, revealing the profile of the critical period of darkness will be essential for building a framework that can be used for the targeted search for molecular mechanism(s) and for outlining the period during which darkness exerts the most therapeutic benefit.

4.2 – Dark-induced anatomical plasticity appears absent in the dLGN of adult cats

The fact that 10 days of darkness imposed in adulthood was unable to alter either cell size or NF-H expression in deprived layers of the dLGN provides additional support for the suggested link between alterations in NF-H label and alterations in dLGN cell size (Kutcher and Duffy, 2007; Duffy and Slusar, 2009). The conditions examined in the anatomical portions of this study offer several instructive pieces of information regarding the lack of darkness-induced plasticity in adulthood. First, that NF-H labeling density remained stable under the influence of darkness in adulthood contrasts with results from young kittens (< 40 days old) that demonstrated significant reduction in NF-H label in

both the dLGN (Kutcher and Duffy, 2007; Duffy and Slusar, 2009; O'Leary et al., 2012) and in the visual cortex after 10 days of darkness (Duffy and Mitchell, 2013). This result suggests that the molecular mechanisms that mediate effects of darkness in kittens are unable to be similarly affected by darkness experienced in adulthood.

It is important to note that the alteration of NF-H by 10-days of darkness has thus far only been reported in young kittens (<40 days old), and correlates rather well with the severe reduction in the vision of the nondeprived eye of monocularly deprived kittens that is observed only when darkness is experienced during the peak of the critical period at about 5 weeks of age (Duffy and Mitchell, 2013). It remains unclear whether or not 10-days of darkness effects NF-H expression in the visual system when imposed on juvenile kittens (>40 days of age), and whether or not dark-induced changes to NF-H expression is linked to the subsequent fast recovery of the vision of the deprived-eye of monocularly deprived kittens in the delayed dark condition (Duffy and Mitchell, 2013). Elucidation of the possible role of changes in NF-H expression in mediating darkness-induced plasticity in juvenile life is further complicated by the fact that anatomical recovery of both cell size and NF-H label in the dLGN occurs on simply opening the eye of monocularly deprived animals (O'Leary et al., 2012). However, although the vision in the deprived eye improves somewhat under similar conditions, it never fully recovers. It is possible that the changes in vision that follow short early periods of monocular deprivation result from cortical imbalances in deprived-eye connections, rather than by a system-wide depression of functional circuitry as may follow longer periods of deprivation.

Because the anatomy of the dLGN recovers when monocular deprivation is relieved at 41 days of age (O'Leary et al., 2012), it could be that juvenile amblyopic cats

which demonstrate full recovery of deprived eye acuity when darkness is introduced after 8 weeks of binocular visual exposure possess an anatomically near- normal dLGN at the time darkness is introduced which may allow the latter to reinstate cortico-thalamic plasticity and fast recovery of deprived-eye vision. It remains to be seen whether the effects of darkness on the vision of amblyopic juvenile kittens are lessened with longer periods of monocular deprivation that extend beyond the time when substantial anatomical recovery from monocular deprivation occurs with binocular visual exposure. Examination of the response of NF-H expression to darkness in juvenile cats will provide a crucial test of the putative role of NF-H as a molecular brake, and will lend clarity to the potential interaction between the anatomical recovery of dLGN and the subsequent promotion of dark-induced fast visual recovery. Second, that darkness appears unable to promote the recovery of either NF-H label or cell size in the dLGN of long-term monocularly deprived adult cats suggests that expression of some or all molecular brakes are immune to darkness (dark-immune).

That the overall immunity to darkness in adulthood could be a consequence of immunity in extracellular or intracellular molecular brakes can be demonstrated by the following hypothetical argument. Because the recovery of cell size requires that cells expand in space, recovery necessarily requires that the extracellular scaffolding which surrounds adult neurons (PNNs) allow room for this growth. It may be the case that darkness is unable to promote plasticity in the extracellular framework, thereby constraining deprived-eye recipient cell size. If changes in NF-H expression are dependent on changes in cell size, this extracellular constraint on cell-size could subsequently suppress NF-H expression. Alternatively, darkness might be unable to initiate the

transcription of NF-H, due to the potential existence of transcription factors which may only be accessible early in life. However, it cannot be ruled out that the long-term monocularly deprived adult cats in this study experienced a reduction of NF-H expression in non-deprived eye recipient layers of the dLGN during darkness, followed by its recovery during the binocular recovery period, although behavioral results suggest this is unlikely to be the case. To rule out the possibility entirely, an additional control could be added where anatomical investigations were made immediately after 10 days of darkness.

Third, that darkness was unable to induce alterations to either cell size or NF-H expression resulting from a subsequent period of monocular deprivation in normal adults adds further support to the argument that dark-immune molecular brakes on plasticity exist in adulthood. In kittens, the loss of NF-H label expression occurs between 5 and 10 days of darkness (O'Leary et al., 2012; Duffy and Mitchell, 2013). It would be helpful to know the mechanism by which this loss of NF-H label occurs, though one candidate could be enzymatic activity. If enzymatic activity is unable to be initiated by darkness in adulthood, the cytoskeleton would remain stable and therefore likely resistent to changes in cell size as a result of monocular deprivation, given that cytoskeletal instability is correlated with changes in cell size in younger animals (Duffy et al., 2012). Alternatively, requisite enzymatic activity could be initiated by darkness in adulthood, but the increased phosphorylation of NF-H with age (Liu et al., 1994) could render NF-H immune to similar enzymatic degradation in adulthood, given that the phosphorylation of proteins can significantly strengthen the binding energy between constituent subunits (Nishi et al., 2011).

While the anatomical results of this study were unable to provide clarity on the independent consequences of NF-H expression and dLGN cell size to vision, the results remain strikingly clear that 10 days of darkness experienced in adulthood is unable to promote cytoskeletal plasticity in the dLGN.

4.3 – Ten days of darkness reinstates ocular dominance plasticity in adult rodents, but not in adult cats

That 10 days of darkness was unable to restore ocular dominance plasticity in adult cats contrasts with demonstrations of the ability of darkness to restore normal ocular dominance in adult monocularly deprived rats (He et al., 2006; He et al., 2007; Montey and Quinlan, 2011). These contrasting results highlight the existence of crucial differences between the animal models used and the parameters set by the two types of studies.

First, it is important to recognize that several differences in visual processing exist between rodents and cats. In rodents, ~90% of retinal afferents cross at the optic chiasm to innervate contralateral brain regions, whereas this number is reduced to ~50% in cats and humans. Rodents also lack the cortical ocular dominance columns that are characteristic of both cats and humans. Instead, ocular dominance varies at the level of each cortical neuron and these neurons are scattered throughout the cortex in a characteristic ‘salt-and-pepper’ type organization (Ohiki and Reid, 2007).

Second, the severity of the visual deficit resulting from monocular deprivation differs significantly in magnitude between cats and rodents. In kittens at PD30 a short (7d) period of monocular deprivation early in life results in a complete loss of form vision in the deprived eye and eventually a long-standing reduction of the acuity of the deprived eye

of 4 octaves (6.0c/deg – 0.3 c/deg; Duffy and Mitchell, 2013). By contrast, the typical reduction in VEP amplitude or behaviourally measured visual acuity in the deprived-eye of chronically-deprived rodents differs by only by 2 octaves (0.8 - 0.2c/deg; He et. al, 2007). Additionally, some reported acuity measurements from rodents have not been acquired through behavioural tests, but are instead extrapolated from VEP response amplitudes to grating stimuli (Montey and Quinlan, 2011). As the latter are thought to reflect activity in the granular layers of V1, changes in VEP amplitude may not be reflective of changes in conscious perception. Further, a large contralateral ocular dominance bias is present in cortical neurons of both hemispheres, which gives rise to minimal binocular interaction in the cortex. Thus, reported changes to rodent ocular dominance represent decreases in VEP amplitudes in the deprived hemisphere as compared to the non-deprived hemisphere. In the case of the dark-promoted recovery from long-term monocular deprivation and the reintroduction of susceptibility to short periods of monocular deprivation in adult rodents, changes in ocular dominance have been attributed to LTP and / or LTD at the level of individual synapses (Montey and Quinlan, 2011). However, dark-induced promotion of LTP and LTD are unlikely to be sufficient to promote ocular dominance plasticity in adult cats.

Third, rodents retain some susceptibility to monocular deprivation in adulthood, where periods of monocular deprivation lasting >5 days can induce significant shifts in ocular dominance towards the non-deprived eye (Lehmann and Lowel, 2008). The induction of juvenile-like plasticity in adult rats by darkness resembles the significant shifts in ocular dominance that occur after only 4 days of monocular deprivation in juvenile animals. In contrast, even a period of deprivation lasting as long as 5 years

appears unable to induce a shift in ocular dominance in cats (Hubel and Wiesel, 1970). It may be the case that the residual plasticity in adult rodent visual cortex is facilitated, in part, by the lack of ocular dominance segmentation into cortical columns, thereby permitting a heightened frequency of ocular dominance interactions between neurons, whereas in cats interactions of this kind can only occur at the borders of ocular dominance columns.

That darkness appears unable to reinstate plasticity in adult cats, but is capable of reinstating plasticity in adult rodents is not the first report of a therapeutic intervention proven effective in rodents and unable to be translated with similar benefit to cats. For example, the digestion of CSPGs by local application of enzymes was reported to reinstate ocular dominance in adult rodents (Pizzorusso et al., 2002), yet similar application in cats was only able, at best, to produce only slight changes in ocular dominance plasticity (Vorobyov et al., 2013).

Section 4.4 – Alternative attempts to reinstate plasticity in adult visual cortex

There have been several past attempts to re-instate ocular dominance plasticity in normal adult cats, as reflected by a restoration of susceptibilty of cortical ocular dominance to monocular deprivation. These include the electrical stimulation of the locus coeruleus (Kasamatsu et al, 1985), oral administration of a noradrenaline pre-cursor (Mataga et al., 1992), surgical transplantation of immature astrocytes directly onto the visual cortex (Muller and Best, 1989), and cortical infusion of NGF (Gu et al., 1994; Galuske et al., 2000). All of the aforementioned manipulations induced a modest susceptibility to monocular deprivation in adulthood which could be detected

electrophysiologically, although in no case did the changes to ocular dominance reflect the profound changes caused by monocular deprivation experienced early in life. However, that a variety of pharmacological treatments have proven effective in producing some degree of susceptibility to monocular deprivation in normal adult cats suggests that attempts to reinstate ocular dominance plasticity in adulthood is not futile. It is important to bear in mind the practical application of the results from those studies which investigate pharmaceutically-induced plasticity in adulthood.

These studies are instructive in revealing some of the various molecular brakes which constrain plasticity in adulthood, although in and of themselves are not directly translatable to an effective clinical therapy for human amblyopes. For all cases examined previously in cats, the ability of various molecular species to induce plasticity in adulthood have been examined with respect to the ability to promote susceptibility to monocular deprivation. However, it is posible that those molecular mechanisms may not be involved in mediating recovery from monocular deprivation in adulthood. For example, critical period plasticity in mice has been shown to be governed through circuit-wide homeostatic synaptic scaling mechanisms (Mrsic-Flogel et al., 2007; Kaneko et al., 2008; Ranson et al., 2012), which shape the capacity of neurons to express stable functions within their network, i.e. firing rates (Chen et al., 2008). Homeostatic synaptic scaling mechanisms are downregulated with age (Huupponen et al., 2007) and recent work in mice has demonstrated that adult plasticity is dependent on a mechanism that promotes the specific autophosphorylation of αCaMKII, independent of those mechanisms governing homeostatic synaptic scaling and critical period plasticity (Ranson et al., 2012). That different mechanisms can govern plasticity during the critical period and in adulthood, is

congruent with the results from adult cats where any induction of plasticity in adulthood seems to rely on changes in the strength of local synapses, but not a larger-scale reorganization of network connectivity that would be consistent with the profound deficit that results from deprivation imposed during the critical period. Together, these reports suggest that the promotion of both homeostatic and synaptic plasticity mechanisms may be required to facilitate the full recovery from amblyopia in adulthood.

While reports on pharmacological approaches can be instructive with respect to their ability in their capacity to shed light on molecular mechanisms of plasticity, their contribution to the development of clinical therapies for amblyopia is somewhat more complicated. First, the local infusion of any pharmacological agent touted to provide relief from the molecular constraints of plasticity is likely too invasive to be considered a viable treatment for human amblyopia. Second, an oral administration of such agents inherently lacks the specificity required to target only visual plasticity; the risks of reintroducing system-wide neural plasticity far outweigh the benefits of a potential improvement to vision. Third, that a constellation of molecules, and multiple mechanisms, appear to be involved in regulating the capacity for adult plasticity, suggests that it will be necessary to develop therapies capable of simultaneously affecting various plasticity mechanisms in order to promote full recovery from amblyopia in adulthood. Finally, approaches which target the reinstatement of ocular dominance plasticity only in primary visual cortex, neglect the contribution that higher order visual areas have on perception. There have been a few reports (Kiorpes et al., 1998; Barnes et al., 2001; Mitchell and Lomber, 2013) that indicate that amblyogenic rearing exerts large effects beyond the primary visual cortex

suggesting that therapies capable of promoting plasticity in the entire visual system will be necessary to promote the full recovery from amblyopia in adulthood.

Recently, the targeted genetic manipulation of paired immunoglobulin-like receptor B (PirB) proved capable of reinstating ocular dominance plasticity in adult mice (Bochner et al., 2014). PirB has been demonstrated through targeted cre-mediated genetic deletion to be an upstream regulator of multiple constrictions on plasticity in adulthood (Bochner et al., 2014). That a post-natal genetic therapy has proven effective in reinstating plasticity to specific cortical areas in adult mice (Bochner et al., 2014) suggests a potentially viable therapeutic method for the relief of a similarly acting upstream dark-immune molecular brake on plasticity. Currently, there are a significant number of clinical trials being conducted around the world which aim to test the treatment of various human disorders including amblyopia by targeted gene therapy or pharmacological interventions (for reviews, see Ginn et al. 2013; Sengpiel, 2014), implying that a genetic approach to reinstating cortical plasticity in human adults may be feasible.

4.5 – Darkness as an adjunct therapy for the treatment of amblyopia in adulthood

In combination with the capacity of darkness to simultaneously affect a constellation of molecules (Castren et al., 1992; Chen et al., 2000; Lein and Shatz, 2000; Fagiolini et al., 2003; Chattopadhyaya et al. 2004; O'Leary et al., 2012; Ye and Miao, 2013) and promote widespread visual plasticity in kittens and juvenile cats (Duffy and Mitchell, 2013), its relative non-invasiveness makes it a prime candidate for potential use in human therapies. Future work that identifies the molecular brakes which render the adult visual system immune to the effects of 10-days of darkness will be instructive for

developing viable genetic therapies which could unlock the potential for darkness to be effective in adulthood.

A number of rodent studies have now demonstrated that environmental enrichness can enhance cortical ocular dominance plasticity in adult and aged rodents, promoting the full recovery from amblyopia in adulthood through the modulation of GABAergic inhibition, BDNF expression and extracellular matrix stability (Baroncelli et al., 2010; Mainardi et al., 2010; Togini et al., 2012; Scali et al., 2012; Baroncelli et al., 2013) and caloric restriction (Spolidoro et al., 2011). One component commonly used for providing environmental enrichment to rodents is the addition of an exercise wheel to their home cage. Recently, it has been demonstrated that increased locomotion in combination with visual stimuli has been shown to enhance the recovery from amblyopia in adult mice (Kaneko and Stryker, 2014). Interestingly, this recovery of visual function can be measured only for those stimuli experienced during locomotion, leading researchers to hypothesize that an increase in the gain of specific activity in the visual cortex is responsible for the subsequent enhancement of function (Kaneko and Stryker, 2014).

While the experience of environmental enrichness and locomotion alone is not likely to promote substantial recovery from amblyopia in humans, it bears consideration that studies of amblyopic patients who play video games as part of their therapeutic regimine show a markedly increased level of improvement compared to those patients who undergo patching alone (Li et al., 2011). Video game play is implicated in perceptual learning (Green and Bavelier, 2012), and perceptual learning is potentiated by heightened states of arousal (Lee et al., 2012). In one study, adult amblyopes who played 2hr/day of video games with their amblyopic eye demonstrated an improvement in visual acuity of

~30% over the course of 20 days (Li et al., 2011). This degree of recovery is reported to be 5-fold faster than what has been observed in children as a result of patching therapies, and in addition to the marked improvements in visual acuity, video game play promotes improvement in positional acuity, spatial attention and stereopsis (Li et al., 2011). Additional studies have since described the ease of tracking patient compliance to prescribed video game therapies and highlighted the superiority these therapies demonstrate over conventional patching in their capacity to promote significantly increased levels of recovery in adulthood (Hess et al., 2014). While video game play has demonstrated its effectiveness in promoting the improvement of a wide-range of visual functions in human adult amblyopes, their recovery nonetheless remains incomplete. A preceeding period of darkness has the potential to potentiate the positive effects of video game play in human amblyopes to allow for a greater recovery of vision.

Additionally, given that reverse occlusion has been used to potentiate the effects of darkness on promoting the recovery of vision in rodents (Montey and Quinlan, 2011) and that patching has been used in combination with video game therapies (Li et al., 2011; Hess et al., 2014), it cannot be ruled out that the additional use of reverse occlusion following darkness experienced in adulthood could have promoted some recovery of vision. Future research that identifies the most effective visual experiences for promoting recovery from amblyopia will be required in combination with research that identifies the molecular species cruically involved with regulating the interaction of plasticity mechanisms with changes of network activity level across development in order to elucidate the most effective approach for the treatment of amblyopia in adulthood.

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APPENDIX A Figures

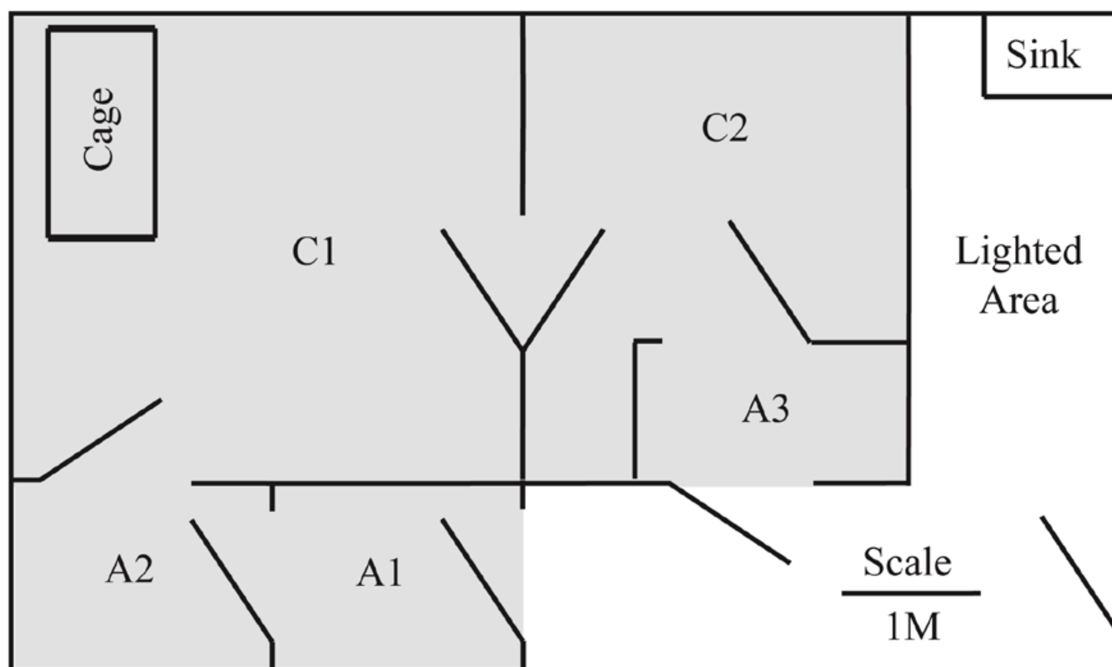

Figure 1. Dark-room facility depicted to scale

Figure 1. Darkroom Facility depicted to scale.

The darkroom was constructed within a larger room to allow for a lighted area as well as a larger light-tight area that is depicted with gray shading. The construct of the darkroom enables animals to be tended to and cages to be cleaned/changed in total darkness. Animals are placed in large caged playpens in the main darkroom (C1), accessible via two light-tight anterooms (A1, A2). A second darkroom (C2), adjacent to the first, could either be entered from the lit area throuh the anteroom A3 or through a pair of light-tight doors from C1. For daily cleaning, animals are placed in carriers and moved into the secondary darkroom (C2), separated from C1 by the two light-tight doors. Cages could be moved for cleaning from C1 to C2 and to the lit area through the anteroom, A3. C1 is illuminated during cleaning, then the lights are turned off, all anteroom doors are closed, and animals returned to their caged playpens in C1.

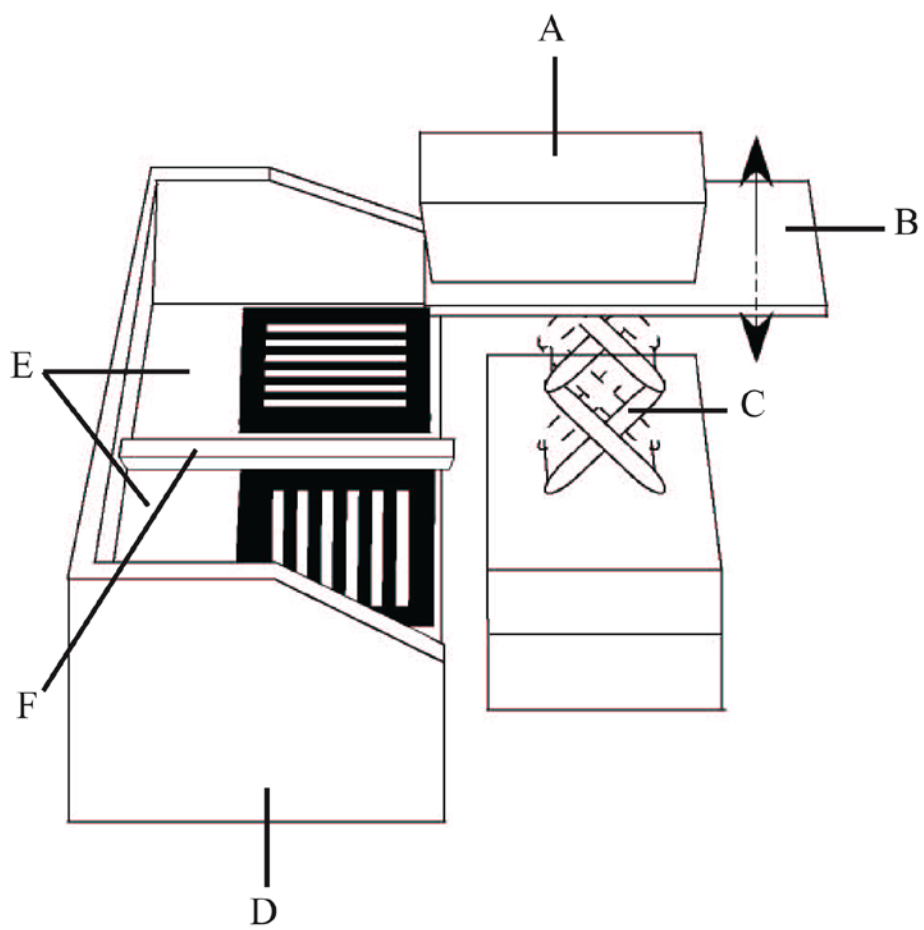

Figure 2. Jumping Stand

Figure 2. Jumping stand used for behavioral measurement of acuity.

The jumping stand consists of an open-ended rectangular starting wooden box (A) attached to a wooden platform (B) that is supported by two yoked laboratory jacks (C) which enable continuous changes in height ranging from flush with the visual stimulus to a maximum height of 72 cm. The stimuli are placed on top of a rectangular box (D) covered by two hinged doors (E). Stimuli are placed on the hinged doors and are separated by a wooden divider (F). Early in training, the starting box is lowered flush with the stimulus, and kittens are presented with the choice of either a vertical grating or an open door that presents a 40 cm drop to the floor. Once kittens gain confidence stepping onto the vertical grating, the open door is closed and a horizontal grating is placed on its lid. The starting box is gradually raised over the course of training in accordance with the development of the kitten's motor skills to the maximum testing height of 72 cm above the stimulus.

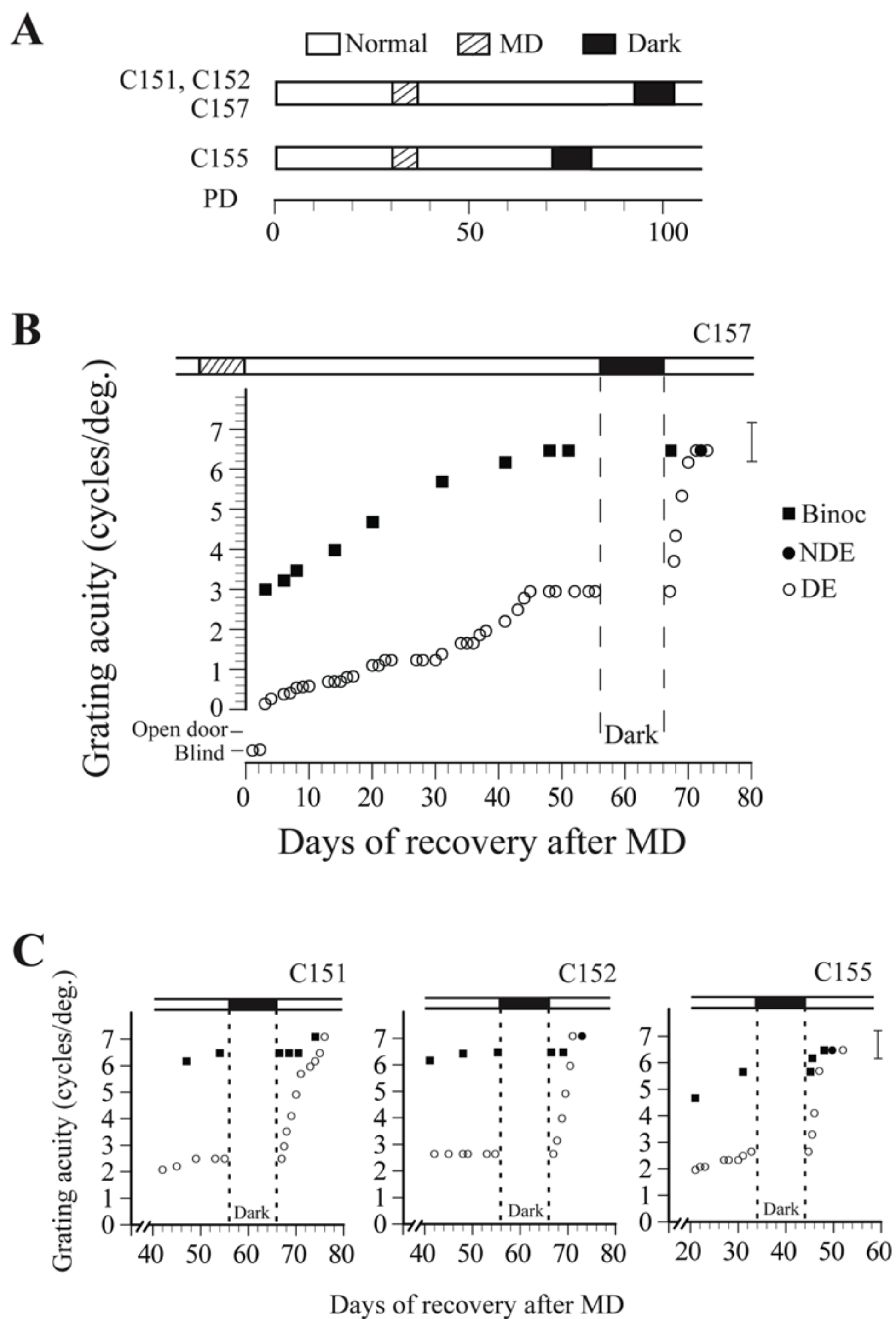

Figure 3. Effects of 10-days of darkness on the vision of juvenile amblyopic kittens (Duffy and Mitchell, 2013)

Figure 3. Effects of a 10-day period of darkness on the vision of the two eyes of four juvenile amblyopic kittens. Data redrawn from the study of Duffy and Mitchell (2013).

Results from this paper are used to highlight the rapid improvement of the deprived-eye acuity in 4 juvenile amblyopic kittens following a 10-day period of total darkness. A schematic of each kitten’s rearing history is presented in (A), detailing the periods of normal vision, monocular deprivation and dark-rearing as a function of post-natal days of age (PD). The results of longitudinal measurements of visual acuity from one animal (C157) shown in (B) demontrate the development of binocular visual acuity (Binoc, black squares) that reflect the acuity of the non-deprived eye and deprived eye visual acuity (DE, open circles) prior to the period of darkness. Immediately following the period of darkness, binocular and monocular acuity measurements were identical to those measured prior to dark exposure, but in the days that followed, the acuity of the deprived-eye improved rapidly to match that of the non-deprived eye (NDE) in the normal range (depicted by the bracket). Panel (C) provides comparison data from 3 other animals in the study, which followed the same pattern exhibited by the animal detailed in (B).

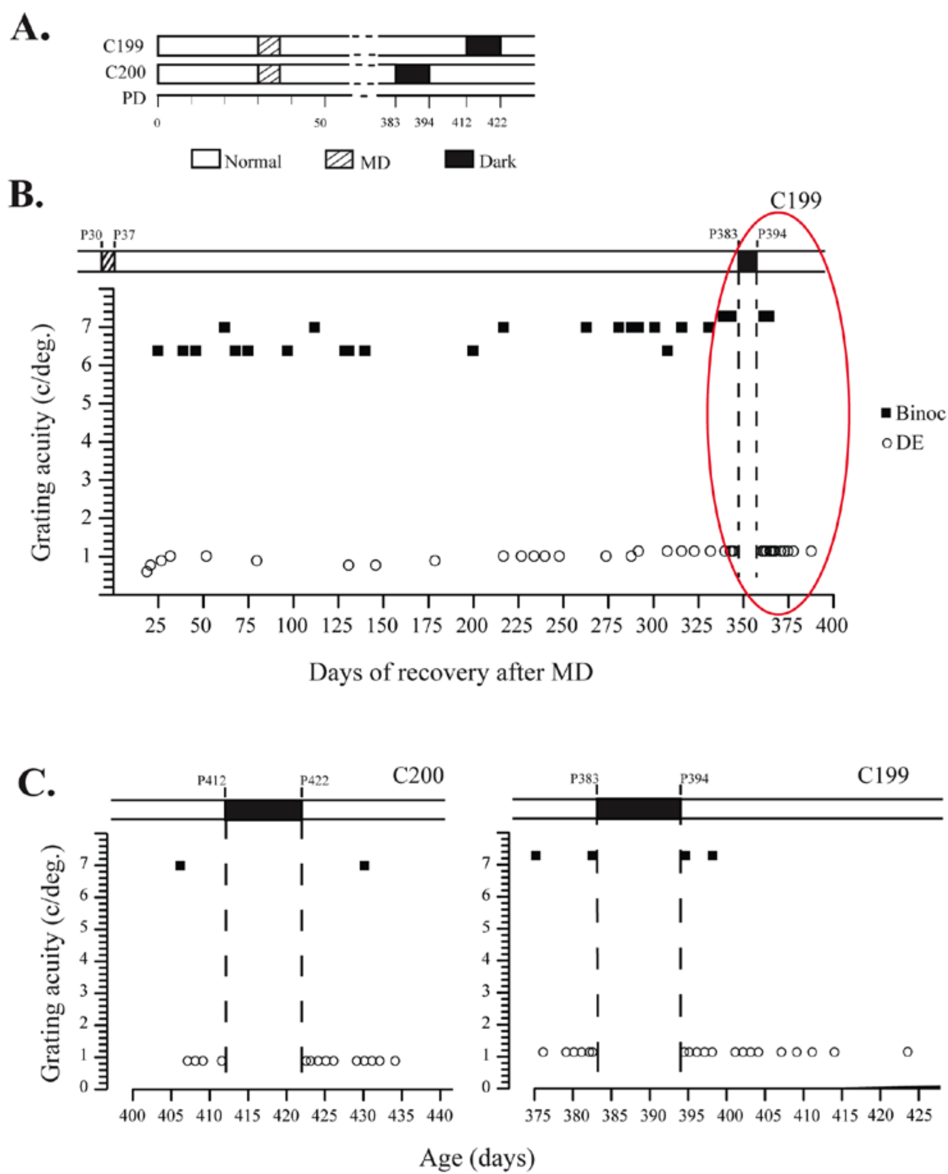

Figure 4. Effects of a 10-day period of darkness on the vision of adult amblyopic cats.

Figure 4. Effects of a 10-day period of darkness on the vision of two amlyopic adult cats.

Rearing histories for the two cats used in this study are depicted schematically in panel A. Periods of normal vision, monocular deprivation and dark-exposure are shown as a function of post-natal days of age. (B) An example of the complete set of binocular (black squares) and deprived eye (open circles) acuity measurements made for one animal (C199) as a function of time from the end of the early period of monocular deprivation. Because behavioural training of this animal was not completed in the initial two weeks of recovery, the immediate effect of the deprivation on the vision of the deprived eye was not documented. However, it is readily apparent that the vision of the deprived eye was severely reduced and remained unchanged for a year. It was also apparent that binocular and monocular acuity remained wholly unchanged by the 10-day period of darkness experienced in adulthood. The period of time encompassed by the ellipse that surrounds the period of darkness is reproduced in (C), which also displays the data for another cat (C200) in this study. C200. The data shown in (C) illustrates the lack of any impovement in the visual acuity of the deprived eye following the period of darkness.

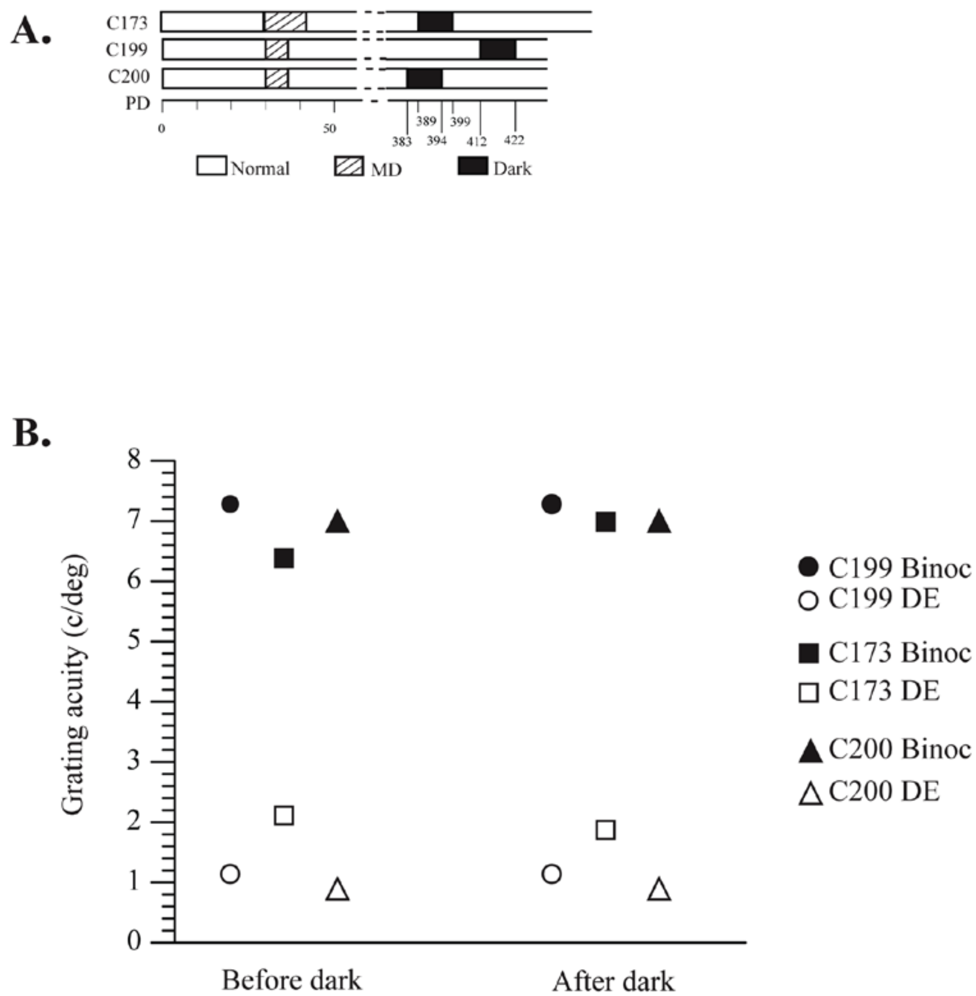

Figure 5. Acuity measures for amblyopic adults before and after a 10-day period of darkness was imposed in adulthood.

Figure 5. Binocular and monocular visual acuities of three amblyopic adult cats before and after a 10-day period of darkness imposed in adulthood.

A schematic reperesentation of the rearing histories of the 3 cats used in this portion of the study is provided in (A). Periods of normal vision, monocular deprivation and dark-exposure are depicted as a function of post-natal days of age. The stable binocular (filled symbols) and monocular (open symbols) grating acuities of each animal are shown before and after a 10-day period of darkness experienced in adulthood (B). The acuity measured after darkness was considered stable if it remained unchanged for a minimum of one week following the period of darkness.

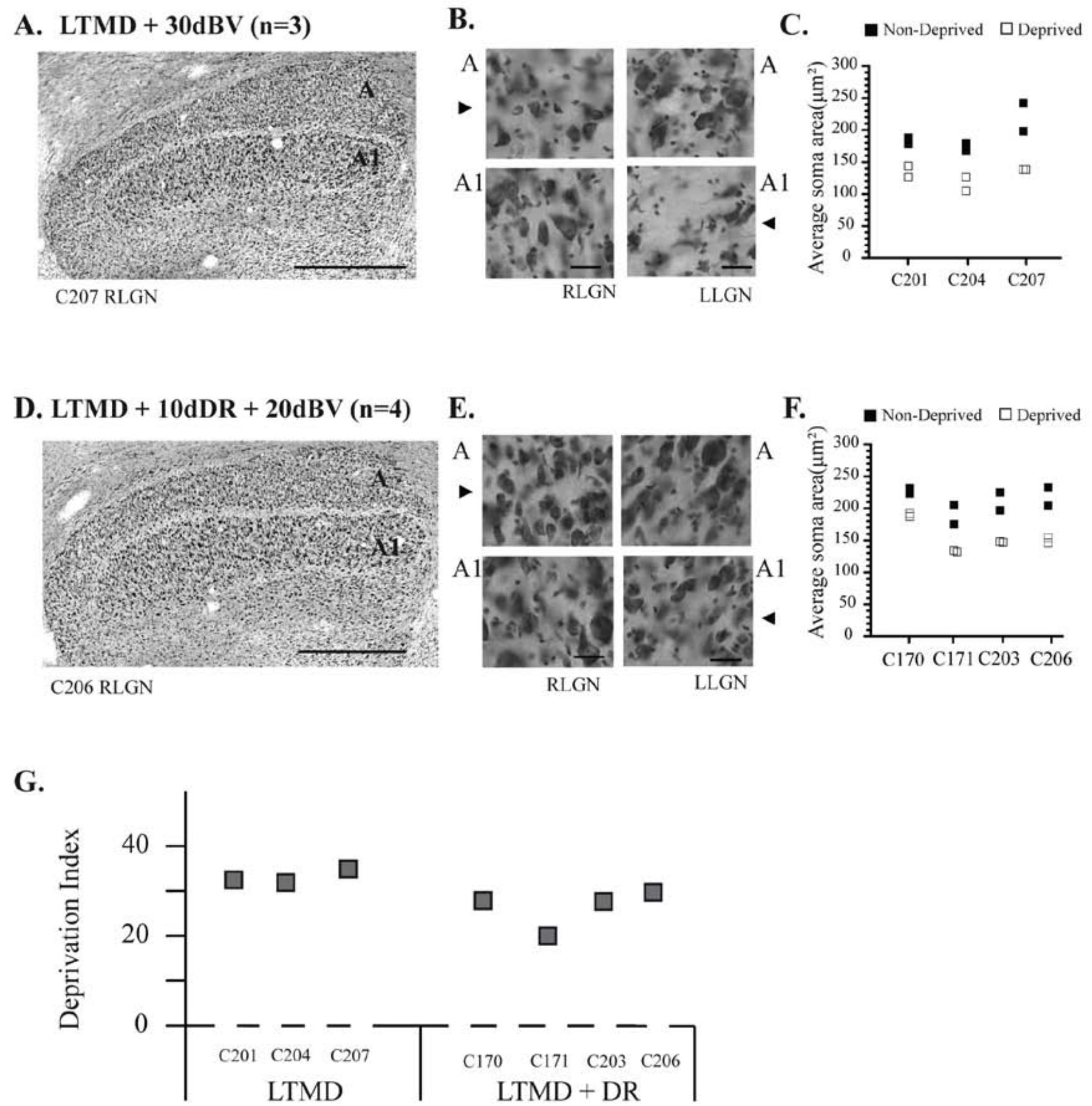

Figure 6. Recovery of cell size in deprived-eye recipient layers of the dLGN of long-term monocularly deprived cats.

Figure 6. Recovery of cell size in deprived-eye recipient layers of the dLGN of 7 long-term monocularly deprived cats.

Seven cats were reared with a long-term monocular deprivation that began on PD30 and lasted until PD365. Following the period of monocular deprivation, animals were assigned to one of two 30-day binocular recovery periods that consisted of either a 30-day period of binocular vision only (30dBV; n=3), or a 10-day period of dark-rearing imposed immediately after the period of monocular deprivation and was then followed by 20 days of binocular vision (10dDR + 20dBV; n=4). Stereological measurements of soma area were made in each dLGN layer for each animal using 50μm Nissl stained sections. Photomicrographs of the left and right dLGN from one animal (C207) from the binocular recovery condition are provided in panel A and B. (A) depicts the entire right dLGN of C207 photographed at 10X magnification. For this dLGN, layer A is deprived, and A1 is non-deprived. Smaller cell bodies of A are visible with the naked eye. (B) Higher power images (60X) of cells in each layer of the right and left dLGN of C207. Arrows point to the deprived layers. Smaller cell bodies are evident in the deprived layers as compared to their non-deprived counterparts. (C) Average soma areas in each layer of the dLGN for each of the three animals in the 30dBV recovery condition. Representative photomicrographs from the dLGN of animals from the second recovery condition (10-day dark exposure + 20-day binocular vision) are provided in panel (D) and (E). (D) A low-power (10X) photomicrograph of the right dLGN of C206. Smaller cell bodies are visible in the deprived A layer as compared to those in the non-deprived A1 layer. (E) High-power (60X) microphotographs of cell bodies in each dLGN of C206. Deprived layers are

marked with an arrow, and smaller cell bodies are evident in deprived layers compared to those in non-deprived layers. (F) Average soma areas in each layer are plotted for each of the 4 cats in the second recovery condition. (G) Comparison of the average soma areas in the deprived and non-deprived dLGN layers in terms of the deprivation metric (1), which reveals the percent difference between the average soma areas from the deprived and non-deprived layers for each animal. The deprivation index for each animal in both recovery conditions is plotted, demonstrating no apparent difference in deprived soma size between groups. Scale bars (A) and (D) = 1mm; (B) and (E) = 100μm

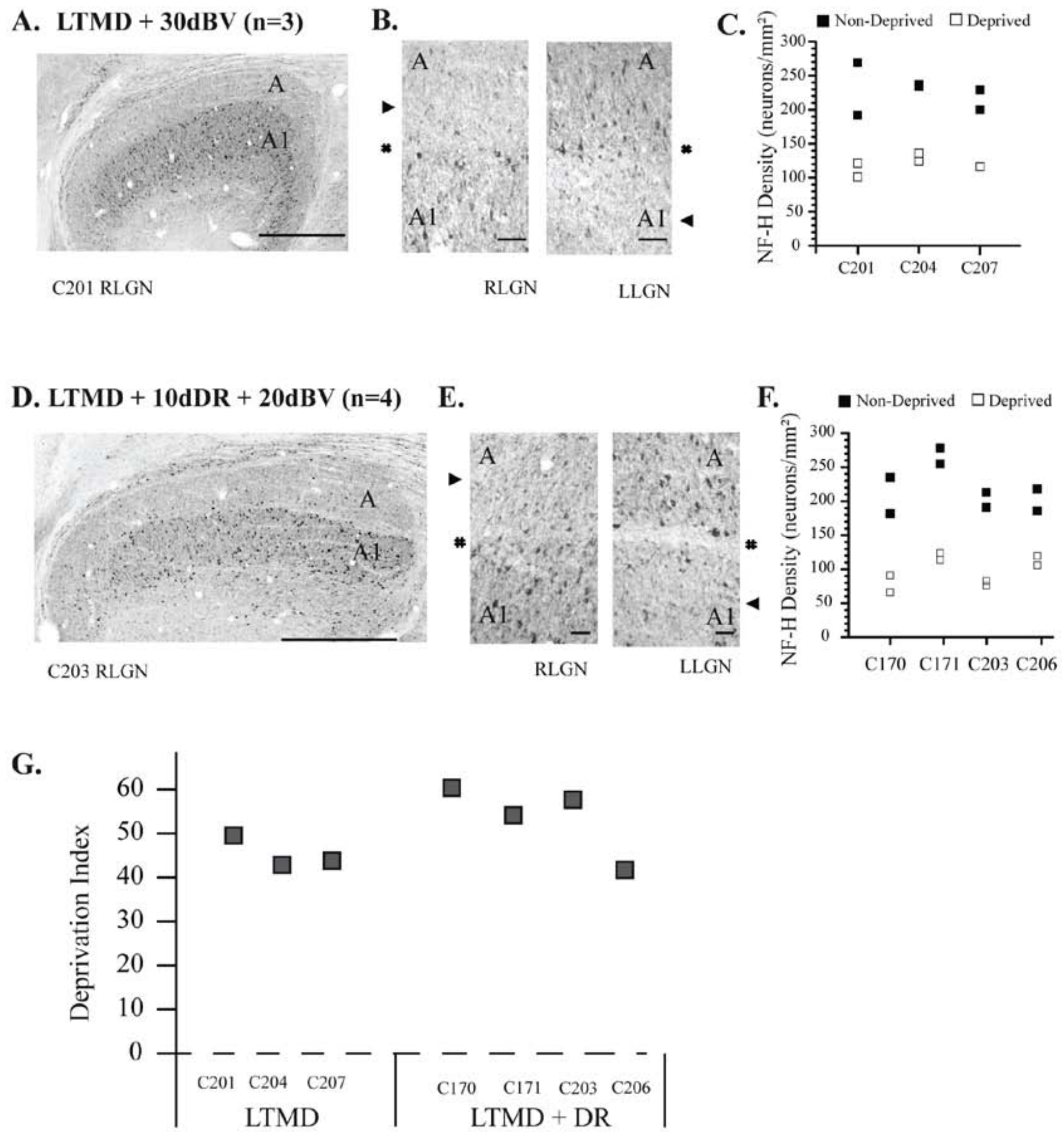

Figure 7. Recovery of NF-H label in deprived-eye recipient layers of the dLGN of long-term monocularly deprived cats.

Figure 7. Recovery of NF-H label in deprived-eye recipient layers of the dLGN of long-term monocularly deprived cats.

Seven cats were reared with a long-term monocular deprivation beginning on PD30 and lasting until PD365 after which they were assigned to either of two 30-day binocular recovery periods. The recovery conditions consisted of either a 30-day period of binocular vision (30dBV; n=3), or a 10-day period of dark-rearing imposed immediately following relief of monocular deprivation followed by a 20 day period of binocular vision (10dDR + 20dBV; n=4). Sterological measurements of NF-H label density were made in each dLGN layer for all animals using 50μm sections immunolabeled for NF-H. Densities are reported as NF-H positive neurons/mm^2. Examples of tissue from one animal (C201) from the 30dBV recovery condition are provided in panel A and B. (A) shows a low –power (10X) microphotograph of the entire right dLGN of C201. For this LGN, layer A is deprived as reflected by a notable lack of NF-H label as compared to the much higher density of label evident in the non-deprived layer, A1. (B) 10X images of the right and left dLGN of C201. Arrows point to deprived layers and the asterisks denote the laminar border between A and A1. Considerably less NF-H label is visible in the deprived layers. (C) Density of NF-H label in both of the A laminae of the dLGN for each of the three animals in the 30dBV recovery condition. Comparable photomicrographs from an animal (C203) from the second recovery condition (10dDR + 20dBV) are provided in panels (D) and (E). (D) A low-power (10X) microphotograph of the right dLGN of C203. The lack of NF-H label in the deprived layer A is readily apparent. (E) Photomicrographs (10X) of each dLGN of C203. Deprived layers are marked with an arrow, and the asterisks denotes the laminar border between layer A and Al. (F) Density of NF-H label in each layer is plotted for the 4

cats in the second recovery condition. (G) Comparison of the NF-H density in the deprived and non-deprived dLGN layers for each animal in terms of the deprivation metric (1) expressed as the percentage difference between NF-H label density in the deprived and non-deprived layers of each animal. The deprivation index for each animal in both recovery conditions is plotted and indicates no apparent difference in relative NF-H label density between groups. Scale bars (A) and (D) = 1mm; (B) and (E) = 100μm.

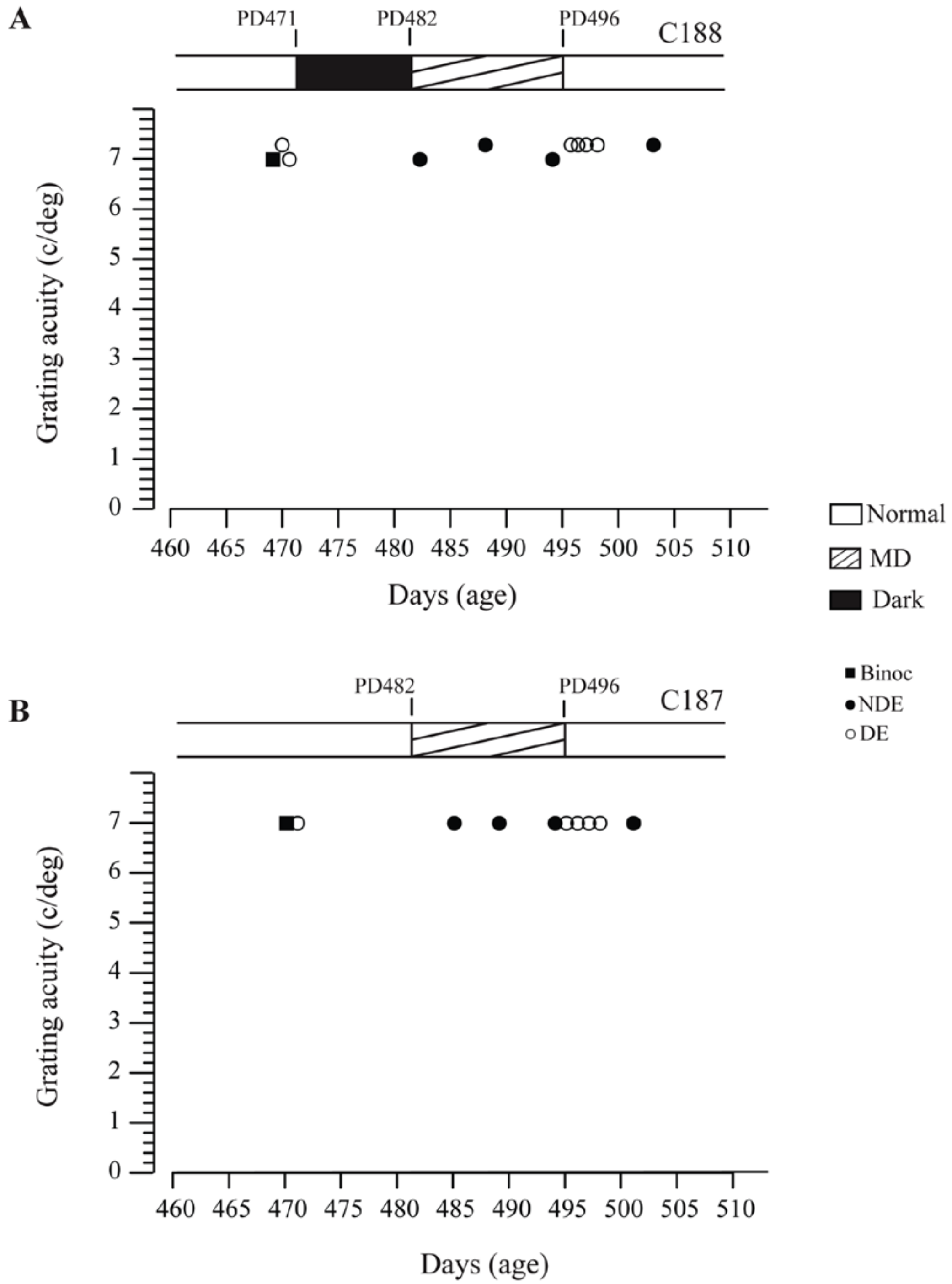

Figure 8. Effects on vision of a 10-day period of darkness imposed before a period of monocular deprivation in adult cats.

Figure 8. Effects on vision of a 10-day period of darkness imposed before a period of monocular deprivation in adult cats.

Binocular and monocular grating acuity measurements for 2 adult cats expressed as a function of days of age. Cats were reared normally until >1yr of age. Both animals experienced a 2-week period of monocular deprivation lasting from post-natal day (PD) 482 to PD496. (A) One animal (C188) experienced a 10-day period of dark exposure prior to the period of monocular deprivation. No change in deprived eye acuity followed the period of monocular deprivation. (B) Acuity measurements from the control animal (C187) that received only the period of monocular deprivation. No change in deprived-eye acuity was noted after the period of monocular deprivation.

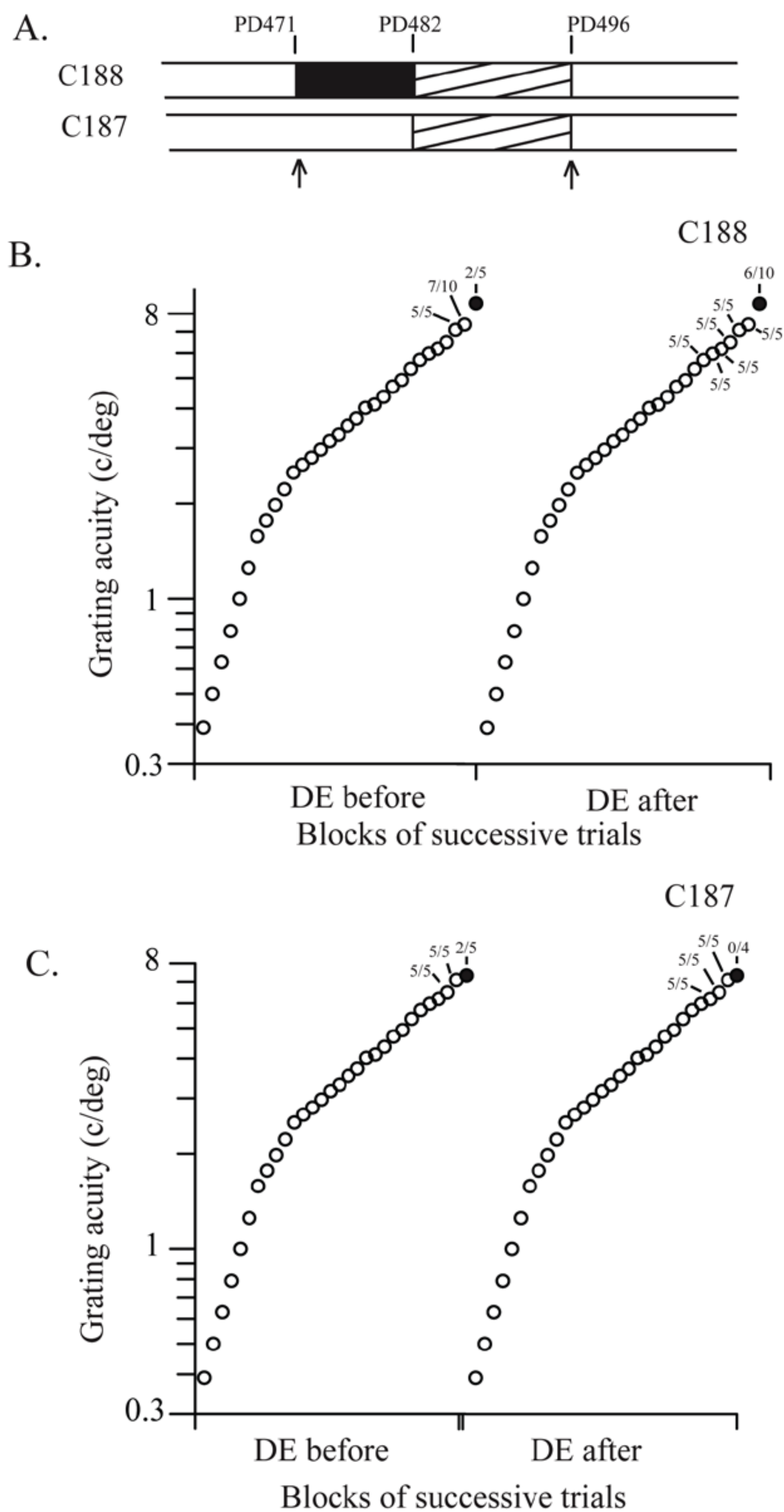

Figure 9. Behavioral performance of C188 and C189 before and after monocular deprivation experienced in adulthood.

Figure 9. Behavioural performance of C188 and C199 before and after monocular deprivation experienced in adulthood.

Performance on successive blocks of trials with gratings of progressively higher spatial freqiuency. Open symbols represent blocks of trials for which criterion performance was achieved and closed symbols represent the first trial block on which the animal failed. Fractions adjacent to the last few blocks of trials indicate the fraction of correct jumps in the given trial block. Grating acuity is expressed on a logarithmic scale to demonstrate that the steps between trials was approximately equal and that above 1 cycle/degree the steps between trials were much smaller. (A) A schematic representation of the rearing history for C188 and C187. Arrows indicate the points in time when the acuity measurements shown below were made. (B) Performance of C188 after monocular deprivation was indiscernable from that immediately before. Deprived eye acuity before = 6.49c/deg; acuity after = 7.1 c.deg. (C) Performance of C187 after monocular deprivation was indiscernable from the peformance before. Acuity before and after monocular deprivation was 7.1c/deg.

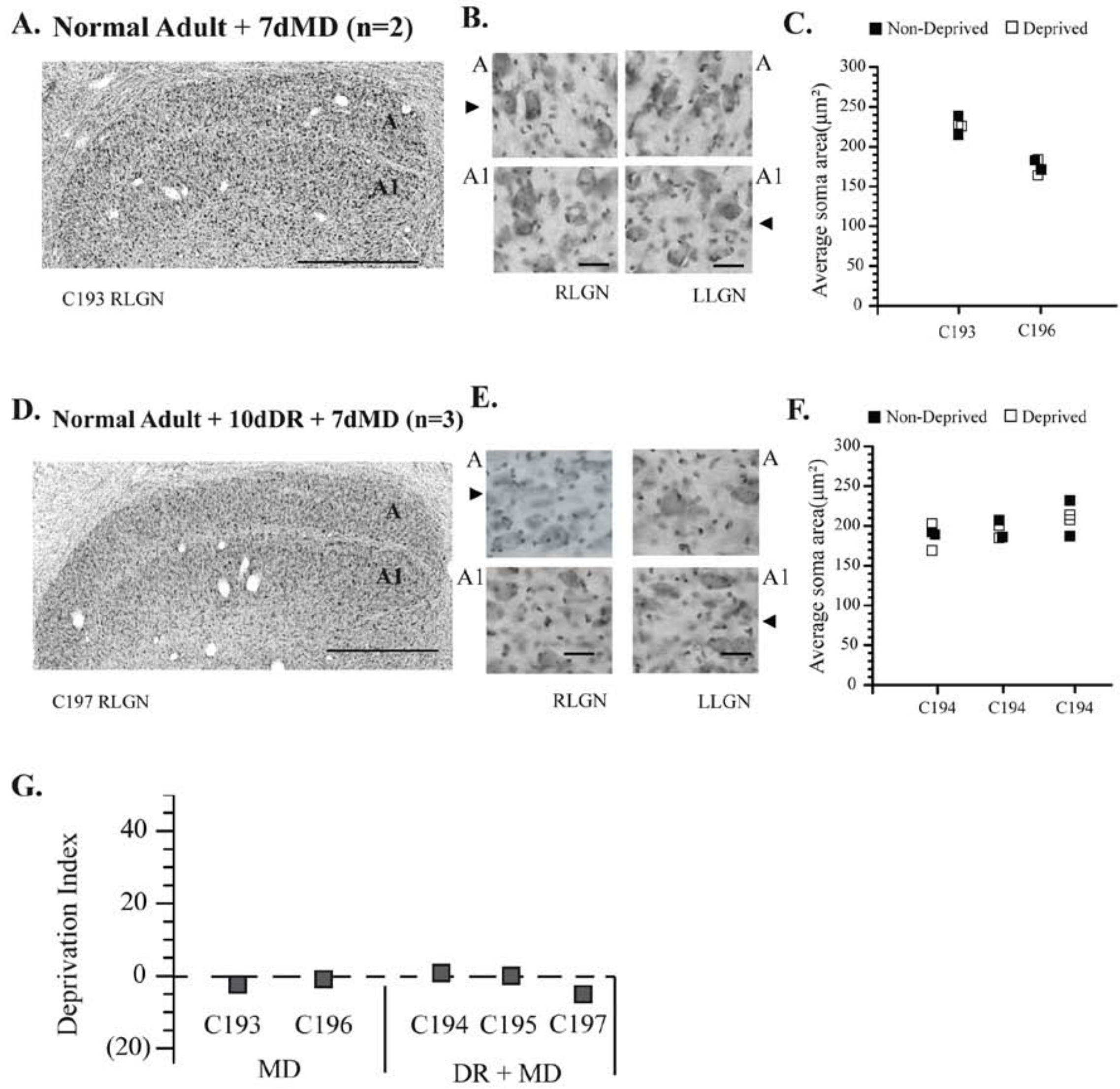

Figure 10. Effect on dLGN cell size of monocular deprivation imposed in adult cats.

Figure 10. Effect on dLGN cell size of monocular deprivation imposed in adult cats.

Five cats were reared normally until either PD365 or PD375. At PD365, 3 cats experienced a 10-day period of darkness followed by 7-days of monocular deprivation, while 2 cats experienced only the 7-day period of monocular deprivation beginning at PD375. Effects on cell size in deprived-eye recipient layers of the dLGN was assessed sterologically by measuring soma areas in each dLGN layer for each animal using 50µm Nissl stained sections. Digital photomicrographs of the dLGN from one animal (C193) that experienced only a 7-day period of monocular deprivation are provided in panels A and B. (A) depicts the entire right dLGN of C193 photographed at 10X magnification. For this LGN, layer A is deprived, and A1 is non-deprived. The pattern of soma size and staining density in the two layers was similar to that observed in normal cats. (B) Higher power (60X) images of the right and left dLGN of C193. Arrows point to deprived layers. No differences in the soma areas between deprived layers and non-deprived layers is visible. (C) Average soma area in each layer of the dLGN for each of the 2 animals in the 7dMD condition. Equivalent photomicrographs of the dLGN of a representative animal (C197) from the second condition (10dDR + 7dMD) are provided in panel (D) and (E). (D) A low-power (10X) photomicrograph of the right dLGN of C197. The pattern of soma size and staining density in the two layers was similar to that observed in normal cats. (E) Higher power (60X) photomicrograph of each layer of the two dLGNs of C197. Deprived layers are marked with an arrow. No difference in the soma areas between deprived and non-deprived layers was evident. (F) Average soma areas in each dLGN layer is plotted for each of the 3 cats in the second condition. (G) Comparison between the average soma areas in deprived and non-deprived dLGN layers for each animal displayed in terms of the

deprivation metric (1), which reveals the percent difference between the average soma areas from the deprived and non-deprived layers. The deprivation index for each animal in both recovery conditions is plotted, and no apparent difference in deprived soma size was evident between groups. Scale bars (A) and (D) = 1mm; (B) and (E) = 16.67μm.

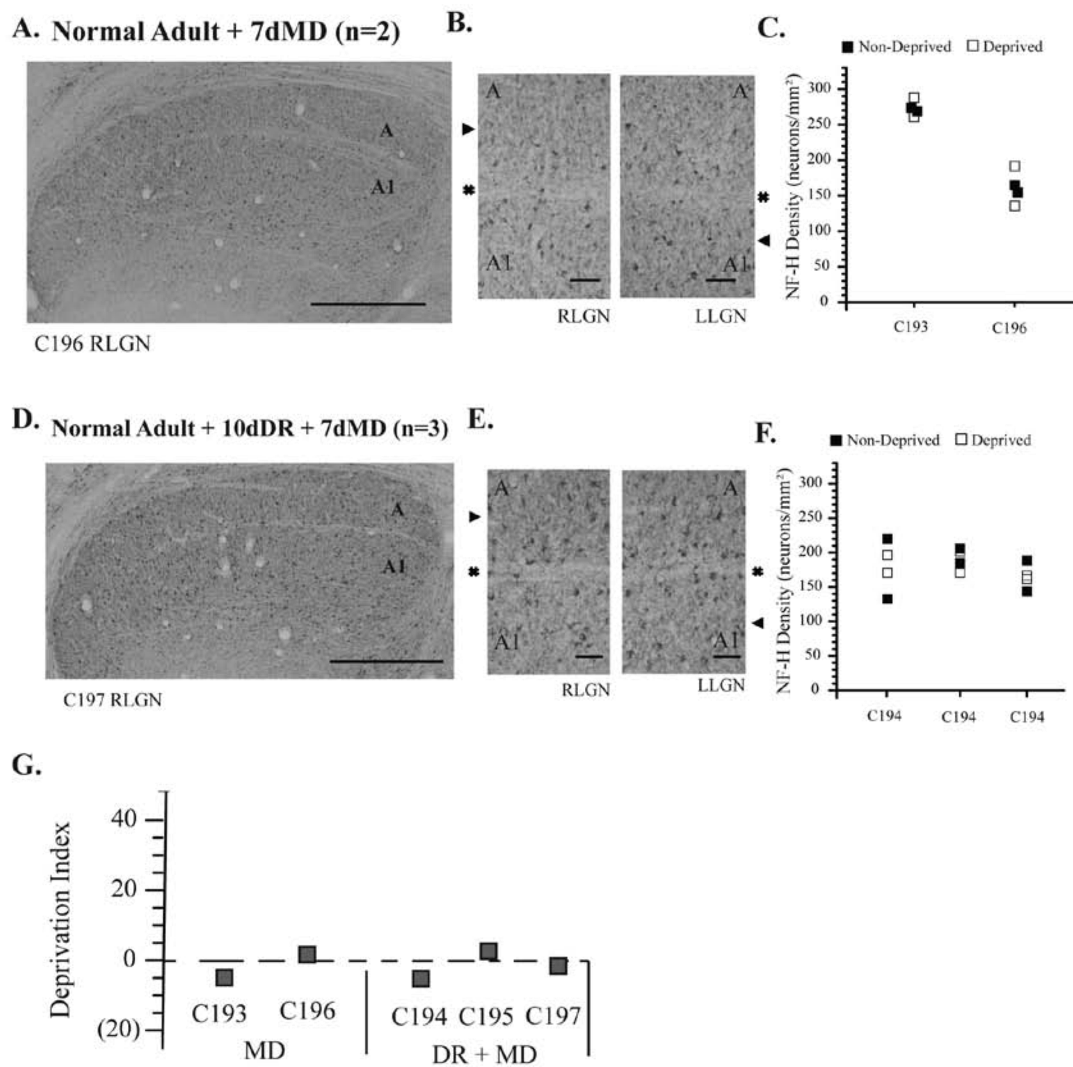

Figure 11. Effect on NF-H labeling in the dLGN of monocular deprivation imposed in adult cats

Figure 11. Effect on NF-H label in the dLGN of monocular deprivation imposed in adult cats.

Five cats were reared normally until either PD365 or PD375. At PD365, 3 cats experienced a 10-day period of darkness followed by 7-days of monocular deprivation, while 2 cats experienced only the 7-day period of monocular deprivation beginning on PD375. Effects on NF-H label density in deprived-eye recipient layers of the dLGN were assessed stereologically. NF-H label density is expressed in terms of NF-H positive neurons per square millimeter. Photomicrographs of the dLGN from one animal (C196) that experienced only a 7-day period of monocular deprivation are provided in panel A and B. (A) depicts the entire right dLGN of C196 photographed at 10X magnification. Layer A is deprived, and A1 is non-deprived. At this low power magnification no difference in NF-H label density between layers was visible. (B) Photomicrographs (10X) of the right and left dLGN of C196. Arrows point to deprived layers, asterisks denote the laminar border between layer A and A1. No difference in NF-H label density was visible between deprived layers and non-deprived layers. (C) NF-H label density of each layer of the dLGN for each of the 2 animals in the 7dMD condition. Examples of tissue from the second condition (10dDR + 7dMD) are provided in panel (D) and (E). (D) A low-power (10X) photomicrograph of the right dLGN of C197. No difference in NF-H label density between deprived A and non-deprived A1 was visible. (E) Photomicrographs (10X) of the right and left dLGN of C197. Deprived layers are marked with an arrow, asterisks denotes the laminar border between A and A1. No apparent difference in NF-H label density between deprived and non-deprived layers was visible. (F) NF-H label density for each layer is plotted for each of the 3 cats in the second condition. (G) Comparison of NF-H

label density in the deprived and non-deprived dLGN layers of each animal in terms of the deprivation metric (1), which reveals the percent difference between NF-H label density in the deprived and non-deprived layers. The deprivation index for each animal in both conditions is plotted, and no apparent difference in NF-H label density was evident between groups. Scale bars in (A) and (D) = 1mm; (B) and (E) = 16.67μm

APPENDIX B Tables

Table 1. Grating acuity of 3 adult amblyopic cats before and after 10 days of darkness imposed in adulthood (cycles/degree of vision)

Cat	Binoc Acuity Before	Binoc Acuity After	DE Acuity Before	DE Acuity After
C173	6.49	7.1	2.22	1.88
C199	7.39	7.39	1.12	1.12
C200	7.1	7.1	0.88	0.88

Table 2. Average soma areas (μm^2) of cells in deprived and non-deprived dLGN layers of adult cats following two different recovery conditions from long-term monocular deprivation in adulthood

Cat	Condition	Deprived	Non-Deprived	DI
C201	30dBV	140	188	34%
C204	30dBV	120	178	33%
C207	30dBV	142	224	37%
C170	10dDR + 20dBV	194	245	21%
C171	10dDR + 20dBV	137	194	29%
C203	10dDR + 20dBV	152	215	29%
C206	10dDR + 20dBV	154	222	31%

Table 3. Neurofilament density (neurons/mm^2) in deprived and non-deprived dLGN layers of adult cats in two different recovery conditions following long-term monocular deprivation.

Cat	Condition	Deprived	Non-Deprived	DI
C201	30dBV	115	235	51%
C204	30dBV	134	240	44%
C207	30dBV	120	219	45%
C170	10dDR + 20dBV	83	213	61%
C171	10dDR + 20dBV	123	271	55%
C203	10dDR + 20dBV	84	206	59%
C206	10dDR + 20dBV	118	206	43%

Table 4. Grating acuity before and after monocular deprivation imposed in adulthood (cycles/degree of vision)

Cat	Condition	Binoc Before	DE Before	Binoc After	DE After
C187	10dDR + 14dMD	7.1	7.1	7.1	7.1
C188	14dMD	7.1	6.49	7.1	7.1

Table 5. Neurofilament density (neurons/mm^2) in deprived and non-deprived dLGN layers of adult cats following monocular deprivation imposed in adulthood.

Cat	Condition	Deprived	Non-Deprived	DI
C193	7dMD	168	164	-2%
C196	7dMD	231	240	4%
C194	10dDR+7dMD	188	181	-4%
C195	10dDR+7dMD	190	199	5%
C197	10dDR+7dMD	169	171	1%

Table 6. Average soma areas (μm^2) of cells in deprived and non-deprived dLGN layers of adult cats following monocular deprivation imposed in adulthood

Cat	Condition	Deprived	Non-Deprived	DI
C193	7dMD	231	232	0%
C196	7dMD	179	181	1%
C194	10dDR+7dMD	190	195	3%
C195	10dDR+7dMD	197	200	2%
C197	10dDR+7dMD	220	214	-3%