IS THE RAPID VISUAL RECOVERY IN THE AMBLYOPIC EYE OF KITTENS

FOLLOWING A SHORT PERIOD OF DARKNESS GUIDED BY VISUAL ACTIVITY

IN THE NON-DEPRIVED EYE?

by

Philip Bobbie-Ansah

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DEDICATION PAGE

To Bruce Evans who inspired me by the ideas he expressed in his book, *Pickwell's*

Binocular Vision Anomalies. My interest in the field of binocular vision and amblyopia

has never been the same from when I first read this book.

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ABSTRACT

Recent study has shown that 10-days of complete darkness imposed 2-months after monocular deprivation in kittens can provoke full and rapid recovery of vision in the deprived eye without affecting the acuity of the fellow eye (Duffy & Mitchell, 2013). This study determined whether the non-deprived eye and its vision played any critical role in dark-mediated amblyopic recovery. Four kittens were deprived of 1-week of monocular vision beginning at postnatal day 29. At 102 days of age, two kittens received 11-day period of reverse occlusion preceded by 10-days of complete dark exposure. The other animals were reverse occluded at equivalent age (P102) without prior exposure to darkness. The acuities for square-wave gratings were assessed on jumping stand. The extent and rate of recovery of vision for both groups of animals were very minimal. Thus, dark-mediated amblyopic visual recovery is largely guided by visually-driven neural activity of the non-deprived eye.

LIST OF ABBREVIATIONS USED

BDNF: brain-derived neurotrophic factor

Binoc: binocular

BLS: bilateral lid suture

cc: cubic centimetres

cd/m^2: Candela per metre square

cm: centimetres

cyles/deg.: cycles/degree

DD: delayed darkness group

DE: deprived eye

dLGN: dorsal lateral geniculate nucleus

DR: dark imposed and reverse occlusion group

GABA: gamma-aminobutyric acid

logMAR: logarithm of minimum angle of resolution

LUMA: light deprivation utilized to mitigate amblyopia

MD: monocular deprivation

Otx2: orthodentine homeobox 2

NDE: non-deprived eye

OD: open door

P: postnatal day

ROC: reverse occlusion group

VEP: visual evoked potential

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CHAPTER 1: INTRODUCTION

1.1 Amblyopia

The term "amblyopia" ("dull vision" or "blunt sight") is used to describe

unilateral and occasionally bilateral reduction in visual acuity that arises from abnormal

visual experience in childhood (Daw, 1998; Rahi, Logan, Timms, Russell-Eggitt &

Taylor, 2002) and usually not attributable to an eye disease (Webber & Wood, 2005).

Amblyopia is the most common visual disorder in children and it is estimated to affect

1-5% of the global population (Attebo et al, 1998; Brown et al., 2000; Drover, Kean,

Courage, & Adams, 2008; Elfein et al., 2015, Friedman et al., 2009; Hashemi et al., 2014;

Newman & East, 2000; Polling, Loudon & Klaver, 2012). In addition to the loss of visual

acuity, amblyopia is often accompanied by severe loss of binocularity, particularly,

stereoacuity (McKee, Levi, & Movshon, 2003). The condition also imposes significant

cost on the individual, the health system and community (Membreno, Brown, Brown,

Sharma & Beauchamp, 2002)

Clinically, amblyopia is usually categorized as either strabismic, anisometropic

and/or visual deprivation depending on the presumed amblyogenic event or on the basis

of an existing condition at the time of initial presentation (McKee et al., 2003). An

additional category, mixed amblyopia, is used to describe the concurrent effects on vision

of two or more amblyogenic factors.

A recent comprehensive review of the functional deficits associated with

amblyopia in humans have led to its consideration as a "complex condition" characterized

by a constellation of deficits (Levi, 2013). In addition to established sensory impairment

in visual acuity, contrast sensitivity, stereoacuity and contour interaction (Bonneh, Sagi,

& Polat, 2007; Hess, Bradley, & Piotrowski, 1983; Hess & Howell 1977; Levi, Yu, Kuai

& Rislove, 2007; McKee et al., 2003; Thompson & Nawrot, 1999; Volkers, Hagemans, Van Der Wildt, & Schmitz, 1987), amblyopes may show ocular motility deficits in initiation and execution of saccadic eye movements (fast coordinated eye movement) (Niechwiej-Szwedo, Chandrakumar, Goltz, & Wong, 2012), eye position stability (González, Wong, Niechwiej-Szwedo, Tarita-Nistor, & Steinbach, 2012) and tracking of moving targets in the field of vision (Ho et al., 2006). The psychosocial effects of amblyopia and the limitations it places on an individual in respect to employment and career opportunities are well documented (Packwood, Cruz, Rychwaslski & Keech, 1999; Webber, Wood, Gole & Brown, 2008). The lifetime risks of bilateral visual incapacitation (blindness) from disease or trauma to the fellow eye, has been reported to be higher for amblyopes than for non-amblyopes (Rahi et al., 2002; Tommila & Tarkkanen 1981; Van Leeuwen et al., 2007). In one such study, the reported risk of visual impairment to the non-amblyopic eye (1.76 %) was found to be three times the rate in normal adult and almost 17 times that of a normal child (0.11%) (Tommila & Tarkkanen, 1981). By these accounts, there is no ambiguity that the cumulative effects of amblyopia on the quality of life can be enormous, thus, providing strong arguments for diagnosis and treatment.

1.2 Treatment for Amblyopia

Clinical management of amblyopia has long been a challenge. Although records of occlusion therapy date as far back as the 10th century (Loudon & Simonsz, 2005; Von Noorden, 1996), the careful documentation of treatment outcomes by Worth from the late 19th century has had significant impact on therapy (Worth, 1929). The goal of treatment has always been the recovery of vision in the affected eye as a pre-requisite for binocular vision development (fusion and stereopsis), binocular summation of acuity and insurance

for useful vision in the event of sight loss to the non-amblyopic eye later in life (Rahi et al., 2002).

The age of initiation of amblyopia therapy has been considered as one of the most significant predictive factors (aside compliance) to successful treatment outcome (Flynn & Cassady, 1978; Mohan, Saroha & Sharma, 2004). The dominant idea over the years has been the commencement of treatment before age 7 (Fronius, Cirina, Ackermann, Kohnen, & Diehl, 2014; Lewis & Maurer, 2009; Worth 1929), during which time plasticity of the visual system may allow optimum visual recovery. However, evidence of successful treatment outcomes in children over 7 years of age (Mintz-Hittner & Fernandez, 2000; Park, Hwang & Ahn, 2004), adolescents (Khan, 2015; Mohan et al., 2004; Sheiman et al., 2005), and adults consequent to vision loss to the fellow eye (El Mallah, Chakravarthy & Hart, 2000; Vereecken & Brabant, 1984), suggest that some level of visual plasticity may exist beyond the conventional "critical period" (Hess, Mansori & Thompson, 2010; Moseley, & Fielder, 2001).

Historically, a large number of therapeutic modalities have been tried with varying successes, including the very recent clinical trial involving the use of oral levodopa as ancillary treatment (Pediatric Eye Disease Investigator Group: PEDIG, 2015). Results from a series of randomized multi-centred clinical trials conducted by the (PEDIG) in the last one and half decade have outlined the importance of elimination of amblyogenic factors (example: extraction of cataract, repair of ptosis), provision of appropriate refractive corrections, and reversal of competitive disadvantage to the amblyopic eye (PEDIG, 2002; 2008; 2010; Gunton, 2013). The latter, which may involve the use of occlusion (patching), optical or pharmacological agents (topical cycloplegic drops) to penalise the sound eye, ensures "forced utilization" of the amblyopic eye in an attempt to

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strengthen its neural connections and bolster recovery of vision. However, the existence of residual amblyopia and the propensity for regression of recovered vision on termination of occlusion therapy (~ 24 – 27%: Bhola, Keech, Kutschke, Pfeifer & Scott, 2006; Holmes et al., 2004; Walsh, Hahn, & LaRoche, 2009) coupled with issues of compliance (Sheiman et al., 2005; Smith, Thompson, Woodruff & Hiscox, 1995), risk of development of occlusion amblyopia in the fellow eye (~ 19 – 25%: Longmuir, Pfeifer, Scott & Olson, 2013; Scott et al., 2005; Varadharajan & Hussaindeen, 2012) and decompensation of small angles of eye misalignments (Charney & Morris, 1984; Lee & Kim, 2012; PEDIG, 2002) have occasionally encouraged suggestions for alternative treatments (PEDIG, 2015). The argument that monocular treatments present disincentive for the use of both eyes and risk the possibility of developing the second (fusion) and third degree (stereopsis) of binocular vision during therapy, is a very a important one (Hess et al., 2010a; Mitchell & Duffy, 2014)

Current treatment models exploring binocular cooperation between the eyes have shown promising results in adult human amblyopes (Hess, Mansouri &Thompson, 2010a, b, 2011; Hess, Thompson & Baker, 2014; Li et al., 2013) and in cats (Duffy & Mitchell 2013, Kind et al., 2002; Murphy et al., 2015). By reducing the luminance contrast of images presented to the non-strabismic eye to match the perceived contrast of stimuli presented to the amblyopic eye under dichoptic viewing conditions, Hess et al. (2010a) have demonstrated that elimination of inter-ocular suppression could promote significant recovery of visual acuity and stereoscopic vision in humans after just 6 weeks of treatment.

While our understanding of the subject of amblyopia, the treatment options and timing of interventions have been enriched over the years through extensive research in

animal and human subjects, challenges associated with some current interventions and successes on emerging binocular treatment approaches, particularly in animals (example: dark rearing in rats and cats), call for continued research to elucidate the underlying neural mechanisms and key parameters in order to support and guide clinical translation in humans. This thesis project, in particular, explores further one of the emerging binocular treatments based on recent evidence of exposure of amblyopic kittens to a brief period of complete darkness. The results from this endeavour provide an understanding of one of the key parameters for dark-induced visual recovery, the nature of which may serve as a guide for possible clinical application of darkness and/or its mechanisms in the treatment of human deprivation amblyopia.

CHAPTER 2: LITERATURE REVIEW

2.1 Animal Models of Amblyopia

Over half a century of research into the development of the central visual pathways has deepened our understanding of early postnatal visual plasticity and the importance of "critical periods" in visual system development (Daw, 1998; Hübener, & Bonhoeffer, 2014; Levi 2012; Mitchell & Mackinnon, 2002; Mitchell & Timney, 1984; Sengpiel, 2014; Wiesel & Hubel, 1963b). The modifiability of the visual system of cats, monkeys and rodents have been demonstrated by use of several experimental manipulations of early visual exposure, including; monocular and binocular deprivation by eyelid suture (Hubel Wiesel, 1970; Wiesel & Hubel, 1963a, b, 1965a), creation of optical defocus with monocular diffusers or lenses (Jaffer, Vorobyov & Sengpiel, 2012; Wensveen et al., 2006; Wiesel & Hubel, 1963a, b), and strabismus induced by prisms (Kumagami, Zhang, Smith & Chino, 2000; Zhang et al., 2005) or surgical sectioning of one or more extra-ocular muscles (Delvin, Jay & Morrison, 1989; Fenstemaker, Kiorpes & Movshon, 2001; Gingras, Mitchell & Hess, 2005; Wiesel & Hubel, 1965b).

Disruption of binocular coordinated visual input in frontal-eyed animals by monocular eyelid suture, an extreme form of biased visual exposure, is known to produce behavioural deficits that closely parallels human stimulus or form deprivation amblyopia (Hess, France, & Tulunay-Keesey, 1981; Von Noorden, Dowling & Fergurson, 1970). Although less prevalent (< 3% of all cases of amblyopia: Antonio-Santos, Vedula, Hatt, & Powell, 2014), deprivation amblyopia can produce severe visual loss as it is frequently found associated with congenital cataract (Maurer & Lewis, 1993), congenital ptosis

(Stein, Kelly & Weiss, 2014) and media opacities of the cornea, aqueous and vitreous humour (Mitchell & MacKinnon, 2002; Von Noorden & Maumenee, 1968).

2.2 Anatomical And Neurophysiological Changes Induced By Early Selective Visual Deprivation In Animals

The pioneering work of Hubel and Wiesel that described the receptive fields properties of cells in the Lateral Geniculate Nucleus (Hubel & Wiesel, 1959) and subsequent description of the cortical architecture and characterization of neuronal response attributes of cells in the visual cortex of cats and monkeys (Hubel & Wiesel, 1962, 1968) set the stage for a plethora of investigations of how these various properties developed in normal animals and those subjected to abnormal early visual experience. The consequences on the retina, geniculate nucleus and visual cortex of alteration of binocular visual inputs during early postnatal development are sequentially considered below.

Deprivation-driven effects on the retina continue to generate some contradictory views. While no profound modifications in retinal histology and gross physiology (electroretinogram or evidence from single unit responses from retinal ganglion cells) were found in monocularly deprived kittens (Cleland, Crewther, Crewther, & Mitchell, 1982; Sherman & Stone, 1973; Wiesel & Hubel, 1963a) or monkeys (Crawford, Blake, Cool, von Noorden, 1975), there are some reports of morphological changes. In kittens, significant morphological changes have been observed in the ipsilateral retina following more severe interventions, such as induction of leukoma (dense white opacity of the cornea) and cataracts (Taktarov, Bekchanov, 1990), or artificial esotropia (Chino,

Shansky & Hamasaki, 1980; Delvin et al., 1989). In a more recent publication, Mwachaka and colleagues also described variation in the retina of young rabbits deprived of vision in one eye by eyelid suture (Mwachaka, Saidi, Odula & Mandela 2015). These notwithstanding, the general view is that alterations at the level of the retina are minimal in comparison to the monumental changes at the thalamic and cortical layers in the visual pathway (Cleland et al., 1982; Wiesel & Hubel, 1963b).

In the dorsal Lateral Geniculate Nucleus (dLGN) of kittens and monkeys, early monocular deprivation (MD) by eyelid suture is known to induce a profound reduction (25% to 40%) of soma size in the layers that receive projections from the deprived eye in comparison to layers fed exclusively by the fellow non-deprived eye (Duffy & Slusar, 2009; Dürsteler, Garey & Movshon, 1976; Hubel, Wiesel & LeVay, 1977; Wiesel & Hubel, 1963a).

While some minor functional changes in response to early MD have been reported in the thalamus, the most profound and consistent deprivation-induced modifications in the visual pathway are those described in the striate cortex (also known as visual cortex, Brodmann area 17 or V1). In normal cats and monkeys, about four-fifths of neurones in the visual cortex are binocularly influenced, in that they can be excited by stimulating either eye (Freeman & Olson, 1982; Hubel & Wiesel, 1962, 1963). Monocular deprivation by eyelid suture during early postnatal development produces marked changes in both the physiological properties of cortical neurones and the organization of the visual cortex. In a remarkable series of studies, Hubel & Wiesel (1970) demonstrated that even a period of MD as short as 3 days in early postnatal life could change the relative responsiveness of cortical cells to the two eyes. In contrast to normal kittens

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where most cells in the visual cortex could be excited by stimulating either eye, kittens that received 1-4 months of MD beginning at the time of eyelid opening, had very few binocularly influenced cells and, majority of cells were driven by the non-deprived eye (Wiesel & Hubel, 1963b). The large influence of the non-deprived eye on cortical neurones induced by early period of MD has been subsequently verified in many studies (Blakemore & Van Sluyters 1974; Blakemore, Garey, & Vital-Durand, 1978; Duffy & Livinstone, 2005; Freeman & Olson, 1982; LeVay, Wiesel & Hubel, 1980; Movshon, 1976a; Wiesel & Hubel, 1965b)

The physiological changes following MD are accompanied by changes in the anatomical organization of the striate cortex. In normal animals, there is an anatomical segregation of cells with respect to the eye of physiological dominance that takes the form of columns that run the full depth of the cortex (Hubel & Wiesel, 1959; Wiesel & Hubel, 1963b; LeVay et al., 1980). In primates, this segregation takes the form of alternating bands or stripes of ocular dominance, while in cats, the segregation takes the form of patches of irregular shape. However, following an early period of MD, columns corresponding to the non-deprived eye expand into territory that would, under normal conditions, be controlled by the deprived eye (Hubel et al., 1977; LeVay et al., 1980; Shatz, & Stryker, 1978). This is often associated with corresponding aggregation of deprived eye columns into a smaller region, especially in layer IV (Hubel et al., 1977; Kind et al., 2002; Shatz, & Stryker, 1978; Wiesel & Hubel, 1963b) and concurrent expansion of geniculate afferents sub-serving the non-deprived eye.

On the basis of electrophysiological studies on bilaterally deprived as well as surgically induced exotropic animals, Hubel and Wiesel introduced the notion of binocular competition during visual development. In kittens (Wiesel & Hubel, 1965a) and

young monkeys (Hubel & Wiesel, 2004) deprived of pattern vision by bilateral lid suture very early in postnatal life, a large proportion of cortical neurones (~50%) were found to exhibit electrophysiological responses comparable to those observed in normal animals and far better than those expected on the basis of comparable periods of monocular deprivation (Wiesel & Hubel, 1963b). Hubel and Wiesel postulated that the difference between the effects of monocular and binocular deprivation arose through a process of competition for synaptic space on cortical neurones during development. Whereas in monocularly deprived animals the deprived eye was at a competitive disadvantage and so lost connections in the cortex, in binocularly deprived animals, neither eye was at a competitive disadvantage during development. An even more profound effect was observed in the visual cortex of exotropic kittens (Hubel & Wiesel, 1965). Although cursory behavioural assessment ruled out amblyopic visual acuity deficits consistent with the ability to alternate fixation between the eyes, the electrophysiological results were very substantial, as there was an almost complete loss of binocularly driven neurones without any bias in the distribution of ocular preference toward one eye. Similar observations of reduced binocular-responsive cells have been reported for normally pigmented kittens (non-albino) with naturally occurring congenital esotropia (Grünau & Rauschecker, 1983; Hoffmann & Schoppmann, 1984).

The idea that strabismus rendered the non-fixating eye and its cortical connections essentially non-competitive, received support from a comparable result observed in animals for which daily periods of monocular occlusion with an opaque contact lens was alternated between the two eyes (Freeman & Olson, 1980; Hubel & Wiesel, 1965). The observed virtual absence of binocular neurones implied an absence of the neural substrate responsible for binocular vision, depth perception (stereopsis) and binocular summation

(Blakemore, Fiorentini, & Maffei, 1972). Such a conclusion was reinforced by later reports of reduced binocular summation in esotropic kittens (Sclar, Ohzawa, & Freeman, 1986) and the absence of stereopsis in experimentally induced exotropic cats (Mitchell & Sengpiel 2009).

On the basis of physiological studies on the cortex of visually inexperienced kittens (Wiesel & Hubel, 1963), ferrets (Crowley & Katz, 2000) and naïve monkeys (Horton & Hocking, 1996; LeVay et al., 1980), a strong argument has been made that at birth the mammalian visual cortex is by no means a *tabula rasa* (Levi, 2012 p.827) with respect to ocular dominance. In fact, it is known that most of the functional properties of adult neurones pertaining to arrangement of columns, orientation selectivity, directional selectivity and binocularity are innately present at birth or develop in the first few weeks after birth (Barlow & Pettigrew 1971; Crowley & Katz, 2000; Horton & Hocking, 1996; Hubel & Wiesel, 1963; LeVay et al., 1980; Mitchell & Timney 1984; Rathjen & Löwel, 2000). The functional importance of normal visual input appears to allow refinement of these cortical properties. Disruption and degeneration of these properties after a period of deprivation are the direct consequences of abnormal visual experience early in life (LeVay et al., 1980; Wiesel & Hubel, 1963, 1965a).

Additionally, visual evoked potential (VEP) responses on stimulation of the deprived eye in juvenile cats for which MD was initiated early in life have been reported to be markedly sluggish (increased latency) and of low amplitude (Shapley & So, 1980; Synder & Shapley, 1979; Wiesel & Hubel, 1963b). The fact that some response could be elicited on stimulation of the deprived eye during the electrophysiological studies suggests the existence of some residual neural connections of this eye, a finding that was

supported in later studies (Freeman & Ohzawa, 1988; Sclar et al., 1986). Freeman and

Ohzawa (1988) discovered that concurrent presentation of stimuli to both eyes of

monocularly deprived animals resulted in phase-specific excitatory or inhibitory

binocular responses which were greater at peaks and lower at troughs in comparison to

the results elicited by stimulating either eye alone. Similar binocular inhibitory

(suppressive), and to a smaller extent, excitatory neural connections have been reported in

prism-induced esotropic and exotropic cats (Chino, Smith, Yoshida, Cheng & Hamamoto,

1994) as well as esotropic monkeys (Kumagami et al., 2000; Zhang et el., 2005) when

tested under dichoptic viewing conditions. These residual neural connections have been

considered as potential substrates for anatomical re-innervation and functional recovery

when amblyopia therapy is initiated within the sensitive period. In fact, elimination of

these inhibitory mechanisms by microiontophoretic injection of selective GABAergic

blocker such as bicuculline in strabismic cats was reported to have restored binocular

responsiveness to monocular cells (Mower, Christen, Burchfiel, & Duffy, 1984).

The idea of a "critical period" for susceptibility to the effect of early abnormal

experience has been widely expressed. In sharp contrast to the marked effects of a 2 –3

month period of MD beginning at about the time of eyelid opening in kittens, adult

animals that received 3 months of monocular eyelid suture showed no morphological

alterations at the dLGN (Wiesel & Hubel, 1963a) or detectable physiological changes at

the visual cortex (Wiesel & Hubel, 1963b). That no significant alterations in geniculate

morphology were observed in adult cats (Wiesel & Hubel, 1963b) that had undergone

comparable lengths of MD (3 months) as those experienced by young kittens, suggested

that the negative consequences of deprivation were limited to early postnatal life. This

provided the first evidence for an early "critical period" of plasticity to the effects of MD,

the profile of which was defined in subsequent studies (Hubel & Wiesel, 1970; Olson & Freeman, 1980).

Hubel and Wiesel (1970) reported that the susceptibility of the cat's visual cortex to the effect of MD reached a summit suddenly during the 4th week of postnatal development, and showed gradual decline between the 6th and 8th week until about 3 months of age. Although their data was complicated by the varying duration of monocular lid closure applied to animals of the different age groups, the general conclusion spurred other studies. Notably, in their application of a consistent period (10 days) of MD at different ages of development, Olson and Freeman (1980) demonstrated that, in cats, the vulnerability of the visual cortex to deprivation peaks at 4-5 weeks of age after which there is a slow but progressive and irreversible decline until about 6 months of postnatal life (Olson & Freeman, 1980). Generally, the critical period in cats is relatively shorter (6 -8 months of age: Daw et al., 1992; Jones, Spear & Tong, 1984; Mitchell & Timney, 1984; Olson & Freeman, 1980) than monkeys (approximately 1-2 years: LeVay et al., 1980) or humans (~5.75 – 6 years: Keech & Kutschke, 1995; Von Noorden, 1981). Although at the peak of the critical period of visual development in cats, as little as 4 hours of MD provokes some changes in ocular dominance (Freeman & Olson, 1982), more perceptible morphological and physiological alterations have been reported following 1- 4 days (Antonini & Stryker, 1996; Freeman & Olson 1982; Hubel & Wiesel 1970; Movshon, & Dürsteler, 1977). However, longer periods (extending up to 16 months) of MD in adult cats produce little or no effect (Hubel & Wiesel, 1970), thus, underscoring the immutable nature of cortical ocular dominance at adulthood.

The current idea on the critical period is expanded to incorporate three different intervals: the time for normal visual development, the period of vulnerability to early visual deprivation, and a time during which recovery is possible from a prior period of deprivation (Blakemore & Van Sluyters, 1974; Lewis & Maurer, 2005). The idea of multiple critical periods has been widely expressed (Harwerth et al. 1986; Mitchell & Mackinnon, 2000), and in most cases, these periods have been found to vary in onset and duration based on species, cortical layer, visual history and visual function under study (Daw, 1998; Daw, Berman & Ariel, 1978).

2.3 Behavioural Consequences of Selective Visual Deprivation

There have been a large number of studies on the behavioural consequences of selective visual deprivation in frontal-eyed animals. Consistent with the anatomical and physiological findings, the severity of the visual deficits varies with the onset and duration of deprivation (Giffin & Mitchell, 1978). The visual sequelae of early MD were noted in Hubel & Wiesel's original investigation. Although they (Wiesel & Hubel, 1963b), together with other earlier researchers (Blakemore & Van Sluyters, 1974; Movshon, 1976b) were unable to formally quantify the extent of visual deficit in the deprived eye, qualitative assessments of various visuomotor behaviours, such as visual placing, visual startle, visual following and ability to negotiate visual cliff (depth perception) were found to be completely absent when animals were forced to use the deprived eye.

The introduction of quantitative methods of assessing visual acuity in cats (Mitchell, Giffin, Wilkinson, Anderson & Smith 1976; Giffin & Mitchell, 1978) have extended our understanding of the magnitude of the behavioural consequence of

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deprivation in a manner which could be related to human visual deficit assessed on clinical optotype (log MAR) or preferential–looking charts. To appreciate the extent of this deficit, it is important to document the time course of development of visual function in cats. In normally reared kittens, development of vision is limited by two major postnatal factors. First, kittens are born with their eyelids shut and remain so until P3 to P12 (Blakemore & Cummings, 1975). Second, the optical media are cloudy for the next 2-3 weeks (Freeman & Lai, 1978; Thorn, Gollender & Erickson, 1976) as the hyaloid artery that surrounds the crystalline lens at birth is gradually resorbed (Bonds & Freeman, 1978). Behavioural measurements of acuity indicate that visual resolution improves gradually from 0.75 cycles/degree at 1 month of age until adult levels of 6.4 - 8.6 cycles/ degree are reached at about 3 - 4 months of age (Mitchell, 1991; Mitchell & Mackinnon, 2002; Mitchell & Timney 1984).

The immediate behavioural effect of monocular deprivation can be as severe as functional blindness (Dews & Wiesel, 1970; Giffin & Mitchell, 1978; Movshon, 1976b; Wiesel & Hubel, 1963a). Although some vision may be recovered in the initially deprived eye, substantial deficits in visual acuity may exist into adulthood. For instance, the square-wave grating acuity of the deprived eye for kittens that receive monocular eyelid suture early in life may be reduced by 3 octaves or more, depending on the length of the deprivation and the age at which it is imposed (Mitchell, 1988; Mitchell 1991; Mitchell et al., 2016; Smith & Holdefer, 1985). By sharp contrast, animals deprived of pattern vision in one eye close to or at about adulthood (~1 year) have shown little or no behavioural signs of vision loss in the inexperienced eye. A comparable behavioural consequence of MD for the deprived eye has been reported for *Macaca mullata* tested with Landolt rings (Von Noorden, Dowling, & Ferguson, 1970). In their study (Von Noorden et al., 1970),

monocular eyelid closure for the first 4 weeks of monkey's life resulted in a profound

inability of the deprived eye to resolve 270 minutes of arc stimuli. However, the

behavioural consequence for animals for which MD began at 3 months of age was

unremarkable (Von Noorden et al., 1970). Significant deficit in contour integration

(crowding phenomenon), one of the characteristic symptoms of amblyopia in humans, has

also been demonstrated in some amblyopic monkeys, alongside minimal and occasional

defects in the fellow eye (Kozman & Koirpes, 2003).

  Although exotropic cats (Wiesel & Wiesel, 1965b) and monkeys (von Noorden &

Dowling, 1970) rarely demonstrate behavioural evidence of amblyopia, their esotropic

counterparts (cats: Jacobson & Ikeda, 1979; Cleland et al., 1982; monkeys: von Noorden

& Dowling, 1970) show reduced vision in the deviating eye. In one such study on kittens

(Cleland et al., 1982), despite reduction in absolute acuities for the esotropic and fixating

eyes, the general pattern of visual deficits in the deviating eyes was found to be consistent

with those observed in kittens following an early period of MD. Exotropic kittens have

been reported to demonstrate loss in stereoscopic vision even in the face of good visual

acuity in either eye (Mitchell & Sengpiel, 2009).

  The large spatial localization deficits (alignment acuity /Vernier acuity) exhibited

by human anisometropic and strabismic amblyopes (Hess & Holiday, 1992; Mayer,

Fulton, & Rodier, l984) have also been demonstrated in kittens reared with strabismus or

following MD (Gingras et al., 2005). In an elaborately designed study using Gaussian

blobs as stumuli, Gingras and associates discovered that in same amblyopic animals, the

alignment deficits were far greater than the grating acuity defects (Gingras et al., 2005). A

similar propensity exists for human amblyopes to show greater deficits for hyperacuities

and optotype acuities than grating acuity (Mayer et al., l984; McKee et al., 2003), a finding that informs the clinical notion that grating acuity may underestimate amblyopia.

Unsurprisingly, there are reports on kittens (Gingras et al., 2005; Mitchell, 1991; Sherman, 1972; Movshon, 1976b) and infant macaque monkeys (Quick, Tigges, Gammon & Boothe, 1989) suggesting spontaneous development of nystagmus (involuntary oscillation of the eyes) and/ or strabismus following MD in a fashion analogous to human sensory strabismus secondary to deep amblyopia.

In summary, the behavioural ills of selective visual deprivations are largely consistent with the anatomical and physiological deficits. The close parallels between the amblyopic visual loss in cats and humans provide good face validity for the use of the former in behavioural studies on amblyopia (Mitchell & Duffy, 2014).

2.4 Recovery From The Effects Of Early Monocular Deprivation

In view of the profound changes provoked by abnormal visual experience during early postnatal life, there have, and continue to be growing numbers of studies exploring procedures and mechanisms for reversing these alterations. The degree and speed of any recovery from MD has been studied most extensively in two conditions. The simplest situation is one where the deprivation is terminated without any concurrent manipulation of the fellow eye, a paradigm commonly referred to as binocular recovery (Giffin & Mitchell, 1978; Kind et al., 2002; Mitchell, 1988). Equally studied is the condition of reverse occlusion where the fellow eye is closed immediately following re-opening of the deprived eye. It has been shown that the anatomical modifications in the dLGN and physiological ills in the visual cortex of monocularly deprived kittens could be restored or almost completely reversed if this manipulation is imposed sufficiently early in life

(Blakemore & Van Sluyters 1974; Dürsteler et al., 1976; O'Leary et al, 2012). For instance, following 9 weeks of reverse occlusion imposed on 5 or 6 weeks old kittens that had earlier been subjected to monocular eyelid closure at about the time of eye opening, the originally deprived eye came to dominate most cells in the visual cortex such that the distribution of ocular dominance were the mirror opposite of the situation that existed just after the initial period of MD (Blakemore & Van Sluyters, 1974). A concordant result was observed with respect to the laminar differences in cell soma sizes in the LGN (Dürsteler et al., 1976). However, the ability of a period of reverse occlusion to bring about a shift in ocular dominance in the cortex appeared to be restricted to the first 14 weeks of postnatal life (Blakemore & Van Sluyters 1974), a period shorter than that during which the visual cortex is susceptible to the effects of MD (Olson & Freeman, 1980a).

Shortly thereafter, it was discovered that the rate at which reverse occlusion could restore both the morphological and physiological alterations imposed by a previous period of MD was very fast. In 5-week old kittens deprived of vision in one eye, Dürsteler et al. (1976) revealed that some amelioration of the effects on the LGN could be observed following just 3 days of reverse occlusion, and after only 12 days, the initial effects of MD were completely switched between the eyes. Corresponding rapid shifts of ocular dominance of cells in the visual cortex were observed in response to identical periods of reverse occlusion in kittens (Movshon, 1976a).

Just as in kittens, reverse occlusion can promote recapture of cortical cells connected to the initially deprived eye of young monkeys when the process is initiated very early (Blakemore, Vital-Durand & Garey, 1981). One such finding in young monkeys led Blakemore et al. (1981) to conclude that "the process of recapture of the

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cortex after reverse suturing at about 4 weeks is virtually complete 2 weeks later" (p.411). However, little or no change in cortical ocular dominance has been observed in monkeys for which monocular eyelid closures was followed by binocular visual input (Blakemore et al., 1981; Hubel et al., 1977; LeVay et al., 1980). The absence of recovery in monkeys after passive binocular exposure may be attributed to a myriad of factors including the preponderance of strabismus (Kiorpes, Boothe, Carlson & Alfi, 1985; Quick et al., 1989), increased sensitivity of cortical neurones to micro-deviations of the eye (Kind et al., 2002), as well as deprivation-induced myopia in the amblyopic eye (Hanverth, Smith, Crawford, & Von Noorden 1989; Qiao-Grider, Hung, Kee, Ramamirtham & Smith, 2004; Raviola & Wiesel, 1978).

As mentioned earlier, some behavioural recovery of visual acuity in the initially deprived eye has been observed consequent to simple restoration of normal binocular input in monocularly deprived kittens (Giffin & Mitchell, 1978; Kind et al., 2002; Mitchell, 1988). This recovery can be rapid in the initial stages (Mitchell, Gingras, Kind, 2001) and seems to rely on the relative alignment of the eyes (Kind et al., 2002). It appears that the extent of visual gain following binocular exposure in kittens is less than that achieved by active suturing of the eyelids of the experienced eye during the critical period (Mitchell 1988; Mitchell & Timney, 1984). However, the substantial recovery of vision observed in the initially deprived eye following a period of reverse occlusion proceeded mostly at the expense of the acuity of the non-deprived eye. In addition, the gains in the deprived eye were usually not retained on termination of treatment (Mitchell, 1988, 1991; Mitchell, Murphy & Kaye, 1984a, b; Murphy & Mitchell, 1987), a finding reminiscent of visual regression reported in human amblyopes after termination of occlusion therapy (Bhola et al., 2006; Hertle et al., 2007; Walsh et al., 2009). Although

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the results in cats differed in some respects according to the specific timing of the initial period of MD and the period of reverse occlusion, the common occurrence was that much of the gain in the vision was lost very quickly (< 10 days) with the onset of binocular visual input accompanied by a slower but incomplete recovery of the vision of the recently occluded eye. As an overall consequence, kittens were left with subnormal vision in either eye, or bilateral amblyopia (Murphy & Mitchell, 1987; Mitchell, 1991; Mitchell & MacKinnon, 2002). In addition, there are reports of the development of strabismus and nystagmus consequent to reverse occlusion of the experienced eye in kittens (Mitchell, 1991) and monkeys (Sloper, Headon & Powell, 1988; Tusa, Repka, Smith & Herdman, 1991) when imposed very early within the sensitive period of visual development. Thus, collectively, these studies demonstrate that the onset and duration of reverse occlusion may have a complex relationship with the speed, extent and stability of visual recovery in the deprived eye.

In view of the unstable nature of vision recovered from reverse occlusion in cats, exploration of alternative occlusion paradigms that maximised recovery and stability of acuity in the amblyopic eye were examined in *seriatim* (Mitchell, 1991; Mitchell, Ptitos & Lepore 1994; Mitchell, Kind, Sengpiel & Murphy, 2003, 2006). Various regimens of part-time occlusion, which allowed some binocular exposure each day, were pitted against full-time occlusion in amblyopic cats. By allowing some proportion (30%-50%) of simultaneous and concordant visual exposure to the two eyes each day during a period of reverse occlusion (Mitchell, 1991; Mitchell et al., 2003, 2006), more stable recovery of the visual acuity of both eyes have been demonstrated, together with the development of normal contrast sensitivity and Vernier acuity (Mitchell, 1991). An equivalent period of mixed binocular and monocular exposure each day has been reported to preserve contrast

sensitivity in monkeys at almost normal levels (Wensveen et al., 2006). The recovery of vision after a short period of binocular exposure during reverse occlusion suggests potential development of some fusional and depth perceptual capabilities. This possibility was investigated in a study conducted 2 decades ago (Mitchell et al., 1994). In a group of 5 kittens that had fully recovered visual acuity from 3.5-5 hours daily part-time occlusion (representing 50-70% of occlusion time), 60% of the animals demonstrated binocular superiority on a test of local stereopsis; however, no benefits were observed on tests of global stereopsis on random-dot stereo displays (Mitchell, et al., 1994).

Finally, Smith and Holdefer (1985) observed some improvement in the visual acuity of the deprived eye of adult cats following enucleation of the experienced eye. Such a finding was consistent with previous physiological evidence of increased cortical responsiveness of the deprived eye from an animal that had undergone similar experimental manipulation (Kratz, Spear & Smith, 1976). Together, these two reports could be linked to evidence in human amblyopes where substantial recovery of vision in the amblyopic eye has been observed in adulthood following vision loss or retinal degeneration in the fellow eye (El Mallah et al., 2000; Vereecken & Brabant, 1984). In turn, the results suggest a continuing role for inhibition of neural signals from the amblyopic eye by the non-deprived eye.

2.5 Dark-Induced Recovery From Monocular Deprivation

Complete elimination of visual input to the eyes from near birth for extended periods by rearing animals in absolute darkness has been employed frequently in the past to explore the extent to which the development of the morphological and physiological responses (properties) of cortical neurones are influenced by the absence of visual

exposure (Cynader & Mitchell, 1980; Freeman and Olson, 1982; Fregnac & Imbert, 1978; review: Mitchell & Timney, 1984). Notably, the proportion of visually-responsive cells in the cortex of normally and dark-reared animals have been found to differ significantly from each other at the third and fourth week of cortical development (Fregnac & Imbert, 1978). After 4 weeks of age, the proportion of orientation selective cells in the visual cortex of light-reared kittens increased in number (Fregnac & Imbert, 1978); whereas those of dark-reared animals showed a preponderance of non-selective cells. As a consequence, at 6 weeks of age, the cortex of dark-reared animals showed less maturity in comparison to those of normally reared animals at 2-weeks of age (Fregnac & Imbert, 1978)

The results from a number of studies that have examined the effects of MD on animals dark-reared from birth have provided empirical evidence to support the idea of extended sensitivity of the visual system to a prior period of darkness (Beaver, Ji & Daw, 2001; Cynader & Mitchell, 1980; Timney, Mitchell & Cynader, 1980). Cynader and Mitchell (1980) demonstrated that 4 to10 months of dark rearing of kittens could increase the susceptibility of the visual cortex to the physiological effect of 3 months of monocular eyelid closure. Subsequently, Cynader (1983) demonstrated that dark rearing extended for as long as 2 years could render the visual cortex of a cat in a plastic state for the effect of monocular eyelid suture and produce consequences well beyond the conventional critical period for deprivation induced depression.

In monocularly deprived adult Long Evans rats, a brief period of darkness (10 days) preceding either reverse occlusion or binocular visual exposure promoted reversal of the ocular dominance shift induced by the prior period of MD (He et al., 2007). The restoration of changes of ocular dominance in the visual cortex was accompanied by

considerable recovery of the spatial acuity of the formerly deprived eye as assessed by VEPs, though the extent of visual recovery (by behavioural assessment) was much less after binocular visual exposure than after reverse occlusion (He et al., 2007). The visual gains observed on these measures was consistent with recovery of dendritic spine density reported in adultm rats reared under comparable conditions (Montey & Quinlan, 2011). Recent immuno-histochemical and behavioural studies on cats have shown that a short period (10 days) of complete darkness could stimulate reversal of mature neurofilament proteins in the visual cortex of normally reared one-month-old kittens, and/or promote amblyopic visual recovery in animals subjected to a 1-week period of MD at an equivalent age (P30) (Duffy & Mitchell, 2013). Interestingly, the behavioural recovery in deprived cats occurred without recourse to the period of dark experience, be it continuous or delayed (7-8 weeks later) with respects to the period of MD. These behavioural findings were replicated in a more recent study (Mitchell et al., 2016) where MDs in kittens were initiated near birth and extended until 5 to 8 weeks of age. In addition to the complete recovery of vision in animals irrespective of the length of deprivation or age of initiation of dark experience, about a quarter of animals for which depth perception was assessed demonstrated superiority using both eyes together than when either eye was tested alone (Mitchell et al., 2016). It appears that the benefits of a brief period of complete darkness on the visual acuity of the deprived eye in kittens is restricted to an as yet unspecified period in early life as it is absent in adulthood (~1 year old). Whereas 10 days of darkness seemed to have produced unequivocal recovery in young and juvenile kittens (Duffy & Mitchell, 2013; Duffy et al., 2015; Mitchell et al., 2016), the same period of darkness was unable to stimulate complementary anatomical or visual recovery in adult cats (Holman, 2014).

As an alternative to dark rearing, which eliminates all light and thus all visually-driven neural activity in the retina, Duffy et al. (2015) examined the possible benefits of the same period (10 days) of bilateral eyelid suture (BLS) that allows light transmission but severely reduces transmission of spatial detail except at low spatial frequencies (Duffy et al., 2015). Although BLS promoted anatomical recovery of neurone soma size in the dLGN, little or no recovery of visual acuity was observed in the deprived eye. Interestingly, subsequent exposure of the same animals to a 10-day period of darkness was found to promote full recovery of the visual acuity of the deprived eye (Duffy et al., 2015). So strict are the parameters of darkness required to promote recovery of the visual acuity of the deprived eye that only a very brief period of light (15- 30mins) could eliminate any recovery of vision (Mitchell et al., 2016).

Although the mechanistic underpinnings of the dark-mediated visual recovery are as yet poorly understood, it has been suggested that the suspension of visually-driven activity by darkness effectively sets back levels of various key proteins in the visual cortex that serve as "brakes" to reduce neural plasticity of the visual cortex (Duffy & Mitchell, 2013; Gianfranceshi, 2003; O'Leary et al., 2012). Consistent with this view are the results of immuno-histochemical and biochemical studies in the LGN or visual cortex of cats and rodents which have shown that darkness reduces neurofilament stability (Duffy & Mitchell 2013; O'Leary et al., 2012), causes down-regulation of brain-derived neurotrophic factor (BDNF) expression (Gianfranceshi, 2003), reduction in cortical presence of Otx2 positive cells (Sugiyama et al., 2008) and decrease in GABAergic inhibition (Gianfranceshi, 2003; He et al., 2006), all of which have been linked to delayed maturation of the visual cortex.

2.6 Problem Statement

As summarized earlier, behavioural studies on two groups of monocularly deprived kittens exposed to 10 days of total darkness revealed two quite different outcomes, depending on whether the dark exposure occurred immediately after termination of the period of MD (at P37; Immediate Darkness group) or was delayed for about 7- 8 weeks (Delayed Darkness group: Duffy & Mitchell, 2013; Mitchell et al., 2016). Whereas the recovery of acuity in the deprived eye of the kittens in the Immediate Darkness group occurred very slowly over a period of 2 months, the recovery in the Delayed Darkness group was quick, such that normal acuity was achieved in just 5 to 7 days (Duffy & Mitchel, 2013; Mitchell et al., 2016). In addition to the different outcomes with respect to the effects of darkness on the acuity of the deprived eye, there were also some significant differences in the effects on the fellow eye. For the animals in the Immediate Darkness group, the immediate effects of dark exposure on the vision of both eyes was substantial, as the animals appeared unable to see with either eye. The subsequent recovery to normal acuity occurred in tandem for the two eyes but very slowly over a period of about 2 months. By contrast, the visual acuity of the non-deprived eye in animals assigned to the Delayed Darkness (DD) group remained unaffected by the period of darkness. Although differences in the ages of the kittens and the presence of a long period of binocular vision for kittens in the delayed darkness (DD) group could have contributed to the very different speeds of recovery, the existence of partially established binocular connections prior to the dark exposure in the latter group may be significant. In particular, it raised the possibility that the rapid recovery of vision in the deprived eye when darkness was delayed may have been guided by the previously established connections with the non-deprived eye.

Earlier, Kind et al. (2002) had suggested that the effectiveness of recovery following early MD in kittens was dependent on the degree of correlation of neural activity between the two eyes, and suggested that the non-deprived eye and its neural connections acted as a "teacher" to the deprived eye. The study described in this thesis was designed to test whether the non-deprived eye served a critical role in mediating the fast recovery following a period of darkness imposed 8 weeks after an early period of MD. To investigate this possibility, the non-deprived eye was occluded for 11days immediately after kittens were removed from the darkroom, thereby eliminating the ability of this eye and its neural connections to contribute to any recovery of the vision of the deprived eye. In addition, a group of kittens were reared that received just the period of occlusion of the non-deprived eye at the same age, but without the preceding period of darkness. The outcome in the control animals would indicate the extent of visual recovery promoted by late occlusion of the non-deprived eye alone. The results from these two groups of kittens, while not illuminating the entire mechanism of visual recovery following darkness, may serve as an important guide to the mechanism of dark-induced rapid visual recovery, and also help shape treatments that may aid clinical translation to human amblyopes.

2.7 Research Questions And Hypotheses

The aim of the research of this thesis was twofold. First, to determine the role of the non-deprived eye and its visual activity on dark-mediated rapid visual recovery; second, to compare the "rate" and "extent" of amblyopic visual recovery in reverse occluded animals to age-matched counterparts that receive 10 days of dark immersion prior to reverse occlusion.

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The following hypotheses were made with respect to the two aims. Visual recovery in animals that receive sequential exposure to complete darkness and occlusion of the non-deprived eye would not only be incomplete, but also proceed at a rate slower than the 5-7 days reported in earlier study by Duffy & Mitchell (2013). This result would support the notion that dark induced visual recovery after a period of MD is guided by visual activity in the non-deprived eye. Second, visual recovery in the deprived eye of animals exposed to both darkness and reverse occlusion would be greater, compared to their counterparts that received reverse occlusion alone, underscoring the effect of enhanced plasticity produced by a short period of darkness in the former.

In summary, it was anticipated that amblyopic visual recoveries in the reverse occlusion (ROC) group will be less with respect to both its rate and extent in comparison to those of animals in the darkness plus reverse occlusion (DR) group, which will in turn be less than those of their companions in the Delayed Darkness (DD) group reported by Duffy and Mitchell (2013).

CHAPTER 3: METHOD

3.1 Overview of Research Design

As illustrated in schematic form in Figure 1 (A, B and C), the animals for this study were reared in a manner that modelled closely those of kittens designated as members of the delayed darkness group in the prior study published by Duffy and Mitchell (2013). Each kitten received a period of MD for one week beginning on postnatal day 29 (P29), followed by a two-month period of binocular visual exposure. Half of the animals were placed in total darkness for 10 days at P92, but unlike those in the prior study, they received a period of reverse occlusion immediately upon removal from darkness at P102, in order to explore the influence of the non-deprived eye and its neural connections to the visual recovery of the deprived eye after darkness. The two other animals were not placed in darkness, but at P102, they received a period of reverse occlusion in order to determine the extent of any recovery that could be promoted by this manipulation alone when imposed at this age. The specific details for each procedure are provided under the respective headings below.

3.2 Kittens And Colony

Four kittens (2 males, 2 females) from 2 litters (C390 from one litter; C391, C392 and C393 from another) were the subjects of this study. All experimental procedures followed protocols approved by the Dalhousie University Committee for Laboratory Animals in accordance with guidelines established by the Canadian Council on Animal Care (CCAC). The kittens exhibited no detectable congenital or developmental anomalies and met normal motor developmental milestones. Apart from any period spent in the darkroom, all four kittens were housed in the normal illuminated colony (12 hours in

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light: 12 hours in darkness) from birth. They were housed together with mothers (queens) and littermates (except C390) in environmentally enriched rooms to stimulate normal social development.

3.3 Monocular Deprivation

The left eye of each kitten was deprived of pattern visual input at the peak of the period of vulnerability of the visual cortex to MD at postnatal day 29 (P29) by use of the minimally invasive procedure for eyelid closure described in earlier publications by Murphy & Mitchell (1987) and Duffy & Mitchell (2013). The procedure involved suturing of the upper and lower palpebral conjunctivae together prior to closure of the eyelids under gaseous anaesthesia (1.5-5% isoflurane in oxygen). Ethicon 6-0 vicryl and Ethicon 5-0 silk were used respectively, for the conjunctival and eyelid sutures. The sewn conjunctivae served as an additional occlusion layer to that provided by the eyelids and together they prevented diffuse light from reaching the retina. Sub-cutaneous injection of an analgesic (Ketoprofen®, 0.2ml/kg) and topical antibiotic-anti-inflammatory (Otomax®) drugs were administered to control respectively, post-operative pain and infection in line with standard operating procedures. The kittens were subsequently transferred from the surgical unit to their colonies after recovery from the effect of the general anaesthesia. The eyelids of the deprived eyes were regularly examined to ensure uncompromised effects ("window" openings) throughout the 7-day period of MD. Surgical removals of the sutures were performed under gaseous general anaesthesia at the end of the period of visual deprivation. The eyelids and conjunctivae were then parted and potential irritants to the cornea (such as polyps) excised.

3.4 Measurement Of Visual Acuity

The acuity for high contrast (Michelson contrast 1.0) square-wave gratings were estimated by use of the jumping stand described three and half decades ago (Mitchell et al., 1976; Mitchell, Giffin & Timney, 1977) and procedures that have been modified by the lab over time (Duffy & Mitchell, 2013; Mitchell et al., 2001; Murphy & Mitchell, 1987). The jumping stand consisted of two main parts: a jumping platform on which was placed a rectangular wooden box with two open ends, and beneath it, a landing area (surrounded on three sides by walls) separated into right and left halves by a 3 cm high wooden divider. The height of the stand could be adjusted in a continuous manner up to the maximum height of 72 cm by adjustment of two yoked laboratory jacks. The stimuli were 19.5cm x19.5cm ink-jet printed square-wave gratings surrounded on all sides by a 4 cm grey border. The gratings were manufactured such that their period differed by amounts that were equated on a logarithmic scale with as many as 12 steps per octave. This stands in contrast to the 3 steps per octave employed in clinical Teller Acuity Cards employed for preferential looking studies with children. The small and regular step size between gratings ensured consistent performance from kittens with change in grating period that in turn results in precise determination of threshold acuity. It was typical of kittens to drop from near flawless performance to chance in just one or two steps comparable to 12% change in spatial frequency (Duffy & Mitchell 2013; Gingras et al., 2005; Mitchell et al., 2016; Murphy et al., 2015). The mean luminance of each grating illuminated by an incandescent lamp was 100 cd/m^2.

In order to elicit reliable responses, kittens were trained on the jumping stand from 29-35 days of age according to their motor development and ability to jump. Animals were first trained to step safely off the jumping platform lowered to its minimum height

(level with the wooden divider) onto a vertical grating of the highest period (32 mm period: spatial frequency of 0.39 cycles/degree at 72 cm) placed on one side of the landing platform, as opposed to stepping onto an adjacent "open door" (a 40cm deep hole). As was the case for all four kittens, the initial step was a careful one made with one paw on the wood divider while using the other to search for the grating on the adjacent closed door. Appropriate responses were immediately rewarded with wet kitten food placed on a wooden popsicle stick or by petting. After the animal had gained confidence and successfully walked onto the grating on 5 trials, the jumping platform was gradually raised a few centimetres so that the animal now had to step or make a small jump onto the grating. After ten such trials, the other half pair (horizontal) grating was introduced. As before, the animal was rewarded for stepping onto the vertical grating. Errors in response resulted in denial of the rewards and immediate repetition of the trial. The pattern of presentation of the stimuli was quasi-random according to a Gellerman Series where no more than two consecutive presentations of positive stimuli are made on the same side to avoid the development of a side preference (Gellerman, 1933).

Over the next few days, the height of the jumping platform was raised gradually in order for the animal to transition between stretching and then leaping onto the vertical grating. A kitten was considered trained after 20 consecutive correct responses were made and this was generally attained in 3-4 days of training. Once kittens could leap onto the grating, the period of the grating was reduced and the animal required to make 5 consecutively correct responses before the next grating was introduced. Pairs of horizontal and vertical gratings of progressively increasing difficulty (smaller period or increasing spatial frequency) were then presented in sucession with minimal adjustments made to the height of the jumping stand until the criterion acuity was no longer met.

When animals had demonstrated an ability to perform the task and appeared to be performing on the basis of visual information, formal measurment of the visual acuity was made. Operationally, the acuity was defined as the highest spatial frequency for which the kitten met the criterion of 5 consecutive correct responses or 7 out of a maximum of 10 trials for each grating period. The highest spatial frequency at which criterion performance was elicited was recorded together with the height at which jump was made, in order to calculate the threshold acuity for a trial session.

As indicated earlier, positive and negative stimuli were presented in a quasi-randomised pattern; alternations of right to left as well as consecutive trials to one or the other side ensured that no single grating orientation was presented on the same side on more than two consecutive occasions (as in Gellerman series) in a trial block. The order of presentation was designed to minimize the adoption of side preferences or an alternating response pattern, the two most common non-visual patterns of behaviour adopted by kittens when visual discrimination becomes difficult. In order to control against the possibility that the animal could detect a particular grating stimulus (using cues such as tiny blobs of food or particles on the grating or its border) rather than its orientation, individual gratings were often re-orientated by 90 degrees rather than a simple change of side. Occasionally, if an animal developed a preference towards one side, presentation of twice as many trials on the opposite side usually eliminated the bias. Once such a bias was corrected, presentations of stimuli were reverted to a pseudo-randomised pattern.

As visual acuity improved, kittens received only a single trial on gratings of very low spatial frequency in order to minimize the length of a daily session and maintain motivation for the entire session, which usually lasted about 20-25 minutes. Typically, the

minimum number of trials per grating size was progressively increased from about an octave from the previously established threshold to 2, 3 and 5. For any spatial frequency, it was necessary for the kitten to reach the established criterion performance of 5 consecutive correct responses or 7 out of 10 trials. In addition to the failure of the animal to achieve this level of performance beyond the threshold acuity, kittens exhibited other signs, such as increase in latency of response, meowing and looking around at the experimenter and/or the ceiling. When they eventually jumped, the responses were usually to the side of last reward or reversion to side preferences.

Binocular measurements of acuities were used as surrogates for the acuity of the non-deprived eye. The acuity of the deprived eye was made by use of an opaque contact lens of appropriate curvature and diameter placed on the cornea of the non-tested eye after instillation of one or two drops of topical proparacaine (Alcaine®) into the lower conjunctival *cul de sac* to anaesthetise the ocular surface. Measurements of the acuity of this eye were made on either the same day or 24 hours after binocular measurement. Once binocular thresholds were achieved at levels comparable to normal kittens of equivalent age, binocular measurements were repeated every 3-5 days starting with a grating having spatial frequency at least 3 octaves lower than the threshold acuity. Monocular measurements were repeated each day or every other day until acuities stabilised and continued until 3 months of age.

3.5 Exposure to Complete Darkness

The 10-day period of darkness occurred in a special darkroom facility located in the Department of Psychology and Neuroscience. The design (depicted in Figure 2) and operation of the facility have been described briefly as part of earlier studies (Beaver, Mitchell & Robertson, 1993; O'Leary et al., 2012) but a more complete account has only been provided recently (Duffy & Mitchell, 2013). Each animal was kept in a special large cage (length: 1.5m; Breadth: 0.7m; Height: 0.9m; and one ledge at both ends raised 0.4m from the floor) equipped with a litter box, 3 feeding troughs, 1 cardboard box for play and an additional one for bedding. The cage contained the daily supply of food and water to be consumed *ad-libitum*. The darkroom facility was also furnished with a radio set timed to turn on and off automatically at 7 o'clock in the mornings and evenings. This provided a 12-hour cycle of sound to establish a circadian rhythm in the absence of light.

Animals were transferred temporarily into a dark holding room (D2) adjacent to the main darkroom (D1) to allow daily cleaning and replacement of food, water, litter box and bedding box. They were transferred back into the main darkroom once the cleaning and replacement processes were completed. Room cleaning and feeding were carried out around the same time each day to maintain a routine for each kitten.

3.6 Reverse Occlusion

The four kittens received a period of reverse occlusion beginning at 102 days of age (P102). For two animals (C390 and C393), this manipulation occurred at the end of a 10-day period of darkness. These kittens were placed in a special opaque box in the darkroom and then moved quickly to the surgical unit for induction of gaseous

anaesthesia. The carrying box was fitted with tubes to permit induction of anaesthesia to occur without the necessity for the animal to be removed, thereby minimizing any visual exposure between the time the kitten was removed from the darkroom and anaesthetisation. After full anaesthesia in 3-5% gaseous isoflurane in oxygen, the kitten was removed from the box and the eyelids of the previously non-deprived eye were sutured together by use of the same procedure as employed for the initial period of MD described earlier.

The two control animals (C391 and C392) also received their period of reverse occlusion at the same age (P102). All four animals received sub-cutaneous ketoprofen injection (Anafen®; 0.1 cc), 0.5% topical proparacaine (Alcaine®) and topical gentamicin-betamethasone-clotrimazole (Otomax®) for post-operative analgesia and prophylaxis, respectively. The period of reverse occlusion was terminated after 11 days for C390 and C393, 17 days for C391, and 24 days for C392 by use of the same procedures as employed to terminate the initial period of MD.

3.7 Documentation of Visual Recovery

Measurement of visual recovery of the formerly deprived and non-deprived eyes began 2 hours after animals had recovered from any surgery. Estimates of sensory visual thresholds (spatial acuity) were made around the same time each day. Behavioural measurements of visual acuity were conducted each day during the reverse occlusion period. These were modified so that tests were conducted daily or on alternate days after cessation of reverse occlusion.

3.8 Objective Refraction

To answer the question as to whether the lack of visual recovery could be due to other confounding factors, including unequal refractive state of the eyes, non-cycloplegic objective refraction (using a hand held Retinomax K-plus 3®, Rigton, Japan) was performed on all kittens at the end of the period of reverse occlusion. The results were also confirmed by streak retinoscopy.

3.9 Assessment of Ocular Alignment

Gross alignment of the visual axes of the eyes was assessed for each animal after termination of reverse occlusion. This was done to rule out possible contribution of manifest strabismus to visual recovery after cessation of interventions. The positions of the corneal light reflexes in both eyes were assessed directly by holding a point source of light (trans-illuminator light of an ophthalmoscope set: Keeler Professional®) midway between the two eyes at about 67cm distance from the frontal plane of the cornea. For one kitten (C392), an indirect assessment was made from a photograph taken later.

3.10 Data Analysis

Raw data (stimuli period in mm) were first converted into spatial frequencies (cycles/degree). The results were plotted graphically using Data Graph 6 ®. The nature of the results allowed for both intra-animal and group comparisons. In effect, each animal served as its own control by virtue of the visual acuity of the non-deprived eye (assessed by binocular means). Due to the limited number of animals in each group and the difficulty in assessing the variance of data for small samples, a non-parametric equivalent

of t-test (Mann Whitney U test) was used to compare the pre-intervention acuities of the

deprived eye. Statistical significance was set at *p-value* of < 0.05.

CHAPTER 4: RESULTS

Behavioural training began during the period of MD and in the days that followed in order to permit accurate assessment of the visual recovery of the deprived eye from the end of the period of MD. Once trained, measurements of the acuity of the deprived eye were made with greater frequency than binocular measurements which served to familiarize the animal with difficult visual discriminations. The results of longitudinal measurements of the visual acuities of the two eyes are displayed separately for each of the two animals in the experimental (DR) and reverse occlusion (ROC) groups in Figures 3 through 8. In view of the extended time scale that was necessary to display the complete data from the end of the early period of MD, this data is shown only for two animals, C390 (Figure 3) and C393 (Figure 4). The results obtained in the period surrounding the two key manipulations, darkness and/or occlusion of the non-deprived eye, are plotted for all 4 animals in Figures 5-8 in order to highlight the effects of these manipulations on the spatial acuity of each eye. Additionally, Figures 9 and 10 are provided to illustrate respectively, the summary of the 'extent' and 'rate' of recovery of vision in the amblyopic eye of animals of equivalent age following the application of reverse occlusion alone (ROC), dark experience prior to reverse occlusion (DR), and dark experience followed by binocular exposure (DD group: Duffy & Mitchell, 2013).

The data of Figure 3 and 4 display a common pattern of recovery of the vision of the deprived eye following the period of MD. Animals appeared blind on the jumping stand with the deprived eye in the first two days after termination of MD. In the course of the next 3-4 days, the vision of this eye recovered to permit first, the detection of an open door from a closed door on the jumping stand, designated as "open door" (OD), followed

by the ability to discriminate between a vertical and horizontal square-wave grating of the same very low spatial frequency. The visual acuity of the deprived eye (open circles) continued to improve at a slow pace to a stable level at about 50 days of age (Figures 3 and 4), by which time deep amblyopia was evident. In contrast, binocular visual acuity (filled squares), an indication of the vision of the non-deprived eye, improved steadily to attain normal adult levels at between 60 to 65 days of age. The visual acuity of the deprived eye of all four animals reached a stable level about 4-6 weeks prior to the commencement of dark exposure and/or occlusion interventions. A summary of the binocular and monocular acuities before interventions for all 4 animals and those of animals which received the same period of MD and complete dark exposure at the equivalent age in an earlier study (Duffy & Mitchell, 2013) is presented in Table 1. The data for the two animals in each group before and after the key interventions of darkness and/or occlusion of the non-deprived eye are described separately below.

4.1 Visual Recovery In Animals That Received A Period Of Reverse Occlusion Immediately After 10 Days Of Darkness

The two animals (C390 and C393) assigned to this group were removed from the darkroom in a light-proof induction box and taken swiftly to the surgical unit where they were immediately anaesthetized, at which time the non-deprived eye was occluded. The initial measurements of the acuity of the deprived eye were made about 2 hours after this surgery and daily thereafter. In stark contrast to the rapid and full recovery of the visual acuity of the non-deprived eye reported earlier for animals with a similar period of early MD and delayed darkness exposure (Duffy & Mitchell, 2013; Mitchell et al., 2016), the acuity of the deprived eye of C390 and C393 changed very little in the 11 days following

darkness. For C390 (Figure 5), the acuity of this eye improved from the baseline level

(0.56 cycles/degree) to only 1.0 cycles/degree in 11 days. Although this represented an

improvement of about one octave, the level achieved was still well below normal levels

(7.4 cycles/degree). Remarkably, no further improvement of the acuity of this eye

occurred following termination of the 11-day period of occlusion of the non-deprived eye.

However, a moderate reduction of the grating visual acuity of the non-deprived eye was

observed as reflected by the measurements of the binocular visual acuity immediately

after termination of reverse occlusion. Whereas the acuity of this eye had attained a value

of 7.39 cycles/degree prior to the period of darkness, a value that would have been

potentially maintained through to the end of the 10 days of darkness (Duffy & Mitchell,

2013; Mitchell et al., 2016), visual acuity dropped to 5.71 cycles/degree immediately after

the ensuing 11-day period during which this eye was occluded. Although the acuity of

this eye improved a little in the next week to 6.16 cycles/degree, no further improvement

was observed in the ensuing 6 weeks of binocular visual exposure. The loss of acuity in

the previously non-deprived eye bears some resemblance to occlusion amblyopia

observed in rare cases during patching therapy in amblyopic children (Scott et al., 2005;

Varadharajan & Hussaindeen, 2012). In agreement with many measurements made in the

past (Duffy & Mitchell, 2013; Mitchell et al., 2016), the monocular visual acuity for the

initially non-deprived eye (filled circles: Figure 5) assessed at the tail end of the

experiment was found to be the same as the binocular visual acuity value. As seen in most

cases of clinical assessment of vision, the binocular visual acuity largely reflects the

acuity of the better of the two eyes.

The recovery of visual acuity of the deprived eye of C393 (Figure 6) closely

paralleled the results described for C390. The acuity of the deprived eye prior to the

period of darkness was 0.56 cycles/degree, a value identical to that of the deprived eye of

C390 at equivalent stage. During the 11-day period of reverse occlusion that immediately

followed, the acuity of this eye improved only marginally to 0. 70 cycles/degree. The

eyelids of the non-deprived eye appeared swollen when they were parted after the 11 days

of reverse occlusion, and in the ensuing days, the swelling was accompanied by a corneal

haze. The eye was treated by frequent application of topical antibiotic ointment first

(Ciloxan®), followed by antibiotic–anti-inflammatory combination (Otomox®).

Although the corneal haze abated gradually, the condition of the cornea and the eyelids

precluded the use of a contact lens occluder in this eye for about 10 days. Consequently,

measurements of the acuity of the deprived eye could not be made in this period. Even

though binocular measurements of visual acuity could be made in order to ascertain the

acuity of this eye, it is possible that the acuity may have been under-estimated due to the

corneal haze. Such measurements were likely to be affected most in the immediate period

following the end of the11-day period of occlusion of the non-deprived eye. A binocular

measurement of acuity made immediately after termination of reverse occlusion indicated

that the acuity of the non-deprived eye was lower (4.4 cycles/degree) than that of the

same eye of C390 (5.7 cycles/degree) at the equivalent stage. However, it is unlikely that

acuity measurements made 10 days later were contaminated as the corneal haze had

retracted from the centre of the pupil and was of lower density. Supporting this assertion

was the fact that the binocular acuities measured at this time point were higher

(5.7 cycles/degree) and similar to the binocular acuity of C390 at the same time.

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A noteworthy aspect of the results following the termination of the relatively short (11-day) period of occlusion of the non-deprived eyes of C390 and C393 was the absence of any improvement in the acuity of the deprived eye once vision was restored to the fellow eye. This occurred just 11 days after the period of darkness, at which time, simultaneous visual input was available to both eyes. However, in dramatic contrast to the results obtained in the prior study (Duffy & Mitchell, 2013) where binocular visual input occurred immediately after the period of darkness and the acuity of the deprived eye recovered quickly to normal levels, a delay of 11 days before binocular visual input was provided prevented any recovery of the acuity of this eye.

4.2 Recovery Promoted By Reverse Occlusion Alone Imposed At Equivalent Age

The extent to which a period of reverse occlusion could stimulate behavioural recovery of the visual acuity of the deprived eye from prior MD was assessed in two animals (C391 and C392) that did not receive a preceding period of darkness. These two animals, to a greater extent, served as controls to the experimental animals (C390 and C393). The pattern of visual recovery in the amblyopic eye of C391 (Figure 7) prior to the key manipulation of reverse occlusion was virtually identical to that described for the experimental animals (C390 and C393) in the same time period (also see Figure 10). In particular, the acuity of this eye recovered slowly to the same low value as observed in all 4 animals prior to the initiation of interventions. As evident from Figures 7 and 8, the acuity of the deprived eye changed very little during the period of reverse occlusion that was imposed at the same age (P102) as that for the animals that received a prior period of darkness (Figures 5 and 6). Even though the period of reverse occlusion was longer (17 days for C391 and 24 for C392) than that for C390 and C393 (11 days), the change in the

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acuity of the deprived eye was minimal (C391: Figure 7) or non-existent (C392: Figure 8). Although the period of reverse occlusion proved of little benefit for the visual acuity of the deprived eye, it did have negative consequences for the acuity of the non-deprived eye in both animals, particularly for C392 for which the acuity was reduced by more than an octave (from 7.39 to 3.14 cycles/degree)

4.3 Rate Of Recovery Of Vision In the Deprived Eye

To compare the rate of recovery of visual acuity of the deprived eye, linear fits were calculated for the recovery data over the 11-day period following the application of darkness and/or occlusion for the experimental and control animals and compared to the two animals for which recovery was slowest (7 days) among those designated as members of the delayed darkness group in an earlier study (Duffy & Mitchell, 2013). Using the equal of a straight line, $y = mx + c$, where 'm' and 'c' represent respectively, the gradient (slope) and y-intercept, the gradient (slope) for each line provides an estimate of the 'rate' of recovery of vision in the deprived eye. The results depicted in Figure 10 indicate that the daily rate of recovery of the acuity of the deprived eye of animals in this study was just 0.015 and 0.027 cycles/degree respectively, for animals that receive reverse occlusion alone (ROC: triangles) or a period of darkness prior to reverse occlusion (DR: circles). By contrast, the acuity of the two animals from the DD group (diamonds) in the prior study of Duffy & Mitchell (2013) that recovered the slowest was 0.813 cycles/degree per day. This indicates, at least, that occlusion of non-deprived eye after the period of dark experience reduces the speed of recovery of vision in the amblyopic eye by as much as 30 fold with respect to the rate of visual recovery of age-matched animals in the delayed darkness group (Duffy & Mitchell, 2013). Similarly, the

rate of recovery of vision for animals that received reverse occlusion alone (ROC) is 54

folds that of the 2 animals in the delayed darkness group (DD) which recovered the

slowest.

CHAPTER 5: DISCUSSION

Previously, it had been shown on monocularly deprived kittens (Duffy &

Mitchell, 2013; Mitchell et al., 2016) that the speed of the improvement of the visual

acuity of the deprived eye induced by a 10-day period of total darkness depended on the

timing of the latter with respect to the prior period of MD. When the period of darkness

followed immediately after an early 7-day period of MD initiated at P30, the animal

appeared blind in both eyes. However, the vision of both eyes recovered slowly thereafter

at an equal rate to eventually attain normal age-matched acuity levels in about 50 days.

By contrast, when the period of darkness was delayed by 2 months with respect to the

same early period of MD, the visual acuity of the deprived eye recovered to the normal

levels previously attained by the non-deprived in just 5-7 days or about 8-10 times faster

than when darkness was imposed immediately after the period of MD. The explanation

for the very different rates of recovery of the acuity of the deprived eye in the two rearing

situations that was made in the literature review (Chapter 2) was that it could follow

directly from the operation of a single rule. Thus, recovery was guided in some way by

cortical neural activity mediated by connections with the non-deprived eye. The simple

hypothesis was that neural activity induced by visual stimulation of the non-deprived eye

guided the recovery of neural connections with the deprived eye, and with refinements of

those connections, the visual acuity of the deprived eye. When darkness followed

immediately after the period of MD, neural connections with both eyes were likely poor,

so that connections with the non-deprived eye could not serve as an active guide for

recovery of connections with the deprived eye. By contrast, when darkness was delayed,

connections with the non-deprived eye were well established and so could guide the

recovery of connections with the other eye. The simple test of this explanation was to occlude the non-deprived eye at the time the animal was removed from the darkroom, so as to eliminate the ability of this eye to serve as a guide for recovery. To this end, two animals were reared in the identical manner to those of the delayed darkness group in the earlier publication (Duffy & Mitchell, 2013), with the exception that the non-deprived eye was occluded for a period of 11 days immediately after dark exposure. The control group for this manipulation consisted of two cats that received the same early period of MD, and later received a period of occlusion of the non-deprived eye at the same age as those of the experimental group but without a prior period of darkness.

5.1 The Extent Of Dark-Induced Visual Recovery In The Absence Of Guidance By The Non-Deprived Eye

The results from the two animals in the experimental group were unequivocal as they demonstrated that elimination of visual input to the non-deprived eye for 11 days after the animal was removed from darkness virtually eliminated any recovery of the visual acuity of the deprived eye. For C390, the acuity of the deprived eye improved in this period from 0.56 to only 1.0 cycles/degree, while for C393, the improvement was even smaller, from 0.56 to 0.7 cycles/degree. In striking contrast, all 4 animals reared in the prior study (Duffy & Mitchell, 2013) in an identical fashion with the one exception that both eyes were open immediately after the period of darkness, recovered normal visual acuity (6.5 to 7.1 cycles/degree) in less than a week. This point is highlighted by Figure 9 which compares the extent of acuity achieved by the deprived eye of C390 and C393 during the 11 days that followed the period of darkness during which the non-deprived eye was closed, to those achieved by the 2 animals that received a period of reverse occlusion only, and the 4 animals in the delayed darkness (DD) group of prior

study (Duffy & Mitchell, 2013). Although the number of animals in the groups are small, the mean visual recovery in the deprived eye of animals that were reverse occluded after a period in the dark (DR group: 0.33 cycles/degree) was about a factor of 11.5 lower than the mean recovery for animals in the earlier study (Duffy & Mitchell, 2013) that received binocular visual exposure preceded by the same period of darkness (DD group: 4.12 cycles/degree). The results for animals that have both eyes open after the period of darkness is bolstered by subsequent observations (Mitchell et al., 2016) of comparable results obtained on many more animals that received even longer periods of prior MD. The lack of substantial recovery of the visual acuity of the deprived eye of the two animals that had the non-deprived eye closed after the period of darkness (DR group) points not just to the fact that restriction of visually-driven neural activity from the fellow eye constraint dark-mediated recovery of vision of the amblyopic eye, but also suggests that occlusion of the fellow eye may convey little or no benefit at the age at which it was initiated (P102). Further data relevant to this particular point was obtained from the two animals in the control group as described below.

The results from animals in the DR group (C390 and C393) represent a test of the idea of the beneficial effects of binocular cooperation (Kind et al., 2002; Smith & Trachtenberg, 2007) and adds to a growing body of evidence that supports binocular approaches to the treatment of amblyopia in cats (Duffy & Mitchell 2013: Kind et al., 2002; Mitchell et al., 2001; Mitchell & Duffy, 2014; Mitchell et al., 2016; Murphy et al., 2015) and humans (Hess et al., 2010a, b; 2011; Li et al., 2013; review by Birch, 2013 p.79 & 80). The findings are also in agreement with reports that have shown a weighted preference of the visual system for binocular visual input in early postnatal visual

development (Mitchell, Kennie & Duffy, 2011; Mitchell & Duffy, 2014).

Kind and colleagues (2002) had earlier demonstrated that the rate and extent of amblyopic visual recovery in cats were dependent on the degree of correlation of neural activity between the two eyes and suggested that the non-deprived eye and its neural connections act as "teacher" to the deprived eye. Although their observations were made on the basis of comparisons of the degree of recovery observed in monocularly deprived and strabismic cats that subsequently received binocular visual exposure, the results from the experimental animals in this thesis demonstrate that the assertion also holds true for the recovery of vision provoked by a period of total darkness.

The idea of the non-deprived eye and its neural connections acting as "teacher" (Kind et al., 2002; Smith & Trachtenberg, 2007) or "guide" for amblyopic visual recovery has been explored in three different scenarios related to the strength of neural connections and, hence, the level of visually driven activity of the non-deprived eye. Depending on the situation, the role of the non-deprived eye can be considered as either "active", "passive" or "temporarily inactivated". The recovery that follows the restored neural plasticity induced by total darkness may represent an example of an active role for the non-deprived eye where binocular visual input following the period of darkness kindles a Hebbian-like associative learning mechanism (Hebb, 2002), culminating in accelerated recovery of vision in the deprived eye. This proposed mechanism suggests that the highly refined neural connections with the non-deprived eye serves as an instructive "guide" for the recovery of the vision of the amblyopic eye and contributes to the speed of the visual recovery.

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A passive role for the non-deprived is provided by the situation where neural connections with the deprived and non-deprived eyes are equally poor, so that no eye can serve as a "teacher" for the other (Smith & Trachtenberg, 2007). The slow and protracted improvement of vision in either eye after darkness is imposed immediately after the period of MD (Immediate Darkness group: Duffy & Mitchell, 2013; Mitchell et al., 2016) could reflect the operation of a passive, "novice-to-novice" cooperative mechanism. The situation that held for the kittens (DR group) in this thesis may represent a third scenario based upon a temporal suspension of pattern vision in the non-deprived eye by eyelid suture (reverse occlusion) immediately following a period of darkness. In such a context, the benefit of a "guide" is eliminated during the period of closure of the non-deprived eye. These results suggest that the rate and extent of visual recovery promoted by 10 days of darkness may be driven not only by the simultaneous presence of pattern vision in both eyes, but also by the quality of the neural activity from the non-deprived eye.

Earlier studies have demonstrated the existence of functional inhibitory (suppressive) connections with the deprived eye (Freeman & Ohzawa, 1988; Sclar, Ohzawa & Freeman, 1986). These residual neural connections may provide anatomical scaffold or template for functional recovery during treatment (Mitchell & Sengpiel, 2009; Mitchell & Duffy, 2014). The neural connections with the deprived eye of the animals in this study may have been strengthened by the 2-month period of binocular exposure that followed the period of MD. Thus, by virtue of these binocular connections, the previously deprived and non-deprived eye could be considered as somewhat "wired together" and therefore can "fire together" on re-activation of juvenile-like plasticity by darkness. It is likely that the reverse occlusion initiated after dark exposure in the experimental group of animals may have disrupted these partially established binocular connections so that there

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was an insufficient binocular neural substrate to guide visual recovery.

The pre-intervention acuities of the deprived eyes for the animals in the present study are significantly lower (Table 1: Mann Whitney U test, $p = 0.017$) than those reported for age-matched animals subjected to equivalent period of MD (Duffy & Mitchell, 2013). This may create a false impression that the failure of substantial visual gain in the deprived eye of the animals in the present study could be attributed to the greater depth of amblyopia. However, the pre-intervention acuity of the deprived eye of one of the animals (identified as C221) described in a recent publication (Mitchell et al., 2016) was as severe and demonstrated rapid and complete recovery after the same period of darkness. Although, the acuities for the non-deprived eyes measured for the current animals prior to interventions appear significant higher (Mann Whitney U test, $p = 0.011$) than those of the delayed darkness group (DD: Duffy & Mitchell, 2013), it is noteworthy that those acuities fell within the normal range for that age (6.5 – 8.6 cycles/degree).

Because the rate and extent of any recovery of the visual acuity of the deprived eye of dark imposed and reverse occluded (DR) animals could reflect the benefits of either darkness or reverse occlusion or both, a control group of two animals was included that were reverse occluded at the same age as the experimental group but without the preceding period of darkness. That the minimal visual recovery observed in the two animals of the control (ROC) group was similar in magnitude to that observed in the two experimental kittens, raises the intriguing possibility that even the minimal gain of acuity of the deprived eye observed in the experimental group could be attributed solely to the period of reverse occlusion rather than the prior dark exposure. In other words, occlusion of the non-deprived eye following the period of darkness effectively eliminated the

benefits of the plasticity induced by the preceding period of darkness.

5.2 Reverse Occlusion After 10 Days Of Darkness Promotes Visual Recovery In Adult Rats But Not In Juvenile Cats

The result from the experimental group of animals presented in the current study is at odds with reports on monocularly deprived adult rats (He et al. 2007; Montey & Quinlan, 2011) for which occlusion of the non-deprived eye following a period of darkness was found to promote remarkable restitution of the visual acuity of the deprived eye as assessed by measurements of visual evoked potentials (VEP). The difference in results between cats and rats for equivalent manipulations could be attributed to several factors. These include the very different decussation patterns of retinal ganglion cell axons at the optic chiasm (~90% versus 50% decussation for rats versus cats), the very different effects of MD, and potential differences in the role of binocular cortical cells in the two species. Whereas it is possible to link the presence of binocular cortical cells to stereoscopic vision in cats, for rodents, binocular cells have been suggested as enabling rats to enhance their overhead visual field for the detection of predators (raptors) at the expense of fusion (Wallace et al., 2013). Second, the range of normal visual resolution in rats as estimated by electrophysiological methods (VEPs) (Dean, 1981; He et al., 2007; Pizzorusso et al., 2006; Silveira, Heywood & Cowey, 1987) or on behavioural tasks (Pizzorusso et al., 2006; Prusky, West, & Douglas, 2000) is about 0.9–1.2cycles/degree. This compares to about one-sixth of the resolving power of the cats' eye. In like manner, the effect of long term MD on the acuity of the deprived eye of rats is minimal (0.2 -0.8 cycles/degree: He at al., 2007; Pizzorusso et al 2006), whereas a 1-week period of monocular eyelid closure in a month old kitten could be dramatic, rendering the visual acuity of the affected eye substantially reduced by as much as 3–4 octaves.

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Finally, bilateral visual exposure after a period in the dark promotes some level of amblyopic visual restitution in adult rats (He et al., 2007), but the same procedure does not stimulate behavioural recovery from the effect of MD in adult cats (Holman, 2014). Thus, it appears that the mechanism of dark-induced visual recovery in rodents and carnivores and non-human primates may differ in terms of the level of binocular cooperation at the visual cortex as well as the age of an animal.

5.3 The Plasticity Induced By Darkness Is Short-Lived

Because the period of reverse occlusion that followed the period of darkness lasted only 11 days, it might be thought that some of the residual plasticity induced by the prior period of darkness would remain at the time that binocular visual input was restored. The lack of any substantive improvement of the acuity of the deprived eye upon restoration of correlated binocular visual input (at P113) raises at least three possibilities.

First, the plasticity induced by 10 days of darkness may be short-lived, not exceeding 11 days. The assumption consistent with this is that there would be little evidence of any amblyopic visual recuperation after 11 days of termination of dark exposure, irrespective of the nature of the succeeding visual experience, whether it be binocular or monocular. Such theory is largely inconsistent with evidence available that demonstrate that dark-mediated cortical plasticity can, indeed, sustain visual recovery beyond 11 days of binocular visual exposure after a period in the dark. In particular, evidence from a recent study (Duffy et al., 2015) that demonstrates that darkness could promote recovery of amblyopic vision within a period spanning 14 to 230 days when imposed on $4^1/_2$ - 5 month old cats, implicitly suggests that the plasticity induced by this

procedure can be enduring, albeit slow, especially in the circumstance where the deprived and non-deprived eye acuities are equally constrained (Duffy et al., 2015).

Alternatively, the period of plasticity induced by darkness may be considered as relatively extended, but its benefits restricted by a discrete time window during which the non-deprived eye can serve as a guide to recovery of the acuity of the amblyopic eye after restoration of visual input to both eyes. The most appealing feature of the hypothesis of limited duration for commencement of binocular exposure (<11 days) is the insight it provides for the rapid amblyopic recovery occasioned by darkness. A third possibility is predicated on the idea that the plasticity induced by darkness peaks suddenly in the first few days and progressively declines over time in a fashion analogous to the pattern of susceptibility of the cat's visual cortex to MD described by Hubel & Wiesel (1970) and Olson & Freeman (1980a). Thus, in the prime stage of dark-induced plasticity, correlated binocular visual experience may "trigger" and actively "sustain" the process for amblyopic recovery until completion, no matter how long it takes. In the absence of binocular visual input, as in the case of reverse occlusion, dark-induced plasticity may be un-sustainable and, therefore, declines rapidly. A low threshold may be reached within the first few days (<11 days), after which time little or no visual recovery may be provoked, in spite of the fact that binocular viewing conditions may still be prevalent.

Under binocular viewing conditions, the presence of visual misalignment (strabismus) is known to affect the extent of amblyopic recovery in cats (Kind et al., 2002). The fact that no gross manifest strabismus was observed in our cats when assessed directly by the position of corneal light reflexes to a point source of light or indirectly from sharp photographs, virtually minimises the possibility that the lack of drastic improvement in spatial resolution (following binocular visual restoration) could be related

to a confounding amblyogenic factor in the form of strabismus. Unlike monkeys, where the prevalence of naturally occurring strabismus has been estimated at approximately 4% (Kiorpes, Boothe, Carlson & Alfi, 1985), spontaneous strabismus in normal pigmented cats apart from those reported for non-Siamese species (Grünau & Rauschecker, 1983; Hoffmann & Schoppmann, 1984), are apparently not very common. In any case, assuming a microtropia (smaller angle of squint < 5°: Lang 1974) was missed due to the obvious challenges associated with the application of 4 prism dioptre base-in/base-out test on kittens, the presence of an *area centralis* in the retina of cats as opposed to *fovea centralis* in monkeys or humans suggests that this small misalignment of visual axis, if present at all in the former, may have had little consequence on recovery of vision. Thus, the lack of functional gain of vision in experimental animals following termination of reverse occlusion and restoration of binocular vision may be more related to reduced neural plasticity and/or binocular substrate than it is to an undetected microtropia.

5.4 Reverse Occlusion Alone Initiated At Around 102 Days Of Age Fails To Promote Any Visual Recovery

The rearing of two animals for reverse occlusion alone provided added benefit for comparison of acuity recovery between the current animals (controls) and those that received a period of complete visual silencing by darkness prior to binocular visual exposure (Duffy & Mitchell, 2013). The fact that 17 or 24 days of reverse occlusion alone initiated at about P102 promoted limited recovery of acuity from MD, as compared to the complete recovery promoted by a shorter period of darkness at P112 (Mitchell et al., 2016) or about 5 months of age (Duffy et al., 2015), provides testament to the efficacy of the latter procedure in not just stimulating much greater recovery, but also doing so even

at a later stage in animals' life.

A rich corpus of studies exists on the effects of timing of reverse occlusion on the morphological, and/or functional effects of an early period of MD (Blakemore & Van Sluyters, 1974; Dew & Wiesel, 1970; Dürsteler et al., 1976; Mitchell 1988; Mitchell 1991; Mitchell et al., 1984a, b; Movshon, 1976a, b; Murphy & Mitchell, 1987). In most of these studies, the prospect of obtaining favourable gain of vision by reverse occlusion was higher (although outcome was not always sustained) when therapy was applied very early (~2-3 months of age). The observation of limited visual recovery in the deprived eye of the adolescent animals that were subjected to reverse occlusion alone (at P102), is largely consistent with the prior evidence of declining capacity of this procedure to stimulate recovery from prior MD, especially when such an intervention is initiated at 3months of age and extended even over a period of one year (Wiesel & Hubel, 1965b). This phenomenon is also generally considered as a reflection of the declining capacity for plasticity with age (Blakemore & Van Sluyters, 1974; Dew & Wiesel; 1970; Hubel & Wiesel, 1970; Wiesel & Hubel, 1965b). However, the concurrent development of occlusion amblyopia in the previously non-deprived eye for the control group of animals points to the existence of yet another form of plasticity within the same visual system. Taken together, these observations suggest that, first, recovery from the effects of MD by a process of reverse occlusion is mechanistically different from that involved in the induction of the effects of sensory deprivation, although they may look procedurally similar. Second, the profile of the critical period for recovery of visual acuity by reverse occlusion in cats may be shorter (less than 15 weeks or 103 days) than the period of susceptibility of the visual system to the debilitating effects of the same manipulation or

MD (6-8 months: Daw et al., 1992; Freeman & Olson, 1980; Jones et al., 1984). This idea is consistent with the general view of different critical periods for recovery and induction of sensory deprivation (Berardi, Pizzorusso & Maffei, 2000; Daw, 1998; Lewis & Maurer 2005). Such a claim is further strengthened by evidence that the capacity of reverse occlusion to engineer recovery or switch in cortical ocular dominance is confined to the first 14 weeks of postnatal life (Blakemore & Van Sluyters, 1974), an interval shorter by several months the documented period of vulnerability of a cat's striate cortex to MD (Freeman & Olson, 1980). Thus, in principle, despite no substantial gain of visual acuity of the deprived eye of animals subjected to reverse occlusion alone, results from previous behavioural studies where this treatment was applied quite earlier in cats (Dew & Wiesel, 1970; Giffin & Mitchell, 1978; Mitchell 1988; Mitchell 1991; Murphy & Mitchell, 1987) give resounding assurance for visual recovery in our reverse occluded animals had non-deprived eye closure been embarked on earlier than $3^{1}/_{2}$ months.

The question as to whether the failure of considerable gain in the acuity of the deprived eye in the animals used for this study could be partly attributed to uncorrected refractive errors, provoked further examination. At least in monkeys, the development of significant myopia has been reported following early MD by eyelid closure (Hanverth et al., 1989; Qiao-Grider et al., 2004; Raviola & Wiesel, 1978). Using the general lens formula of $P = 1/d$ (in metres), where 'P' is the refractive power in dioptres and 'd' is the distance or observation height in metres, it can be argued theoretically that our kittens would have had to develop refractive errors of approximately 1.50 dioptres ($P = 1/0.72$) or greater to have any impact on the visual acuity results at maximum observation (jumping stand) height of 72 cm. The absence of significant bilateral refractive errors or profound

difference in refractive states between the deprived and non-deprived eyes (spherical equivalence of $\sim \pm 0.25$ DS) when assessed by non-cycloplegic retinoscopy and auto-refractometry ruled out possible contribution of uncorrected isometropia or anisometropia. Furthermore, previous studies that employed cycloplegic (atropine) refraction had also not reported significant anisometropia in monocularly deprived or dark reared kittens (Timney et al., 1980) or strabimic cats (Cleland et al., 1982; Jacobson & Ikeda, 1979).

It is not clear whether the unremarkable gain of vision in the deprived eye of animals that received reverse occlusion alone (ROC group) could be partly due to the particular duration of reverse occlusion employed, as evidence exists that suggests better recovery of vision with extended period of reverse occlusion (9-12 weeks: Murphy & Mitchell, 1987; Mitchell 1991) when imposed very early in the critical period. However, the lack of functional recovery of vision demonstrated by C392 (Figure 8) following 24 days of complete closure for the non-deprived eye, seems to suggest that increasing the length of reverse occlusion in that animal would have had very little beneficial consequence for the deprived eye, while accentuating the risk of development of very deep occlusion amblyopia. This result is fairly consistent with earlier report (Movshon, 1976b) which revealed that 24 days of reverse occlusion imposed on far younger kittens (6 weeks old kittens) was less capable of fully promoting normal behavioural visual abilities in the initially deprived eye, while at the same period permitting rapid deterioration of the performance of the fellow eye.

5.5 Occlusion Amblyopia

The negative behavioural consequence of dark experience followed immediately by reverse occlusion for the acuity of the non-deprived eye (in the experimental group) was supported by anatomical results reported in rodents (rats) following an identical set of manipulations (Montey & Quinlan, 2011). In this study, significant reduction in dendritic spine density of the non-deprived cortex was observed when dark exposure was contiguous with reverse occlusion (Montey & Quinlan, 2011) an effect quite characteristic of MD applied during cortical plasticity.

While it seems very attractive to link the development of occlusion amblyopia in the experimental group of animals to the robust plasticity induced by dark exposure, it is important to emphasize that similar or even a worse phenomenon was also observed in the other animals which received reverse occlusion alone (ROC). Thus, given that dark exposure has been reported to "freeze the visual cortex at the same immature state" (Timney et al., 1980, p.1053) or re-activate juvenile-like ocular dominance plasticity (Monty & Quinlan, 2011), one would have expected a very dramatic effect of reverse occlusion on ipsilateral (non-deprived eye) vision, an effect which may be comparable in magnitude to those observed after 1 week of MD at the peak of ocular dominance plasticity. That such a dramatic decline in acuity of the non-deprived eye was not observed in the two animals exposed to darkness followed immediately by reverse occlusion (DR group), raises the question as to whether the development of occlusion amblyopia in these animals proceeded on the back of pre-existing residual plasticity. However, this simplistic line of argument does not necessarily rule out the possibility of a greater deficit of occlusion amblyopia in the experimental group, had the period of closure of the non-deprived eye been extended to match that of animals in the control

group. Such a claim is valid in light of evidence that suggests that the behavioural effect of MD on dark reared animals is far more pronounced than the effects of a similar period of deprivation imposed on light reared counterparts (Beaver, Ji & Daw 2001; Timney et al., 1980).

Although the data presented in this report is not sufficient to determine any correlation between the length of reverse occlusion and the magnitude of occlusion amblyopia that ensues, it demonstrates clearly that at least for 24 days of reverse occlusion (C392: Figure 8), the reduction of acuity in the initially non-deprived eye is greater than the corresponding deficit observed for 17 days of non-deprived eye closure (C391: Figure 7). This is largely consistent with the general view of increased negative consequence of reverse occlusion with length of depression of visual input (Dews & Wiesel, 1970; Mitchell 1991; Movshon, 1976b). In view of the remarkable impairment of vision imposed on the previously non-deprived eye by reverse occlusion, the documented benefits and effectiveness of part-time occlusion or short periods of concordant binocular visual input (30-50% of occlusion time) in offsetting deprivation amblyopia is of utmost importance (Mitchell 1991, Mitchell et al., 2003; 2006).

The development of occlusion amblyopia has been reported in humans undergoing occlusion therapy. Particularly, an incidence as high as 21% to 25.8% has been reported in children (4months -10 year old) undergoing occlusion therapy for amblyopia (Scott et al., 2005; Varadharajan & Hussaindeen, 2012). Subsequently, all except one child regained vision in the affected eye (Scott et al., 2005). Unlike the results from previous studies in cats (Mitchell 1991, Murphy & Mitchell 1987; Mitchell et al., 1984a,b) where gradual but incomplete recovery of vision in the occlusion amblyopic eye (previously non-deprived eye) had been observed following termination of treatment, no vision was

recouped from the current animals that received reverse occlusion alone without prior exposure to a period in the dark. That this lack of recovery of vision in the occlusion amblyopic eye was mainly demonstrated in animals assigned to the control (ROC) group also points to the decline of plasticity for recovery after $3\frac{1}{2}$ months of age.

CHAPTER 6: CONCLUSION

A number of conclusions can be drawn from this study. First, the results support the idea that the rapid visual recovery from amblyopia observed after a short period of total darkness is guided in some way by visually driven neural activity from the non-deprived eye. Second, reverse occlusion promotes far less visual recovery from MD, compared to a shorter period of darkness (followed by binocular visual exposure) when imposed at the same late age. Third, the plasticity induced by 10 days of darkness appears to be short- lived as little or no amblyopic visual recovery occurs when the period of occlusion of the non-deprived eye is terminated after only 11 days.

6.1 Implications For Amblyopia Management

The results from the current study demonstrate the important role of the non-deprived eye to both the extent and speed of recovery of the vision of the deprived eye following a 10-day period of darkness. Little or no recovery of the visual acuity of this eye occurs if the other eye is occluded for the first 11 days following the period of darkness. This result has important implications for the treatment of deprivation amblyopia in children, as it suggests that while darkness alone may be effective, darkness in combination with conventional full time occlusion therapy may not. While conventional occlusion therapy by itself may be effective as a treatment for amblyopia, it appears to be quite ineffective when it follows a period of darkness.

The fact that darkness, followed by binocular visual input, produces far more substantial recovery of amblyopic vision compared to occlusion therapy in juvenile cats, suggests that the former may be a useful treatment for amblyopia in human adolescents at a time when conventional occlusion therapy may be ineffective. The use of complete

darkness as therapy for rapid recovery of amblyopia may require not only that the fellow eye be present but also its visual acuity, and for that matter, neural connections be refined.

Exploration of intensive binocular visual training as an effective addendum to darkness treatment might be a valuable more avenue to consider. That significant recovery of amblyopic vision and stereopsis in some adult humans have been observed following intensive binocular training on visual perceptual learning tasks (Astle, McGraw & Webb, 2011; Ding & Levi, 2011) or with dichoptic treatment (Hess et al., 2010a, 2011; Li et al., 2013; Vedamurthy et al., 2015), heightens the possibility of enhanced effects if such procedures are initiated after dark experience. The proposed treatment approach, while it may not be completely devoid of challenges, may reduce some of the psychosocial effects associated with conventional occlusion therapy (patching) in children (Hrisos, Clarke & Wright, 2004; Koklanis, Abel & Aroni, 2006). Indeed, there is currently a study underway (Project LUMA: Light deprivation Utilized to Mitigate Amblyopia) exploring the beneficial effect of 10 days of dark exposure when combined with binocular treatment approaches in adult human amblyopes (Backus et al., 2016).

The existence of a discrete time frame for the introduction of binocular input (<11 days in cats) following the period of darkness underscores the importance of the sequence of timed events if dark treatment is to be considered in humans. It possibly suggests that intensive binocular treatment (example: specially designed video games for amblyopic children) should be initiated immediately after the period spent in darkness.

With the promise shown in rats and cats, the question as to how long an amblyopic human can be subjected to darkness in an attempt to spur visual recovery would be very crucial for this treatment model. Berardi et al. (2000) attempted to scale

the profile of the development of visual acuity in humans, monkeys, cats and rats as a function of age based on existing literature so that critical periods could be compared between the species. Mitchell and Duffy (2014) have proposed detailed studies on the profile of accumulation of various intracellular and extracellular molecular correlates of cortical maturation as a means of estimating and optimising the timing for amblyopic treatment in humans. Indeed, Song and colleagues (2015) have actually compared the emergence and maturation of neurofilament proteins in the visual cortex of cats and humans and have derived a mathematical equation for extrapolating of timing of events between the two species. As much as it is not presumed that the results from cats may apply in *toto* in human amblyopes, the principle of conservation of molecular mechanisms across species (cats and rats) makes a minimum 10-day period of darkness worthy of consideration in humans. It is noteworthy that this minimum period is considerably shorter than the general rule of thumb for occlusion therapy that suggests one week of full time occlusion for every year of age up to 4 years of age (Hardesty, 1959; Longmuir et al., 2013).

6.2 Study Limitations and Future Questions

Although the results from the few animals seem convincing, the limitation for statistical analysis imposed by such a small number of animals cannot be completely ignored. Further studies with a larger number of animals in each treatment group may prove worthwhile in terms of generating the necessary data to determine a stronger correlation between the type of intervention utilized and the extent of visual recovery observed after amblyopic treatment.

Further studies on the morphological and physiological changes at the geniculate and visual cortex respectively, may help elucidate the mechanisms involved, especially when it has been shown that a manipulation can promote anatomical but not necessarily behavioural recovery (Duffy et al., 2015). Additional investigations into the profile of cortical plasticity following darkness and occlusion of the non-deprived eye may help determine the exact window of time beyond which the non-deprived eye may be unable to guide recovery of the deprived eye even after restoration of normal binocular input. Such data may provide a guide as to the minimum window of time for initiation of binocular treatment models after darkness.

In addition to the practical and logistical challenges of creating patient-friendlier darkrooms for clinical treatment of amblyopia (Duffy et al., 2015), the ethical challenges of placing humans in darkness for longer durations suggest that alternatives to dark rearing have to be explored in the very near future. In line with this, intra-vitreal injection of chemical compounds that temporarily produce visual silencing may be helpful at mimicking the beneficial effects of darkness. One such compound, tetrodotoxin, a Na^+ blocker, has been tested on monocularly deprived cats with profound success (unpublished data: Mitchell & Duffy).

Another question to be answered in the near future pertains to whether dark therapy can promote visual recovery from strabismic amblyopia: to what extent and at what rate? Such exploration may help determine other sub-categories of amblyopes that may be better suited for dark therapy.

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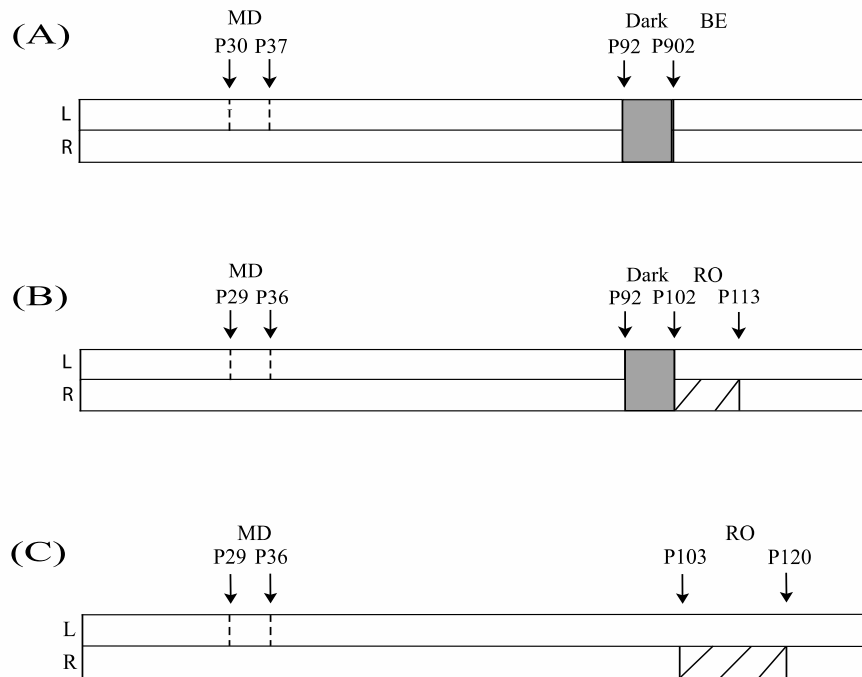
Figure 1. Schematic representation of the rearing histories of animals.

(A) A representation of the rearing profile of animals assigned to the delayed darkness group in earlier study by Duffy and Mitchell (2013). "R" and "L" represent the right and left eye respectively. Animals received 7 days of monocular deprivation (MD: broken lines) at 30 days of age, followed 2 months later (P92) by10 days of complete darkness (grey-coloured column) prior to normal binocular visual exposure (BE).

(B) A representation of the rearing history of 2 animals in the current study which received comparable periods of MD from 29 to 36 days of age (P29-P36). These animals were exposed to10 days of complete darkness (grey-coloured column) beginning at 92 days of age, followed immediately by 11 days of closure (striped column) of the non-deprived eye.

(C) An illustration of the rearing profile of the 2 animals assigned to control group that experienced equivalent period of MD at comparable age followed later (P102) by a period of reverse occlusion (RO) without prior exposure to darkness.
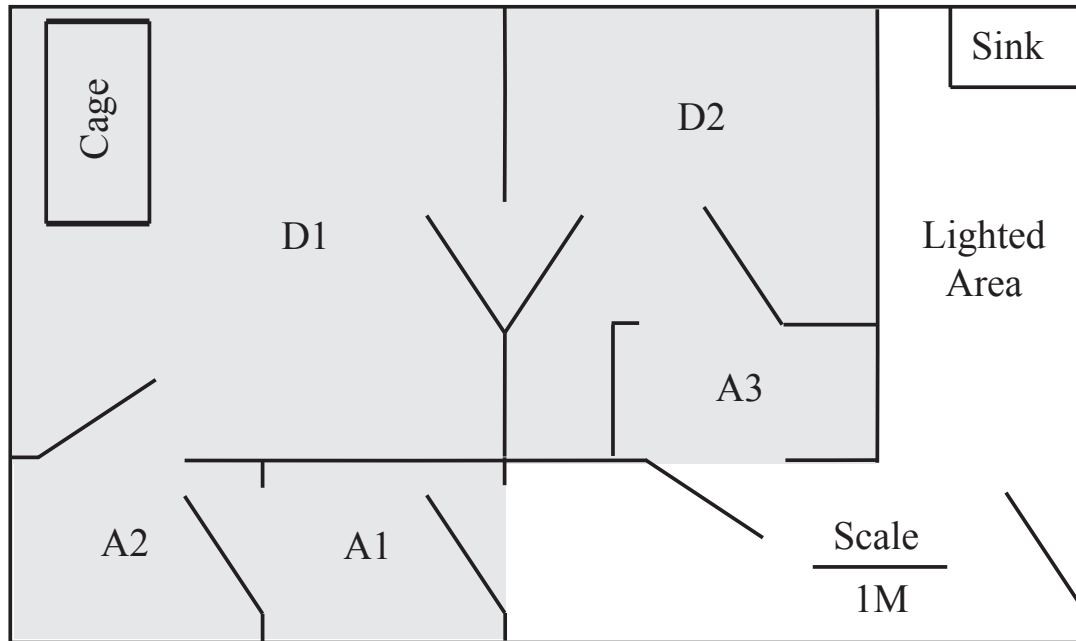
Figure 2. Darkroom facility drawn to scale

The darkroom facility is divided into main areas, a dark area (depicted by the gray-coloured section) with dark-coated walls for absorption of light, and a lighted area from where cage supplies were prepared. The dark area consisted of 3 anterooms (A1, A2 and A3), a main darkroom (D1) and a dark holding room (D2). Access to the main darkroom was provided via 2 anterooms (A1, A2) secured with light-tight doors. Animal was kept in large cage placed in the main darkroom. For daily cleaning and replacement of cage supplies in this room, animal was secured in a carrier and transferred into the temporary dark holding room. The light in the main darkroom was then switched on. Once routine was completed, the light was switched off and animal transferred back into the cage in the main darkroom.
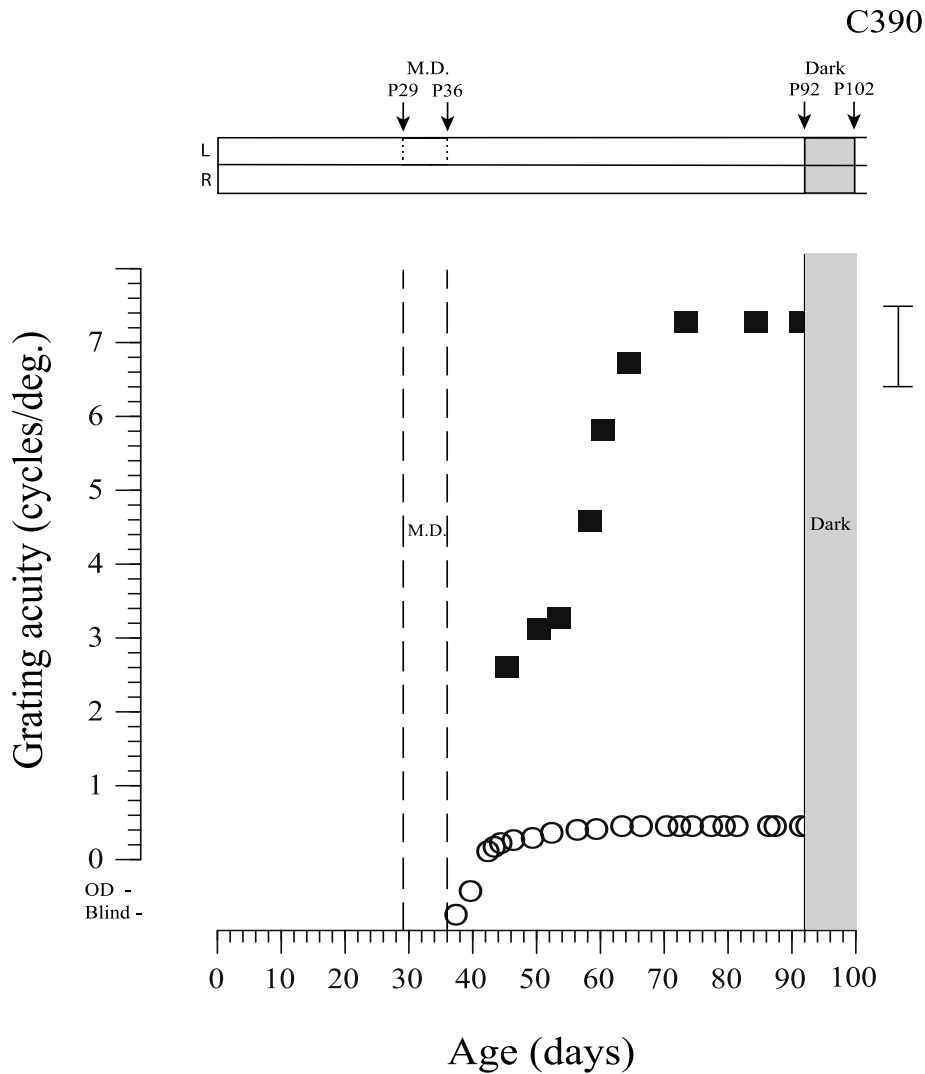
Figure 3. Visual acuity data for C390 after one week of MD.

The rearing history for this animal is schematically depicted in the panel above. MD (vertical broken lines) was imposed at 29 days of age and terminated after 7 days to allow normal visual input to both eyes. Binocular visual acuity (filled squares) improved steadily after MD until peak value of 7.4 cycle/degree was attained. For the 4 weeks period leading to dark exposure, binocular acuity remained stable. Vision of the deprived eyed (depicted in open circles) improved gradually from a blindness (Blind) to open door (OD) to low and stable level. The grating acuity for this eye remained stable and severely reduced for about 6 weeks prior to dark exposure (grey column) and subsequent occlusion on the non-deprived eye. The vertical bracket at the upper right conner represents the normal range of visual acuity for the age.
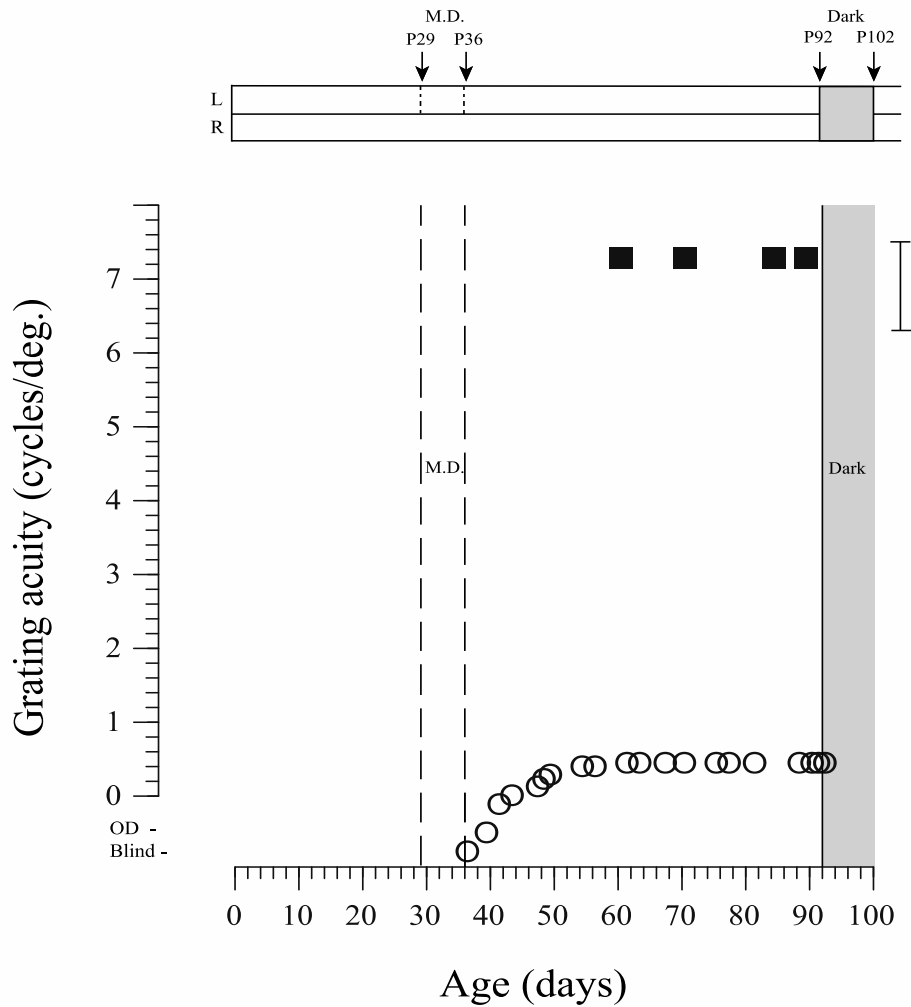
Figure 4. Visual acuity data for C393 after one week of MD.

The specific interval for MD and dark exposure are schematically depicted in the panel above. The left eye was deprived of vision from postnatal day 29 to 36 as illustrated by the vertical hatched lines. Binocular visual acuity (filled squares), which also reflects the acuity of the non-deprived eye, attained a maximum value of 7.4 cycle/degree at about 2 months of age. The vision of the deprived eye improved from blindness (Blind) to open door (OD) and finally, low visual resolution on square wave gratings. The binocular and deprived eye acuities remained stable about 6 weeks prior to the imposition of darkness (at 92 days of age). The vertical bracket (upper right corner) represents the normal range of acuity for the age.
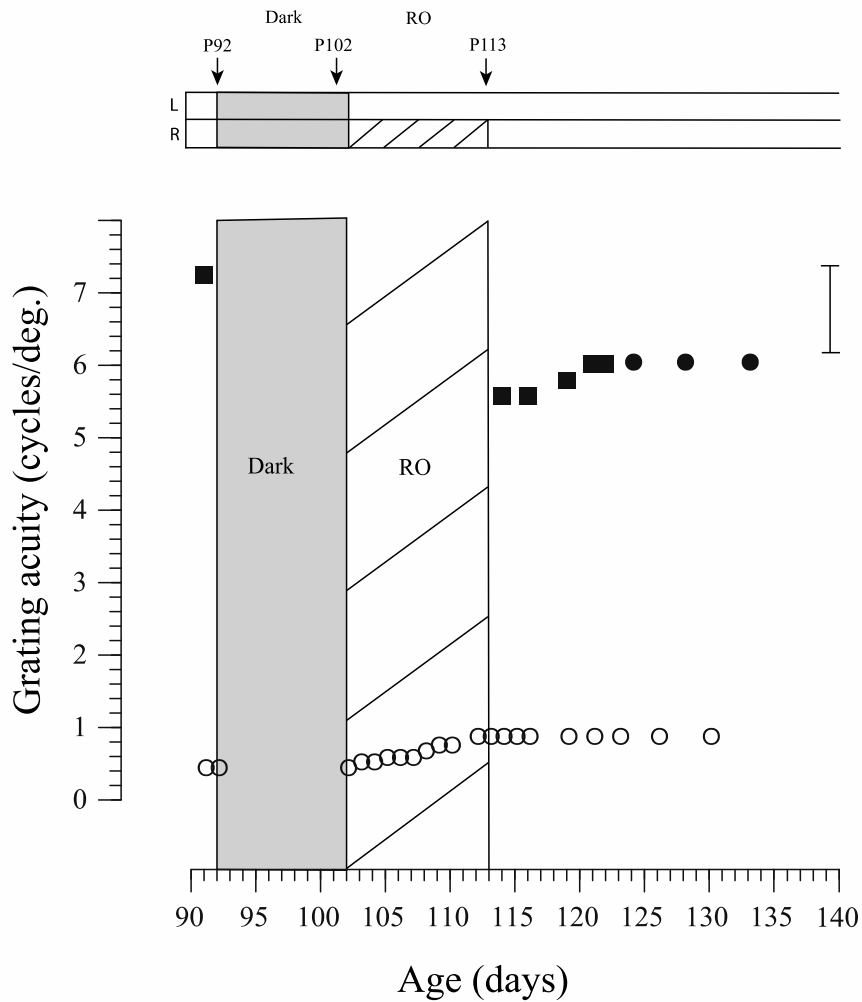
Figure 5. Visual recovery data for C390 following dark exposure and reverse occlusion.

   The timelines for the various manipulations to visual input are represented in the panel above. Ten days of complete dark exposure was initiated at 92 days of age (P92) followed immediately by 11-day period of closure of the non-deprived eye. Visual recovery in the deprived eye (open circles) improved minimally after the two key manipulations and remained unchanged following termination of reverse occlusion (RO). In contrast, binocular visual acuity (filled squares) declined from the optimum level achieved prior to dark experience. Although vision for this eye improves minimally during the period of reverse occlusion, the final acuity remained slightly lower than the normal range for the age (vertical bracket). Filled circles represent the acuity of the non-deprived eye.
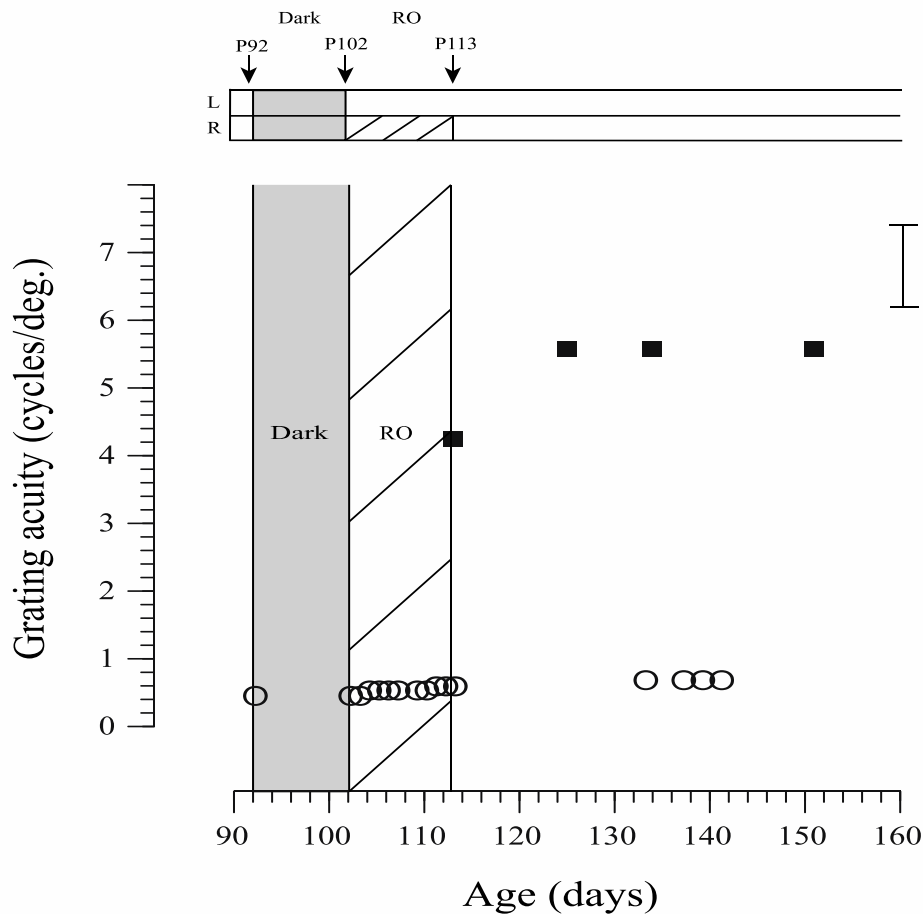
Figure 6. Visual recovery data for C393 following dark exposure and reverse occlusion.

The timelines for the various experimental manipulations are schematically portrayed in the panel above. Grey and diagonal striped areas represent, respectively, the period of dark exposure and reverse occlusion. No substantial change in the vision of the deprived eye (open circles) was evident after sequential exposure to darkness and reverse occlusion (RO). However, the acuity of the non-deprived eye as assessed by binocular measurements (filled squares) demonstrated a marked reduction. The development and subsequent treatment of corneal haze in the non-deprived eye prevented the estimation of acuity of the fellow eye within the 10-day period following cessation of reverse occlusion. However, when it was finally possible to do so, the non-deprived eye demonstrated some improvement in acuity but this was still lower than the optimum level achieved prior to dark experience. Vision in the deprived eye remained unchanged after restoration of normal binocular visual experience.
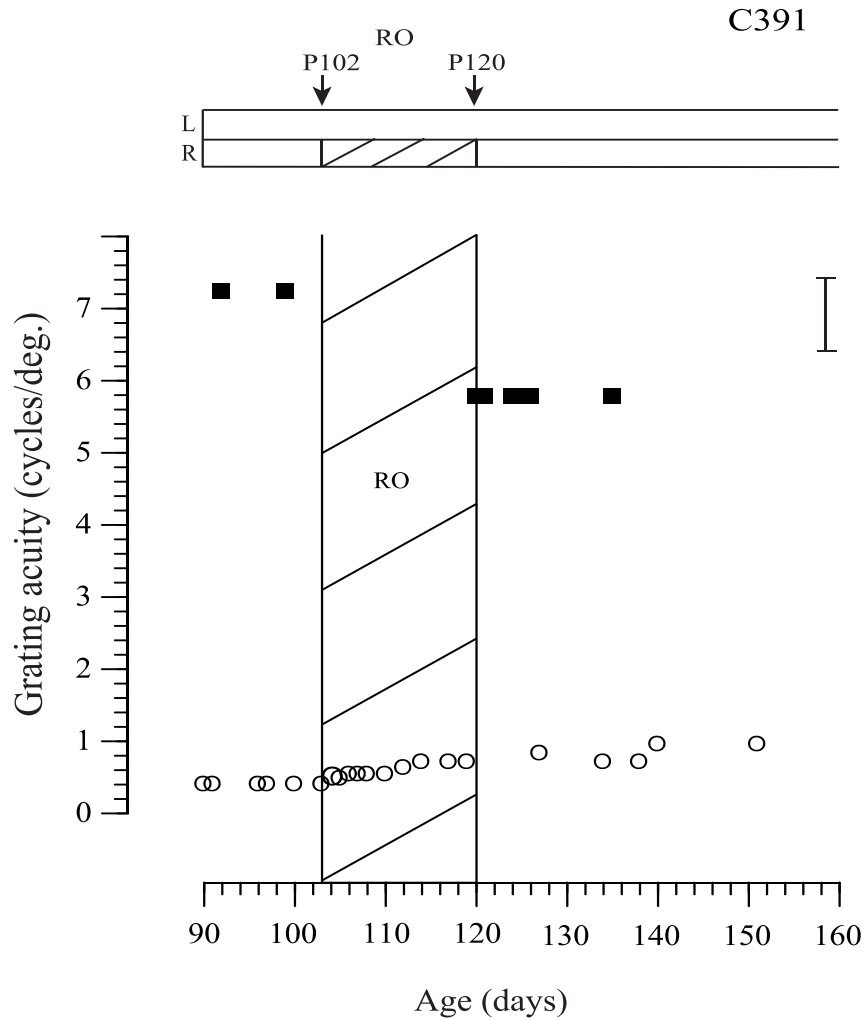
Figure 7. Visual recovery data for C391 following reverse occlusion.

The panel above depicts the timeline for experimental intervention. The deprived eye (open circles) demonstrated no remarkable improvement in acuity during and after 17 days of closure of the fellow eye (hatched region). In contrast, the effect of same period of reverse occlusion (RO) on the acuity of the non-deprived eye was evidenced by the decline in the binocular visual acuity (filled squares). Essentially, the acuity of the non-deprived eye remained lower than age-matched normal values (vertical ledger on the left upper corner)
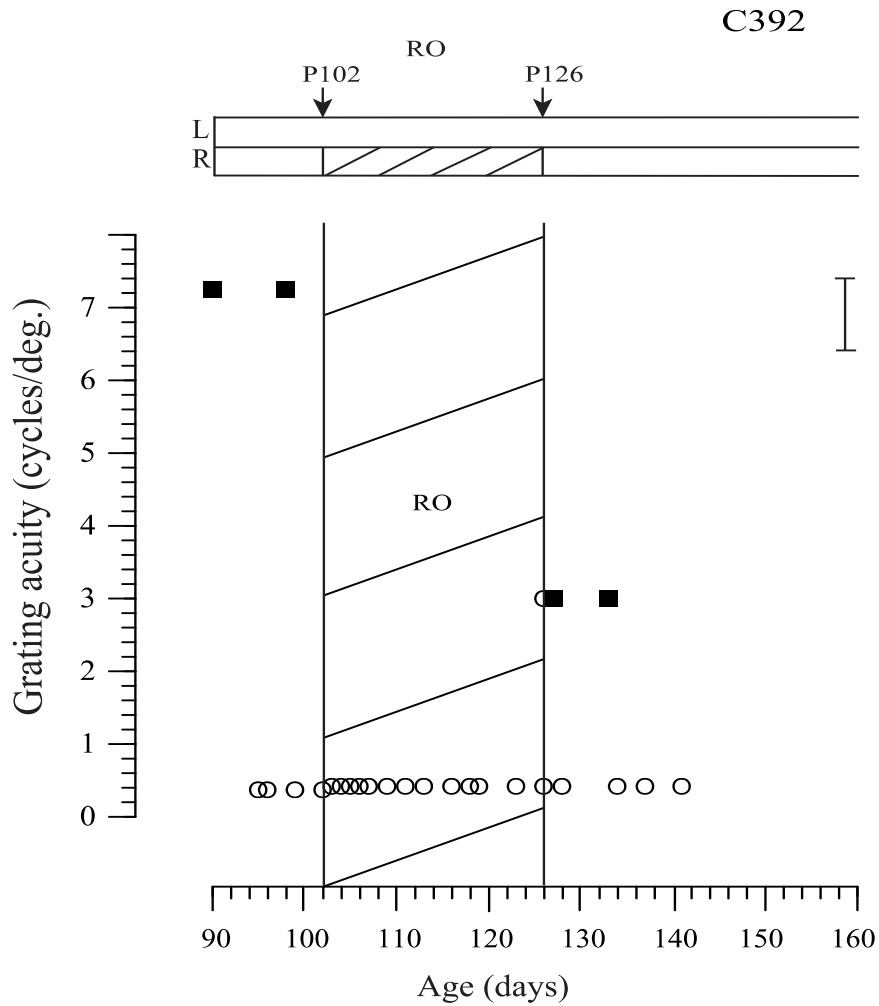
Figure 8. Visual recovery data for C392 following reverse occlusion.

The hatched region represents the period of reverse occlusion (RO). The deprived eye showed no gain in acuity during and after 24 days of closure of the fellow eye. However, the effect of this manipulation on the acuity of the non-deprived eye as assessed by binocular measurements showed a marked decline. The overall monocular and binocular visual acuities demonstrated unequal bilateral amblyopia.
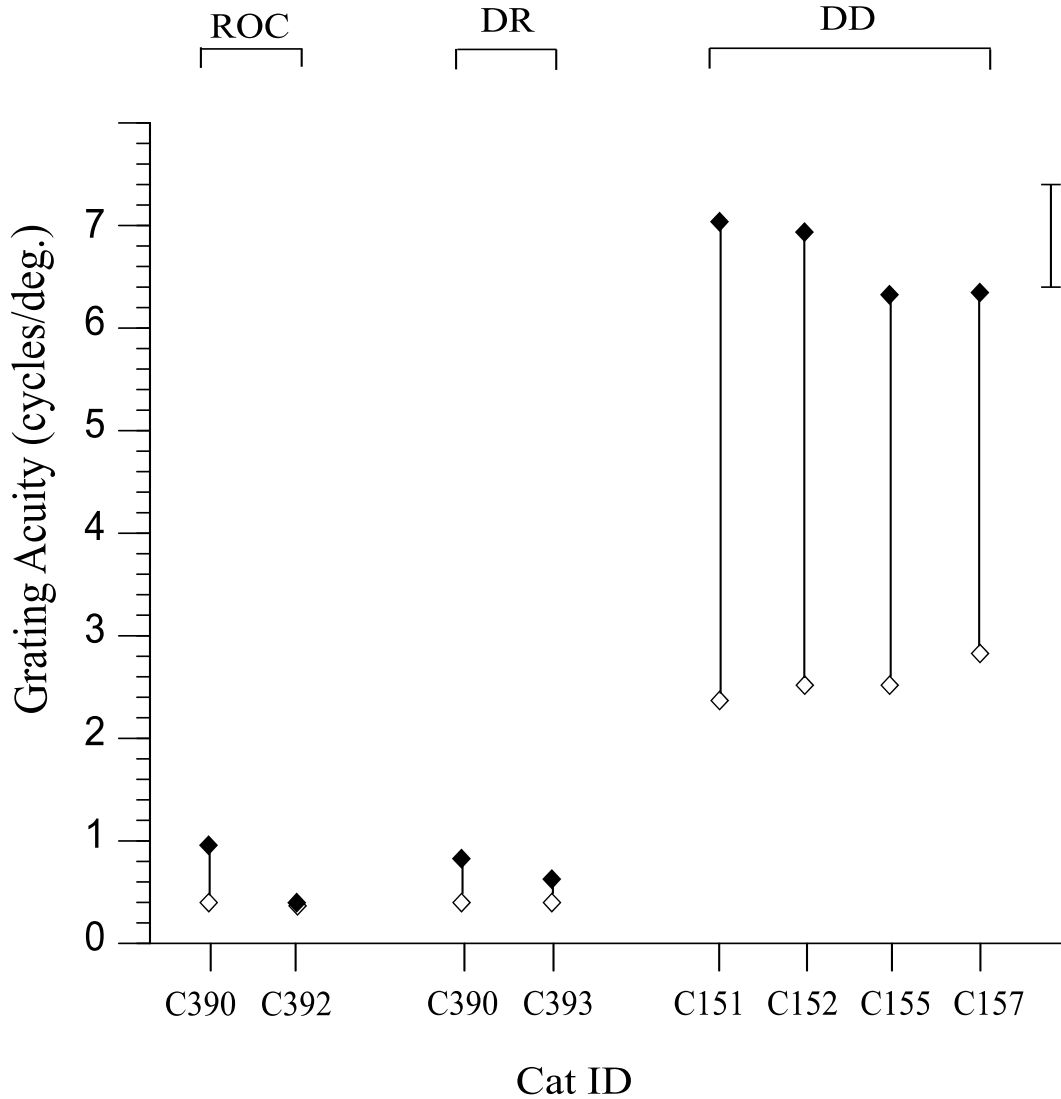
Figure 9. Comparison of extent of visual recovery following interventions

Empty (unfilled) and filled diamonds depict respectively, the pre and post-intervention acuities of the deprived eye. The vertical line between the diamonds indicates the difference in the pre and post-intervention acuities. ROC represents animals in the reverse occlusion group, DR: dark imposed and reverse occlusion group, and DD: delayed darkness group (Duffy & Mitchell, 2013). The mean recoveries of visual acuity for animals in the ROC and DR groups are similar (0.31 versus 0.33 cycles/degree). In contrast, the mean recovery of acuity for animals that had both eyes open after dark experience in prior study (Duffy & Mitchell, 2013) was 4.12 cycles/degree.
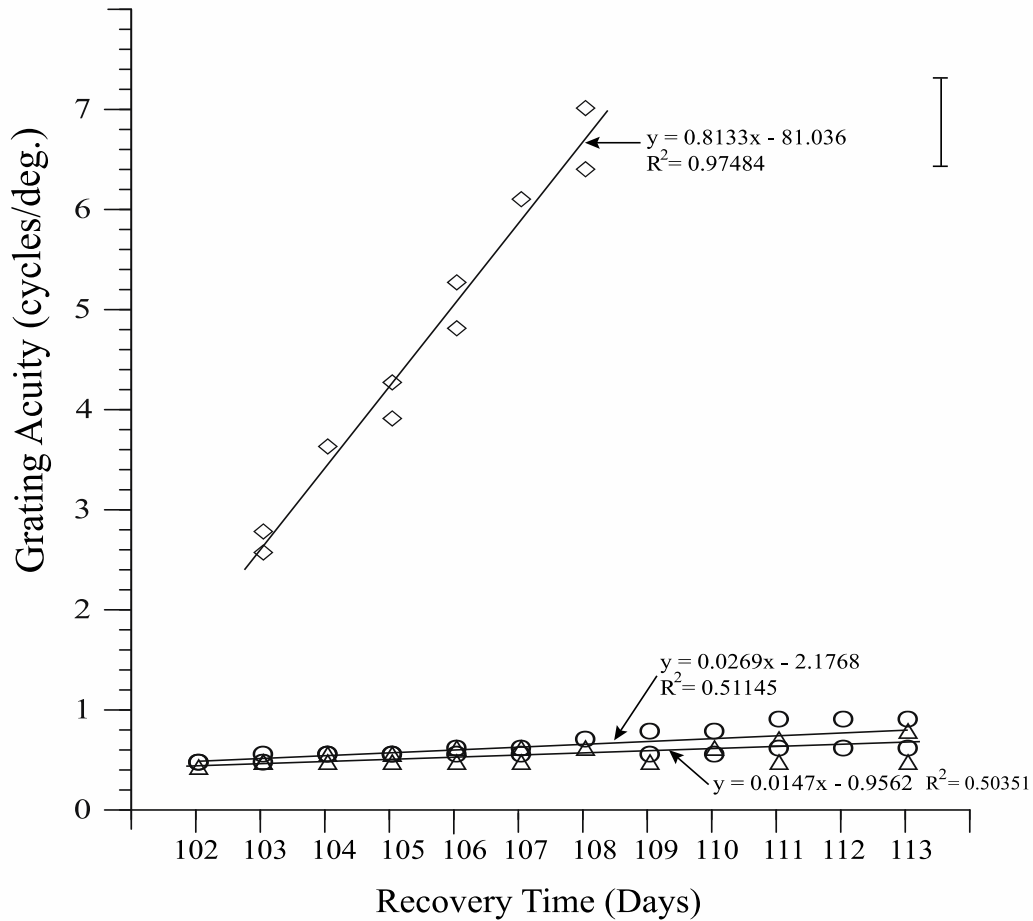
Figure 10. Comparison of the rate of visual recovery following interventions

The recovery of acuity of the deprived eye of the animals over time is presented as triangle symbols, for the 2 animals in the reverse occlusion group (ROC), circles, for the 2 animals in dark imposed and reverse occlusion (DR), and diamonds, for the 2 animals that recovered the slowest in the delayed darkness group (DD, C157and C152: Duffy & Mitchell, 2013). The equation of each line of fit is given (example: y = 0.8133x – 81.036 for DD group) to reflect the general equation of a straight line y = mx + c, where 'm' is the gradient (slope) and 'c' is the y-intercept. The gradient of the line of best fit indicates a rate of recovery of 0.015, 0.027 and 0.813 cycles degree^{-1}/day of treatment respectively, for ROC, DR and DD groups.

APENDIX B Table

Table 1. Monocular and binocular acuities before interventions.

Group	Cat ID	MD	Visual acuity before Intervention	
			DE (cyc/deg.)	Binoc. (cyc/deg.)
DR	C390	P29-36	0.56	7.39
	C393	P29-36	0.56	7.39
ROC	C391	P29-36	0.56	7.39
	C392	P29-36	0.51	7.39
DD	C151	P30-37	2.51	6.49
	C152	P30-37	2.66	6.49
	C155	P30-37	2.66	5.71
	C157	P30-37	2.97	6.49

DR represents dark imposed and reverse occlusion group, ROC: reverse occlusion group, and DD: delayed darkness group (Duffy & Mitchell, 2013). DE represents the acuity of the deprived eye, and binoc., indicates the binocular acuity. The pre-intervention acuities for animals in the current study (DR+ROC) are almost the same. The acuities of animals in the DD group are equally similar. Mann-Whitney test was significant ($p = 0.017, U=0, Z= -2.381$) for the pre-intervention acuity of the deprived eye of DR+ROC and DD groups.

Table 2. Refractive state of the eyes at about 4 months of age

CAT ID	DE (Dioptres)	NDE (Dioptres)
C390	Plano	Plano
C391	-0.25	-0.25
C392	+0.25	Plano
C393	Plano	-0.25/+0.50 x 60

Retinoscopy was performed on the non-cyclopleged eyes of all animals at about 4 months of age following the termination of the key interventions of dark exposure and/or reverse occlusion. DE represents the refractive state of the deprived eye, and NDE, the non-deprived eye. Except for C393 that developed some mild mixed astigmatism in the non-deprived eye (following the development of cornea haze on termination of reverse occlusion), the refractive states of the eyes were almost equal in all animals.