DARK-REARING PROMOTES DRASTIC IMPROVEMENT OF VISUAL ACUITY IN THE AMBLYOPIC EYE OF LID-SUTURED KITTENS

by

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To Scroogina, Bah, Jaffa, Samantha,

Novak, Sherrington, Hering, Krimsky, and Heidi

who always made me smile at the end of a long day.

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Abstract

This report extends findings (Duffy & Mitchell, 2013) of a dramatic recovery of vision in the deprived eye of amblyopic kittens following a short 'dark-pulse' (a 10 day period of darkness) to situations of clinical relevance. To this end, the initial deprivation began at post-natal day 7 rather than post-natal day 30. As before, the dark-pulse was imposed either immediately after the initial monocular deprivation, or was delayed several weeks after stable amblyopia was establsihed. In some animals, this dark-pulse was shortened, or disrupted by short periods of daily binocular visual experience. The effects on the visual acuity and alignment acuity of the two eyes were documented as well as the effects on binocular depth perception. The benefits of a short dark-pulse were identical to those of the prior study. A dark-pulse of 5 days was ineffective as was a dark-pulse interrupted daily by light for 30-minutes.

List of Abbreviations and Symbols Used

CRT	cathode ray tube (computer monitor)
ΔD	distance between depth stimuli
D	distance to nearest stimulus
dLGN	dorsal lateral geniculate nucleus
LGN	lateral geniculate nucleus
DR	dark-rearing
MD	monocular deprivation
IOL	intra-ocular lens

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CHAPTER 1: INTRODUCTION

1.1 Animal Models for Amblyopia

Amblyopia, colloquially known as 'lazy-eye', has long been thought of as a developmental visual disorder in which usually one or occasionally both eyes suffer from poor vision as a result of being disadvantaged during early development (von Noorden & Campos, 2002). If not treated promptly, amblyogenic factors including congenital cataract, strabismus, and anisometropia, can disrupt normal development of the visual system during its immaturity, resulting in lasting visual impairment. The underlying mechanisms which cause amblyopia when these factors are present is an intriguing question. There has been a long history of assertions as to the aetiology of amblyopia based on clinical observations of the associated conditions that may serve as predisposing factors (Duke-Elders & Wybar, 1973). Initially, these observations prompted the emergence of a variety of terms such as amblyopia *ex anopsia* (amblyopia of disuse), amblyopia of extinction, amblyopia of arrest, and of suppression (Duke-Elders & Wybar, 1973). These titles served to both classify different types of amblyopia and to embody notions of their aetiology. In time, these terms were replaced by others, such as strabismic amblyopia or anisometropic amblyopia, that specified an associated condition (respectively, strabismus or anisometropia) that presumably lead to the development of amblyopia in early life. Early clinical observations also identified approaches that result in substantial improvement of the vision of the amblyopic eye and, most importantly, recognized times in life (critical periods) when these methods were most successful. A

key component of amblyopia treatment has been a period during which the vision of the non-amblyopic eye has been disadvantaged by such interventions as patching, lens blur or the use of pharmaceutical interventions in order to force use of the so-called 'bad' eye. Detailed documentation of the success of these interventions began well over a century ago. In 1903, Worth published the first edition of his landmark book that summarized his lifetime's experience with the treatment of over two-thousand patients with strabismus and strabismic amblyopia by a combination of several interventions that included refractive correction, patching, surgical correction of the deviation, and finally orthoptic training that focused upon the development of binocular fusion. He noted with respect to the latter that such training was most successful when applied before the age of seven, although occasionally good results could be obtained for older patients (Worth, 1903).

A century of clinical studies has allowed refinement of the traditional methods of treatment particularly with respect to the timing of these interventions and awareness of the importance and documentation of the degree of compliance with patching treatment. Moreover, the genesis of the collaborative research group, the Pediatric Eye Disease Investigators Group (PEDIG), has had a powerful impact on the conduct of clinical trials of various testing and treatment procedures, and has improved uptake of new treatment strategies among clinicians (Beck, 2002). Arguably, the most important influence on clinical treatment of amblyopia has emerged in the last half-century from neurophysiological investigations of the adult cat visual cortex and subsequent studies of experiential influences on its anatomical and functional development. This work has allowed a greater appreciation of the neurophysiological underpinnings of amblyopia and

resulted in a modification of the ways in which amblyopia is categorized, notably with the addition of categories such as deprivation amblyopia that can be linked closely to the results of animal studies.

Neurophysiological and Anatomical Studies

Studies on animals with both normal as well as abnormal visual systems have been essential to our understanding of the anatomy and physiology of the visual system. Investigation of the normal functions of the visual pathway using animal models essentially began with studies at the level of the retina. Adrian and Matthews (1927a; 1927b; 1928) were first to describe excitation in frog and eel retinas, and found that neural activity from several retinal cells converged onto optic nerve fibers that then form the optic nerve. Later work established that the converging input onto retinal cells beyond the photoreceptors was characterized by receptive fields that defined regions of visual space; the latter when stimulated caused a modulation of the frequency of action potentials ("firing rate") of specific optic nerve fibers (Hartline, 1940a; Hartline, 1940b). Subsequently it was discovered that single cells in the retina and higher visual structures were specialized to recognize more complex stimuli than simple responses to spots of light. Barlow (1953) first emphasized that retinal ganglion cells and optic fibers in the frog visual system recognized complex forms such as the detection of movement. He found more sophisticated responses, indicating that not all cells within a particular class behaved in the same way to common stimuli (Barlow, 1953). Coincidently, Kuffler (1953), working on the cat retina, characterized "on" and "off" centre-surround receptive

field properties of retinal ganglion cells. These early studies of neural excitiation and inhibition within the retina were essential to investigations of higher visual areas in the mammalian visual system.

Because amblyopia has long been thought to arise from imbalanced early visual input between the two eyes, an understanding of its neural basis was not possible before the pioneering investigations of the visual cortex by Hubel and Wiesel in the 1960s. In species such as monkeys and cats with frontal eyes like humans, the visual cortex is the first level in the visual pathway where input from two eyes converge upon single cells in the brain. Their long-standing collaboration that began in the late 1950s ultimately won Hubel and Wiesel the Nobel Prize in Physiology and Medicine for their work on the cat and monkey visual cortex. They began by characterizing the receptive fields of single cells in the primary visual cortex (striate cortex) of anesthetized cats (Hubel & Wiesel, 1959). Their initial findings, published in 1959, presented the first recordings of what would later be called 'complex cells' in the cat primary visual cortex. These cells had one of the most astonishing attributes of primary visual cortex cells, that is orientation selectivity (Hubel & Wiesel, 1959). Their initial study also found that many cortical cells could be stimulated by either eye (binocular cells), although in many instances the response was better to input from one eye than the other (ocular dominance) (Hubel & Wiesel, 1959). This work was followed in the next decade by studies of the adult cat (Hubel and Wiesel, 1962) and monkey visual cortex (Hubel & Wiesel, 1968), and importantly by investigations of the state of the cortex in newborn kittens (Hubel & Wiesel, 1963) as well as kittens reared with unusual early visual experience (Wiesel &

Hubel, 1963a; Wiesel & Hubel 1963b). The latter work established the framework for future research into the development and plasticity of the brain.

By the early 1960's, it had been established that many single cells in the primary visual cortex receive varying degrees of binocular input, and show highly selective responses to particular visual stimuli, such as orientation (Hubel and Wiesel, 1962). This work was closely followed by studies that employed a variety of rearing conditions to investigate morphological and physiological changes in the dorsal lateral genicualate nucleus (dLGN) (Wiesel & Hubel, 1963a) and the visual cortex (Wiesel & Hubel, 1963b). By depriving kittens of vision in one or both eyes at different times in early postnatal life, they were able to determine whether, and to what extent, the physiological properties of cells observed in adulthood depended upon normal early visual experience. By use of single-neuron analysis of visual responses in the primary visual cortex of newborn kittens, or else in kittens that were binocularly deprived of patterned vision using binocular eyelid-suture, they sought to unravel the extent to which the response selectivity of cells in the visual cortex required visual experience. It was discovered that newborn kittens as well as kittens deprived of normal visual experience for the first few weeks of life, posessed some cortical cells with similar responses to cells found in adult primary visual cortex (Hubel & Wiesel, 1962). Both simple and complex receptive fields were present and showed orientation selectivity, and most cells showed binocular responses similar to those in adult cats (Hubel & Wiesel, 1962). However, the cells of the kittens' visual cortex were found to respond more sluggishly than in the adult cat (Hubel & Wiesel, 1962). Their responses habituated quickly to repeated stimulation, and

selectivity for orientation was poorly tuned as compared to those of adult animals (Hubel & Wiesel, 1962). Hubel and Wiesel reasoned that many of the connections between visual cortical cells responsible for the highly specific visual responses must be present at birth, or at least within a few days of it, since they occurred even in the absence of patterned visual experience (Hubel & Wiesel 1962; Wiesel & Hubel, 1963a; Wiesel & Hubel, 1963b). This finding flew in the face of previous assertions that visual connections were experience-dependent, developing completely after birth (Berger, 1900; Goodman, 1932). Instead, Hubel and Wiesel suggested that neural connections within the visual cortex are present prior to visual exposure, requiring visual experience only to strengthen and to fine-tune connections for greater feature selectivity.

Employing a variety of rearing conditions, Hubel and Wiesel, and later others, investigated the effects of abnormal early visual experience on the morphology and physiology of the dorsal lateral genicualate nucleus (Wiesel & Hubel, 1963a) and the visual cortex (Wiesel & Hubel, 1963b). Kittens were reared with an imbalance of visual input between the two eyes by monocular deprivation, a condition closely mimicking 'deprivation amblyopia' in humans that may result from amblyogenic factors such as unilateral congenital ptosis or unilateral congenital cataract, for example. The first study (Wiesel & Hubel, 1963a) using this animal model examined kittens deprived of visual experience in one eye by eyelid-suture from birth. After three months of monocular deprivation, histological analysis was performed on the lateral geniculate nucleus (LGN), a thalamic structure with distinct eye-specific layers that receive separate monocular input from either eye, and project onto the visual cortex. Previous studies on the

projections of the retina to the LGN in the cat had demonstrated that after eye enucleation, alternate cell laminae in the LGN atrophied (Minkowski, 1913), revealing the eye-specific nature of their input. In the cat, the LGN can be separated into 3 distict laminae; after enucleation the contralateral LGN showed degeneration in the pars dorsalis A, while the pars dorsalis A1 remained normal, and the pars dorsalis B showed degeneration as well (Minkowski, 1913). In the ipsilateral LGN, the reverse was seen; pars dorsalis A appeared normal, pars dorsalis A1 showed degeneration, and pars dorsalis B remained normal (Minkowski, 1913). Histological analysis of kittens after early monocular deprivation revealed similar marked shrinkage of cell soma size in layers receiving input from the deprived-eye (Wiesel & Hubel, 1963a). Cell areas of deprived-input layers showed an average of 40% shrinkage compared to non-deprived layers (Wiesel & Hubel, 1963a). Despite this marked change in the LGN, there were no histological changes apparent in the deprived retina, optic nerve, superior colliculi, or primary visual cortex (Wiesel & Hubel, 1963a). A kitten monocularly deprived for only two months showed similar results, although less marked histological changes in the LGN (Wiesel & Hubel, 1963a). Conversely, when an adult cat was monocularly deprived for a similar duration of time, no histological changes were seen in the LGN (Wiesel & Hubel, 1963a). This animal model revealed that monocular deprivation in early life causes structural abnormality in the LGN of kittens, and that longer deprivation produces a more severe effect. However, there must be a critical period during which monocular deprivation causes shrinkage of cell size within the LGN since no effect was seen when deprivation was induced for adult cats.

By use of the same monocular deprivaiton model, Hubel and Wiesel also looked at the animals' visual behaviour, and performed electrophysiological investigations to analyse any functional cortical changes that might occur despite absent histological findings (Wiesel & Hubel, 1963b). As before, kittens were deprived of form vision in one eye by eyelid-suture for the first few months after birth. After the eyelid-sutures were removed, and the kittens forced to use the previously deprived eye by placing an opaque contact lens in the non-deprived eye, they behaved as if blind (Wiesel & Hubel, 1963b). Normal visual placing and following behaviours were absent, and there was no indication that the animals could perceive form at all (Wiesel & Hubel, 1963b). Electrophysiological investigations revealed that most cortical cells were actively driven by only the non-deprived eye, suggesting a drastic shift in ocular dominance within the primary visual cortex to respond primarily to input from this eye (Wiesel & Hubel, 1963b). Adult cats deprived in the same way exhibited no detectable physiological abnormality in the visual cortex and the vision of the deprived eye appeared normal on casual observations of the animal's behaivour (Wiesel & Hubel, 1963b). Variations of this monocular deprivation model, including suture of the nictating membrane, were also investigated. The LGN of kittens deprived by this less extreme form of monocular deprivation showed no pronounced atrophy within the LGN, but yet the cortical dominance showed a pronounced shift to the non-deprived eye (Hubel & Wiesel, 1962). Later work showed that the cortical effects of eyelid-suture could be attributed to the monocular reduction of form information (blur) and not to the reduction of light (Blakemore, 1976; Jaffer, Vorobyov, Kind & Sengpiel, 2012). In an important experiment, Wiesel and Hubel (1965a) examined the effects of binocular eyelid-suture,

expecting that this would result in greater cortical change than those observed after monocular occlusion. To their surprise, the results were far more subtle as cells could be excited, albeit sluggishly, by visual stimulation of either eye (Wiesel & Hubel, 1965a). They introduced the idea that the effect of monocular deprivation was a result of binocular competition between the eyes for synaptic space on cortical cells during visual development. In other words, the bulk of projections pertaining to either eye in the central pathway may partly depend on whether or not the other eye is stimulated (Wiesel & Hubel, 1963b; Wiesel & Hubel, 1965a). With these experiments, Hubel and Wiesel broke early arguments to show abnormal visual experience in early life can permanently disrupt any initially formed circuits. Later elegant research supported this hypothesis that atrophy in LGN arises from competition between the eye-specific inputs to the cortex (Guillery, 1972). And more recent studies have confirmed using optical imaging and visually-evoked potentials that eyelid-suture during early development induces an ocular dominance shift in the primary visual cortex of cats (Jaffer, Vorobyov & Sengpiel, 2012).

The notion of the importance of binocular competition during development received further support from experiments on the results of artificial strabismus induced surgically in kittens by unilateral medial rectus myotomy to induce exotropia at the time of natural eye-opening (Hubel & Wiesel, 1965). On casual observation, the cats did not seem visually impaired in either eye when tested monocularly, suggesting that they were not amblyopic as a result of induced exotropia. Electrophysiological examination of the visual cortex then revealed that the vast majority of cells were driven exclusively by just one eye with an equal number of cells driven by each eye (Hubel & Wiesel, 1965). This

lack of binocular cortical cells suggests that the kittens, though not amblyopic, would lack binocular functions such as stereopsis. In parallel studies, the major findings from investigations of monocularly deprived cats were confirmed on infant monkeys (von Noorden, Dowling & Ferguson, 1970; LeVay, Wiesel & Hubel, 1980). Artificial strabismus induced surgically in young monkeys showed similar behavioural results; exotropic monkeys showed no sign of amblyopia in either eye (von Noorden & Dowling, 1970). Further research into kittens reared with early artificial exotropia have found that although there is no ill-effect on visual acuity of either eye, it is accompanied by a loss of stereoscopic vision (Mitchell, 1988). Following the subsequent development of anatomical techniques that permitted visualization of eye-specific regions in the cortex by autoradiography, it was possible to map the distribution of so-called ocular dominance columns in the visual cortex in both normal and monocularly deprived animals (Hubel, Wiesel, & LeVay, 1977). As could be predicted on the basis of the electrophysiological findings, the amount of cortical territory dominated by the deprived eye in layer IV of the visual cortex (the cortical layer labelled by the first techniques) was much reduced following early monocular deprivation, and there was a corresponding expansion of territory pertaining to the non-deprived eye (Hubel, Wiesel & LeVay, 1977).

Studies of Visual Recovery

Once it was established that an imbalance in cortical input during early life results in significant visual impairment and structural anomalities within the visual pathway, it was important from a clinical perspective to explore possible experimental manipulations

to promote recovery from the consequences of the early abnormal visual input. Hubel and Wiesel (1965b) first sought to examine the extent of recovery from early monocular visual deprivation. Kittens with monocular eyelid-suture were deprived to three months of age, at which time they were allowed normal visual input to both eyes, a condition referred to as binocular recovery (Wiesel & Hubel, 1965b). Despite receiving normal visual input through the deprived eye for an extended amount of time, the kittens showed only minimal behavioural recovery; they remained severely visually impaired in this eye and never learned to move freely based on visual cues to the previously deprived eye alone (Wiesel & Hubel, 1965b). Furthermore, the abnormal cortical response and shrinkage of cell soma size within the LGN that had been seen in previous monocular deprivation studies (Wiesel & Hubel, 1963a; Wiesel & Hubel, 1963b), remained abnormal, even after 15 months of visual input to the deprived eye (Wiesel & Hubel, 1965b). It seemed that either connections lost during visual deprivation were incapable of re-establishing with restored normal visual input, or else failed to recover simply as the animal aged (similar to the natural history of untreated amblyopia).

Later animal studies investigated post-deprivation recovery other than passive (binocular) recovery, most notably reverse-occlusion by full-time occlusion of the previously normal eye (equivalent to full-time patching therapy for human amblyopia), and part-time reverse-occlusion, in which periods of binocular exposure were deliberately provided each day in addition to the daily periods of occlusion of the normal eye. Hubel and Wiesel's studies of binocular visual recovery (Wiesel & Hubel, 1965; Hubel & Wiesel, 1970) found that if monocular deprivation had extended beyond the critical

period, large interlaminar cell size differences in the LGN of kittens persisted even with as much as 5 years of normal visual exposure. Passive binocular recovery was therefore ineffective in the treatment of deprivation amblyopia at this late stage in development. In a series of studies conducted in both the dLGN and the visual cortex (Blakemore & van Sluyters, 1974; Movshon, 1976; Dursteler, Garey & Movshon 1976), the effects of reverse-occlusion were investigated. Remarkably, when deprivation was reversed early, at between four and six weeks of age, the pattern of ocular dominance among cortical cells could be switched completely (for reversal at four or five weeks), or nearly so, from dominance by the originally non-deprived eye to dominance by the formerly deprived eye (Blakemore & van Sluyters, 1974; Movshon, 1976). However, in kittens that were monocularly deprived to between 8 and 14 weeks of age, and then reverse-occluded, the effects of the initial period of monocular deprivation largely remained. Delaying the age at which reverse-occlusion was implimented reduced the rate and the extent of recovery of ocular dominance (Movshon, 1976). The changes in the viusal cortex were accompanied by similar effects in the dLGN; whereas early reverse-occlusion had reversed the morphological effects, animals that were reverse-occluded at this later stage of development showed persistent interlaminar difference in LGN cell size (Dursteler, Garey & Movshon, 1976).

A remarkable feature of reverse-occlusion was the speed at which the effects occurred. Even within only three days of reverse-occlusion, most of the physiological effects of deprivation in the visual cortex and the accompanying morphological changes in the dLGN were reversed so long as it was implemented at four weeks of age

(Movshon, 1976; Dursteler, Garey & Movshon, 1976). Moreover, the effects of reverse-occlusion were complete in just 12 to 21 days, depending on when it was implemented (Movshon, 1976; Dursteler, Garey & Movshon, 1976). The effects of reverse occlusion observed in animals closely mimics what may occur in a human condition known as 'occlusion amblyopia', occuring in patients typically lost to follow-up during aggressive patching therapy, who return with improved vision in the previously amblyopic eye, but with their 'normal' eye visually impaired (Scott, et al., 2005). These findings expand the concept of a critical period in visual development, as one not only during which amblyopia may be induced by abnormal visual input, but also a period during which treatment by occlusion therapy may be effective. Similarly, the severity of the consequences of early monocular deprivation for the deprived eye depends on when deprivation begins, and how long it is imposed (Giffin & Mitchell, 1978).

An important experiment compared the two recovery conditions of passive binocular recovery and reverse-occlusion (Mitchell, Cynader & Movshon, 1977). Kittens initially monocularly deprived to post-natal day 45 or 60 were given seven to eight weeks of either passive binocular recovery, or reverse-occlusion therapy. Surprisingly, kittens in both recovery conditions showed similar improvement of vision in the previously deprived eye (Mitchell, Cynader & Movshon, 1977). However, the cortical ocular dominance shift was smaller for animals after passive binocular recovery. This suggested a shift in ocular dominance that allowed for a greater binocular cortical response. Research into passive binocular recovery suggested that it may be possible to not only improve vision of amblyopic eyes, but also to gain binocular function.

Modifications of reverse-occlusion treatment then sought to determine to what extent vision in the previously deprived eye could improve, with hopes of promoting binocular input as well. With the realization that reverse-occlusion as employed in animal studies of amblyopia often had detrimental effects on the previously normal eye, it was suggested that current treatments were unsatisfactory and that amblyopia treatment should be optimized to promote the best vision possible for *both* eyes (Mitchell, Murphy, Dzioba & Horne, 1986). An elegant experiment was then conducted with kittens monocularly deprived by eyelid-suture from the time of natural eye opening until six weeks of age; a condition now known to produce a significant deprivation amblyopia effect. For the next five to six weeks, the kittens were allowed only 7 hours of vision each day (for the rest of the day they were placed with their mother in a darkroom). Each day, they were reverse-occluded for different periods of time (1-2, 3.5, 5 or 7 hours/day) with the help of a removable mask worn by the kittens (described in Dzioba, Murphy, Horne, & Mitchell, 1986), while for the rest of their time in the light both eyes were open. Visual acuities for square-wave gratings were measured for both eyes (method described by Mitchell, Giffin, & Timney, 1977) throughout the occlusion regimen, and for a period of time following termination of treatment. For animals receiving the longest period of reverse-occlusion, 7-hours per day, vision of the deprived eye initially improved to normal levels (Mitchell, et al., 1986). However, as reported in previous eyelid-suture studies (Mitchell, Murphy, & Kaye, 1984a; Mitchell, Murphy & Kaye, 1984b) this gain was not maintained following the termination of treatment. After the occlusion regimen was terminated, the visual acuity of the previously deprived eye

regressed significantly (Mitchell, et al., 1986). Substantial visual improvement in the deprived eye was also seen for kittens deprived for intermediate amounts of time (5 or 3.5 hours), however, after termination of occlusion therapy, visual acuity continued to improve (Mitchell, et al., 1986). Surprisingly, it seemed that some process of recovery was set in motion by part-time reverse-occlusion, which continued even after occlusion treatment was terminated. Remarkably, this sustained improvement in visual acuity of the previously deprived eye did not regress as was seen for the full-time reverse-occlusion condition, and any reduction of visual acuity of the normal eye by occlusion treatment disappeared following termination of treatment, resulting in equal normal visual acuity for both eyes (Mitchell, et al., 1986). A regimen of part-time reverse-occlusion was therefore recommended as an optimal treatment to improve vision of amblyopes (Mitchell, et al., 1986).

Successful animal models of deprivation amblyopia have lead to an understanding of not only the disease-process, but how any subsequent visual impairment is best treated. Most studies examining recovery from amblyopia have employed the model of deprivation amblyopia, an extreme form of amblyopia that occurs in humans. In such cases, amblyopia may result from unilateral congenital cataract or unilateral congenital ptosis, for example, and can result in lasting severe visual impairment without quick surgical and theraputic intervention. Visual outcomes for patients with unilateral congenital cataract, for example, have historically been dismal without aggressive and immediate treatment (Drummond, Scott & Keech, 1989). The information gained from animal models of deprivation amblyopia is highly applicable to the clinical assessment

and treatment of amblyopia in humans, and has helped clinicians to better treat their amblyopic patients. Today, the most common treatment for amblyopia is occlusion therapy; occluding the 'good' eye to force use of the 'bad' eye. Because of extensive animal studies, it is now known that this treatment acts to reverse the imbalance of cortical input between the eyes to favour input from the amblyopic eye to the visual cortex. As shown in animal models, if occlusion therapy is imposed in early life, it can be extremely effective in reversing the effects of monocular deprivation during the critical period for visual development.

1.2 Neural Mechanisms of Amblyopia

Using first cats, and then monkeys, the groundbreaking research of Hubel and Wiesel established that with even brief periods of abnormal vision, such as monocular deprivation, there are structural changes in the LGN, a shift in cortical ocular dominance columns, and reduced orientation selectivity of primary visual cortex cells (Wiesel & Hubel, 1963a; Wiesel & Hubel, 1963b; Wiesel & Hubel, 1965b, Hubel, Wiesel & LeVay, 1977). These physical changes were found to persist with even as much as 5 years of normal visual exposure (Wiesel & Hubel, 1965; Hubel & Wiesel, 1970). This period of susceptibility to permanent changes in the visual system is defined by an animal's 'critical period' for visual development. The critical period has been defined in many species, and typically begins at eye opening (Daw & Wyatt, 1976). Plasticity often peaks soon after visual experience begins, and is followed by a gradual decline until sometime after physical maturity (Daw & Wyatt, 1976). Research into plasticity within

these critical periods has found that the peak of plasticity occurs at slightly different times for different visual perceptual abilities. The critical period for ocular dominance, for example, overlaps but extends considerably later than that for direction-selectivity for primary visual cortex cells in the cat (Daw & Wyatt, 1976). The molecular mechanisms behind amblyopic changes that occur during critical periods of visual development are of great interest in brain plasticity research. Currently, excitement surrounds the exploration of possible molecular brakes which act to terminate the brain's plasticity, thus ending a critical period for development. Many clinicians hope that once these mechanisms are understood, it may be possible to return brain areas to a more plastic state and thereby provide therapies for numerous developmental disorders, including amblyopia.

Over the past two decades, there has been a flurry of research into the molecular changes that occur in the visual pathways during established critical periods in early visual development. A host of molecular changes including the regulation of receptor synapses (such as AMPA, NMDA and GABA receptors), protease activity, neurofilament protein levels, and many more molecular markers have been investigated (Daw, 2006; chapter 11). Because the critial periods for visual development, and the effects of abnormal input during that time, are well documented, studies of brain plasticity continue to use animal models for deprivation amblyopia and strabismus. Recently, the functional effects of monocular deprivation were shown to be mirrored by changes in protein expression in the cat primary visual cortex (Jaffer, Vorobyov, Kind, & Sengpiel, 2012). The synaptic protein expression of the GluR1 subunit of AMPA receptors, as well as downstream regulation of signalling molecules from N-methyl-D-asparate (NMDA)

receptors was found to be regulated by visual exposure of the deprived eye (Jaffer, Vorobyov, Kind, & Sengpiel, 2012). Previous studies had also found that visual experience triggers the formation of NMDA receptor insertions in the visual cortex of the rat (Quinlan, Philpot, Huganir & Bear, 1999). These findings suggest one mechanism by which visual input may regulate plasticity within the visual cortex, by regulating the expression of synaptic receptor sites.

The close relationship between behavioural and structural changes in the LGN of the cat, particularly the discovery that there is a loss of neurofilament protein following monocular occlusion, has led to research into the possible role of neurofilament and brain plasticity. Following monocular lid-suture, labelling of neurofilament protein (using monoclonal antibody SMI-32) is reduced significantly in the deprived laminae of the cat LGN (Bickford, Guido & Godwin, 1998). These results demonstrate how abnormal input by lid-suture influences the organization of the neuronal cytoskelton, known to provide stability to cell structures. Similar findings were found in the primary visual cortex of monocularly deprived and strabismic monkeys (Duffy & Livingstone, 2005; Fenstemaker, Kiorpes & Movshon, 2001). However, when monocular deprivation was induced in adult animals, no reduction of neurofilament was seen in the cat LGN (Bickford, Guido & Godwin, 1998) or the monkey primary visual cortex (Duffy & Livingstone, 2005). Since no effect could be produced beyond the critical period for visual development, these findings suggest that early monocular deprivation and strabismus causes a restructuring of neural circuitry only in an immature visual system. It is speculated that neurofilament may play a role in the development of amblyopia by

structurally supporting abnormal neural circuitry if amblyogenic factors are present during heightened visual pathway plasticity.

The relationship between neurofilament and amblyopia has recently been investigated in monocularly deprived kittens. As noted by Bickford et al. (1998), neurofilament labelling is remarkably reduced in deprived layers of the dorsal LGN immediately after monocular deprivation (O'Leary, Kutcher, Mitchell, & Duffy, 2012). The recovery of neurofilament levels was then observed during either passive binocular recovery, or reverse-occlusion. Levels of neurofilament immunoreactivity as well as cell soma size increased equally and substantially in deprived laminae within 8 days (O'Leary, et al., 2012). As a control condition, some monocularly deprived kittens were placed in complete darkness for 8 days at the same time as the other animals received visual input through their deprived eye. Surprisingly, neurofilament immunoreactivity dropped drastically in all layers of the dLGN, and cell soma size recovered immediately following this brief period of total binocular occlusion, or 'dark-pulse' (O'Leary, et al., 2012). Interestingly, this loss of neurofilament labelling was not only seen in the previously deprived layers of dLGN, but also extended to the non-deprived layers (O'Leary, et al., 2012). An important, recent investigation of the effects on vision of a 10-day 'dark-pulse' imposed either immediately or 8 weeks after a 7-day period of monocular deprivation imposed at postnatal day 30 (p30) revealed surprising and substantial improvements in the vision of the deprived eye (Duffy & Mitchell, 2013). Kittens placed in darkness immediately after the period of monocular deprivation initially appeared blind in both eyes on initial re-exposure to light. Subsequently, however, the

visual acuity of both eyes improved in synchrony to normal levels in 7 weeks. In other words, amblyopia (defined as the relative visual loss with respect to the fellow eye) never developed in the deprived eye. On the other hand, when kittens were placed in darkness 8 weeks after termination of the period of monocular deprivation, the substantial amblyopia that had developed in the deprived eye disappeared rapidly in just 7 days following re-exposure to light. Incidently, the vision of the non-deprived eye was not affected by the dark-pulse indicating that the effects of darkness on the vision of this eye are observed during a very short critical period. Thus, no matter whether the dark pulse occurred immediately after the period of monocular deprivation or else delayed the animals developed normal vision in both eyes. It is of interest that an experimential manipulation (continual exposure to white noise for 7 weeks) has been reported to restore plasticity in the primary auditory cortex of adult rats (Zhou, Panizzutti, de Villers-Sidani, Madeira & Merzenich, 2011).

1.3 Objectives and Thesis Overview

Over the past half-century, animal models for amblyopia have provided invaluable knowledge of the underpinnings of amblyopia, and have aided in the optomization of therapeutic treatments. The animal model of monocular deprivation in particular has high face validity with respect to the human condition of deprivation amblyopia, a condition that occurs most frequently in patients with congenital cataract or ptosis. Historically, patients with monocular congenital cataract are known to have extremely poor visual outcome for the cataractus eye, even after surgical removal and

amblyopia therapy. In recent years, the push for early cataract removal and use of contact lenses in very early life, as well as proactive amblyopia treatment, have improved the outcomes, although visual acuity remains poor for most patients (Drummond, Scott & Keech, 1989). The majority of these patients achieve vision of only 20/60 on Snellen or poorer (Drummond, Scott & Keech, 1989), a level of vision (in the better seeing eye) below driving standards in Canada (Yazdan-Ashoori, & ten Hove, 2010) and much of the United States (Johnson, & Wilkinson, 2010). Despite the push for early intervention, visual acuity outcomes for these patients remains highly correlated with excellent compliance with contact lens and patching therapy following surgery (Drummond, Scott & Keech, 1989). Amblyopia remains the major cause of residual visual deficit after surgery for these patients (Ledoux, Trivedi, Wilson, & Payne, 2007). The high incidence of amblyopia in these patients is likely due to poor compliance with amblyopic treatments, including patching. Many subsequently develop sensory strabismus, another amblyogenic factor. The search for new and effective treatments for deprivation amblyopes is therefore ongoing, and we turn to animal models once again in search of ways to enhance treatment.

The amazing effectiveness of a 'dark pulse' to prevent or eliminate amblyopia in monocularly deprived kittens (Duffy & Mitchell, 2013) leads naturally to the need to extend the study to animals deprived in a fashion that is arguably closer to the amblyogenic events observed clinically. In the earlier study (Duffy & Mitchell, 2013) monocular deprivation was imposed for a week beginning at postnatal day 30 (p30). Reasonably, it could be argued that the sudden onset of deprivation and its brief duration

is quite different from the situation in congenital cataract which is thought to begin early and typically persists for many months or even years. The study described in this thesis represents an extension of the earlier study to investigate the visual consequences of a dark-pulse imposed on animals deprived earlier (from the time of natural eyelid-opening) and lasting longer (1 month). Not only is this model more representative of the human clinical condition of deprivation amblyopia, but it answers a key question brought up by the earlier study. The visual recovery observed following the dark-pulse could reflect a process by which the restored plasticity promoted by darkness permitted restoration of a pre-existing normal architecture laid down prior to the initial monocular deprivation at p30. Alternatively, the restored plasticity due to darkness may be sufficient to promote recapitulation of the sequence of development in the visual system. In the latter case, darkness would be just as effective if imposed on animals in which the initial period of monocular deprivation began at or prior to eyelid-opening in which situation the cortical architecture would little change from the immature state dictated prior to modification by visually driven neural activity.

Further issues examined in this thesis include a preliminary investigation of the minimum duration of the dark-pulse required to achieve a therapeutic effect, as well as the strictness of the requirement for darkness. Some kittens were therefore given a dark-pulse of just five days duration, or else received small daily periods of light exposure (30-minutes) each day throughout 10-day dark-pulse. Finally, this thesis reports investigation of the effects of a dark-pulse on other visual abilities such as depth and vernier (alignment) acuity. Kittens who achieved normal visual acuities in both eyes following

the dark-pulse were then tested on a depth discrimination task as well as a vernier alignment (spatial localization) task. It was hoped that the results of this report would answer some important questions raised by the initial study (Duffy & Mitchell, 2013), which must be answered in order for clinicians to consider whether total binocular deprivation may enhance the treatment of their amblyopic patients.

CHAPTER 2: THE ANIMAL MODEL

2.1 Cat Colony and Housing

Nine purpose-bred kittens from 3 litters born and raised in the closed cat-breeding colony of Dalhousie University (Studley campus) served as subjects for this study. Protocols for breeding, surgery, rearing and behavioural testing were approved by the Dalhousie Committee on Laboratory Animals, in accordance with Canadian Council on Animal Care regulations. All kittens were raised with their mother and litter-mates under normal colony lighting conditions except when experimental manipulation of binocular light-exposure was required. For 6 of the animals, the left eyelid was sutured closed at post-natal day 7; in some cases, where the eyelids were still closed, the eyelids were first opened before immediate eyelid-suture. For the remaining 3 kittens the left eyelid was sutured closed at post-natal day 30. At post-natal day 37 the eyelids were re-opened. The surgical procedures from eyelid closure and re-opening were performed under general gaseous anesthesia (1-3% isoflurane in oxygen) in the surgical suite at Dalhousie University (Studley campus) by use of procedures described in detail by Murphy & Mitchell (1987). Post-operatively, all animals were given analgesics and antibiotics in accordance with the protocol guidelines.

After close observation of their post-operative recovery, animals receiving an immediate dark-pulse were placed directly into a specialized darkroom for 10 days with their mother, and littermates. One kitten was allowed daily binocular light-exposure with

its mother, during which it was removed to an adjacent room illuminated by fluorescent lights that simulated normal colony lighting conditions. After the designated dark-pulse, kittens and their mother were relocated together to the colony room, illuminated by flurescent lights on a 12-hour light/dark cycle. Animals that received a delayed dark-pulse were returned to the colony room following surgery and their littermates and remained under normal colony lighting conditions for five to eight weeks, at which time they were placed in the darkroom for 10 days.

The specially constructed darkroom in the Life Sciences Centre at Dalhousie University (Studly campus) was used to provide total binocular deprivation for the dark-pulse treatment. The darkroom (*figure 1*), described in detail in a previous study (Beaver, Mitchell & Robertson, 1993), is separated from an illuminated anteroom by a series of sealed doors and hallways. The walls and doors within the room and surrounding halls are painted black in order to absorb light. Within the room, the animals were held in a large rearing cage (measuring 93cm high, 66.5cm deep, and 153cm wide). The rearing cage contained a litter box, food, water, and cardboard boxes and towels for bedding. A radio within the darkroom was put on a timer set to turn on and off on a 12-hour cycle, to stimulate the light/dark cycle experienced under normal colony conditions.

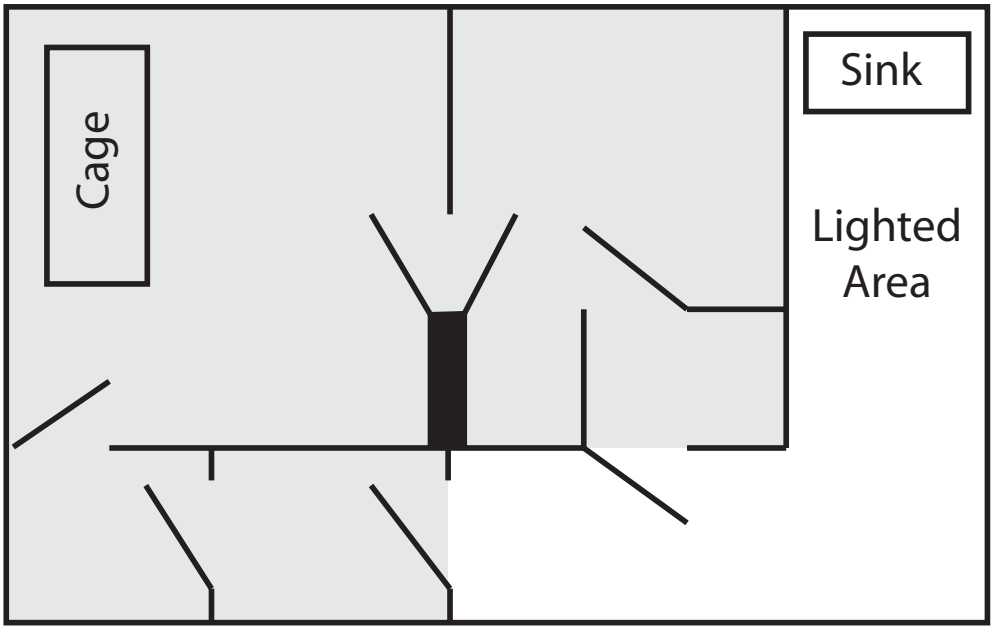

Figure 1. This specially constructed darkroom was used to deprive subjects of light. The housing room and dark anteroom (used for cleaning procedures) is separated from lit rooms by a series of sealed doors and halways. All walls and doors within the room and surrounding halls are painted black to absorb light. Within the housing room, there is a large rearing cage (measuring 93cm high, 66.5cm deep, and 153cm wide). The rearing cage contains a litter box, food, water, and cardboard boxes and towels for bedding. The lighted area contains a sink area used to refill water bowls and to prepare food.

2.2 Rearing Conditions

A total of 9 kittens were subjects of this study. Six kittens were monocularly deprived (MD) by eyelid suture from post-natal day 7 (p7), and the remaining three from post-natal day 30 (p30). All kittens had their deprived eyelids opened on post-natal day 37 (p37). Immediately following eyelid-opening, three kittens (two MD p7-37, and one MD p30-37) were placed in the darkroom for 10 consecutive days. Three kittens (two MD p7-37, and one MD p30-37) were housed under normal colony lighting conditions until the visual acuity had stabilized in the deprived eye for 3 weeks, at which time (approximately 8 weeks later), they were placed in the darkroom for 10 consecutive days. Two kittens (one MD p7-37, and one MD p30-37) received a shortened dark-pulse of five days. Of the kittens receiving the shortened dark-pulse; the first (MD p7-37) was placed in darkness immediately after the period of MD at p37, while for the second animal (MD p30-37) the pulse was imposed 8 weeks later, after vision of the deprived eye had stabilized. Finally, a single animal (MD p7-37) was immediately placed in the darkroom for 10 days after the period of MD but was removed to an illuminated anteroom for 30-minutes at the same time each day.

CHAPTER 3: BEHAVIOURAL TESTING : SQUARE-WAVE GRATINGS

3.1 Method

To test visual acuity for square-wave gratings, a specialized jumping stand was used consisting of an adjustable platform (P) from which the kittens were made to jump onto one of two landing areas (L) (*Figure 2*). All kittens were tested using this apparatus and were trained to jump toward a vertically oriented, high contrast, square-wave grating stimulus. Training began when the kittens' motor function allowed them to walk out of a lowered platform onto the landing platform. Kittens were made to walk from the lowered platform onto the right or left landing area, one of which was the vertical grating stimulus, and the other an 'open door' revealing a hole in the apparatus. When kittens correctly walked onto the vertically oriented grating (the only platform available), they were positively rewarded with petting and food. As their motor systems developed, and the animals became more comfortable with the task, the adjustable platform was increased gradually in height to make the kittens jump onto the platform. When kittens seemed comfortable with the task of jumping to the vertical grating, the 'open door' was replaced by an adjacent horizontal grating stimulus. This second stimulus was used instead of simply leaving the platform 'blank' (as is done in the Preferential Looking Task in humans) in order to prevent luminance cues to the animal. The kittens were then reinforced by petting and food for jumping to the vertical grating. When the animal achieved 10 consecutively correct jumps to the vertical grating, the gratings were increased in spatial frequency very gradually until a spatial frequency was reached where

the animal was no longer able to make the discrimination. The spatial frequency steps were equated logarithmically with 12 steps per octave, as compared to three steps per octave for the conventional clinical logMar acuity chart. At the beginning of a session, the spatial frequency was set very low and was increased after only one or two errorless trials. Within approximately three steps of threshold, the minimum number of trials was increased to as much as 5. After an error, the kitten had to achieve 5 consecutively correct trials or else 7 or more correct out of the maximum 10 trials allowed. The threshold was defined as the spatial frequency where this minimum criterion could not be met. Most kittens behaved nearly flawlessly until near threshold, where they showed signs of difficulty that included increased latency and crying. At threshold, many animals would revert to a position habit.

Training of the kittens began at around four weeks of age, and testing was intensive subsequently in order to ensure that they were sufficiently well trained to permit measurement of the acuity of the non-deprived eye prior to the end of the period of monocular deprivation. This early training was important in order to permit longitudinal measurement of the acuity of the deprived eye as soon as normal visual input was restored, as well as follow any subsequent recovery of vision. When kittens in the immediate dark-pulse condition were removed from the darkroom, visual acuity was measured daily until measurements stabilized. It was then monitored daily or every two days until it reached normal levels for an 8-week-old kitten (between 6 and 7 cycles per degree). For kittens in the delayed dark-pulse conditions, visual acuity measurements for the deprived eye began as soon as vision was restored to the deprived eye, and were

followed closely regularly daily or on alternate days until the acuity stabilized. After a delayed dark-pulse, their visual acuity was monitored closely until it reached normal levels for an 8-week-old kitten. For kittens' whose deprived-eye never reached normal levels of visual acuity, their vision was monitored two to three times weekly until it stabilized.

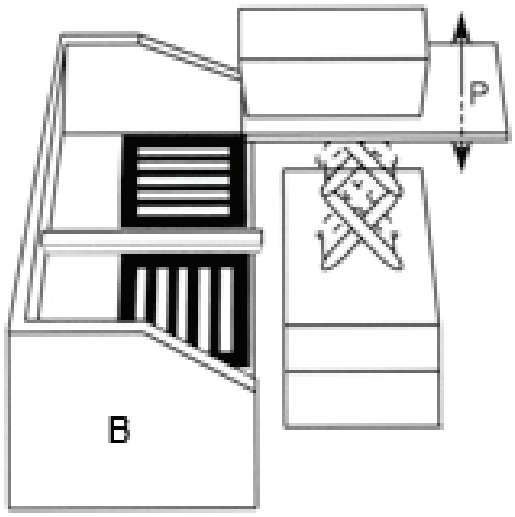

Figure 2. This jumping stand apparatus consists of an adjustable platform (P) from which the kitten jumps. Kittens are trained to jump onto the vertical square-wave grating stimulus presented on the rectangular landing box (B) measuring about 56cm high, 81cm long and 51cm wide.

3.2 Results

Visual acuity for square-wave gratings was assessed using a well-established behavioural method (Mitchell, Giffin, & Timney, 1977). Training on the jumping stand apparatus (*figure 2*) began at approximately four weeks of age to ensure acurate assessment of visual acuity following light exposure to the deprived eye. Testing was conducted on all kittens following monocular deprivation, and was monitored closely until visual acuity measurements stabilized.

Surprisingly, the three kittens (one MD from p30 to 37, and two from p7 to 37) that were placed in the darkroom for 10 days immediately following monocular deprivation initially appeared blind in *both* eyes when removed from the darkroom (*figure 3, 4, and 5*). While this was expected for the deprived eye, this detrimental effect on the "previously normal eye" was completely unexpected. However, visual acuity for both eyes improved gradually thereafter in lock-step, eventually reaching normal levels (6 to 7 cycles/deg) after 7 weeks (*figure 3, 4* and *5*). Importantly, monocular amblyopia did not develop in any of these kittens, as amblyopia would be expected based on studies of early monocular deprivation. The dark-pulse therefore prevented the development of deprivation amblyopia that became evident in the one kitten of this study yet to be placed in darkness (see C189, *table 1*). This animal has an acuity of only 1.25cycles/deg in the deprived eye.

Two kittens (one MD from p30 to 37, and one MD from p7 to 37) were placed in a darkroom for 10 consecutive days after amblyopia was allowed to develop and stabilize following monocular deprivation. Unlike kittens in the immediate dark-pulse conditions, the non-deprived eye did not show any loss of vision following the short period of binocular light deprivation (*figure 6 and 7*). The effect of the dark-pulse on the fellow amblyopic eye, however, was strikingly different as the visual acuity of this eye improved rapidly to normal levels, equal to the non-deprived eye within 5 to 7 days (*figure 6* and *7*). The speed and extent of recovery in the amblyopic eye was surprising given that the dark-pulse was imposed at approximately 3 months of age, when traditional therapeutic treatments are known to produce slow, incomplete recovery of vision (Mitchell, 1988). It is for this reason that a third animal of the delayed dark-pulse condition (C189) has yet to undergo a dark-pulse treatment. After long-term monocular deprivation (p7-37), its visual acuity was monitored closely to observe normal visual acuity in the non-deprived eye, and severe amblyopia in the deprived eye (C189; *table 1*). This animal will receive 10-days of darkness after it is an adult (over 1 year of age) in order to determine if therapeutic effects are possible in kittens at an age well past established critical periods for visual development.

Two kittens (one MD from p30 to 37, and one MD from p7 to 37) were placed in the darkroom for only five days following monocular deprivation in order to discover if any therapeutic dark-pulse effect could still be achieved with a shorter period of deprivation. For the short-term monocular deprivation animal, amblyopia was allowed to stabilize, and then the 5-day dark-pulse was imposed. No therapeutic effect was seen for

the amblyopic eye following this intervention (*figure 8*). The fast and complete recovery of vision that was seen in kittens after a 10-day period of darkness delayed dark-rearing condition (*figure 6 & 7*) was not replicated. A similar result was found for the long-term monocular deprivation animal for which the 5-day dark-pulse was imposed immediately following monocular deprivation. Similar to the other immediate dark-pulse condition animals, both the non-deprived and deprived eyes initially appeared blind (*figure 9*). Within 6 weeks, the visual acuity of the non-deprived eye improved gradually to normal levels (*figure 9*). However, the visual acuity of the deprived eye quickly plateaued at a low level and remained (*figure 9*).

Finally, a single kitten (monocularly deprived from p7 to 37) that received a 10-day dark-pulse immediately upon eyelid-opening, was provided with short daily periods (30-minutres) of normal binocular exposure throughout darkrearing. This condition was created with clinical applicability in mind as short periods of normal light exposure would presumably benefit patients by allowing social interaction and allow for eye examinations throughout treatment. Unlike kittens who received an immediate dark-pulse of 10 days, the visual acuity of the non-deprived eye did not show a loss of visual acuity, but remained at typical values for kittens of about 7 weeks of age and thereafter improved gradually to normal levels within 7 weeks of recovery after monocular deprivation (*figure 10*). As with other animals that received an immediate 10-day dark-pulse, the deprived eye initially appeared blind (*figure 10*). However, the subsequent improvement of acuity for this eye was minimal so that it developed severe amblyopia (*figure 10*). A very short period of binocular light exposure during the 10-day dark-pulse

therefore prevented the detrimental effect on the previously normal eye, but completely abolished the therapeutic effect to the deprived eye and was unable to prevent amblyopia from developing for that eye.

Cat ID	NDE	DE	MD	DR	
C156	**6.49c/deg**	**6.49c/deg**	**P30-37**	**Immediate**	**10 days (P37-47)**
C155	**6.49c/deg**	**6.49c/deg**	**P30-37**	**Delayed**	**10 days (P64-74)**
C169	**6.19c/deg**	**2.09c/deg**	**P30-37**	**Delayed**	**5 days (P92-97)**
C166	**6.49c/deg**	**6.49c/deg**	**P07-37**	**Immediate**	**10 days (P37-47)**
C192	**7.10c/deg**	**7.10c/deg**	**P07-37**	**Immediate**	**10 days (P37-47)**
C165	**6.49c/deg**	**6.49c/deg**	**P07-37**	**Delayed**	**10 days (P93-103)**
C189	**7.10c/deg**	**1.25c/deg**	**P07-37**	**Delayed**	**10 days (to be adult)**
C191	**6.49c/deg**	**1.4c/deg**	**P07-37**	**Immediate**	**5 days (P37-42)**
C190	**6.49c/deg**	**1.25c/deg**	**P07-37**	**Immediate**	**10 days *1/2 hr light/day (P37-47)**

Table 1. Visual acuity outcome data for all animals with their conditions (MD). NDE = non-deprived eye, DE = deprived eye, DR = dark-rearing condition.

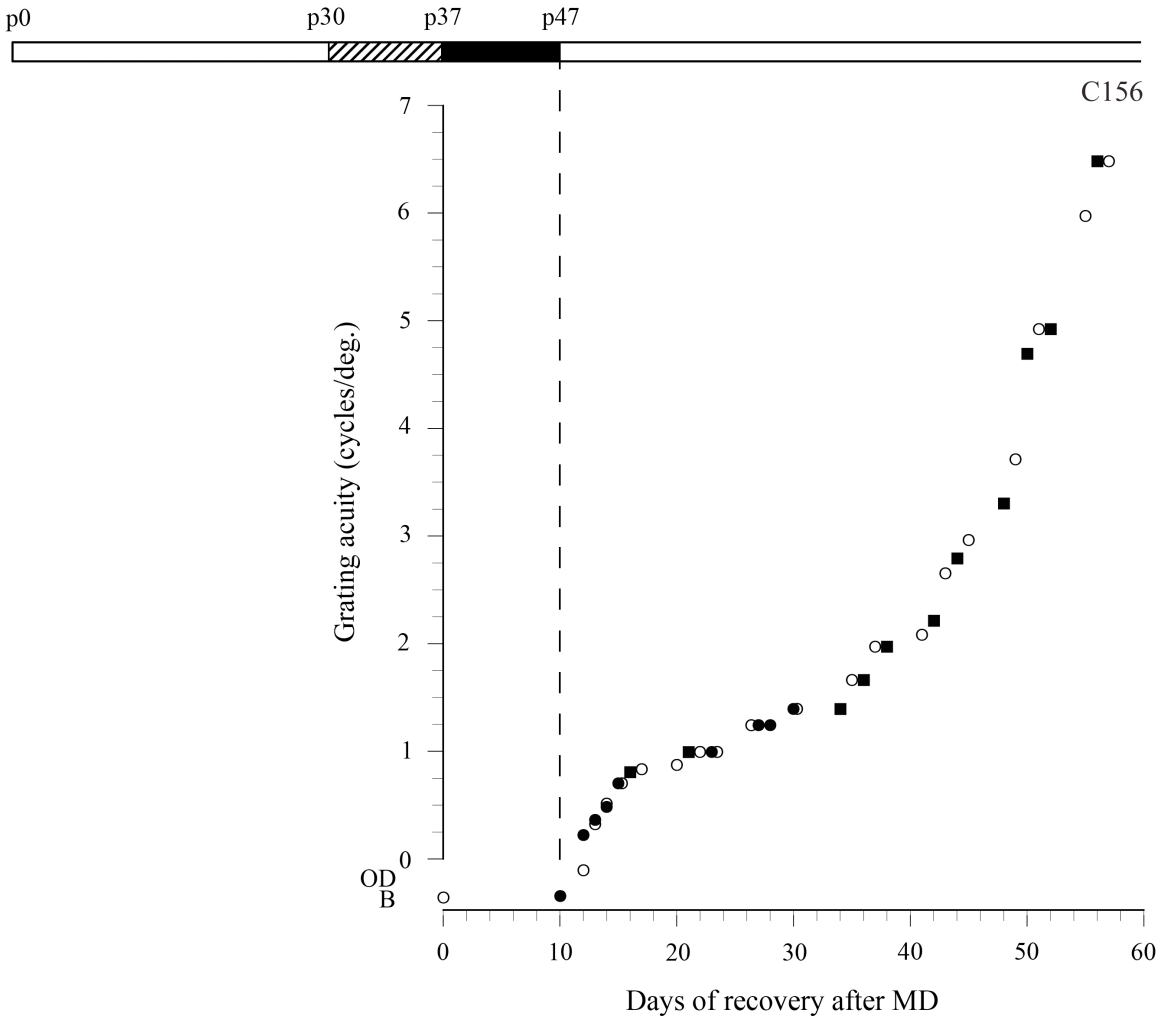

Figure 3. Visual acuity data for C156. Treatment condition displayed above the figure: monocular deprivation from postnatal day 30 (p30) to p37, and then dark-reared for 10 days to p47. Empty circles and solid circles symbolize respectively, deprived eye (DE) acuities, and non-deprived eye (NDE) acuities. Solid squares symbolize binocular trials. The symbol B on the ordinate depicts an inability to find a closed door on the jumping stand by visual cues alone. The ability to solve this task is depicted by the symbol OD (Open Door descrimination).

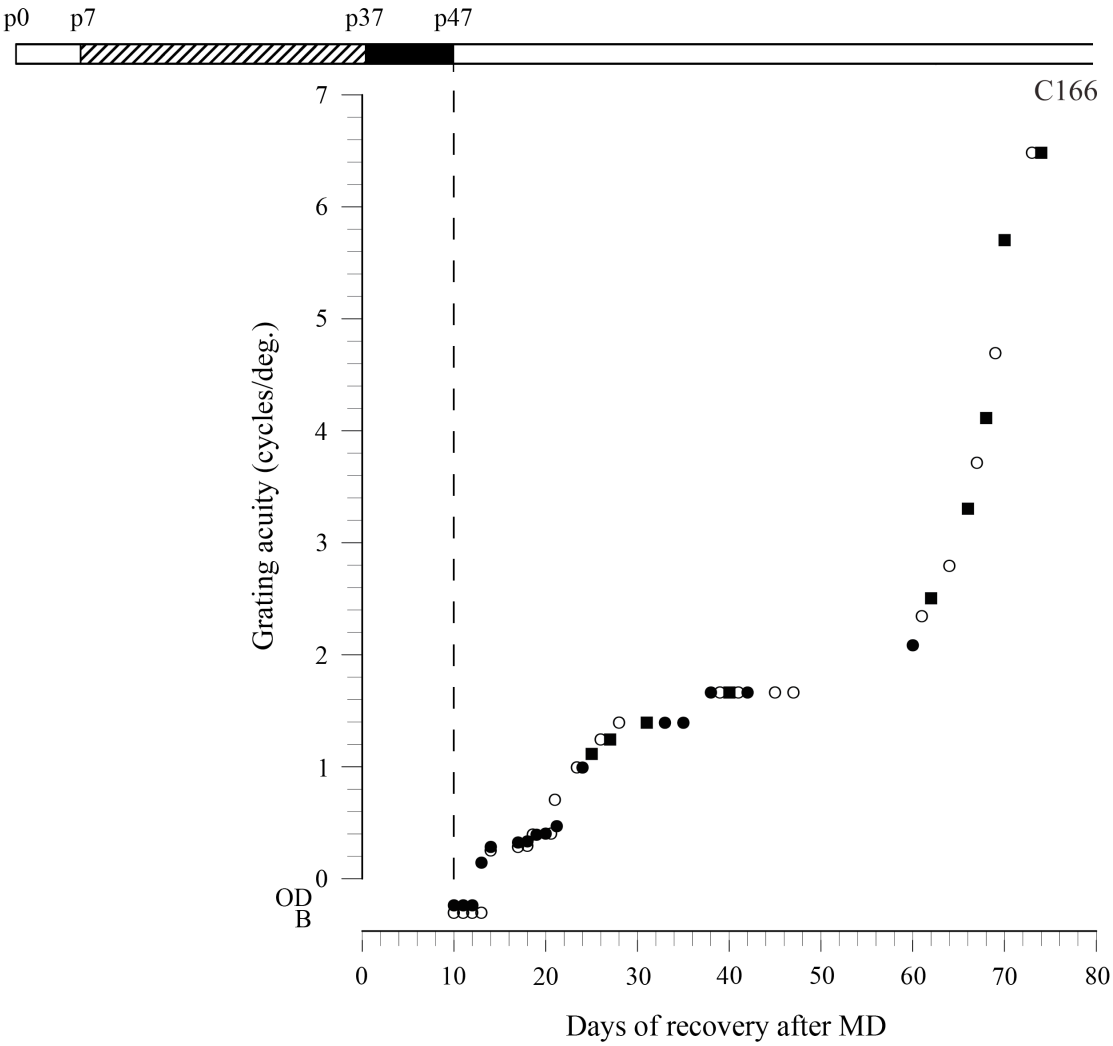

Figure 4. Visual acuity data for C166. Treatment condition displayed above the figure: monocular deprivation from the time of natural eye-opening (p7) to p37 and then dark-reared for 10 days to p47. Empty circles symbolize deprived eye (DE) acuities, while solid circles and squares symbolize the acuity of the non-deprived eye (NDE) measured respectively, either monocularly or binocularly.

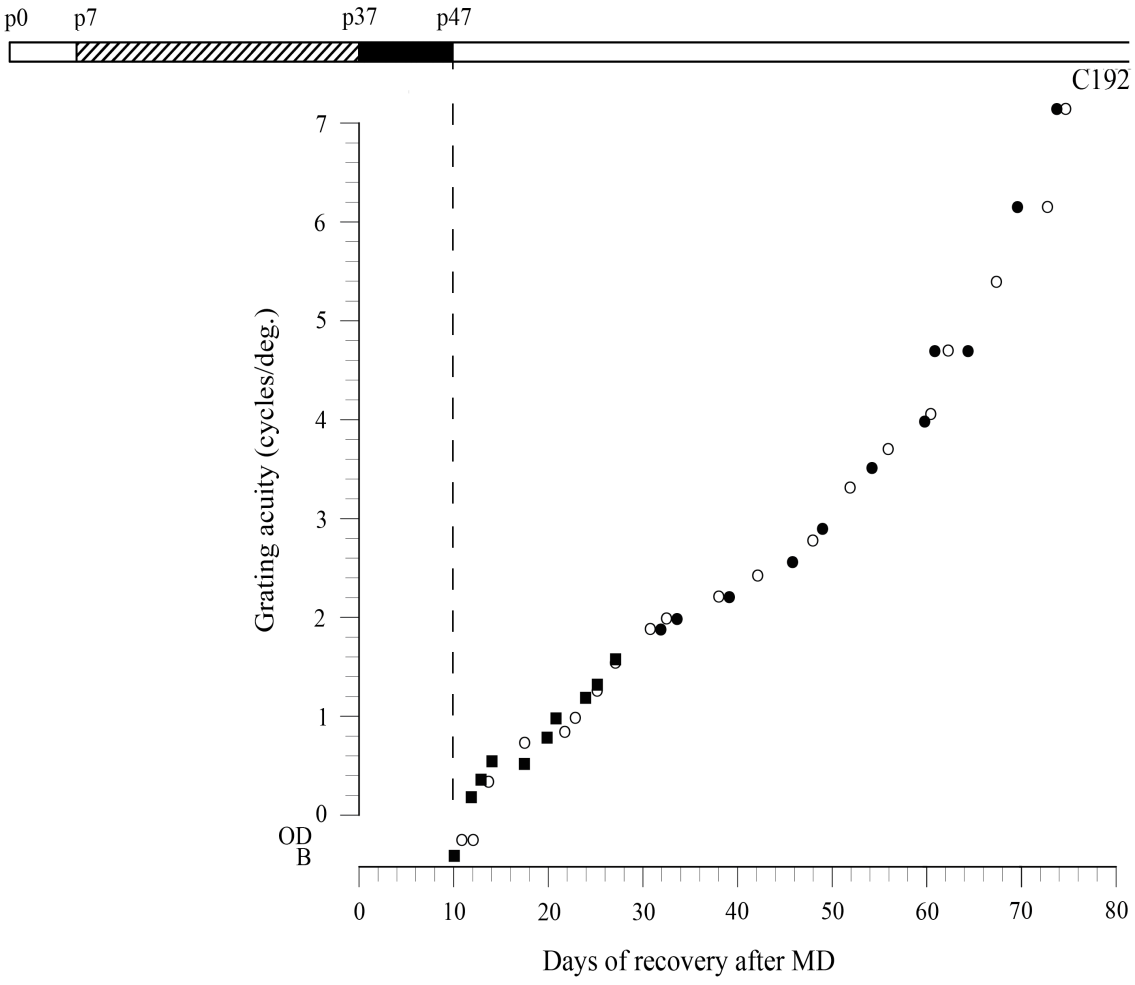

Figure 5. Visual acuity data for C192. Treatment condition displayed above the figure: monocular deprivation from natural eye opening (p7) to p37, and then immediate dark-reared for 10 days. Empty circles symbolize deprived eye (DE) acuities, while solid circles and squares symbolize the acuity of the non-deprived eye (NDE) measured either monocularly or binocularly (squares).

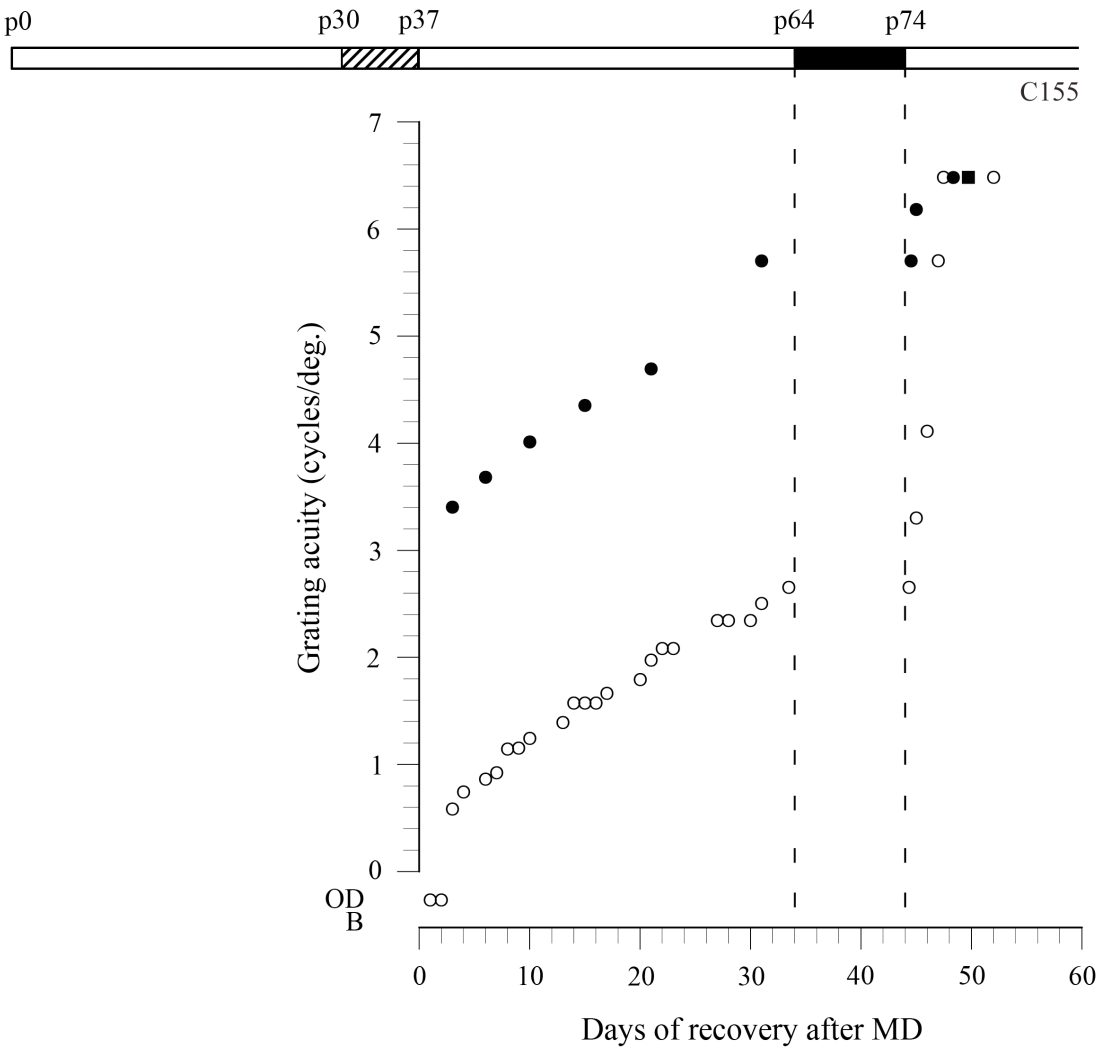

Figure 6. Visual acuity data for C155. Treatment condition displayed above the figure: monocular deprivation from postnatal day 30 (p30) to p37, and then delayed dark-reared for 10 days from p64-74. Empty circles symbolize deprived eye (DE) acuities, while solid circles and squares symbolize the acuity of the non-deprived eye (NDE) measured either monocularly or binocularly (squares).

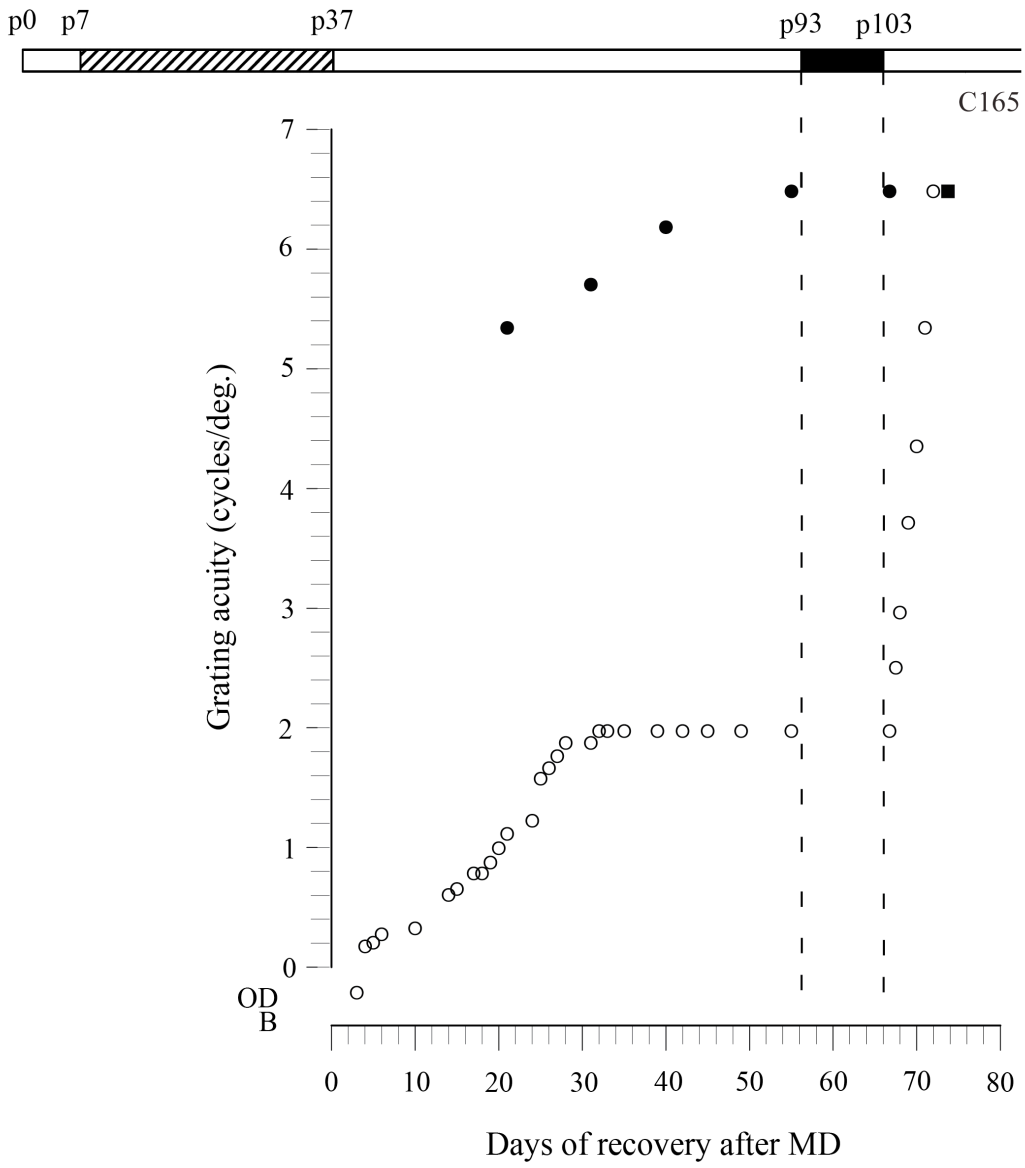

Figure 7. Visual acuity data for C165. Treatment condition displayed above the figure: monocular deprivation from natural eye opening (p7) to p37, and then delayed dark-reared for 10 days from p93-103. Empty circles symbolize deprived eye (DE) acuities, while solid circles and squares symbolize the acuity of the non-deprived eye (NDE) measured either monocularly or binocularly (squares).

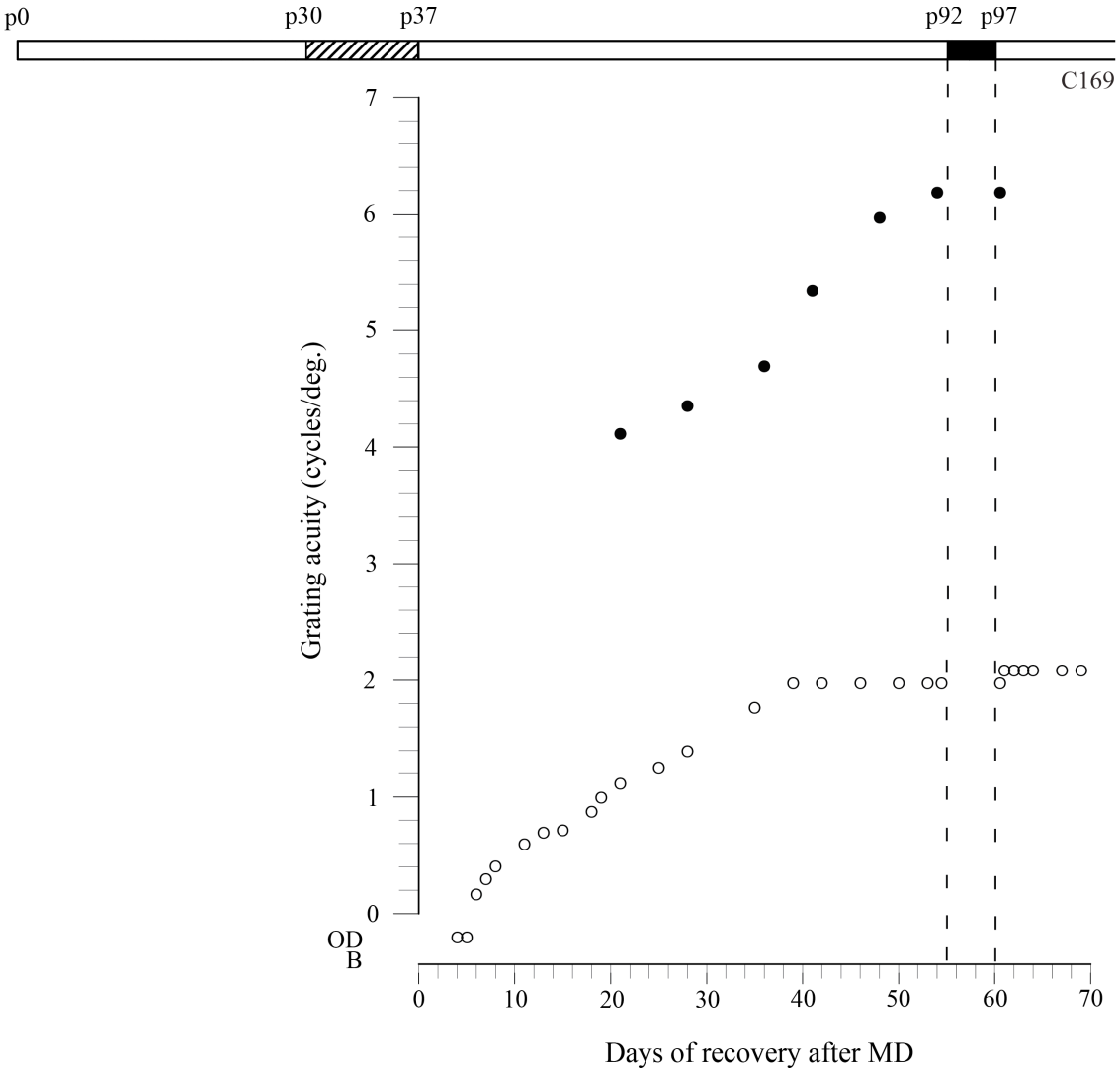

Figure 8. Visual acuity data for C169. Treatment condition displayed above the figure: monocular deprivation from from postnatal day 30 (p30) to p37, and then delayed dark-reared for 5 days from p93-103. Empty circles symbolize deprived eye (DE) acuities, while solid circles and squares symbolize the acuity of the non-deprived eye (NDE) measured either monocularly or binocularly (squares).

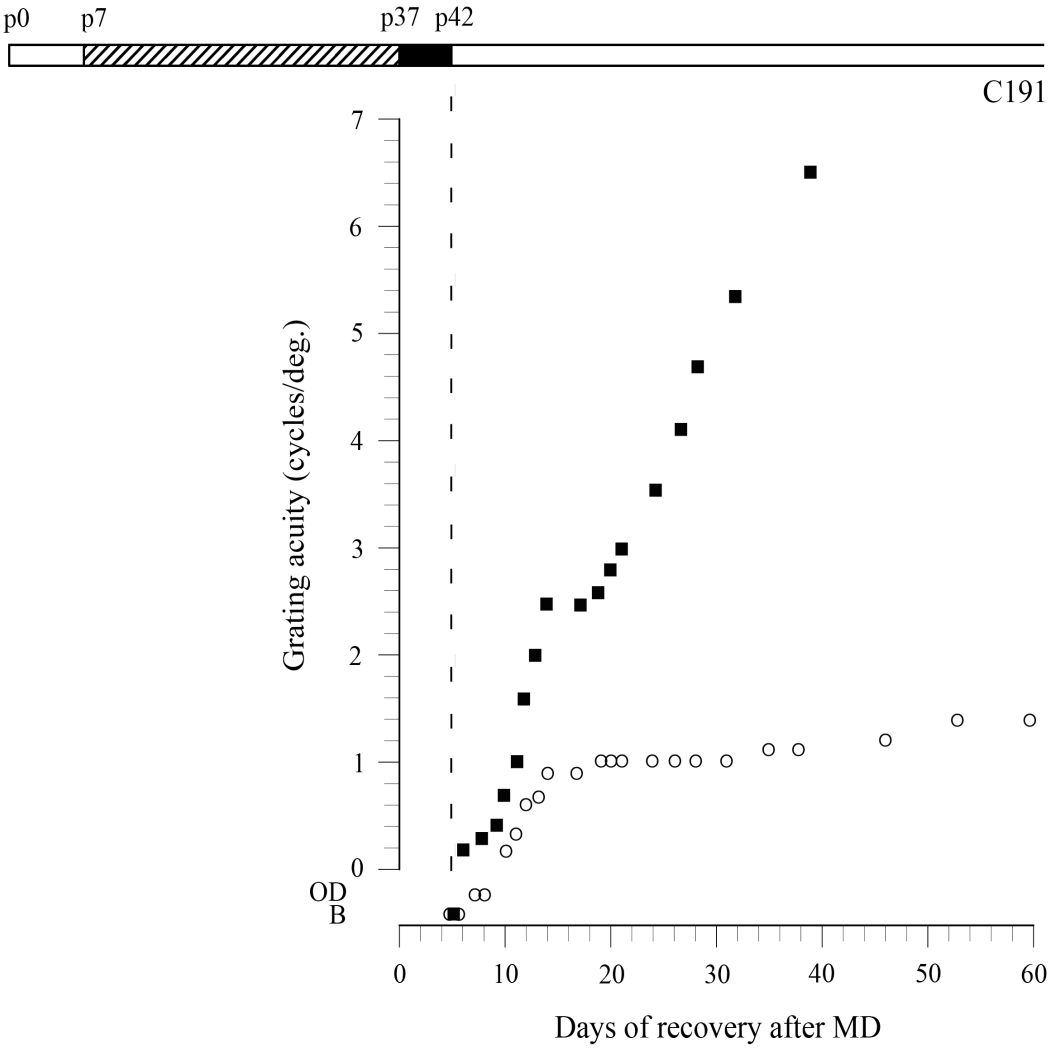

Figure 9. Visual acuity data for C191. Treatment condition displayed above the figure: monocular deprivation from natural eye opening (p7) to p37, and then immediate dark-reared for only 5 days Empty circles symbolize deprived eye (DE) acuities, while solid circles and squares symbolize the acuity of the non-deprived eye (NDE) measured either monocularly or binocularly (squares).

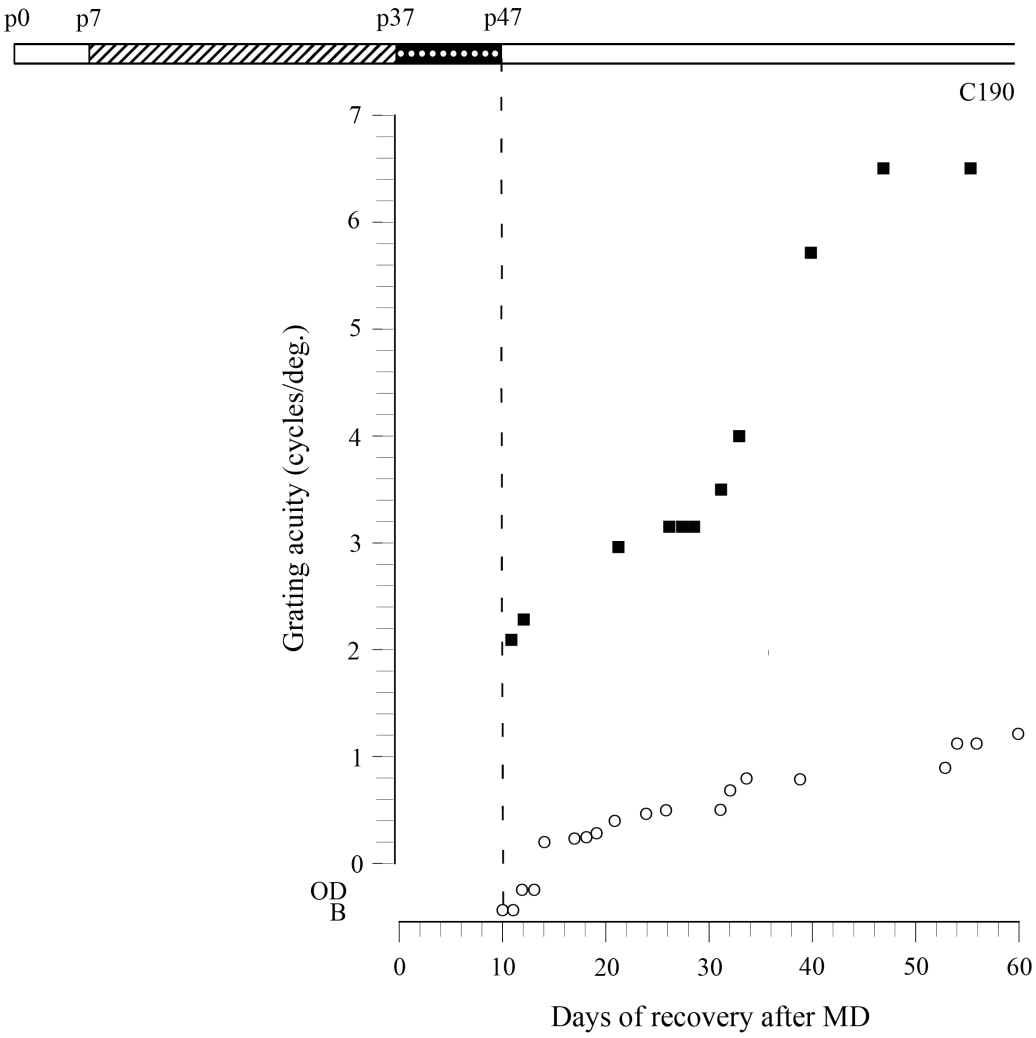

Figure 10. Visual acuity data for C190. Treatment condition displayed above the figure: monocular deprivation from natural eye opening (p7) to p37, and then immediate dark-reared for 10 days with 30-minutes of binocular light exposure daily. Empty circles symbolize deprived eye (DE) acuities, while solid circles and squares symbolize the acuity of the non-deprived eye (NDE) measured either monocularly or binocularly (squares).

CHAPTER 4: BEHAVIOURAL TESTING : STEREOACUITY

4.1 Method

To test stereoacuity in cats, a unique behavioural method was used to assess their ability to descriminate stimuli with reduced monocular depth information located at different distances from them. A specialized jumping stand was used from which the cats made judgements of depth between two adjacent stimuli (described in detail by Mitchell, Kaye & Timney, 1979). Normal animals with forward-facing eyes, are able to discriminate much smaller separations of depth when performing binocularly than with just one eye. To test this in cats, they were trained to jump onto the closer of two stimuli presented beneath a plexiglass landing area (*figure 11)*. The stimulus plates seen beneath the landing areas consisted of two panes of glass covered by difference sized black dots, similar to the stumuli used in the Howard-Dolman box to test gross stereoacuity in humans (Mitchell, Kaye & Timney, 1979). The position of each plate could be adjusted in height relative to the other in order to determine distance discrimination thresholds. Only the kittens who reached normal levels of visual acuity in both eyes were tested using this apparatus following behavioural testing for square-wave grating acuity. Similar to grating acuity methods, kittens were made to jump onto either the right or left landing area. Unlike the training required for grating acuity testing, this test takes advantage of the innate preference for a kitten to jump onto the closer of two stimuli. When kittens correctly jumped toward the closer stimulus (the higher plate), they were

rewarded with petting and food reward. Testing began with the two stimulus plates at the most extreme distance difference (approx. 22cm), the difference in height between the two plates was then decreased until the animal was no longer able to distinguish which was the closer stimulus. The kitten was required to make at least 7 of 10 correct discriminations before the plates were adjusted. The distance between the two plates was decreased slowly until the kitten found it difficult to judge which plate was closest. Distance discrimination threshold was determined when the animal was behaving at 'chance', set at less than 7 correct out of 10 jumps, or 3 incorrect jumps in a row. As occurred with visual acuity testing, animals typically began crying or exhibited increased latency when approaching threshold, and then showed position habit when they could no longer perform the task. Testing was performed with both eyes open initially, to determine the kitten's binocular performance on the task. After the animal was familiar with the apparatus, performing well to exhibit a depth discrimination threshold, an opaque contact lens was placed in one eye to assess their monocular performance on the same day of binocular testing. Testing was done over 10 subsequent days, each day assessing binocular ability, and then placing the contact lens in one eye for monocular testing. The occluded eye was alternated each day for a total of 5 monocular thresholds for each eye.

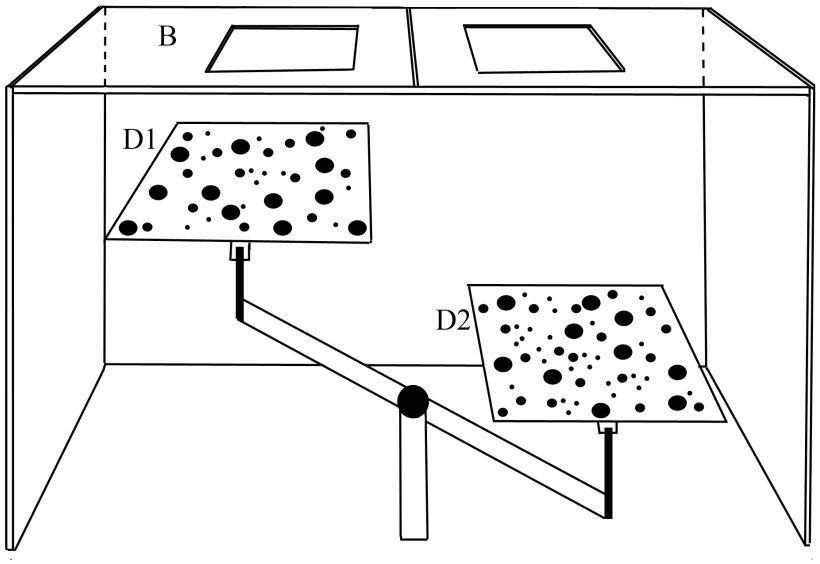

Figure 11. This jumping stand apparatus consists of a platform from which the kitten jumps, similar to that used for grating acuity methods. Kittens are trained to jump toward the closer of two plates (D1 & D2) presented beneath the landing box (B).

4.2 Results

Stereoacuity, as measured by depth discrimination, was conducted on all kittens that achieved normal visual acuity in each eye following experimental intervention. Training on the jumping stand apparatus began after visual acuity stabilized at normal levels as measured by square-wave gratings. Three kittens (one monocularly deprived from p30 to 37, the other two from p7 to 37) that were placed in the darkroom for 10 consecutive days immediately following monocular deprivation did achieve normal visual acuity in each eye. Depth discrimination testing revealed that the single kitten who underwent short-term monocular deprivation (C156; MD p30-37) appeared to have stereopsis; its binocular performance was excellent and it was unable to perform the task at all using either eye alone (*figure 12*). The depth thresholds are expressed as a ratio of the smallest difference in depth between the two plates that the cat could detect ΔD divided by the distance to the farthest stimulus ($D + \Delta D$) where D is the distance to the nearest stimulus. When using two eyes, C156 could discriminate differences in the distances of the two plates of about 1cm. The two kittens with longer monocular deprivation (C166 & C192; MD p7-37) appeared to have either marginal or no stereopsis. The first (C192; *figure 14*) performed moderately well binoculalry, but not as well as did C156 (MD p30-37; *figure 12*) but could not perform the task at all monocularly implying that there was a cue to depth available only when using both eyes. The second animal (C166; *figure 13*) was stereoblind, as both its binocular and monocular performance were equally poor. Depth discrimination thresholds were also measured on the two kittens (one MD p30-37, and one MD p7-37) for which the dark-pulse was delayed. The animal

with short-term MD (C155) performed as if it had marginal stereopsis, performing only slightly better with both eyes than with just one (*figure 15*). However, the animal with longer MD (C165) was stereoblind, as both its monocular and binocular perfromance was equally poor (*figure 16*). Although the results of depth discrimination testing were variable between subjects, it was encouraging that one animal (C156;Figure 12) appeared to acquire good stereopsis similar to that observed in normal animals and 2 others (C192 and C155) appeared to possess marginal stereopsis. On the other hand, two animals (C166 and C165) were clearly stereoblind.

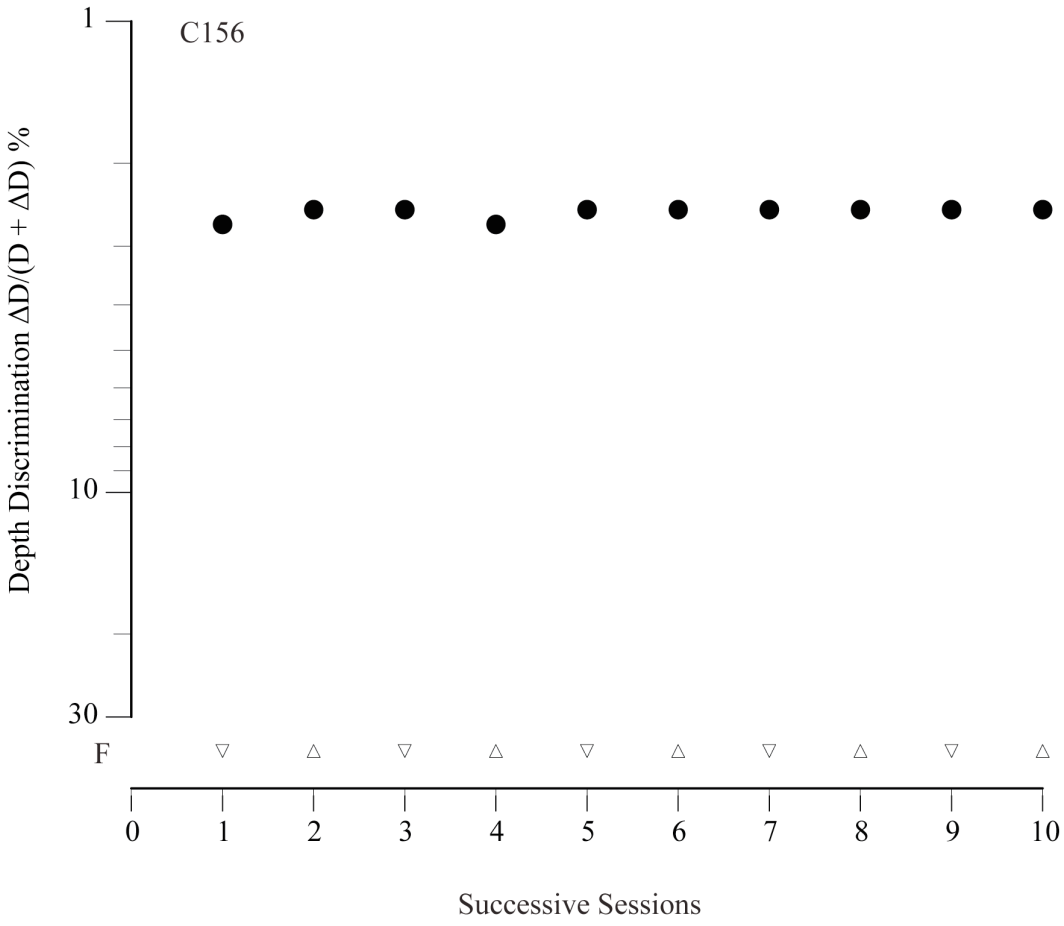

Figure 12. Stereoacuity data for C156 (MD p30-37, DR p37-47). Depth discrimination thresholds are expressed as a ratio of the smallest difference in depth between the two plates that the cat could detect; ΔD/(D + ΔD). F indicates failure to perform the task at the largest depth discrimination. Solid circles symbolize binocular outcomes, while open triangles symbolize alternating monocular outcomes. Testing was performed over 10 subsequent days to acquire a total of 10 binocular measures, and 5 monocular measures for each eye.

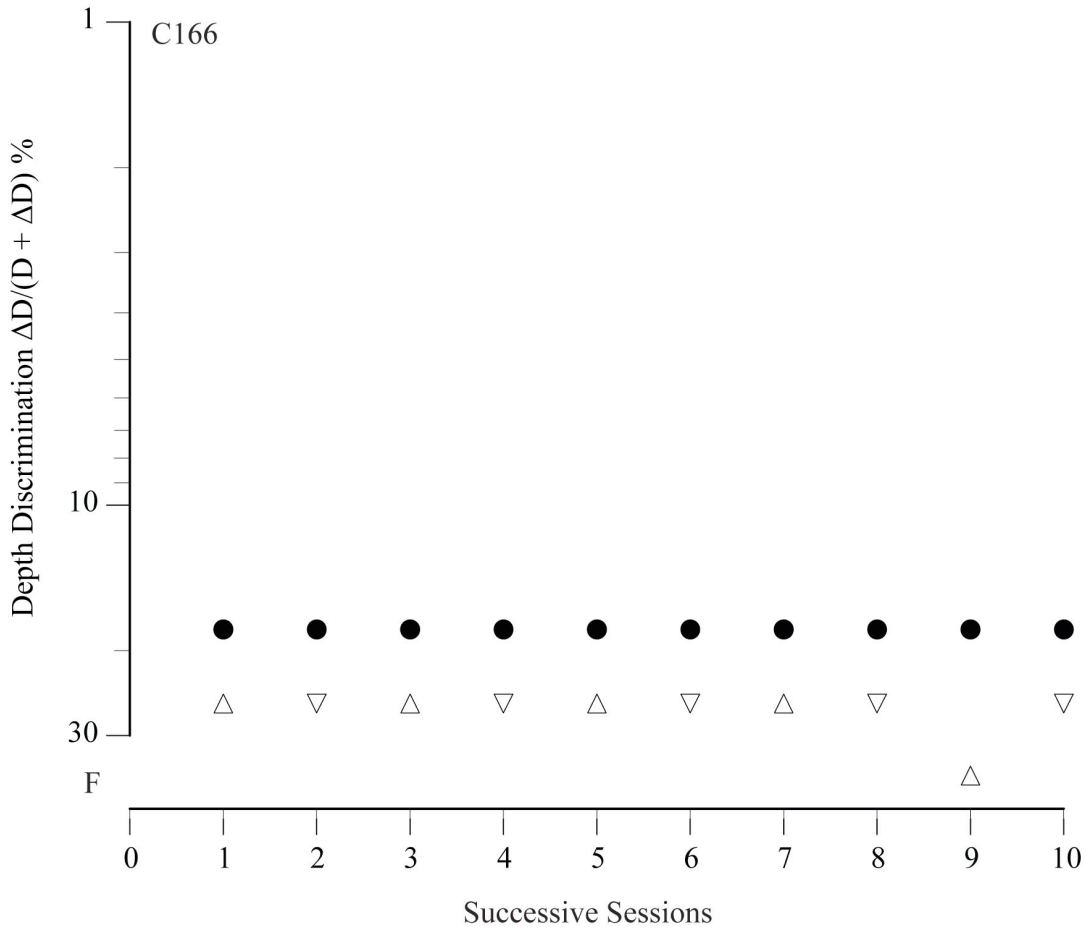

Figure 13. Stereoacuity data for C166 (MD p7-37, DR p37-47). Depth discrimination thresholds are expressed as a ratio of the smallest difference in depth between the two plates that the cat could detect; ΔD/(D + ΔD). F indicates failure to perform the task at the largest depth discrimination. Solid circles symbolize binocular outcomes, while open triangles symbolize alternating monocular outcomes. Testing was performed over 10 subsequent days to acquire a total of 10 binocular measures, and 5 monocular measures for each eye.

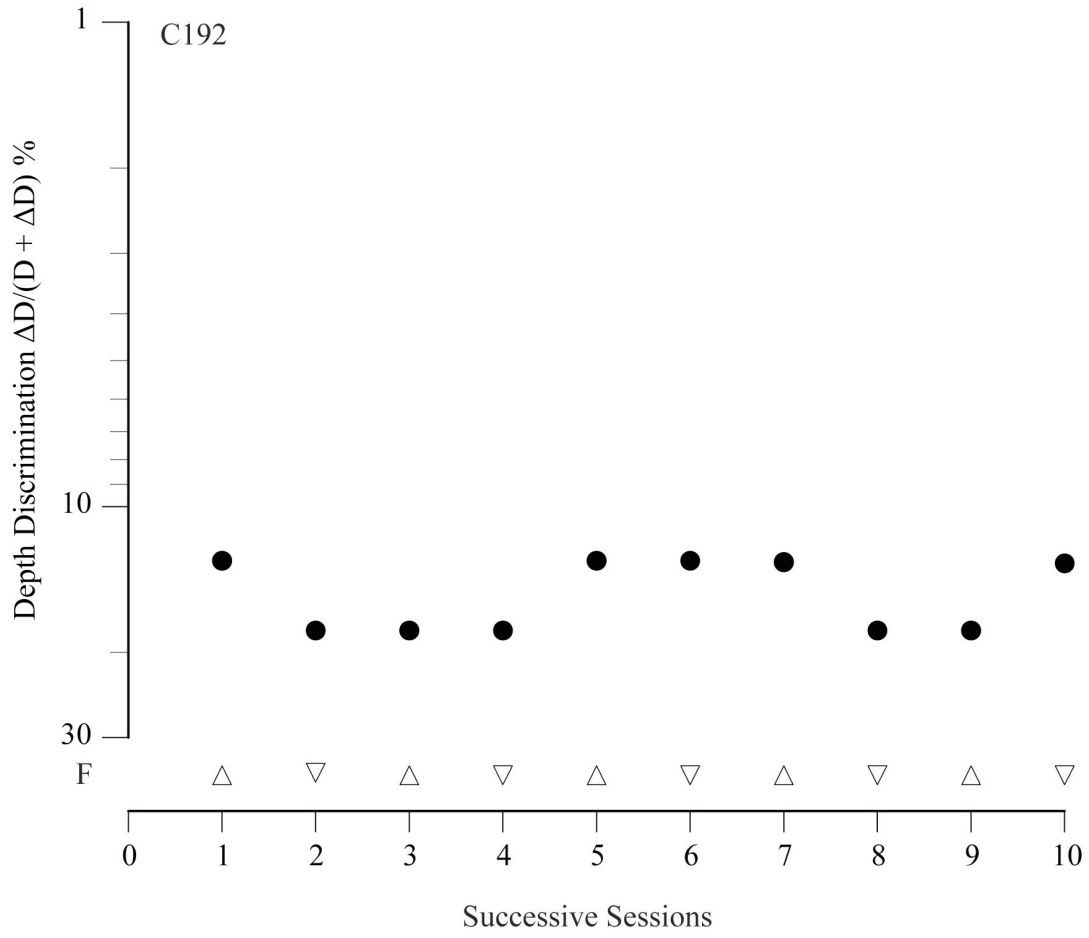

Figure 14. Stereoacuity data for C192 (MD p7-37, DR p37-47). Depth discrimination thresholds are expressed as a ratio of the smallest difference in depth between the two plates that the cat could detect; ΔD/(D + ΔD). F indicates failure to perform the task at the largest depth discrimination. Solid circles symbolize binocular outcomes, while open triangles symbolize alternating monocular outcomes. Testing was performed over 10 subsequent days to acquire a total of 10 binocular measures, and 5 monocular measures for each eye.

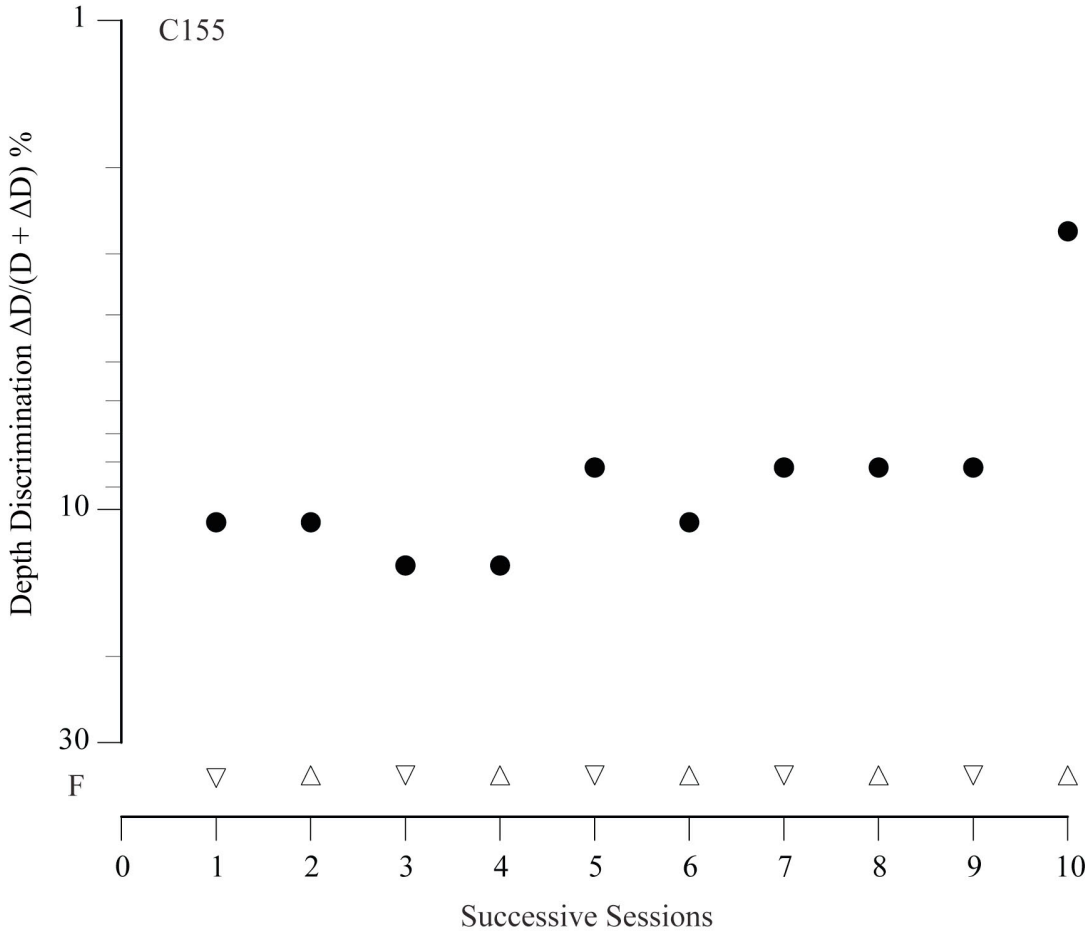

Figure 15. Stereoacuity data for C155 (MD p30-37, DR p64-74). Depth discrimination thresholds are expressed as a ratio of the smallest difference in depth between the two plates that the cat could detect; ΔD/(D + ΔD). F indicates failure to perform the task at the largest depth discrimination. Solid circles symbolize binocular outcomes, while open triangles symbolize alternating monocular outcomes. Testing was performed over 10 subsequent days to acquire a total of 10 binocular measures, and 5 monocular measures for each eye.

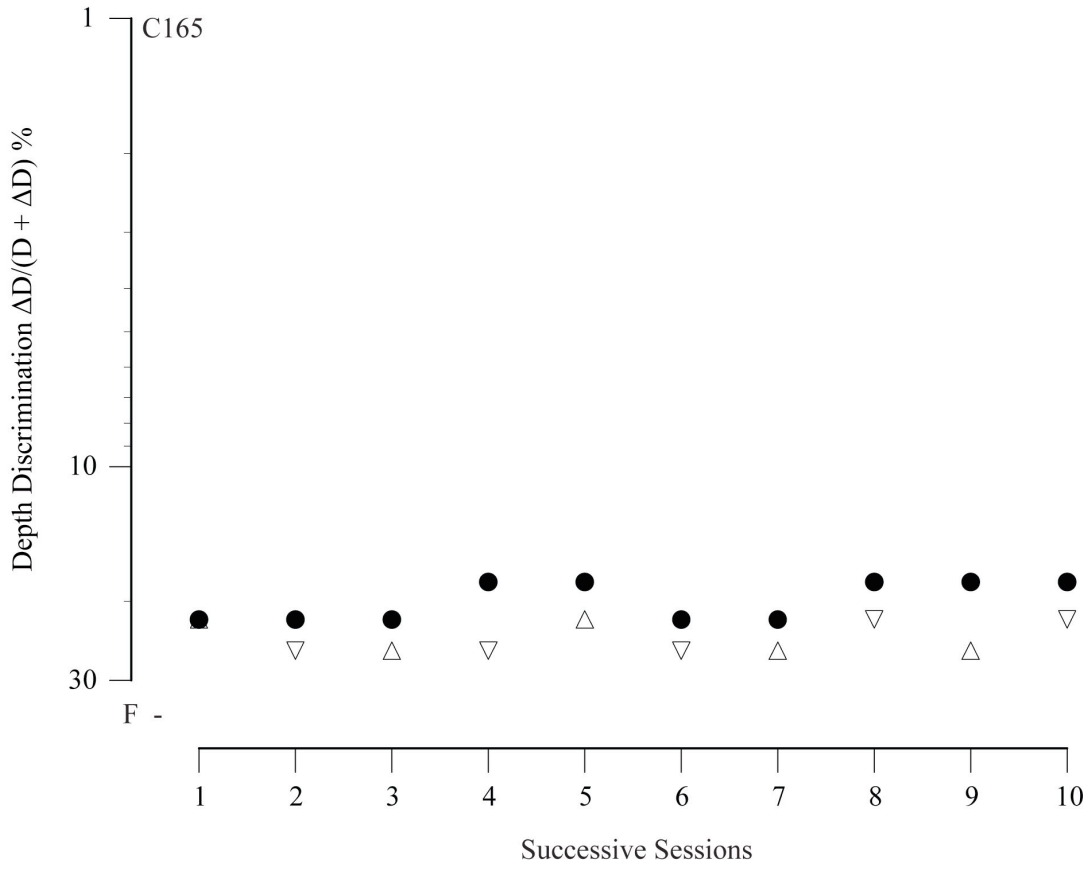

Figure 16. Stereoacuity data for C165 (MD p7-37, DR p93-103). Depth discrimination thresholds are expressed as a ratio of the smallest difference in depth between the two plates that the cat could detect; ΔD/(D + ΔD). F indicates failure to perform the task at the largest depth discrimination. Solid circles symbolize binocular outcomes, while open triangles symbolize alternating monocular outcomes. Testing was performed over 10 subsequent days to acquire a total of 10 binocular measures, and 5 monocular measures for each eye.

CHAPTER 5: BEHAVIOURAL TESTING : ALIGNMENT ACUITY

5.1 Method

Due to the complexity of the task, alignment acuity was tested on only 4 animals to determine their spatial localization abilities, much like tests for vernier acuity in humans. As with stereoacuity testing, the landing areas consisted of a plexiglass top on a rectangular platform underwhich stimuli could be presented from below on the face of a CRT monitor. A wooden divider 3cm high on the glass top separated the left and right halves of the computer screen to create two landing areas (*figure 17*). Stimuli on the left and right halves of the CRT monitor were controlled by a Macintosh computer running a MatLab program. The size, separation, and contrast of the blobs to the right and left could be altered by the computer program.

The stimuli consisted of two sets of three circularly symmetric gaussian blobs presented to the right or the left of the display monitor beneath the landing platforms. For one set, the blobs were presented in perfect vertical alignment, and for the other, the centre blob was offset horizontally from the top and bottom blobs in vertical alignment. Three sets of gaussian blob sizes were used as stimuli, each different in size and vertical separation by a factor of 4. Kittens tested using this apparatus were trained to jump toward the mis-aligned stimulus, with the centre blob offset horizontally from a hypothetical vertical line that joined the centre of the top and bottom blob (*figure 17*). Training began after visual acuity had stabilized in both eyes, and after stereoacuity

testing was completed. Testing began with the largest offset of the biggest gaussian blob. Kittens were made to jump from the platform onto the right or left landing area, and were positively rewarded by pats and food reward for jumping toward the offset stimulus. Once the kittens were comfortable with the task, the offset of the mis-aligned stimulus was reduced. At the beginning of a session, the offset was set very large and was decreased in small logarithmic steps after only one or two errorless trials. Within approximately three steps of threshold, the minimum number of trials was increased to as much as 5. After an error, the kitten had to achieve 5 consecutively correct trials or else 7 or more correct out of the maximum 10 trials allowed. The threshold was defined as the offset size where this minimum criterion could not be met. Similar to testing on visual acuity and stereoacuity tasks, most kittens behaved nearly flawlessly until near threshold. Approaching threshold, they typically showed signs of difficulty that included increased latency and crying. At threshold, many animals would revert to a position habit. Once binocular threshold was achieved, a contact lens was placed in one eye for monocular testing. For each gaussian blob size, six thresholds were obtained for each eye (three with the offset blob to the right, and three with the offset blob to the left of the hypothetical vertical). A total of 16 thresholds were therefore obtained for each animal.

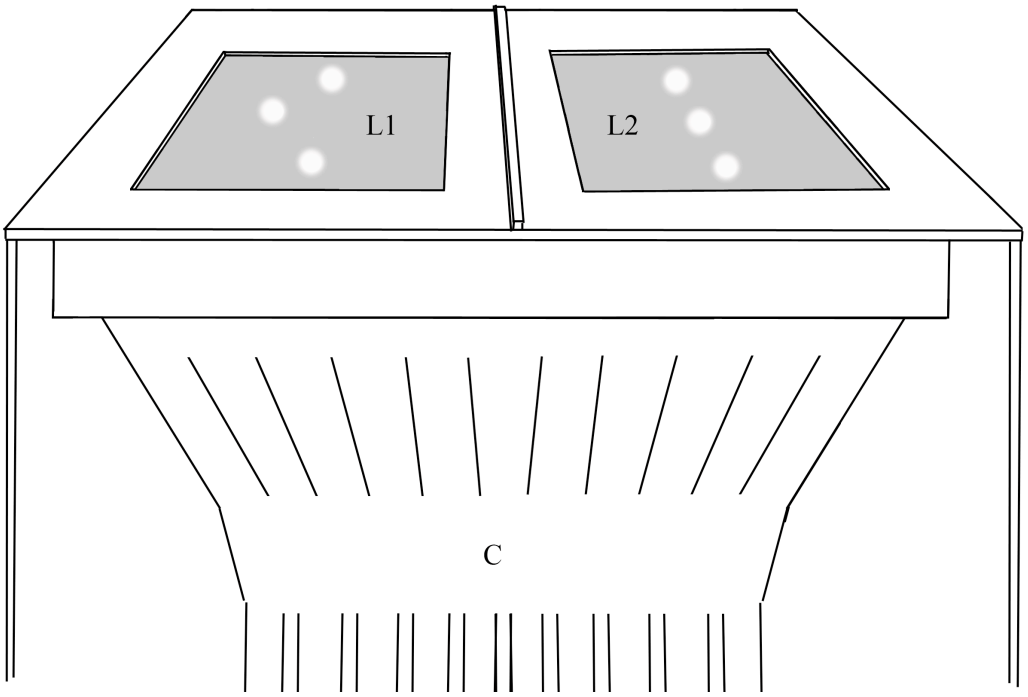

Figure 17. This jumping stand apparatus consists of a platform from which the kitten jumps, identical to that used for stereoacuity methods. Kittens are trained to jump onto the right or left landing area (L1 & L2), below which an aligned or offset set of 3 gaussian blobs were presented on a CRT monitor (C) within the rectangular box.

5.2 Results

Spatial localization deficits have been well-documented in kittens deprived of normal visual development. Using kittens monocularly deprived in early life, or with induced esotropia, studies have compared performance for alignment accuracy between normal and amblyopic eyes (Gingras, Mitchell, & Hess, 2005). As with amblyopic humans, amblyopic kittens showed profound deficits that are proportional to gaussian blob size, and independent of contrast sensitivity loss also typical for amblyopic eyes (Gingras, Mitchell, & Hess, 2005). The results of this report confirm previous findings in the one amblyopic animal tested (C191). This kitten, which received an immediate dark-pulse of only 5-days after a long-term monocular deprivation (MD p7-37), developed severe amblyopia during binocular recovery. The amblyopic eye showed a profound spatial localization deficit on alignment acuity testing, which could only perform the task with the largest gaussian blob stimulus. This eye's performance was in striking contrast to the ability of the kitten's non-deprived eye to perform the task acurately, which was similar to the threshold for a normal cat (Gringas, Mitchell & Hess, 2005).

The three other cats tested on this apparatus had all achieved normal visual acuity for each eye prior to testing, and had completed stereoacuity testing. Two animals from the immediate dark-pulse condition (both MD p7-37), and two of the delayed dark-pulse condition (MD p30-37, and MD p7-37), achieved normal alignment acuity in both their previously deprived and non-deprived eyes. No amblyopic deficit could be found using

this testing technique, as would be expected given previous studies of monocularly deprived kittens (Gingras, Mitchell, & Hess, 2005).

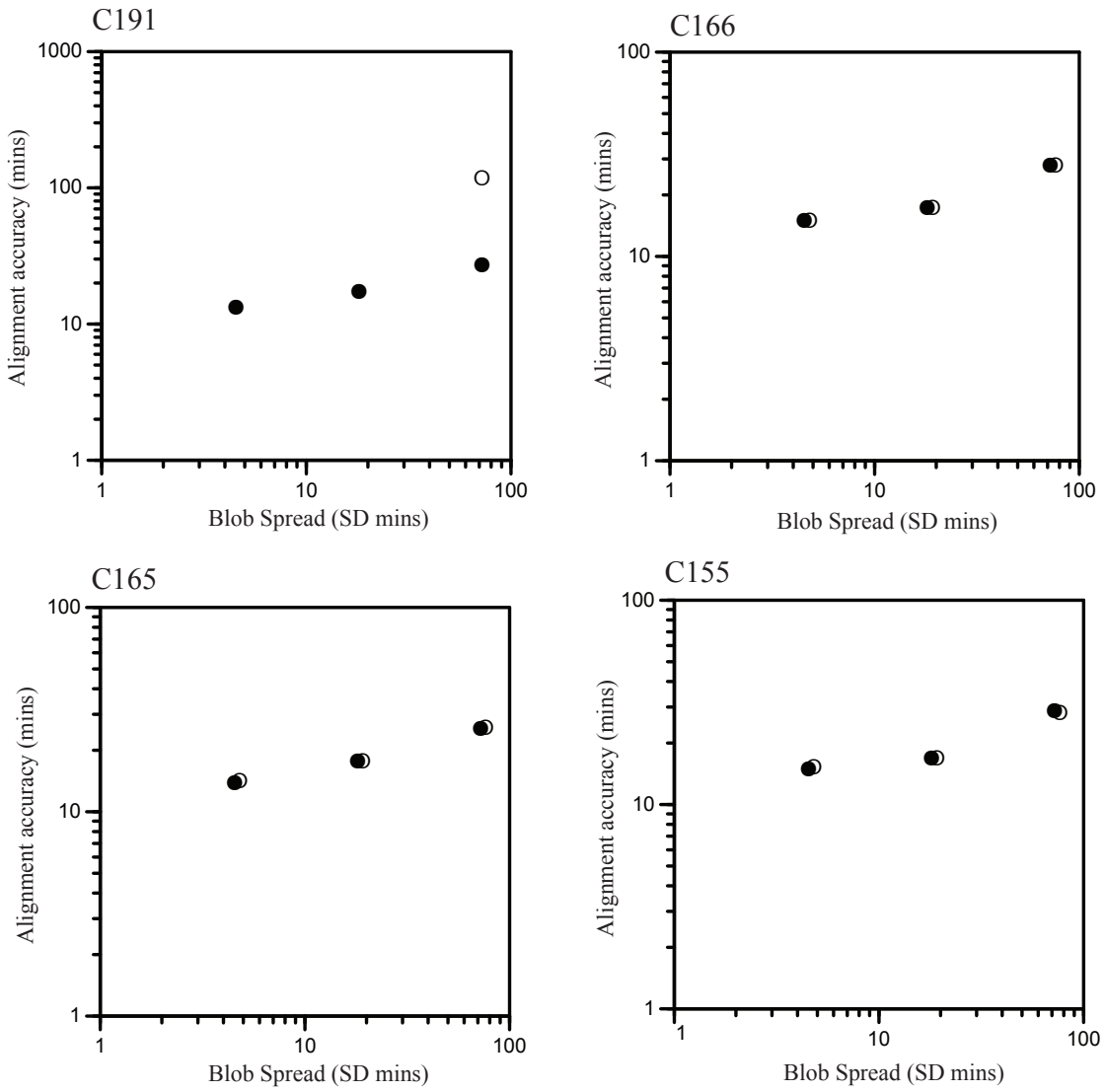

Figure 18. Alignment accuracy (spatial localization) in min of arc of the two eyes of C191 (MD p7-37, DR p37-42), C166 (MD p7-37, DR p37-47), C165 (MD p7-37, DR p93-103), and C155 (MD p30-37, DR p64-74). Presented as a function of the spatial scale of blob size expressed in terms of their standard deviation (SD in min arc). Empty circles symbolize deprived eye (DE) accuracy, while solid circles symbolize the accuracy of the non-deprived eye (NDE). For C191 measurements were possible for only the largest blob size with the deprived eye.

CHAPTER 6: DISCUSSION & CONCLUSIONS

6.1 Important Findings

The findings of this report confirmed and extended the surprising results of previous studies of drastic visual improvement of amblyopic eyes in kittens after a short period of dark-rearing (Duffy & Mitchell, 2013). With regard to the primary outcome measure of this study (visual acuity), there were three important findings. First, all animals who received 10 days of complete darkness showed full recovery of vision in both eyes. These animals were subsequently investigated for stereoacuity and alignment (vernier, or spatial localization) acuity. Results from these secondary measures found varying levels of stereoscopic vision, however, acuity for spatial localization was normal. Second, established amblyopia dramatically resolved within five to seven days after a delayed dark-pulse. Recovery of vision in the deprived eye has not been shown to occur to this extent in previous research that employed the monocular deprivation model for amblyopia in kittens (Mitchell, 1988). Finally, the therapeutic effect on visual acuity was seen for animals whether the 10-day dark-pulse was applied immedately after eyelid-opening, or if it was delayed for several weeks. The extent of recovery seen in both of these conditions was equal, but the rate at which vision recovered was much faster following a delayed dark-pulse. The reason for this is unclear. It is possible that speed of visual recovery may be linked to the amount of time given for recovery following deprivation, or else may be related to the age of the animal when they are put in the dark (Duffy & Mitchell, 2013).

This study also confirmed the surprising detrimental effect that 10 days of dark-rearing had on the previously normal eye of kittens who received a dark-pulse immediately after eyelid-opening (Duffy & Mitchell, 2013). Following a short period of dark-rearing, both the non-deprived eye and the deprived eye initially behaved as if blind upon removal from the dark-room. This detrimental effect on the previously normal eye was not seen for the animals that received a delayed dark-pulse. With clinical applicability in mind, this adverse effect on the normal eye is not favourable, despite ultimately recovering normal vision. Consequentially, it is important to investigate at what stage of development the therapeutic effect for the amblyopic eye can be maximized, with no ill-effect to the 'normal' eye. This proposed study has now been conducted in the Mitchell Lab (Psychology Department, Dalhousie University), the results of which will be published shortly.

The long-term monocular deprivation (MD p7-37) condition used for this study answered one particularly important question proposed by earlier work; whether visual recovery only occurs if normal binocular input is present prior to deprivation (Duffy & Mitchell, 2013). The conditions employed in the earlier study produced acute amblyopia by means of a short period of monocular deprivation (p30-37) during the peak of the critical period for visual development in the kitten (Duffy & Mitchell, 2013). As mentioned previously, following only 10 days of dark-rearing, visual acuity for all animals improved equally in both eyes to normal levels. It is possible that the visual recovery seen for these animals may have occurred because amblyopia was induced after

normal binocular cortical architecture had been established during the first 30 days of life (before monocular deprivation). Given the results of this study, it is unlikely that any pre-existing cortical architecture was responsible for the recovery observed because eyelid-suture was performed at the time of natural eyelid-opening (p7), therefore preventing any normal binocular experience prior to experimental manipulation. It is possible that the drastic improvement of vision seen in amblyopic eyes after dark-rearing reflects a re-capitulation of development, allowing for new neural architecture to support normal connections despite early presence of an amblyogenic factor (monocular deprivation).

6.2 Further Questions

At the conclusion of this report, one question still remains regarding whether the therapeutic effect of a short period (10 days) of dark-rearing may be catalyzed well past the end of the critical period for visual development in the cat. All kittens that received dark-rearing in this study were still visually immature. It is for this reason that a single animal (C189), monocularly deprived from p7 to p37, has yet to undergo treatment by a dark-pulse. This experimental animal may help to answer whether dark-rearing may be effective in treating amblyopic eyes in a visually mature system. Nonetheless, the drastic effect that a short period of binocular deprivation has on amblyopic eyes indicates a possible application to enhance the treatment of amblyopia in children.

With the investigation of an interrupted dark-pulse treatment, it was discovered that very short periods of normal light exposure (in a room illuminated by fluorescent lighting) each day prevented the therapeutic effect of dark-rearing. Further investigation is required to determine if lower levels of light exposure may be tolerated. It is possible that very dim lighting conditions may allow a therapeutic effect to persist. The ability to provide short 'breaks' from such a treatment would be encouraging to clinicians. Not only would physicians benefit from the ability to perform ophthalmic examinations, but it would also provide patients and their families with valuable visual interaction. For the same reasons, another investigation of interest is the degree of visual deprivation required to produce the therapeutic effects of the dark-pulse. At this time, it is unknown whether binocular patching (24 hours per day) could produce the same effect, a condition which would allow varying degrees of diffuse light exposure. It is possible that total light diffusion, the lack of any form or contrast perception, may provide the same lack of competition between the eyes, thus providing a therapeutic effect similar to the experience of dark-rearing.

The kittens in this study had a variety of fur colours, and therefore received varying degrees of light deprivation during eyelid-suture for monocular deprivation. A black-furred kitten, for example, would experience more light deprivation with eyelid-suture than a white-furred kitten. Previous research using this animal model has documented a varience between 4 and 5 log units depending on fur colour (Wiesel & Hubel, 1963a). All kittens were therefore deprived of patterned light stimulation, but to varying degrees of diffuse light stimulation. Due to the low number of amblyopic

subjects in this study, a correlation could not be made between levels of amblyopia and eyelid fur colour. The animal population consisted of white, black, orange, and tabby-coloured kittens.

Another uncontrolled factor in this study was the environmental experience of each animal, both during light and dark-rearing. During dark-rearing procedures, all animals were housed in the same cage with the same contents, but the activities of each animal, including playing behaviour or amount of time spent sleeping, for example, were not monitored. Each animal also experienced similar colony conditions to one-another. The kittens were all group-housed with their mother and litter-mates, as well as other cats in the closed-colony at Dalhousie University (Studley Campus). However, the amount of exploration of their rooms or cages was not monitored, nor was their play behaviour or other activities. It is possible that the kittens interacted differently with their environment, some may have explored the counter or sink area, while others preferred playing on the floor, for example. It could be argued that some kittens had more depth-stimulating experiences that required the use of depth cues for accurate movement. The activities of each animal were not monitored in the colony room, and so any effect of this type of behaviour on the outcome measures of this study is unknown.

6.3 Clinical Applicability

The popular animal model used in this study reflects a severe form of deprivation amblyopia that occurs in humans. The conditions examined in this report included

kittens monocularly deprived from birth, a model reflective of the origin of early amblyogenic precursors in humans with untreated congenital unilateral cataract, or congenital ptosis, for example. The results regarding a possible enhancement to traditional amblyopia treatment presented in this report is important to Orthoptists in particular, who work to quickly improve visual acuity of amblyopic eyes. The use of binocular visual deprivation, or binocular occlusion, as a *treatment* for amblyopia has not been identified in published literature. Binocular patching has, however, been used preventatively in cases of congenital unilateral cataract (Wright, Wehrle & Urrea, 1987; Taylor, et al., 2001) and may enhance development of binocular fusion and stereoacuity for some patients (Wright, Matsumoto & Edelman, 1992). Binocular patching in these instances is used essentially as a means to maintain the *status quo* and prevent additional deprivation effects as a result of the continuing discordant visual input until proper optical correction can be provided (IOL or a contact lens) following surgical cataract removal. Other studies have found no ill-effects of short-term binocular occlusion on infants undergoing treatment for jaundice, suggesting no increased incidence of strabismus or abnormal stereoacuity (Hoyt, 1980).

Even after early surgery, amblyopia remains the major cause of residual visual impairment for patients with congenital unilateral cataract (Ledoux, Trivedi, Wilson, & Payne, 2007). Follow-up exams with orthoptists and ophthalmologists are therefore critical to continue care for these patients. Amblyopia treatment compliance is known to vary greatly between patients. Often treatments such as patching therapy are attempted over the course of many years, with varying degrees of success. For patients with very

poor compliance, alternative treatments include penalizing the "good" eye with atropine drops; arguably an invasive pharmacological treatment as compared to an adhesive patch placed over the eye. Many studies have demonstrated poor compliance with patching, especially in older, school-aged children for which patching therapy is commonly associated with psychological distresss (Hrisos, Clarke & Wright, 2004). Patching therapy becomes even more challenging if patients have restricted access to treatment. Longer distance required to travel for follow-up appointments is associated with poor compliance with patching therapy (Smith, Thompson, Woodruff & Hiscox, 1995). Not surprisingly, the outcome of amblyopia treatment is significanlty related to levels of compliance (Fielder, et al., 1994), making compliance one of the most challenging aspects of treating amblyopic children. Current therapies for amblyopia treatment must be further optimized to quickly treat patients with these compliance issues in particular.

Not only is poor compliance associated with poor visual outcomes, but loss to follow-up is also a problem during patching therapy. With good compliance, full-time occlusion by patching may produce excellent visual acuity outcomes, with no long-term complications for the 'normal' eye as long as treamtent is carried out as directed by a certified eye care specialist. However, if patients are lost to follow-up procedures and treatment continues unmonitored, or else patch for longer than prescribed, occlusion amblyopia may develop in the previously normal eye. Occlusion amblyopia has been shown to occur in up to to 25.8% of patients undergoing patching therapy for amblyopia (Scott, Kutschke, Keech, Pfeifer, Nichols & Zhang, 2005). The importance of follow-up examinations is therefore critical to monitor for this risk to the non-amblyopic eye.

Psychosocial difficulties associated with amblyopia and strabismus are not just a problem for school aged children, but also for teenagers and adults. Corrective strabismus surgery may offer people of all ages improvement in psychosocial functioning (Satterfield, Keltner & Morrison, 1993). Surgery to improve ocular alignment has been shown to result in major improvements in quality of psychosocial functioning for the majority of adult strabismic patients (Burke, Leach & Davis, 1997). However, poor visual acuity has been associated with recurrence of strabismus in some patients, and may result in multiple surgeries (Faridi, Saleh, Ewings & Twomey, 2007). A significant negative social prejudice against patients with strabismus has been well documented in the literature. Treatment of strabismus positively alters preceived characteristics of individuals and improves their ability to socialize normally and obtain employment (Olitsky, Sudesh, Graziano, Hamblen, Brooks & Shaha, 1999). The treatment of amblyopia may therefore directly affect outcomes of strabismus surgeries, the number of surgical procedures required, and subsequent social functioning throughout life.

In addition to the direct benefit of improving vision with amblyopia treatment, having functional vision in the amblyopic eye during childhood is a valuable strategy against incapacitating vision loss later in life (Rahi, Logan, Timms, Russell-Eggitt & Taylor, 2002). Most amblyopic patients who become injured in their 'normal' eye later in life are no longer able to keep their employment as a result (Rahi, Logan, Timms, Russell-Eggitt & Taylor, 2002). Because current amblyopia therapies, such as patching,

are effective only during visual immaturity, any compliance or follow-up issues must be addressed quickly to provide each patient with the best quality of vision possible.

6.4 Conclusions

Over the past half-century, neuroanatomical and physiological studies of the cat visual cortex, and of monocularly deprived kittens in particular, has accelerated our understanding of the molecular mechanisms of amblyopia, and has optimized the treatment of amblyopia in children. Recent studies of brain plasticity point to several possible molecules involved as brakes to plasticity. The heightened instability of neurofilament protein induced by dark-rearing may be just one of many molecular changes that occur with cortical plasticity (Duffy & Mitchell, 2013). Following a short period of dark-rearing, neurofilament levels are drastically reduced in the cat visual cortex and dLGN (O'leary et al., 2012). Behavioural studies, including this report, have found an equally drastic improvement of visual recovery in the amblyopic eyes of kittens (Duffy & Mitchell, 2013). A dark-pulse immediately after monocular deprivation prevents the subsequent development of predicted amblyopia. If an equal dark-pulse is delayed after amblyopia is established, the present amblyopia resolves completely within only five to seven days. The swift and complete recovery from amblyopia produced by just a short period of dark-rearing may enhance the current treatments of amblyopia in humans.

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