MICROGLIAL ACTIVATION IN THE CAT DORSAL LATERAL GENICULATE NUCLEUS FOLLOWING RETINAL INACTIVATION

by

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Dalhousie University is located in
Mi'kma'ki, the ancestral and unceded
territory of the Mi'kmaq.
We are all Treaty people.

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ABSTRACT

Amblyopia is the leading cause of monocular vision loss in children. Success in treating amblyopia using conventional methods such as patching and atropine penalization are hindered by compliance issues, rigid treatment schedules, and a short treatment window during young childhood. Thus, alternative treatments addressing these barriers are necessary to improve visual outcomes. Retinal silencing, by administering intravitreal injections of tetrodotoxin (TTX), is a potential treatment for amblyopia in lieu of patching or penalization. Fellow eye retinal silencing has shown good results in promoting visual recovery of the deprived eye, with no permanent detriment to the silenced eye. However, the underlying mechanisms that protect the eye during silencing are not fully understood. Microglia are a subtype of glial cells involved in mediating the development of the synaptic network in the central nervous system. The current study sought to determine if microglia are involved in a homeostatic response that protects the synaptic network against lowered neural activity elicited by retinal silencing. In other words, how are synapses-serving the inactivated eye protected from perturbation? Microglial activation was investigated as a possible protective mechanism by measuring ionized calcium binding adaptor molecule 1 (Iba1) immunolabeling within the silenced-eye layers of the dorsal lateral geniculate nucleus. The results of this study revealed that retinal silencing in young animals activates microglia, and that retinal silencing in older animals did not elicit such activation. These findings raise the possibility that activation of microglia mediates a homeostatic response to protect against retinal silencing-induced synaptic modifications. The current study also showed that retinal silencing in younger animals activates microglial transiently, as activation is not sustained following TTX with a period of binocular vision. As TTX treatment stands as a possible alternative treatment for human amblyopia, it is important to understand how TTX affects the synaptic network of the visual system.

LIST OF ABBREVIATIONS USED

ADP: adenosine diphosphate
ATP: adenosine triphosphate
BCM: Bienenstock-Cooper-Munro
BV: binocular vision
CCAC: Canadian Council on Animal Care
CD47: cluster of differentiation 47
CNS: central nervous system
CX3CL1: CX3C ligand 1
CX3CR1: CX3C receptor 1
C1q: complement component 1q
C3b: complement component b
dLGN: dorsal lateral geniculate nucleus
GABA: gamma-aminobutyric acid
GAD: glutamic acid decarboxylase
GFAP: glial fibrillary acidic protein
Iba1: ionized calcium-binding adaptor molecule 1
IL-1β: interleukin 1 beta
LE: left eye
LTP: long term potentiation
MD: monocular deprivation
M-CSF: macrophage colony-stimulating factor
MI: monocular inactivation
mRNA: messenger ribonucleic acid
ODC: ocular dominance column
PBS: phosphate-buffered saline
P2Y12R: P2Y purinoreceptor 12
RE: right eye
RGC: retinal ganglion cell
RO: reverse occlusion
TNFα: tumor necrosis factor alpha
TNT: tunneling nanotubules
TTX: tetrodotoxin
UCLA: University Committee for Laboratory Animals
VEP: visually evoked potential

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CHAPTER 1: INTRODUCTION

1.1 - Amblyopia

Amblyopia is the leading cause of visual impairment in childhood, affecting 2-4% of the world's population (Hu et al., 2022). It is defined as decreased visual acuity in one or both eyes, despite the eye itself appearing healthy upon physical examination (von Noorden, 1985). It is thought to be a result of conditions that cause imbalances in visual experience between the eyes that precipitate alterations within the visual system (Hubel and Weisel, 1970). These conditions include, but are not limited to, obstruction to the visual axis (deprivation), misalignment of the eyes (strabismic), and/or unequal refractive states (anisometropic (von Noorden, 1978). Strabismic amblyopia and anisometropic amblyopia are more common and moderate forms of amblyopia. Although deprivation amblyopia is less common, it is the severest form and most resistant to conventional treatment (Cheng et al., 1991). Deprivation amblyopia results from obstructions to the visual axis, caused by congenital cataracts, lid abnormalities, and retinal pathologies such as retinoblastomas (Hamm et al., 2017; Warda et al., 2022). Generally, developing earlier in life than other forms, the diagnosis and treatment of deprivation amblyopia fall into a smaller window of opportunity for good outcomes.

1.1.1 - Treatments for amblyopia

Penalization of the non-amblyopic eye is the most common clinical treatment for amblyopia. Amblyopia is treated by penalizing the non-amblyopic eye to stimulate the use of the amblyopic eye (Wallace et al., 2018). Penalizing the non-amblyopic eye reduces the influence of the dominant (non-amblyopic) eye and promotes visual recovery of the amblyopic eye (Zhou et al., 2019). Penalization can be achieved by occlusion

(patching), optical (lenses), and pharmacological (topical atropine) measure (Kaur et al., 2023). Although considered the of amblyopia treatment, occlusion therapy produces similar amounts of visual recovery as pharmacological penalization (Pediatric Eye Disease Investigator Group, 2002). Occlusion therapy is not without its downfalls, including poor compliance, psychosocial considerations, risk of reoccurrence amblyopia, risk of occlusion amblyopia, and unreliable outcomes (Holmes et al., 2004; Al-Zuhaibi et al., 2009; Haine et al., 2025). The age of the patient, timing of diagnosis and intervention, and length of treatment are all critical factors that influence the outcome of amblyopia treatment and visual development (Kaur et al., 2023). A younger visual system responds better to intervention and treatment as the juvenile brain is highly plastic and adaptable. Therefore, earlier diagnosis and intervention is essential to promote sustained visual recovery (Daw, 1998). Occlusion therapy is typically considered for children up to the age of 10 years, after which recovery is not as reliable or even likely with this treatment (DiSantis, 2014).

Various treatment methods have been explored outside of penalization. One method is binocular treatment, otherwise known as dichoptic training, which has been extensively explored as an alternative to treat amblyopia. Dichoptic training focuses on simultaneous binocular stimulation to promote binocular viewing and hopefully improve visual acuity in the amblyopic eye (Li et al., 2011). Most dichoptic treatments nowadays rely on engaging video games to promote simulatenous use of both eyes. Dichoptic training has been shown to improve stereoacuity, a measure of binocular vision, in patients with mild anisometropic amblyopia (Liu et al., 2021). Furthermore, there are studies that have shown that dichoptic training is more effective in treating amblyopia

when compared to monocular training, such as occlusion therapy (Li et al., 2013; Liu and Zhang, 2018). However, these studies were performed in a small sample size of less than 20 participants and were not conducted as randomized controlled studies. Based on two large randomized controlled trials of over 300 participants, binocular therapy is not as effective as standard treatment (patching) (Pediatric Eye Disease Investigator Group, 2016). This suggests that dichoptic training is not more effective, or even as effective, as traditional occlusion therapy.

Determining what amblyopia treatment should be implemented clinically remains a primary topic of debate as each treatment comes with its own benefits and limitations. However, occlusion therapy remains the preferred treatment in most clinical practices due to its low cost, moderate success rate, and generally good compliance in comparison to other treatments (Chen and Cotter, 2016; Zagui, 2019; White and Walsh, 2022). Studying the visual system in animal models provides an opportunity to test and better understand other amblyopic treatments that may address the limitations of occlusion therapy (Duffy et al., 2023).

1.2 - Animal models of amblyopia

Our current understanding of the human visual system is drawn mainly from rodent, cat and monkey models. Cats and rodents share a similar developmental profile (Hubel and Wiesel, 1970; Cragg, 1975; Wang et al., 2023). Cats and monkeys have forward-facing eyes with a large binocular zone with binocular integration allowing for stereopsis. Additionally, the dorsal lateral geniculate nucleus (dLGN) of cats and monkeys is laminated and contains monocular zones, and their visual cortices has well-defined ocular dominance columns (ODC) (Crair et al., 1998). ODCs are striped patterns

of cortical neurons organized in a manner to facilitate development of binocular vision (LeVay et al., 1980). These columns of neurons are highly susceptible to changes in visual experience, especially during neurodevelopment (Hubel and Wiesel, 1965). Rodents have lateral facing eyes, a small binocular zone providing poor stereopsis, and a visual system dominated by monocular visual processing (Baroncelli et al., 2013). Rodents' dLGN contains binocular cells, with a salt-and-pepper pattern of contralateral eye-favoring cells (Okhi and Reid, 2007; Seabrook et al., 2017). The rodent visual cortex has poorly differentiated ODCs (Laing et al., 2015). The effects of deprivation amblyopia on the visual system differs between forward-facing and lateral-facing animals. In rodents, the effects of deprivation are subtle and diffuse changes that are more localized to subcortical regions (Jaepel et al., 2017; Huh et al., 2020) However, in cats and monkeys, deprivation causes a more dramatic effect in the visual cortex, specifically as a shift in the ocular dominance columns and a disruption to the balance of excitatory and inhibitory synapses (Tieman, 1984). Additionally, rodent visual acuity is extremely low compared to cats (Prusky and Douglas, 2004). Therefore, the cat is arguably a more suitable model for amblyopia studies because it more closely resembles the primate visual system.

1.2.1 - Visual system in cats

As a vital part of the visual pathway in both cats and primates, the dLGN is organized into layers based on monocular visual input among other factors. The dLGN processes visual input before relaying it to the primary visual cortex (Shatz, 1983). The connection between the retina and dLGN during visual development is critical to refining the higher-order connections between the dLGN and visual cortex (Dougherty et al.,

2018). In cats, the segregation of layers in the dLGN is heavily influenced by the visual experience that begins at natural eye opening (Stryker and Harris, 1986). Contralateral-serving synapses occupy most of the dLGN during prenatal and early postnatal development in cats, however their occupancy decreases to similarly match the ipsilateral-serving synapses to form the dLGN layers A and A1 (Kliot and Shatz, 1982; Shatz, 1983; Shatz and Kirkwood, 1984). Layer A receives input from the ipsilateral retinogeniculate afferent fibers, whereas layer A1 receives input from the contralateral fibers (Guillery and Colonnier, 1970). The dLGN's organization of layers based on visual input from either eye serves as a unique way to study amblyopia, as the effects of amblyopia are evident in dLGN anatomy (Duffy et al., 2018).

1.2.2 - Monocular deprivation

One common method to model deprivation amblyopia in cats is by suturing closed the eyelids of one eye, which is called monocular deprivation (MD). The anatomical and physiological effects of MD in kittens were published by Wiesel and Hubel in 1963. Measures of anatomical hallmarks from deprivation include neuronal soma size and neurofilament protein. Neurofilament protein is integral to the stability and size of neurons. Therefore, a change in neuronal soma size or expression of neurofilament protein can indicate neuronal damage or stress (Gaetani et al., 2019). MD during the critical period in kittens causes decreased neuronal soma size and neurofilament in the dLGN layers corresponding to the deprived eye (Wiesel and Hubel, 1963a; Bickford et al., 1998). A decrease in neuronal soma size and expression of neurofilament suggests that deprivation reduces neuronal durability and synaptic strength of the neurons serving the deprived eye. Thereby, reducing the likelihood of neurons responding to visual input.

Diminished visually evoked potentials (VEPs) recorded from the primary visual cortex of cats is observed with stimulation of the deprived eye after MD (Fong et al., 2021). Furthermore, MD results in severe impairment of visual acuity and a dramatic rearrangement of cortical circuitry, with non-deprived eye-serving neurons occupying most of the synaptic space in the visual cortex. This phenomenon is described as ocular dominance plasticity, which highlights how visual input from either eye affects the sensitivity of cortical neurons to visual stimuli (Hubel and Wiesel, 1963).

In addition to mimicking amblyopia, animal models are used to assess treatment method efficacy and outcomes. Occlusion therapy is modeled with a research procedure called reverse occlusion (RO). Reverse occlusion consists of suturing the non-deprived eye closed after the deprived eye is opened, to promote use and visual recovery of the deprived eye. In cats, the success of RO as a treatment for the effects of MD is limited to the critical period, as the visual system is more responsive to RO when implemented during the critical period when plasticity capacity is high. As the cat ages out of the critical period, the success of RO reduces significantly (Blakemore and Sluyters, 1974). Alternative amblyopic treatments for MD include rearing in complete darkness, restoring clear binocular vision, and binocular retinal inactivation. However, these treatments all only cause limited recovery in visually mature animals (Duffy et al., 2018). One of the only treatments for notable recovery beyond the critical period following MD is fellow eye enucleation or significant damage to the fellow eye's optic nerve (Kratz and Spear, 1976; Cragg et al., 1976). Fellow eye enucleation involves enucleating (removing) the fellow (stronger) eye to drive visual stimulation of the deprived weaker eye. Studies show that fellow eye enucleation during or after the critical period in MD subjects results in a

dramatic shift in the ODCs towards the deprived eye (Kratz and Spear, 1976). These results support the belief that deprivation effects are actively suppressing neuronal responses to the deprived eye by the fellow eye, as removal of this dominance by the fellow eye by enucleation relieves this suppression on the deprived eye. Ocular dominance plasticity is the phenomenon that explains both the active suppression driven by the fellow eye, and the dramatic shift in visual input from the two eyes in the ODCs (Kasamatsu and Imamura, 2020). This phenomenon as part of the visual cortex's role in refining the neural circuitry is confined to critical periods during neurodevelopment (Mitchell and Maurer, 2022).

1.2.3 - Critical period

A collection of findings conducted by Wiesel and Hubel (1963a, 1963b, 1965) first identified ocular dominance plasticity by examining the effects of MD on cortical visual responses. Their study reveals that depriving one eye of visual stimulation during early postnatal development, before 5 weeks of age, causes a shift in ocular dominance to favor the non-deprived eye. Following MD, most neurons within the visual cortex respond exclusively to input from the non-deprived eye (Wiesel and Hubel, 1963). These findings suggest that the visual system is the most sensitive to visual manipulations, such as monocular lid closure, during a specific time in early development. This period is suggested to start shortly after eye opening and last until approximately 3 months of age in kittens (Daw, 1998). During this time, ocular dominance plasticity peaks as cortical neurons compete for dominance in the visual cortex (Hubel and Wiesel, 1963). When MD is induced around the peak of the critical period, its effects are more severe and last longer than when induced past the critical period (Wiesel and Hubel, 1963; Olson and

Freeman 1980). Cortical plasticity peaks at P30 in cats with a slow decrease in cortical plasticity past roughly age 3 months (Daw, 1998; Olson and Freeman 1980). The timing of when in the critical period lid suturing is performed influences the severity of deprivation amblyopia, with MD at ages past P48 eliciting a much smaller shift in ocular dominance compared to MD at younger ages. MD performed at P109 showed only a marginal loss of function in the deprived eye. For instance, MD at P48 does not elicit as severe of decreased synaptic connections than when MD is performed at P30 (Olson and Freeman, 1980). Interestingly, the effects of peak cortical plasticity are so intense that a 10-day period of MD between P28 to P38 elicits a detrimental response comparable to MD lasting from birth to adulthood (Wiesel and Hubel, 1963, 1965). The effects from MD are more pronounced during the critical period and are at its most dramatic at the peak of the critical period when ocular dominance plasticity is at its strongest. It is important to note that the beginning of the critical period coincides with the start of postnatal synaptogenesis in kittens. Synaptic formation increases rapidly from P8 to P37, with a peak comparable to the critical period (Cragg, 1975). Thus, it makes sense that cortical plasticity is at its strongest at the peak of synaptic refinement, as the formation and refinement synapses contribute to the organization of ODCs and cortical neurons' response to visual stimulation from either eye.

Normal visual development requires binocular concordant visual input during the critical period. Discordant visual input, as is observed in conditions that produce amblyopia, leads to uncoordinated synaptic activity and shifting of ocular dominance that favors the non-deprived eye. This results in weakened synapses serving the deprived eye-

and alters the perception of visual stimuli presented by the deprived eye (Birch and Duffy, 2024).

The natural tug-of-war response that ocular dominance creates during the critical period is driven by balanced visual input from either eye. The development of a balanced cortical network is based on extensive refinement of excitatory and inhibitory neuronal connections. MD weakens excitatory connections from the deprived eye to the visual cortex (Tsumoto and Suda, 1978). There is reduced glutamate uptake seen in the regions of the dLGN serving the deprived eye (Schliebs et al., 1984). MD heightens the GABAergic inhibitory process on the cortical neurons serving the deprived eye in the visual cortex. This is seen as both an increase in presynaptic GABA release and postsynaptic GABAergic synapses (Kannan et al., 2016). These cortical neurons have a reduced response to visual stimuli compared to the neurons serving the fellow eye. A study by Hensch et al., 1998, showed that in mice with a knockout of GABA-synthesizing enzyme glutamic acid carboxylase (GAD), there was a loss of cortical response to MD that was seen in wildtype mice. Specifically, the shift in ocular dominance to favor the non-deprived eye seen in wildtype mice was not seen in GAD knockout mice. Furthermore, the cortical effects caused by MD in cats were reversed when cats were injected with a GABA antagonist (Mower and Christen, 1989). This suggests that MD reduces excitatory neurotransmission while heightening inhibitory neurotransmission in this cortical circuitry. Due to MD's detrimental effects on the visual system, it is imperative to investigate therapeutic treatments for deprivation amblyopia.

1.3 - Retinal inactivation

An emerging novel treatment for recovery from the effects of MD is monocular inactivation (MI) using intravitreally administered tetrodotoxin (TTX). This procedure involves temporarily inactivating retinal ganglion cells (RGCs) using TTX, a potent voltage-gated sodium channel blocker (Duffy et al., 2018). In very small amounts, TTX silences RGC activity for at least 48 hours, after which normal retinal activity is restored (Stryker and Harris, 1986; Fong et al., 2016; Duffy et al., 2018; Kwaw et al., 2025). Diminished VEPs measured during MI confirm its physiological effect on visual activity (DiCostanzo et al., 2020). MI differs from MD in that MD attenuates visually-driven patterned activity, whereas MI silences all neuronal activity (spontaneous and visually-driven) (Chapman et al., 1986; Rittenhouse et al., 1999). A study by Duffy et al., 2023 shows MI produces a more dramatic structural change in the dLGN compared to MD. Surprisingly, TTX administered at ages past the critical period still produce structural changes in the dLGN, even when MD at that age does not produce any changes. These results suggest that MI restores synaptic plasticity in the dLGN, at ages when synaptic plasticity is normally dampened. Therefore, MI may play a role in recovery from deprivation by reintroducing juvenile plasticity in animals at ages past the critical period. Rittenhouse et al. (1999) reveals that a MD-induced shift in ODCs is reduced when the deprived eye is inactivated. Recovery from deprivation is also seen following a period of inactivation in the fellow eye for 10 days (Duffy et al., 2018). This recovery is seen even in visually mature cats. These cats underwent MD of varying lengths at P30, followed by fellow eye MI at ages after the critical period. Recovery from MD is shown by complete recovery of neuronal soma size, neurofilament recovery, and restoration of normal VEP levels (Duffy et al., 2018; Fong et al., 2021). When compared to RO, MI

promotes substantially better recovery in the deprived eye-related VEPs (Hogan et al., 2023). Remarkably, this recovery occurs without apparent detriment to the anatomical and functional state of the inactivated eye (Fong et al., 2021). There are no known ocular pathologies seen in the inactivated eye following a 10-day period of inactivation (DiCostanzo et al., 2020; Hogan et al., 2023; Duffy et al., 2023). Furthermore, there are no known systemic effects or behavioral changes following MI in kittens (Stryker and Harris, 1986). These findings suggest that fellow eye MI induces cortical plasticity previously only seen in juvenile brains, at a time after the critical period, when plasticity is waning.

1.3.1 – BCM Model

The changes in synaptic activity caused by MI differ from MD and are best described in relation to the Bienenstock-Cooper-Munro (BCM) model. The BCM model proposes a sliding modification threshold, where both the active synaptic input and postsynaptic response influence the strength of the synapse, otherwise known as 'bidirectional synaptic modification' (Bienenstock et al., 1982). With this model, high levels of activity increase the modification threshold, whereas low levels of activity decrease the threshold. A sliding threshold keeps synapses functioning within a range that prevents synaptic saturation or synaptic weakening. Lowering the threshold for synaptic activity lowers the amount of pre-synaptic activity necessary for neurons to fire, thereby increasing the likelihood of long-term potentiation (LTP) and synaptic strengthening (Kirkwood et al., 1996). Silenced visual input, during MI, decreases synaptic activity sufficiently to cause a lowering of the modification threshold, making it more likely for synaptic strengthening (Rittenhouse et al., 1999). However, during MD, there is still

some spontaneous activity firing even without visual stimuli, therefore the modification threshold does not need to be lowered as significantly as it does during MI. As the threshold is not lowered dramatically, there is less likelihood of synaptic strengthening of the deprived-eye neurons, contributing to further synaptic depression (Cooper and Bear, 2012). This difference between MI and MD partially explains why substantial synaptic recovery is observed following MI, but not following MD.

Although the BCM model may explain why recovery is observed following MI, fellow eye MI promotes recovery from MD by relieving an active suppression process driven by the fellow eye. Retinal silencing of the fellow eye relieves the inhibition of deprived eye-serving neurons, further promoting a strengthening of synapses serving the originally deprived eye (Kasamatsu and Pettigrew,1976; Duffy et al., 2018). This also suggests that the inhibition of deprived eye-serving cortical neurons is driven by an active inhibitory process originating from the fellow eye (Duffy et al., 2018; Fong et al., 2021).

The mechanisms that protect the inactivated eye from long-term detriment following MI are not well understood. One possibility is the involvement of glial cells, which are important mediators of neural plasticity in the visual system (Venturino et al., 2021; Wang et al., 2023). A recently published study by Duffy (2025) suggests that activated astrocytes, a subtype of glial cells, are involved in the protection of neural connections during MI. Elevated levels of glial fibrillary acidic protein (GFAP), expressed by astrocytes, are seen in dLGN layers serving the inactivated eye (Duffy, 2025). However, this study shows that in MI-treated 4-6 week old kittens, astrocyte activation is smaller as compared to older animals. Therefore, there may be another glial cell, besides astrocytes, that mediate the neuroprotective response during inactivation

imposed at ages around the peak of the critical period. Microglia represent an alternative type of glial cell that have the capacity to regulate neural plasticity and that may be recruited under conditions of retinal inactivation (Cornell et al., 2021).

1.4 - Microglia

Microglia, first discovered and confirmed in the early 1900s, are known as the resident immune cell and the 'glue' of the central nervous system (Penfield, 1925; Oehmichen et al., 1983). They play a vital role in protecting the brain against injury and disease (Davalos et al., 2005). Microglia are plentiful, comprising up to 10% of brain cells (Katsumoto et al., 2014). They have diverse roles in the brain, all requiring a different morphology. Previous studies thought microglia transition from a quiescent ramified state to an active amoeboid state during infection or injury (Streit et al., 1998). However, dichotomic terminology excludes the diverse range of microglia in relation to their roles in development, aging, disease, and plasticity. Research over the past two decades has shifted from this narrative to one where microglia are constantly active, adopting different morphologies based on the conditions (Nimmerjahn et al., 2005; Tremblay et al., 2010; Paolicelli et al., 2011).

1.4.1 - Microglial roles and morphology

Amoeboid-shaped microglia are comprised of a highly rounded cell body with little to no branching, providing them with their phagocytic and migratory capabilities required for clearing cellular debris and engulfing apoptic cells (Zusso et al., 2012). Amoeboid microglia are seen in embryonic and early postnatal development in the central nervous system (CNS), as they help control the neuron population through phagocytosis of excess or apoptic cell bodies (Cunningham et al., 2013).

The previously thought quiescent state that microglia adopt is their ramified morphology. Tremblay et al. (2010) reveals that in homeostatic conditions, ramified microglia actively survey their local environment. Ramified microglia have elongated cell bodies, extended dendritic processes and a highly motile cytoskeleton (Nimmerjahn et al., 2005). Ramified microglia's branched morphology lets them survey a large area, with highly sensitive protrusions that allow them to respond rapidly to potential injury or pathology. These protrusions also physically interact with presynaptic axonal terminals, post-synaptic dendritic spines, and other glial cells such as astrocytes (Davalos et al., 2005; Wake et al., 2009). Ramified microglia are also shown to either prune synapses through the complement cascade and/or protect synapses through CD47 signaling (Elward and Gasque, 2003; Schafer et al., 2012).

Other forms of microglia include hyper-ramified, seen in chronic stress models; jellyfish microglia, seen in response to traumatic brain injury; honeycomb formation of microglia, seen in response to leakage from the blood-brain barrier; and rod microglia, seen in response to injury (Vidal-Itriago et al., 2022).

The microglia cytoskeleton comprises filamentous actin, which contributes to microglia's dynamic motility (Stence et al., 2001). Actin is a cytoskeletal protein necessary for the growth and motility of most cells (Ohsawa et al., 2000). Disruptions to actin production leads to unstable cytoskeletons, causing neurons to become vulnerable (Kessels et al., 2025). Furthermore, disorganization of the cytoskeleton, due to loss of actin, leads to phagocytosis impairment, reduced synaptic pruning, and instability of the neuronal network (Kessels et al., 2025). Therefore, the actin cytoskeleton that makes up microglia plays a key role in how microglia mediate the local neuronal circuits.

Microglia are involved in modulating CNS homeostasis; through there are several neuroprotective roles they can adopt in different morphological states (Nimmerjahn et al., 2005). When microglial dendritic processes sense changes to local neuronal activity, this triggers microglial morphological changes, and activation of microglia to maintain synaptic homeostasis (Cornell et al., 2021). One of these homeostatic roles is synaptic pruning. Microglia are critical to refining synaptic circuitry, relying on the synaptic pruning process to eliminate weakened synapses, subsequently allowing space for strengthening of other synapses. Microglia physical interaction with neurons is another essential part of their homeostatic response. Microglia increase their frequency of interactions with synapses, expand their processes to increase protective coverage of synapses, and use these physical attachments to directly provide nutritional materials to distressed neurons (Tremblay et al., 2010; Scheiblich et al., 2024). Lastly, microglia modulate this homeostatic response by relying on the purinergic signaling pathway and releasing necessary neurotrophic factors. Microglia release pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα), interleukin-1β (IL-1β) and interferon γ, to enhance neurogenesis and oligodendrogenesis (Shigemoto-Mogami et al., 2014). They rely on purinergic signaling through P2Y12R, a receptor expressed by microglia during their homeostatic response (Morillas et al., 2021). Microglia's diverse homeostatic response is vital to protecting local neural networks in the brain (Chen and Trapp, 2015).

1.4.2 - Synaptic pruning via microglia-mediated complement cascade

An important role for microglia is synaptic pruning, a process vital to the refinement of synaptic connections during neurodevelopment. In the visual pathway,

microglia shift their focus to promoting synapse organization and other glial cell activation in the period before eye opening and the peak of the critical period (Wang et al., 2023). During periods of synaptic remodeling, microglia enter a homeostatic active state, increasing their contact with synapses to protect or prune synapses required for synaptic refinement (Paolicelli, 2011). A way microglia protect and prune synapses is through the classical complement cascade signaling, where less-active synapses can be tagged for pruning through a signal recognized by microglia. Impaired synaptic pruning, by disrupting the complement cascade, causes weakened synaptic connections, deficient microglial function, and overall neural deficits (Paolicelli, 2011). The complement cascade is initiated by astrocytic activation of C1q, a protein that activates C3b. C3b is the marker that tags synapses for pruning. C3b is recognized by receptors expressed by microglia, signaling the microglia to these tagged synapses, kickstarting microglia-mediated phagocytosis of synapses (Schafer et al., 2012). Deletion of either C1q or C3b mRNA results in failed synaptic pruning (Stevens et al., 2007). The complement cascade is important to the segregation of eye-specific layers in the dLGN during early development, as immature weak synapses are pruned to provide space for the maturation of stronger synapses (Zabel and Kirsch, 2013). Disruption to any step of the cascade results in sustained deficits in segregation of dLGN layers (Schafer et al., 2012). The cascade is important during early development, in the period before eye-opening, when the eye-specific layers are formed by elimination of inappropriate retinogeniculate synapses and strengthening of the appropriate synaptic connections (Stevens et al., 2007). However, inappropriate upregulation of this cascade is a factor in synaptic loss seen in neurodegenerative diseases (Stevens et al., 2007). Thus, a balance in this microglia-

mediated process is vital to the healthy development and homeostasis of the visual system. Although other cell types express C3b and participate in the complement cascade, microglia are the only phagocytic cells to express the C3b receptor during the visual critical period (Schafer et al., 2012).

1.4.3 - Microglia-neuron interactions facilitate neuronal development

Physical interactions between microglia and neuronal synapses are critical during development. Imaging has confirmed that microglia physically interact closely with synapses and associated elements in a healthy mouse visual cortex during the critical period (Tremblay et al., 2010). Microglia express CX3CR1, a receptor that binds to CX3CL1, expressed by neurons during physiological and pathological states. CX3CR1 knockout mice demonstrated decreased excitatory activity, deficits in synaptic pruning, and reduced functional connectivity, implicating this microglia-synapse interaction in the overall strengthening of synaptic connectivity (Paolicelli et al., 2011). Another neuroprotective role performed by microglia is the physical connections made with neurons using tunneling nanotubes (TNTs), which is seen in both physiological and pathological states. The TNTs allow for microglia to exchange mitochondria, organelles, vesicles, and necessary proteins to neurons to promote healthy neuron function (Chakraborty et al., 2023). Disrupting microglia mitochondrial function before TNTs can form between microglia and neurons prevents this restoration of health in the neurons (Neher and Simons, 2024). Furthermore, co-culturing neurons with microglia shows that TNT formations can recover neuronal activity (Scheiblich et al., 2024). Microglia's unique ability to interact and influence neurons and other glial cells make them an

interesting cell to study in neurodevelopmental and neurodegenerative disorders (Liu et al., 2021).

1.4.4 - Microglia-mediated purinergic signaling during neurodevelopment

Microglia can take on a homeostatic role during neurodevelopment, maintaining the excitatory/inhibitory neurotransmission balance through purinergic signaling (Yu et al., 2019). Purinergic signaling relies on P2Y12R, a receptor expressed exclusively on the ramified process of CNS microglia (Haynes et al., 2006). P2Y12R expression remains relatively stable through development and adulthood (Crain et al., 2009; Mildner et al., 2017). P2Y12R is expressed by homeostatic microglia, in their housekeeping role, and is an indicator of a healthy brain (Peng et al., 2019). Upregulation of P2Y12R reflects a ramified microglial state, often in response to changes to neuronal activity (Kettenmann et al., 2011; Savage et al., 2019). Conversely, in mice lacking P2Y12R, there is decreased microglial activity and ocular dominance plasticity in the visual cortex (Sipe et al., 2016). P2Y12R interacts with ATP, triggering a morphological change in microglia that involves membrane ruffling and increased motility (Sipe et al., 2016; Davlos et al., 2005). ATP is necessary to the basal velocity of microglial process and motility as cortical application of apyrase, which converts ATP to ADP, results in reduced microglial motility (Davalos et al., 2005). In vivo studies showed that disrupting ATP signaling results in reduced microglial motility, whilst ATP application increases microglial motility and further extends processes (Davalos et al., 2005; Haynes et al., 2006). Application of glutamate receptor agonists results in increased velocity, motility, and size of microglial processes. These studies suggest that increased excitatory neurotransmission increases microglial motility. Furthermore, deletion of microglia

abolishes LTP, suggesting their role in maintaining long-term synaptic potentiation (Saw et al., 2020). Silencing neural activity, by TTX administration, reveals conflicting results regarding microglia responses. An in vivo study in the neocortex shows that silencing activity does not change microglial motility (Nimmerjahn et al., 2005), whereas another study in vivo in the retina shows silencing activity reduces microglial activity (Tremblay, 2012). However, microglia's effect on the inhibitory circuit is slightly more defined, as microglia depletion results in the disruption of inhibitory neurons, leading to an imbalance in the excitatory/inhibitory neurotransmission and impaired ocular dominance plasticity during the critical period (Wang et al., 2023). As amblyopia is a neurodevelopmental disorder, microglial involvement is highly possible. There is currently research into microglia's role in the visual system, but their role in amblyopia is not yet fully defined.

1.4.4 - Experience-dependent activation of microglia

Microglia facilitate the maturation of the various synaptic networks through an experience-dependent process (Rogerson-Wood et al., 2025). Altering visual experience during the critical period elicits a change in how microglia interact with synapses (Schafer et al., 20112). In a study using dark rearing in the critical period, an increased area of microglial processes was observed, with an increase in microglia contact with neurons and astrocytes, suggesting microglia are activated by changes to visual activity such as dark rearing (Tremblay et al., 2010). However, reversal of dark rearing (brief-exposure to daylight), reverses microglial motility and synaptic contact to normal, suggesting microglia do not remain activated once activity levels have returned to normal (Tremblay et al., 2010). These findings suggest that microglia are activated by changes to

visual activity levels to potentially mediate synaptic activity by enhancing their surveillant role. Other forms of sensory deprivation studied include whisker trimming, as it is a method used to suppress neuronal activity. Microglia show increased surveillance and coverage of synaptic material following whisker trimming, confirming that sensory deprivation activates microglia (Liu et al., 2019). Furthermore, studies have also investigated microglial response following anesthetic-induced changes in neuronal activity (Yang et al., 2024).

Application of anesthetics produces hypoactivity in the administered region, noted by suppressed neuronal activity, which is thought to cause an increase in microglial surveillance (Umpierre and Wu, 2021). Hypoactivity caused by administration of common general anesthetics, including isoflurane, ketamine/xylazine, fentanyl, muscimol, or urethane, during rodent surgery increases overall microglial surveillance activity (Liu et al., 2019). In regards to TTX as a local anesthetic, one study shows that TTX has no effect on microglia process dynamics (Nimmerjahn et al., 2005); whereas another study shows TTX application increases the area of microglial processes and their contact with neurons and astrocytes (Liu et al., 2019). Another study using TTX shows microglia expand their processes and associated extracellular space, increasing their contact with synaptic elements (Tremblay et al., 2010). Reduced neuronal activity, by general anesthetics, sensory deprivation, and pharmacological local inhibition (TTX), increase microglial surveillance and protection over neurons, suggesting microglial dynamics are experience-dependent.

Other glial cells are shown to respond in an experience-dependent manner (Lawal et al., 2022). Microglia have a ‘bidirectional’ relationship with astrocytes (Bhusal et al.,

2023). Microglia are active sensors in the CNS, so changes to local activity levels can activate microglia to secrete factors that activate astrocytes (Gao et al., 2023). Conversely, astrocytes release factors that activate P2Y12R on microglia, initiating phagocytosis and release of pro-inflammatory cytokines (Koizumi et al., 2007; Liu et al., 2017). This close relationship between microglia and astrocyte suggest glial cells work together to modulate synaptic homeostasis in response to changes to activity levels.

A recent study by Duffy (2025) reveals a neuroprotective role for astrocytes during MI. As MI does not cause permanent neuronal loss, and visual function is recoverable following MI, shown by recovered VEPs, glial cells may be mediating the synaptic network to prevent neuronal and synaptic loss. Therefore, a neuroprotective role for microglia during MI is possible due to their close relationship to astrocytes.

1.5 – Microglia in the dLGN

The dLGN serves as the intermediary in relaying retinal input to the visual cortex, and so it is a fair assumption that manipulations to retinal activity will reflect directly into changes in the dLGN and its activity (Rittenhouse et al., 1999). Therefore, studying microglia in the dLGN can reveal how dLGN microglia alters genicular-cortical connections to modulate synaptic activity in the visual cortex. Microglial activation is commonly immunolabeled with ionized calcium binding adaptor molecule 1 (Iba1).

1.6 – Iba1 as a marker of activated microglia

Iba1 is one of the most commonly used markers for immunohistochemical analysis of microglia. It is a reliable marker of the different microglial morphologies and is widely expressed throughout the brain. Iba1 is a small 17-kDa calcium binding protein specifically and densely expressed by microglia (Imai et al., 1996). As an adaptor protein,

Iba1 binds actin to mediate calcium signaling. Microglial calcium signaling is increased following TTX application or administration of general isoflurane anesthesia (Brawek et al., 2014; Umpierre et al., 2020). Thus, a marker of calcium signaling such as Iba1 can be indicative of microglial activation. Calcium signaling is required to initiate membrane ruffling in microglia (Ohsawa et al., 2004), characterized by the formation of irregular, actin-rich protrusions of the microglial outer membrane (Liu et al., 2008). This process helps the dynamic remodeling of microglia's highly motile cytoskeleton, required for its proliferation, migration, and fast morphological transitions. Cell motility relies on the reorganization of the actin cytoskeleton, mediated by Iba1 binding to actin (Stossel, 1993). Additionally, Iba1 supports microglial-mediated phagocytosis, by colocalizing with other factors required for phagocytosis (Ohsawa et al., 2000). The expression of Iba1 by microglia is facilitated by the release of Macrophage-Colony Stimulating Factor (M-CSF), a cytokine facilitating the growth and proliferation of activated microglia. Throughout neurodevelopment, M-CSF is released to prompt the proliferation and maturation of microglia (Smith et al., 2013). Removing M-CSF disrupts the downstream cascades that leads to the expression of Iba1 and organization of the actin cytoskeleton (Imai et al., 2002). Silencing Iba1 expression dramatically reduces activated microglia features such as enhanced motility and neuronal interaction, proliferation, and migration (Gheorghe et al., 2020). This supports the notion that the highly motile actin cytoskeleton that microglia require in their activated state relies on the expression of Iba1.

Upregulation of Iba1 by microglia has been seen in the healthy brain as well as following injury, ischemia, inflammation, and infection (Hwang et al., 2006; Hopperton et al., 2018;

Lafrenay et al., 2020). Therefore, studying Iba1 immunoreactivity can indicate the extent of microglia activation and its associated morphological changes.

The current study aims to assess dLGN microglia activation in kittens subjected to monocular retinal inactivation. Elevated Iba1 immunolabeling in the dLGN layers serving the inactivated eye would suggest that silenced retinal input activates microglia to maintain synaptic homeostasis in the dLGN. This study also aims to observe if elevated Iba1 immunolabeling is sustained following a period of subsequent binocular vision (BV). If microglia remain activated after MI, when retinal input is recovered, this suggests microglia continue to regulate synaptic activity in healthy conditions as well. However, it is not expected that elevated Iba1 immunolabeling is observed following a period of MD. During MD, there is still active retinal input, therefore the synaptic threshold is not lowered enough to prompt protection of synapses by glial cells.

This current study may indicate a role for activated microglia in mediating the homeostatic synaptic response elicited by MI. As MI is being investigated as an effective treatment for amblyopia, these results further support MI as a more effective manner in triggering a homeostatic response in comparison to RO.

CHAPTER 2: MATERIALS AND METHODS

2.1 - Experimental design

This study was conducted on thirty-two kittens reared from birth in a closed colony at Dalhousie University. All experimental procedures described were approved by the Dalhousie University Committee for Laboratory Animals (UCLA) and followed the Canadian Council on Animal Care (CCAC) Guidelines. The rearing history of animals is described in detail in Table 1. Kittens were divided into five experimental groups: 1. a control group (Normal) in which kittens were reared binocularly for a range of 4 weeks to 9 years (adulthood) (n=12); 2. an experimental group (RE TTX) in which kittens underwent 10 days of retinal inactivation by intraocular injections of TTX in the right eye at various ages ranging from 4 to 22 weeks (n=12); 3. an experimental group (RETTX + BV) in which kittens underwent 12 days of retinal inactivation by intraocular injections of TTX in the right eye at 10 weeks followed by 8 days of binocular vision (BV) (n=2); 4. an experimental group (LETTX + BV) in which kittens underwent 7 days of retinal inactivation by intraocular injections of TTX in the left eye at 4 weeks followed by binocular vision for 4 weeks (n=2); 5. an experimental group (RE MD) in which kittens were monocularly deprived in their right eye at 8 weeks for 10 days (n=4).

2.2 - Experimental procedures

2.2.1 - Retinal inactivation

Animals in RETTX, RETTX + BV, and LETTX + BV groups were anesthetized with 3-4% gaseous isoflurane in oxygen and a heating pad at 37^{o}C was used to maintain their body temperature. Retinal inactivation was performed with an intravitreal microinjection of TTX (3mM dissolved in citrate; 0.5 µL/100-gram body weight)

(ab120055; abcam, USA). Prior to injection, a small puncture hole was made using a sterile 30-gauge needle into the sclera at the ora serrata. The total volume of TTX solution was administered slowly using a sterilized Hamilton syringe (Hamilton Company, USA) with a fixed 30-gauge needle (point syle 4) through the puncture hole 5-10mm into the vitreous chamber angled away from the anterior chamber. Afterwards, the needle was held for a one-minute waiting period before being retracted. Following the injection, a topical antibiotic (1% chloromycetin; CDMV, Canada) and a local anesthetic (Alcaine solution) were applied to the eye to prevent post-injection complications. Metacam (0.05mg/kg) was administered for post-procedure analgesia. The full period of retinal inactivation required for each group was achieved by administering injections every 48 hours as TTX produces retinal inactivation for roughly 48 hours (Duffy et al., 2018). The original puncture hole was used for the following injections. During inactivation, inactivation was confirmed by observing animals for an absence of pupillary light reflex, visuomotor behaviors (including visual startle, visual placing, following moving objects or laser pointers), absence of VEPs, and lack of vision-mediated interaction with littermates or staff. During these assessments, the fellow eye was occluded with an opaque contact lens. Animals from RETTX + BV group were given binocular vision for a period of 8 days, starting 2 days after the last TTX injection was administered. Animals from LETTX + BV group were given binocular vision for a period of 4 weeks, starting 2 days after the last TTX injection was administered.

2.2.2 - Monocular Deprivation

MD by eyelid closure was performed at 8 weeks. Animals were anesthetized with 3-4% gaseous isoflurane in oxygen and a heating pad at 37ºC was used to maintain their

body temperature. The upper and lower palpebral conjunctivae were closed with a sterile 5-0 vicryl suture and the eyelids were closed with 5-0 silk suture. The lid closure procedure lasted 15 minutes and then Metacam (0.05mg / kg) was administered for post-procedure analgesia. Topical Alcaine sterile ophthalmic solution (1% proparacaine hydrochloride; CDMV, Canada) was applied for local anesthesia. Broad-spectrum topical antibiotic (1% chloromycetin) was applied to the closed lid to prevent post-operative infection. The kittens were monitored daily for postoperative infection and to ensure proper lid closure.

2.2.3 – Histology

The animals from RETTX group were sacrificed two days after the final TTX injection was administered, whereas the animals from RETTX + BV and LETTX + BV following the period of binocular vision. The animals from RE MD were sacrificed following 10 days of MD. Animals were anesthetized with isoflurane (5% in oxygen) and then euthanized with a lethal dose of sodium pentobarbital (euthanyl; 150 mg/kg). Afterwards, the animals were exsanguinated by transcardial perfusion with 150 mL of phosphate-buffered saline (PBS) followed by a solution of PBS containing 4% dissolved paraformaldehyde. Following perfusion, brain tissue was extracted from the skull, and the thalamus was dissected from the cerebral cortex for dLGN sectioning and processing. Resected tissue with the dLGN was cryoprotected in a solution containing PBS and 30% sucrose for several days and then cut coronally into 50 micrometer-thick slices using a sliding microtome. The tissue sections were stored at –20^{o}C in an antigen preservation solution. All tissue used for this study underwent the same extraction, preparation, storage, and immunolabeling procedures.

2.2.4 - Iba1 immunolabeling

Tissue sections containing the right and left dLGN were immunolabeled for Iba1 using a rabbit polyclonal antibody (ab178846; abcam, USA). Sections were immersed into a PBS solution containing the anti-Iba1 antibody (1:4000) for at least 12 hours. The sections were washed three times with PBS solution before being placed in a PBS solution containing biotinylated goat anti-rabbit secondary antibody (1:500) (111-065-003; Jackson ImmunoResearch, USA) for 1 hour. Sections were washed in PBS 3 times before being placed in a PBS solution containing avidin and peroxidase-conjugated biotin for 1 hour. The sections were washed again with PBS before exposing the sections to a PBS solution containing hydrogen peroxide (1:1000) and chromogen substrate, 3,3'-diaminobenzidine (0.5 mg/ml) for 4 minutes. Following the reaction, the sections were washed again with PBS before being mounted on gelatin-coated glass slides to dry. After the sections were dry on the slides, they were immersed in a graded series of ethanol, cleared with Histo-Clear (National Diagnostics, USA), and cover slipped with Permount medium (Fisher Scientific, Canada).

2.2.5 - Morphologic identification

Microglia adopt an ameboid shape to reflect a phagocytically active phenotype (Svahn et al., 2013; Vidal-Itriago et al., 2022). Figure 2A and 2B display the amoeboid shape and identifying amoeboid morphology with Iba1 immunolabeling. When microglia differentiate, they can enter a transitional state between amoeboid and ramified where they still have a circular cell body but start to extend more processes. This transitional shape is displayed in Figure 2C. Ramified microglia have elongated cell bodies, with

extended processes that aid in surveying their surroundings and synaptic maturation (Wake et al., 2009), seen in Figure 2E.

2.2.6 – Quantification

Quantification was performed without knowledge of rearing condition. The measurements were made from dLGN sections that spanned coronal plane 6-7, positioned midway along the anterior-posterior axis of the nucleus. Quantification was performed on dLGN layers A and A1. Each tissue section contained two dLGN therefore providing four measurements: two from left dLGN (layer A: input from right eye and layer A1: input from left eye) and two from right dLGN (layer A: input from left eye and layer A1: input from right eye). Using the polygon tool in QuPath software, a sampling region was marked around each dLGN layer. Only cells clearly exhibiting Iba1 immunolabeling were selected for counting, as presented in Figure 1 (Tremblay et al., 2010). For each dLGN layer, the density of Iba1-positive cells was calculated by dividing the number of cells counted by the region area sampled.

2.2.7 - Statistical analysis

The developmental profile of Iba1 immunolabeling in normal animals was characterized by an exponential plateau curve, where YM is the maximum value for Iba1-positive cell density, Y0 is the starting value for Iba1-positive cell density, and k is the rate constant.

$$Y = YM - (YM - Y0)*exp(-k*x)$$

To make a statistical comparison between eye-specific dLGN layers in each condition, an average measurement was taken from each layer in each animal. A Wilcoxon matched pairs signed rank test (two-tailed, non-parametric) was used to determine if the

inactivated-eye dLGN layers had higher Iba1 labeling compared to the normal-eye dLGN layers. The same statistical test was used to determine if the deprived-eye dLGN layers had higher Iba1 labeling compared to the normal-eye dLGN layers.

CHAPTER 3: RESULTS

3.1 - Normal development of Iba1 labeling in the cat dLGN

Morphology-based observations revealed microglia adopt different shapes throughout development in normal cat dLGN. Fig. 2 illustrates the different microglia morphology seen through normal development in the dLGN. In early postnatal development, at 5 weeks old, microglia appeared to have an amoeboid shape, characterized by little to no processes extending from a circular cell body (Fig. 2A, 2B). In 10-week-old kittens, microglia were in a transitional state, with more, but short, processes and a circular soma (Fig. 2C & D). In normal adult animals, microglia adopted a ramified shape, with elongated cellular bodies and long, branching processes closely connected to other microglia (Fig. 2E & F).

Observation of Iba1 immunolabeling within dLGN layers of normal cats revealed very few immunoreactive microglia at 5 weeks old, the earliest age examined. At this age there was a low level of Iba1 immunolabeling observed in layer A and layer A1 of the dLGN, evident at low (Fig. 3A) and high magnification (Fig. 3B). Clear Iba1 labeling was observed outside of dLGN layers, confirming that the reaction was successful in labeling Iba1-positive cells. From 5 to 10 weeks, there was an increase in Iba1 immunolabeling and this increases even further into adulthood. Quantification of Iba1-positive cell density within the dLGN was plotted for layers serving the right eye (unfilled circles) and left eye (filled circles). Data showed an increase in Iba1-positive cell density from 5 weeks old to 22 weeks old, before plateauing into adulthood (Fig. 3G). Iba1-positive cell density reached near adult levels at around 22 weeks old (Fig. 3G). The quantification of Iba1 immunolabeling through development was fit by an

exponential curve (R = 0.64) that showed a steady increase in Iba1 immunolabeling before reaching approximately 85% of adult values at around 22 weeks old (Fig. 3G).

3.2 - Iba1 immunolabeling following monocular inactivation

The current study also examined Iba1 immunolabeling in the dLGN following MI for a period of 10 days at varying ages ranging from 4 to 22 weeks. Microglia within inactivated-eye dLGN layers from 4-week-old animals resembled the ramified state seen in microglia of normal adult animals (Fig 2F; Fig. 4B). However, microglia in normal (fellow) eye dLGN layers adopted the amoeboid shape seen in the young normal animals (Fig 2B; Fig. 4B). Furthermore, at 4 weeks old, MI produced an observable increase in Iba1 immunoreactivity within inactivated-eye dLGN layers compared to normal-eye dLGN layers (Fig. 4A). Quantification of Iba1-positive cell density following MI at 4 weeks and 6 weeks revealed that inactivated-eye layers have a higher cell density (mean = 252 cells / mm^2) compared to normal-eye dLGN layers (mean = 189 cells / mm^2) (Fig. 4C). This increase was statistically significant ($p < 0.01$) according to the Wilcoxon matched pairs signed rank test on the combined density data from both age groups. When 10 days of inactivation was administered at 10, 17 or 22 weeks of age, there was no observable difference in Iba1 immunoreactivity or morphology between the inactivated- and normal-eye dLGN layers (Fig. 4D, 4E). Quantification of Iba1-positive cell density following MI at 10, 17, and 22 weeks confirmed that there was no difference in cell density between the inactivated dLGN layers (mean = 167 cells / mm^2) and the normal dLGN layers (mean = 175 cells / mm^2). Furthermore, there was no statistical significance between the inactivated- and normal-eye dLGN layers (combined dataset - Wilcoxon matched-pairs signed rank test; $p > 0.05$) (Fig. 4F).

3.3 - Durability of elevated Iba1 labeling

To investigate the durability of elevated Iba1 immunolabeling after 4 weeks, the LETTX + BV group underwent MI in the left eye at 4 weeks old for a period of 7 days. Following MI, the animals were given a period of 4 weeks of normal binocular vision. Microglia in the formerly inactivated-eye and normal-eye dLGN layers adopted an amoeboid shape similar to the 4 week old normal animals. Additionally, observed elevated Iba1 immunolabeling seen at 4 weeks old, described above, in the inactivated-eye dLGN layers, did not persist with a period of normal binocular vision (Fig. 5A, 5B). Quantification of Iba1 cell density showed that there was no increase in the inactivated-eye dLGN layers (mean = 264 cells / mm^2) compared to the normal-eye dLGN layers (mean = 246 cells / mm^2). Although MI at 10 weeks old did not elicit a microglia response, as described above, a recovery of visual input provided by BV, after a period of MI, may modify the synaptic threshold enough to activate microglia. Therefore, an additional experimental group was investigated to observe if there was a difference in Iba1 immunolabeling following MI at 10 weeks old, an age past the critical period. The RETTX + BV group underwent MI in the right eye at 10 weeks old for a period of 12 days. Following MI, these animals were given a period of 8 days of normal binocular vision. The microglia adopted a similar transitional shape to the 10-week-old normal animals. There was no observable change in Iba1 immunolabeling between the layers. Quantification of Iba1-positive cell density shows there was no difference between the inactivated eye dLGN layers (mean = 115 cells / mm^2) and the normal-eye dLGN layers (mean = 111 cells / mm^2) (Fig. 5C, 5D). The combined data sets of these two experimental groups revealed there was no statistical difference in the Iba1-positive cell

density between inactivated- and normal-eye dLGN layers (Wilcoxon matched-pairs signed rank test; $p > 0.05$) (Fig. 5E). These results from both experimental groups suggest that microglial activation, in the younger animals, is a transient effect that is not sustained after inactivation.

3.4 - Iba1 immunolabeling following monocular deprivation

To investigate Iba1 immunolabeling following MD, an experimental group that underwent 10 days of MD at 8 weeks old was investigated. This same group of animals exhibited reduced neuronal size within the deprived-eye layers in comparison to the normal-eye layers (Duffy, 2025), verifying that lid suturing was effective in inducing deprivation. In this study, microglia in both the deprived- and normal-eye layers appeared to have similar morphologies, adopting a transitional shape with short processes and a circular cell body. Furthermore, there was no observable change in Iba1 immunolabeling between the deprived- and normal-eye dLGN layers. (Fig. 6A, 6B). Quantification of Iba1-positive cell density showed no difference between inactivated eye dLGN layers (mean = 88 cells / mm^2) and normal eye dLGN layers (mean = 89 cells / mm^2). The Wilcoxon matched pairs signed rank test ($p > 0.05$) confirmed there was no statistical difference in Iba1-positive cell density between the deprived- and normal-eye dLGN layers (Fig. 6C). There was still important insight provided by observing microglia at an age between 4 and 10 weeks old, as there was not an experimental cohort of 8 week old MI-treated animals.

CHAPTER 4: DISCUSSION

The current study sought to determine how visual manipulations, specifically MI and MD, affects microglial activation in the cat dLGN. Iba1 immunolabeling is used to observe and quantify the density of Iba1-positive microglial in the dLGN A and A1 layers. A postnatal developmental profile of Iba1 immunolabeling was established within the dLGN of visually normal cats. Our study showed that Iba1 immunolabeling in normal cats increases steadily from early postnatal development to adulthood. Immunolabeling was the lowest among our youngest animals, at 5 weeks old, and then increased up to 22 weeks of age. The highest level of Iba1 immunolabeling was observed in adult cats.

Second, our study quantified the amount of Iba1 labeling within the dLGN eye-specific layers following MI administered at varying ages during postnatal development. Interestingly, in younger animals at 4 weeks old, MI produced a substantial increase in Iba1 within the dLGN layers serving the inactivated eye. However, this prominent increase in Iba1 was not seen at the older ages examined. Furthermore, the MI-induced elevation of Iba1 seen at 4 weeks of age did not persist after the effects of TTX wore off and binocular vision was provided. Elevated Iba1 immunolabeling, revealing activated microglia, did not extend to MD because no change in Iba1 immunolabeling was measured in dLGN layers serving the deprived eye in 8 week old animals.

4.1 - Microglia during normal development

Relatively little is known about the development of Iba1-positive microglia in the visual system of primates and cats. A study by Askew et al. (2017) showed that although microglia turnover frequently throughout development, the number of microglia present is relatively stable from early development into aging. The current study showed an

increase in Iba1-positive microglia throughout development, suggesting that microglia upregulate Iba1 expression throughout development rather than an increase in proliferation of microglia. One study in the mouse visual cortex revealed Iba1 immunolabeling increases in early postnatal development before stabilizing into late postnatal development (Wang et al., 2023). This study also showed that microglia underwent a change in their branching morphology, a transition from amoeboid to ramified in postnatal development as they adopt to their newly required roles after development (Wang et al., 2023). The amoeboid shape was adopted in embryonic and early postnatal development to reflect a phagocytically active phenotype required to remove excess synaptic-associated elements (Svahn et al., 2013; Hattori, 2022; Vidal-Itriago et al., 2022). However, in late postnatal and further development, microglia adopted the ramified shape needed to maintain homeostasis during disruptions to their local neural environment (Lier et al., 2021). The current study loosely follows this morphological transition in microglia, as microglia in the younger animals appeared to adopt the amoeboid shape, whilst microglia in the older animals adopted the ramified shape.

As there are very few studies profiling Iba1 expression over development, the current study provides a new perspective on how Iba1 expression changes over development. Fewer Iba1-positive microglia were observed in younger animals, suggesting that young microglia do not express Iba1 unless there is distress to their local environment, wherein which they upregulate Iba1 expression to mediate their response. One explanation as to why there was a steady increase in Iba1 immunolabeling in older animals is that Iba1 accumulates overtime so that older animals have higher levels of

Iba1. Another possibility is that normal adult microglia in their ramified state express higher levels of Iba1 compared to in their amoeboid state. Adult microglia rapidly alter the degree of ramification in their surveillant role, which requires an increase in Iba1 expression to facilitate these morphological changes (Ito et al., 1998; Sasaki et al., 2001). Whereas amoeboid microglia remain in a more constant state, that may not require an increased expression of Iba1 to facilitate their roles (Vidal-Itriago et al., 2022). Overall, the current study addresses the gap in literature pertaining to Iba1 expression in the dLGN throughout development by providing a developmental profile showing an increase in Iba1 expression over time with a change in microglial morphology.

4.2 - Microglial activation following monocular inactivation

MI at 4 weeks old elicited a microglia response shown by elevated Iba1 immunolabeling and morphological changes in the inactivated eye-serving dLGN layers. Previous studies have shown that microglia adopt the ramified shape in response to changes in local activity levels (Liu et al., 2019). This morphology allows microglia to increase surveillance through physical contact with neurons and other glial cells, as well as molecular factor secretion, all to promote neuronal health (Liu et al., 2019; Tremblay et al., 2010; Kettenmann et al., 2011; Wake et al., 2013). Low activity levels brought on by MI are apparently detected by microglia, leading to an increased expression of Iba1. As previously noted, microglia upregulate expression of Iba1 in response to changes in calcium signaling brought on by activity level fluctuations (Kanazawa et al., 2002; Brawek et al., 2013; Umpierre et al., 2020). When microglia upregulate Iba1 expression, there are changes such as increased motility, extension of microglial processes, and secretion of neurotrophic factors (Imai et al., 1996). These collective findings support the

ramified morphology and elevated Iba1 immunolabeling observed in the inactivated eye dLGN layers. The current study revealed MI at 4 weeks old caused elevated Iba1 immunolabeling resembling the activated microglial phenotype, but this was not observed with MI imposed at older ages.

4.2.1 - Microglial activation during the critical period

It is possible that microglia are exclusively activated by low activity levels when MI is administered during the critical period peak. Many studies have shown that experience-dependent microglial interaction with synapses occur in the early postnatal period (Schafer et al., 2012; Stevens et al., 2007; Tremblay et al., 2010). These studies demonstrated that microglia modulate synapses in an experience-dependent developmentally regulated fashion, as homeostatic roles including synaptic pruning were upregulated when activity is disrupted during the critical period, but not so much so when activity was disrupted in later developmental periods (Schafer at al., 2012). The current study fits with the narrative that microglia are activated in an experience-dependent manner, sensitive to changes in synaptic activity mainly during the critical period.

4.2.2 - Microglia work in a complementary manner with astrocytes

Further evidence supports an experience-dependent role for microglia in early postnatal development, and one that possibly involves astrocytes in later postnatal development. Both microglia and astrocytes are believed to have active states during physiological conditions, though they can be activated by changes in sensory stimuli as well (Fakhoury, 2018). Microglia are among the first macrophages to differentiate and migrate in embryogenesis. The colonization of microglia in the brain begins earlier than that of neurons, astrocytes, and oligodendrocytes (Hattori, 2022). As they are already

established in the CNS, microglia help to set up the spatial patterning of astrocytes as astrocytes begin differentiating and colonizing later in the embryogenic timeline. Microglia formation peaks in early embryogenesis, followed by astrogenesis in the late prenatal and early postnatal periods (Reemst et al., 2016). As microglia are active sensors, they sense and react to changes in activity earlier than astrocytes (Jha et al., 2018). Although astrocytes are essential for synapse maturation, microglia are the only glial cell present at the time synaptogenesis begins in embryogenesis (Kettenmann et al., 2013). Astrocytes spend the first part of early postnatal period undergoing maturation, a process aided by microglia. Microglia modulate purinergic factors to activate astrocytes, and prompt the release of neurotrophic factors from microglia (Pascual et al., 2012). Further studies have shown that microglia mediate astrocyte-synapse interactions in a manner that is activity-dependent (Faust et al., 2024). The experience-dependent mechanism that activates microglia and astrocytes may occur at different periods in postnatal development. Changes to activity levels in early postnatal development evoke a microglial response that provides neuroprotective measures to regulate neuronal plasticity. Microglia play a primary role at the beginning of synaptogenesis, pruning excess immature synapses while secreting molecular factors to protect the stronger synapses (Tremblay et al., 2010). Pharmacological depletion of microglia during the visual critical period causes excessive synaptic growth resulting in a disorganized visual cortical network and diminished ocular dominance plasticity (Ma et al., 2020). Astrocytes have been shown to promote the stabilization of the developed synaptic network and drive the closure of the critical period closure for visual plasticity (Ribot et al., 2021). Astrocytes modulate both excitatory and inhibitory synapses to control the timing of the

critical period (Takano et al., 2020). The current study suggests that different glial cell types are activated dependent on the age in which activity levels are disrupted. At early ages, during the peak of the critical period, such as 4 week old cats, low activity levels activate a microglial-driven response to protect the synaptic network. This may be due to microglia playing a predominant role in experience-dependent postnatal development, as their surveillant feature alerts them to activity changes earlier than other glial cells (Hanisch et al., 2007; Vainchtein et al., 2020). However, at older ages examined, from 10 weeks onward, microglia may no longer be activated because astrocytes assume the predominant role following closure of the critical period. Further support of this is that marked astrocytic activation was observed following MI at older ages examined, in the same animals used for this study (Duffy, 2025) but not at ages during the critical period peak. Duffy (2025) observed evidence of a small activation of astrocytes at 4 and 6 weeks old, however, the effect was much smaller than at the older ages examined. These findings suggest that astrocytes are not key participants in the synaptic homeostasis elicited by MI during the critical period peak (Duffy, 2025). In aggregate, these findings suggest that early in the critical period, microglia in the dLGN are highly sensitive to decreased levels of retinal output activity, possibly responding to protect the geniculate synaptic network by modulating synaptic strength to balance reduced visual input produced by MI. However, microglia may play a diminished role in later development and adulthood when astrocytes become key participants in the protection of the synaptic network (Clarke and Barres, 2013). The current study suggested that microglia and astrocyte take on a complementary role in their contribution to maintaining homeostasis upon disruptions to local neural activity.

4.2.3 - Synaptic pruning mediated by microglia

Both astrocytes and microglia are implicated in synaptic pruning and phagocytosis during neural development in both healthy and pathological conditions (Schafer et al., 2012; Nguyen et al., 2020; Cahoy et al., 2008). Astrocytes eliminate both excitatory and inhibitory synapses to refine the retinogeniculate network (Chung et al., 2013). During early development, microglia preferentially prune weaker retinogeniculate synapses (Schafer et al., 2012), and following changes to visual experience in early development they increase their contact and coverage with synapses, however synaptic density remains unchanged (Tremblay et al., 2010). This suggests a neuroprotective role, with little to no synaptic loss, that microglia play during their homeostatic response to activity disruptions in early development. It is possible that microglia contribute minimally to synaptic pruning that is not enough to produce a considerable loss of neurons in the inactivated eye dLGN layers (Duffy, 2025). Any pruning performed by microglia was insufficient to induce functional deficits in the visual cortex as diminished VEPs recorded during MI were recoverable with a period of BV following MI (Duffy et al., 2023).

4.2.4 - Prolonged microglial activation may disrupt synaptic homeostasis

It is important to note that microglia-mediated synaptic homeostasis can become detrimental. Persistent microglial activation can lead to a continuous release of pro-inflammatory cytokines, forcing the CNS into a chronic state of neuroinflammation (Muzio et al., 2021). Continuous microglial-driven synaptic pruning results in excessive synaptic loss, contributing to cognitive deficits and neurodegenerative disease. Repetitive physical interactions with synapses can stunt synaptic growth, resulting in neuronal loss

(Wang et al., 2023). Therefore, prolonged periods of MI may result in sustained microglial activation that produces detrimental, rather than protective, effects on the synaptic networks. Further studies should investigate whether the structure and function of the dLGN is recoverable following longer periods of MI, as prolonged glial cell activation may prevent this recovery.

4.3 - Microglial activation is not sustained once inactivation wears off

Microglia are often the first to respond to changes in neuronal activity levels, due to the active sensors on their dendritic processes (Kreutzberg, 1996; Borst et al., 2021). In the current study, microglial activation was transient in nature and was not sustained when activity levels were recovered following inactivation. Previous studies support the suggestion that microglial activation is transient, rather than a prolonged process. In a study by Tremblay et al. (2010), microglia underwent morphological changes indicative of a ramified state, with increased physical interactions with synapses and astrocytes, following a period of dark rearing in juvenile mice. However, these changes were not sustained following a period of light re-exposure, with those altered features reversing to normal. Another study showed that microglia transiently upregulate expression of complement cascade-associated receptors during a developmental wave of neuronal apoptosis, and this upregulation was not sustained following this apoptotic period (Ghena et al., 2025). During disruptions to photoreceptor activity in the retina, microglia temporarily increased their processes in the injury field, returning to normal once photoreceptor activity was restored (Miller et al., 2019). These findings, in addition to the current study, support a transient microglial response, lasting the period of disruption of activity, that is not sustained once activity levels are restored to normal.

4.4 - Monocular deprivation does not activate microglia

The current study demonstrated that MD at 8 weeks old did not activate microglia as made evident by the lack of observable change in Iba1 immunolabeling or morphological characteristics between the inactivated-eye and normal-eye dLGN layers. One possibility is that MD does not meet the threshold required to activate microglia. MD by lid suturing still allows for spontaneous activity in the retina, whereas MI by TTX eliminates spontaneous neuronal activity in the retina (Rittenhouse et al., 1999). Therefore, the difference in their effects is that the visually deprived neurons still receive active input through spontaneous activity from the deprived eye. It was previously thought that active suppression from the non-deprived eye could drive the deprivation effects, such as the shift in ODCs towards the non-deprived eye. However, a study by Rittenhouse et al. (1999) uses the BCM theory to suggest deprivation effects may be due to homosynaptic (driven by the deprived eye) depression, instead of heterosynaptic (driven by the non-deprived eye) depression. As previously noted, the BCM model suggests a sliding modification threshold regulates synaptic activity. The modification threshold is lowered when postsynaptic activity is diminished to facilitate LTP and strengthening of the synapses. However, a low level of postsynaptic activity during MD is maintained, as the visually deprived neurons still receive spontaneous retinal input from the deprived eye. Therefore, Rittenhouse et al. (1999) concluded that homosynaptic (driven by the deprived eye) depression causes deprivation effects from MD. Homosynaptic depression results in a reduced cortical response to input from visually deprived neurons leading to deprived eye-serving neuronal loss and overall functional deficits, comparable to amblyopia. By contrast, TTX eliminates spontaneous retinal

activity, reducing postsynaptic activity. When postsynaptic activity is reduced, the modification threshold is lowered to prevent synaptic depression as part of the homeostatic response (Rittenhouse et al., 1999). The current study suggests that dLGN microglia were activated by the lowered modification threshold, thereby performing neuroprotective roles, including enhanced surveillance and coverage of synapses, secretion of neurotrophic factors to help mitigate synaptic loss, and recruitment of other glial cells. MI produced significant recovery from the effects of previous fellow eye deprivation, even at an age when recovery was not possible with reverse occlusion. MI restored synaptic plasticity in the visual system, resulting in structural and functional recovery from the effects of previous deprivation (Fong et al., 2021). As previously noted, this recovery did not result in structural or functional detriment to the inactivated eye (DiCostanzo et al., 2020). Although the mechanisms underlying recovery from MI are still unknown, the current study supports a role for microglia in protection of the visually inactivated neurons from synaptic weakening during a period of MI in the critical period.

4.5 - Microglial activation may be implicated with use of other anesthetics

As TTX is a local anesthetic, it begs the question of whether other anesthetics can produce an inactivation effect of the same magnitude, in which there is a homeostatic response in the visual system. As the current study established a role for microglia in this homeostatic response, it is important to investigate the effect of other anesthetics on microglia. There is conflicting literature regarding anesthetics' effects on microglia in animal studies. One study by Haruwaka et al. (2024) showed that a period of gaseous isoflurane anesthesia increased the frequency of microglia-neuron interactions.

Furthermore, the study found that hypoactivity brought on by isoflurane activated microglia to transiently protect synapses during the anesthesia. Interestingly, another study showed that synaptic pruning was not part of the microglia-mediated response to anesthetic effects, specifically ketamine, pentobarbital, or propofol. This study revealed that microglia regulated the anesthesia response through P2Y12 signaling, a receptor expressed by microglia in their homeostatic state (He et al., 2023). These collective findings showed that anesthesia activated a homeostatic microglial response through purinergic signaling, instead of a phagocytic driven response. The current study fits into these findings by suggesting a microglial homeostatic-mediated response that protect neurons during inactivation, rather than a response that eliminates neurons. Low dose TTX is currently under study for its potential as a therapeutic pain agent, as it works similar to other anesthetics but with a longer duration effect compared to other more common anesthetics (Padera et al., 2006).

4.6 - Tetrodotoxin as a therapeutic agent

TTX's therapeutic uses have generated interest over the past few decades. Low doses of TTX have a possible role in pain management, as it acts as an analgesic in varying pain models including cancer-related, acute, chronic, and neuropathic pain (Nieto et al., 2012; Bucciarelli et al., 2021). Low doses of TTX were shown to block action potentials without affecting the axoplasmic transport *in vitro* (Ochs and Hollingsworth, 1971). This current study, in addition to several others, supports a therapeutic role for TTX in treating amblyopia, acting as a replacement for fellow eye occlusion to promote recovery from amblyopia. Low-dose intravitreal TTX injections in cats did not affect the cat's visual or general health (DiCostanzo et al., 2020; Hogan et al., 2023). Furthermore,

intravitreal injections are used in common clinical practice to treat maculopathies, inflammation, and infection in adults (Arevalo et al., 2007; Peyman et al., 2009). Therefore, intravitreal administration of TTX is supported on a clinical basis as TTX is used in clinical practice.

4.7 - Limitations to quantifying microglia

The current study used cell counting to quantify Iba1-positive microglia. Other methods of quantifying microglia and their morphology include pixel density coverage, fractal analysis, sholl analysis, and individual skeletal analysis. Pixel density coverage was not used in the current study as it is very sensitive to stain quality, background staining, and any changes to stain protocol. Additionally, averaging pixel density across multiple images can introduce bias (Green et al., 2022). Therefore, cell counting was performed as it is less sensitive to changes in staining. Additionally, the protocol for identifying cell soma in cell counting allowed for consistency among multiple images. Fractal, sholl, and individual skeletal analysis all provide quantitative measures of cell span, branching, and identifies hallmark features of microglia morphology (Green et al., 2022). However, these three methods are more relevant when focusing on specific cell interactions and microglia complexity, therefore they were not necessary for the current study.

4.8 - Clinical Implications

Amblyopia is the leading cause of monocular vision loss in children and young adults, affecting up to 2% of the world's child population (Hu et al., 2022). Untreated amblyopia can have long-term effects such as reduced binocular vision and risk of severe visual function loss if there is loss of the non-amblyopic eye (Nilsso, 2007). Diagnosing

and treating amblyopia faces several barriers including lack of pre-education screening, psychosocial impacts, and adherence to treatment (Yoo et al., 2025). Deprivation amblyopia produces more dramatic deficits to the visual system, with earlier onsets and poorer visual prognosis, in comparison to other forms of amblyopia (Epelbaum et al., 1993; Keech and Kutschke, 1995). Deprivation amblyopia primarily occurs before one year of age, due to its etiologies in congenital cataracts, retinal pathologies, and lid abnormalities (Hamm et al., 2017). A good visual prognosis requires earlier intervention, often before the age of 2 months old, and a more rigorous patching regimen (Cheng et al., 1991).

Amblyopia is commonly treated with occlusion therapy; however, its success is hindered by several barriers, including compliance, reoccurrence amblyopia, rigid treatment regimen, and being completed by an early age within the critical period (Holmes and Levi, 2018; Jia et al., 2022; Wallace et al., 2018). Additionally, there is increasing evidence that suggests there is residual visual impairment even with successful patching treatment (Pediatric Eye Disease Investigator Group, 2002). Furthermore, there is risk of occlusion amblyopia during occlusion therapy, where visual acuity decreases in the fellow eye during treatment (Searle et al., 2002; Agervi et al., 2010). Other methods of treating amblyopia such as pharmacological penalization are not beneficial for deeply reduced vision that is often seen with deprivation amblyopia (Tejedor and Ogallar, 2008). Therefore, there is still a need for alternative amblyopia treatment to address some of the barriers faced with the current therapies.

Retinal inactivation provides an alternative treatment that can address some of the limitations of occlusion therapy. Retinal inactivation through intravitreal administration

of TTX was shown to improve visual function in a previously amblyopic eye in various animal models, including mice, cats, and monkeys (Fong et al., 2016; Duffy et al., 2018; Fong et al., 2021; Kwaw et al., 2024). Interestingly, retinal inactivation promoted recovery from deprivation even when the animal was considered visually mature, an outcome not seen often with conventional treatments (Duffy et al., 2018; Fong et al., 2021). These findings suggest retinal inactivation restores juvenile synaptic plasticity in visually mature animals, allowing the deprived eye to visually recover. Although MI produces a larger impact on the structure and function of the visual system, these effects are temporary whereas MD produces effects of a smaller magnitude but are long-lasting (Frenkel and Bear, 2004; Rittenhouse et al., 1999). However, the specifics of how inactivation restores synaptic plasticity, including promoting recovery from deprivation whilst not permanently affecting the visual function of the fellow eye, are not yet fully understood.

The current study, along with previous literature, suggests a role for glial cells in protecting the synaptic network serving the inactivated eye during inactivation. Microglia and astrocytes work in complementary fashions to provide a homeostatic response to inactivation. Microglia provides a transient homeostatic response to inactivation in younger animals, whereas astrocytes take on the role of mediating this response in visually mature animals. These findings aid in explaining how synaptic activity in the visual system is protected following MI.

As TTX emerges as a strong candidate for an alternative to occlusion therapy, it is important to understand how and why the eye remains uncompromised following inactivation. Administering TTX intravitreally can address patching treatment limitations

with compliance and could provide a more flexible treatment regimen. Furthermore, as TTX appears to promote recovery after visual immaturity, inactivation addresses the issues surrounding pre-school screening as amblyopia could be treated in older patients. Overall, the efficacy of fellow eye MI addresses some of the current limitations of conventional amblyopia treatment, presenting a novel treatment that produces recovery of the amblyopic eye without detriment to the fellow eye.

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APPENDIX A: Tables

	Rearing Conditions			
	Normal	Inactivation	BV	MD
Normal				
n=2	P0-P40	-	-	-
n=1	P0-P47	-	-	-
n=2	P0-P70	-	-	-
n=2	P0-P98	-	-	-
n=3	P0-P154	-	-	-
n=2	Adult (≥1yr)	-	-	-
Monocular Inactivation				
n=4	P0-P30	P30-P40	-	-
n=2	P0-P42	P42-P52	-	-
n=3	P0-P70	P70-P80	-	-
n=1	P0-P119	P119-129	-	-
n=2	P0-P154	P154-P164	-	-
Monocular Inactivation + BV				
n=2	P0-P30	P30-P37	P37-P56	-
n=2	P0-P70	P70-P82	P82-P90	-
Monocular Deprivation				
n=4	P0-P56	-	-	P56-P66

Table 1. Animal rearing conditions

APPENDIX B: Figures

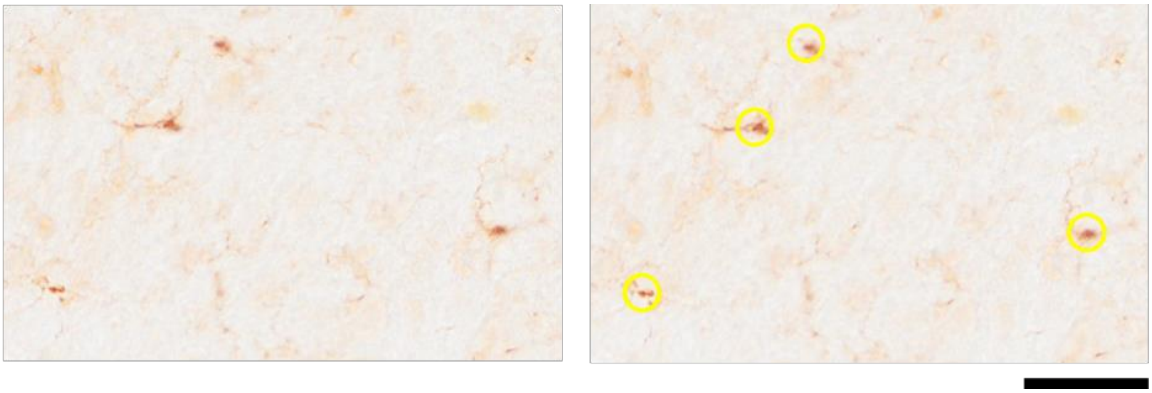

Figure 1. Microglia counting

Microglia were selected for counting if there was a distinct cell body observed following Iba1 labeling. This figure illustrates how microglia were counted based on the immunolabeling of the cell body, and these cells were marked with a yellow circle. Iba1 Scale bar = 25µm

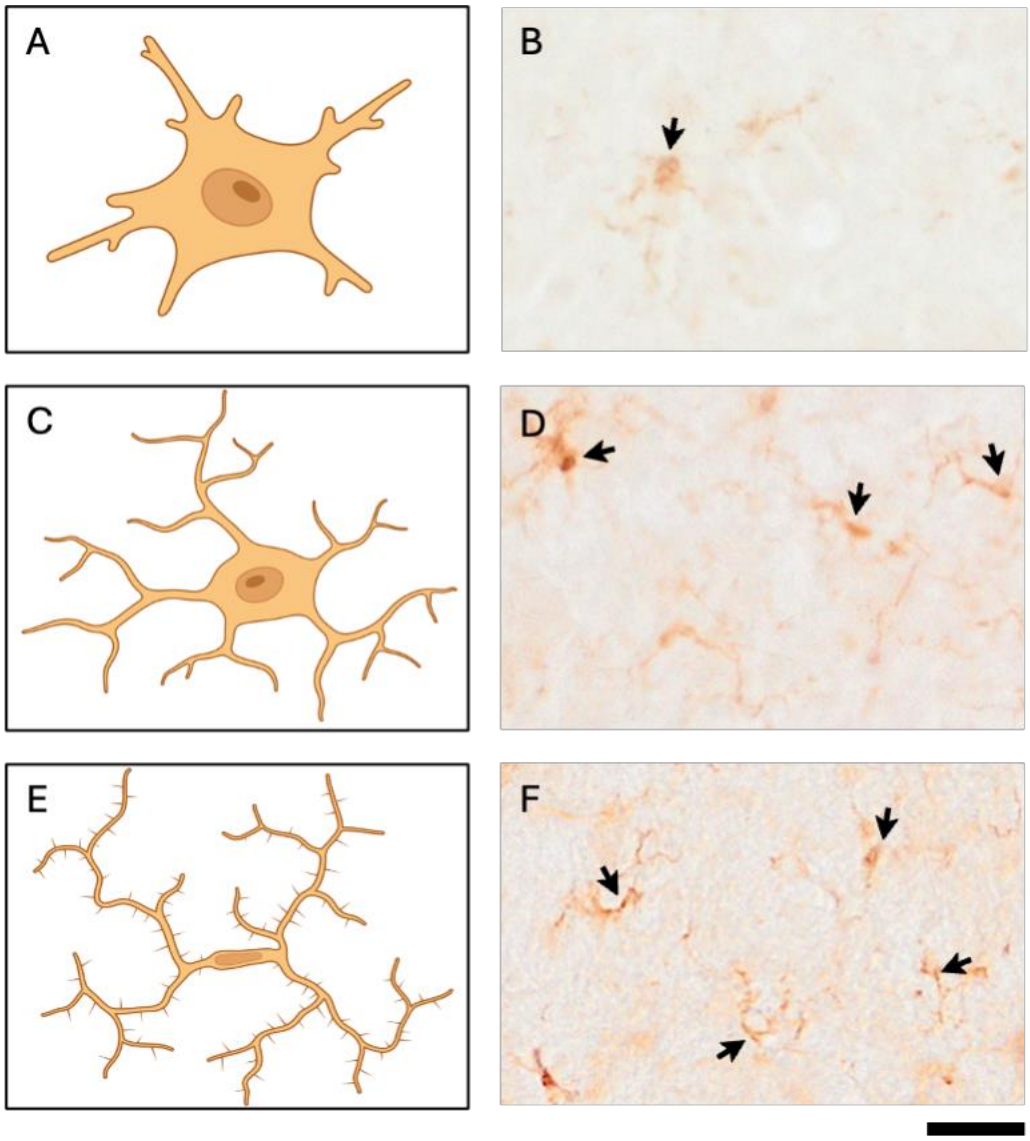

Figure 2. Comparison of microglial morphologies during development.

The amoeboid microglia shape, illustrated in A, is characterized by a circular large cell body and little-to-no processes. The amoeboid microglia are observed in the earliest age examined, 5-week-old normal subjects, shown in B. The transitional shape, illustrated in C, is seen in older animals, such as the 10-week and 14-week-old subjects, as observed in D. The ramified shape, illustrated in E, is characterized by elongated cell bodies and extended processes with branching. Adult ramified microglia are observed in F. Black arrows identify microglia cell bodies. Scale bar = 25µm

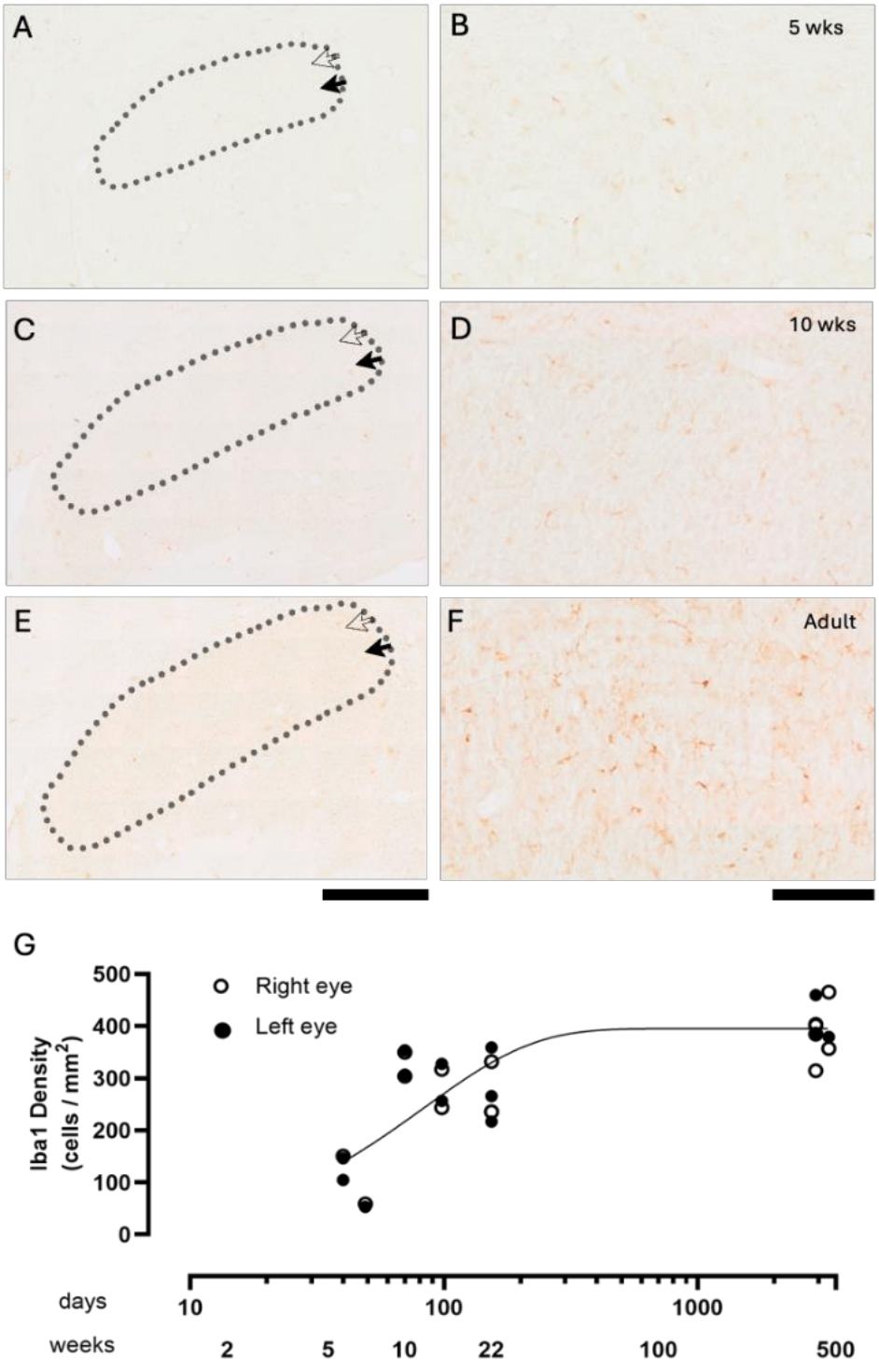

Figure 3. Development of Iba1-labeled microglia in the LGN

Low magnification images of the dLGN (dotted boundary) are displayed on the left. High magnification images are displayed on the right. At 5 weeks of age, Iba1 immunoreactivity throughout layer A (unfilled arrow) and layer A1 (filled arrow) was low. At low (A) and high (B) magnification, very few Iba1-positive microglia were evident. There was an increase in Iba1 immunolabeling from 5 to 10 weeks of age (C and D), and a further increase into adulthood (E and F). The Iba1-positive cell density was calculated for layers serving the right eye (unfilled circles) and the left eye (filled circles) of each animal. This quantification revealed a steady increase up to 22 weeks old, when they reached near adult levels. Data in BG were fit with an exponential plateau curve. Scale bar = 1mm (A, C, and E); 125 μm (B, D, and F).

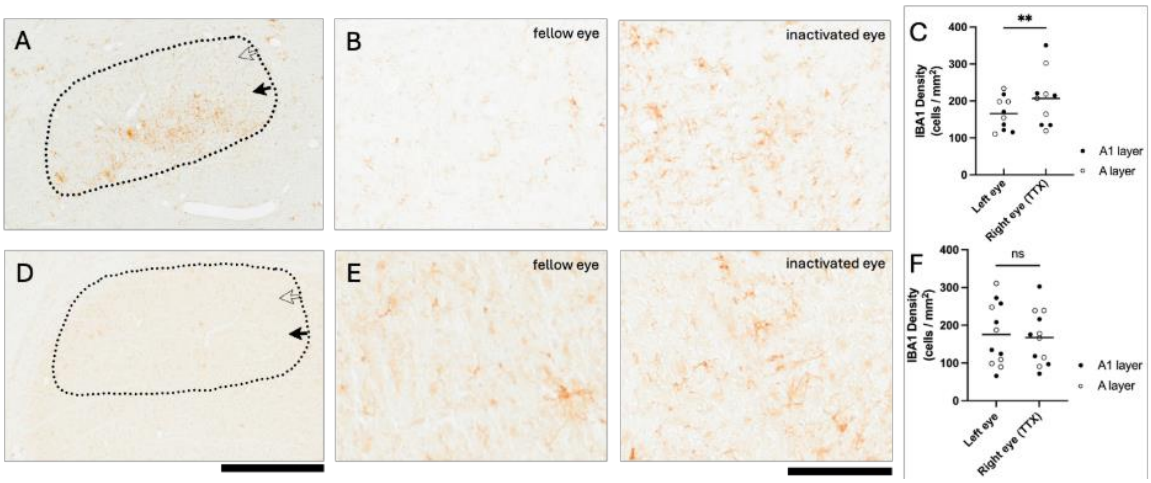

Figure 4. Increased Iba1 immunolabeling in the LGN after MI

Low magnification images of the dLGN (dotted boundary) at 4 weeks (A) and 22 weeks (D) are displayed. High magnification images of the fellow eye and inactivated eye dLGN layers at 4 weeks (B) and 22 weeks (E) are displayed. Iba1 immunoreactivity at 4 weeks was elevated within inactivated-eye layers (B). Iba1-positive cell density was statistically significantly higher within inactivated-eye layers compared to normal eye layers in a combined dataset of 4 and 6 weeks old (C) (Wilcoxon matched-pairs signed rank test; $p < 0.05$). There was no clear change in Iba1 immunoreactivity at 22 weeks (E), and Iba1-positive cell density was not significantly higher within the inactivated eye-layers compared to the normal eye layers (F) (Wilcoxon matched-pairs signed rank test; $p > 0.05$). Unfilled and filled circles represent A and A1 layers, respectively. Scale bars = 1mm (A, D); 125 µm (B, E). Double asterisk indicates statistical significance less than 0.01.

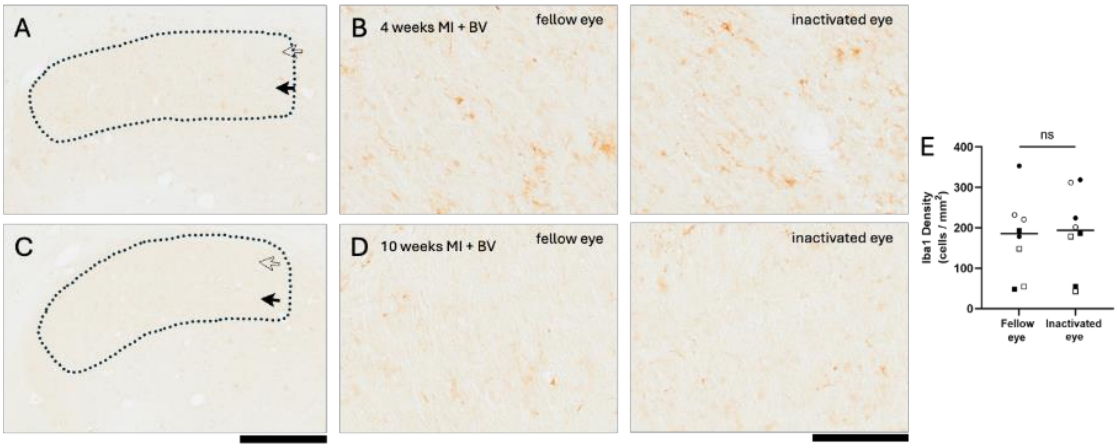

Figure 5. Durability of elevated Iba1 labeling

Low magnification images of the dLGN (dotted boundary) at 4 weeks MI + BV (A) and 10 weeks MI + BV (C) are displayed. High magnification images of the fellow eye and inactivated eye dLGN layers of both groups (B and D) are displayed. Elevated Iba1 immunolabeling does not persist following a period of MI at 4 weeks of age (B). Iba1-positive cell density (E) was not significantly higher within inactivated eye-layers compared to the normal eye layers in a combined dataset of both groups (Wilcoxon matched-pairs signed rank test; $p > 0.05$). Unfilled and filled circles represent 4 weeks MI + BV A and A1 layers, respectively. Unfilled and filled squares represent 10 weeks MI + BV A and A1 layers, respectively. Scale bar = 1mm (A, C); 125 µm (B, D).

Figure 6. MD does not alter Iba1 labeling

MD at 8 weeks does not increase Iba1 immunolabeling. A low magnification image of the dLGN (dotted boundary) at 8 weeks is displayed in A. There was no apparent difference in Iba1 labelling between normal and deprived LGN layers (B). Quantification of Iba1-positive cell density (C) revealed no statistically significant difference between the normal and deprived layers (Wilcoxon matched-pairs signed rank test; $p > 0.05$). Unfilled and filled circles represent A and A1 layers, respectively. Scale bar = 1mm (A); 125 µm (B).