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(Article begins on next page)

HARVARD UNIVERSITY
Graduate School of Arts and Sciences



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presented by Allison Rosen Bialas

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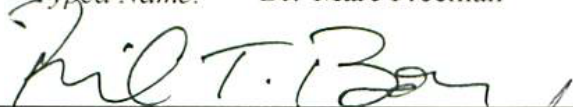
Typed Name: Dr. Michela Fagiolini

Signature: _____

Typed Name: Dr. Thomas Schwarz

Signature: _____

Typed Name: Dr. Marc Freeman



Dr. Richard Born, Program Head

Date: April 29, 2013



Dr. David Lopes Cardozo, Director of Graduate Studies

**Initiating Complement-dependent Synaptic Refinement: Mechanisms of Neuronal C1q
Regulation**

A dissertation presented

By

Allison Rosen Bialas

To

The Division of Medical Sciences

In partial fulfillment of the requirements

For the degree of
Doctor of Philosophy

In the subject of

Neuroscience

Harvard University

Cambridge, Massachusetts

April 2013

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Initiating Complement-dependent Synaptic Refinement: Mechanisms of Neuronal C1q Regulation

Immune molecules, including complement proteins, C1q and C3, have emerged as critical mediators of synaptic refinement and plasticity. Complement proteins localize to synapses and refine the developing retinogeniculate system via C3-dependent microglial phagocytosis of synapses. Retinal ganglion cells (RGCs) express C1q, the initiating protein of the classical complement cascade, during retinogeniculate refinement; however, the signals controlling C1q expression and function remain elusive. RGCs grown in the presence of astrocytes significantly upregulated C1q compared to controls, implicating an astrocyte-derived factor in neuronal C1q expression. A major goal of my dissertation research was to identify the signals that regulate C1q expression and function in the developing visual system. In this study, I have identified transforming growth factor beta (TGF- β), an astrocyte-secreted cytokine, as both necessary and sufficient for C1q expression in RGCs through an activity-dependent mechanism. Specific disruption of retinal TGF- β signaling resulted in a significant reduction in the deposition of C1q and downstream C3 at retinogeniculate synapses and significant synaptic refinement defects in the retinogeniculate system. Microglia engulfment of RGC inputs in the lateral geniculate nucleus (LGN) was also significantly reduced in retinal TGF β RII KOs, phenocopying the engulfment defects observed in C1q KOs, C3 KOs, and CR3 KOs. Interestingly, in C1q KOs and retinal TGF β RII KOs, microglia also failed to preferentially engulf less active inputs when retinal activity was manipulated, suggesting that retinal activity and TGF- β signaling cooperatively regulate complement mediated synaptic refinement. In support of this hypothesis, blocking spontaneous activity in RGC cultures significantly reduced C1q upregulation by TGF- β . Moreover, manipulating spontaneous retinal activity in vivo modulated C1q expression levels in RGCs and C1q deposition in the LGN. Together these findings support a model in which retinal activity and

TGF- β signaling control expression and local release of C1q in the LGN to regulate microglia-mediated, complement-dependent synaptic pruning. These results provide mechanistic insight into synaptic refinement and, potentially, pathological synapse loss which occurs in the early stages of neurodegenerative diseases concurrently with aberrant complement expression and reactive gliosis.

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DEDICATION

This dissertation is dedicated to my parents, Robert and Marilyn Rosen. They encouraged my love of science, taught me to never shy away from a challenge, and emphasized the importance of always making time for the people and things I love most. I am so blessed to have them in my life as a constant source of love and enthusiastic support.

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celebrate my PQE, they never hesitated to do anything they could to encourage me and show me how proud they are of me. I could not have made it through graduate school without them.

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GLOSSARY OF TERMS

RGC: Retinal ganglion cell

LGN: Lateral geniculate nucleus

dLGN: Dorsal lateral geniculate nucleus

TGF- β : Transforming growth factor-beta

TGF β RII: Transforming growth factor-beta receptor II

C1q: Initiating protein of the classical complement cascade

C3: Complement protein 3

CR3: Complement receptor 3

TTX: Tetrodotoxin

CPP: NMDA receptor blocker

NBQX: AMPA receptor blocker

MHC I: Major Histocompatibility Complex Class I

NP1: Neuronal Pentraxin 1

CTB: cholera toxin beta subunit

ASD: Autism Spectrum Disorders

nAChR: Nicotinic acetylcholine receptor

CHAPTER 1:

Introduction

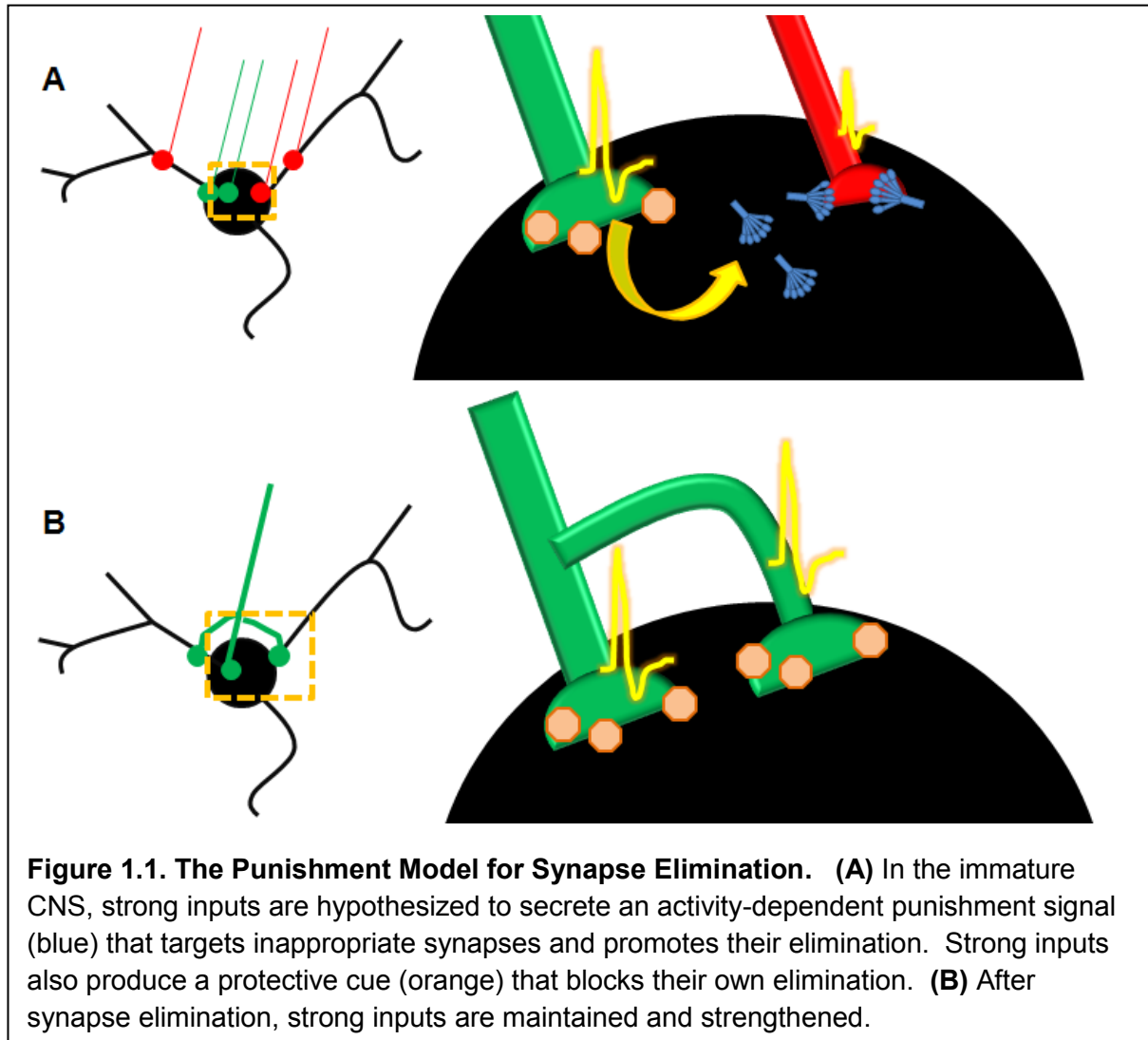
Immune molecules have emerged as critical mediators of CNS development; however, the mechanisms regulating the expression and function of immune molecules in the CNS remain a mystery. As a site of immune privilege, the developing CNS is an unexpected place to encounter the expression and function of many immune system genes. Previous reports had characterized upregulation of immune system components primarily during injury and disease. More recent studies have revealed that immune molecules are not only present in the normal CNS, but also, they are functionally relevant for brain development (Bjartmar et al., 2006; Huh et al., 2000; Stevens et al., 2007). Processes ranging from programmed cell death, neurite outgrowth, and polarization to synapse development, function, and plasticity can be modulated at some level by proteins traditionally associated with the immune system. In most of these processes, immune molecules execute functions in the CNS that are very different from their conventional immune system roles. One intriguing exception is the classical complement cascade. Proteins from this innate immune system pathway are traditionally responsible for tagging and eliminating pathogens and apoptotic debris in the immune system. Surprisingly, the nervous system has adopted this pathway to tag and eliminate inappropriate synapses in the developing CNS. Recent work has shown that complement proteins localize to synapses during development and promote their elimination by the resident immune cells and phagocytes of the brain, microglia (Schafer et al., 2012a; Stevens et al., 2007).

Thus, the CNS and the immune system use similar mechanisms to eliminate unwanted and potentially harmful material. In the immune system, failure to clear pathogens can result in cell death and widespread infection. In the CNS, failure to prune inappropriate connections results in dysfunctional circuitry and potentially hyperexcitability and seizures. Indeed, several immune system components that have been implicated in normal brain development, including complement proteins, have been linked to neurodevelopmental disorders such as Autism Spectrum Disorders (ASD) and epilepsy (Aronica et al., 2007; Vargas et al., 2005; Voineagu et al., 2011). Despite the importance of these molecules for CNS function, very little is known

about the molecular mechanisms regulating the expression and function of immune system molecules in the CNS. For my dissertation research, I have investigated the cellular and molecular components required for the developmental process of complement-dependent synapse elimination. This work provides insight into the normal molecular mechanisms driving this process in development and suggests how these mechanisms may go awry in neurodevelopmental disorders, such as ASD and epilepsy.

Mechanisms Driving Synaptic Refinement in the CNS

One of the developmental processes utilizing immune system molecules is synaptic refinement, which is the process by which circuits become mature and precise. Synaptic refinement is an activity-dependent process encompassing the strengthening and maturation of some synapses and the weakening and elimination of others. This process has been extensively studied in the peripheral nervous system at the neuromuscular junction where motorneurons compete for synaptic territory on muscle fibers. Activity-dependent synaptic competition has also been studied in the brain, most notably in the dorsal lateral geniculate nucleus in the developing visual system (Boulanger and Shatz, 2004; Hua and Smith, 2004; Huberman, 2007; Katz and Shatz, 1996; Sanes and Lichtman, 1999). The maturation of nervous system circuitry is driven in part by Hebb's rule, which states that synapses which successfully and reproducibly evoke responses in the postsynaptic cell are maintained and mature (Butts et al., 2007; Hebb, 1949). CNS circuits initially are connected in an imprecise manner, however, and contain many inappropriate connections that must be removed for proper brain function. It is unclear why this "overwiring" occurs in the vertebrate nervous system, but one idea is that this process is a product of evolution (Lichtman and Coleman, 2000). In simpler invertebrate nervous systems, such as *C. Elegans*, neurons are unique and simple circuits of only one or two neurons regulate most functions. In more complex nervous systems, however, a function such as photosensitivity requires an entire visual system comprised of thousands of

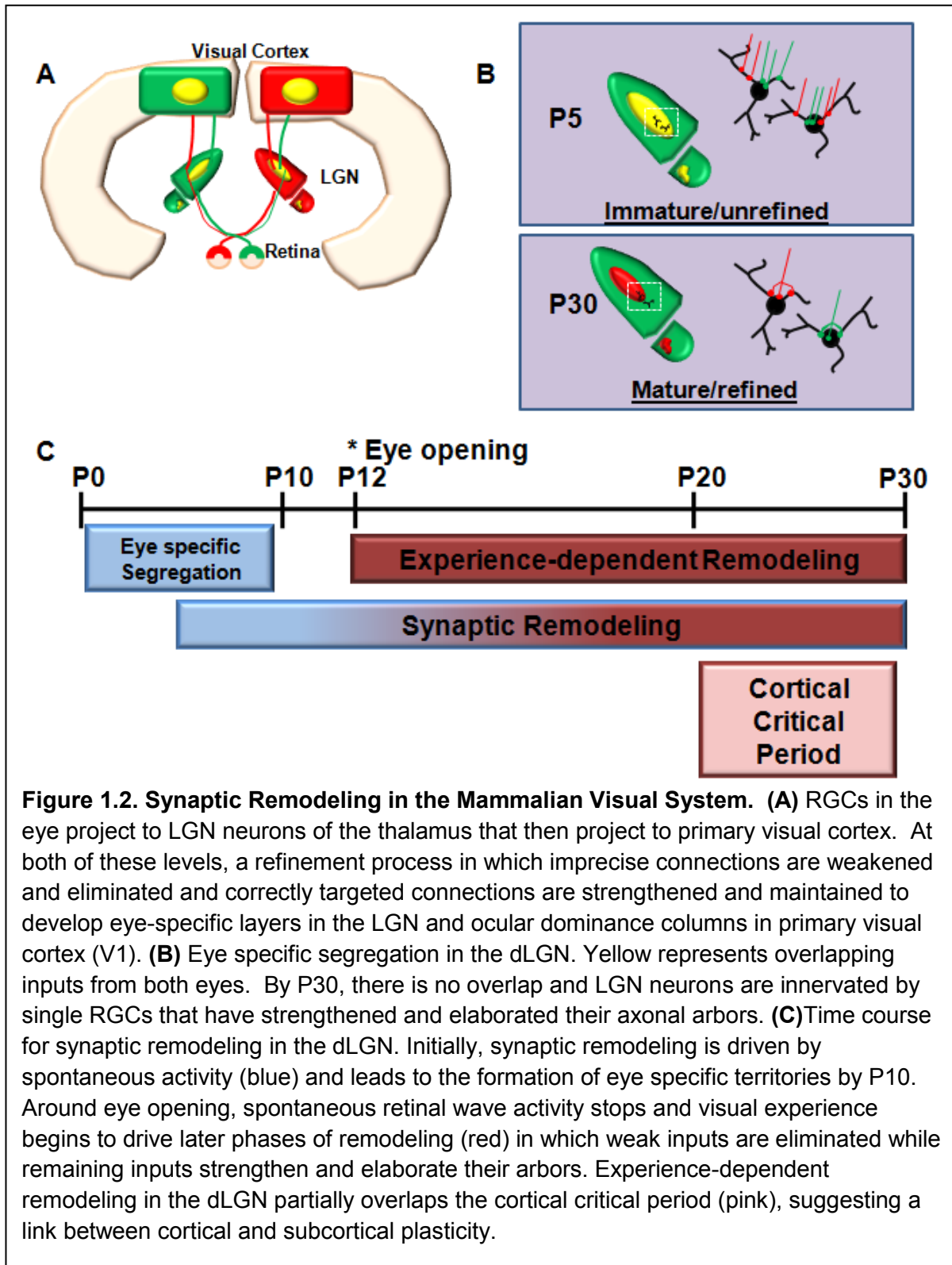


neurons and synapses, while a single neuron is charged with this function in the worm. Thus, the vertebrate nervous system can be thought of as having a large degree of redundancy compared to the invertebrate. This redundancy occurs both pre- and post-synaptically, therefore if we think of the mouse retinogeniculate system as evolving from a simpler visual system in which each neuron only has one postsynaptic target, we can imagine that each presynaptic neuron, or for example a retinal ganglion cell (RGC), is equally capable of innervating all postsynaptic cells, for example lateral geniculate nucleus (LGN) relay neurons. As a result, many RGCs project to the same LGN relay neurons and overlapping circuits are established. Of course, the generation of many identical circuits confers no advantage to the mouse over the worm, but refinement of these circuits to generate thousands of unique connections results in the ability to discern objects and colors in space, which can help vertebrates to find food and avoid predators. This overwiring allows for the animal to test out multiple circuits to determine which are the most appropriate based on its environment and sensory experience. Given the importance of synaptic refinement in generating the complex circuitry in the vertebrate nervous system, a longstanding question in neurobiology is *what determines which synapses will be eliminated versus maintained in the developing brain?*

One model for synapse elimination, based on the pioneering work of Lichtman et al. at the NMJ, is the punishment model (Jennings, 1994; Lichtman and Colman, 2000) (Figure 1.1). This model proposes that this developmental synaptic competition is mediated by the production of two activity-dependent signals: a punishment signal and a protective signal (Jennings, 1994; Lichtman and Colman, 2000). Strong connections that successfully fire the postsynaptic cell are proposed to secrete an activity-dependent punishment signal that eliminates nearby inappropriate connections, while concurrently expressing a protective signal that prevents elimination of these strong inputs (Figure 1.1A). It is not known whether this model is correct or relevant to the developing CNS and the identity of the potential punishment and protective signals have not been identified.

The mouse visual system has served as an excellent model for addressing questions related to how specific synapses are eliminated in the postnatal brain. A classic example of synaptic pruning in the CNS occurs in the postnatal visual system at the retinogeniculate synapse between retinal ganglion cells (RGCs) and the thalamic relay neurons of the dorsal lateral geniculate nucleus (dLGN) (Hooks and Chen, 2006; Jaubert-Miazza et al., 2005; Kano and Hashimoto, 2009) (Figure 1.2A). In adult mammals, after retinogeniculate refinement, RGC axons terminate in distinct, non-overlapping eye-specific domains in the dLGN (Figure 1.2A). In the neonatal retinogeniculate system (P0-P10), however, each dLGN relay neuron initially receives many weak, immature RGC inputs (Figure 1.2B) from both contralateral and ipsilateral eyes. RGC inputs in the dLGN segregate into eye specific territories by P10 such that each dLGN relay neuron is only innervated by immature inputs from a single eye. Within eye specific territories, synaptic refinement or remodeling continues to occur during a period spanning eye opening (P10-P30 in mouse). During this period, certain inputs strengthen and mature while others that fail to strengthen are eliminated such that by P30 each dLGN neuron receives stable inputs from an average of 1-2 RGC axons (Chen and Regehr, 2000; Hooks and Chen, 2006; Jaubert-Miazza et al., 2005) (Figure 1.2C).

This later stage of synapse elimination is driven initially by spontaneous retinal wave activity that persists until P15 (Demas et al., 2003), and after by visual experience (Figure 1.2C) (Hooks and Chen, 2006; Huberman, 2007). Interestingly, later phases of synaptic remodeling after eye opening are significantly disrupted if mice are placed in the dark, deprived of visual experience, after one week of vision (after P20). This late dark rearing can actually revert the dLGN to a less refined state: synapses that have strengthened on relay neurons weaken and additional immature RGC inputs can again be detected innervating relay neurons after 7-12 days of visual deprivation (Hooks and Chen, 2006). Visual experience also regulates the maturation of the visual cortex which acquires exquisite organization as it matures. The period



of sensory experience dependent remodeling in the dLGN closely matches the timing of the critical period for plasticity in visual cortex, suggesting that these processes may be linked (Hooks and Chen, 2006, 2007). Complex cortical characteristics develop during the critical period including visual receptive fields, as well as ocular dominance and orientation selectivity columns (Hensch, 2004; Sur & Rubenstein, 2005). Disruption of neuronal firing at any of these stages of visual development has profound effects on synaptic connectivity, yet surprisingly little is known about the molecular mechanisms that drive the elimination of some synapses and the maintenance and strengthening of others at each of these phases.

Retinal Wave Activity and Synaptic Refinement

Pioneering studies have revealed that synaptic refinement is driven by correlated bursts of action potentials in RGCs that initiate in random RGCs and then stream across the retina. These spontaneous “waves” of activity start around birth in the rodent and continue until eye opening. Waves occur in stages and each stage has different properties thought to drive different aspects of visual development. In the rodent, stage I waves begin before birth and can be partially blocked by nicotinic acetylcholine receptor (nAChR) inhibitors (Bansal et al. 2000). Stage II waves begin around birth and continue for the first two postnatal weeks during the retinogeniculate refinement period (Bansal et al. 2000, Syed et al. 2004). These waves are driven by acetylcholine released from starburst amacrine cells (Feller et al. 1996, Zheng et al. 2004). Desensitizing nAChRs with intraocular injection of the cholinergic agonist epibatidine disrupts eye stage II waves (Penn et al., 1998), and mice lacking $\beta 2$ -subunits of neuronal nAChRs ($\beta 2nAChR^{-/-}$) lack stage II retinal waves and exhibit uncorrelated retinal spiking (Bansal et al., 2000; Muir-Robinson et al., 2002). Conversely, intraocular injections of pharmacological agents that elevate intracellular levels of cAMP, such as forskolin or dbcAMP, increase wave activity within the retina (Stellwagen et al., 1999). Blocking nAChRs pharmacologically or genetically results in a failure to segregate into eye specific territories

(Grubb et al., 2003; Huberman, 2007). Intriguingly, increasing wave activity by raising cAMP levels also disrupts eye specific territories such that the more active eye occupies more dLGN territory than the less active eyes (Huberman, 2007; Stellwagen and Shatz, 2002). Stage III waves occur later and last until eye opening. These waves are thought to drive the specification of retinal circuits into ON-OFF and direction selective classes as well as the later stages of synaptic remodeling in the dLGN during which innervations of dLGN relay neurons is reduced 1-2 strong RGC inputs (Hooks and Chen, 2006; Huberman, 2007) (Figure 1.2).

Although manipulating retinal wave activity has clear effects on eye specific segregation and synaptic refinement, uncertainty remains over whether activity plays an instructive or permissive role in refinement and what aspects of activity are most important for driving refinement (Blankenship and Feller, 2010; Feller, 2009). Early studies showed that spiking in dLGN neurons is required for eye-specific segregation by blocking dLGN activity with intracranial infusions of TTX and disrupting eye specific segregation (Sretavan et al., 1988). The first evidence that activity might be instructive came from studies in which one eye was removed before eye specific segregation and RGC axons from the intact eye continued to innervate the entire dLGN (Morgan and Thompson, 1993; Sretavan and Shatz, 1986). The effects of increasing retinal wave activity with cAMP on eye specific segregation further support an instructive role for activity in the retina (Stellwagen and Shatz, 2002). The relative levels of spontaneous activity in the two retinas, rather than a required threshold level of retinal activity, dictate how much LGN territory axons from a given RGC will occupy. Results from $\beta 2nAChR^{-/-}$ mice also support an instructive role for the patterns of activity in the retina since RGCs in these mice still exhibit firing but not in a correlated fashion (Bansal et al., 2000). Thus, the importance of neuronal activity in retinogeniculate refinement is clear; however, *the molecules required for the pruning of retinogeniculate synapses and the mechanism linking differences in neural activity with the removal of a synapse remain elusive.*

The Role of Activity-dependent Gene Transcription in Synapse Development

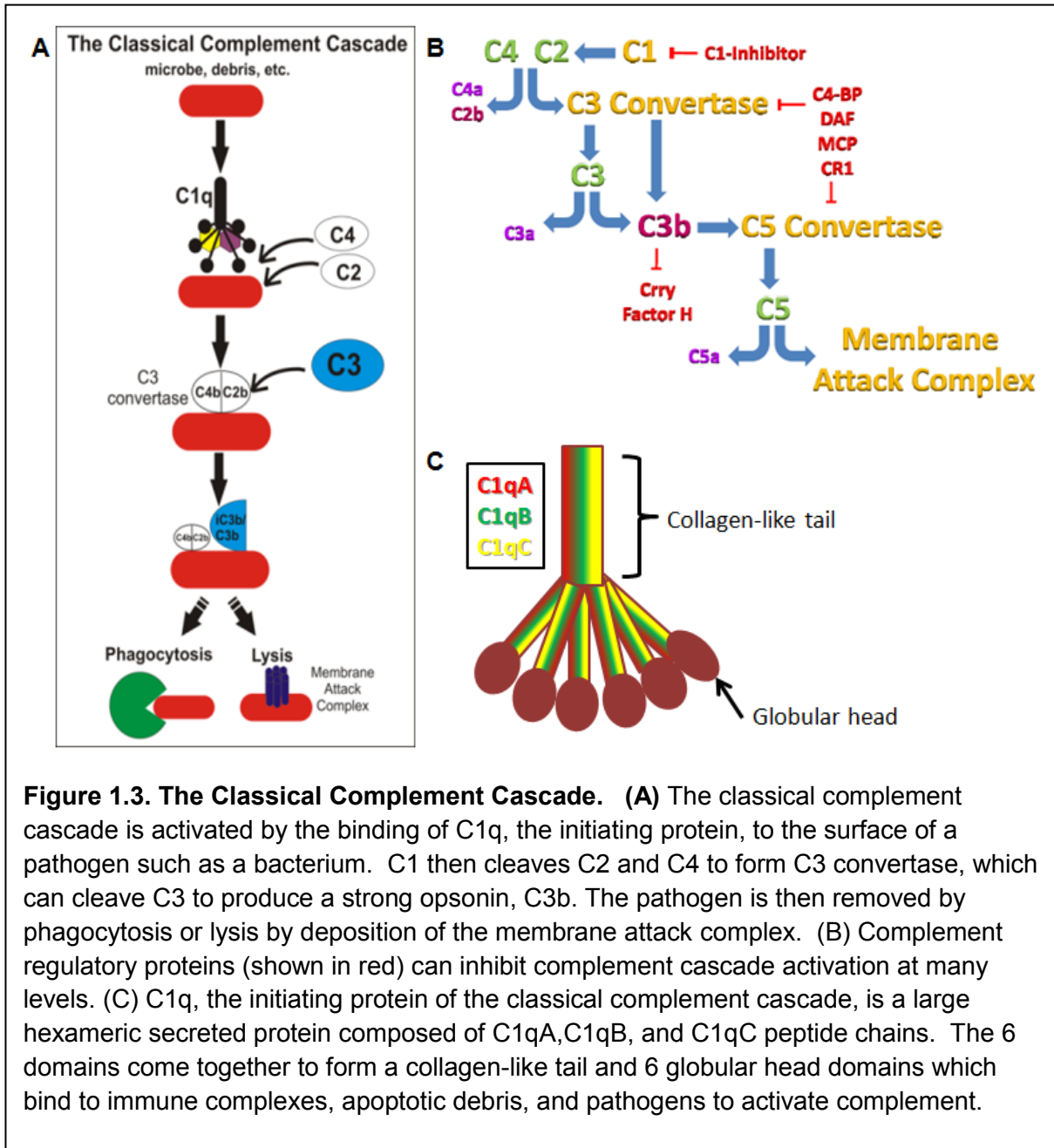
How can neuronal activity be translated to the physical removal of inappropriate synapses? One possible mechanism is that activity drives the expression of genes encoding proteins that execute synapse elimination. Activity-dependent gene transcription is initiated when neurons fire action potentials, resulting in calcium influx through voltage gated calcium channels or glutamate receptors (Greer and Greenberg, 2008; Sheng et al., 1990). Rises in intracellular calcium can activate pathways such as the Ras/MAPK pathway, the calcium/calmodulin-dependent protein kinases, the phosphatase calcineurin, and Rac GTPases (Greer and Greenberg, 2008). Differences in patterns of activity preferentially drive certain pathways and consequently activate the transcription of a specific subset of activity-dependent genes. Several hundred genes have been identified as calcium- and activity-dependent and have been implicated in many stages of nervous system development. Surprisingly, however, only a handful of activity-dependent genes have been implicated in synapse elimination.

Interestingly, two of these genes encode components of the innate immune system, MHC class I and neural pentraxins. MHC class I molecules are expressed on the surface of every cell in the body and present peptides from within the cell to T cells. Healthy cells are ignored while infected cells can be identified and targeted for removal. In the immune system, pentraxins are acute phase proteins which can recognize pathogens and help to activate complement and phagocytic cells to clear these pathogens. Both MHC I and neural pentraxins are expressed in an activity-dependent manner in the retinogeniculate system and mice deficient in MHC I signaling or neural pentraxins exhibit defects in eye specific segregation in the dLGN (Bjartmar et al., 2006; Corriveau et al., 1998b; Huh et al., 2000). However, it remains unclear how these molecules lead to the specific targeting and removal of inappropriate synapses.

The Classical Complement Cascade Expression and Function in CNS

The classical complement cascade is a branch of the innate immune system that traditionally is responsible for tagging or opsonizing apoptotic debris or pathogens to promote rapid elimination of this material from the body. Although most complement components are synthesized by hepatocytes, many other cells in the body locally produce complement components at low levels and can be induced to express higher levels of complement during inflammation. Additional functions for complement components have also been identified in the immune system during B-cell proliferation (Ambrus et al., 1990; Peters et al., 1988), in liver regeneration (Strey et al., 2003), during neurogenesis (Rahpeymai et al., 2006), and recently in synaptic refinement in the CNS (Stevens et al., 2007). In the CNS, some of the over forty complement proteins are synthesized by neurons, microglia, astrocytes, and oligodendrocytes (Alexander et al., 2008b; Barnum, 1995; Gasque et al., 2000).

The complement cascade includes three branches: the classical pathway, the alternative pathway, and the lectin pathway (Degn et al., 2007; Fujita, 2002; T.J. Kindt, 2006). The proteins that have been implicated in synaptic refinement are components of the classical pathway. In this classical branch, C1, composed of the proteins C1q, C1r, and C1s, initiates the classical complement cascade when it binds to immune complexes and coats (opsonizes) dead cells, pathogens, or debris. The C1 complex is secreted by circulating macrophages in the bloodstream and is only activated when C1q binds to the surface of a target. C1q is the recognition and binding domain of C1 and is a large hexameric protein composed of 3 peptide chains, C1qA, B, and C (Kishore and Reid, 2000). C1q structurally consists of a collagen-like domain and six globular head domains which can recognize and bind immune complexes, apoptotic debris, or pathogens (Eggleton et al., 1998; Kishore and Reid, 2000). Binding of C1q causes activation of the two associated enzymes, C1r and C1s, that cleave downstream



components C2 and C4, leading to formation of C3 convertase. This enzyme cleaves inert C3 protein into activated C3a (an anaphylatoxin) and C3b (a potent opsonin) fragments, which recruit phagocytic cells and tag material for phagocytosis, respectively (Klos et al., 2009; Zhou, 2011). Macrophages and other phagocytic cells express receptors for C3b, including complement receptor 3 (CR3), allowing these cells to engulf any particles or cells coated with this protein. Also, C3b initiates formation of the membrane attack complex (MAC). This complex of proteins can lyse cells when it is deposited in the cell membrane (T.J. Kindt, 2006).

While these complement proteins are responsible for eliminating pathogens and apoptotic debris from the body, another class of complement proteins, known as complement regulatory proteins, is responsible for protecting healthy cells from damage during complement activation. These regulatory proteins can be membrane bound or secreted and prevent cascade activation at several different levels of the cascade (Figure 1.3) (Kraus et al., 2006; Sahu and Lambris, 2000). C1-inhibitor irreversibly binds C1r and C1s to inhibit spontaneous activation of the complement cascade (Gasque et al., 2000). Downstream, secreted inhibitors C4 binding protein (C4-BP) and Factor H interfere with C3 convertase formation and function. Inhibitors of C3 and C5 formation can also be membrane bound (Elward and Gasque, 2003; Gasque et al., 2000). These inhibitors including Crry and CSMD1, which prevents C3 and C4 deposition, DAF and CR1, which inhibit C3 and C5 convertases (Iida and Nussenzweig, 1981), and a co-factor for C3 and C4 inactivation known as MCP (Elward and Gasque, 2003). The expression and function of complement regulatory proteins in the brain are not well understood; however, in the immune system these regulatory proteins are critical for homeostatic regulation of complement cascade function and protection against aberrant complement mediated elimination in health and disease.

The Classical Complement Cascade Mediates Retinogeniculate Refinement

Recent work has implicated this classical complement cascade in synaptic refinement in the retinogeniculate system. This result was surprising since previously reports of classical complement

cascade proteins in the CNS were associated with injury and disease. Perhaps even more surprising was the finding that C1q expression was detected in developing neurons of the retina (Stevens et al., 2007). Microglia, the resident phagocytic immune cell of the brain, are the main source of C1q in the CNS; however in the postnatal retina, C1q is enriched in developing RGCs during the peak of synaptic refinement, suggesting C1q as a candidate activity-dependent gene regulating the development and refinement of retinogeniculate synapses. Indeed, complement deficient mice (C1q or C3 KO) exhibit sustained defects in CNS synapse elimination, as shown by the failure to segregate into eye-specific territories and the retention of multi-innervated LGN neurons (Stevens et al., 2007); however, many mechanistic questions remain: 1) How do secreted complement proteins regulate the elimination of specific synapses at the right time and place? 2) What are the contribution and function of neuronal C1q in retinogeniculate synapse elimination? 3) What are the signal(s) that regulate and restrict neuronal C1q expression in the postnatal retina? These questions have been the focus of my dissertation research.

The immune system continues to provide important mechanistic insight into these questions. Based on published and ongoing studies in the lab, the overarching hypothesis is that the classical complement cascade regulates the selective elimination of specific synapses using similar activation and regulatory pathways as seen in the immune system (Figure 1.3). In the innate immune system, C1q and C3 opsonize unwanted cells and debris for rapid removal by macrophages. If the complement cascade were working in its traditional manner, in the developing brain, I hypothesize that activity-dependent release of soluble C1q from active synapses would lead to local C3 cleavage to opsonize neighboring 'weaker' synapses by C1q and/or C3 (iC3b). In response, phagocytic cells would be recruited to the area to engulf the tagged material. In the CNS, microglia express many of the same phagocytic receptors as macrophages, including complement receptor 3, CR3, suggesting that these cells could work to clear inappropriate synapses by complement-dependent engulfment. Recent work has shown

that, indeed, C1q and C3 localize to retinogeniculate synapses (Stevens et al., 2007) and microglia engulf retinogeniculate projections during the refinement period in a complement-dependent manner (Schafer et al., 2012a). However, the mechanisms regulating this process are unknown. Given that C1q is the initiating protein of the classical complement cascade and that C1q is developmentally expressed in RGCs during the retinogeniculate refinement period, *I hypothesize that C1q also initiates complement-dependent synapse elimination. Thus, understanding how C1q is regulated and functions at CNS synapses is critical and the focus of my thesis.*

Microglia: Novel Mediators of Synapse Development

In the immune system, macrophages express phagocytic receptors including C3 receptors (CR3/CD11b), which promote engulfment of complement-tagged material (Carroll, 2004; Gasque, 2004; van Lookeren Campagne et al., 2007). In the healthy postnatal CNS, microglia are the primary immune and phagocytic cells and the only cells to express CR3 (Bobak et al., 1987; Graeber, 2010; Guillemin and Brew, 2004; Ransohoff and Perry, 2009; Schafer et al., 2012a). Microglia are myeloid-derived cells and, until recently, they were thought to develop from peripheral macrophages that entered and colonized the brain throughout development and into adulthood. We now know that most microglia arise from a unique pool of yolk-sac derived myeloid progenitors and migrate into the CNS by embryonic day E10.5 (Ginhoux et al., 2010). Following migration into the CNS, microglia undergo a slow maturation and differentiation process that persists through late postnatal development (Ransohoff and Perry, 2009). Microglia start as highly phagocytic, amoeboid cells and maintain this morphology through the period of programmed cell death when they actively remove debris and dead cells (Ferrer et al., 1990). By the first postnatal week in mouse, these cells develop branches which are used to continually survey the environment and downregulate their phagocytic receptors (Ling and Wong, 1993). In the event of injury or infection, microglia quickly adopt a more

classically “reactive” state and migrate to the site of injury where they shield the injury site, engulf pathogenic material or debris, and release many cytokines and chemokines (Davalos et al., 2005; Nimmerjahn et al., 2005). While past work had focused on the role of microglia during disease, recent studies have revealed dynamic interactions between microglia and synapses in the healthy brain, particularly during developmental synaptic pruning (Schafer et al., 2012c; Tremblay et al., 2011).

The process of synaptic refinement occurs corresponding to a specific time in microglial development when these cells have acquired processes but remain phagocytic (Schafer et al., 2012a). These special process-bearing phagocytic microglia have been observed in the dLGN and several other postnatal brain regions including hippocampus, cerebellum, and olfactory bulb (Dalmau et al., 1998; Perry et al., 1985), suggesting a role in synaptic remodeling, but until recently, the function of microglia in normal brain had remained a mystery.

Microglia Sculpt Postnatal Neural Circuits in an Activity- and Complement-dependent Manner

Since Microglia express an array of phagocytic receptors, including complement receptor 3 (CR3/CD11b/CD18) that could mediate the engulfment of complement-tagged material, these cells were a prime candidate to execute complement-dependent synapse elimination. Using the mouse retinogeniculate system as a model, microglia were found to engulf RGC presynaptic inputs during the peak of retinogeniculate refinement in the developing dLGN (Schafer et al., 2012a). Moreover, genetic or pharmacological disruptions in microglia-mediated engulfment during the postnatal period resulted in sustained functional deficits in eye-specific segregation. Furthermore, microglia-mediated engulfment of synaptic inputs was dependent upon signaling between CR3, expressed specifically by microglia, and complement component C3, which is present at high levels and localizes to synapses in the postnatal dLGN (Schafer et al., 2012a).

Interestingly, microglia-mediated engulfment was found to be regulated by neuronal activity. When competition between RGC inputs originating from the two eyes was enhanced by monocular injection of TTX or forskolin, microglia preferentially engulfed inputs from the eye with reduced neuronal activity relative to the other eye (Schafer et al., 2012a). These data are consistent with previous work demonstrating a decreased synaptic territory of the “weaker” inputs and increased territory of “stronger” inputs within the dLGN (Cook et al., 1999; Del Rio and Feller, 2006; Hooks and Chen, 2006; Huberman et al., 2008a; Penn et al., 1998; Stellwagen and Shatz, 2002). *Although it is not yet known how microglia target specific “weaker” synapses, complement is partially required for microglial engulfment, suggesting a link between neuronal activity and complement.*

Recent studies also suggest that microglia associate with postsynaptic elements during synaptic remodeling in the hippocampus and juvenile visual cortex, raising the question of whether microglia-dependent pruning is a global mechanism of synaptic remodeling in the CNS (Paolicelli et al., 2011b; Tremblay et al., 2010). Paolicelli et al. demonstrated a role for the fractalkine receptor (CX3CR1), expressed on the surface of microglia, in hippocampal synapse development and maturation. *Cx3cr1*^{KO} mice have a transient reduction in the number of microglia in the postnatal brain; thus, fractalkine signaling could interact with complement and other signals to regulate microglia-mediated developmental pruning by influencing microglia number or, possibly, recruitment to synaptic sites in the postnatal brain (Ransohoff and Stevens, 2011). In addition, recent electron microscopy studies in visual cortex have revealed significant changes in microglia dynamics in response to sensory experience (Tremblay et al., 2010), providing further evidence that microglia can sense changes in neuronal activity. Together these new findings raise several fundamental questions related to the underlying mechanisms of microglia- and complement-mediated pruning including how neural activity, complement, and microglia may interact to sculpt developing visual circuits

Other Roles for Microglia in the CNS

Microglia are well positioned to influence many nervous system functions in addition to synaptic remodeling. Fine microglial processes are dynamic surveyors of the extracellular milieu and contact neuronal cell bodies, synapses, and astrocytes (Nimmerjahn et al., 2005). Recent work has demonstrated repeatedly, however, that these glial cells are particularly important at synapses (Ransohoff and Stevens, 2011; Schafer et al., 2012c; Tremblay et al., 2011). Microglia have been implicated in synapse maturation (Roumier et al., 2004), synaptic remodeling (Paolicelli et al., 2011b; Schafer et al., 2012a), synaptic transmission (Coull et al., 2005; Pascual et al., 2012), and synaptic plasticity, including long-term potentiation (LTP) (Roumier et al., 2004) and synaptic scaling (Stellwagen and Malenka, 2006).

At developing synapses, in addition to helping to prune inappropriate connections, microglia also influence synaptic plasticity and maturation. Mice deficient in the microglia-expressed protein KARAP/DAP12, a 12 kDa transmembrane polypeptide associate with a variety of cell-surface receptors including MHC-I receptors (Lanier, 2001; Olcese et al., 1997; Lanier et al., 1998), show enhanced LTP as well as changes in glutamate receptor content, by physiology and biochemical methods. Specifically, expression of the AMPA receptor GluR2 subunit was decreased in the postsynaptic densities versus cell membrane fractions, demonstrating specific impairment of synaptic receptor accumulation (Roumier et al., 2004; Roumier et al., 2008). Additional studies have also supported a role for microglia in synapse maturation. Mice deficient in fractalkine signaling, *Cx3cr1*^{KO} mice, showed increased spine density, enhanced hippocampal long-term depression (LTD), and increased pentylenetetrazol (PTZ)-induced seizure susceptibility, consistent with immature circuitry (Paolicelli et al., 2011b). These mice have a transient decrease in microglial density in the hippocampus; however, abnormalities in microglia number and synapse density in postnatal *Cx3cr1*^{KO} mice returned to normal levels by adulthood, suggesting that the observed effects are likely a more general effect caused by reduced microglial density rather than a specific *Cx3cr1*-dependent effect.

Microglia also participate in the regulation of glutamatergic and GABAergic synaptic transmission, particularly after injury or inflammation (Coull et al., 2005; Pascual et al., 2012; Tsuda et al., 2003). Triggering microglia activation with Lipopolysaccharide (LPS), a bacteria-derived endotoxin, elevated AMPA receptor-mediated spontaneous EPSC frequency in organotypic hippocampal slices (Pascual et al., 2012). Microglia were required for this effect in that slices prepared from *PU.1* null mice, which lack lymphoid and myeloid lineage cells, showed no LPS-induced changes (McKercher et al., 1996; Pascual et al., 2012). Interestingly, this microglia-specific effect on EPSCs is thought to be indirect. Microglia release ATP in response to LPS, which is thought to bind P2Y1 receptors on astrocytes and mediate an increase in excitatory transmission via a previously described metabotropic glutamate receptor 5 (mGluR5)-dependent mechanism (Pascual et al., 2012). Activated microglia have also been linked to the development of allodynia after peripheral nerve injury. One mechanism for allodynia after injury or inflammation is the conversion of GABAergic synaptic transmission from hyperpolarizing (inhibitory) to depolarizing (excitatory) (Coull et al., 2003). Recent work has shown that microglia, activated in response to ATP released during nerve injury, contribute to this effect by releasing brain-derived neurotrophic factor (BDNF) (Coull et al., 2005). Consequently, BDNF downregulates the K^+ - Cl^- -cotransporter KCC2 via TrkB signaling on dorsal horn neurons and impairs neuronal Cl^- extrusion. Blocking microglial production of BDNF by RNAi can prevent this shift in GABA transmission and prevents allodynia (Coull et al., 2005). While both of these studies revealed a role for microglia in synaptic transmission in response to injury, it remains unclear if similar microglia-mediated mechanisms may modulate neurotransmission in the healthy brain.

Microglia continue to modulate synaptic plasticity and function in the mature CNS. *In vitro* studies have demonstrated that when either cultured microglia or conditioned media from cultured microglia was added to cortical or hippocampal slice cultures, NMDA-receptor mediated excitatory postsynaptic currents (EPSCs) were larger in amplitude and longer in

duration (Hayashi et al., 2006; Moriguchi et al., 2003). Microglia-secreted glycine and l-serine were required for this effect (Hayashi et al., 2006). Further supporting a role for microglia in adult plasticity, organotypic hippocampal slices prepared from adult (3 month) *Cx3cr1*^{KO} mice showed reduced LTP induction compared with WT littermates (Rogers et al., 2011). These mice also exhibited learning and memory defects in the Morris water maze and in contextual and cued fear conditioning (Rogers et al., 2011). Defects in adult neurogenesis have also been reported in these mice, suggesting that these observed phenotypes may be indirect effects of microglial deficits. *Taken together, these studies support an integral role for microglia in synapse development and function in the normal and diseased brain; however, the molecular mechanisms regulating microglia function and microglia-synapse interactions, in most cases, remain a mystery.*

What Are the Mechanisms Regulating Complement Expression and Function?

Proteins of the complement cascade represent a class of proteins that could regulate microglia function and synapse development. Could regulatory pathways controlling complement expression during synaptic refinement modulate downstream microglial functions such as engulfment during retinogeniculate refinement? Expression of C1q in RGCs is developmentally restricted in the postnatal retina to the period of acetylcholine-dependent retinal wave activity that drives retinogeniculate refinement (Stevens et al., 2007). C1q, as the *initiating protein of the classical cascade*, controls the cleavage of downstream C3 to generate C3b, a phagocytosis cue to cells expressing CR3, including macrophages in the periphery or microglia, the only cells to express CR3 in the CNS (Figure 1.3A) (Graeber et al., 1988; T.J. Kindt, 2006). Thus, the mechanisms that control C1q expression in the brain ultimately control complement function. I hypothesize that C1q expression in developing RGCs is a critical mediator of microglia pruning of synapses and complement-mediated synapse elimination. Thus, a major goal of my dissertation

research is to understand the signals that control C1q expression in the postnatal retina and its function at retinogeniculate synapses in the dLGN.

What Are the Mechanisms Regulating C1q Expression?

What are the signals that regulate C1q transcription in developing retinal ganglion cells? Most components of the complement cascade are known to be synthesized in hepatocytes of the liver; however, C1q is unique in that it is produced locally by circulating macrophages in the bloodstream (Lu et al., 2008). Pathways have been identified regulating transcription of other complement components, but little progress has been made in characterizing the regulation of C1q transcription in any cell type, including peripheral macrophages. C1q levels in the blood are known to rise with inflammation and many pro-inflammatory cytokines have been known to induce C1q transcription. In the CNS, however, C1q does not enter the brain from the bloodstream and is instead produced primarily by microglia, the primary phagocytic cells of the brain. During retinogeniculate refinement, RGCs express C1q, but these neurons downregulate C1q expression around eye opening (Stevens et al., 2007). *This developmental expression of C1q in RGCs suggests that neuron-derived C1q may be regulated differently and may have unique functions, including synaptic refinement.*

The expression of C1q in RGCs corresponds with the onset of spontaneous retinal activity, suggesting that neuronal activity could modulate C1q expression in RGCs. Furthermore, Havik et al. recently showed genes involved with innate immunity, including several complement genes (*c2*, *c3*, *c4a/b*, *seizure-related gene 6 (sez6)*, *cfb*, *crry/cr1* and *itgb2/cd18*), were upregulated in non-anesthetized adult animals in response to *in vivo* high frequency stimulation, demonstrating that complement can be upregulated by activity (Håvik et al., 2007). Interestingly, blocking electrical activity *in vivo* results in incomplete synaptic pruning, giving a phenotype similar to what is seen in C1q and C3 KO mice (Huberman et al., 2008b). These data suggest a link between activity and complement; however, neurons fire action potential throughout life, while C1q expression in RGCs is confined to a tight developmental

window. Interestingly, at the same time that C1q is expressed in RGCs and retinal wave activity is driving refinement, immature astrocytes also arrive in the retina, suggesting that these developmental processes may be linked.

Additional Functions for C1q in the Healthy and Diseased CNS

In addition to the role of C1q in synapse elimination, other functions have been discovered for C1q in the healthy brain, during neurodegeneration, and after spinal cord injury and stroke. C1q expression in response to injury and neurodegeneration suggested that C1q may have a neuroprotective role in these contexts. A recent study addressed this question in vitro and found that adding exogenous C1q, in the absence of other complement proteins, improved neuronal viability and neurite outgrowth and prevented β -amyloid-induced neuronal death (Benoit and Tenner, 2011). C1q effects on neurite outgrowth were blocked with siRNAs targeting C/EBP- δ , NGF, or NT-3. In addition, exogenous C1q significantly upregulated expression of genes associated with cholesterol metabolism and transiently decreased cholesterol levels in neurons, known to facilitate neurite outgrowth. C1q also increased the nuclear translocation of cAMP response element-binding (CREB) protein and CCAAT/enhancer-binding protein- δ (C/EBP- δ), two transcription factors involved in nerve growth factor (NGF) expression. These data directly show that, in addition to clearing apoptotic debris during injury by activating the classical complement cascade, C1q can also directly effect changes in neuronal gene expression to promote survival and outgrowth (Benoit and Tenner, 2011). In addition, in vivo studies using laser-capture microdissection and microarray analysis after stroke show that inosine treatment, known to potently improve recovery after stroke, upregulated the expression of genes associated with axon growth and the complement cascade (Zai et al., 2009), suggesting that complement proteins contribute to inosine's effects. Conversely, many studies have also suggested that C1q can have a harmful role in injury and disease (Alexander et al., 2008a). Most recently, C1q was implicated in the development of inflammation-induced

persistent pain (Simonetti et al., 2013). In this scenario, C1q was downregulated in response to increased nuclear calcium signaling in spinal neurons, which contributed to activity-dependent spine remodeling on spinal neurons after inflammation and the development of allodynia (Simonetti et al., 2013). In addition to identifying a role for C1q in the development of pain hypersensitivity, this work also suggests that activity is an important regulator of C1q expression and function in neurons.

Intriguingly, proteins with structural similarity to C1q, known as C1q-like molecules, are highly expressed in cells of the CNS, including neurons, and have also been implicated in synapse development (Bolliger et al., 2011; Yuzaki, 2010). The C1ql subfamily of the C1q family includes small, secreted proteins that are expressed in differential patterns in the brain (Iijima et al., 2010). These proteins have similar globular head domains resembling C1q but lack the N-terminal collagen domain found in C1q (Bolliger et al., 2011; Ghai et al., 2007). Adding any of the four C1ql molecules to cortical neuron cultures reduced synapse density, suggesting that these molecules regulate synapse number. Recent work has shown that these proteins are ligands for BAI3 protein, a member of the cell-adhesion class of G protein-coupled receptors that has recently been linked to schizophrenia in human genome wide association studies (DeRosse et al., 2008). The globular domains of all four C1ql proteins bind to the extracellular thrombospondin-repeat domain of BAI3, and adding the thrombospondin-repeat fragment of BAI3 to neuronal cultures blocked the effects of C1ql proteins on synapse density, demonstrating that the effect was specific for C1ql proteins (Bolliger et al., 2011). It is not clear how C1ql proteins function to limit synapse density, or if BAI3 is required for this effect, but it is intriguing to speculate whether C1q can also bind BAI3 and has similar functions.

Another member of the C1q family that is highly expressed in the CNS is Cbln1, which functions as a synaptic organizer at the parallel fiber-purkinje cell synapse in the cerebellum (Matsuda et al., 2010). Cbln1 is a small, secreted protein similar to the C1q-like proteins and lacking the collagen domain found in C1q. The globular domains of Cbln1 closely resemble

those found in C1q; however, while C1q is a hexameric protein composed of six trimers (C1q A, B, C) (Figure 1.3), Cbln1 functions as a dimer composed of two trimers. The genes encoding Cbln1 are expressed by granule cells in the cerebellum and secreted Cbln1 rapidly induces parallel fiber synapse formation. Cbln1 signaling also is required to maintain mature synapse function as well. The binding partner for Cbln1 was recently identified as glutamate receptor $\delta 2$ (GluD2) (Matsuda et al., 2010; Yuzaki, 2009). Secreted Cbln1 released from parallel fibers binds to neurexin presynaptically and GluD2 postsynaptically to coordinate synaptogenesis (Yuzaki, 2010). In evaluating the functions for the known C1q family members in the CNS, it becomes clear that this family of molecules is an important regulator of synapse development and suggests that C1q itself may have other roles in synapse development aside from activating the complement cascade which may involve interactions with some of these receptors for C1q-like molecules.

Astrocytes Regulate Synapse Development and Function

Recent work has shown that astrocytes have an active role in many nervous system processes, including synapse development; however, their role in synapse elimination is not well understood. The arrival of immature astrocytes in the CNS is highly correlated with the formation of synapses. In the rodent visual system, RGC axons innervate the superior colliculus by birth. However, there is a 1-week delay before the majority of synapses are formed (Lund, 1969). This delay coincides with the birth and proliferation of astrocytes. The establishment of methods to purify and culture rodent RGCs allowed Pfrieger and Barres to ask whether synapses could form in the absence of astrocytes (Pfrieger and Barres, 1997). Purified RGCs were healthy, elaborated dendrites and axons but formed few synapses when cultured without astrocytes. In contrast, addition of a feeding layer of astrocytes or astrocyte-conditioned medium significantly increased RGC synaptic activity and the number of structural synapses that formed (Pfrieger and Barres, 1997; Ullian et al., 2001).

These and other findings indicated that secreted factors from astrocytes strongly enhance pre- and postsynaptic function in developing RGCs and prompted further exploration into the specific molecules that control this process. One class of astrocyte-secreted molecules that was recently identified is a family of extracellular matrix proteins, thrombospondins (TSPs) (Christopherson et al., 2005). TSPs were found to be required for synaptogenesis in vitro and in vivo (Christopherson et al., 2005), and recent work identified the calcium channel subunit, $\alpha 2\delta$ -1, as the receptor for TSP required for synaptogenesis (Eroglu et al., 2009). Recent work has identified additional astrocyte-secreted proteins, hevin and SPARC, that regulate synaptogenesis (Kucukdereli et al., 2011). Hevin, like TSP, promotes the formation of ultrastructurally normal synapses that are postsynaptically silent. Conversely, SPARC inhibits synaptogenesis by specifically antagonizing hevin (Kucukdereli et al., 2011). Furthermore, Glypican-4 and -6 recently were identified as molecules sufficient to induce fully functional synapses in RGC cultures and in vivo (Allen et al., 2012). Understanding how these astrocyte-derived factors cooperate to regulate synapse formation is an area of active investigation. The discovery of these molecules demonstrates that astrocytes can both positively and negatively regulate synaptogenesis to influence synapse number.

In addition to the function for astrocytes in synapse formation and function, a question that remains is whether and how astrocytes affect synapse elimination. Astrocytes show evidence of phagocytic capabilities, like microglia (Al-Ali and Al-Hussain, 1996; Bechmann and Nitsch, 1997; Cahoy et al., 2008), suggesting they may also function to prune synapses during development. Previous studies also suggested a role for microglia and astrocytes in engulfing axonal debris during large-scale cortical pruning (Berbel and Innocenti, 1988). Although it is not yet clear whether astrocytes can refine circuitry on a fine scale like microglia, a specialized class of astrocytes in the optic nerve head myelin transition zone (MTZ) has been shown to normally express a common phagocytic marker, the galactose-specific lectin Mac-2 (also known as Lgals3 or galectin-3) (Nguyen et al., 2011; Sun et al., 2009). In normal, healthy mice, large

inclusions of axonal material have been observed in these astrocytes, suggesting that MTZ astrocytes may phagocytose axonal components as a form of axonal maintenance in the normal animal (Nguyen et al., 2011).

Immature Astrocytes Upregulate C1q in Developing Retinal Ganglion Cells

The role of astrocytes in directly engulfing synapses remains unclear; however, recent work has implicated astrocytes in regulating complement-dependent synapse elimination. Neuronal C1q expression in RGCs was initially discovered in an unbiased *in vitro* screen for neuronal genes that are upregulated upon exposure to immature astrocytes (Stevens et al., 2007). Purified rodent RGCs have proven a useful model system with which to investigate the role of astrocytes in synapse development, as RGCs can be immunopurified and cultured for weeks in the complete absence of glia and other cell types (Barres et al., 1988a; Meyer-Franke et al., 1995). As discussed earlier, neurons form few synapses unless they are cultured with a feeding layer of astrocytes, or astrocyte-conditioned medium (ACM) (Ullian et al., 2001). These findings led to the question of whether astrocyte-derived signals regulate neuronal genes involved in synapse development. Surprisingly, gene chip studies revealed that one of the most highly upregulated genes in purified RGCs exposed to astrocytes was the complement component C1q (Stevens et al., 2007). Given the correlation between the arrival of astrocytes in the retina and C1q expression, I hypothesize that this astrocyte-derived factor is an important molecular switch to initiate C1q expression and downstream complement activation.

What Is the Astrocyte-derived Factor Regulating C1q Expression?

Astrocytes secrete a number of neuroactive substances shown to regulate neuronal gene expression, synapse development, and plasticity. These glial cells are also a major source of cytokines in the brain, which are known to modulate immune system function. IFN- γ , IL-6, IL-10, IL-1, TGF- β 1, 2, 3, and TNF- α are all known to be produced by astrocytes under

some conditions; however, it is not known what of these cytokines are present in the brain during retinogeniculate refinement.

There are two basic requirements for the candidate astrocyte-derived signal that regulates C1q expression in RGCs. First, the molecule must be secreted by astrocytes during development. Second, there must be a corresponding receptor for this molecule on RGCs. Any connection with C1q regulation in the periphery could suggest a role for this molecule in the CNS as well. IFN- γ is a well-known pro-inflammatory cytokine in the immune system shown to increase C1q expression in macrophages (Lu et al., 2008). Rat astrocytes secrete it, particularly in response to TNF- α (Xiao and Link, 1998). Neurons are also known to express type II interferon receptors, which bind IFN- γ (Neumann et al., 1997). IL-6 is known to increase C1q expression in peritoneal macrophages and is secreted by astrocytes especially in the diseased brain (Dong and Benveniste, 2001; Lu et al., 2008). Neurons of the CNS do express the IL-6 receptor, but specific expression by developing RGCs has not been shown (Qiu et al., 1998; Schobitz et al., 1993). IL-10 is an anti-inflammatory cytokine shown to cause C1q upregulation and expressed by astrocytes (Dong and Benveniste, 2001; Moosig et al., 2006). In neurons, IL-10 receptor expression has been shown to provide an anti-apoptotic signal (Watkins and Maier, 2003). IL-1 and TNF- α are also both expressed by astrocytes and can influence C1q expression in other systems (Dong and Benveniste, 2001; Lu et al., 2008). They have been shown to influence neuronal plasticity and outgrowth (Stellwagen and Malenka, 2006; Temporin et al., 2008).

The isoforms of TGF- β represent strong candidates for C1q regulation. The characteristics of the TGF β signaling pathway make it well-suited to the task of regulating C1q in neurons. First, TGF β interacts with TGF β receptors expressed in neurons and throughout the body (Lacmann et al., 2007; Massague, 2000). Second, published reports indicate that TGF β 2 and TGF β 3 are enriched in astrocytes, which trigger neuronal C1q upregulation *in vitro* (Cahoy et al., 2008; Unsicker et al., 1991). Third, TGF β has been shown to influence C1q expression in

other cell types (Morgan et al., 2000). TGF β signaling is also connected with synapse development. The three mammalian isoforms of TGF β (β 1, β 2, and β 3) have each been connected with synapse formation, function, or maintenance in recent studies (Chin et al., 2002b; Heupel et al., 2008b). TGF β 1 plays a role in synaptogenesis at the frog neuromuscular junction. Interestingly, Schwann cells, the “astrocyte-like” glial cells of the peripheral nervous system, are the source of TGF β 1 in this system (Feng and Ko, 2008a). It is not known if TGF β 1 similarly affects CNS synapses. TGF β 2 and β 3 have been linked to synapse function and dysfunction. Loss of TGF β 2 causes central synapse impairment and TGF β 3 becomes highly upregulated in astrocytes during seizure (Heupel et al., 2008b; Kim et al., 2002). In addition, TGF β signaling has been implicated in neurodegenerative diseases, which exhibit synapse loss in the early stages of the disease (Flanders et al., 1995).

Another class of candidates I considered as potential regulators of C1q were toll-like receptor (TLR) ligands. Signaling through toll-like receptors (TLRs) has been shown to upregulate C1q (Lu et al., 2008). One TLR2 ligand, CD14, is known to be expressed by astrocytes, and neurons have been shown to express TLR2 especially in response to ischemia (Bsibsi et al., 2007; Tang et al., 2007). Other astrocyte-derived secreted molecules have been shown to upregulate C1q as well. Prostaglandin E2 increases C1q expression in macrophages and has been shown to influence plasticity in visual cortex (Akaneya and Tsumoto, 2006; Trinder et al., 1995). Thrombospondins, a known activator of latent TGF- β (Schultz-Cherry et al., 1994), are secreted by astrocytes and are known to influence the formation of structural synapses in RGC cultures (Ullian et al., 2004). Astrocytes also produce amyloid precursor protein (APP) as well, and β -amyloid, a cleavage product of APP has been shown to upregulate C1q (Li et al., 2008).

A number of these cytokines and other neuroinflammatory factors have been shown to affect plasticity in the adult brain (Stellwagen and Malenka, 2006; Temporin et al., 2008; Vikman et al., 2001). Because of their ability to influence neuronal activity in the adult, their secretion

from astrocytes, and their ability to upregulate C1q, neuroinflammatory signals are potential candidates for the C1q-upregulating factor during synapse elimination.

In this dissertation, I have focused on the following questions with particular focus on C1q, the initiating protein of the classical complement cascade: 1) *How do secreted complement proteins regulate the elimination of specific synapses at the right time and place?* 2) *What are the contribution and function of neuronal C1q in retinogeniculate synapse elimination?* 3) *Does neuronal activity regulate and restrict neuronal C1q expression in the postnatal retina?* Chapter 2 details the methods used to address these questions. Chapter 3 will present my experimental approach to determine the role of neuronal C1q in complement-dependent synapse elimination identify TGF- β as a key signal neuronal C1q expression in the postnatal retina. In chapter 4, I focus on how activity may guide complement-dependent synapse elimination to elimination specific synapses at the right time and place. Chapter 5 discusses the implications of my findings for the field of synaptic refinement and suggests future directions for this study.

Significance

This purpose of the work presented here is to advance our understanding of the molecular mechanisms that regulate CNS synapse elimination. Identifying the signals and molecular pathways that regulate C1q in neurons may allow one to target neuronal complement upregulation to manipulate synapse elimination *in vivo*. Given the importance of astrocytic signaling in the CNS, disrupting astrocyte signaling globally is not an option; therefore, identifying the factor and the neuronal receptor it binds is crucial for *in vivo* manipulation of this neuron-glia signaling pathway. The molecular mechanisms of synapse elimination have yet to be characterized; thus, understanding whether and how C1q regulates complement cascade activation and function in the developing retinogeniculate system could have broad implications

in understanding the role of complement in establishing precise synaptic connectivity in other sensory systems.

In addition to providing insight into normal CNS development, these experiments have implications for neurodevelopmental disorders such as Autism Spectrum Disorders (ASD) that are thought to involve aberrant synapse development and synaptic connectivity. Recent work has shown elevated cytokine levels and other signs of neuroinflammation in the brains of autistic individuals (Li et al.; Vargas et al., 2005). Particularly of interest are increases in specific cytokines with the potential ability to upregulate C1q: IL-6, TNF- α , IFN- γ , and TGF β isoforms. Inappropriate regulation of C1q and the complement cascade during brain development could negatively influence synaptic refinement and brain wiring. A synapse elimination defect could cause an imbalance in excitation and inhibition in the brain, which is one theory about the etiology of ASD (Chao et al., 2010; LeBlanc and Fagiolini, 2011; Rubenstein and Merzenich, 2003). Unraveling the mechanistic details of complement-dependent synapse elimination may also provide insight into the mechanisms underlying the synaptic dysfunction reported in Autism Spectrum Disorders (Careaga et al., 2010; Enstrom et al., 2009; Garay and McAllister, 2010).

Complement proteins are also highly upregulated in the early stages of many CNS neurodegenerative diseases, such as Alzheimer's disease, in which early synapse loss is a hallmark. In CNS injury or disease, C1q is rapidly upregulated in microglia and neurons, concurrently with the appearance of reactive astrocytes (Cowell et al., 2003; Shen et al., 1997). As reactive astrocytes antigenically resemble immature astrocytes (Cahoy et al., 2008), I hypothesize that reactive astrocytes re-express immature signals, including the signal responsible for C1q expression in neurons. Thus, astrocytes and complement could have an early role in the pathophysiology ultimately leading to synapse loss and neuronal death. Consistent with this hypothesis, in the DBA/2J glaucoma mouse model, C1q expression and localization to synapses corresponded with the appearance of reactive astrocytes early in the

disease prior to substantial synapse loss and neuronal degeneration (Howell et al., 2011; Rosen and Stevens, 2010; Stevens et al., 2007). The parallels between disease and development suggest that the signals regulating synapse elimination in the immature brain may be part of the pathway leading to synapse loss in diseased states. Identification of the signals associated with C1q upregulation could lead to several potential therapeutic targets in the treatment of neurodegenerative diseases such as glaucoma and Alzheimer's disease.

Growing evidence also supports a link between complement-dependent synapse elimination and epilepsy (Aronica et al., 2007; Vargas et al., 2005; Vezzani, 2008). Studies have shown that defects in synapse elimination can promote seizure activity. Importantly, a recent study has reported seizure activity in C1q KO mice (Chu et al., 2010). This phenotype is consistent with the model that synaptic refinement helps to set the balance between excitation and inhibition in the brain. A nervous system that fails to prune away inappropriate connections can become hyperexcitable with a lowered threshold for seizure activity and epileptogenesis. In this context, understanding the mechanisms regulating complement-dependent synapse elimination may be important for identifying therapeutic targets that can be anti-epileptogenic, correcting the potential hyperinnervation underlying seizures instead of simply suppressing seizures.

In addition, complement has been shown to become chronically upregulated and activated during epileptogenesis in both animal models of epilepsy and in human temporal lobe epilepsy (TLE) (Aronica et al., 2007). This upregulation and activation of complement could play an early role in destabilizing neural networks and promoting the development of more severe and frequent seizures. Understanding the role of activity in complement regulation could provide insight into its upregulation during seizure activity as well. Taken together, the results presented in this dissertation will advance our understanding of the mechanisms of synaptic refinement and may ultimately help to determine the mechanisms of synapse loss and dysfunction in several disorders and diseases of the nervous system.

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CHAPTER 2:

Materials and Methods

Mice

Floxed TGF- β 2 mice (B6.129S6-*TGF- β 2^{tm1Hlm}*) were obtained from the NCI Mouse Repository and crossed to the CHX10-Cre line, Tg(Chx10-EGFP/cre,-ALPP)2Clc/J (Jackson Lab), to generate retina-specific TGF- β 2KO mice. C1qA KO mice (C57BL6 background) were generously provided by M. Botto (Botto et al., 1998). Experiments were approved by the institutional animal use and care committee in accordance with NIH guidelines for the humane treatment of animals.

Neuron and Astrocyte Cultures

Retinal ganglion cells were cultured from P8 Sprague-Dawley rats after serial immunopanning steps to yield >99.5% purity as described in (Barres et al., 1988c). Cells were maintained in serum-free media as described (Meyer-Franke et al., 1995). Cortical astrocytes were prepared from P1-P2 rat cortices as previously described (Ullian et al., 2001). Retinal astrocytes were prepared from P8 Sprague Dawley rat or mouse retinas by adapting described methods for purification of cortical astrocytes (Foo et al., 2011). After first immunopanning away microglia and RGCs, an anti-ITGB5-coated petri plate was used to isolate astrocytes from remaining cells in suspension. Purified cortical astrocytes were prepared as described in (Foo et al., 2011). Purified retinal and cortical astrocytes were maintained in a defined serum-free medium supplemented with hbEGF as described (Foo et al., 2011). Astrocyte conditioned medium (ACM) was prepared as previously described [Ullian et al., 2001]. Astrocytes were switched to minimal media (Neurobasal + glutamine, Pen/Strep, Sodium Pyruvate) once confluent and media was collected after 5 days and concentrated to 10X using Vivaspin columns (Sartorius).

qPCR

RGCs, microglia, and astrocytes were acutely isolated using immunopanning as described above from either mice or rats at indicated ages. For acute isolation experiments, RNA was

collected directly from the immunopanning plate without culturing the cells. For cultured RGC experiments, total RNA was prepared, cDNA was synthesized, and qPCR performed using the Applied Biosystems Cells to Ct Power SYBR green kit as described by the manufacturer. Briefly, cell lysates were collected from 80,000 RGCs in provided lysis buffer and cDNA was synthesized directly from this lysate. QPCR reactions were assembled for the genes of interest (*c1qa*, *c1qb*, *tieg*, *gapdh*) using 4ul of cDNA per reaction and samples were run on the Rotogene qPCR machine (QIAGEN). Expression levels were compared using the ddC_T method normalized to GAPDH.

Immunohistochemistry

Brains and eyes were harvested from mice after transcardial perfusion with 4% paraformaldehyde (PFA). Tissue was then immersed in 4% PFA for 2 hours following perfusion, cryoprotected in 30% sucrose, and embedded in a 2:1 mixture of OCT:20% sucrose PBS. Tissue was cryosectioned (12-14 microns), sections were dried, washed three times in PBS, and blocked with 2% BSA+ 0.2% Triton X in PBS for 1 hr. Primary antibodies were diluted in antibody buffer (+ 0.05% triton + 0.5% BSA) as follows: C1q (undiluted culture supernatant), C3 (Cappel, 1:300), vglut2 (Millipore, 1:2000), TGFβRII (R&D Systems goat anti-human, 1:200), psmad (Millipore, 1:200), Calretinin (Millipore, 1:1000), arc (Synaptic Systems, 1:300), PSD95 (Zymed, 1:500), Iba1 (Wako, 1:500), Neurofilament (Sigma, 1:1000), and MAP2 (Chemicon, 1:500) and incubated overnight at 4°C. Secondary Alexa-conjugated antibodies (Invitrogen) were added at 1:200 in antibody buffer for 2 hr at room temperature. Slides were mounted in Vectashield (+DAPI) and imaged using the Zeiss Axiocam, Zeiss LSM700, or Perkin Elmer Ultraview Vox Spinning Disk Confocal.

ELISA

The 7-plex Mouse Inflammatory Cytokine kit (MSD) was used to profile cytokines in ACM. Freshly prepared ACM was profiled according to manufacturer's provided protocol. Standards

were diluted in minimal media used to make ACM for greater accuracy. Plates were read and data were acquired and analyzed using the MSD Sector Imager 2400. ELISA kits were obtained for TGF- β 1, 2, and 3 separately (R&D systems, MSD) and were performed according to manufacturer's instructions.

Western Blot

RGC and whole retina lysates were collected and homogenized in RIPA buffer with complete protease inhibitors (Roche). Samples were boiled for 5 min in SDS sample buffer, resolved by SDS PAGE, transferred to PVDF membranes, and immunoblotted. Antibodies were diluted in 5% milk in PBS + 0.1% Tween. Antibodies: Rabbit anti-C1qA polyclonal (Epitomics, 1:5000); Goat anti-TGF β RII (R&D Systems, 1:1000).

In situ hybridization

In situ hybridization for C1qb was performed on 12 μ m retinal sections as previously described in (Stevens et al., 2007). Probes targeting the entire C1qb coding sequence (Open Biosystems clone: 5715633) were generated by digesting the plasmid with EcoRI, and performing *in vitro* transcription with T7 polymerase using the DIG RNA labeling kit (Roche Applied Science) as per the manufacturer's instructions. 1.8kb probes were then cleaved to form 300 bp probes by alkaline hydrolysis before use.

LGN analysis

Mice received intraocular injection of cholera toxin-b subunit (CTB) and were sacrificed the following day. Tissue was processed and analyzed as previously described (Jaubert-Miazza et al., 2005; Stevens et al., 2007). Mouse pups were anesthetized with inhalant isoflurane. Mice received intravitreal injections of cholera toxin-b subunit (CTb) conjugated to Alexa 488 (green label) in the left eye and CTb conjugated Intraocular injection to Alexa 594 (red label) into the right eye as described in (Bjartmar et al., 2006). Images were digitally acquired using the Zeiss

Axiocam. All images were collected and quantified “blind,” and compared to age-matched littermate controls. Gains and exposures were established for each label. Raw images of the dLGN were imported to Photoshop (Adobe), and the degree of left and right eye axon overlap in dLGN was quantified using the multithreshold protocol as previously described (Bjartmar et al., 2006) and using threshold independent R value analysis as described by (Torborg and Feller, 2004). For threshold independent analysis, we performed background subtraction using a 200 pixel rolling ball radius filter and normalized the images. We then calculated the R value ($\log(F_i/F_c)$) for each pixel and determined the variance of the R value distribution for each image (4 images/animal). Pseudocolored images representing the R value distribution were generated in ImageJ.

Retinal cell counts

Retinal flat mounts were prepared by dissecting out retinas whole from the eyecup and placing four relieving cuts along the major axis, radial to the optic nerve. Each retina was stained with DAPI (Vector Laboratories, Burlingame, CA) to reveal cell nuclei. Measurements of total cell density in the ganglion cell layer (which includes both ganglion cells and displaced amacrine cells) were carried out blind to genotype from matched locations in the central and peripheral retina for all four retinal quadrants of each retina. Quantification was limited to P30 retinas, which is an age subsequent to ganglion cell genesis and apoptosis in the mouse. For each retina (1 retina per animal; n=3 mice per treatment condition or genotype), 12 images of peripheral retina and 8 images of central retina were collected. For each field of view collected (20 per retina), Macbiophotonics ImageJ software (NIH) was used to quantify the total number of DAPI using the nuclei counter plugin and TUJ1-positive cells were counted using the cell counter plugin. All analyses were performed blind to genotype or drug treatment.

Zstack Image and Microglial Engulfment Analysis

Mice received intraocular injections of anterograde tracers at P4. All mice were sacrificed at P5 and brains were 4% PFA fixed overnight (4°C). Only those brains with sufficient dye fills were analyzed. For each animal, two sections of medial dLGN were chosen for imaging for reconstruction of RGC inputs and C1q staining as well as for microglia engulfment analysis. Images were acquired on a spinning disc confocal at 60X using 0.2 µm z-steps. For each dLGN, 4-8 fields were imaged in the ipsilateral territory and 4-8 fields were imaged in the contralateral territory (minimum of 8 fields per dLGN, 16 fields per animal). Subsequent images were processed and quantified using ImageJ (NIH) and Imaris software (Bitplane). For subsequent acquired z-stacks, ImageJ (NIH) was used to subtract background from all fluorescent channels (rolling ball radius=10) and a mean filter was used for the EGFP channel (stained for Iba1) of 1.5. Subsequently, Imaris software (Bitplane) was used to create 3D volume surface renderings of each z-stack. Surface rendered images were used to determine the volume of the microglia, all RGC inputs, and the volume of C1q staining. To visualize and measure the volume of engulfed inputs, any fluorescence that was not within the microglia volume was subtracted from the image using the mask function. The remaining engulfed/internal fluorescence was surface rendered using parameters previously determined for all RGC inputs/C1q and total volume of engulfed/internal inputs was calculated. To determine % engulfment (or %C1q-positive terminal), the following calculation was used: Volume of internalized RGC input (or volume of C1q) (µm³)/Volume microglial cell (or RGC inputs) (µm³). For all KO engulfment experiments, all analyses were performed blind.

Microglia density quantification

For quantification of cell density, 2 dLGN were imaged per animal (n=3 per treatment condition or genotype). To capture the entire dLGN, a 10X field was acquired. Microglia were subsequently counted from each 10X field. To calculate the density of microglia, the area of the

dLGN was measured using ImageJ software (NIH). All analyses were performed blind to genotype or drug treatment.

Quantification of Synapse Number in RGC cultures

For synapse quantification of RGCs, we followed a previously developed immunohistochemistry (IHC)-based method described and validated in Christopherson et al. (2005) and Ullian et al. (2001). Briefly, synapse numbers were quantified for RGCs at DIV 10. RGCs grown on glass coverslips were washed three times with warm PBS and then briefly fixed (7 min.) in 4% paraformaldehyde. Coverslips were blocked for 1 hr. with 5% BSA +0.2% triton diluted in antibody buffer. Primary antibodies for vglut2 (Millipore, 1:1000) and PSD95 (Zymed, 1:500) were diluted as indicted in antibody buffer and added to coverslips to incubate overnight at 4°C. Coverslips were washed with PBS and secondary antibody were diluted as described above and added to coverslips for 2 hours. After washing, coverslips were mounted on slides and at least 10 RGCs, randomly selected based on DAPI staining, were imaged per coverslip. Images were then analyzed using the PunctAnalyzer ImageJ macro as previously described.

Statistical Analysis

For all statistical analyses, GraphPad Prism 5 software (La Jolla, CA) was used. Analyses used include one-way or two-way ANOVA with Bonferroni post hoc test and Student's t-test. Igor was used to calculate the R value variance in Chapter 3. All n and p values and statistical tests are indicated in figure legends.

CHAPTER 3:

TGF- β Signaling Regulates Complement C1q Expression and Developmental Synaptic Refinement

Contributions: All experiments were designed by Beth Stevens and Allison Bialas. Experiments and data analysis were performed by Allison Bialas. Retina and LGN co-cultures were prepared by Ryuta Koyama.

Introduction

Increasing evidence implicates immune molecules in synapse development and refinement. Several molecules best known for their functions in the immune system, including MHC I (Huh et al., 2000), neuronal pentraxins (NP1 and NP2) (Bjartmar et al., 2006), and proteins of the classical complement cascade (Stevens et al., 2007) mediate synapse remodeling in the developing visual system, yet surprisingly little is known about the signals that regulate the expression and function of these immune molecules at developing synapses.

The classical complement cascade proteins C1q and a downstream complement protein, C3, localize to synapses in the LGN during the synaptic refinement period and mediate synapse elimination by a mechanism involving microglia-mediated synaptic phagocytosis (Schafer et al., 2012b; Stevens et al., 2007). Prior to synaptic refinement (before P10 in the mouse), the relay neurons in the dorsal lateral geniculate nucleus of the thalamus (dLGN) receive inputs from RGCs from both eyes that compete for synaptic territory. After P10, the dLGN becomes segregated into discrete eye specific territories, and by P30, each relay neuron receives innervations from only 1-2 RGCs. Genetic deletion of C1q, C3 and the microglia-specific CR3 results in sustained defects in synaptic refinement indicated by a failure to fully segregate into eye specific territories (Schafer et al., 2012b), indicating that these molecules function in a common pathway to refine synaptic circuits. Importantly, complement localization to synapses and microglial engulfment of retinogeniculate synapses is restricted to a narrow window of postnatal development (P5-P8), suggesting that the complement-dependent pruning process is tightly regulated. C1q, the initiating protein of the classical complement cascade, is developmentally expressed in retinal ganglion cells (RGCs), peaking during the synaptic refinement of retinogeniculate synapses; however, the signals that control the expression and function of C1q at retinogeniculate synapses remain elusive.

As the initiator of the classical complement cascade, C1q is a critical point of regulation in the classical complement cascade. C1q, a large, hexameric, secreted protein composed of

C1qA, C1qB, and C1qC peptide chains, is the recognition domain of the initiating protein, C1, in the classical complement cascade. In the immune system, C1q binds to membranes of apoptotic cell or pathogens and triggers a proteolytic cascade of downstream complement proteins resulting in C3 opsonization and phagocytosis by macrophages that express complement receptors. Recent work suggests that microglia in the developing CNS function like macrophages in the immune system and engulf inappropriate retinogeniculate inputs in a complement-dependent manner during the retinogeniculate pruning period (Schafer et al., 2012b). Given the precise expression of C1q in RGCs during retinogeniculate refinement, I hypothesize that RGC expression and the secretion of C1q from RGC inputs in the dLGN initiates the complement cascade and activates microglia-mediated synaptic pruning. In the current study, I sought to identify the signals that regulate C1q expression in RGCs, and to then manipulate those signals to determine whether C1q expression in RGCs is required to initiate complement-dependent synaptic refinement and microglia-mediated pruning of retinogeniculate synapses.

Surprisingly little is known about the signals that regulate C1q transcription in any cell. Gene profiling revealed C1q genes (*c1qa*, *c1qb*, and *c1qc*) were among the genes most highly upregulated in purified RGCs upon exposure to astrocytes (Stevens et al., 2007), implicating a secreted factor; however, the identity of the astrocyte derived signal(s) that regulate C1q expression has not been identified. In the immune system, expression of complement and other immune genes can be modulated by rapid cytokine signaling pathways that regulate the inflammatory response. In the developing brain, astrocytes are a major source of cytokines several of which modulate synapse function and plasticity (Boulangier and Shatz, 2004) and are among the many astrocyte-derived factors that potently regulate synapse development and function (Allen et al., 2012; Christopherson et al., 2005; Ullian et al., 2001).

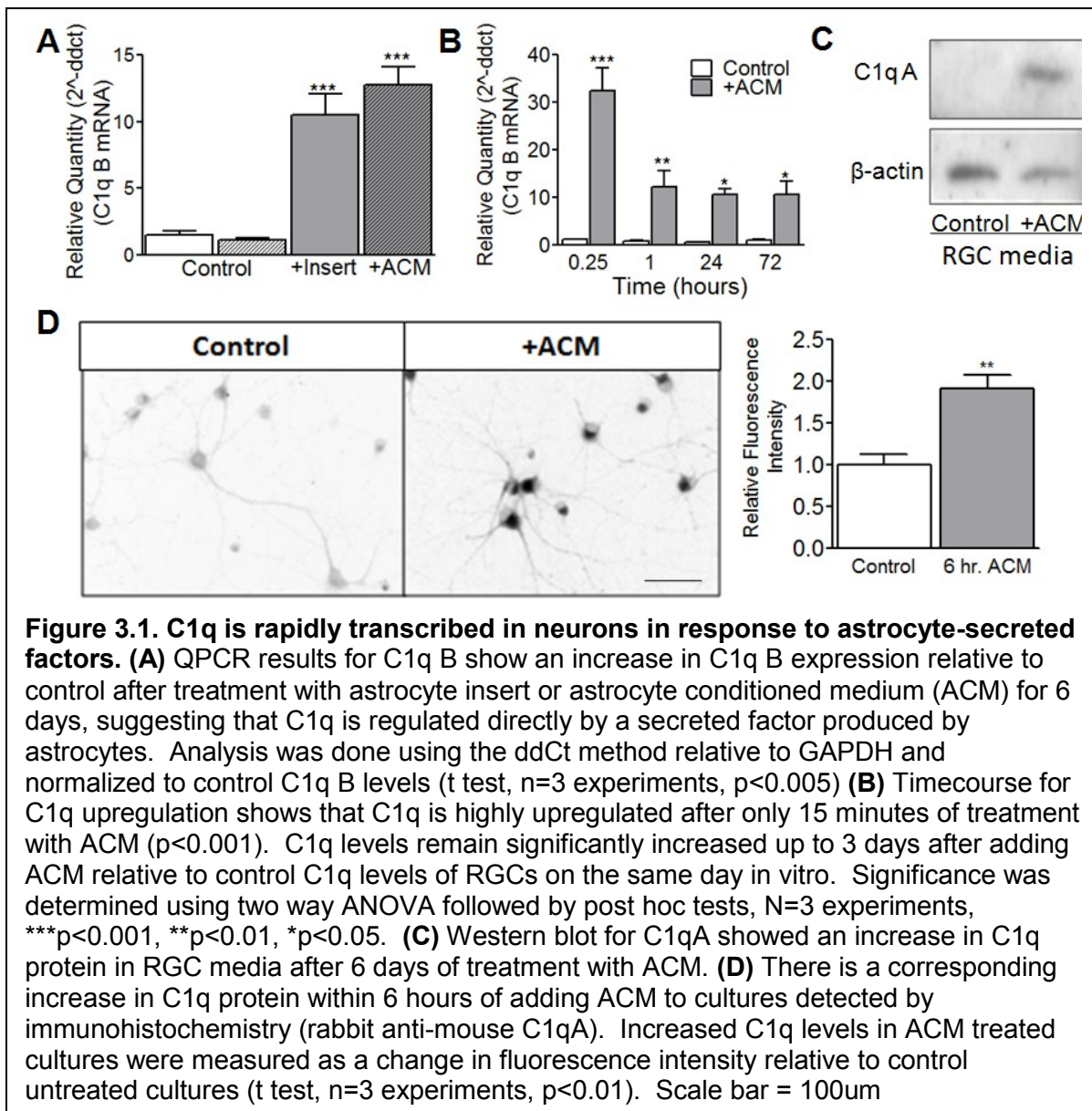
The presence of immature astrocytes in the retina corresponds with the period of C1q expression in RGCs in vivo, suggesting that the astrocyte-derived factor that upregulates C1q in RGC cultures may also regulate C1q expression in RGCs in vivo. In the current study, I

identified transforming growth factor beta (TGF- β) as the factor secreted by astrocytes necessary and sufficient for C1q expression in RGCs. Astrocyte-derived TGF- β induces rapid phosphorylation of receptor-associated transcription factors known as R-Smads in RGC cultures and promotes C1q transcription. Blocking TGF- β signaling in retinal neurons resulted in a significant reduction in C1q expression in postnatal RGCs as well as reduced synaptic localization of complement in the dLGN. Moreover, retinal TGF β RII KO mice showed decreased synaptic engulfment by microglia and defects in refinement of retinogeniculate synapses, suggesting that TGF- β -dependent regulation of neuronal C1q in the retina regulates complement-dependent synapse elimination in the dLGN. Taken together, my data reveal a novel role for the TGF- β cytokine signaling pathway in regulating C1q expression in neurons and in initiating complement-dependent synaptic refinement in the developing CNS.

Results

C1q expression is rapidly and directly upregulated in retinal ganglion cells (RGCs) by a secreted factor.

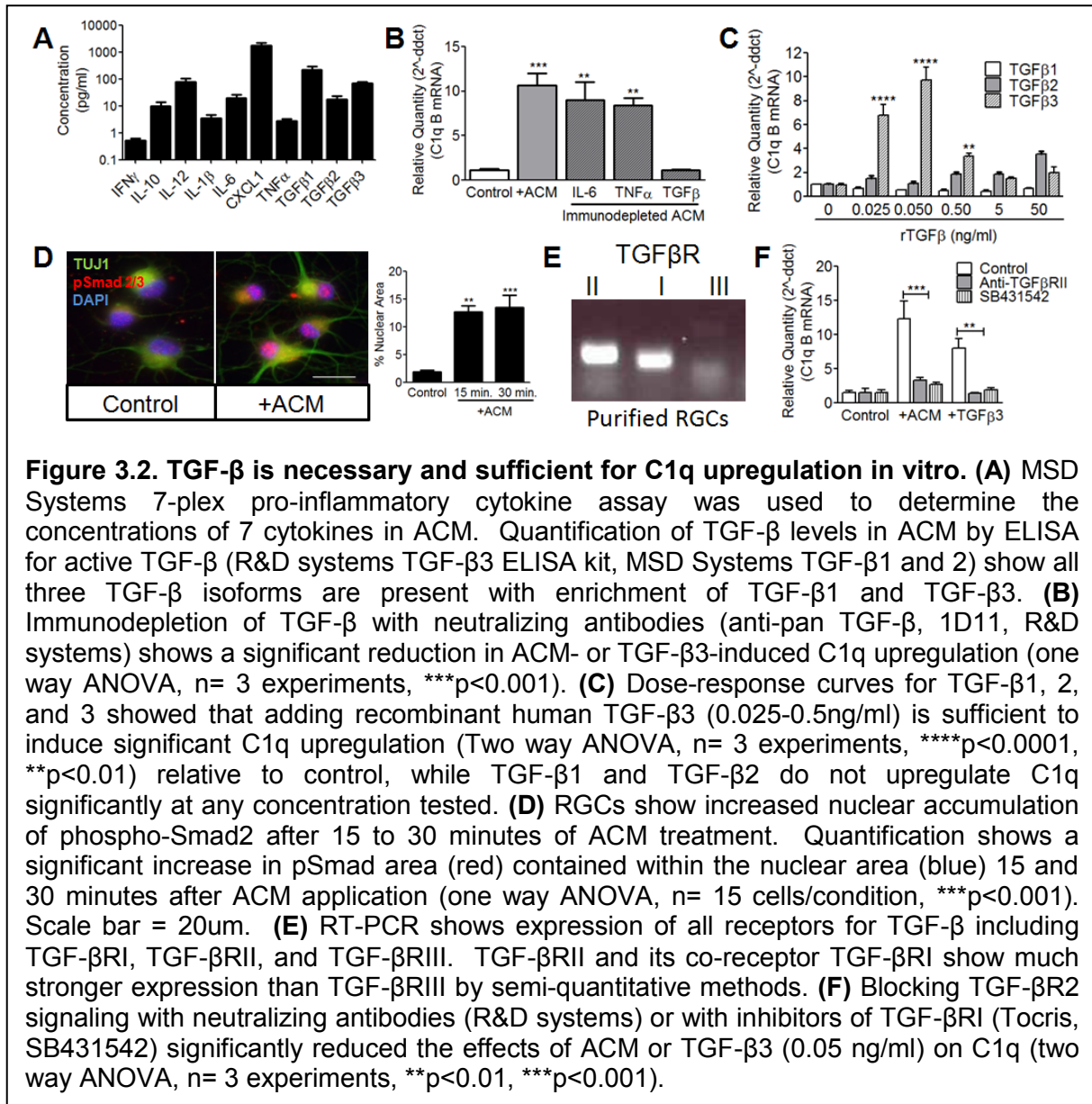
Previous findings suggested that an astrocyte-derived factor triggers neuronal C1q expression. The C1q genes (*c1qa*, *c1qb*, and *c1qc*) were among the most highly upregulated genes in purified RGC neurons upon chronic exposure to cortical astrocytes (7 days) that were grown on tissue culture inserts (Stevens et al., 2007) (Figure 3.1A). To determine if bidirectional signaling between astrocytes and neurons was required for C1q upregulation, I measured C1q mRNA levels in purified postnatal (P8) RGC cultures treated with conditioned medium (ACM) collected from cortical astrocytes. Treatment of RGCs with ACM and astrocyte inserts resulted in a comparable 10-20 fold upregulation of C1q in RGCs, implicating a direct, astrocyte-secreted factor in neuronal C1q upregulation (Figure 3.1A). ACM collected from purified postnatal (P8) retinal astrocyte cultures (Foo et al., 2011) resulted in a similar upregulation compared to purified or standard cortical astrocytes (Supplemental Figure 1A).



Surprisingly, C1q was upregulated after only 15 minutes of ACM treatment (Figure 3.1B; Supplemental Figure 1B,C). I also observed rapid upregulation of C1r and C1s, the enzymes that associate with C1q to initiate the complement cascade (Supplemental Figure 1D). This rapid C1q upregulation was blocked by actinomycin, a transcriptional inhibitor, confirming that this effect was a result of transcription (Supplemental Figure 1E), and treating cells with boiled ACM failed to upregulate C1q, suggesting that a protein in ACM triggered C1q upregulation (Supplemental Figure 1F). Rapid increases in C1q protein levels were also detected by immunostaining in purified RGC cultures using anti-C1q antibodies and were quantified after 6 hours of ACM treatment (Figure 3.1D). Media samples from RGC cultures following ACM treatment (1 week) showed increased C1q protein levels by western blot (Figure 3.1C). Media samples of ACM alone or from RGCs treated with ACM acutely showed undetectable levels of C1q by western blot (data not shown), suggesting that the C1q I detect is produced by the RGC cultures and accumulates in the media over time in response to ACM. Thus, I used this purified culture system as a robust assay to screen for secreted signals that rapidly upregulate and sustain C1q expression in RGCs.

TGF- β is necessary and sufficient to induce C1q upregulation.

To identify the astrocyte-derived factor responsible, several candidate molecules were screened for the ability to upregulate C1q in RGC cultures. Cytokines, potent modulators of immune and neural function, were the main class of molecule tested since astrocytes are a major source of cytokines in the brain. Candidate cytokines measures included IFN- γ , IL-10, IL-12, IL-1 β , IL-6, CXCL1, TNF α , and TGF- β 1, 2, and 3. Concentrations of candidate cytokines present in ACM were determined using the MSD mouse 7-plex inflammatory cytokine assay and individual ELISAs for TGF- β 1, 2, and 3 (Figure 3.2A). TGF- β 1, 2, and 3 were among the most enriched cytokines in ACM, while IFN- γ , IL-1 β , and TNF α were detected at the lowest levels.



Immunodepletion of individual cytokines from ACM was performed to determine if specific cytokines were required for astrocyte-dependent C1q upregulation. Although several cytokines, including TNF α and IL-6, induced modest C1q upregulation when added directly to RGC cultures (Supplemental Figure 2A), immunodepletion of the latter cytokines using neutralizing antibodies (R&D systems) failed to affect ACM-induced C1q upregulation (Figure 3.2B). In contrast, specific immunodepletion of transforming growth factor beta (TGF- β) from ACM completely prevented ACM-induced C1q upregulation as measured by qPCR (Figure 3.2B). RGCs were treated for 15 min. with either control ACM or ACM immunodepleted of TGF- β using an anti-pan TGF- β neutralizing antibody (1D11, R&D Systems) or antibodies specific to TGF- β 1, 2, or 3 (Supplemental Figure 2B). Depletion of TGF- β from ACM was confirmed by ELISA (Supplemental Figure 2C). Together these data show that TGF- β is necessary to upregulate neuronal C1q in the purified RGC culture system.

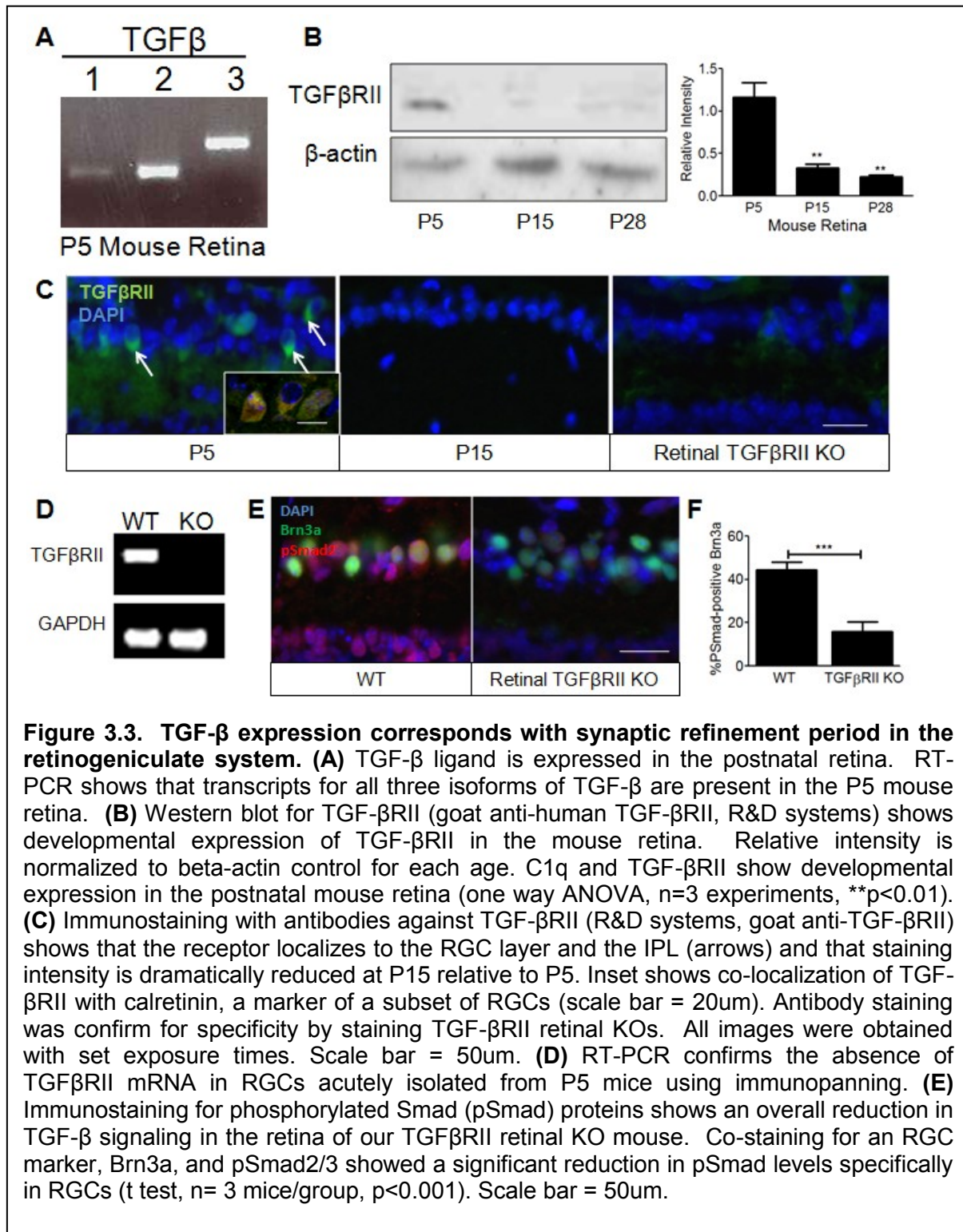
To determine which TGF- β isoform was responsible for upregulating C1q at the concentrations measured in ACM, dose-response curves (50ng/ml-25pg/ml) were generated for each of the TGF- β isoforms and C1q levels were measured by qPCR. TGF- β 3 was most effective at upregulating C1q at concentrations similar to those measured in ACM, whereas TGF- β 2 treatment resulted in modest upregulation (Figure 3.2C). In addition, glycine elution to release bound TGF- β from anti-pan or anti-TGF- β 3 neutralizing antibodies produced an eluate which, when added to RGC cultures, could upregulate C1q to the same extent as ACM (Supplemental Figure 2D). Although these isoforms can bind the same receptor and may have somewhat redundant functions, I found that TGF- β 3 is enriched in retinal astrocytes compared with RGCs, cortical astrocytes, and microglia (Supplemental Figure 2E). These results together with my in vitro results that TGF- β 3 is most effective at upregulating C1q (Figure 3.2C) suggest that TGF- β 3 may be the key isoform regulating retinal C1q expression in vivo.

I next addressed whether activation of TGF- β receptor signaling in RGCs was required for developmental expression of C1q in postnatal RGCs. In the canonical TGF- β signaling

pathway, TGF- β 1, 2, or 3 binds to TGF β RII which then phosphorylates TGF β RI. TGF β RI then phosphorylates a Smad transcription factor to alter gene expression. A third receptor, TGF β RIII, can also associate with TGF β RII and alters ligand affinity (Massague, 2000). All of the components required for TGF- β signaling are expressed in purified RGCs (Figure 3.2D,E). Treatment of RGCs with ACM resulted in rapid (15-30 min.) nuclear accumulation of phosphorylated Smads (pSmad 2/3), as measured by immunostaining with phospho-Smad (Millipore; AB3849) antibody (Figure 3.2D), consistent with the timing of C1q upregulation. In addition, transcripts for TGF β RII, TGF β RIII, and TGF β RI were present in purified RGCs (Figure 3.2E). These data indicate that RGCs express functional TGF- β receptors that can be activated with a timecourse consistent with C1q upregulation. To determine whether the TGF- β receptors, I and II, were responsible for this upregulation, TGF β RI and II were blocked in RGC cultures with anti-TGF β RII neutralizing antibodies and a TGF β RI-specific inhibitor (Inman et al., 2002) (SB431542, Tocris, 30uM) for 1 hour followed by a 15-minute treatment with either TGF- β 3 (50 pg/ml) or ACM. Both TGF- β -induced and ACM-induced upregulation of C1q were blocked in the presence of TGF β RII neutralizing antibodies or TGF β RI inhibitors (Figure 3.2F). Thus, TGF β RII signaling is required for ACM-induced C1q upregulation, in developing RGCs.

TGF- β signaling is required for regulation of C1q expression in the developing retina.

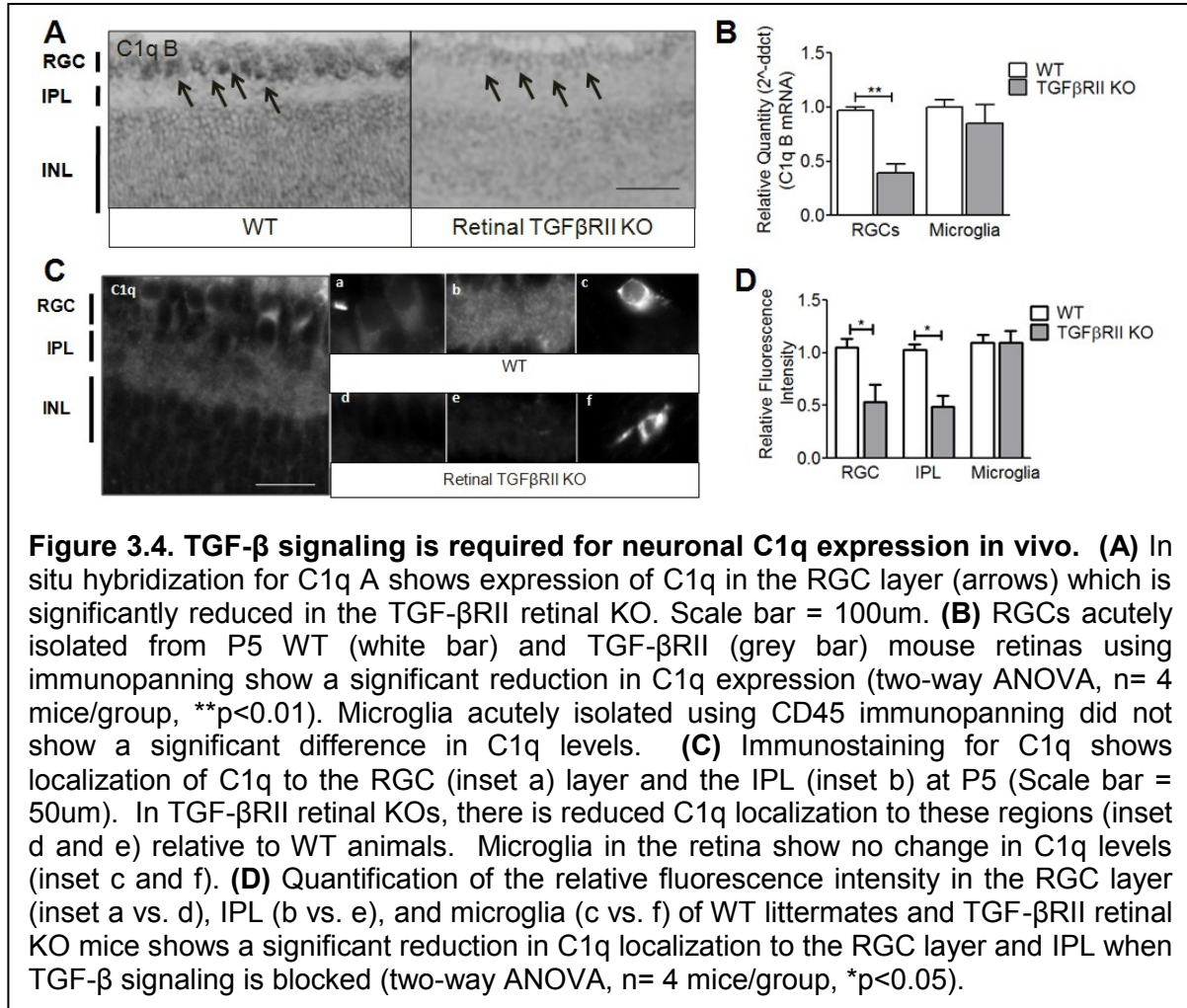
C1q is developmentally regulated in the postnatal retina showing a peak expression around P5 and sharply decreasing by P10-P15 (Stevens et al., 2007), corresponding with the period of synaptic refinement in the retinogeniculate system and with the presence of immature astrocytes throughout the developing CNS (Supplemental Figure 3A). Expression of all three isoforms of TGF- β can be detected by RT-PCR at P5 and in the mature retina (Figure 3.3A). In addition, TGF β RII protein can be detected in whole retinal lysates by western blot throughout



life; however, the level of TGF β RII protein is highest at P5 and sharply decreases by P15, correlating with C1q expression in the postnatal retina (Figure 3.3B). Immunostaining with antibodies against TGF β RII showed TGF β RII localization to the RGC and inner plexiform layers (IPL) of the retina at P5 and TGF β RII levels dramatically decreased by P15 (Figure 3.3C). Co-staining with anti-calretinin (Millipore), a marker of a subset of RGCs, confirmed localization of TGF β RII to RGCs (Figure 3.3C inset). Taken together, these data show that TGF- β ligands and TGF- β receptors are present at the right time in the retina to regulate C1q expression.

To determine if TGF- β signaling regulates C1q expression *in vivo* during retinogeniculate refinement, I used genetic and pharmacological approaches to block TGF- β signaling and measured C1q expression *in vivo*. Retina-specific TGF β RII KO mice were generated by crossing floxed TGF β RII mice (MMRC) with CHX10-Cre mice that express Cre in all neurons of the retina at E13.5 (de Melo et al., 2003; Rowan and Cepko, 2004). Knockout was confirmed by immunostaining for TGF β RII (Figure 3.3C) and by RT-PCR for TGF β RII using RNA from acutely isolated RGCs from P5 transgenic mice (Figure 3.3D). In addition, indicators of active TGF- β signaling were reduced as well. Transcripts for TIEG, a known TGF- β -dependent gene, were significantly reduced in RGCs from the TGF β RII retinal KO compared to WT RGCs (Supplemental Figure 3B), and immunostaining for pSmad 2/3 was reduced in the retina and specifically in Brn3a-positive RGCs (Figure 3.3E). These results indicate that my retina-specific knockout was successful and I observed no morphological abnormalities in these transgenic retinas (Supplemental Figure 3C,D). Cell numbers, axon specification, and retinal stratification showed no significant difference from WT retinas.

To test the requirement of retinal TGF- β signaling in developmental C1q expression in RGCs, retinas and RGCs from P5 TGF β RII retinal KOs and mice receiving intraocular injection of anti-TGF- β were assayed for changes in C1q expression relative to WT or vehicle treated littermates. *In situ* hybridization for *c1qb* on P5 retina cryosections revealed a decrease in signal for *c1qb* in the RGC layer of the retina in TGF β RII KO compared to WT (Figure 3.4A;

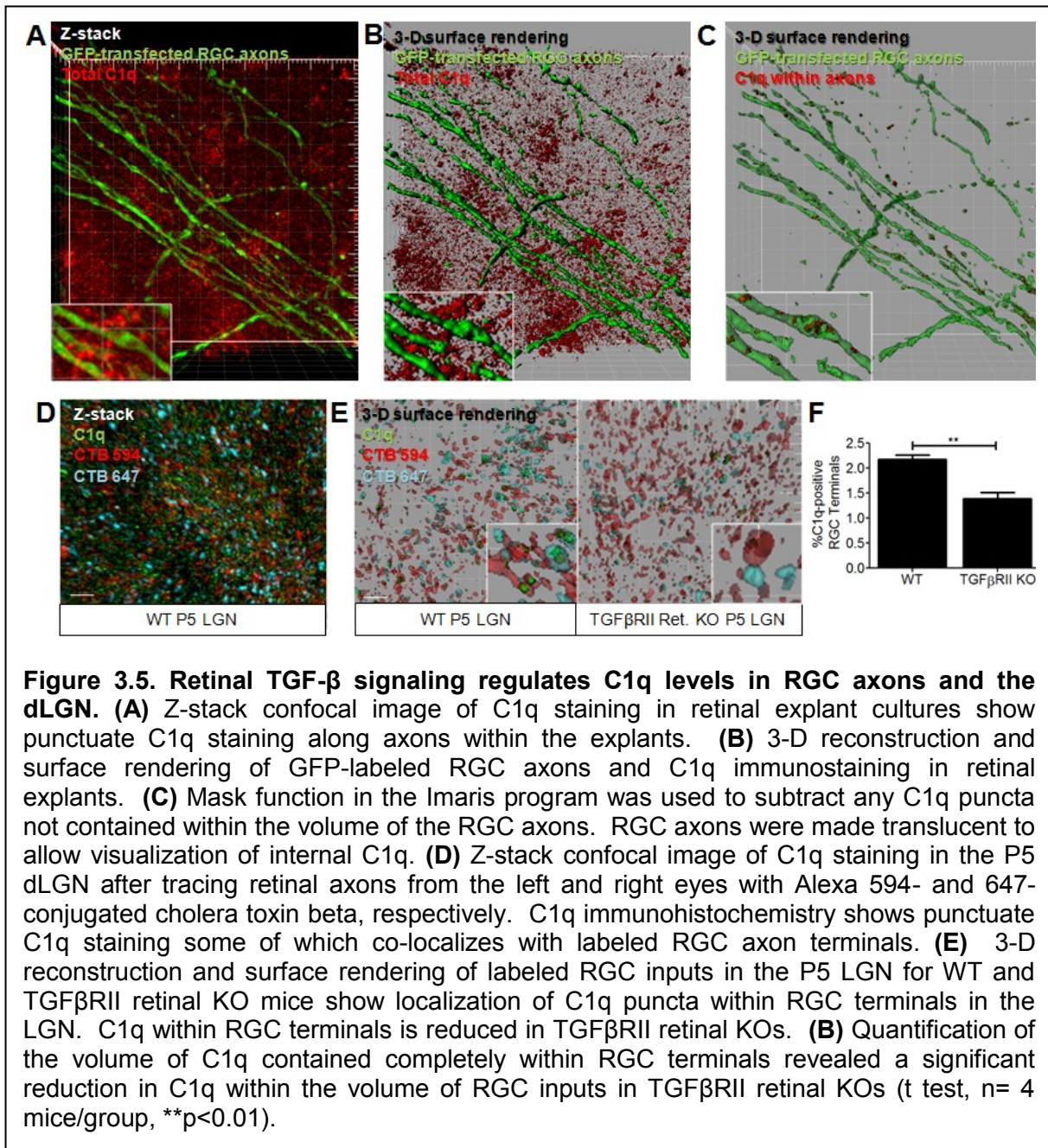


anti-TGF- β : Supplemental Figure 4A). To verify that the reduction in C1q expression was in RGCs and not other cell types in the RGC layer, such as microglia, RGCs and microglia were acutely isolated and purified from P5 retinas using established immunopanning techniques (Barres et al., 1988c; Foo et al., 2011). Acutely isolated RGCs from the retinal TGF β RII KO showed a significant reduction in C1q expression by qPCR while microglia showed no change in C1q levels (Figure 3.4B), as expected as microglia do not express the retinal neuron-specific gene, *Chx10*. Interestingly, P5 acutely isolated RGCs from the anti-TGF- β injected retinas also showed a significant reduction in C1q expression, while acutely isolated retinal microglia showed no change in C1q, suggesting TGF- β regulation of C1q may be specific to neurons (Supplemental Figure 4B). Cell purity was confirmed by qPCR for neuron and microglia specific genes, *nse* and *iba1*, respectively (Supplement Figure 4C). Immunostaining for C1q at P5 in TGF β RII retinal knockout and anti-TGF- β injected mice also showed a 40-50% reduction in C1q immunostaining in the IPL and RGC layers of the retina, but showed no change in microglial staining (Figure 3.4C, Supplemental Figure 4D). Taken together, these data show that TGF- β signaling is required for postnatal expression of C1q in RGCs and demonstrate that TGF- β upregulates C1q in RGCs, but not microglia.

Retinal TGF- β signaling regulates complement levels in retinogeniculate axons and in the LGN.

C1q and C3 localize to synapses in the dLGN during the period of synaptic refinement, but whether retinal ganglion cell neurons (RGCs) are a key source of C1q in the LGN is not known. One possibility is that neuronal C1q is transported along RGC axons and locally secreted into the dLGN to initiate complement-dependent synaptic pruning. Alternatively, microglia, although sparse in the dLGN at this time, may be the key source of secreted C1q in the postnatal dLGN during refinement.

To determine if C1q can be trafficked along RGC axons, I first used retinal explant-LGN co-cultures with GFP-labeled RGCs to clearly visualize individual RGC axons. I immunostained



these cultures for C1q and used confocal imaging to create z-stacks of the co-cultures (Figure 3.5A). 3-D reconstructions and surface rendering were done to define the volume of GFP-labeled RGC axons and the volume of C1q staining (Figure 3.5B). I used the mask function in Imaris to identify the volume of C1q staining contained completely within the volume of RGC axons (Figure 3.5C). I find that C1q immunopositive puncta can be observed completely within the volume of RGC axons (Figure 3.5C), suggesting that C1q can be transported along the axon.

To determine if C1q derived from RGC axons regulates C1q levels in the LGN during pruning in vivo, C1q protein levels and localization were examined in the LGN and within RGC terminals in the LGN during development in WT and TGF β RII retinal KOs. I first traced RGC axons from the left and right eyes with cholera toxin beta conjugated to Alexa 594 and 647, (CTB-594 and CTB-647) respectively, and then performed C1q immunohistochemistry on LGN sections. Confocal imaging was used to create z-stacks of RGC inputs and C1q staining in the LGN (Figure 3.5D). I then used Imaris software as described above to create a volume of RGC inputs and a volume of C1q staining and used the mask function to identify all C1q staining contained within the volume of the RGC inputs (Figure 3.5E). To verify that this technique could accurately detect proteins within the terminal versus those closely apposed to cell membranes, I also stained for vglut2, a vesicular protein enriched in RGC terminals, and PSD95, a postsynaptic protein which can be found closely apposed to RGC terminals. I found that my analysis accurately localized these proteins as expected and vglut2 could be detected within RGC terminals, while PSD95 puncta were rarely detected within the volume of RGC terminals (Supplemental Figure 5). As shown in Figure 3.5E, C1q puncta (shown in green) could be detected within the CTB-labeled RGC terminals in the LGN (red and cyan). These puncta disappeared in C1q KO animals, confirming the specificity of this staining (data not shown). Importantly, there was a significant decrease in the amount of C1q contained within the RGC axonal terminals in retinal TGF β RII KO mice (Figure 3.5E,F) which specifically have a reduction

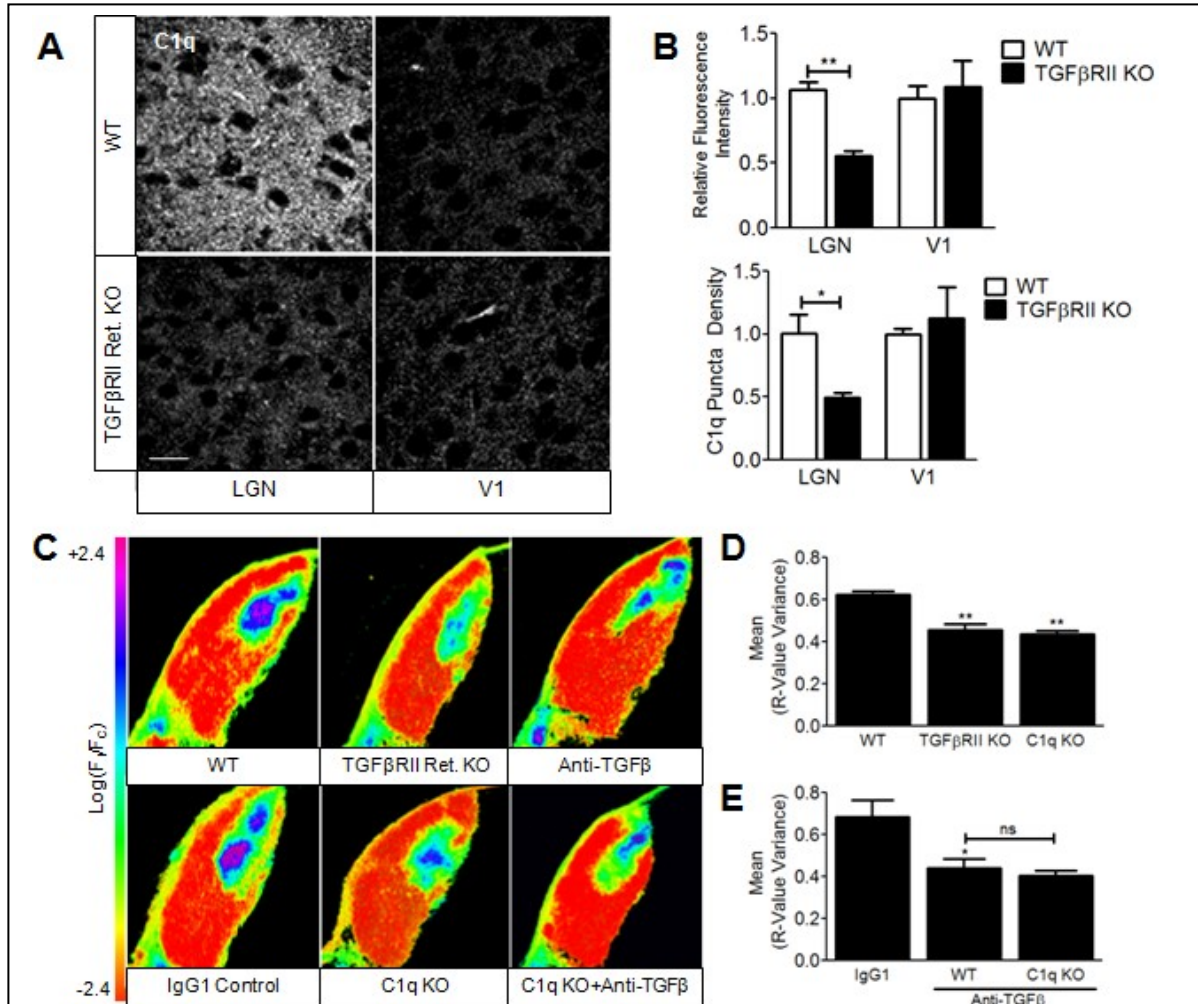
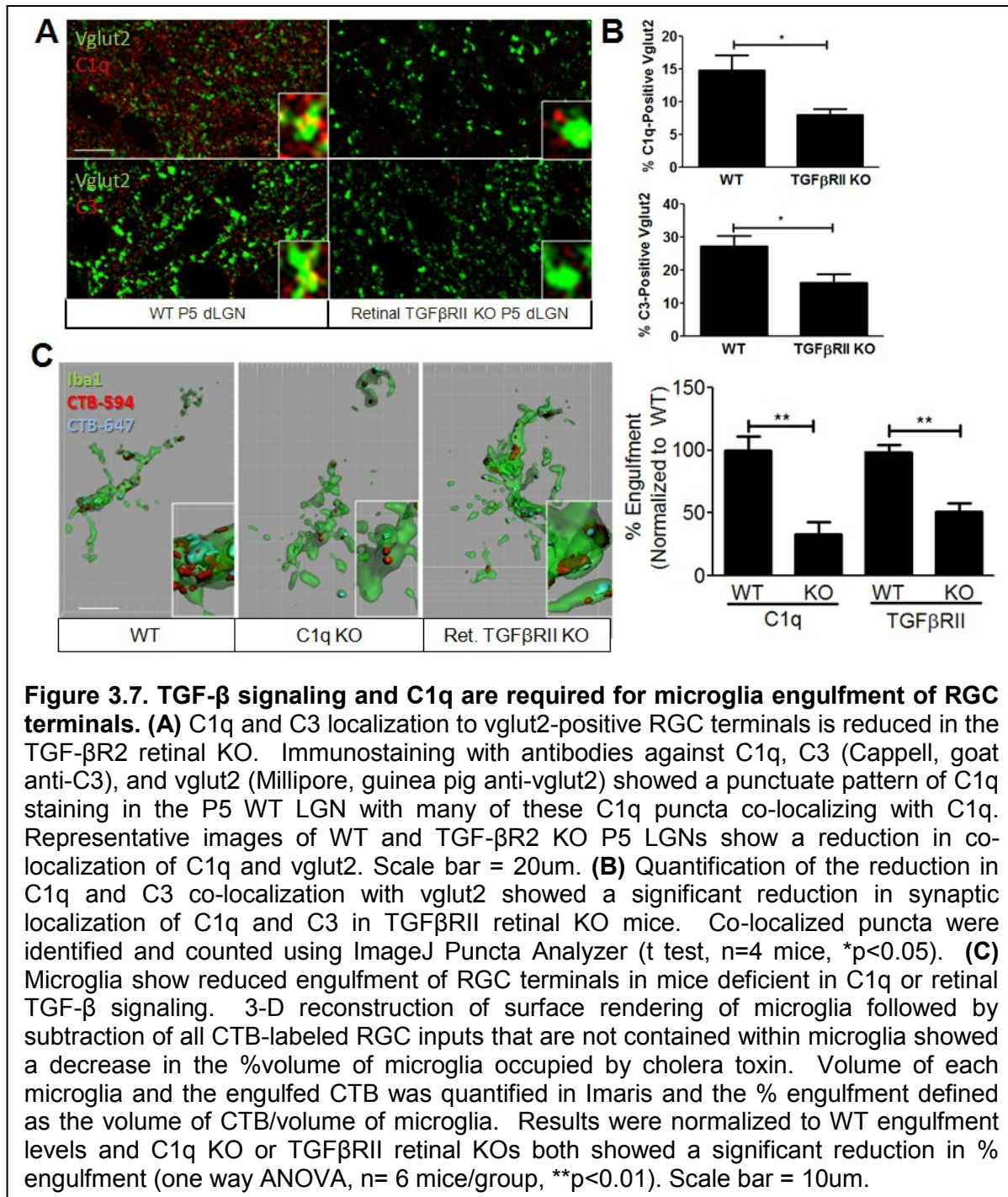


Figure 3.6. TGF- β signaling is required for eye specific segregation in the retinogeniculate system. (A) Immunostaining for C1q in the LGN and primary visual cortex (V1) shows a reduction in C1q fluorescence intensity in the LGN but not in V1 (scale bar = 100 μ m). **(B)** Quantification of the relative fluorescence intensity and density of C1q puncta shows a significant reduction in the intensity and the number of puncta per field of view in the LGN of TGF β RII retinal KO vs. WT (two-way ANOVA, $n=4$ mice/group, $**p<0.01$, $*p<0.05$). C1q puncta density was normalized to WT for each group and analysis was done using ImageJ puncta analyzer. No difference in intensity or C1q puncta density was observed in V1. **(C)** Representative dLGN images for WT, TGF β RII retinal KO, C1q KO, IgG1 control, and anti-TGF- β injected WT and C1q KO mice pseudocolored to show the R -value for each pixel. $R=\log_{10}(F_{ipsi}/F_{contra})$. **(D)** Quantification of the mean variance of the R -value for each group. A significant reduction in the mean variance of the R value is seen in mice deficient in TGF- β signaling or C1q, indicating a decrease in dark blue/purple pseudocolor pixels. (one way ANOVA, $n=6$ animals/group, $**p<0.01$). **(E)** There is no additional decrease in the variance when TGF- β signaling is blocked in C1q KO mice. Data shown as mean R value variance \pm SEM (one way ANOVA, $n=6$ animals/group, $*p<0.05$).

in C1q expression in RGCs. These results suggest that C1q from the retina is transported to RGC terminals in the LGN, since when TGF- β signaling is blocked only in retinal neurons, C1q cannot be found within RGC terminals.

If C1q in the LGN is supplied by RGCs, I would predict the retinal TGF β RII KO to show a reduction in C1q immunostaining throughout the LGN. Indeed, immunohistochemistry with a C1q antibody revealed a significant decrease in the intensity of C1q staining in the dLGN but not in primary visual cortex (V1) (Figure 3.6A), which is not a direct target for RGCs. Quantification showed a significant reduction in fluorescence intensity in the retinal TGF β RII KO as well as a significant decrease in the density of C1q puncta in the LGN but not in V1 (Figure 3.6B,C). TGF β RII retinal KOs showed no difference in dLGN size or the number of dLGN relay neurons as determined by SMI32 staining (data not shown).

My data suggest that RGC inputs secrete C1q into the dLGN, and previous work has shown that C1q localizes to synapses in the dLGN. While I see a reduction in total C1q levels in the dLGN in retinal TGF β RII KO mice, I wondered if there was a significant decrease in synaptic localization of complement, or if the amount of C1q remaining in the dLGN was sufficient to label the same number of synapses. To address whether retinal TGF- β regulation of C1q regulates complement localization to retinogeniculate synapses, I examined synaptic localization of complement in the LGN in TGF β RII retinal KO mice by immunostaining in the LGN using antibodies for C1q, C3, and, to mark RGC terminals, vglut2 (Figure 3.7A). I observed approximately 15% of vglut2-positive puncta co-labeled with C1q (Figure 3.7B). Only 2% of puncta contained C1q within terminals (Figure 3.5F), suggesting that the rest of the C1q staining is extracellular. Also, 25% of vglut2 puncta co-stained with the downstream complement protein, C3 (Figure 3.7B). C1q is upstream of C3 activation in the complement cascade and I find that C3 deposition in the LGN is completely absent in the C1q KO mouse (data not shown). In TGF β RII retinal KO dLGNs, I observed a significant reduction in synaptic localization of both C1q and C3 (Figure 3.7B), suggesting that retinal TGF- β signaling is



required for synaptic localization of complement proteins at retinogeniculate synapses. Given that TGF β RII retinal KOs exhibit specific reduction in TGF- β signaling and C1q in the retina, a reduction in C1q and C3 co-localization with synaptic puncta in the dLGN suggests that RGC derived C1q, and not local C1q from microglia in the LGN, is required to activate C3, allowing it to localize to synapses.

TGF- β signaling is required for eye specific segregation.

In C1q KOs, RGC axons do not segregate into eye specific territories normally as assessed by anterograde tracing techniques (Stevens et al., 2007). If regulation of C1q by retinal TGF- β signaling is required for eye specific segregation, then TGF β RII retinal KO mice should exhibit a phenotype similar to the C1q KO. To test this hypothesis, I used established anterograde tracing techniques to visualize the formation of eye specific territories in the LGN and to assay eye specific segregation in TGF β RII retinal KO mice and in mice injected with anti-TGF- β . Mice received intraocular injections of CTB-488 and CTB-594 at P9 in the left and right eyes respectively and mice were sacrificed at P10. I assayed eye specific segregation using an unbiased assay in which the degree of segregation is defined as the variance of the distribution of the logarithm of the ratio of fluorescence intensity from each fluorescence channel (R value) (Torborg and Feller, 2004). Using this assay, high variance signifies a high degree of segregation in the LGN, while low variance corresponds to increased overlap between contralateral and ipsilateral territories. Compared to WT controls, my results showed a significantly lower variance in C1q KO mice, as expected, and in TGF β RII retinal KOs (Figure 3.6D,E) and mice injected with anti-TGF- β , indicating a defect in eye specific segregation in these mice (Figure 3.6F). I also confirmed these results by quantifying the percent overlap using the original method used to identify the C1q KO phenotype (Supplemental Figure 6A,B).

To address whether TGF- β regulates synaptic refinement independently of C1q, I performed intraocular injection of anti-TGF- β neutralizing antibodies in WT and C1qA KO

animals to determine if blocking TGF- β disrupts synaptic refinement in the absence of C1q. If TGF- β and C1q regulate pruning by different mechanisms, blocking TGF- β in the C1qA KO should lead to a more severe phenotype, or increased overlap compared to vehicle-injected C1qA KOs. If TGF- β and C1q are in the same pathway, blocking TGF- β signaling in the C1qA KO mouse should have no effect. Blocking TGF- β in the C1qA KO did not result in a more severe phenotype, consistent with TGF- β exerting its effect on synaptic refinement via regulation of C1q (Figure 3.6D,F; Supplemental Figure 6A-B). Taken together, these results show that retinal TGF- β signaling is required for eye specific segregation and that TGF- β and C1q likely work in the same pathway regulating this process.

Retinal TGF- β signaling and C1q regulate microglia engulfment of RGC terminals.

Recent work has demonstrated that microglia engulf RGC terminals during the pruning period in a complement-dependent manner (Schafer et al., 2012b). Microglia engulfment of retinogeniculate synapses is thought to be the final step in complement-dependent synapse elimination; therefore, if RGC-derived C1q regulates this process, I predict TGF β RII retinal knockout mice to show a reduction in microglial engulfment of RGC terminals. To test this hypothesis, I used an established microglia engulfment assay to quantify microglial phagocytosis of RGC inputs in C1qA KO mice and retinal TGF β RII KO mice. P4 mice received intraocular injection of CTB-594 and CTB-647 in the left and right eyes respectively. Mice were sacrificed at P5 and brains were sectioned and stained with antibodies against Iba1, a microglial marker. Using established methods, CTB labeled RGC inputs within microglia in the dLGN were imaged using confocal microscopy and the volumes of the microglia and RGC inputs were reconstructed in the Imaris program to determine the percentage of microglia volume occupied by CTB (Schafer et al., 2012b). I found a significant reduction in the engulfment of RGC inputs in C1q KO and TGF β RII KO mice as compared to littermate controls (Figure 3.7C). I found no

differences in the number, distribution, or morphology of microglia in the LGNs in WT vs. C1q or TGF β RII KO conditions (supplemental Figure 5D). These results show that reduction of retinal TGF- β signaling, and hence C1q expression in RGC neurons, significantly reduced the microglial phagocytosis of RGC terminals in the LGN. Together these data support the hypothesis that RGC-derived C1q is required to initiate the process of complement-dependent synapse elimination.

Discussion

This study establishes that TGF- β signaling in RGC neurons plays a key role in refinement of RGC synapses on their target relay neurons in the dLGN neurons. Taken together these experiments demonstrate that retinal TGF- β signaling is necessary for C1q- and microglia-mediated pruning to occur in the LGN. I demonstrated that TGF- β signaling is necessary and sufficient for transcriptional upregulation of C1q in purified RGCs (Figure 3.2B,C). Consistent with in vitro studies, conditional knockout of TGF β RII in retinal neurons led to reduced C1q expression in RGCs during the period of active refinement of retinogeniculate synapses in the thalamus (Figure 3.4). Importantly, inhibition of TGF- β signaling in the postnatal retina resulted in significant defects in eye specific segregation in the dLGN that mimicked the phenotype observed in global C1q KOs (Figure 3.6). My findings support a model in which retinal TGF- β signaling controls expression and local release of C1q in the dLGN to regulate microglia-mediated, complement dependent synaptic pruning (Figure 5.1). In support of this model, specific disruption of retinal TGF- β signaling resulted in a significant reduction in the deposition of C1q and downstream C3 (Figure 3.7) at retinogeniculate synapses. Microglia engulfment of RGC terminals was also significantly reduced in retinal TGF β RII KOs, which phenocopied the engulfment defects observed in C1q, C3, and CR3 KOs (Schafer et al.,

2012b). Together, these data suggest that TGF- β regulation of C1q expression in the retina is critical for complement-dependent synapse elimination in the developing CNS.

C1q Regulates Microglia-Mediated Pruning in the Thalamus

Increasing evidence implicates microglia in developmental synaptic pruning (Paolicelli et al., 2011a; Schafer et al., 2012b; Tremblay et al., 2010); however, the mechanisms that control the timing and location of microglia-mediated engulfment remain elusive. Process bearing phagocytic microglia in the postnatal LGN engulf RGC inputs during this period in a manner dependent on complement C3/CR3 signaling and neuronal activity (Schafer et al., 2012b). These findings suggest that microglia, expressing complement receptor 3, recognize and phagocytose inappropriate synapses that are tagged by the opsonin and ligand for CR3, C3. C3 becomes an active opsonin and ligand for CR3 as a result of activation of the classical complement cascade. C1q is the initiator of the classical complement cascade, and thus, a critical regulator of C3 activation. My findings suggest that retinal C1q activates the classical complement cascade in the dLGN resulting in C3 deposition and microglia-mediated pruning. In support of this model, microglial engulfment of RGC terminals decreases when retinal C1q expression is reduced in TGFBR1I retinal KO mice (Figure 3.7C). Moreover, I observed a significant decrease in microglial engulfment of RGC inputs and a complete absence of C3 deposition at synapses in C1q KOs (Figure 3.7). These findings are consistent with the normal role for C1q in the immune system, where C1q is required for C3 deposition on apoptotic debris or pathogens, tagging this material for engulfment by phagocytic macrophages. In the developing CNS, I find that neuronal C1q is required to activate C3 and localize it to synapses, initiating microglia-mediated, complement-dependent synaptic refinement.

Previous work revealed that microglia-mediated pruning is regulated by neuronal activity (Schafer et al, 2012) raising the question of whether neuronal activity regulates the expression, local trafficking, or targeting of C3 to specific synapses. During the retinogeniculate pruning

period, microglia preferentially engulf less active RGC inputs (Schafer et al, 2012) in the dLGN, suggesting microglia can sense/read out local changes in synaptic activity. Later in development during experience dependent refinement, microglia dynamics and association with synapses and dendritic spines in visual cortex are modulated by visual experience (Tremblay et al., 2010), suggesting that microglia can 'sense' local changes in neuronal activity. Many of the molecules implicated in synaptic refinement, such as MHC I (Corriveau et al., 1998a) and neural pentraxins (Tsui et al., 1996), show activity-dependent transcriptional regulation. C1q expression corresponds with the onset of retinal wave activity and the phenotype observed in C1q KO mice closely mimics the phenotype observed when spontaneous activity is disrupted in the retina. Thus, I envision a model in which C1q is locally secreted from synaptic terminals in an activity-dependent manner to regulate C3-CR3 dependent synaptic phagocytosis by microglia. Whether and how neuronal activity regulates complement expression and function are open areas of investigation.

Retinal C1q is Required for Retinogeniculate Refinement

In the postnatal retinogeniculate system, C1q expression can be detected in both microglia, macrophage-like cells of the brain, and in retinal ganglion cells. Intriguingly, my results demonstrate that RGCs contribute significantly to C1q levels in the LGN for complement-dependent synaptic refinement. In TGF β RII retinal KO mice, which only have defects in C1q expression in RGCs and not microglia, I observe defects in retinogeniculate refinement and microglial engulfment (Figures 3.6 and 3.7), suggesting that microglial expression of C1q cannot compensate for the loss of RGC-derived C1q. Microglia, like peripheral macrophages, express C1q throughout life and do not change expression levels in response to TGF- β (Supplemental Figure 3.4B). Although microglia are present in the LGN during the refinement period and express C1q at this time, microglia are relatively sparse in the postnatal dLGN (Supplemental Figure 3.6A) versus older ages. As development progresses, microglia density increases in the

LGN and throughout the brain (Schafer et al, 2012), yet circuits stabilize over time, suggesting that microglial C1q expression is insufficient to initiate synaptic refinement.

In support of a role for retinal C1q in synaptic refinement, retinal ganglion cells (RGCs) exhibit a unique developmental expression of C1q restricted to the first week of postnatal development during the peak of complement-dependent synaptic refinement in the dLGN (Stevens et al, 2007; Supplemental Figure 3A). Indeed, C1q levels in the LGN are significantly reduced in the retina-specific TGF β RII KO mice, demonstrating that a significant portion of C1q is supplied by RGCs (Figure 3.5C). Moreover, I observe C1q within RGC terminals in the dLGN (Figure 3.5A) and can detect C1q within RGC axons in retinal explant cultures (Figure 3.4D). While utilizing local C1q secreted by microglia in the dLGN would seem to be a simpler mechanism for complement-dependent synaptic refinement, my results suggest that a significant portion of C1q in the LGN comes from RGC inputs and that this portion of C1q is critical for synaptic refinement. Given the importance of spontaneous retinal activity in driving synaptic refinement, it is intriguing to speculate that secretion of C1q from RGC inputs is a molecular cue linking information about the retina to the LGN to ensure proper circuit development.

Interestingly, C1q and TGF- β expression are also expressed in embryonic and postnatal spiral ganglion neurons (Lu et al., 2011), raising the question of whether TGF- β signaling regulates complement expression in other neurons and brain regions as well. Very little is known about synaptic refinement in the auditory system; however, there is a characterized elimination of spiral ganglion inputs to outer hair cells that occurs in the first postnatal week (Huang et al., 2007). C1q expression increases in spiral ganglion neurons around this window of refinement, suggesting that C1q may have a more general role in refining sensory systems throughout the CNS. Future studies using conditional neuronal and microglia C1q KO mice will be needed to definitively answer this question in the developing visual system and throughout the CNS.

TGF- β : a key regulator of neuronal C1q expression and function in the developing CNS.

In the immune system, TGF- β is characterized as an anti-inflammatory cytokine that dampens the immune response. In the mammalian nervous system, TGF- β signaling pathways regulate diverse developmental processes, including regulation of neuronal survival and programmed cell death, axon specification, and synaptogenesis. Embryonic and postnatal periods of programmed cell death in the mouse retina are modulated by TGF- β signaling (Beier et al., 2006) and also the efficacy of neurotrophic factors critical for cell survival, such as GDNF, is amplified by TGF- β signaling (Peterziel et al., 2002). In the developing rodent neocortex, TGF- β signaling is necessary and sufficient for axon specification. Recent work has also shown that TGF- β regulates synaptogenesis in cortical neurons in vitro (Diniz et al., 2012; Yi et al., 2010) and at the *Drosophila* and *Xenopus* NMJs (Feng and Ko, 2008b). At the *Drosophila* NMJ, glia are the source of the TGF- β ligand (Fuentes-Medel et al., 2012) and they direct synaptogenesis via regulation of a neuron-derived TGF- β family member and the downstream RacGEF Trio (Ball et al., 2010). TGF β also affects synaptic function at excitatory and inhibitory synapses in the pre-Bötzing complex in the mouse brainstem (Heupel et al., 2008a) and modulates sensory neuron excitability and synaptic efficacy at sensorimotor synapses in the sea slug *Aplysia* (Chin et al., 2002a).

In the present study, TGF- β signaling in RGCs is a key regulator of developmental synaptic refinement through regulation of C1q expression. I found TGF β RII signaling in the retina did not dramatically affect the number of retinal neurons or retinal morphology. Moreover, disruption of TGF β RII signaling does not alter axon specification in transgenic retinas, despite the role for TGF β RII in axon specification in cortex. I observe normal axon initial segment staining (Supplemental Figure 3D) and can effectively use anterograde tracers in retinal TGF β RII KO mice (Supplemental Figure 6A), suggest that either RGCs use a different mechanism to specify axons or that the process is complete by time TGF β RII is deleted in my TGF β RII retinal KO mice (E13.5). Although I have not quantified synapses in the dLGN of my

TGF β RII KO mice, it is unlikely that defective synaptogenesis would contribute to decreased synapse refinement measured as increased overlap between eye specific territories. In addition, previous studies have reported that TGF- β is not synaptogenic in RGC cultures (Christopherson et al., 2005), further supporting that synaptogenesis is likely to be normal in these mice.

Astrocytes Regulate Synaptic Refinement via TGF- β

Astrocytes potently regulate synapse development and function. Astrocytes secrete a number of synaptogenic factors, including thrombospondins, glypicans, hevin, and SPARC. In the postnatal mammalian CNS, astrocytes are a major source of cytokines including TNF α , TGF- β , IL-6, IL-1 beta, IL-2, IL-18, IL-8, and interferon-alpha and -beta (Boulanger and Shatz, 2004) many of which have been implicated in synapse development and plasticity. At the *Drosophila* NMJ, glia modulate synaptogenesis by releasing a TGF- β family ligand, Maverick, that stimulates neuronal expression of Gbb, a retrograde signal from the postsynaptic cell that triggers synaptogenesis (Fuentes-Medel et al., 2012). My results suggest that astrocytes also modulate synaptic refinement in the mammalian CNS through the regulation of C1q expression.

I provide evidence that astrocyte-secreted TGF- β is necessary and sufficient to upregulate C1q in purified RGCs in vitro. Immunodepleting TGF- β from astrocyte conditioned media prevented C1q upregulation and TGF- β 3 in particular was sufficient to upregulate C1q at concentrations measured in ACM. My in vivo data demonstrate that postnatal retinal astrocytes produce all isoforms of TGF- β , but TGF- β 3 is enriched in postnatal astrocytes compared to RGCs and microglia, implicating astrocytes as the likely source of TGF- β required for C1q expression in RGCs. I did find that RGCs and microglia purified from the postnatal retina express TGF- β to a lesser extent than retinal astrocytes and that astrocytes from other regions can also produce TGF- β . My data show that retinal TGF- β plays a key role in regulation of C1q expression and complement-mediated synaptic pruning in the dLGN; however, the contribution

of local astrocyte and microglia-derived TGF- β and the specific isoforms of TGF- β that are required to regulate neuronal C1q expression and function in vivo are still open questions

In addition to relevance in remodeling of circuits in the healthy brain, my findings have important implications for understanding mechanisms underlying synapse elimination in the diseased brain. Dysregulation of immune system components including complement components and cytokines has been demonstrated in many CNS disorders and diseases including epilepsy, schizophrenia, and neurodegenerative disorders such as glaucoma and Alzheimer's disease. Many of these disorders have been associated with synapse loss or dysfunction, suggesting that aberrant complement upregulation may reactivate the developmental synapse elimination pathway in disease to promote synapse loss. In particular, TGF- β localizes to beta-amyloid plaques and has been linked to the formation of these plaques in Alzheimer's disease (Wyss-Coray et al., 1997) and blocking TGF- β and Smad2/3 signaling mitigates plaque formation in mouse models of Alzheimer's (Town et al., 2008). C1q has been found associated with plaques in Alzheimer's brain as well (Afagh et al., 1996), and in mouse models of Alzheimer's, C1q-deficiency has been shown to be neuroprotective (Fonseca et al., 2004b). My work demonstrating a new link between TGF- β signaling, complement, and synapse elimination opens up new avenues of investigation into the role of this regulatory mechanism for C1q in these disorders and in other regions of the healthy CNS during development.

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CHAPTER 4:

Retinal Ganglion Cells Exhibit Developmental Activity-dependent Expression of C1q

Contributions: All experiments were designed by Beth Stevens and Allison Bialas. All experiments and data analysis were performed by Allison Bialas.

Introduction

During development, patterns of spontaneous and experience driven neuronal activity sculpt developing neural circuits, in part, by regulating neuronal gene expression. Calcium- and activity-dependent transcription have been implicated in multiple neuronal processes including neuronal survival (Mao et al., 1999), dendrite formation (Vaillant et al., 2002), regulation of synapse number (Flavell et al., 2006; Lin et al., 2008; Margolis et al., 2010), and synaptic plasticity (Barco et al., 2002; Bourtchuladze et al., 1994; Etkin et al., 2006). Patterned spontaneous retinal activity plays an instructive role in the refinement of retinogeniculate synapses, yet surprisingly little is known about how differences in neuronal activity are linked to the specific removal of certain synapses. Activity-dependent transcription of synaptic refinement cues is thought to be critical for this process; however, the identities of the activity-dependent genes required for synaptic refinement are largely unknown.

Recently, components of the classical complement cascade, a critical innate immune pathway, were implicated in synapse elimination (Stevens et al., 2007). The first clue that complement proteins played a role in synapse development came when the genes encoding C1q, the initiating protein of the classical complement cascade, were identified in an unbiased screen for genes that were upregulated in purified postnatal retinal ganglion cell neurons (RGCs) by astrocytes (Stevens et al., 2007). Chronic exposure to astrocytes promotes synaptogenesis and increases spontaneous activity in RGCs, suggesting a link between synaptic activity, C1q, and synapse development.

In the developing retina, expression of C1q in RGCs is restricted to the period of activity-dependent synaptic remodeling in the retinogeniculate system, implicating this immune molecule in developmental retinogeniculate refinement. Based on the role of the classical complement cascade in the innate immune system (Figure 1.3), complement proteins were thought to be critical for eliminating inappropriate synapses. Indeed, C1q and the downstream

complement component, C3, localize to synapses in the LGN during this period (Stevens et al., 2007) and mediate synapse elimination in the retinogeniculate system by a mechanism involving microglia-mediated synaptic phagocytosis (Schafer et al., 2012a). Importantly, in vivo manipulations of spontaneous retinal activity revealed that microglia in the dLGN preferentially engulf RGC inputs projecting from the less active eye, demonstrating that microglia-mediated pruning is an activity-dependent process (Schafer et al., 2012a). A major question raised by these findings is whether complement expression and complement mediated synapse elimination are regulated by neuronal activity. My recent work has revealed that RGC-derived C1q is critical for initiating the process of complement-dependent synapses elimination (Chapter 3), therefore C1q, the initiating protein of the classical complement cascade, could be a key point of activity-dependent regulation. In Chapter 3, I also identified astrocyte -derived TGF- β as a key signal regulating C1q expression in RGCs (Chapter 3; Figure 3.3, 3.4); however, it remains unclear how TGF- β signaling throughout the retina can result in complement-dependent elimination of specific synapses.

I hypothesize that activity-dependent regulation of C1q is necessary for complement-dependent synapse elimination in the retinogeniculate system. Indeed, there are several key pieces of evidence that support link between complement and activity. First, developmental expression C1q in the postnatal retina corresponds to the period of spontaneous retinal wave activity, an established regulator of retinogeniculate refinement (Feller, 2009; Penn et al., 1998; Stellwagen and Shatz, 2002; Stevens et al., 2007). Moreover, C1q KO and C3 KO mice show sustained defects in synapse elimination that closely resemble the phenotype observed when spontaneous retinal wave activity is perturbed (Huberman, 2007; Rossi et al., 2001; Stevens et al., 2007). In addition, chronic astrocyte exposure is known to trigger increases in neuronal activity and C1q upregulation triggered by chronic astrocyte exposure corresponds with these increase in neuronal firing (Pfrieger and Barres, 1997; Ullian et al., 2004). Finally, several components of the complement system, including C3, C2, and multiple complement regulatory

proteins were recently found to be upregulated in the hippocampus after *in vivo* high frequency stimulation (Håvik et al., 2007). Interestingly, C1q and C3 are both upregulated during periods of aberrant neuronal activity in epilepsy (Aronica et al., 2007; Vezzani, 2008); however, it is not clear whether activity directly triggers this upregulation.

Here, I show for the first time that expression of C1q, the initiating protein of the classical complement cascade, can be rapidly induced in an activity-dependent and calcium-dependent manner in neurons *in vitro* and *in vivo*. I find that acutely exposing RGCs to astrocyte-secreted factors leads to increased neuronal activity and intracellular calcium levels that mediate, in part, astrocyte- and TGF- β -induced upregulation of C1q. I also show that neuronal activity modulates the expression of C1q in the retina and LGN during the period of synaptic refinement in the retinogeniculate system. Lastly, I find that the activity-dependent process of microglia-mediated synaptic pruning requires C1q, suggesting that complement is an important cue to help microglia properly target RGC inputs for engulfment. Together, these results identify a new activity-dependent molecule in synaptic refinement and provide novel insight into the mechanism of activity-dependent microglial synaptic pruning.

Results

Astrocytes Trigger Acute Changes in Neuronal Activity

One of the many features distinguishing neurons from glia is that neurons are electrically excitable and can fire action potentials. Interestingly, adding astrocytes or ACM to RGC cultures has been shown to increase neuronal firing over the course of several days, further suggesting a link between neuronal activity and C1q upregulation (Pfrieger and Barres, 1997; Ullian et al., 2001). However, my recent findings revealed that C1q can be induced in RGCs within 15 minutes of adding ACM and acute astrocyte-induced increases in neuronal firing have not been previously reported. This finding was unexpected and suggested that C1q transcription in neurons can be induced in the order of minutes, similar to classic activity-

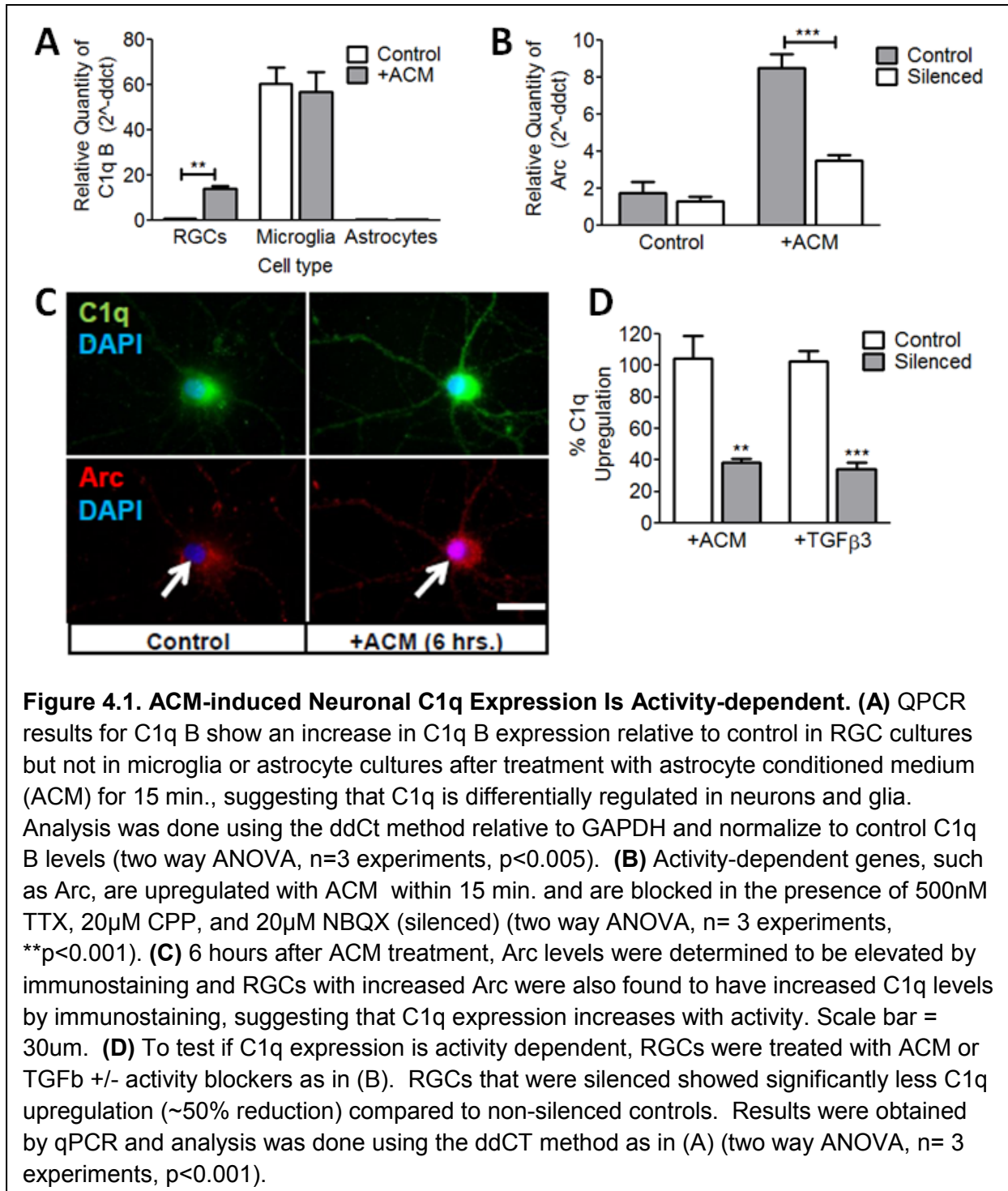
dependent transcription factors such as cFos (Greenberg et al., 1986). To determine if unique pathways were regulating C1q expression in neurons and glia, I added astrocyte conditioned medium (ACM) to purified RGC, microglia, and astrocyte cultures acutely (15 minutes) and assayed changes in C1q expression by qPCR. ACM treatment only triggered C1q upregulation in RGCs (Figure 4.1A), and not glial cells, suggesting activity-dependent pathways may contribute to C1q expression in neurons.

To determine if acute treatment with ACM changed neuronal firing in RGCs, I performed qPCR to assay changes in a known activity-dependent immediate early gene, *arc* (Lyford et al., 1995). I observed significant increases in *arc* expression 15 min. after the addition of ACM (Figure 4.1B) that were blocked in the presence of activity blockers (500nM TTX, 20 μ M CPP, and 20 μ M NBQX). By immunostaining, I detected a significant increase in *arc* protein levels within the first few hours of exposing RGCs to ACM (Figure 4.1C). Interestingly, there was a strong correlation between robust immunostaining for C1q and increased *arc* staining, suggesting that C1q is more highly expressed in actively firing neurons.

Neuronal C1q Upregulation Is Activity-dependent and Calcium-dependent

To determine if neuronal activity is required for C1q upregulation, I treated RGCs +/- ACM with a cocktail of activity blockers (500nM TTX, 20 μ M CPP, and 20 μ M NBQX) to completely silence neuronal firing. I confirmed that ACM-induced upregulation of *Arc* and cFos were significantly reduced in silenced neurons as a control for activity-dependent gene expression (Figure 4.1B). I found that silenced neurons treated with ACM showed a significantly reduced upregulation of C1q by qPCR (Figure 4.1D).

In addition, I previously found that TGF- β was necessary and sufficient for C1q upregulation (Figure 3.2), therefore, I hypothesized that TGF- β -dependent regulation of C1q



is also activity-dependent. To address this question, I treated RGCs with recombinant TGF- β 3 (50pg/ml) with the same cocktail of activity blockers (500nM TTX, 20 μ M CPP, and 20 μ M NBQX) to completely silence neuronal firing. My results showed that upregulation of C1q by adding recombinant TGF- β 3 (50pg/ml) was also significantly blocked when neurons were silenced (Figure 4.1D), suggesting that the TGF- β pathway of C1q upregulation in neurons is modulated by neuronal activity. The rapid kinetics of C1q upregulation and the effects of activity on C1q expression levels suggested that calcium-dependent pathways could be regulating C1q expression. Calcium is a well-known modulator of activity-dependent gene transcription, but it was unknown whether acute ACM application could trigger calcium increases in neurons. To address this question, I used the ratiometric calcium-sensitive dye Fura-2 to measure changes in calcium levels in RGCs during ACM application. I found that ACM induced a significant increase in intracellular RGC calcium levels within 1 minute of ACM application (Figure 4.2A). To test if C1q transcription was calcium-dependent, I treated cultures with ACM in the presence or absence of 2 mM EGTA to chelate calcium in culture media. In the presence of EGTA, neither ACM nor TGF- β produced a significant upregulation of C1q (Figure 4.2B). Similar effects were observed when 100 μ M CdCl₂ was added to the culture medium to block calcium influx through voltage-gated calcium channels (Figure 4.2B). In addition, I found that treating RGC cultures with 55mM KCl, which depolarizes neurons and promotes calcium-influx through voltage-gated calcium channels, also upregulated C1q in a calcium-dependent manner (Figure 4.2B). I verified that this result was not specific for KCl-induced depolarization by treating with 20 μ M glutamate, 20 μ M forskolin, and by transfecting RGCs with the Designer Receptors Exclusively Activated by Designer Drugs (DREADD) HM3d construct to drive GPCR activated calcium-increases (Armbruster et al., 2007)(Figure 4.2C). All 3 treatments gave a similar, significant upregulation of C1q by qPCR, suggesting that increasing intracellular calcium levels is sufficient to upregulate C1q expression. Increased C1q protein levels also were observed by immunohistochemistry in KCl-depolarized versus control RGC cultures after a six hour

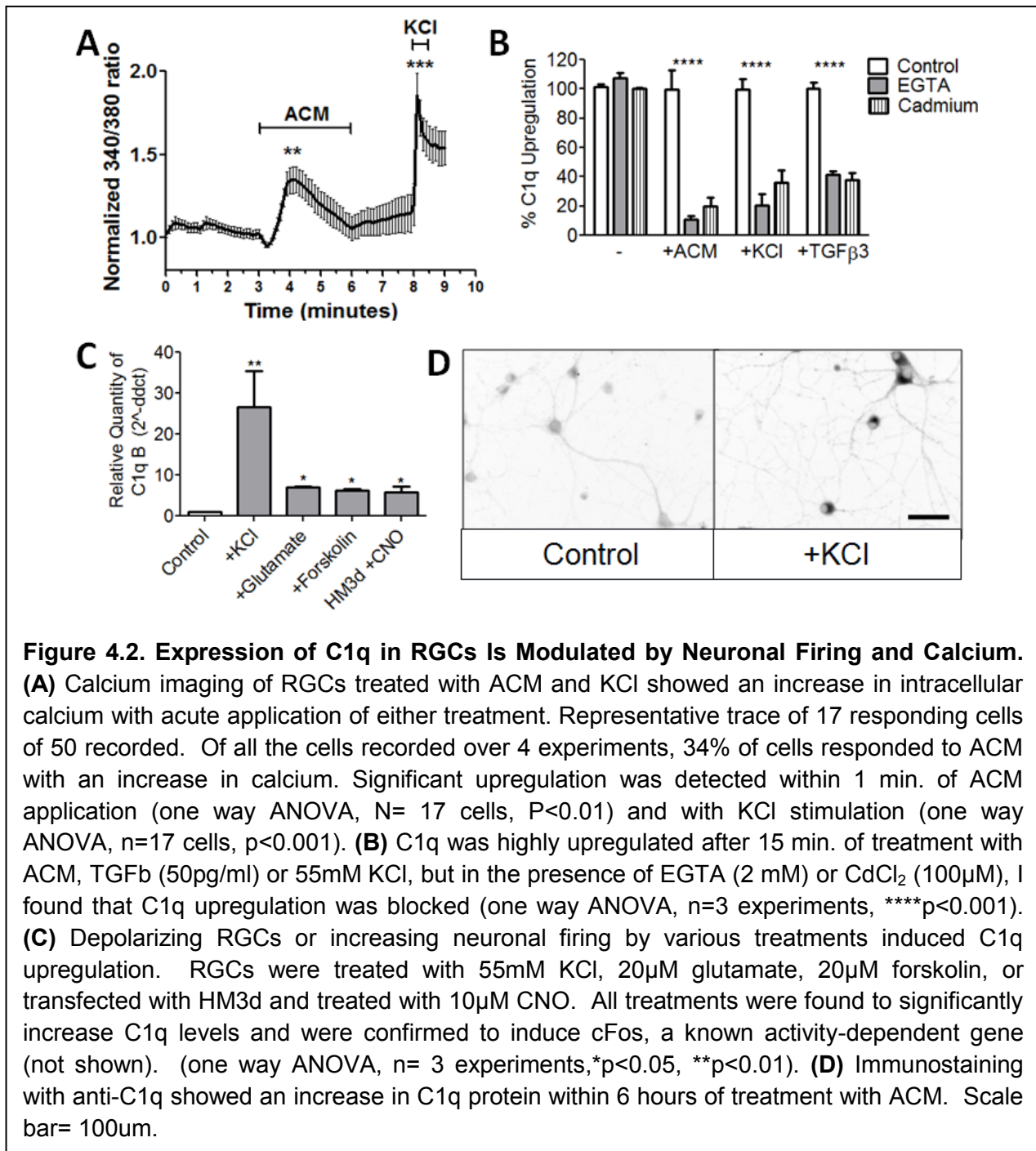


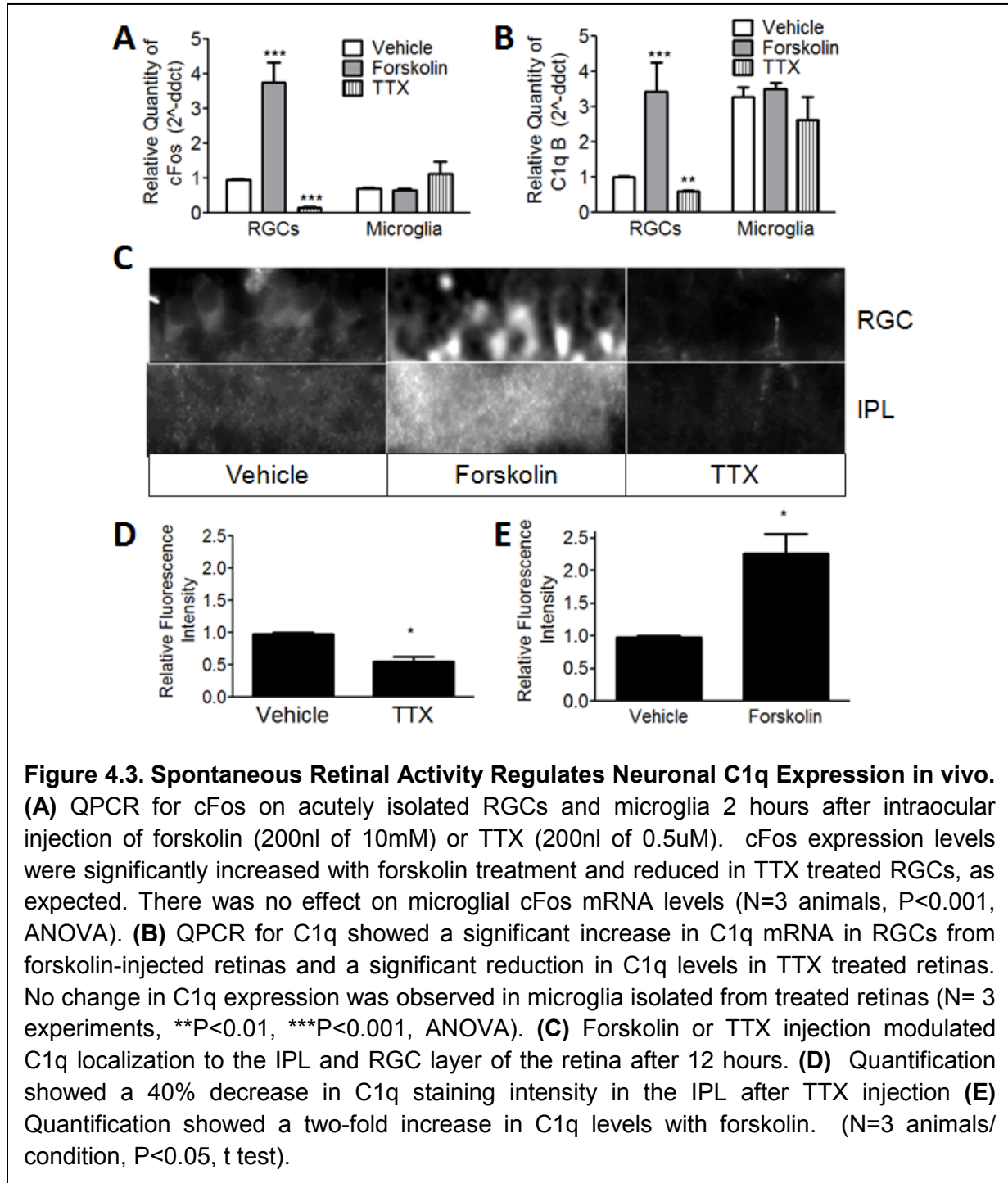
Figure 4.2. Expression of C1q in RGCs Is Modulated by Neuronal Firing and Calcium.

(A) Calcium imaging of RGCs treated with ACM and KCl showed an increase in intracellular calcium with acute application of either treatment. Representative trace of 17 responding cells of 50 recorded. Of all the cells recorded over 4 experiments, 34% of cells responded to ACM with an increase in calcium. Significant upregulation was detected within 1 min. of ACM application (one way ANOVA, N= 17 cells, $P < 0.01$) and with KCl stimulation (one way ANOVA, $n = 17$ cells, $p < 0.001$). **(B)** C1q was highly upregulated after 15 min. of treatment with ACM, TGF β (50pg/ml) or 55mM KCl, but in the presence of EGTA (2 mM) or CdCl $_2$ (100 μ M), I found that C1q upregulation was blocked (one way ANOVA, $n = 3$ experiments, **** $p < 0.001$). **(C)** Depolarizing RGCs or increasing neuronal firing by various treatments induced C1q upregulation. RGCs were treated with 55mM KCl, 20 μ M glutamate, 20 μ M forskolin, or transfected with HM3d and treated with 10 μ M CNO. All treatments were found to significantly increase C1q levels and were confirmed to induce cFos, a known activity-dependent gene (not shown). (one way ANOVA, $n = 3$ experiments, * $p < 0.05$, ** $p < 0.01$). **(D)** Immunostaining with anti-C1q showed an increase in C1q protein within 6 hours of treatment with ACM. Scale bar= 100 μ m.

treatment (Figure 4.2D). To determine if activity-dependent C1q expression was a general phenomenon in all neurons, I treated cultures of hippocampal neurons and cortical neurons, along with my purified RGCs, with 55mM KCl for 15 minutes to stimulate activity-dependent transcription in all three cell types. To verify that neurons were depolarized with this treatment, qPCR was used to assay cFos levels. As expected, cFos levels were significantly increased in all three neuronal cultures (Supplemental Figure 8A), but qPCR for C1q showed a dramatic, significant upregulation of C1q only in RGCs (Supplemental Figure 8B). My results suggest that activity-dependent C1q upregulation may be specific to RGCs or that my purification method, which exclusively isolates RGCs (Barres et al., 1988b), excitatory neurons in the retina, may help to bring out C1q changes in these cultures versus mixed cortical or hippocampal cultures.

Neural Activity Modulates C1q Expression in the Developing Retina

To determine if neuronal activity regulates C1q expression in vivo at the peak of the retinogeniculate refinement period (P5), I first blocked or increased spontaneous retinal activity via intravitreal injection of 200nl of 500 μ M TTX or 20mM forskolin in P4 animals. I assayed C1q levels using multiple techniques at P5. First, I confirmed that my activity manipulations were effective by performing qPCR for the known activity-dependent gene, cFos, on cDNA prepared from P5 RGCs acutely isolated by immunopanning 24 hours after intraocular injection. In initial experiments, cFos levels were increased with forskolin treatment as expected, but TTX-treated mice showed no change in cFos, suggesting that the effects of TTX may have worn off (data not shown). I then shortened my treatment time to 2 hours, and at this time point, cFos levels were increased in forskolin-treated mice and decreased in TTX-treated mice (Figure 4.3A). C1q expression levels assayed by qPCR in acutely isolated P5 RGCs also showed a similar increase and decrease with respective forskolin and TTX treatments at 2 hours (Figure 4.3B). Interestingly, C1q expression levels in microglia were not affected by these treatments



(Figure 4.3A,B). By immunostaining, I observed a 40% reduction in C1q protein levels in TTX-treated retinas and a two-fold increase in fluorescence levels in forskolin-treated retinas (Figure 4.3C-E). Taken together, these results suggest that C1q expression levels in the retina are modulated by neuronal activity during the peak of developmental synaptic refinement.

Retinal Activity Modulates Complement Localization in the dLGN during Synaptic Refinement.

I next determined if activity-dependent regulation of C1q in the retina affected C1q localization and function in the LGN. Immunostaining for C1q in the P5 LGN, at the peak of synaptic refinement, showed a significant reduction in C1q staining in mice 12 hours after binocular TTX injection (200nl of 500 μ M), while binocular intraocular injection of forskolin resulted in increased C1q staining in the LGN (Figure 4.4A), suggesting that manipulating spontaneous retinal activity modulates C1q secretion from RGC axons into the LGN.

As shown in Figure 4.4A, immunostaining for C1q resulted in a punctate staining pattern in the LGN. Many of these puncta co-localized with RGC terminals, as shown in the previous chapter (Figure 3.7A), consistent with the idea that C1q tags synapses for removal. However, I also found that C1q can be detected within CTB-labeled RGC inputs in the LGN (Figure 3.5A). Given my data that C1q expression increases in RGCs in vivo with forskolin treatment, I wondered if forskolin treatment would also result in increased C1q levels within RGC terminals in the LGN. To address this question, I injected forskolin (200nl of 20mM) and vehicle into the left and right eyes of P4 mice, respectively, and also injected CTB-594 and CTB-647 to the respective eyes to label RGC projections from forskolin and vehicle treated eyes (Figure 4.4B). I then performed immunostaining for C1q in the LGN at P5 and collected Z-stack confocal images of the LGN in regions innervated by both forskolin (red labeled) and vehicle (blue labeled) RGC inputs (Figure 4.4C). Using Imaris (Bitplane) software, I then performed 3-D reconstructions and surface rendering of the terminals and C1q staining within the LGN as in

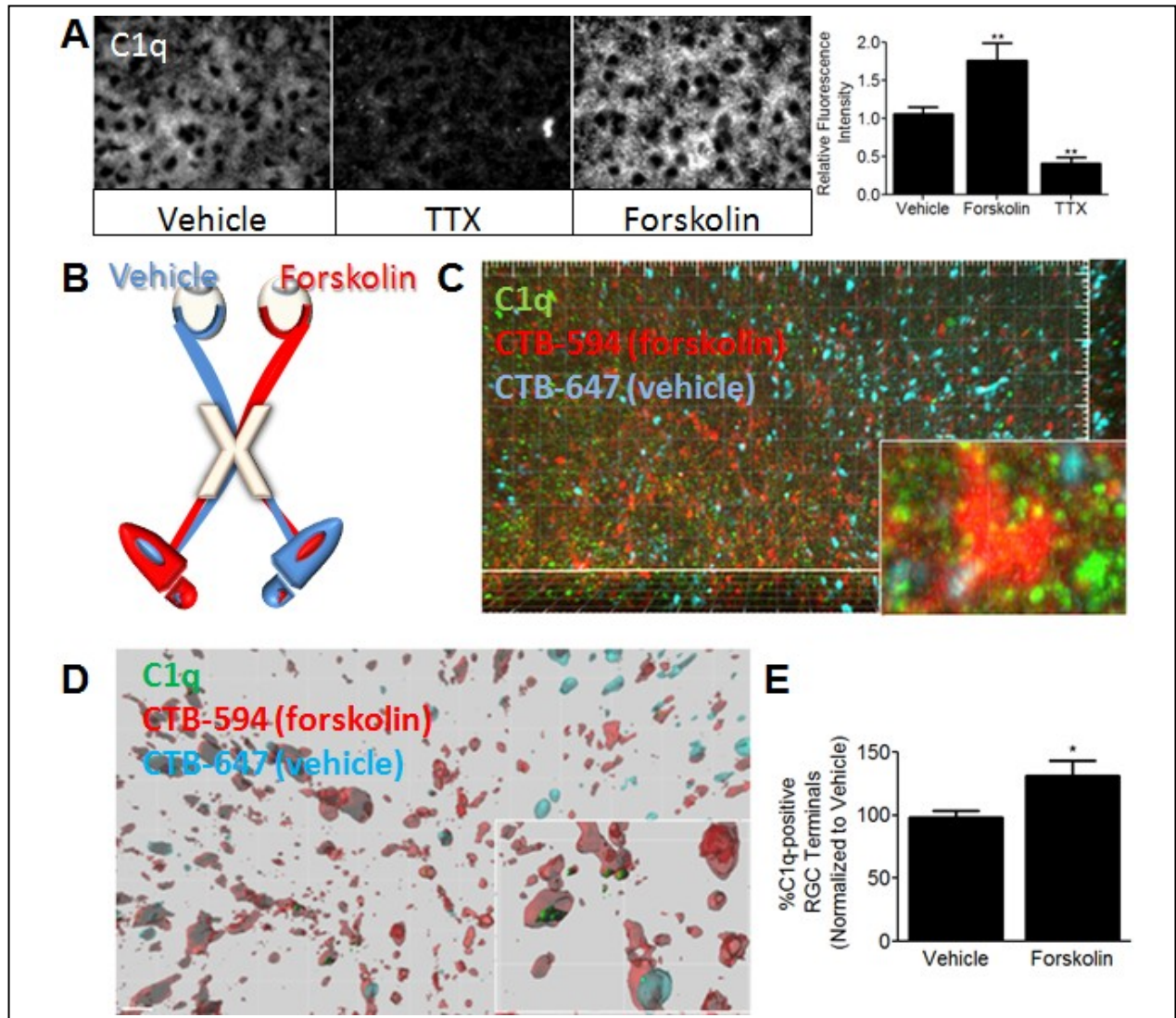


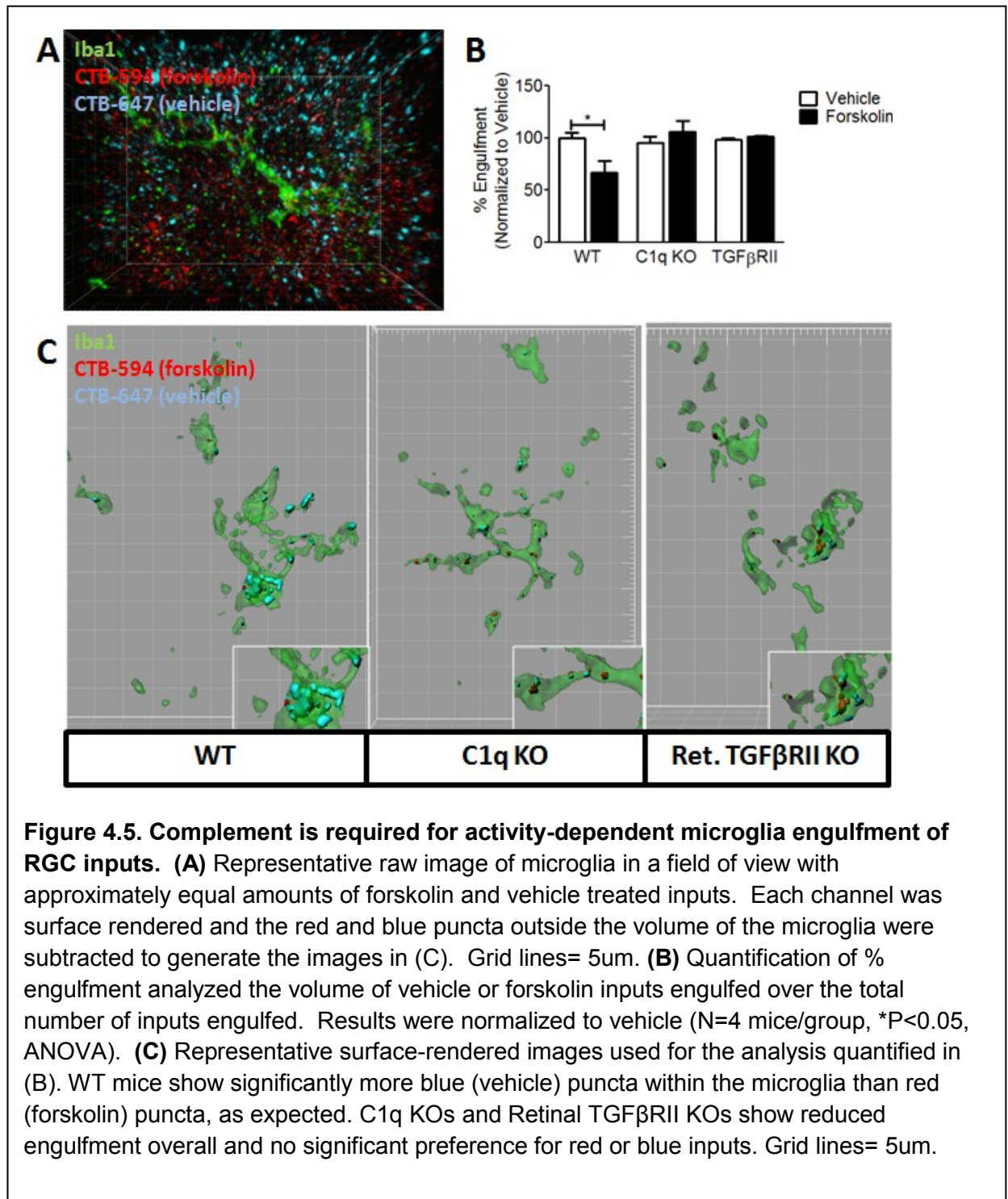
Figure 4.4. Neuronal Activity Modulates C1q Levels in the dLGN and within RGC

Terminals. (A) Immunostaining for C1q in the P5 mouse dLGN showed a strong reduction in C1q when retinal activity was reduced with TTX and increased with forskolin compared to vehicle controls (N= 4 mice, **P<0.01, ANOVA). (B) Monocular injections of forskolin and vehicle (DMSO) were done at P4 and eyes were labeled with CTB-594 (red) and CTB-647 (blue), respectively, to create a difference in retinal activity between the two eyes. At P5, projections from more active (forskolin; red) and less active (vehicle; blue) could then be observed in the dLGN. (C) Immunostaining was done for C1q (green) in the P5 dLGN after monocular forskolin (red) and vehicle (blue) injections to label RGC projections. Z-stack confocal images were collected. Inset shows co-localization of C1q puncta with red and blue puncta. Gridlines= 10um. (D) To determine if C1q was localized within RGC terminals, 3-D reconstruction and surface rendering were done as described in chapter 3. (E) Quantification of the volume of RGC terminal occupied by C1q showed significantly more C1q in terminals from forskolin-treated RGCs. (N=4 mice, P<0.05, t test).

chapter 3. Using the mask function, I determined the volume of C1q staining completely within the volume of forskolin-treated or vehicle-treated terminals (Figure 4.4D). To verify that this technique could accurately measure C1q within the terminal and exclude C1q that could be closely apposed to the cell membrane, I also stained with antibodies against vglut2 and PSD95. Vglut2, a presynaptic protein, could be detected completely within RGC terminals using this method, while PSD95, a postsynaptic protein, though closely apposed to RGC terminals, was detected at very low levels within CTB-labeled terminals using my method of analysis (Supplemental Figure 5). My results showed that increasing spontaneous retinal activity with forskolin injection significantly increased the volume of C1q puncta within the RGC terminals (Figure 4.4E), suggesting that more active RGCs transport more C1q to the LGN than their less active counterparts.

C1q Is Required for Activity-dependent Microglial Engulfment of RGC Terminals

Microglia have recently been identified as critical mediators of activity-dependent synapse elimination (Schafer et al., 2012a). When retinal activity was perturbed by intraocular injection of forskolin or TTX, microglia preferentially engulfed CTB-labeled RGC terminals from the less active eye (Schafer et al, 2012). Microglia engulfment of RGC terminals is also dependent on complement C3-CR3 signaling; however, whether complement was required for the observed activity-dependent engulfment was still not clear. To address this question, I performed forskolin injections in one eye as in Figure 4.4B and quantified the CTB-labeled RGC inputs from forskolin- and vehicle-treated eyes engulfed by microglia in C1q KOs and WT littermates. I immunostained LGN sections for Iba1 (Wako, 1:500) to label microglia and collected Z-stack images to measure microglia engulfment as in the TGFb chapter. I focused on regions of the LGN with equal innervations from the contralateral and ipsilateral eyes where microglia had equal chances of contacting either vehicle or forskolin treated inputs



(Figure 4.5A). In WT mice, I found that, consistent with published work, microglia preferentially engulfed RGC inputs from the vehicle-treated eye (Figure 4.5B,C); however, in C1q KO mice, microglia showed no preference for inputs from either eye and engulfed vehicle- and forskolin-treated inputs with equal frequency. Further, TGF β RII retinal KO mice that show reduced C1q levels (Chapter 3) also showed a similar phenotype in which microglia had no preference for weaker RGC inputs (Figure 5B,C). Taken together, these results suggest that C1q is required for activity-dependent microglial engulfment of RGC inputs.

Discussion

My results demonstrate for the first time that C1q expression in neurons is upregulated by neuronal activity and suggest that C1q may be a critical link between neuronal activity and microglia-mediated pruning of inappropriate synapses. In purified RGC cultures, I found that C1q upregulation by astrocytes or by TGF- β , the previously identified component of ACM required for C1q upregulation, are both blocked when cells are silenced with a cocktail of activity blockers (Figure 3.1D). Furthermore, I found that C1q upregulation is calcium-dependent and that many treatments that increase intracellular calcium levels can upregulate C1q in RGCs (Figure 4.2). Moreover, developmental retinal C1q expression in RGCs in vivo was found to be modulated by spontaneous retinal activity (Figure 4.3). Importantly, C1q levels in the dLGN were also modulated by activity and analysis of C1q levels within RGC terminals in the dLGN showed that C1q was specifically increased in RGC terminals of more active neurons (Figure 4.4). Finally, my results provided evidence that activity-dependent microglial engulfment of RGC inputs is C1q-dependent. In both the C1q KO and in TGF β RII retinal KOs that have reduced C1q expression in RGCs, microglia showed no preference for less active RGC inputs while WT microglia preferentially engulfed RGC inputs from the less active eye (Figure 4.5). Together, my results show that C1q expression is activity dependent and suggest that activity-dependent regulation of C1q guides microglial engulfment of RGC terminals.

C1q: a Link between Retinal Activity and the Elimination of Specific Synapses

Although there are other molecules that have been implicated in synaptic refinement, it is still unclear how activity-dependent regulation of these molecules is linked to the elimination of specific synapses. One hypothesis that has been proposed for how synapse elimination could occur is known as the “Punishment Model” (Jennings, 1994; Lichtman and Colman, 2000) (Figure 1.1). This model suggests that synapse elimination could be accomplished by the activity-dependent expression of two signals: a punishment signal secreted by strong inputs to punish weaker neighbors and a protective signal that shields strong inputs from this punishment signal. Interestingly, the complement cascade fits nicely into the framework of this punishment model. My results show that C1q expression is activity-dependent (Figure 4.3), that more C1q can be found within the terminals of more active RGCs (Figure 4.4), and that microglia lose their preference for less active RGC inputs in the absence of C1q (Figure 4.5). While we still do not know if C1q is directly tagging synapses for elimination or “punishing” weaker inputs by activating C3 to tag weaker inputs, my results suggest that C1q is required for microglia to properly target less active inputs. Ongoing work in the lab, using a newly developed in vitro model of activity-dependent synaptic competition, will allow us to further investigate whether C1q and/or C3 are specifically targeting ‘weaker’ retinogeniculate synapses.

An open question is whether there is also a protective signal in complement-dependent synapse elimination. In the immune system, there are several “don’t eat me’ signals that protect healthy or self cells from inappropriate macrophage-mediated phagocytosis. In addition, there are a number of complement inhibitor proteins in the immune system and many of these proteins are expressed in the CNS (Elward and Gasque, 2003), including CD59, DAF, Crry, and CSMD1 (Davoust et al., 1999; Kim and Song, 2006; Kraus et al., 2006). Moreover, C1q itself may have additional roles in the nervous system outside of just triggering complement deposition. C1q-like molecules, such as Cbln1 and C1ql1-4, are expressed in the CNS and have been demonstrated to regulate synapse formation and synapse density (Bolliger et al.,

2011; Iijima et al., 2010; Yuzaki, 2010). Could C1q have similar properties to the C1q-like molecules and actually work to strengthen some synapses and eliminate others? This idea is intriguing given the broad distribution of C1q staining that I observed throughout the dLGN (Figure 4.4). C1q does not seem to localize exclusively to inappropriate synapses, suggesting that it may have other functions. C1q also does not appear to be regulated in the same way in all neurons, consistent with my results that depolarizing cortical or hippocampal neurons did not trigger significant C1q upregulation (Supplemental Figure 8B). Recent work has shown that C1q expression in dorsal spinal neurons decreases slightly with activity, suggesting that an alternative mechanism may be at work in this system (Simonetti et al., 2013). RNAi experiments knocking down C1qC in spinal neurons led to an increase in spine number while adding C1q reduced spine number (Simonetti et al., 2013), suggesting that C1q may function to limit synapse number in these cells. Interestingly, in RGC cultures, I have noticed a similar effect, discussed further in chapter 5 (Supplemental Figures 9-12), although I observe an increase in C1q expression with neuronal activity.

C1q Is Required for Activity-dependent Microglia-mediated Synaptic Engulfment

Microglia have recently emerged as critical mediators of synapse development and function. Work from our lab revealed that microglia prune retinogeniculate projections C3-CR3 dependent phagocytosis. Interestingly, when retinal activity was modulated during microglial mediated pruning, microglia preferentially engulfed inputs originating from the less active eye, suggesting a link between neuronal activity and microglial engulfment. Thus, both complement signaling and activity were established as regulators of microglia-mediated engulfment of RGC inputs, but it was not clear if the activity-dependent component of engulfment was also complement dependent. Moreover, it was unclear if C1q was needed for this process or if C3 could auto-activate via alternative complement pathway signaling to eliminate synapses.

My recent findings revealed defects in microglial engulfment of RGC inputs in C1q KO mice and in TGF β RII retinal KO mice (Figure 3.7), suggesting that initiation of the complement cascade by TGF- β dependent expression of C1q in RGCs is the trigger upstream of microglia-mediated synaptic refinement. This finding, combined with my discovery that C1q expression is activity-dependent (Figure 4.3, 4.4), suggested that C1q may be a cue linking differences in neuronal activity levels with microglial engulfment. To test this idea, I assayed microglial preference for weak inputs after retinal activity manipulation in C1q KO mice and in TGF β RII retinal KO and found that microglia lost their preference for less active inputs in these mice (Figure 4.5). Given that C3 is downstream of C1q in the complement cascade, it will be intriguing to determine if C3 deficient mice exhibit similar defects or if activity-dependent expression of C1q is enough in the absence of C3 to accurately guide microglia to preferentially engulf less active inputs.

Interestingly, roles for microglia in synapse development and function have been described in other brain regions as well. In visual cortex, recent *in vivo* live imaging studies have highlighted the highly dynamic way in which fine microglial processes survey the CNS environment (Nimmerjahn et al., 2005; Wake et al., 2009). Many recent studies have supported changes in microglial dynamics in response to neuronal activity and sensory experience (Ransohoff and Stevens, 2011; Schafer et al., 2012c; Tremblay et al., 2011). In visual cortex, manipulating sensory experience through short-term light deprivation and re-exposure led to changes in microglial dynamics such that light re-exposed mice had an increase in microglial apposition to synaptic clefts and increased microglial engulfment of “synapse-associated elements” in an electron microscopy study (Tremblay et al., 2010). Interestingly, light deprivation was associated with reduced microglial motility and large dendritic spines contacted by microglia during light deprivation shrank over time (Tremblay et al., 2010). These results suggest intriguing experience-dependent changes in microglial dynamics; however, the molecular mechanisms driving this process are unknown.

In the hippocampus, transient deficits in synaptic pruning have been observed in the CX3CR1 KO mice corresponding with a transient decrease in microglial density in these mice (Paolicelli et al., 2011a). Importantly, this phenotype was thought to be attributed to decreased microglial numbers rather than to a specific role for CX3CR1 signaling in synapse development. Indeed, while many studies have reported interesting connections between microglia, neuronal activity, and synapse development, few molecular mechanisms have been proposed to explain these interactions. Thus, whether and how activity-dependent expression of complement proteins influences microglial dynamics in other brain regions is an active area of research.

TGF- β and Activity Cooperatively Regulate C1q

I previously found that TGF- β regulates C1q (Chapter 3), but here I have reported that C1q expression can also be stimulated directly by depolarizing RGCs and increasing intracellular calcium. These findings suggest three possibilities: 1) TGF- β signaling pathways can be directly stimulated by neuronal activity in the absence of TGF- β ligand. 2) TGF- β ligand is secreted by neurons in an activity-dependent manner. 3) TGF- β - and activity-dependent pathways represent two parallel mechanisms for C1q induction. While my experiments have not completely ruled out any of these possibilities, I find that TGF- β upregulation is activity dependent and calcium dependent, suggesting that these pathways are likely to be linked and not two alternative mechanisms of C1q expression.

If TGF- β signaling and activity can upregulate C1q by some common pathway, there are many possible points of intersection, which are illustrated in Figure 4.6. One possibility is that TGF- β ligand is secreted by neurons or neighboring astrocytes in response to increased neuronal activity. Indeed, activity-dependent secretion of TGF- β has been reported in mixed cortical cultures, but given the speed at which C1q upregulation is observed upon depolarization (within 15 minutes), this option seems unlikely. Another possibility is that activity modulates the

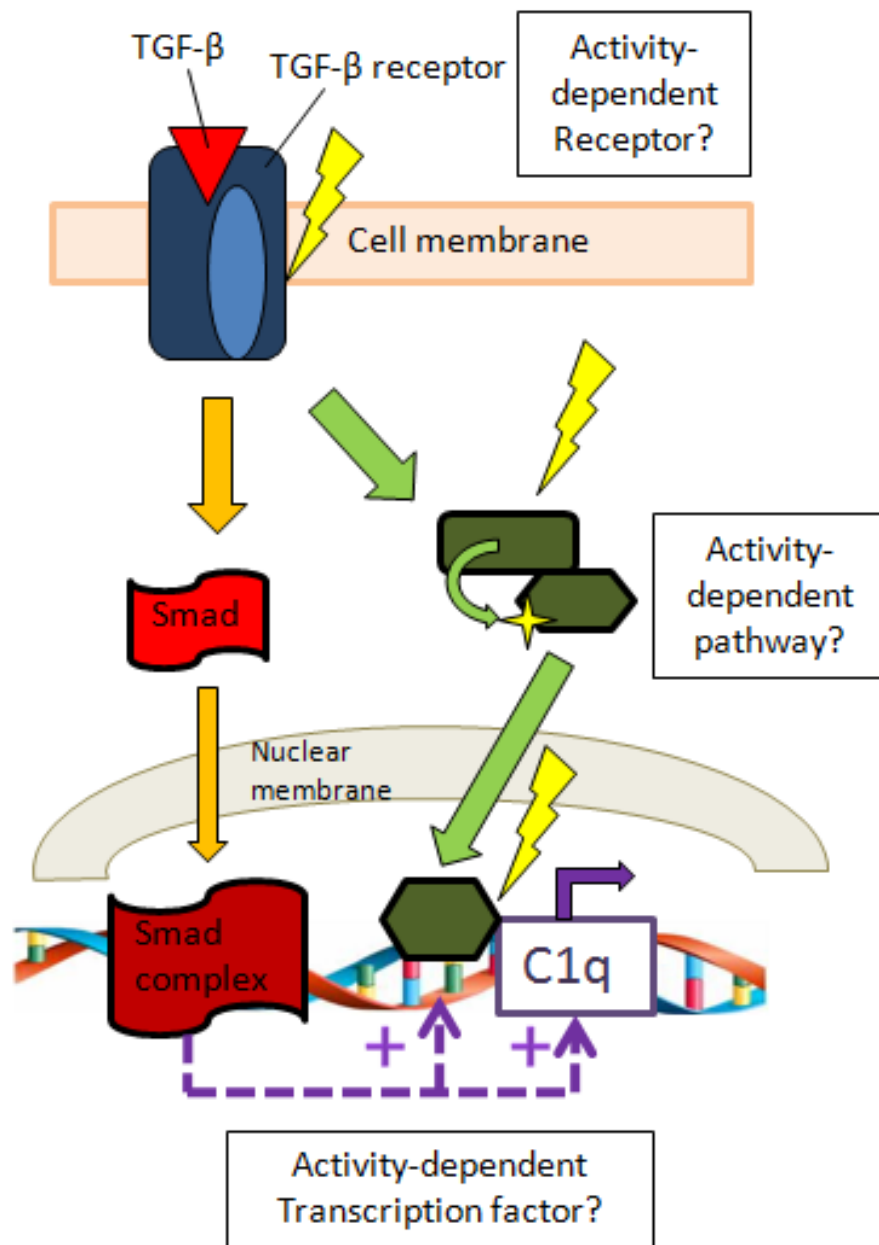


Figure 4.6. Model for Cooperative Regulation by TGF- β and Activity. There are several possible levels at which TGF- β and neuronal activity may interact to regulate C1q transcription. Activity may change receptivity for TGF- β by modulating receptor expression levels or trafficking to the cell membrane. Alternatively, TGF- β and neuronal activity may stimulate common downstream kinase pathways or activate transcription factors that interact in the nucleus to drive C1q transcription.

receptivity for TGF- β by increasing the number of TGF β R_s on the RGC cell membrane. My experiments have shown that TGF β R_{II} expression does not change with activity in RGCs in vivo; however, I have not ruled out the possibility that activity enhances TGF β R_{II} trafficking. Downstream of TGF β R_{II} signaling, it is possible that Smad transcription factors are activated by activity. Indeed, certain activity-dependent kinases can directly phosphorylate Smad proteins (Derynck and Zhang, 2003; Engel et al., 1999). Furthermore, Smads can interact with other activity-dependent co-factors in the nucleus, such as cfos and CREB (Topper et al., 1998; Zhang et al., 1998). In particular, Smads can interact with cFos and cJun to regulate gene transcription at AP-1 sites (Zhang et al., 1998). Very little is known about the promoter elements driving expression of the three C1q genes, but it is intriguing to speculate that specific sequences recruiting both Smads and activity-dependent co-factors, such as AP-1 sites, may be present in the C1q promoter regions. In addition, TGF- β and activity both activate some common kinase pathways, including MAPK, which could explain my results that either TGF- β or activity can stimulate C1q transcription (Derynck and Zhang, 2003; Massague, 2000). Although the work presented here does not distinguish at which level TGF- β and activity are working together, my results do show that activity is required for TGF β -dependent upregulation of C1q and the level at which TGF- β and activity work together is an open area of research.

Calcium-dependent Transcription in Synapse Development

Neuronal activity enhances and represses distinct subsets of genes to drive many neurodevelopmental processes. During the initial phases of synapse development, activity-dependent gene transcription helps to establish neuronal networks and, later, it functions to adapt connectivity based on sensory experience. Activity-dependent transcription is initiated when evoked potentials in the postsynaptic cell trigger calcium influx, which activates pathways such as the Ras/MAPK pathway, the calcium/calmodulin-dependent protein kinases, the phosphatase calcineurin, and Rac GTPases (Greer and Greenberg, 2008). Differences in

patterns of neuronal activity preferentially drive certain pathways over others and consequently activate the transcription of specific subsets of activity-dependent genes to effect synaptic changes (Greer and Greenberg, 2008; Itoh et al., 1995).

Previous work has implicated a number of activity-dependent genes in synapse formation and development, but just a handful of activity-dependent genes have been implicated in synaptic refinement. Interestingly, two of these genes are associated with immune system function, MHC I and neuronal pentraxins (Bjartmar et al., 2006; Corriveau et al., 1998a; Huh et al., 2000). Class I MHC molecules were first identified as activity-dependent when infusion of tetrodotoxin to block all action potential activity in the LGN significantly reduced expression of class I MHC molecules (Corriveau et al., 1998a). Furthermore, increasing neuronal firing with kainic acid induced seizure resulted in dramatic increases in class I MHC expression (Corriveau et al., 1998a). The role for class I MHC in activity-driven structural remodeling and synaptic plasticity was discovered by examining mice lacking three molecules required for class I MHC signaling: $\beta 2$ – microglobulin, CD3 ζ and TAP1. All three mouse mutants showed defects in synaptic refinement in the retinogeniculate system (Huh et al., 2000).

Neuronal pentraxins (NP1 and 2) were also found to be involved in retinogeniculate refinement. As with class I MHC molecules, mice deficient in neuronal pentraxins showed defects in eye specific segregation at P10, but these defects were not maintained into adulthood and the RGC inputs to the LGN became segregated by P30 (Bjartmar et al., 2006). A similar age-dependent phenotype results when spontaneous activity in the retina is blocked from P1-P10 and then allowed to recover. When retinal activity was measured in mice deficient in neuronal pentraxins, levels of RGC spiking activity were dramatically increased which may explain the phenotype in eye specific segregation (Bjartmar et al., 2006). Neuronal pentraxins represent an immune system molecule that is required for normal acetylcholine mediated retinal wave activity during development. NP1 can also interact with another neuronal pentraxin, Narp, to influence synaptogenesis and synaptic plasticity (Xu et al., 2003). Narp was identified as an

activity-dependent immediate early gene and is itself synaptogenic (O'Brien et al., 1999; Tsui et al., 1996). When complexed with NP1, Narp has an enhanced synaptogenic effect, mediating both activity-dependent and activity-independent clustering of AMPA receptors (Xu et al., 2003). These interesting acute phase proteins seem to have critical roles in both synaptogenesis and synaptic refinement.

My work has identified C1q as another activity-dependent immune system gene required for synaptic refinement; however, it is unclear whether or how C1q interacts with MHC1 or other immune system proteins during retinogeniculate refinement. Recent work has shown that C1q co-localizes with MHC I at synapses in the LGN during the pruning period, suggesting that these proteins might be interacting (Datwani et al., 2009). Another possibility, however, is that these proteins co-localized because they were both working by independent mechanisms at an inappropriate synapse to eliminate it. In the immune system, MHC I and C1q are not known to interact, thus any nervous system interactions would be a novel finding for both the CNS and the immune system. Intriguingly, a class of pentraxins has been shown to interact with C1q in the bloodstream and can work to inhibit C1q, although nothing is known about interactions between pentraxins and complement in the CNS (Jeon et al., 2010). An exciting future area of research will be to determine whether and how these immune molecules work together to sculpt neural circuits.

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CHAPTER 5:

Discussion

The formation of precise mature neural circuits requires selective pruning of inappropriate synapses as well as the strengthening and maintenance of appropriate synaptic connections. Synapse elimination is thought to result from competition between neighboring axons for postsynaptic territory based on differences in patterns or levels of activity. Hebb's rule applies in this context such that inputs that successfully and reproducibly drive the postsynaptic cell are maintained (Butts et al., 2007; Hebb, 1949; Hooks and Chen, 2006; Katz and Shatz, 1996; Sanes and Lichtman, 1999). Synaptic maintenance and maturation are accomplished via activity-dependent gene products, including neurotrophins such as BDNF (Tao et al., 1998), cell adhesion molecules such as NCAM (Fields and Itoh, 1996), and cytoskeletal associated proteins such as Arc (Lyford et al., 1995). A longstanding mystery in the field of synaptic refinement, however, is the mechanism by which neural activity drives the selective elimination of weaker synapses. Based on studies at the NMJ, the "Punishment Model" proposes that synapse elimination could be accomplished by the activity-dependent expression of two signals: a punishment signal to eliminate nearby weaker inputs and a protective signal to block removal of strong inputs (Figure 1.1) (Jennings, 1994; Lichtman and Colman, 2000). The identities of these signals have remained elusive; however, my findings support a "punishment signal"-like role for C1q in complement-dependent synapse elimination.

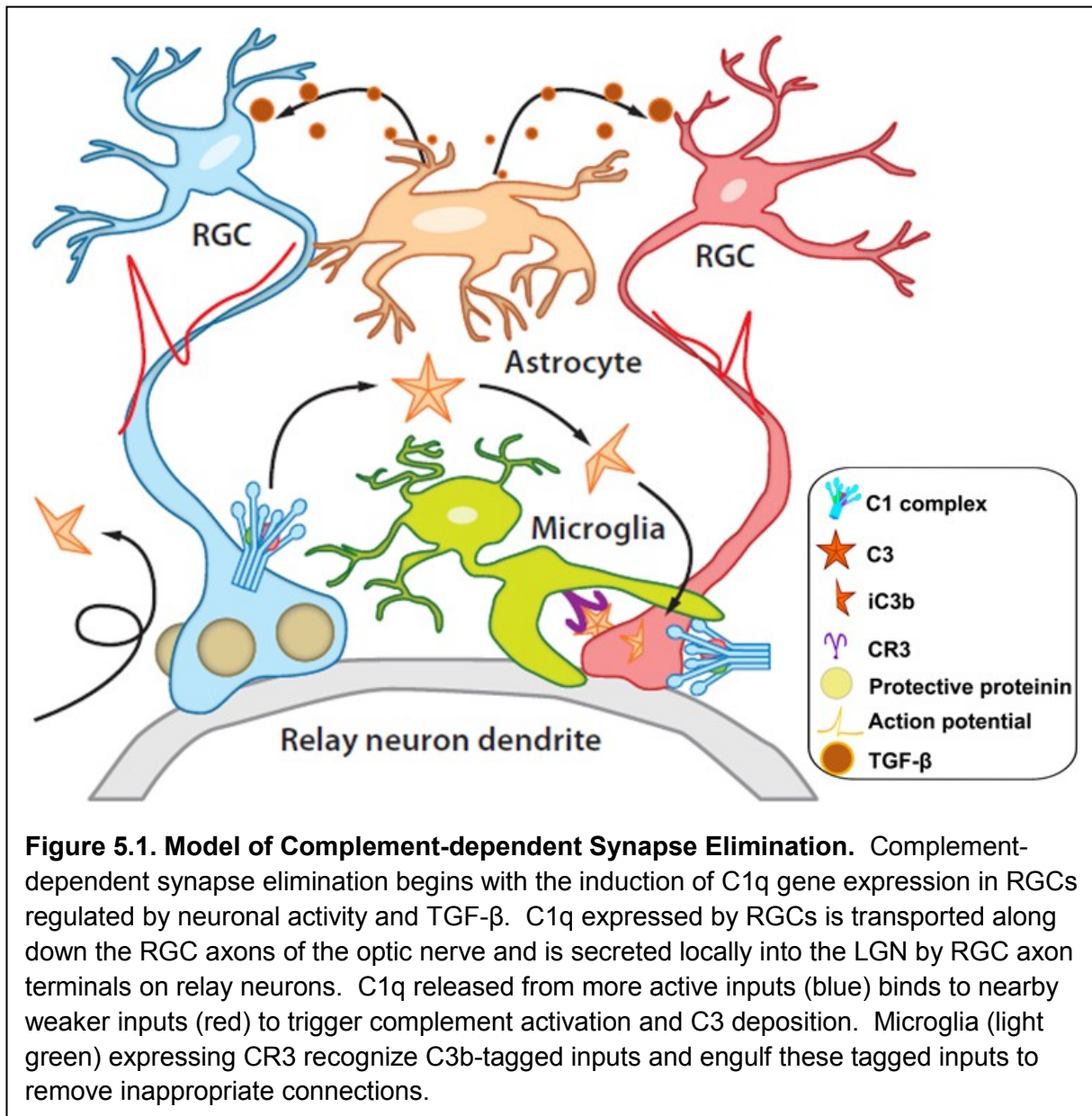
The findings presented in this dissertation identified C1q as a critical link between retinal activity and the selective elimination of less active RGC inputs. I found that C1q expression by RGCs is regulated by activity- and TGF- β - dependent signaling pathways and that modulating C1q expression in RGCs by manipulating spontaneous retinal activity or TGF- β results in significant local changes in C1q levels in the LGN. Furthermore, mice deficient in C1q showed a failure to preferentially target weaker inputs during microglia-mediated engulfment of RGC inputs. Taken together, these results suggest that C1q is required for microglia to sense differences in neuronal activity and, consequently, C1q links differences in retinal activity with the selective elimination of less active synapses. Many intriguing questions remain including: 1)

How does activity regulate complement-dependent tagging of synapses? 2) What are the protective signals and other punishment signals in synaptic pruning? 3) Is C1q unique in its retinal expression and trafficking to the LGN to mediate refinement or is this a general characteristic of other refinement cues? 4) Is neuronal activity an important modulator of TGF- β signaling throughout the CNS? 5) Does complement mediate synaptic refinement throughout the CNS? 6) What role does complement play in CNS injury and disease? These questions, which I will discuss in this chapter, represent some of the exciting future directions for my dissertation research. Given the many associations between immune molecules such as complement and nervous system dysfunction, my results identifying novel regulatory mechanisms for C1q expression in neurons may have broad implications for basic neurodevelopmental research and clinical studies.

Model for Complement-dependent Synapse Elimination

In this dissertation, I proposed the hypothesis that C1q acts as an activity-dependent "punishment signal" that triggers local complement-dependent synapse elimination at less active synapses. My results, presented in Chapters 3 and 4, support regulation of RGC-derived C1q as a critical point in complement-dependent synaptic refinement and identify astrocyte-derived TGF- β and neuronal activity as modulators of neuronal C1q expression. Moreover, I find that C1q deficient mice fail to preferentially target weaker RGC inputs during microglia-mediated engulfment of synapses and show reduced microglial engulfment overall. This reduction in microglial engulfment and preference for weaker inputs is thought to underlie the retinogeniculate refinement defects I observe in C1q KOs and TGF β RII retinal KOs.

These data suggest a model, illustrated in Figure 5.1, in which expression of C1q in RGCs is triggered by astrocyte-secreted TGF- β and neuronal firing in the retina.



C1q is then transported to the dLGN in the thalamus where it is secreted from axon terminals, in the form of the entire C1 complex, and localizes to synapses. Binding of C1q at synapses then activates downstream C3, generating the C3b opsonin thought to selectively tag inappropriate connections to promote CR3-dependent engulfment by phagocytic microglia.

Spontaneous Activity and Complement-dependent Synaptic Refinement

The focus of my dissertation has been to determine how C1q becomes upregulated in RGCs and whether expression of C1q by RGCs is critical for complement-dependent retinogeniculate refinement. My data demonstrate that C1q is regulated by neuronal activity and that C1q is required for activity-dependent targeting of less active synapses by microglia. In our experimental paradigms, we drive competition such that one eye is more active than the other; however, during normal development, the “winner” and “loser” axons may not have such dramatically different levels of activity. During postnatal development prior to eye-opening and the development of visual responses, the retina exhibits highly correlated spontaneous firing patterns termed retinal waves. These waves of spontaneous activity are required for eye specific segregation and the refinement of retinogeniculate synapses (see Introduction). Although all RGCs are spontaneously active during waves, the bursts of action potential of two nearby RGCs are more highly correlated than two distant RGCs. Thus, individual RGC patterns of activity are quite different between eyes, and even within eyes. The retinogeniculate system exhibits Hebbian plasticity during this period and activity is thought to have an instructive role in shaping neural circuits, but the molecular mechanisms of Hebbian plasticity that eliminate a less active synapse over a more active synapse are not clear.

Given my results that C1q expression is activity-dependent, the complement cascade could be one of the molecular mechanisms driving this activity-dependent process. My results demonstrate that activity regulates C1q transcription in the retina and affects overall C1q levels in the LGN (Figures 4.3,4.4); however, we still do not know whether or how activity regulates

complement-mediated elimination of specific (ie. less active) retinogeniculate synapses. There are several levels at which neuronal activity could regulate complement-dependent synapse elimination: 1) Transcription of C1q and downstream complement components could be activity-dependent, (Chapter 4); 2) Secretion of complement proteins could be activity-dependent, and 3) The localization or targeting of complement proteins to specific synapses may be activity-dependent. These three levels of regulation are not mutually exclusive and it is possible that complement is regulated by activity at all of these levels. We hypothesize that activity-dependent regulation of C1q expression could be a mechanism to initiate and activate complement at specific synapses in response to changes in neuronal firing.

In this dissertation, my data support the hypothesis that neuronal activity and TGF- β cooperatively regulate C1q transcription to initiate complement-dependent retinogeniculate refinement. In Chapter 3, I found that TGF- β signaling is required for the developmental expression of C1q in the postnatal retina, and in Chapter 4, I found that C1q transcription is also activity-dependent. Interestingly, when neuronal activity was silenced, TGF- β was far less effective at upregulating C1q (Figure 4.1), suggesting that TGF- β and neuronal activity work together to regulate C1q expression (see Figure 5.1). Based on my data, we hypothesize that TGF- β is the developmental cue that turns C1q on at the right time and place, but that activity-dependent regulation of C1q expression helps to guide C1q function in eliminating less active synapses. My data show that perturbing retinal C1q expression results in changes in C1q levels in the LGN, supporting the idea that regulation of RGC-derived C1q is critical for retinogeniculate refinement and suggesting that C1q is secreted from RGC axons into the LGN. It remains unclear, however, how and if C1q selectively targets less active synapses. Is activity-dependent regulation of C1q transcription alone sufficient to accurately target the right synapses for elimination? This is an especially intriguing question since during the retinogeniculate refinement period, all RGCs are spontaneously active and do not differ greatly in their levels of activity. Their patterns of activity differs greatly, however, and could potentially lead to

differential C1q expression, but how less active synapses could then specifically get eliminated remains unclear. Given that C1q is a secreted protein and that C1q can interact with many other complement molecules, it is likely that the precise elimination of less active synapses is accomplished by activity-dependent regulation of C1q and the process of complement-dependent synaptic refinement at many different levels.

The role of neuronal activity in regulating C1q trafficking and secretion is still an active area of research in the lab. My data in Figures 3.5 and 4.4 suggest that C1q is transported along RGC axons and is secreted into the LGN by RGC terminals; however, direct evidence of C1q transport and secretion happen in RGC axons requires additional experiments. To further validate my findings in Figures 3.5 and 4.4, I am currently validating that C1q can be found within RGC terminals using Immuno Electron Microscopy for C1q. In addition, our lab is developing constructs to express fluorescently-tagged C1q protein under a CMV promoter in our RGC cultures, our retina-LGN co-cultures, and potentially in vivo. These constructs will take activity-dependent transcription out of the equation, since C1q transcription will be driven by a constitutively active promoter. We then can directly ask if just transport and secretion change in response to activity manipulations. With these constructs, we hope to observe C1q transport in real time by live imaging or to show that when we transfect labeled C1q into RGCs, we indeed can detect this labeled C1q in the LGN or secreted into the culture medium from axons. Once we establish that we can observe transport and secretion, we can then use activity manipulations to ask how both transport and secretion are affected when neuronal activity is perturbed. We can also use various blockers of active axonal transport to determine which if any of these mechanisms are required for C1q transport. In the immune system, C1q transport and secretion have not been rigorously investigated since C1q is constitutively secreted by macrophages and this question is not especially relevant to the immune response. In neurons, however, and particularly in this case where RGCs are secreting this protein into the LGN far

away from the RGC cell body, this question becomes extremely interesting and important to understanding C1q function in retinogeniculate refinement.

Another important unanswered question is whether and how C1q works directly as a tag at synapses to indicate synapses meant for elimination. My results and published work have shown that C1q localizes to synapses during the pruning period, and in Chapter 4, I showed that C1q-deficient mice have a failure to preferentially engulf less active RGC inputs. While these results suggest that C1q helps in recruiting microglia to engulf less active synapses, we still do not know if C1q directly tags synapses or whether downstream molecules like C3b (see Figure 5.1) are the actual tag required for engulfment. We hypothesize that C1q is an activity-dependent “punishment signal” that locally cleaves C3 and activate the complement cascade at or near specific synapses (see Figure 1.1). To address these questions, our lab has turned to a newly-developed retina-LGN co-culture system (see Figure 3.5) that allows imaging of activity-dependent synaptic competition in real time. Immunostaining for C1q and C3 can be performed retrospectively ask whether the less active or the “losing” axon is specifically labeled or tagged with complement C1q and/or C3. We can also manipulate activity in subsets of RGCs and determine if C1q and/or C3 specifically localize to the less active axons. In addition, C1q KO and C3 KO mice have defects in synapse elimination and microglial engulfment, but microglia still do engulf some RGC inputs and some degree of synaptic refinement still occurs in these mice. Thus, it is likely that there are other complement-dependent and -independent synapse elimination cues. Using this new co-culture system, our lab also will be able to screen for novel candidate molecules. The results presented in this dissertation will inform these future studies to determine what additional cues regulate synaptic refinement and how they interact with C1q.

Alternative Punishment Signals

My results support a role for C1q in initiating complement-dependent synapse elimination as in my model (Figure 5.1); however, there are several alternative molecules that could cooperate with or inhibit C1q during synapse elimination. I have focused on C1q and C3 as punishment factors, but there could be additional complement factors that work as punishment signals. There are two potential classes of complement molecules that I predict could act as “punishment” signals: opsonins (such as C3b or C4b) and anaphylatoxins (such as C3a and C5a) (Figure 1.3 B). While C3b is considered the primary opsonin in the immune system, C4b can also opsonize and potentially tag synapses for elimination. I have not investigated the role for other opsonins in this process. Anaphylatoxins, including C3a or C5a, could work to recruit phagocytic microglia to the sites of synaptic refinement and potentially could induce apoptosis at synapses expressing the C3a or C5a receptors (Mattson and Duan, 1999; Nataf et al., 1999). In this case, the anaphylatoxin could trigger apoptosis in a weak synapse (or a process containing a weak synapse) and microglia would phagocytose the apoptotic blebs. Intriguingly, activated caspases, mediators of apoptosis, have been detected locally within dendrites, axons, synaptic terminals, and growth cones in non-apoptotic cells (Kuo et al., 2006; Li et al., 2010; Williams et al., 2006). Activation of apoptotic pathways locally at synapses has been proposed as a potential mechanism of synapse loss in neurodegenerative disorders (Mattson et al., 1998); however, recent work has implicated apoptotic signaling pathways involving caspase-3/7 and caspase-9 in the weakening of synapses by long-term depression (LTD) (Li et al., 2010), suggesting a potential role for local apoptosis in developmental synapse elimination. It is not yet known whether a similar mechanism is at play during developmental axonal pruning; however, preliminary data from our lab suggests a local ‘synaptosis’ of weaker retinogeniculate synaptic boutons involving caspase-dependent signaling. In future studies it will be important to investigate whether C1q or C3 selectively target less active boutons through this or other mechanisms.

Lastly, the specific binding partners for C1q and C3b on the surface of RGC inputs have yet to be identified. In the immune system, antigen-antibody complexes, including IgG and IgM complex, bind C1q to trigger cascade activation, but C1q can also be activated by some proteins on the surface of bacteria and by some viruses in an antibody-independent manner. Some of these proteins bind the C1q globular domain but other activating molecules such as DNA, C-reactive protein (CRP), serum amyloid protein (SAP), decorin, and some putative C1q receptors are thought to bind C1q via the collagen domain (Kishore et al., 2004; Kishore and Reid, 2000). Perhaps some of these non-traditional activators of C1q are regulated on RGC inputs in an activity-dependent manner and work to eliminate weaker synapses. Alternatively, C1q can recognize apoptotic cells by the presence of phosphatidylserine on the cell membrane (Païdassi et al., 2008). If, as discussed earlier, local apoptotic pathways become activated at synapses meant for elimination, perhaps phosphatidylserine could appear on the cell membrane specifically at weak synapse and promote C1q deposition and cascade activation.

Complement Regulatory Proteins as “Protective Signals”

The “Punishment Model” also suggests that an activity-dependent protective cue may prevent elimination of strong connections. In this case, the production and secretion of complement proteins by neurons may not be the only cue controlling the “tagging” process. Instead, complement secreted during the pruning period may non-specifically bind to all or most synapses and the protective cues may regulate which synapses are engulfed. Complement regulatory proteins are good candidates for protective signals. There are two classes of complement regulatory proteins: Membrane bound (such as CR1, DAF, MCP, and CD59) and secreted (C1 INH, Factor H, and C4BP) (Elward and Gasque, 2003; Iida and Nussenzweig, 1981). Some of these molecules including CR1 were recently shown to be upregulated in response to *in vivo* high frequency stimulation, suggesting activity-dependent regulation (Håvik et al., 2007). Based on the broad distribution of C1q immunostaining that I observe in the

dLGN, C1q may be deposited at all synapses; however, my data demonstrating that microglia cannot distinguish between weak and strong inputs in the absence of C1q suggests that C1q conveys some information about neuronal activity to microglia.

Molecules outside of the complement system could also work as punishment or protective cues in complement-dependent synapse elimination via regulation of microglial activation state. Resting microglia, which predominate in the mature brain, downregulate their phagocytic receptors, while earlier in development and in response to injury or inflammation, microglial phagocytic capacity is greatly increased. Many cytokines such as TNF- α and IFN- γ (Meda et al., 1995; von Zahn et al., 1997), as well as classes of molecules known as “eat me” and “don’t eat me” signals (Elward and Gasque, 2003), such as CD47 and CD200, function to regulate phagocytosis by immune cells in the periphery. In the brain, many of these same cues regulate microglial activation state and phagocytosis (von Zahn et al., 1997), which could prevent microglia from engulfing too many or too few synapses.

The models I have outlined detailing specific ways in which punishment and protective cues modulate synaptic refinement are by no means mutually exclusive mechanisms. In our lab, we hypothesize that these various punishment and protective cues work together to regulate synaptic refinement at multiple levels. Future studies will determine which of these punishment and protective cues are required for synaptic refinement and how their functions are coordinated during this process.

Retina-derived versus Local LGN Cues in Retinogeniculate Refinement

My findings implicate TGF- β as an activity-dependent retinal cue that regulates synapse elimination locally in the dLGN during retinogeniculate refinement. In my model for complement-dependent synaptic refinement, TGF- β cytokine signaling and neuronal activity regulate C1q expression in RGCs, which modulates the targeting of C1q and C3 to synapses and microglia-mediated pruning in the dLGN. These findings raise the question as to whether

RGCs are a critical source of other refinement cues. Other molecules, such as MHCII and NP1, have also been implicated in retinogeniculate refinement. Interestingly, in the fly, TGF- β signaling triggers the dramatic neuronal remodeling that occurs during metamorphosis (Awasaki et al., 2011). Could retinal activity and TGF- β work together to initiate a synaptic refinement gene program specifically in RGCs or is this regulatory mechanism unique to C1q? Furthermore, are cues expressed by RGCs that function in the LGN trafficked there along the axon or is there local axonal translation to produce some of these proteins during the refinement period (Brittis et al., 2002; Martin, 2004)?

Molecules required for retinogeniculate refinement could drive this process in several different ways. First, RGC terminals could secrete the molecule that promotes refinement into the dLGN, as I observed with C1q. Another possibility is that cues are locally secreted from neurons and glia in the dLGN. Lastly, a molecule could function to regulate retinal activity, and indirectly influence refinement in the dLGN. My data suggest that C1q expressed by RGCs is required for synaptic refinement and that local secretion of C1q during the refinement period in the dLGN, from microglia or other sources, cannot compensate for the loss of RGC-derived C1q. I hypothesize that C1q is transported along RGC axons to the dLGN, but local translation and synthesis of C1q within RGC axons in the optic nerve or dLGN is also a possibility, although local translation of C1q has not previously been reported. I have not assessed retinal wave activity in retinal TGF β RII KO or C1q KO mice, so I cannot rule out the possibility that C1q regulates retinal activity. However, my findings that microglia lose their preference for engulfing less active inputs in both C1q KO mice and retinal TGF β RII KO mice provide evidence that C1q has a local function in the LGN that cannot be accounted for by any potential functions that C1q might have in the retina (Figure 4.5). Thus, even if C1q and TGF- β have other functions in the retina coinciding with the refinement period, C1q would still need to be functioning in the LGN for microglial targeting of weaker inputs to be disrupted in C1q-deficient mice.

Interestingly, other immune molecules implicated in refinement do not necessarily follow this same pattern of expression in the retina and function in the LGN. For example, C3 and CR3, which work downstream of C1q, are thought to be locally expressed in the LGN (Schafer et al., 2012a). CR3 is expressed on the surface of microglia, but the source of C3 remains unclear. Other immune proteins implicated in synaptic refinement, MHC I and NP1, seem to have local regulation and functions as well. MHC I was first identified as an activity-dependent gene in the LGN, not the retina. MHC I was downregulated after infusion of TTX into the fetal cat LGN (Corriveau et al., 1998a), suggesting that postsynaptic relay neuron expression of MHCI may be more important than RGC-derived MHCI. A receptor for MHCI, however, CD3zeta, is expressed by RGCs and is required for eye specific segregation as well (Huh et al., 2000; Xu et al., 2010). CD3zeta deficient mice have defects in GluR-mediated synaptic transmission in RGCs (Xu et al., 2010), which suggests that the role of CD3zeta in the retina versus at RGC terminals in the LGN may be critical for pruning. Whether MHC I also regulates retinal activity is unclear, but other receptors for MHC I, such as PirB, could be more important for their local functions in the LGN (Syken et al., 2006). NP1 functions as a regulator of spontaneous acetylcholine-dependent retinal activity, which drives a transient pruning phenotype in the LGN in NP1-deficient mice (Bjartmar et al., 2006); however, no clear function for NP1 in the LGN has been described. Thus, these data suggest that RGCs are not the only source for refinement molecules and suggest that there are multiple mechanisms that work in parallel to refine retinogeniculate circuits. It will be interesting to investigate if these pathways somehow work together and to determine if C1q is indeed the only cue regulated in this manner to directly link spontaneous retinal activity with synaptic pruning in the LGN.

TGF- β and Activity-dependent Transcription.

My results demonstrate that TGF- β and activity-dependent signaling pathways interact to regulate C1q expression and complement-dependent synapse elimination. Does neuronal

activity also modulate TGF- β signaling in other contexts during development throughout the nervous system? Activity-dependent gene transcription has been implicated in many of the same processes as TGF- β , including neuronal survival and proliferation (Lu et al., 2005; Spitzer, 2006), synaptogenesis (Diniz et al., 2012), and synapse function and plasticity (Fukushima et al., 2007; Greer and Greenberg, 2008; Heupel et al., 2008a).

As discussed in Chapter 4, there are many possible points of intersection between activity-dependent pathways and TGF- β signaling pathways (Figure 4.6). It has been proposed that many cytokines, including TGF- β , signal in a cell-specific manner such that TGF- β itself serves only as a source of activated Smads and the downstream effect of Smad phosphorylation is based on the convergence of many other cues (Massague, 2000), such as potentially activity-dependent factors. Patterns of neuronal activity change dramatically over development; for example, in the retina, RGCs undergo waves of spontaneous activity before the onset of vision, but change their firing pattern after eye opening based on photoreceptor activation (Huberman et al., 2008a; Katz and Shatz, 1996; Penn and Shatz, 1999; Stellwagen et al., 1999). Thus, in the retina and throughout the brain, it is possible that the pool of transcriptional co-factors that can interact with Smads may change dramatically over development and can help to diversify neuronal responses to TGF- β . Interestingly, Smads alone have a low affinity for their DNA binding sequence and therefore seem to require co-factors to successfully stimulate transcription. Smads have been shown to interact with activity-dependent co-factors in the nucleus, such as cFos, Mef2 and CREB (Quinn et al., 2001; Topper et al., 1998; Zhang et al., 1998). In addition, TGF- β and activity both activate some common kinase pathways, including the MAPK pathway (Massague, 2000). Although it is not yet clear at what level neuronal activity and TGF- β signaling are interacting to regulate C1q transcription, it is intriguing to speculate that many activity-dependent pathways may be cooperatively regulating other critical TGF- β dependent processes throughout the nervous system.

Evidence for Complement-dependent Synaptic Refinement in Other Brain Regions

Synaptic refinement occurs throughout the brain at different time points. The developing visual system is one of the best studied systems; however, recent work has characterized synaptic refinement in other brain regions as well, including the cerebellum and the auditory system. In the cerebellum, climbing fiber projections from the inferior olive initially synapse onto purkinje cell bodies (Hashimoto et al., 2009; Hashimoto and Kano, 2005). In the second and third postnatal weeks in the mouse, climbing fibers undergo refinement resulting in a single climbing fiber driving activity in a single purkinje cell, much like the 1:1 connectivity between RGCs and relay neurons that is generated in the mature dLGN. The “winning” climbing fiber elaborates and moves distally, “climbing” to the purkinje cell dendrites, while remaining “losing” fibers are eliminated (Hashimoto et al., 2009; Hashimoto and Kano, 2005). Roles for complement and microglia have yet to be explored in the cerebellum; however, microglia do regulate purkinje cell survival (Marin-Teva et al., 2004) in the cerebellum and branched, phagocytic microglia are present in the cerebellum around the time when synapse elimination is occurring. Moreover, the C1q-like protein, Cbln1, has been implicated in synapse formation and maintenance at parallel fiber-purkinje cell synapses, but, interestingly, Cbln1 KO mice also exhibit persistent multiple climbing fiber innervations onto purkinje cells (Hirai et al., 2005). This intriguing result suggests a role for this C1q-like molecule in cerebellar synaptic refinement. Given this finding, perhaps C1q does not regulate refinement globally, but rather C1q family members, including Cbln1 and C1qL1-4 (Bolliger et al., 2011; Yuzaki, 2010), regulate this process in specific brain regions.

A stereotyped refinement process also occurs in the auditory system. Spiral ganglion neuron inputs onto outer hair cells undergo refinement in the first postnatal week (Huang et al., 2007). Initially, neurons from both classes of spiral ganglion neurons innervate all hair cells, but from P3-P6 neurites from type I spiral ganglion neurons retract and synapses on outer hair cells are eliminated while inner hair cell innervation is maintained (Huang et al., 2007). Interestingly,

C1q and TGF- β expression are also expressed in embryonic and postnatal spiral ganglion neurons (Lu et al., 2011), raising the question of whether TGF- β signaling regulates complement expression and synapse elimination in spiral ganglion neurons as well. Very little is known about the mechanisms of synaptic refinement in the auditory system; however, developmental expression of C1q is detected in spiral ganglion neurons, the sensory neurons that transmit auditory signals from the cochlea to the brain much like RGCs connect the retina with the brain, around the time when synapse elimination occurs from outer hair cells (Lu et al., 2011). Interestingly, although C1q is enriched in microglia throughout CNS, C1q expression in sensory neurons, both RGCs and spiral ganglion neurons, is developmentally regulated and restricted to periods of developmental synaptic remodeling. These results are consistent with my findings that neuron-derived C1q is specifically required for complement-dependent synaptic refinement (Chapter 3).

Recent work has also implicated C1q in the refinement of cortical circuits in that C1q KO mice show increased synaptic connectivity and experience spontaneous seizures (Chu et al., 2010). In particular, the axonal bouton density was significantly increased in layer V pyramidal neurons of C1q KO mice, and C1q KOs had frequent seizures consisting of behavioral arrest associated with bihemispheric spikes and slow wave activity (Chu et al., 2010). This hyperexcitability is thought to be attributed to a failure to prune excessive excitatory synapses during development, although it is difficult to rule out the possibility that C1q could alternatively work as a negative regulator of synaptogenesis in the cortex as well.

Cascade-dependent and -independent Functions for C1q in the CNS

C1q-like molecules regulate synapse density and act as synaptic organizers in the cerebellum (Bolliger et al., 2011; Yuzaki, 2010), as discussed briefly above. These interesting proteins closely resemble the globular domains found in C1q, but lack the C1q collagen domain (Kishore and Reid, 1999) (Figure 1.3). The putative receptors thought to bind C1q-like family

members to regulate synapse development, including BAI3 and GluD2, recognize the globular domains of these proteins (Bolliger et al., 2011; Matsuda et al., 2010), suggesting that C1q may also bind to these receptors or similar proteins to potentially execute functions independently of complement cascade activation. My findings support a role for C1q in synapse elimination; however, the process of synaptic refinement involves the strengthening and maturation of certain synapses with the concurrent elimination of weak, immature synapses. Could C1q play a role in the selective strengthening and maturation of synapses as well? One of the C1q-like molecules, Cbln1, binds to Neurexin presynaptically and GluD2 postsynaptically to act as a synaptic organizer in the cerebellum (Yuzaki, 2009). Cbln1 rapidly induces synapse formation but is also required during synapse maturation and maintenance (Yuzaki, 2010), processes which are a critical part of synaptic refinement in the LGN. Other C1q-like molecules are also expressed throughout the developing CNS and work to limit synapse density *in vitro* (Bolliger et al., 2011), although their functions *in vivo* remain unclear.

To explore whether C1q had functions independent of cascade activation for neurons, I characterized the formation and development of synapses in purified RGCs isolated from global C1qA KO mice. These purified RGC cultures are devoid of microglia, suggesting that any results I observed could not be the result of complement-dependent microglia engulfment of synapses. I developed RNAi constructs and C1qA rescue constructs to specifically knock down or rescue C1qA expression in RGCs *in vitro*. My results showed that RGCs deficient in C1qA have significantly more synapses per cell compared with WT RGCs (Supplemental Figure 9). I detected synapses by immunostaining for the presynaptic protein, vglut2, and a postsynaptic protein, PSD95, and quantified the number of synapses as co-localized puncta. Co-localized puncta were quantified in ImageJ using the PunctAnalyzer macro. In normal WT cultures, RGCs do not form many synapses; however, my C1q KO RGCs showed a six- to eight-fold increase in synapse number compared to WT cultures (Supplemental Figure 9A), suggesting that a normal function for C1q might be to inhibit synapse formation or to eliminate synapses in

the absence of microglia. Adding back C1qA expression using a rescue construct expressing C1qA under the control of a CMV promoter (Open Biosystems, Clone 3592169) resulted in almost WT synapse numbers in C1qA transfected C1q KO neurons (Supplemental Figure 9B), suggesting that this effect is specific for C1q. Furthermore, sparse transfections of purified RGCs with C1qA-shRNA resulted in an increase in synapses only in C1qA-shRNA transfected neurons, but not in other non-transfected control neurons in the same culture (Supplemental Figure 9C). These results suggest that the effects of C1q are either locally restricted and/or cell autonomous. To address this question, I added WT RGC media to C1q KO RGCs and was able to partially rescue the increase in synapse number, suggesting that the effects I observed in transfected cells were not cell-autonomous (Supplemental Figure 10). A possible explanation for this result is that in the transfected cultures, C1q does not diffuse very far in undisturbed cultures in the incubator, while adding C1q exogenously to the cultures gives a more even distribution of C1q protein.

Given that I observed novel functions for C1q in RGC cultures, I next wanted to determine if TGF- β regulated this function for C1q in RGC cultures as well. To address this question, I cultured WT RGCs in the presence of anti-TGF β neutralizing antibodies. Although I hypothesize that astrocytes are the main source of TGF- β in the retina, given that they express much higher levels of TGF- β than RGCs (Supplemental Figure 2E), RGCs do express TGF- β and could potentially maintain the basal level of C1q expression I observe in purified RGC cultures. Interestingly, I found that blocking TGF- β chronically with neutralizing antibodies partially phenocopied the increase in synapses that I observed in the C1q KO RGC cultures (Supplemental Figure 11), suggesting that TGF- β regulates C1q function in this context as well. These findings suggest that the refinement defects I observed in C1q-deficient mice may be partially attributed to a novel microglia-independent refinement process.

I also looked at neurite outgrowth and polarization in C1q KO RGCs and RGCs transfected with C1q shRNAs as a control to determine if any morphological defects in RGCs

could account for the phenotypes I observed *in vivo*. Surprisingly, I found that RGCs deficient in C1q had a similar number of total neurites and overall outgrowth; however, many of these RGCs had multiple axons, as indicated by immunostaining for Map2 (to label dendrites), neurofilament, and β IV spectrin (axon initial segment marker). In C1q KO RGCs (Supplemental Figure 12) and in RGCs transfected with C1q-shRNA (data not shown), there was a significant increase in the number of cells with multiple axons identified by positive immunostaining for β IV spectrin and neurofilament, but Map2 negative. These results suggest that C1q may play a role in neurite polarity, although multiple axons were not observed in C1q KO mice *in vivo*, suggesting that this phenotype may be an artifact of the culturing process. RGCs are known to sprout additional axons after axotomy (Cho and So, 1992) and recent findings suggest a role for C1q in stroke and optic nerve crush (Ohlsson et al., 2003; Zai et al., 2009). Perhaps the cell culture process mimics some aspects of injury and, in that case, my data would be consistent with a role for C1q to inhibit sprouting.

Together these findings suggest several possible cascade-independent roles for C1q, raising the intriguing question of whether C1q has functions independent of complement cascade activation *in vivo*. Could the presence of downstream complement proteins such as C3 help to drive C1q function toward synapse elimination, while, in the absence of C3, C1q may serve other functions? This possibility is particularly interesting in the context of injury and disease in which there are opposing reports showing that C1q is neuroprotective in some cases and harmful in other cases. Dysregulation of immune system components, including complement components and cytokines, has been demonstrated in many CNS disorders and diseases including epilepsy (Aronica et al., 2007), schizophrenia (Mayilyan et al., 2008), and neurodegenerative disorders such as glaucoma (Howell et al., 2011; Rosen and Stevens, 2010; Stevens et al., 2007) and Alzheimer's disease (Afagh et al., 1996; Fonseca et al., 2004a; Zanjani et al., 2005; Zhou et al., 2008). Many of these disorders have been associated with synapse loss or dysfunction (Arnold, 1999; Rosen and Stevens, 2010; Selkoe, 2002),

suggesting that aberrant complement upregulation may reactivate the developmental synapse elimination pathway in disease to promote synapse loss. In glaucoma, C1q localizes to the synaptic layers of the retina and the RGC layer early in the disease before significant loss of RGCs is observed (Stevens et al., 2007). Complement upregulation in Alzheimer's disease also occurs very early in the disease around the onset of mild symptoms. The rapid upregulation of C1q that I observed (Figure 3.1), as well as reports of complement activation in the early stages of neurodegeneration suggest that complement upregulation may be one of the earliest pathological events in these disorders (Rosen and Stevens, 2010; Zanjani et al., 2005). Thus, C1q upregulation and perhaps TGF- β signaling may be an important target for early therapeutic intervention. Indeed, TGF- β localizes to beta-amyloid plaques and has been linked to the formation of these plaques in Alzheimer's disease (Wyss-Coray et al., 1997) and blocking TGF- β and Smad2/3 signaling mitigates plaque formation in mouse models of Alzheimer's (Town et al., 2008). C1q has been found associated with plaques in Alzheimer's brain as well (Afagh et al., 1996), and in mouse models of Alzheimer's, C1q-deficiency has been shown to be neuroprotective (Fonseca et al., 2004b).

In the above cases, C1q and complement activation are thought to contribute to neuropathology. Conversely, other results have shown that complement activation after injury or in disease may help to clear harmful debris to promote recovery (Alexander et al., 2008a). For example, inhibiting C3 with a soluble form of the complement inhibitor, Crry, exacerbated some aspects of neuropathology, including neurodegeneration and plaque formation, in Alzheimer's disease (Wyss-Coray et al., 2002; Zhou et al., 2008). Moreover, recent work has shown that adding C1q to neuronal cultures upregulated genes that promote survival and outgrowth and also increased the nuclear translocation of cAMP response element-binding (CREB) protein and CCAAT/enhancer-binding protein- δ (C/EBP- δ), two transcription factors involved in nerve growth factor (NGF) expression (Benoit and Tenner, 2011). The conflicting reports of harmful and helpful roles for complement in injury and disease are difficult to

reconcile; however, my results suggesting both cascade-dependent and -independent functions for C1q may be one explanation. Another possibility is that complement proteins have detrimental roles in some stages of injury and disease, but may have protective roles at different stages. For example, in neurodegenerative disease, complement proteins may contribute to synapse loss early but help to clear debris at later stages. Thus, complement-deficient mice may show an overall slower progression of the disease because of inhibited synapse loss; however, adding inhibitors for complement after the onset of symptoms may result in more severe neuropathology since this approach may only block this later-stage complement function.

Interestingly, the functions that have been identified for C1q in the CNS during development, disease, and injury are largely based on studies conducted using C1q-deficient mice. These mice were made by deleting *C1qa* (Botto et al., 1998), one of the three C1q genes. Interestingly, *c1qb* and *c1qc* are still expressed in these mice, but the C1 complex which activates the classical complement cascade cannot be formed. It is not clear, however, whether C1qB and C1qC peptides are still produced and secreted. If so, could these peptides have unique functions? Individual C1qB and C1qC peptide monomers have not been reported in the literature, but given the novel functions recently identified for C1q-like molecules, it would be interesting to investigate whether C1q A, B, and C triple knockout mice have phenotypes not observed in the C1qA-deficient mice.

Complement Dysregulation in Neurodevelopmental Disorders

In addition to the roles for complement in neurodegeneration and injury, complement dysregulation and microglial dysfunction have also been linked to neurodevelopmental disorders such as autism, epilepsy, and schizophrenia. Recent work has shown elevated cytokine levels and other signs of neuroinflammation in the brains of autistic individuals (Li et al.; Vargas et al., 2005). Particularly of interest are increases in specific cytokines with the potential ability to

upregulate C1q: IL-6, TNF- α , IFN- γ , and TGF β isoforms. If one of the factors elevated in ASD triggers C1q upregulation in neurons and influences pruning, then synaptic refinement may not occur properly. One theory about the etiology of ASD is that there is a cortical imbalance in excitation and inhibition (Chao et al., 2010; LeBlanc and Fagiolini, 2011; Rubenstein and Merzenich, 2003). In support of this theory, recent work has suggested abnormal development of inhibitory circuits in two mouse models of autism, suggesting hyperexcitability contributes to autism (Gogolla et al., 2009). Conversely, in mouse models of Rett Syndrome, a genetic disorder in which patients exhibit many autistic-like symptoms (Amir and Zoghbi, 2000; Chahrour and Zoghbi, 2007), MeCP2 deficiency results in decreased excitation, but normal levels of inhibition (Dani et al., 2005). This deficit results in an overall shift in the neocortical excitatory/inhibitory balance in favor of inhibition. Interestingly, recent work has shown that the progressive visual system defects in MeCP2 KO mice can be rescued by decreasing levels of NR2A, a glutamate receptor subunit highly upregulated in these mice, and consequently, helping to restore the excitatory/inhibitory balance (Durand et al., 2012).

These results indicate that shifting activity in either the excitatory or inhibitory direction can have dramatic effects on cortical development, thus precise regulation of synaptic pruning is required to prevent this imbalance. This fact is particularly evident in a recent study revealing that C1q KO mice have increased synaptic connectivity in neocortex, as shown by both physiological and anatomical analysis, and experience spontaneous seizures (Chu et al., 2010). This finding suggests that complement-dependent pruning defects can result in the type of hyperexcitability thought to underlie epilepsy and other developmental disorders. Interestingly, a major risk factor for autism is maternal inflammation, and many autistic-like behaviors can be induced in mice by inducing inflammation in the pregnant dam. Many cytokines increase as a result of this inflammatory insult, including TGF- β , suggesting that complement dysregulation may account for some of the defects observed in these mice. In support of this hypothesis, a recent microarray study analyzing changes in gene expression in human autistic brain samples

versus controls revealed several immune and inflammatory response genes, including the C1q genes and components of the TGF- β signaling pathway, that were upregulated in autistic brains relative to controls (Voineagu et al., 2011). Intriguingly, genes associated with synapses were among the downregulated genes in this same study (Voineagu et al., 2011). Given these intriguing genetic associations between immune dysfunction and autism, unraveling the mechanistic details of complement-dependent synapse elimination may provide insight into a new type of auto-immune, neurodevelopmental defect (Careaga et al., 2010; Enstrom et al., 2009; Garay and McAllister, 2010).

Conclusion

My findings and the work of many others have shattered the dogma of the brain as a traditional site of “immune privilege” and suggest a new view of the CNS as an “immune specialized” site. This immune specialization is particularly evident in the case of complement-dependent synapse elimination. Here, the cytokine TGF- β is “specialized” in the retina to trigger activity-dependent upregulation of C1q, while in the immune system this cytokine traditionally suppresses inflammatory cues like C1q. Furthermore, C1q expression and secretion are “specialized” in retinal ganglion cells to locally activate complement in the LGN and selectively tag synapses meant for elimination. In the bloodstream, C1q is constitutively produced and secreted by circulating macrophages and is prevented from binding to healthy cells of the body by complement inhibitors, but in the brain it actually seeks to elimination parts of healthy, non-apoptotic cells. Interestingly, local secretion of C1q from microglia in the LGN does not compensate for the loss of RGC-derived C1q, and the cues that regulate C1q in RGCs, TGF- β and neuronal activity, have no effect on microglial C1q expression, again emphasizing “specialized” roles for neurons and microglia in this system. In addition, postnatal microglia, which can be approximated as process-bearing macrophages, are uniquely “specialized” to phagocytose RGC inputs in an activity- and complement-dependent manner precisely during

the retinogeniculate refinement period and not in the mature visual system (Schafer et al., 2012a).

Despite these specializations, it is quite striking how similar complement-mediated synapse elimination in the brain is to complement function in the immune system. Both processes use some the same proteins (C1q, C3, and CR3) and similar phagocytic cells, macrophages in the periphery and microglia in the brain. Further study is required to identify the synaptically localized binding partner for C1q; however, the mechanism of complement-dependent synapse elimination currently represents one of the most comprehensive molecular mechanisms for synaptic refinement in the CNS yet to be described. For this reason, determining to what extent this process is a global mechanism for synapse elimination throughout the brain will be a priority for future studies. If C1q is indeed regulated similarly and functioning to initiate synapse elimination throughout the brain, future work to conditionally knock out C1q or inhibit TGF- β and disrupt the pruning process in key brain regions systematically could have broad implications.

In addition to the direct parallels between complement function in the immune system and the developing CNS, my results demonstrating cascade-independent roles for C1q in polarity and controlling synapse number hint toward evolved mechanisms that may be unique to the brain. These findings suggests that, despite identifying C1q as the initiator of complement-dependent synapse elimination and determining the key regulators of neuronal C1q expression, we have only begun to understand the complexities of the many neural-immune interactions that regulate almost every stage of nervous system development.

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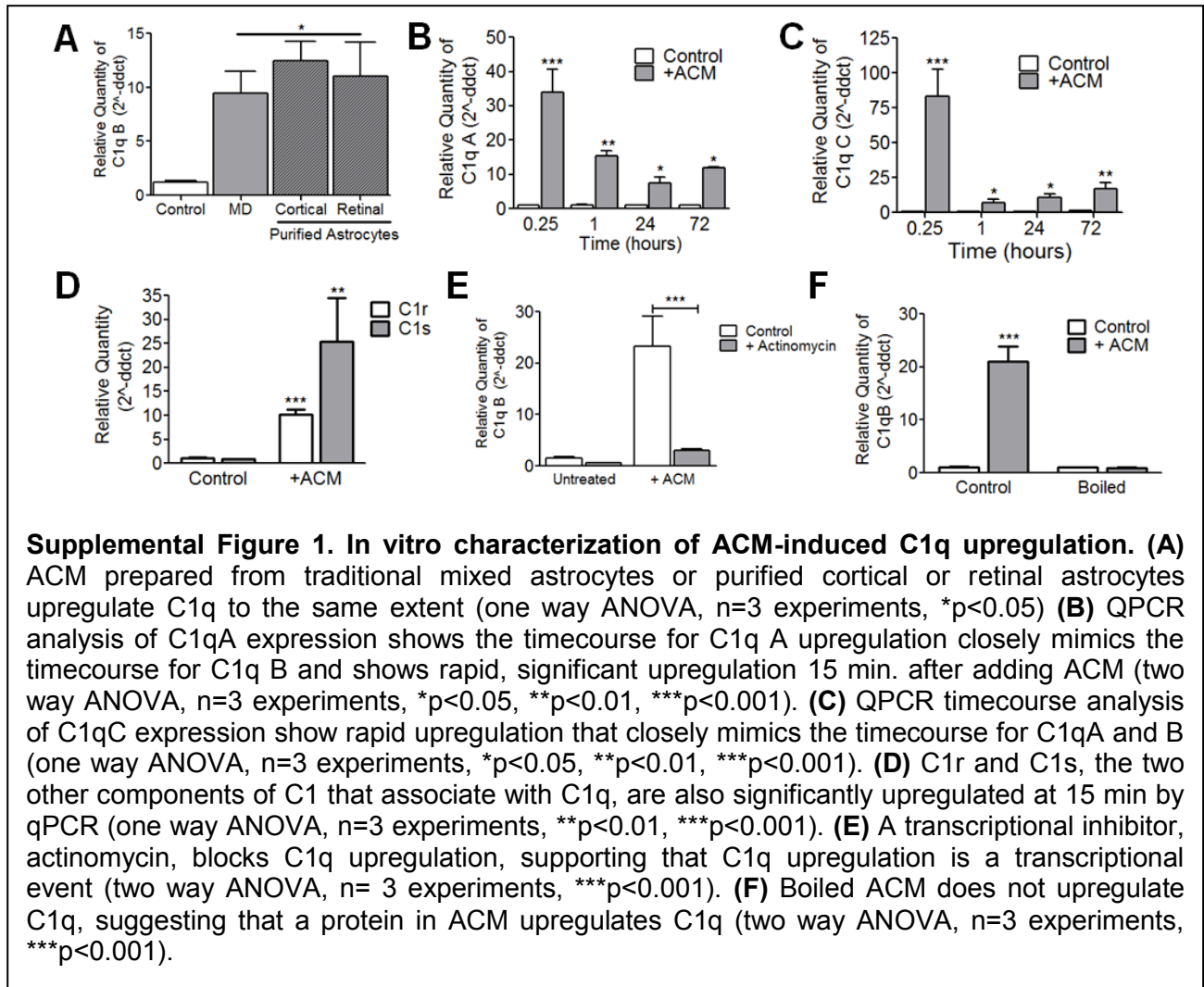
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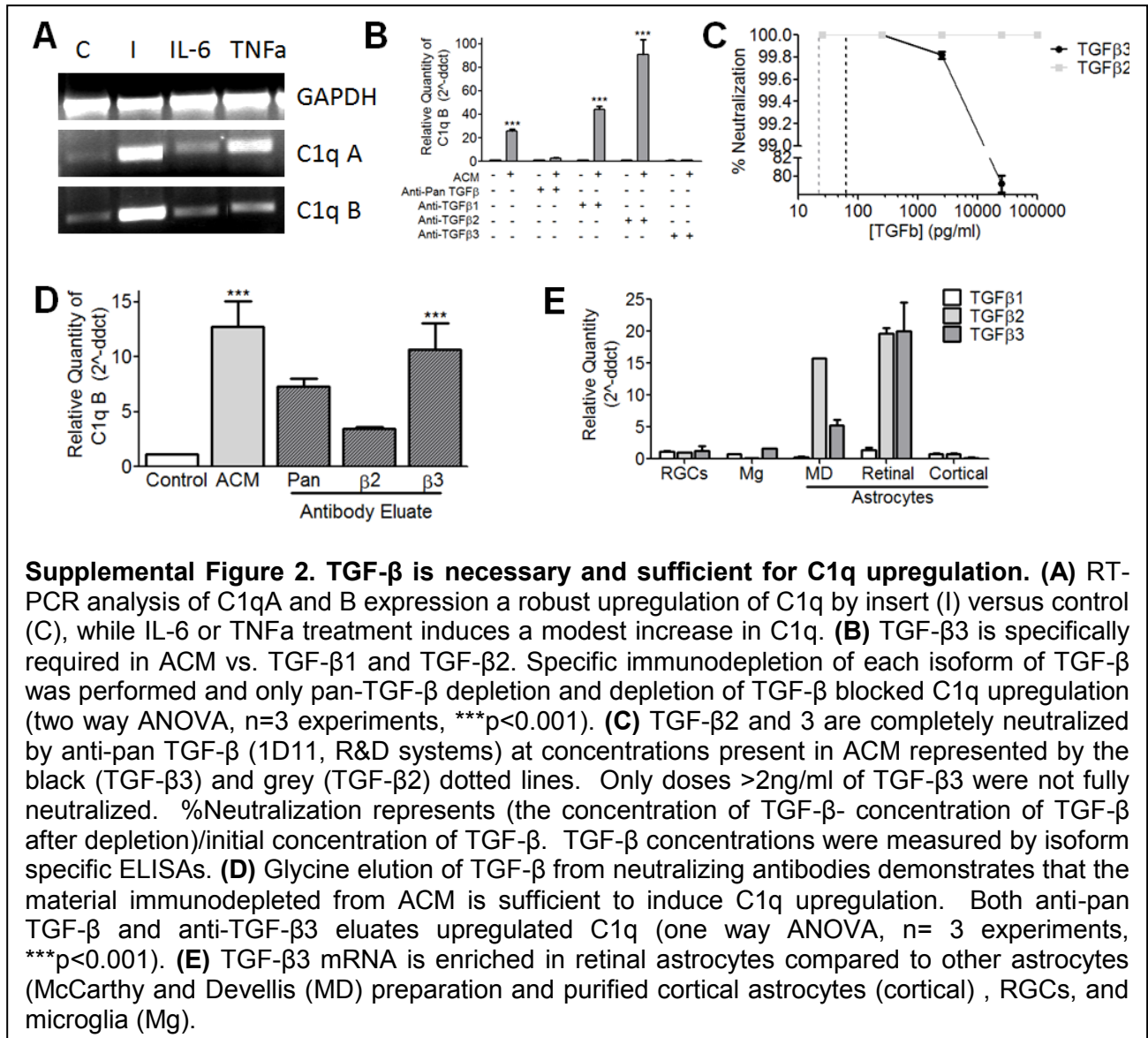
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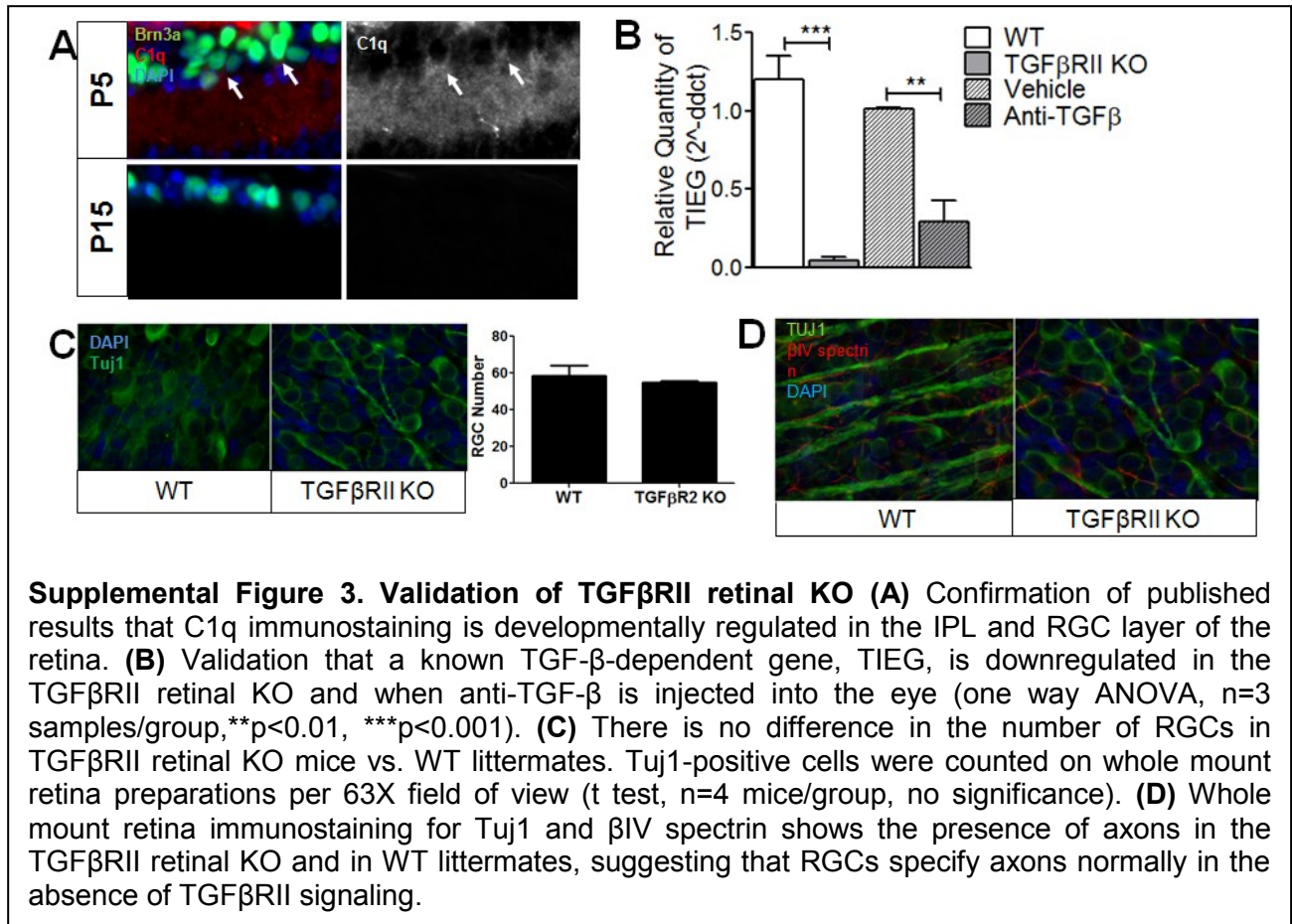
APPENDIX I:

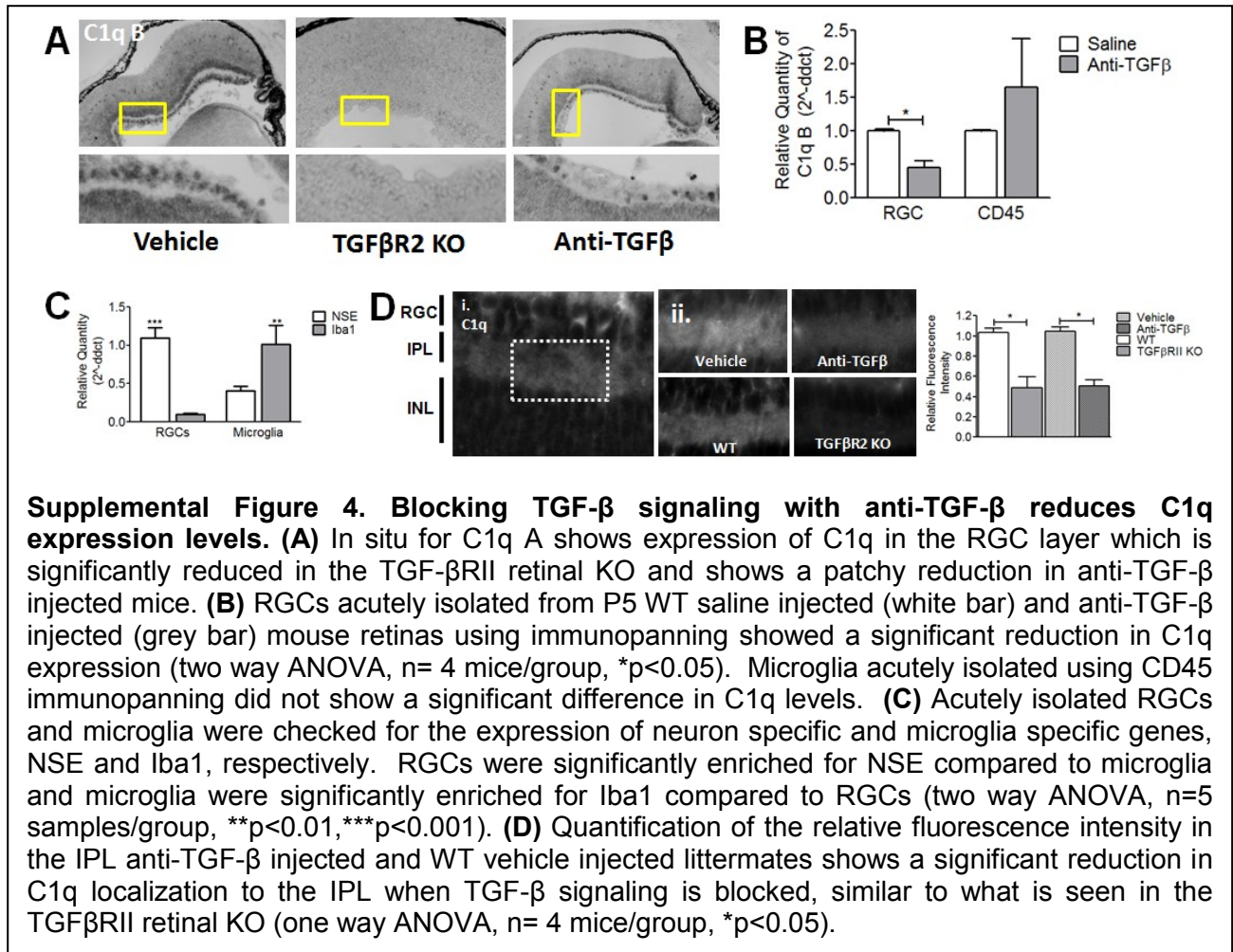
Supplemental Figures

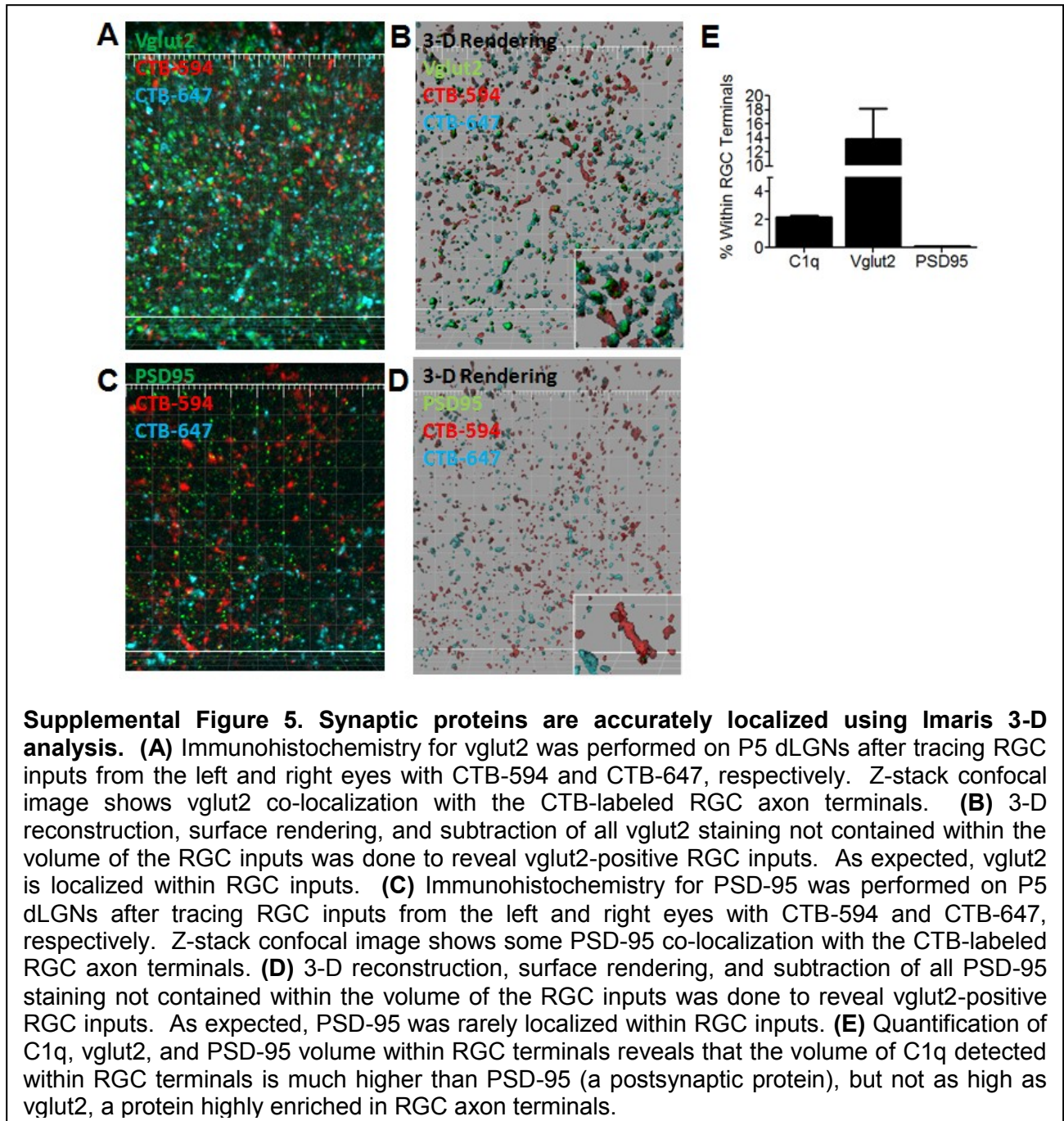
Contributions: All experiments were designed by Beth Stevens and Allison Bialas. All experiments and data analysis were performed by Allison Bialas. Arnaud Frouin assisted in preparing C1q-shRNA and C1qA overexpression constructs.

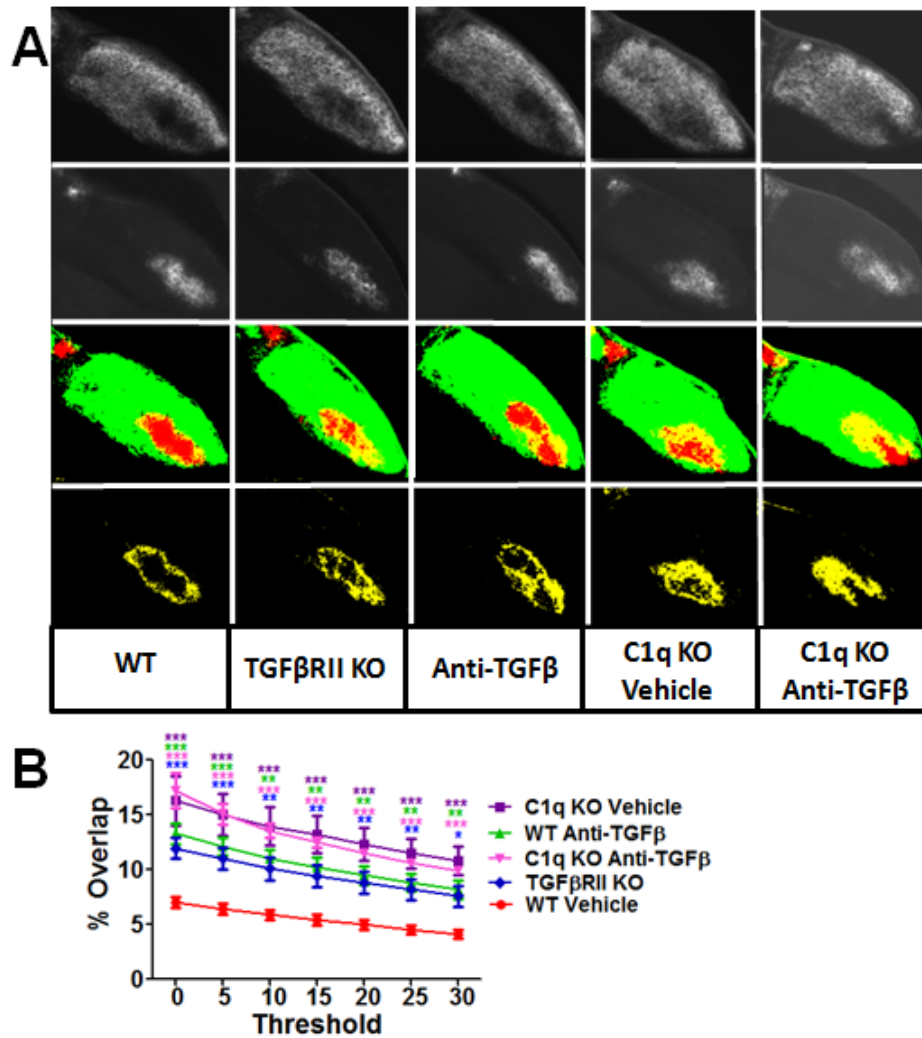






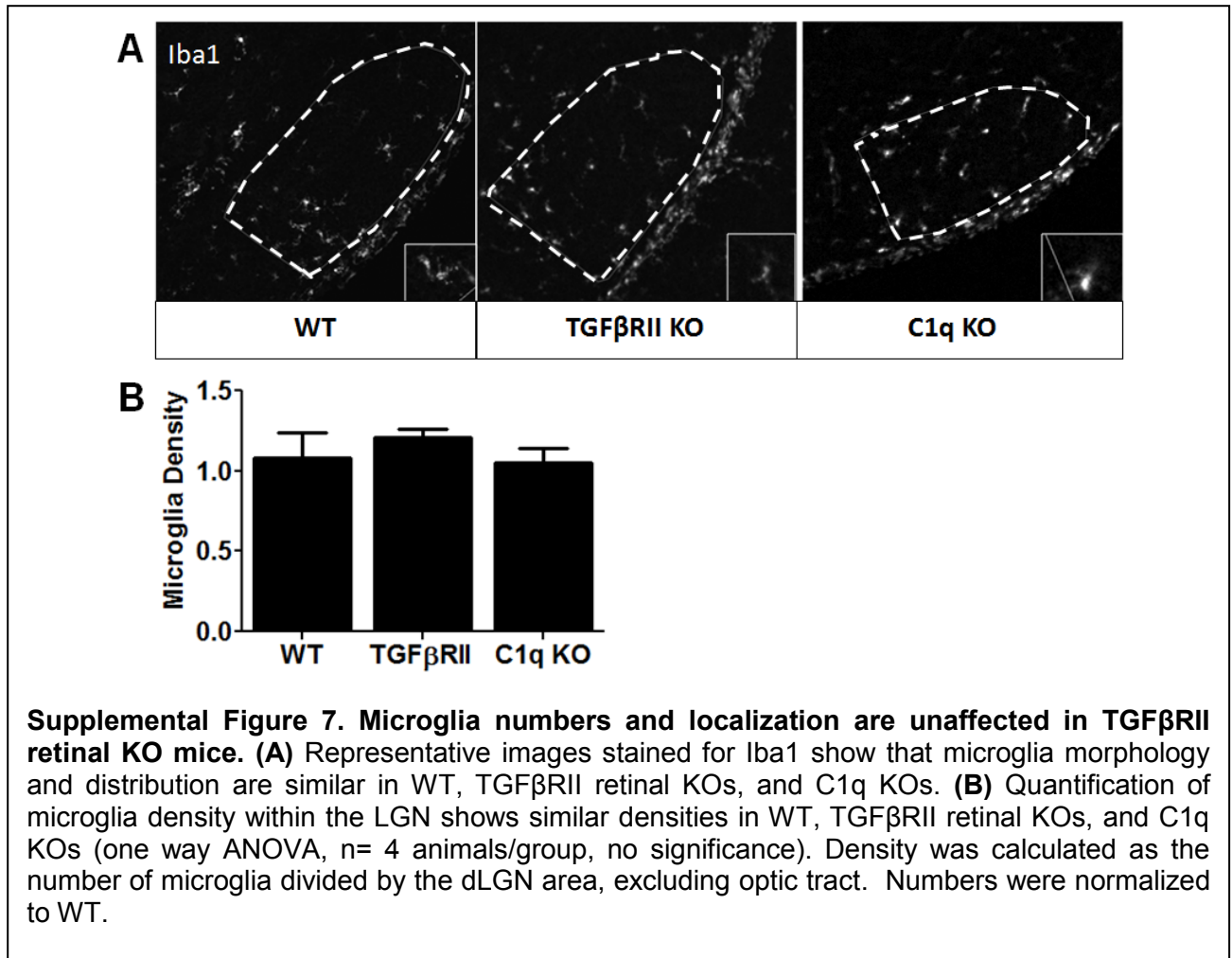


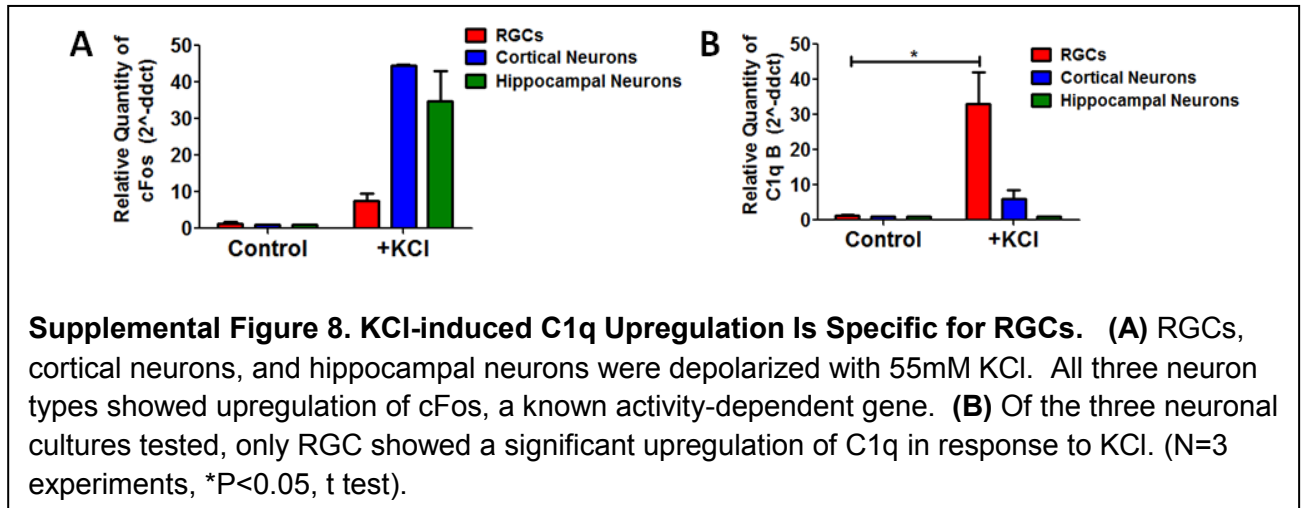


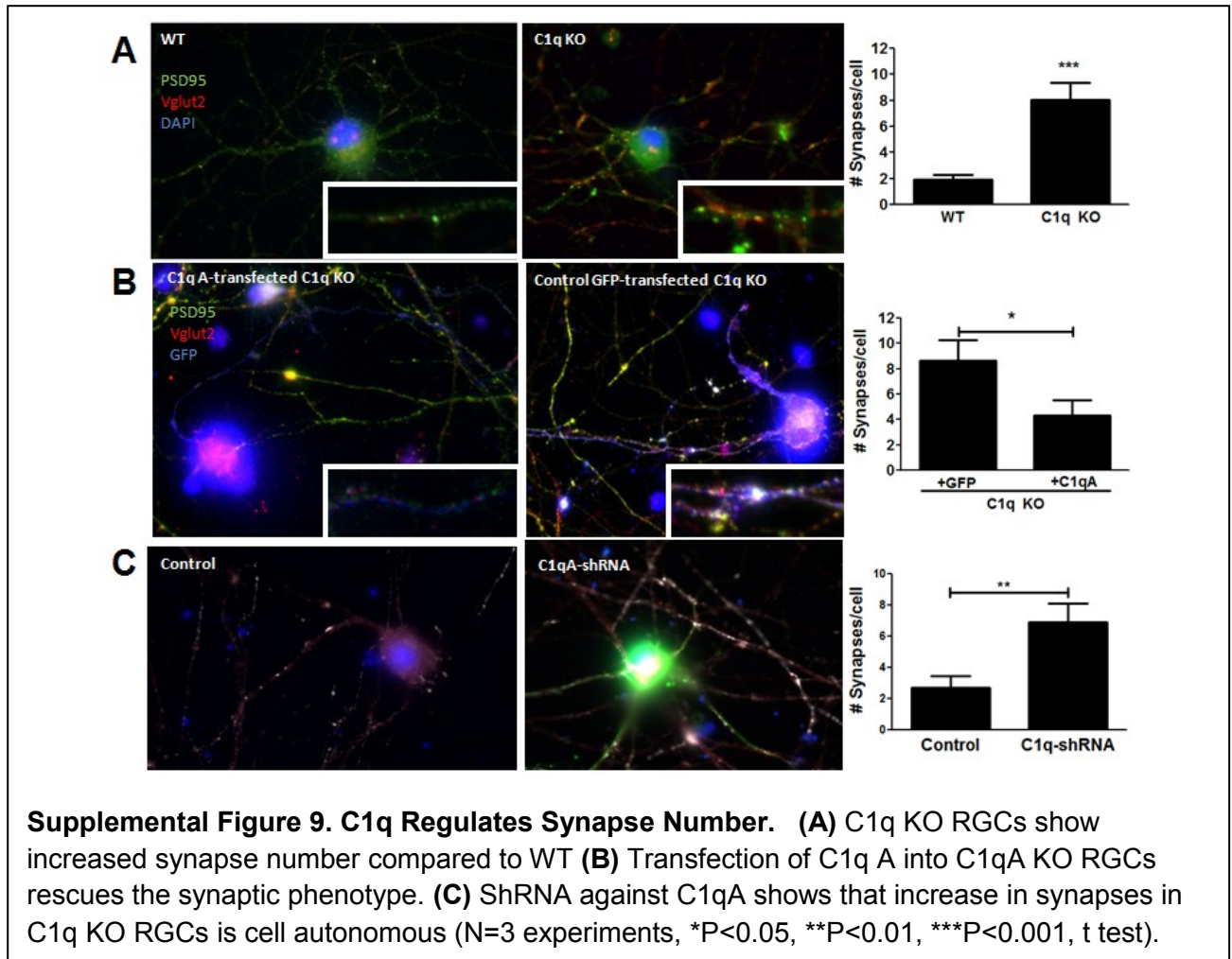


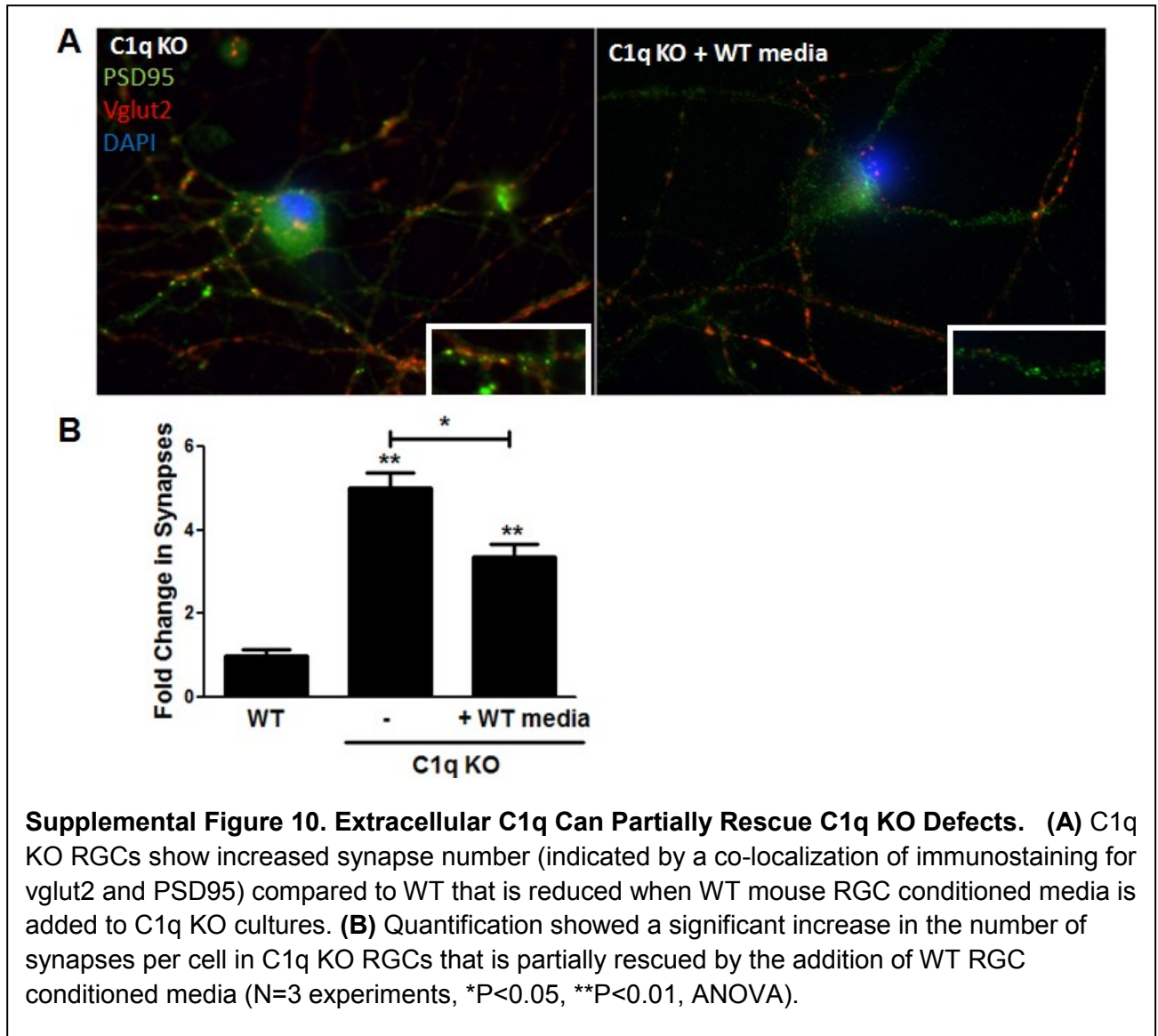
Supplemental Figure 6. Retinal TGF- β signaling is required for eye specific segregation.

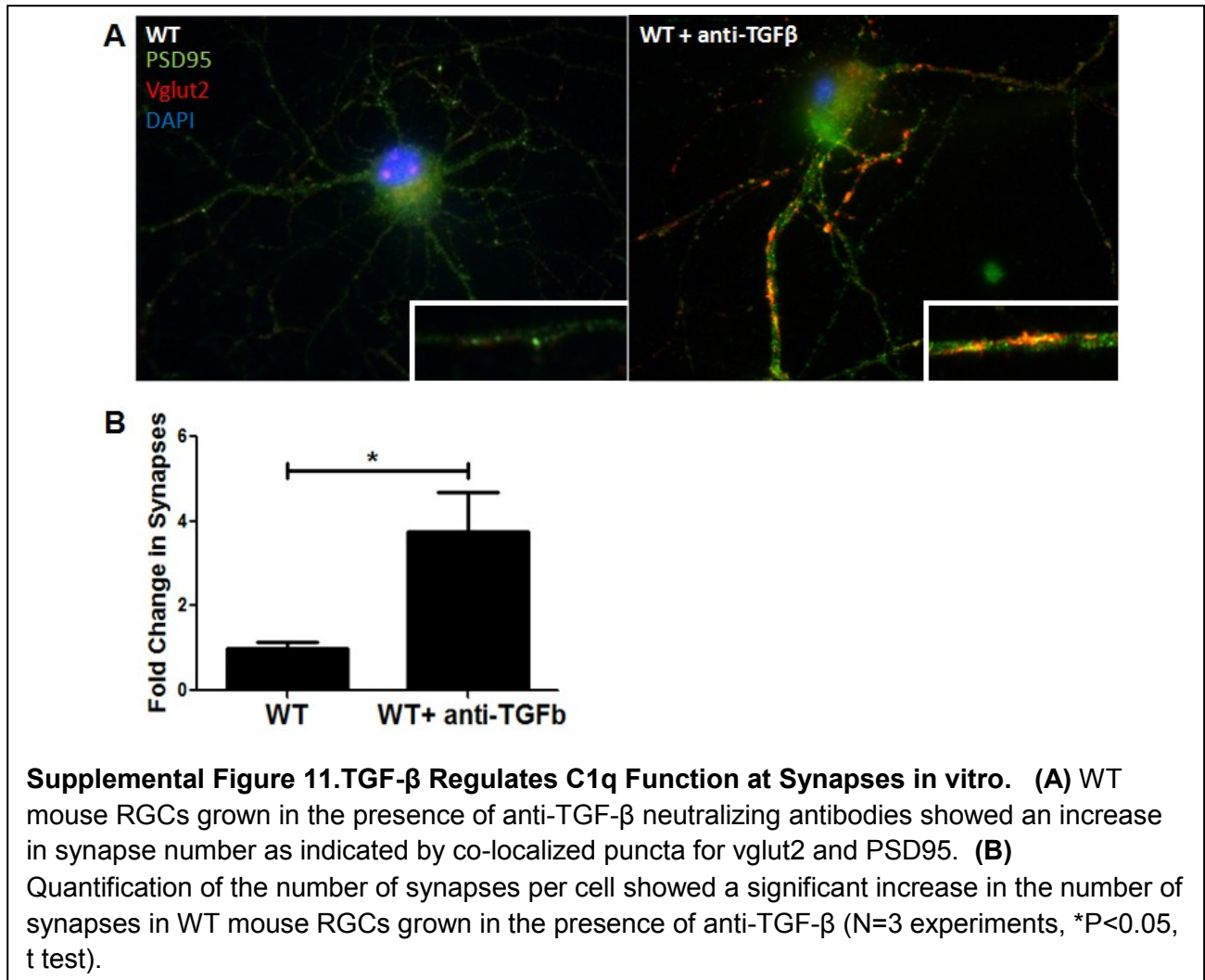
(A) Representative images of anterograde tracing (Alexa conjugated b-cholera toxin) of contralateral (green, top row) and ipsilateral (red, second row) retinogeniculate projections and their overlap (yellow, bottom row) in the dorsal LGN for WT, TGF- β retinal KO, C1q KO, and anti-TGF- β injected WT and C1q KO mice. **(B)** Quantification of percentage of dLGN area receiving input from both contralateral and ipsilateral eyes (yellow area). Data shown as mean yellow area \pm SEM. Mice deficient in TGF- β signaling (blue and green lines) show significantly more overlap between contralateral and ipsilateral inputs at P10 at every threshold measured compared to WT vehicle injected littermate mice (red) (two way ANOVA, $n = 6$ animals/group, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$). Mice deficient in C1q only, TGF- β only, and both C1q and TGF- β show similar amounts of overlap (green, pink, and purple lines) show significantly more overlap between contralateral and ipsilateral inputs at P10 at every threshold measured compared to WT littermate mice (red) (two way ANOVA, $n = 6$ animals/group, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$), but C1q KO mice injected with vehicle do not differ from C1q KO mice injected with anti-TGF- β .

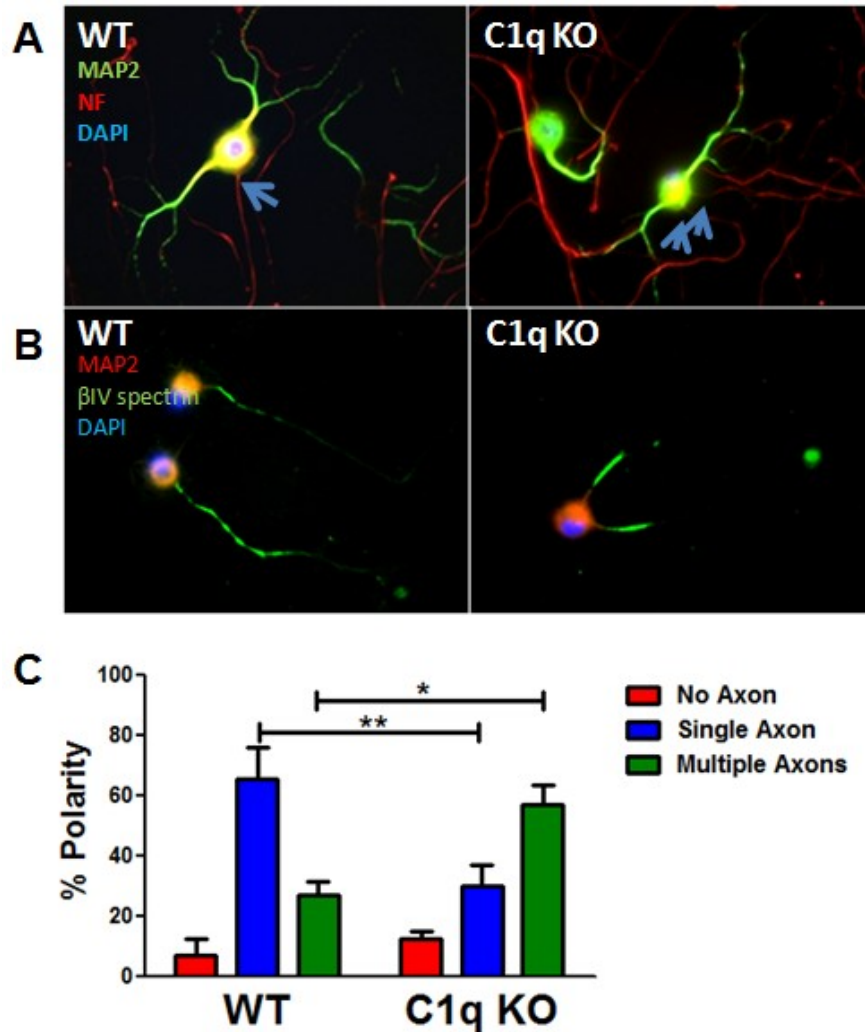












Supplemental Figure 12. C1q Regulate Neuronal Polarity in RGC Cultures. (A)

Immunostaining for MAP2 and neurofilament (NF) showed an increase in axons in C1q KOs.

(Axons =MAP2-negative, NF-positive neurites). **(B)** Immunostaining for a marker of axon initial segments, βIV spectrin, and MAP2, a dendritic marker, was used to validate that the neurites in

(A) were indeed functional axons. **(C)** Quantification of the percentage of cells with no axon (red), a single axon (blue), and 2 or more axons (green) in WT and C1q KO RGC cultures showed a significant increase in the % of cells with multiple axons and a significant decrease in the number of cells with one axon in the C1q KOs. (N=3 experiments, *P<0.05, **P<0.01, Two way ANOVA).

APPENDIX II:

Role of Glial Cells and Immune Molecules in Visual Development

Contributions: This review was written by Allison Bialas and Beth Stevens.

Publications: This review on the role of glial cells and immune molecules in visual development will appear as chapter 96 in the New Visual Neurosciences published by MIT press.

Introduction

The proper development of the visual system is dependent on communication between remarkably diverse cell types with specialized roles at each developmental stage. Until recently, visual development had been viewed largely through a “neurocentric” lens. However, new research is revealing that glia, the “other” cells of the visual system, actively communicate with neurons and one another to influence nervous system development and brain wiring. Much of our general knowledge of glial function has come from seminal studies carried out in the visual system. In particular, the mammalian retina and optic nerve have been critical model systems for studying neuron–glia communication during development for many reasons. First, all major glial cell types are represented (see figure 96.1), and their anatomical interactions and biological functions can be studied *in vitro* and *in vivo*. Second, the accessibility of the eye is particularly amenable to pharmacological and molecular manipulations of neuron–glia interactions *in vivo*. Importantly, development of pioneering methods to purify and culture retinal neurons and each of the main glial cell types has been a major advance in the field (B. Barres et al., 1988; Foo et al., 2011; Mi & Barres, 1999; Shi, Marinovich, & Barres, 1998). This approach allows for investigation of specific questions about how neurons and glia influence each other and the mechanisms involved, which are often difficult to address *in vivo*. Isolation of highly purified visual system neurons and glia has led to the elucidation of the astrocyte, oligodendrocyte, neuron, and Muller glial transcriptomes (Cahoy et al., 2008; Lovatt et al., 2007; Roesch et al., 2008), rich resources that continue to provide important insight into molecular mechanisms governing glial roles in the visual system. Together, these and other studies have revealed a prominent role for glia in wiring the visual system by regulating diverse functions including neuronal survival, axon guidance, myelination, synapse development, and plasticity. The goal of this chapter is to introduce glia, their functional roles in visual system development, and critical neuron–glia signals involved, including several immune-related signaling pathways

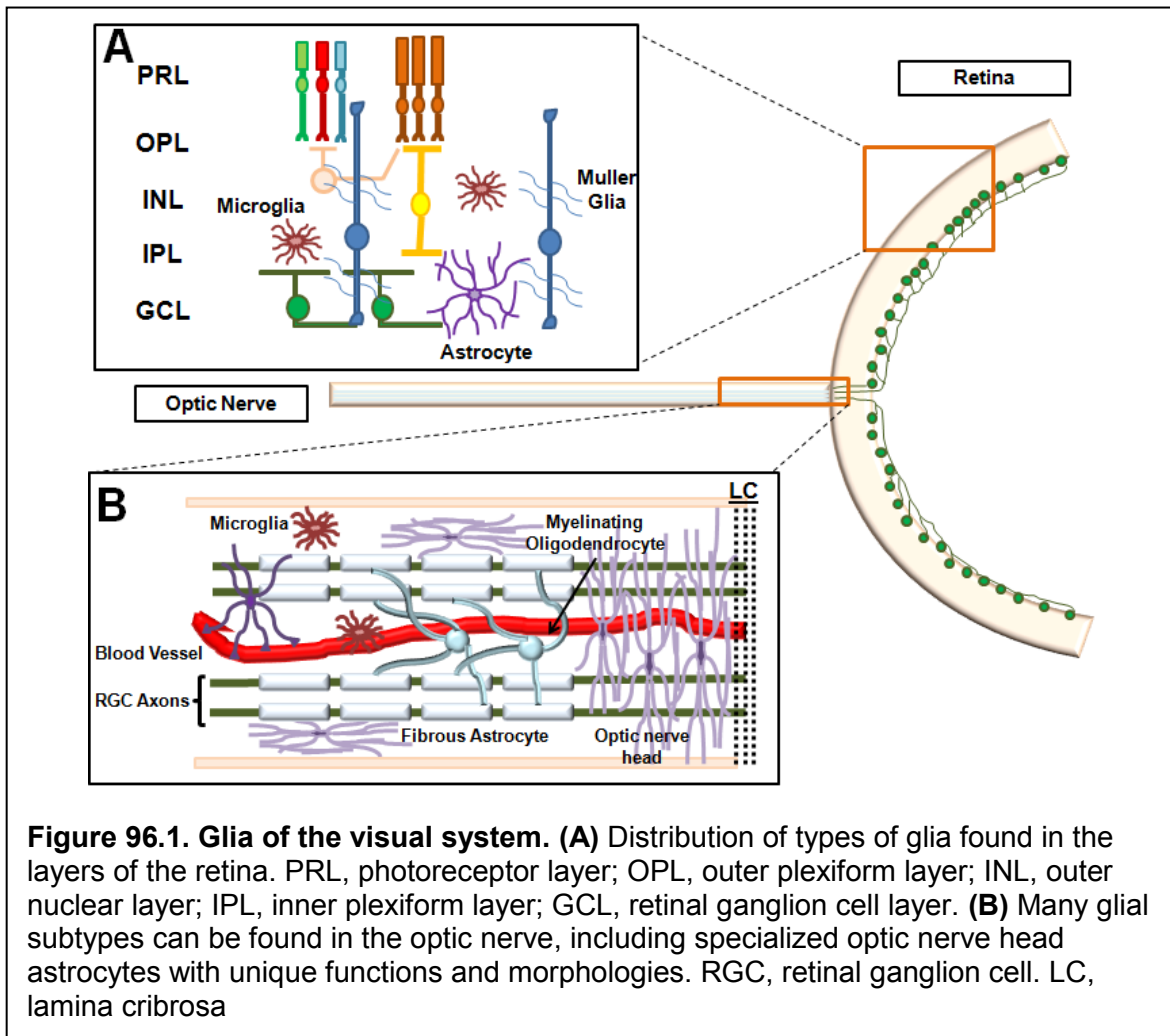


Figure 96.1. Glia of the visual system. (A) Distribution of types of glia found in the layers of the retina. PRL, photoreceptor layer; OPL, outer plexiform layer; INL, outer nuclear layer; IPL, inner plexiform layer; GCL, retinal ganglion cell layer. **(B)** Many glial subtypes can be found in the optic nerve, including specialized optic nerve head astrocytes with unique functions and morphologies. RGC, retinal ganglion cell. LC, lamina cribrosa

that help refine immature synaptic circuits. Here, we will highlight some of the recent discoveries that brought these long-overlooked cells into the spotlight.

Glia of the Visual System

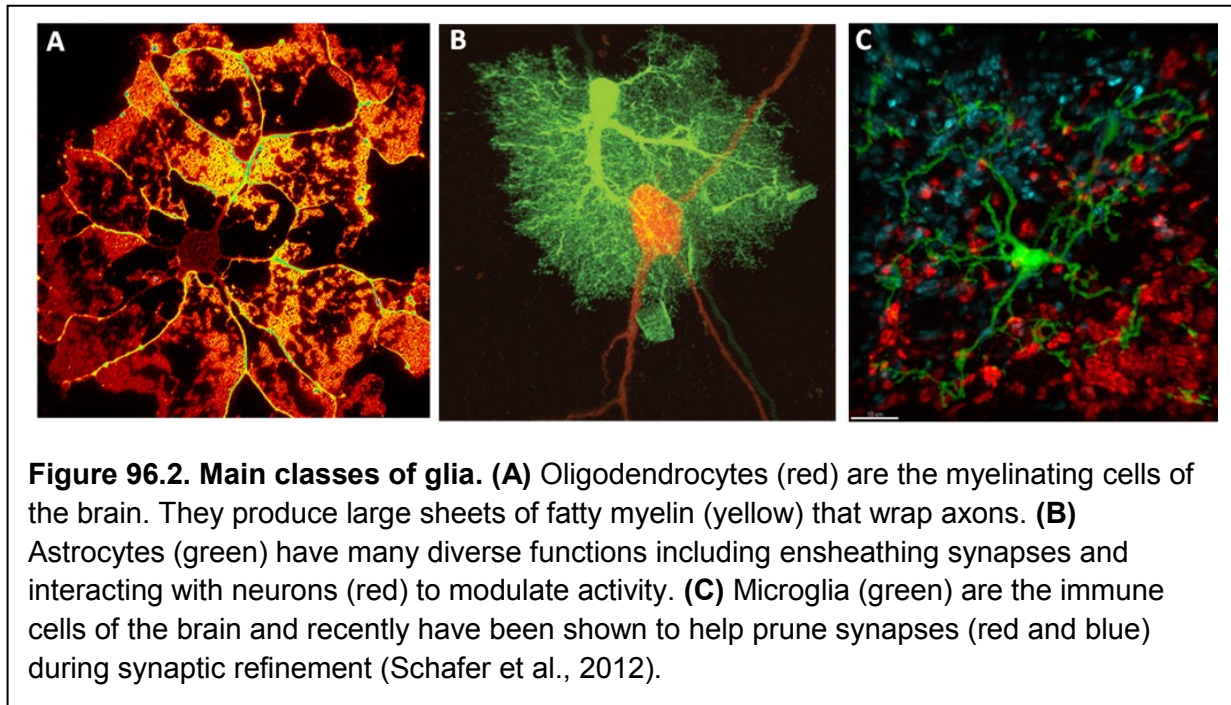
From the specialized Muller glia of the retina that act as living optic fibers to direct light to the photoreceptors (Franze et al., 2007) to the astrocytes of the visual cortex that help define receptive fields (Schummers, Yu, & Sur, 2008), virtually every level of visual processing is in some way dependent on glia. Unlike neurons, glia are electrically inexcitable cells; however, they still actively communicate with neurons and one another by chemical signals, secreted proteins, and contact-dependent molecular interactions. Before exploring glial function during visual development, it is important to define the types of glia in the visual system, which exhibit remarkable diversity with respect to their origin, morphology, and function.

In general, glia fall into three main classes (see figure 96.2): (1) Astrocytes, which extend processes that intimately associate with synapses, blood vessels, and axons; (2) oligodendrocytes, which ensheath and myelinate axons; and (3) microglia, the resident “immune” cells in the brain that rapidly respond to changes in the extracellular milieu. These general classes and functions of glia have been observed and characterized in many model systems, from flies and worms to vertebrates, suggesting that many glial functions are widely conserved (Freeman et al., 2003; Freeman & Doherty, 2006). Although it is convenient to place glia into morphological groups, it is becoming increasingly clear that the current categories fail to represent the extraordinary diversity and heterogeneity of glial morphology and function. This issue will be discussed in the sections below.

Astrocytes

Astrocytes, named for their star-like appearance, are a numerous and diverse class of glia. In addition to performing “neural support roles,” such as buffering the ionic environment and removing excess glutamate via glutamate transporters, astrocytes are active participants at

each major stage of neural development. They maintain neuronal survival, contact vasculature to modulate blood flow, stimulate neurite outgrowth, guide axons, and promote the formation and plasticity of synapses.



Astrocytes come in two general classes: fibrous (white matter/type 2) astrocytes and protoplasmic (gray matter) astrocytes. Fibrous astrocytes associate longitudinally with white matter tracts, such as the optic nerve (see figure 96.1B), whereas protoplasmic astrocytes are uniformly distributed throughout gray matter and synaptic regions (see figures 96.1 and 96.2B) (Oberheim, Goldman, & Nedergaard, 2012). Until recently, our understanding of astrocyte morphology was based largely on immunostaining for a protein highly expressed by astrocytes, glial fibrillary acidic protein (GFAP). While this protein is found in most astrocytes in the brain, its localization is hardly representative of astrocytic morphology, as previously assumed. Recent *in vivo* imaging and three-dimensional reconstructions of astrocytes revealed that, despite their name, both fibrous and protoplasmic astrocytes are not “star-shaped” after all and have complex branching patterns (see figure 96.2B).

Protoplasmic astrocytes are heterogeneous and diverse in their functions, many of which have yet to be discovered (Oberheim, Goldman, & Nedergaard, 2012; Zhang & Barres, 2010). They classically buffer ions and neurotransmitters in the extracellular space, but astrocytes also express receptors for most neurotransmitters and can release a variety of neuroactive, synaptogenic, and trophic factors at synapses. The term “tripartite synapse” has become a well-known phrase to denote the important contribution of an synapse-ensheathing astrocytic process to synapse function (Haydon, 2001). A single protoplasmic astrocyte can extend thousands of processes that intimately associate with synapses. In fact, it has been estimated that a single astrocyte can associate with an average of four neurons (Halassa et al., 2007) and contact over 100,000 synapses (Bushong et al., 2002)! Moreover, the densely packed processes of adjacent astrocytes are organized into large, nonoverlapping anatomical domains, the functional significance of which is unknown. It has been hypothesized that astrocytic tiling helps connect neurons into microcircuits, especially as astrocytes are tightly coupled via gap junctions (Nagy & Rash, 2000). Astrocytic processes from a single protoplasmic astrocyte can contact blood vessels as well as synapses, placing them in the perfect position to regulate blood brain barrier development and neurovascular coupling as well (Haydon & Carmignoto, 2006; Rubin & Staddon, 1999).

Fibrous astrocytes can be distinguished by morphology and molecular markers, including ganglioside and GFAP expression, and they are also quite heterogeneous (Raff et al., 1983; Zamanian et al., 2012). Even in the optic nerve, one can observe substantial morphological diversity (see figure 96.1B). Recent imaging studies using GFAP reporter mice have identified that individual optic nerve head (ONH) astrocytes are more elaborate and extensive than most fibrous astrocytes. In fact, the processes of ONH astrocytes span most of the width of the nerve and overlap domains of other astrocytes (see figure 96.1B). The function of these specialized astrocytes has yet to be characterized during development, but they are

particularly of interest in glaucoma research, where abnormalities in the ONH are thought to be early signs of pathology (Sun et al., 2010).

Oligodendrocytes

Oligodendrocytes, the myelinating glia of the CNS, are active participants in visual system development (see figures 96.1B and 96.2A). The myelin sheath regulates action potential propagation, axon diameter, as well as the expression and distribution of voltage-gated sodium channels (Rasband & Shrager, 2000; Sánchez et al., 1996; Schafer et al., 2006). Myelinating oligodendrocytes also provide trophic support for axons (Nave, 2010) and are potent regulators of neurite outgrowth and structure (Bandtlow, Zachleder, & Schwab, 1990; McKerracher et al., 1994). Moreover, myelin has been proposed to play a role in limiting regeneration and visual system plasticity (McGee et al., 2005; Schwab, 1990).

During postnatal development, different types of oligodendrocytes associate with axons in discrete waves of myelination throughout the CNS. Myelinating oligodendrocytes are derived from oligodendrocyte precursor cells (OPCs), which are born during late embryonic and early postnatal development. OPCs undergo a precise developmental program driven by environmental and intrinsic cues described later in this chapter that regulate their development into myelinating oligodendrocytes (Dugas et al., 2006). Interestingly, early studies suggest that OPC proliferation may be regulated by neuronal activity (Barres & Raff, 1993), among other molecular cues.

Recent studies also demonstrate that a subset of OPCs expressing the chondroitin sulfate proteoglycan, NG2, have unique characteristics. These “NG2” cells are multipotent and can give rise to neurons, astrocytes, and oligodendrocytes (Nishiyama et al., 2009; Zhu, Bergles, & Nishiyama, 2008), but perhaps their most unique property is the fact that they can receive synaptic input from neurons and may be integrated into the neural circuitry of the brain (Bergles et al., 2000; Lin & Bergles, 2003). These synaptic junctions can be associated with either glutamatergic or GABAergic neurons and exhibit all the characteristics of a typical

neuron–neuron synapse. The significance of these synapses is still unknown, but it has been suggested that these connections enable rapid communication between neurons and these glial progenitors to alter the behavior of these cells (Bergles, Jabs, & Steinhäuser, 2010).

Mature myelinating oligodendrocytes are also remarkably heterogeneous. They are generally classified into subtypes based on their branching pattern (del Río-Hortega, 1928), the type of axon they myelinate, and the thickness of myelin formed (Butt et al., 1995).

Oligodendrocytes are excluded from the retina; therefore retinal ganglion cell (RGC) axons within the eye are unmyelinated (Boiko et al., 2001). There are also nonmyelinating oligodendrocytes throughout the CNS, but their role in visual system development is unclear, although the close proximity of these cells to neurons and synapses suggests they may help to regulate the microenvironment around neurons.

Microglia

Microglia are the primary immune and phagocytic cells of the CNS (see figures 96.1 and 96.2C). Microglia are myeloid-derived cells and, until recently, they were thought to develop from peripheral macrophages that entered and colonized the brain throughout development and into adulthood. We now know that most microglia arise from a unique pool of yolk-sac derived myeloid progenitors and migrate into the CNS by embryonic day E10.5 (Ginhoux et al., 2010). Following migration into the CNS, microglia undergo a slow maturation and differentiation process that persists through late postnatal development (Ransohoff & Perry, 2009). Microglia start as highly phagocytic, amoeboid cells and maintain this morphology through the period of programmed cell death when they actively remove debris and dead cells (Ferrer et al., 1990). By the first postnatal week in mouse, these cells develop branches which are used to continually survey the environment and down-regulate their phagocytic receptors (see figure 96.2C) (Ling & Wong, 1993). In the event of injury or infection, microglia quickly adopt a more classically “reactive” state and migrate to the site of injury where they shield the injury site, engulf pathogenic material or debris, and release many cytokines and chemokines (Davalos et

al., 2005; Nimmerjahn, Kirchhoff, & Helmchen, 2005). While past work had focused on the role of microglia during disease, recent studies have revealed dynamic interactions between microglia and synapses in the healthy brain, particularly during developmental synaptic pruning (Schafer, Lehrman, & Stevens, 2012; Tremblay et al., 2011).

The preponderance of glia throughout the visual system and their seemingly endless array of functions have led developmental neurobiologists to ask how these diverse cells influence visual system development. Understanding how glia communicate with neurons and each other to wire up the visual system has evolved into a broad and prolific area of research in developmental neurobiology. In the following sections, we will explore some of the major neuron–glia signaling interactions that help guide each major stage of visual system development.

How Are the Diverse Neurons and Glia of the Visual System Generated?

One of the earliest roles for glia in the developing brain is the generation of neurons and other glial cells. In fact, it is well established that radial glia in the ventricular zone serve as precursors to neurons and some glia, including astrocytes and oligodendrocytes (Alvarez-Buylla & García-Verdugo, 2002; Doetsch, 2003). Radial glia, as their name suggests, each extend a long process toward the pial surface that guides the outward migration of newly born neurons. In the retina, specialized radial glia called Muller cells give rise to many of the cells of the retina, including amacrine cells, horizontal cells, photoreceptors, Muller glia, and bipolar cells (Prada et al., 1991) (see figure 96.1A). Unlike radial glia of the ventricular zone which are transient, Muller glia persist throughout life and have unique functions in the retina. Their radial processes extend throughout the layers of the retina and act as “living optic fibers,” allowing light to be transmitted to the photoreceptors without distortion (Franze et al., 2007) (see figure 96.1). In addition to their important role in light transmission, Muller glia, a type of specialized astrocyte, perform many of the functions carried out by astrocytes in the rest of the brain, including modulation of blood flow and neuronal activity (de Melo Reis et al., 2008; Newman & Zahs, 1998).

Interestingly, recent work has also uncovered neurogenic potential for adult Muller cells from many organisms including humans, suggesting novel therapeutic potential for these cells for eye diseases (Bhairavi et al., 2011; Fischer & Reh, 2001; Ramírez & Lamas, 2009; Takeda et al., 2008).

Where do the rest of visual system glia come from, and how are they guided to their appropriate territories? Early in development, pools of glial precursor cells residing in the ventricular zones follow one of several migratory streams to their appropriate location. During gliogenesis, six individual glial precursor cell types have been identified (Lee, Mayer-Proschel, & Rao, 2000); however, rigorous fate-mapping studies following glial development have yet to be performed. Glial precursor cells are derived from the same radial glia that generate neurons. The birth of oligodendrocytes and astrocytes begins as neurogenesis ends; however, astrocytes and oligodendrocytes remain in an immature state in the first few weeks of life.

The arrival and maturation of the various glial subtypes differs significantly. The majority of microglia seem to migrate into the CNS by embryonic day E10.5 (Ginhoux et al., 2010; Saederup et al., 2010); however, they do not reach their mature branched state until the second postnatal week. OPCs are the first glial cells born from radial glia after neurogenesis. OPCs undergo a maturation process that is not fully complete in all areas of the brain until adulthood (see myelination section). Astrocytes are the last cells born in the brain, and they are derived from radial glia. They migrate along the optic nerve and arrive in the optic nerve fiber and RGC layers of the retina, along with the rest of the visual system, toward the end of embryonic and early postnatal development (Watanabe & Raff, 1988). These cells also have an immature phenotype with a distinct gene expression profile including increased expression of intermediate filaments such as nestin and vimentin, some synaptogenic cues, and other neuroactive substances (Cahoy et al., 2008; Christopherson et al., 2005).

As glia populate the nervous system, they profoundly affect neuronal function; however, newborn neurons play an active role in regulating gliogenesis and glial function as well. In the

retina, for example, RGC axons secrete Shh and additional growth factors which promote expansion of astrocyte precursors in the embryonic and postnatal optic nerve (Burne & Raff, 1997; Dakubo et al., 2003). Another progenitor cell pool that is modulated by neuronal signaling is the NG2 cell pool, discussed earlier in this chapter. These cells retain the potential to generate neurons and astrocytes into adulthood, and it is postulated that special synaptic connections between neurons and NG2 cells may be a way in which neurons can rapidly trigger proliferation of this population (Nishiyama et al., 2009; Zhu, Bergles, & Nishiyama, 2008).

How Are Neurons and Axons Guided to Their Proper Locations?

It has long been thought that glia play a key role in neuronal migration and guidance. Indeed, not only do radial glia give rise to neurons but also their processes serve as roadmaps guiding newborn neurons to their appropriate locations. Once neurons populate the visual system, they next must specify axons and dendrites to connect with appropriate targets. Glia play an important role in guiding axons along their path by secreting guidance cues and providing structural support for axons.

Glia-derived cues have been shown to influence axon structure and guidance *in vivo*. In *Drosophila*, for example, photoreceptor axons are guided along their appropriate path by a stream of glial cells, known as retinal basal glia (RBG), migrating from the optic stalk into the optic disc (Rangarajan, Gong, & Gaul, 1999). When migration of RBG cells into the optic disc is blocked, as in the *gish* loss-of-function mutant, photoreceptor cells extend axons away from instead of into the optic stalk (Hummel et al., 2002). Once photoreceptor cell axons reach their targets, another *Drosophila* glial cell type, the lamina glia, help guide these axons to the appropriate lamina of the optic lobe, reminiscent of classic guidepost cells (Bentley & Caudy, 1983; Poeck et al., 2001; Suh et al., 2002). Interestingly, however, lamina glia initially require axonal cues to migrate to their stereotyped laminar positions. They migrate along optic lobe axons to their correct position. Absent or aberrant projections from optic lobe neurons result in a failure of glial migration (Dearborn & Kunes, 2004).

In the mammalian visual system, glia also are critical for RGC axon guidance. Radial glia localized to a region of the ventral midline known as the “glial palisade” secrete molecular cues which guide RGC axons to form the optic chiasm at this position (Mason & Sretavan, 1997). Glial Ephrin-B2 and EphB1 signaling have been identified as critical guidance cues at the chiasm. During development of RGC ipsilateral projections, ephrin-B2 is expressed by radial glial cells at the midline. Ipsilaterally projecting RGCs, which express EphB1, are repelled by ephrin-B2-producing radial glia of the midline. Consistent with a role for ephrin-B2 and EphB1 signaling in axon guidance at the chiasm, blockade of ephrin-B2 prevents the formation of ipsilateral projections in mice and mice deficient in EphB1 show a dramatic reduction in the number of ipsilateral projections (Williams et al., 2003).

The target neurons for RGCs in the lateral geniculate nucleus (LGN) also appear to be guided into their appropriate positions by glia. In most mammals, the LGN is divided into several cell layers including magnocellular and parvocellular layers that receive input from distinct types of RGCs. The cues that regulate the formation of these layers are not well understood; however, laminations in the distribution of glial proteins such as GFAP and vimentin were observed prior to the development of neuronal layers, suggesting that a glial-derived cue may direct neurons to their locations (Hutchins & Casagrande, 1988).

How Does Myelin Form and Function in the Developing Visual System?

Myelination is one of the most complex cellular interactions in biology. The process requires recognition, association, ensheathment, and elaboration of glial membrane around an appropriate axon at precisely the right time in development. The end result is the formation of compact myelin sheath, which is essential for rapid propagation of nerve impulses along axons. Much of our understanding of the formation and function of myelin comes from studies carried out in the visual system. In particular, the vertebrate optic nerve (see figure 96.1B) has served as an excellent model system in which the stages of myelination have been extensively studied both in vitro and in vivo. These and other studies have uncovered critical molecular pathways

regulating oligodendrocyte maturation and myelination (Emery, 2010a; Pogoda et al., 2006). The process begins with the differentiation of an OPC into a postmitotic, premyelinating oligodendrocyte. Once oligodendrocytes mature, environmental cues drive the progression of myelination. The factors promoting myelination, therefore, include both intrinsic oligodendrocyte factors that promote maturation and extrinsic factors from the axon or other cell types (Emery, 2010a, 2010b). A recently developed coculture system that enables rapid myelination of CNS axons offers new opportunities for molecular dissection of multiple stages of myelination (Watkins et al., 2008).

Oligodendrocytes are critically dependent on continual communication with axons during embryonic and early postnatal development. A number of molecular pathways have been identified that regulate myelination either directly or indirectly. For example, Wnt/ β -catenin signaling is critical to drive terminal oligodendrocyte differentiation. Wnt signaling is specifically activated as terminal oligodendrocyte differentiation begins, but in mature oligodendrocytes, down-regulation of Wnt signaling appears to be necessary for myelination as mutations preventing Wnt down-regulation lead to myelination defects (Fancy et al., 2009; Fu et al., 2009). Conversely, cues such as Jagged (Wang et al., 1998), polysialylated-neural cell adhesion molecule (PSA-NCAM) (Grinspan & Franceschini, 1995), and LINGO-1 (Mi et al., 2005), all inhibit either OPC differentiation or myelination.

In addition to these molecular cues, neural activity is thought to regulate myelination. Early studies in the optic nerve demonstrated that mice reared in the dark developed fewer myelinated axons compared with control mice (Gyllenstein & Malmfors, 1963). Hypomyelination was also observed in optic nerve of the naturally blind cape-mole rat (Omlin, 1997) whereas prematurely opening the eyes in rabbit accelerated myelination (Tauber, Waehneltd, & Neuhoff, 1980). A number of recent in vitro studies have provided additional information on activity-dependent myelination. Activity-dependent regulation of axon-derived cues is one proposed mechanism (Itoh et al., 1995). For example, axonal release of ATP and adenosine is thought to

stimulate purinergic receptors on OPCs promoting oligodendrocyte maturation and myelination (Stevens et al., 2002). More recent experiments suggest that astrocytes influence activity-dependent regulation of myelination via ATP-dependent production of leukemia inhibitory factor (LIF). LIF could then promote OPC maturation and myelination (Ishibashi et al., 2006, 2009; Watkins et al., 2008). The precise molecular mechanisms underlying activity-dependent effects on myelination remain an active area of investigation (Emery, 2010a).

What are the intrinsic signals controlling myelination? The oligodendrocyte transcriptome has revealed many highly expressed, oligodendrocyte-specific molecules whose roles are mostly unknown (Cahoy et al., 2008; Nielsen et al., 2006). One such key factor is myelin regulatory factor (MRF). MRF is a nuclear protein developmentally expressed specifically in postmitotic oligodendrocytes. RNA interference knockdown of MRF in oligodendrocytes suppresses expression of CNS myelin genes while overexpression of MRF promotes myelin gene expression. Mice in which MRF has been knocked out specifically in oligodendrocytes fail to generate myelin, display severe neurological abnormalities, and die in the first few postnatal weeks (Emery et al., 2009). Moreover, oligodendrocytes in these mice are arrested in the premyelinating stage, suggesting MRF is a master switch for myelination. Several other transcription factors have been identified that either promote or inhibit maturation of OPCs (Dugas et al., 2010). The transcription factor *Olig2* is initially responsible for specifying the oligodendrocyte lineage (Lu et al., 2002; Zhou, Choi, & Anderson, 2001). Downstream transcription factors including *Olig1*, *Ascl1*, *Nkx2.2*, *Sox10*, *YY1*, and *Tcf4* as well as MRF then promote maturation of OPCs into myelinating oligodendrocytes while cells expressing *Id2*, *Id4*, *Hes5*, and *Sox6* maintain an OPC phenotype (Wegner, 2008).

Other Functions of Myelin

In addition to increasing the efficiency and speed of action potentials, myelinating oligodendrocytes wear many other hats. They appear to regulate axon structure, neurite outgrowth, and the expression and localization of ion channels along axons (Sánchez et al.,

1996; Starr et al., 1996). For example, recent work has shown that contact between RGCs and myelinating oligodendrocytes enhances axon function by altering voltage-gated sodium channel (Na_v) expression and distribution. In mature RGCs, the voltage-gated sodium channel, $\text{Na}_v1.2$, exclusively localizes to unmyelinated portions of RGC axons within the eye while a different type, $\text{Na}_v1.6$, is localized to nodes of Ranvier along the myelinated axon. Interestingly, during development, $\text{Na}_v1.2$ is first expressed throughout the axon, including at nodes of Ranvier. As myelination progresses, however, $\text{Na}_v1.2$ channels are replaced at mature nodes of Ranvier with $\text{Na}_v1.6$ channels, suggesting that myelin-derived cues guide channel expression and localization (Boiko et al., 2001).

Myelin is also a potent inhibitor of neurite outgrowth. As myelination proceeds in waves in the CNS, regions that become myelinated early may act as repulsive guidance cues for CNS tracts that form later in development (Schwab & Schnell, 1991). Candidate myelin-derived proteins that inhibit axon growth include Nogo, NI-35, NI-250, and myelin-associated glycoprotein (MAG) (Caroni & Schwab, 1988a, 1988b; McGee et al., 2005; McKerracher et al., 1994), and these molecules have been extensively studied *in vitro* and *in vivo*. Interestingly, peripheral nervous system myelin does not inhibit regeneration or growth, suggesting that unique components of CNS myelin underlie growth inhibition (Mukhopadhyay et al., 1994). Understanding the mechanisms of growth inhibition in the CNS is an area of active research in the regeneration field and may also be relevant to critical period plasticity, which we will discuss.

What Do Astrocytes Do at Developing Synapses?

Establishment of the correct numbers and types of synapses is crucial for proper visual system development and function. We know a great deal about the neuronal proteins required for the assembly and maturation of CNS synapses; however, pioneering studies in the mammalian visual system have identified astrocytes as critical mediators of CNS synaptogenesis through several secreted signals. The spatiotemporal correlation between the appearance of immature astrocytes at CNS synapses and the onset of synaptogenesis

suggested that astrocytes might provide important synaptogenic cues. For example, in the rodent visual system, RGC axons innervate the superior colliculus by birth (Lund, 1972). However, there is a 1-week delay before the majority of synapses are formed. This delay coincides with the birth and proliferation of astrocytes.

Do astrocytes promote synaptogenesis? The establishment of methods to purify and culture rodent RGCs allowed Pfrieger and Barres to ask whether synapses could form in the absence of astrocytes (Pfrieger & Barres, 1997). Purified RGCs were healthy, elaborated dendrites and axons but formed few synapses when cultured without astrocytes. In contrast, addition of a feeding layer of astrocytes or astrocyte-conditioned medium significantly increased RGC synaptic activity and the number of structural synapses that formed (Pfrieger & Barres, 1997; Ullian et al., 2001). These and other findings indicated that secreted factors from astrocytes strongly enhance pre- and postsynaptic function in developing RGCs (Ullian et al., 2001) and prompted further exploration into the specific molecules that control this process.

What Are the Astrocyte-Secreted Synaptogenic Factors?

One class of astrocyte-secreted molecules that was recently identified are thrombospondins (TSPs) (Christopherson et al., 2005), a large multimeric family of extracellular matrix proteins (see table 96.1). In vivo, TSP-1 and -2 localize to astrocytic processes and synapses during development. Using purified RGC cultures, it was later found that astrocyte-derived TSPs are necessary and sufficient for the assembly of structural synapses. The synapses induced by TSPs are ultrastructurally normal, presynaptically active, but postsynaptically silent. Mice deficient for both TSP-1 and -2 exhibited fewer synaptic puncta by immunostaining, demonstrating the importance of this molecule in normal development in vivo. Further investigation revealed that a calcium channel subunit, $\alpha 2\delta$ -1, is the receptor for TSP required for synaptogenesis (Eroglu et al., 2009). The molecular mechanism by which TSP- $\alpha 2\delta$ -1 interaction mediates synaptogenesis has yet to be fully elucidated. Interestingly, $\alpha 2\delta$ -1 is also the receptor for an anti-epileptic drug, gabapentin. Treating

cultured RGCs or mice with gabapentin during synaptogenesis significantly reduced the number of synapses formed, identifying gabapentin as a potent inhibitor of synaptogenesis (Eroglu et al., 2009).

Are There Other Astrocyte-Derived Synaptogenic Signals?

Recent work has identified additional astrocyte-secreted proteins, hevin and SPARC, that regulate synaptogenesis (Kucukdereli et al., 2011). Both of these proteins are enriched in the superior colliculus coinciding with robust synaptogenesis during the postnatal period. Hevin, like TSP, promotes the formation of ultrastructurally normal synapses that are postsynaptically silent. Conversely, SPARC inhibits synaptogenesis by specifically antagonizing hevin. Although SPARC does not directly interact with hevin, it appears to compete with hevin for a common binding partner. Consistent with in vitro results, mice deficient in SPARC show an increase in

Table 96.1: Astrocyte-derived Molecules Regulate Synaptogenesis

Molecule	Effect on Synapses	Citation
Thrombospondins	+ Structural synapses + Presynaptic function	Christopherson et al. (2005)
Hevin	+ Structural synapses + Presynaptic function	Kucukdereli et al. (2011)
SPARC	– Structural synapses	Kucukdereli et al. (2011)
ApoE/cholesterol	+ Synapse number and efficacy	Mauch et al. (2001)
Glypican	+ Structural and functional synapses +Pre- and postsynaptic function	Allen et al. (2012)

synapse number in the superior colliculus while hevin-deficient mice show a reduction (see table 96.1). The discovery of these molecules and their interaction demonstrates that astrocytes can both positively and negatively regulate synaptogenesis to influence synapse number.

What is the elusive factor that converts silent synapses into fully functional connections? Recent work has identified glypicans as a novel class of astrocyte-derived proteins that regulate the maturation of postsynaptic machinery, specifically by inserting GluR1 subunits into the postsynaptic membrane (Allen et al., 2012). Glypican-4 and -6 (see table 96.1) were identified as molecules sufficient to induce fully functional synapses in RGC cultures and in vivo (Allen et al., 2012). Glypicans are the first astrocyte-derived molecules to be identified that can induce fully functional synapses, raising many questions about the underlying mechanisms.

Since the initial finding that astrocytes regulate synaptogenesis, the list of astrocyte-derived synaptogenic factors continues to grow. For example, cholesterol complexed with apoE enhances presynaptic function in RGC cultures by regulating presynaptic release machinery and potentially enhancing dendritic maturation (Goritz, Mauch, & Pfrieder, 2005; Mauch et al., 2001). Understanding how these astrocyte-derived factors cooperate to regulate synapse formation is an area of active investigation. One hypothesis is that astrocytes regulate distinct phases of synaptogenesis (i.e. initial adhesion vs. maturation) via different factors and signaling pathways. Alternatively, many of these signals could act in a complex to initiate a common downstream pathway required to assemble functional CNS synapses.

How Is Connectivity Refined?

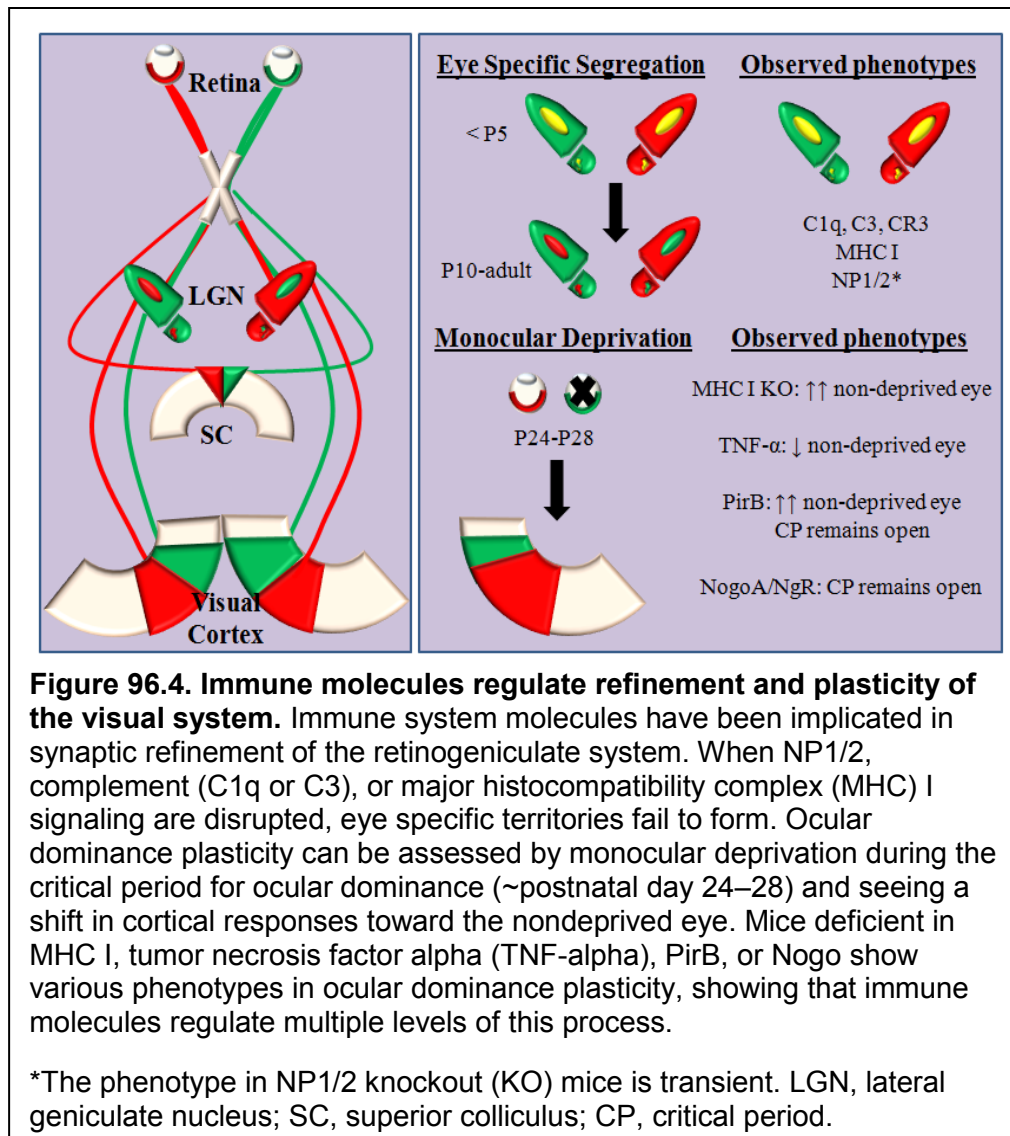
Glia and Immune Molecules Sculpt Developing Circuits in the Visual System

The circuitry established during synaptogenesis is initially imprecise and includes many weak synapses which compete with each other for postsynaptic territory. This activity-dependent competition leads to synaptic refinement—the selective pruning of inappropriate synapses and strengthening of appropriate synaptic connections (reviewed in Hua & Smith, 2004; Huberman, Feller, & Chapman, 2008; Katz & Shatz, 1996). While the role of neuronal activity in developmental synaptic pruning is well established (Hua & Smith, 2004; Katz & Shatz, 1996; Sanes & Lichtman, 1999) (and covered in more detail in other chapters), the molecular

mechanisms linking neuronal activity with synaptic pruning are less clear. Recent studies implicate glia and immune molecules in sculpting synaptic circuits in the visual system.

The previous section discussed a role for astrocytes in forming new synapses. Could they also influence synapse elimination? A screen to determine how astrocytes influence neuronal gene expression first identified the innate immune protein, C1q, as one of the few genes that was highly up-regulated in purified retinal ganglion neurons (RGCs) in response to astrocyte-derived secreted factors (Stevens et al., 2007). This was a surprising finding since C1q was not thought to be expressed by neurons in the healthy brain and microglia were presumed to be the only source of C1q in the brain. Perhaps even more unexpected was the finding that C1q and downstream complement protein C3 localize to subsets of synapses throughout the postnatal brain and retina (Stevens et al., 2007). In the immune system, C1q is the initiating protein in the classical complement cascade. The complement cascade opsonizes or “tags” pathogenic microbes and cellular debris for rapid elimination by phagocytic macrophages or complement-mediated cell lysis. Could these classic immune molecules be similarly opsonizing or tagging immature synapses for elimination?

This idea was tested in the mouse retinogeniculate system—a classical model for studying activity-dependent developmental synapse elimination (reviewed in (Guido, 2008; Hong & Chen, 2011; Huberman, 2007; Sretavan & Shatz, 1986). Early in development, RGCs form transient synaptic connections with relay neurons in the dorsal LGN (dLGN) of the thalamus (see figure 96.4). During the first 2 postnatal weeks, many of these transient retinogeniculate synapses are permanently eliminated (Campbell & Shatz, 1992; Hooks & Chen, 2006; Sretavan & Shatz, 1984). Consistent with a role for the classical complement



cascade in synaptic pruning, mice deficient in C1q and downstream C3 exhibit sustained defects in synapse elimination, as shown by the failure to segregate into eye-specific territories and the retention of multi-innervated LGN relay neurons (Stevens et al., 2007) (see figure 96.4). These mice still undergo a substantial degree of synaptic refinement (Stevens et al., 2007), suggesting that complement proteins cooperate with other pathways and neuronal activity to refine developing retinogeniculate circuits. Together, these findings raise many questions regarding the underlying cellular and molecular mechanisms. For example, if complement is tagging synapses, how then are complement-tagged synapses eliminated?

A Novel Role for Microglia in Developmental Synaptic Pruning

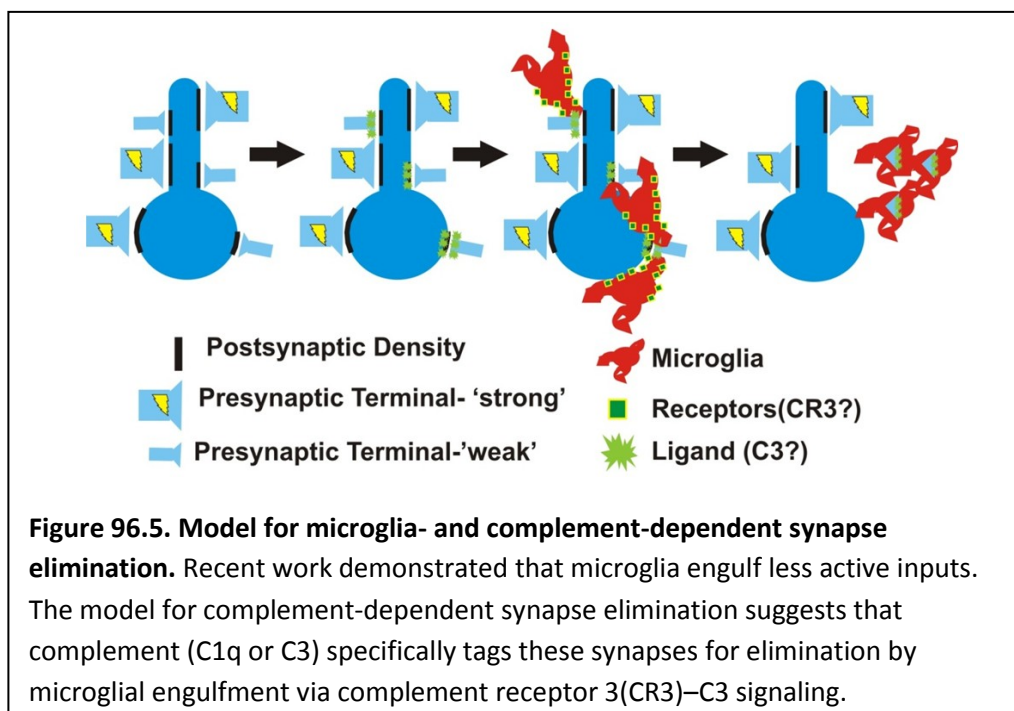
Emerging evidence implicates microglia as key players in developmental synaptic pruning (Ransohoff & Stevens, 2011; Schafer et al., 2012; Schafer, Lehrman, & Stevens, 2012). As discussed earlier, process-bearing phagocytic microglia have been observed in the dLGN and several other postnatal brain regions including hippocampus, cerebellum, and olfactory bulb (Dalmau et al., 1998; Perry, Hume, & Gordon, 1985), but until recently, the function of microglia in normal brain had remained a mystery. Microglia express an array of phagocytic receptors, including complement receptor 3 (CR3/CD11b/CD18) that could mediate the engulfment of synaptic elements during development. In the brain, microglia are the only resident cells expressing CR3 (Bobak et al., 1987; Graeber, 2010; Guillemin & Brew, 2004; Ransohoff & Perry, 2009). In the immune system, the activated C3 fragment C3b (iC3b) opsonizes the surface of cells/debris and “tags” them for elimination by phagocytic macrophages that express C3 receptors (CR3/CD11b) (Carroll, 2004; Gasque, 2004; van Lookeren Campagne, Wiesmann, & Brown, 2007).

Using the mouse retinogeniculate system as a model, microglia were found to engulf RGC presynaptic inputs during a peak pruning period in the developing dLGN (Schafer et al.,

2012). Moreover, genetic or pharmacological disruptions in microglia-mediated engulfment during the postnatal period resulted in sustained functional deficits in eye-specific segregation. Furthermore, microglia-mediated engulfment of synaptic inputs was dependent upon signaling between CR3, expressed specifically by microglia, and complement component C3, which is highly expressed in the postnatal dLGN (see figure 96.4) (Schafer et al., 2012). Interestingly, microglia-mediated engulfment was found to be regulated by neuronal activity. When competition between inputs from the two eyes was enhanced, microglia preferentially engulfed inputs from the eye with reduced neuronal activity. Although it is not yet known whether or how microglia target specific “weaker” synapses, these data are consistent with previous work demonstrating a decreased synaptic territory of the “weaker” inputs and increased territory of “stronger” inputs within the dLGN (see figure 96.5) (Cook, Prusky, & Ramoa, 1999; Del Rio & Feller, 2006; Huberman, Feller, & Chapman, 2008; Penn et al., 1998; Shatz, 1990; Shatz & Stryker, 1988; Stellwagen & Shatz, 2002).

Recent studies also suggest that microglia associate with postsynaptic elements during synaptic remodeling in the hippocampus and juvenile visual cortex, raising the question of whether microglia-dependent pruning is a global mechanism of synaptic remodeling in the CNS (Paolicelli et al., 2011; Tremblay, Lowery, & Majewska, 2010). Paolicelli et al. (2011) demonstrated a role for the fractalkine receptor (CX3CR1), expressed on the surface of microglia, in hippocampal synapse development and maturation. *Cx3cr1*^{KO} mice have a transient reduction in the number of microglia in the postnatal brain; thus, fractalkine signaling could interact with complement and other signals to regulate microglia-mediated developmental pruning by influencing microglia number or, possibly, recruitment to synaptic sites in the postnatal brain (Ransohoff & Stevens, 2011). Together these new findings raise several fundamental questions related to the underlying mechanisms of microglia- and complement-mediated pruning including how neural activity, complement, and microglia may interact to sculpt developing visual circuits.

Microglia may not be the only cells in the CNS phagocytosing material in the healthy, normal brain—astrocytes also show evidence of phagocytic capabilities (Al-Ali & Al-Hussain, 1996; Bechmann & Nitsch, 1997; Cahoy et al., 2008). Although it is not yet clear whether astrocytes refine circuitry like microglia, a specialized class of astrocytes in the ONH myelin transition zone (MTZ) has been shown to normally express a common phagocytic marker, the galactose-specific lectin Mac-2 (also known as Lgals3 or galectin-3) (Nguyen et al., 2011; Sun et al., 2009) (see figure 96.1). In normal, healthy mice, large inclusions of axonal material have been observed in these astrocytes, suggesting that MTZ astrocytes may phagocytose axonal components as a form of axonal maintenance in the normal animal (Nguyen et al., 2011).



Other Immune Molecules

Complement is among several immune-related molecules that have been identified as mediators of synaptic refinement and plasticity in the visual system (reviewed in Boulanger, 2009; Shatz, 2009). These include neuronal pentraxins (e.g., NP1/2, NARP) and components of

the adaptive immune system (e.g., class I major histocompatibility complex [MHC I] family of proteins and receptors) (Bjartmar et al., 2006; Corriveau, Huh, & Shatz, 1998; Datwani et al., 2009; Huh et al., 2000). It is not clear at this point if these pathways interact with glia and/or complement or represent parallel pathways for refinement.

Class I MHC molecules were the first to be identified as mediators of synaptic refinement. These molecules were initially shown to be expressed in an activity-dependent manner in the LGN. Infusion of tetrodotoxin to block all action potential activity in the LGN significantly reduced expression of class I MHC molecules. Furthermore, increasing neuronal firing with kainic acid induced seizure resulted in dramatic increases in class I MHC expression (Corriveau, Huh, & Shatz, 1998). The activity-dependent regulation of this gene suggested a potential role for the class I MHC signaling pathway in synaptic refinement. The role for class I MHC in activity-driven structural remodeling and synaptic plasticity was later demonstrated by examining three mouse strains deficient in class I MHC signaling: beta 2-microglobulin knockout mice, CD3 zeta-deficient mice, and a strain deficient in TAP1 and beta 2-microglobulin (Goddard, Butts, & Shatz, 2007; Huh et al., 2000). All three mouse mutants showed similar defects in synaptic refinement in the retinogeniculate system, assayed by neuroanatomical tracing of eye specific territories (see figure 96.4). Mice deficient in class I MHC also showed enhanced long-term potentiation (LTP) and absent long-term depression in the adult hippocampus (Huh et al., 2000), implicating MHCs in plasticity in other brain regions as well. In the hippocampus, class I MHC was shown to modulate plasticity by inhibiting NMDA receptor-regulated alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor (AMPA) trafficking (Fourgeaud et al., 2010), but it is not clear if this same mechanism plays a role in visual system plasticity.

Understanding the mechanism by which class I MHC signaling promotes synaptic refinement and plasticity are important questions and areas of active investigation; however, recent work demonstrated that MHC I affects even earlier stages of development. MHC I is

present at synapses in visual cortex during the peak of synaptogenesis and works as a negative regulator of excitatory and inhibitory synaptogenesis. In vitro, knocking down MHC I signaling increased excitatory and inhibitory synapse density, while beta 2-microglobulin deficient mice showed an increase in synapse density throughout the brain in vivo (Glynn et al., 2011). The strength of excitatory and inhibitory synapses was also modulated by MHC I, suggesting that this molecule has diverse and important functions throughout development. Interestingly, recent work demonstrated a colocalization of C1q and MHC I proteins at RGC synapses in the postnatal LGN (Datwani et al., 2009), hinting that these proteins may interact during development. Recent work also has revealed enhanced ocular dominance plasticity in mice class I MHC signaling supporting a role for this pathway in experience-dependent plasticity, as discussed in the following section (Syken et al., 2006).

Neuronal pentraxins (NP1 and 2) were found play a role in retinogeniculate refinement. Neuronal pentraxins are synaptic proteins with homology to pentraxins of the peripheral immune system, which are traditionally involved in opsonization and phagocytosis of dead cells in the immune system (Nauta et al., 2003). Mice deficient in neuronal pentraxins, NP1 and NP2, as well as the receptor, NPR, have transient defects in eye-specific segregation in the dLGN (see figure 96.4) (Bjartmar et al., 2006). These defects were not maintained into adulthood, and the eye-specific retinogeniculate inputs became segregated by postnatal day 30 (P30). A similar age-dependent phenotype results when spontaneous activity in the retina is blocked from P1–P10 and then allowed to recover. When retinal activity was measured in mice deficient in neuronal pentraxins, levels of RGC spiking activity were dramatically increased, which may explain the phenotype in eye specific segregation (Bjartmar et al., 2006). Interestingly, PTX3, a long pentraxin, which has homology to neuronal pentraxins, can enhance microglial phagocytic activity (Jeon et al., 2010). In addition, neuronal pentraxins are significantly homologous to short pentraxins such as C-reactive protein, which is a well-described binding partner of C1q. Thus,

neuronal pentraxins could potentially serve as synaptic binding partners for C1q during synapse development.

Neuronal pentraxins also have other functions in the developing visual system. Neuronal pentraxins are required for normal acetylcholine-mediated retinal wave activity during development. NP1 can also interact with another neuronal pentraxin, Narp, to influence synaptogenesis and synaptic plasticity. Narp was identified as an activity-dependent immediate early gene that can promote synaptogenesis and the clustering of AMPARs in other systems (O'Brien et al., 1999; Tsui et al., 1996). When complexed with NP1, Narp has an enhanced synaptogenic effect, mediating both activity-dependent and activity-independent clustering of AMPARs (Bjartmar et al., 2006; Xu et al., 2003). These interesting acute phase proteins seem to have critical roles in both synaptogenesis and synaptic refinement.

Role of Glia and Immune Molecules in Visual Experience-Dependent Plasticity

After eye opening, sensory experience helps to shape developing visual circuitry and tune visual responses. As the visual cortex matures, it develops exquisite organization, and visual receptive fields, along with ocular dominance and orientation selectivity columns, are mapped to the cortex (Hensch, 2004; Sur & Rubenstein, 2005). Recent work has demonstrated that astrocytes not only develop finely tuned responses to visual stimuli that mimic the mapping of neuronal responses but also influence the magnitude and duration of adjacent visually driven neuronal responses via glutamate transporters (Schummers, Yu, & Sur, 2008). The effects of sensory experience on glia and the role of glial cells in opening and closing the critical periods for visual plasticity have yet to be fully explored, although there have been clues that astrocytes, oligodendrocytes, and microglia influence cortical plasticity.

One of the first clues that astrocytes might play an instructive role in ocular dominance plasticity came from a study in the late 1980s by Müller and Best. They showed that introducing kitten astrocytes into the mature cat visual cortex could reopen the critical period for ocular

dominance (Müller & Best, 1989). Dark-rearing kittens, which delays maturation of the visual cortex, has also been shown to delay astrocyte maturation in visual cortex, linking neuronal activity with astrocyte maturation in this area (Müller, 1990). The neuron–astrocyte signaling pathways required for critical period plasticity have yet to be identified, however, and are an open area of active research.

Microglia have also been recently implicated in experience-dependent plasticity. Recent *in vivo* imaging experiments have shown that microglia processes are highly dynamic and often associate with dendritic spines during the critical period for ocular dominance (Tremblay, Lowery, & Majewska, 2010; Tremblay et al., 2011; Wake et al., 2009). In experiments in which retinal activity was reduced via tetrodotoxin or enucleation of both eyes, microglia retracted their processes away from spines and showed reduced motility overall (Wake et al., 2009). Microglia were also shown to participate in the experience-dependent structural remodeling that occurs after dark adapting mice for 1 week and exposing them to light. Dark exposure decreases synaptic strength, and microglia processes were often found associated with subsets of spines. The presence of phagocytic compartments in microglial processes also increased with dark adapting. Upon exposure to light, microglia remained phagocytic, however were less often associated with spines (Tremblay, Lowery, & Majewska, 2010). These experience-dependent changes in microglial dynamics support a role for microglia in experience-dependent structural remodeling.

Myelinating oligodendrocytes, as well as specific myelin proteins, have also been implicated in limiting experience-dependent plasticity in the adult visual cortex. Myelin-related proteins such as Nogo, MAG, and OMgp have been extensively studied as potent inhibitors of axonal growth and regeneration (Filbin, 2003). Nogo, which is expressed on the surface of oligodendrocytes, interacts with the Nogo receptor (NgR) expressed on axons to inhibit axonal growth. Interestingly, these proteins also seem to limit cortical plasticity (GrandPré, Li, & Strittmatter, 2002). When myelination is disrupted as in the NgR^{-/-} mouse, mice enter the

critical period normally; however, experience-dependent plasticity persists into adulthood such that even at P60 or beyond, monocular deprivation can trigger an increase in cortical responses to the nondeprived eye (see figure 96.4) (McGee et al., 2005). Myelin-derived Nogo, MAG, and OMgp, which have a broad distribution throughout the layers of visual cortex in adult versus juvenile mice, are thought to consolidate the neural circuitry established during experience-dependent plasticity.

A critical role for immune molecules has also been discovered in ocular dominance plasticity. In this system, monocular visual deprivation results in the weakening and pruning of inputs projecting from regions innervated by the deprived eye and an expansion of inputs from the nondeprived eye (see figure 96.4). Class I MHC molecules as well as proteins that interact with MHC I, such as paired-immunoglobulin-like receptor B (PirB), have been implicated in limiting ocular dominance plasticity. In mice that lack the transmembrane portion of PirB, thus preventing PirB signaling, ocular dominance plasticity, particularly the expansion of nondeprived inputs, is more robust and can be induced at any age (Syken et al., 2006). Interestingly, tumor necrosis factor alpha (TNF-alpha), a cytokine produced by astrocyte cultures and microglia, is also required for the strengthening of nondeprived inputs. Mice deficient in TNF-alpha exhibit the expected loss of deprived-eye responses (see figure 96.4), but the increase in cortical response to stimulation of the open eye is absent (Kaneko et al., 2008). This result supports the hypothesis that the weakening and strengthening of inputs in response to monocular deprivation are two distinct processes.

The mechanism by which TNF-alpha is acting in ocular dominance plasticity has not been fully elucidated. Beattie and colleagues showed that the proinflammatory cytokine TNF-alpha enhanced synaptic efficacy by increasing surface expression of AMPARs (Beattie et al., 2002), which could explain the failure of synaptic strengthening during ocular dominance plasticity. In addition, TNF-alpha recently has been implicated in homeostatic synaptic scaling, a form of synaptic plasticity that involves uniform adjustments in the strength of all synapses on a

cell in response to prolonged changes in the cell's synaptic activity such as blockade of synaptic function. Stellwagen and Malenka showed that TNF-alpha mediates synaptic scaling in response to prolonged activity blockade (Stellwagen & Malenka, 2006). Another possibility is that TNF-alpha affects plasticity as a result of its ability to induce MHC I expression (Israel et al., 1989). In fact, many proinflammatory cytokines including interleukin (IL-) 6, IL-1 beta, IL-2, IL-18, IL-8, and interferon-alpha and -beta all can inhibit hippocampal LTP, suggesting convergence on some common pathway (Boulanger, 2009). A major missing piece of the puzzle, however, is the identity of the molecules that scale down activity in the projections from the deprived eye.

Discussion

This chapter has highlighted the diversity of glial morphology and function in the visual system. The normal progression of visual development, originally viewed through a "neurocentric" lens, is now understood to be dramatically influenced by the diverse types of visual system glia. Conversely, "activation" and dysfunction of glial cells can be damaging to the CNS and are hallmarks of many CNS diseases. Thus, understanding the role of glia and immune molecules during normal development could provide important insight into the mechanisms underlying neurodevelopmental disorders and neurodegenerative diseases affecting visual system function.

The recent work reviewed here has provided a strong foundation for understanding the many roles that glia play in visual system development and function and has paved the way for many future studies. Many important and open questions remain, including the following: What are the cues that promote the terminal differentiation of glial precursors into their diverse morphological subtypes? What is the significance of "NG2" cells in visual function? Do astrocytes and other glial cells directly regulate development of inhibitory synapses? How do glia help control experience-dependent remodeling of synaptic circuits? How do microglia sense and read out activity-dependent cues to help sculpt developing synaptic circuits? Do immune

molecules, such as class I MHC and complement, have other functions in the developing visual system?

Despite recent advances in understanding the molecules and pathways that underlie neuron–glia and neural–immune communication during visual development, there is still a need for better molecular tools to manipulate subsets of glial cells at the right place and time in vivo. Regardless, the exciting new findings highlighted here lay a strong foundation in a new area of neuroscience, in which there is much to explore and discover.

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