Innate Immune Molecules Direct Microglia-Mediated Developmental Synaptic Refinement

Innate immune molecules direct microglia-mediated developmental synaptic refinement

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Innate immune molecules direct microglia-mediated developmental synaptic refinement

Microglia, the brain's resident immune cells and phagocytes, are emerging as critical regulators of developing synaptic circuits in the healthy brain after having long been thought to function primarily during central nervous system (CNS) injury or disease. Recent work indicates that microglia engulf synapses in the developing brain; however, how microglia know which synapses to target for removal remains a major open question. For my dissertation research, I studied microglia-mediated pruning in the retinogeniculate system and sought to identify the molecules regulating microglial engulfment of synaptic inputs. I discovered that "eat me" and "don't eat me" signals, immune molecules known for either promoting or inhibiting macrophage phagocytosis of cells or debris, localize to the dorsal lateral geniculate nucleus of the thalamus (dLGN) and direct retinogeniculate refinement. We found that "eat me" signal C3 and its microglial receptor, CR3, are required for normal engulfment, and that loss of either of these molecules leads to a reduction in phagocytosis and sustained deficits in refinement. These data suggest that microglia-mediated pruning may be analogous to the removal of non-self material by phagocytes in the immune system. To test this hypothesis, I examined whether protective signals are required to prevent excess microglial engulfment, as they prevent phagocytosis of self cells in the immune system. I found that protective "don't eat me" signal CD47 is required to prevent excess microglial engulfment and retinogeniculate pruning during development. Moreover, another "don't eat me signal", CD200, also prevents overpruning. Together,

these findings indicate that immune molecules instruct microglia as to which synapses to engulf and present a model in which a balance of stimulatory and inhibitory cues is necessary to guide remodeling of immature synaptic circuits. These data shed new light on mechanisms regulating synaptic refinement and microglial function in the healthy, developing CNS, and may have implications for disorders characterized by immune dysregulation and circuit disconnectivity, such as autism spectrum disorder (ASD) and schizophrenia.

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Chapter 1:

Introduction

Microglia, the brain's resident immune cells and phagocytes, have long been thought to execute their primary functions during nervous system disease and injury, where they are known to transform into an activated state capable of phagocytosis and cytokine secretion (Hanisch and Kettenmann, 2007; Ransohoff and Perry, 2009). As defenders of the central nervous system (CNS), they have been considered to be in a "resting" state when the brain is healthy, a state characterized by monitoring the environment and waiting for a call to action. However, a number of studies conducted over the last decade have begun to alter this view. New data indicate that microglia are vital for the development and function of the healthy brain and that genetic disruption of these cells produces defects in neural circuitry and behavior (Eyo and Wu, 2013; Schafer et al., 2013; Wake et al., 2013). Recent work indicates that microglia are required for normal synaptic circuit remodeling and that they phagocytose synaptic elements, demonstrating that microglial engulfment can occur in a non-pathological context (Paolicelli et al., 2011). This important finding actually raises more questions than it answers, and chief among these is how microglia know which synapses to engulf. Synaptic refinement involves the removal of unnecessary or inappropriate connections and the strengthening and maintenance of those that remain (Hua and Smith, 2004; Katz and Shatz, 1996; Sanes and Lichtman, 1999). As this process calls for the removal of specific synapses, signaling mechanisms must be employed to instruct microglia as to which inputs to engulf.

In the immune system, phagocytosis is a carefully orchestrated process, governed by a large array of both pro- and anti-phagocytosis molecular cues (Brown and Neher, 2012; Griffiths et al., 2007; Hochreiter-Hufford and Ravichandran, 2013). Intriguingly, some of these cues are required for synaptic refinement in the developing retinogeniculate

system, although how these immune molecules could mediate the removal of synaptic connections remains largely unknown (Boulanger, 2009; Huberman et al., 2008; Shatz, 2009). The newfound knowledge that microglia phagocytose synaptic connections during brain development may provide the answer. For my dissertation research, I have investigated how immune molecules guide microglial engulfment during retinogeniculate refinement based on the hypothesis that these molecules serve similar functions in the immune and nervous systems. This work provides insight into the mechanisms underlying both synaptic pruning and microglial phagocytosis in the developing CNS and reveals that immune mechanisms are utilized to sculpt the developing nervous system. The insights provided by these data can serve as the basis for future investigation of immune molecules and microglia in disorders involving abnormalities in circuit connectivity, such as autism spectrum disorder (ASD) and schizophrenia, as well as in neurodegenerative disease.

Synaptic refinement is a key event in CNS development

During early nervous system development, neurons send out exuberant processes and form an excess of synaptic connections. These overlapping and redundant connections are unique to the immature vertebrate nervous system, and while the reason for the emergence of this redundancy is unknown, the creation of specific, mature circuits from initially redundant connections could enable information storage and plasticity, perhaps encoding memories or underlying learning (Lichtman and Colman, 2000). To achieve the more precise and organized circuitry that characterizes the adult nervous system, a period of pruning must occur during development to remove inappropriate or unsuccessful connections, leaving only those that are necessary for the mature circuit (Guido, 2008; Huberman et al., 2008; Jaubert-Miazza et al., 2005; Shatz and Kirkwood, 1984). This period

of removal occurs concurrently with a period of arborization, which is thought to establish and strengthen the remaining connections (Dhande et al., 2011; Hahm et al., 1999; Hong and Chen, 2011; Snider et al., 1999). While the molecular mechanisms underlying synaptic refinement in the CNS remain largely unknown, it is well established that neural activity plays a key role (Chen and Regehr, 2000; Del Rio and Feller, 2006; Penn et al., 1998; Shatz, 1990; Stellwagen and Shatz, 2002; Torborg and Feller, 2005), and synapse elimination is often conceptualized as a competition for postsynaptic territory among presynaptic inputs in which the "winning" input is stronger and somehow capable of destabilizing competing weaker inputs (Nguyen and Lichtman, 1996; Wyatt and Balice-Gordon, 2003). Synaptic refinement is a fundamental feature of nervous system development, and has been observed in a variety of regions in both the central and peripheral nervous systems (Hong and Chen, 2011; Kano and Hashimoto, 2009; Sanes and Lichtman, 1999).

Much of our knowledge about synapse refinement has come from pioneering work at the neuromuscular junction (NMJ). In this system, motor neurons innervate muscle fibers, forming relatively simple circuits with large synaptic terminals that are easily visualized using microscopy. Initially, individual muscle fibers are innervated by many motor axons that form synapses in the same location. Over a period of days, all but one input in a synaptic location will lose territory, detach, and withdraw, with the remaining input expanding into the newly vacated territory and transitioning into a more mature morphology (Sanes and Lichtman, 1999). The outcome of this process seems to be regulated, at least in part, by neuronal activity, as synapse elimination accelerates as the disparity in synaptic strength between competing inputs grows, and the remaining motor axon generally has a greater synaptic strength than its competitors (Colman et al., 1997). If

two inputs are equally effective in driving the postsynaptic muscle fiber, both might remain (Herrera and Werle, 1990). The activity-dependent nature of this competition has led to a model describing the types of signals that might mediate this process (Lichtman and Colman, 2000). This model proposes that synapse elimination is dependent upon two signals: 1) a secreted punishment signal capable of destabilizing weak synapses and 2) a local protective signal that shields strong inputs from the punishment signal (Figure 1.1). It is not yet known whether this model is correct or applies to CNS synaptic refinement, and the elusive 'protective' and 'punishment' signals have not been identified. I hypothesize that molecules that normally protect against the inappropriate removal of self cells in the immune system, such as CD47, could act as protective signals to prevent the elimination of appropriate synapses during CNS development (Chapter 4).

Synaptic refinement in the CNS has proven harder to study. Much of this difficulty stems from the fact that CNS synapses are both more complex and harder to visualize than NMJ synapses. The regions that undergo pruning in the CNS are diverse and contain a variety of synapses, and many of these synaptic connections are small, dense, and difficult to resolve individually using microscopy. Additionally, many regions that undergo well-characterized pruning are in deeper brain structures that are not amenable to chronic live imaging. In the CNS, a great deal of what is known about pruning has been elucidated electrophysiologically and supplemented with anatomical tracing and imaging of fixed tissue. While many brain areas are now known to undergo pruning, including the brainstem, cerebellum, and somatosensory system (Kano and Hashimoto, 2009; Lu and Trussell, 2007; Wang and Zhang, 2008), synaptic refinement has been most extensively

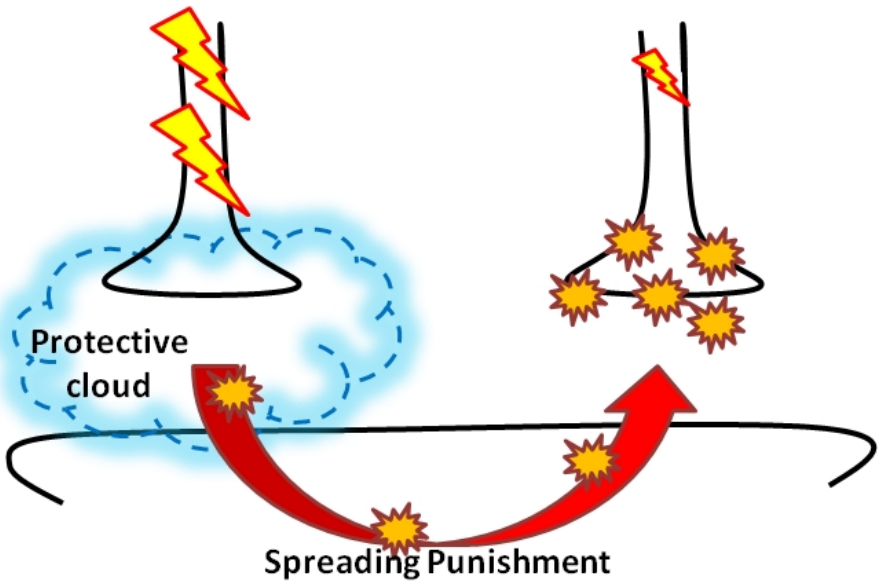

Figure 1.1. The punishment model of synapse elimination. Two signals have been proposed as mediators of activity-dependent synapse elimination: 1) A secreted punishment signal (orange) that binds to and destabilizes weak or asynchronously firing neurons, and 2) A local protective signal (blue) that shields more active neurons from the punishment signal.

characterized in the visual system, making it a good model for further investigation of the molecular mechanisms underlying this process (Feller, 1999; Huberman et al., 2008; Shatz and Kirkwood, 1984).

The retinogeniculate system

Synaptic refinement in the visual system has been primarily studied at the retinogeniculate synapse, which exhibits remarkably precise connectivity in the mature CNS. In this system, retinal ganglion cells (RGCs) that reside at the back of the eye project to relay neurons in the dorsal lateral geniculate nucleus (dLGN) of the thalamus. In the adult CNS, RGCs exhibit retinotopic mapping and eye-specific segregation, meaning that RGCs in specific retinal locations project to defined areas of the dLGN and that RGCs from the left and right eyes send inputs to distinct, non-overlapping territories within the thalamus (Guido, 2008; Hong and Chen, 2011; Huberman et al., 2008; Sretavan and Shatz, 1986). Additionally, RGC-relay neuron connectivity is between one-to-one or three-to-one in the mature circuit, indicating that a single relay neuron obtains all of its visual information from only one to a few RGCs (Chen and Regehr, 2000; Hooks and Chen, 2006; Ziburkus and Guido, 2006).

The immature circuit, however, contains a great deal of redundancy. During early development, RGCs from the two eyes project to overlapping regions in the dLGN and relay neurons are multiply innervated by as many as ten or more weak inputs. The mature circuitry is obtained following two phases of synaptic refinement. The first phase involves the pruning of overlapping connections and the establishment of a retinotopic map, while the second phase is characterized by the elimination of supernumerary inputs (Hong and Chen, 2011; Huberman et al., 2008; O'Leary and McLaughlin, 2005). Although synaptic

refinement in the dLGN is often described in terms of the removal of excess or incorrect connections, it is important to note that this is also a period during which extensive arborization and bouton formation occurs to strengthen the remaining inputs (Dhande et al., 2011; Hahm et al., 1999; Snider et al., 1999).

The first phase of pruning occurs after birth but before eye-opening in the mouse and is largely complete by postnatal day 10 (P10) (Jaubert-Miazza et al., 2005). This period of pruning is driven by spontaneous retinal waves caused by correlated bursts of action potential firing in the retina (Feller, 1999; Stellwagen and Shatz, 2002; Torborg and Feller, 2005). During this period, eye-specific territories are formed such that RGCs from the two eyes project to stereotyped, non-overlapping regions within the dLGN (Figure 1.2). The resolution of overlapping inputs into distinct territories is thought to occur via the weakening and removal of incorrectly targeted inputs and the strengthening and maintenance of correctly targeted projections (Penn et al., 1998; Shatz, 1990). For this process to occur normally, inputs from the two eyes must be able to compete, as removing one eye prevents the pruning of incorrectly targeted connections sent from the remaining eye (Sretavan and Shatz, 1986). Additionally, disrupting retinal activity either genetically or pharmacologically impairs eye-specific segregation, highlighting the necessity of spontaneous activity for this process (Bansal et al., 2000; Demas et al., 2006; Grubb et al., 2003; Shatz and Stryker, 1988; Stellwagen and Shatz, 2002).

The second phase of pruning involves the removal of excess connections from the now primarily monocularly innervated dLGN relay neurons (Figure 1.2). The first part of this phase is thought to occur from P8-P16 and depend on spontaneous retinal activity, including both retinal waves and RGC spiking (Hong and Chen, 2011). During this period,

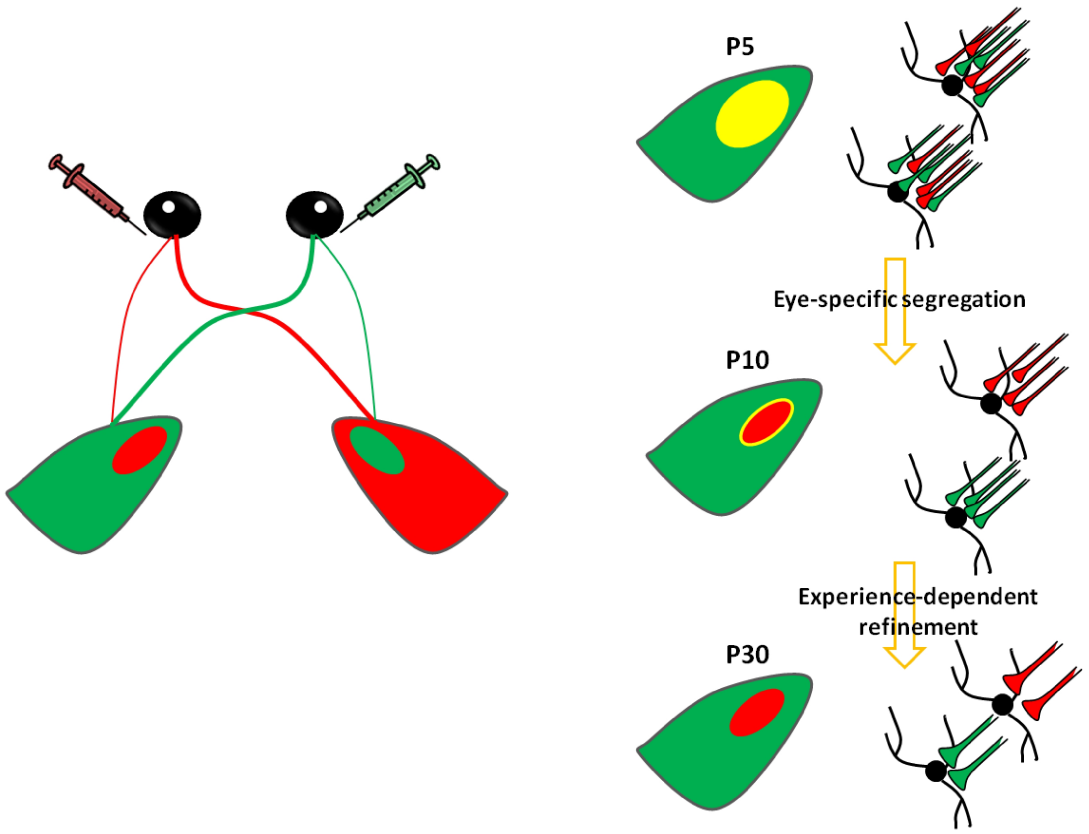

Figure 1.2. Synaptic refinement in the retinogeniculate system. In the mature dLGN, left and right eyes project to distinct, eye-specific territories that can be visualized using anterograde tracers (left). Most RGCs project contralaterally, crossing the optic chiasm, while a small number project ipsilaterally and form a defined patch. During early development (P5), RGC inputs from left and right eyes project overlapping inputs onto relay neurons in the dLGN (right, yellow). By P10, the first phase of pruning has led to the removal of nearly all overlapping inputs to create eye-specific territories. Relay neurons are still multiply innervated at this stage. Over the next few weeks, spontaneous and visually evoked activity drive the removal of supernumerary inputs so that each relay neuron ultimately receives input from only 1-3 RGCs.

the number of inputs received by each relay neuron goes down by half while the strength of each input increases 8-fold (Chen and Regehr, 2000; Hooks and Chen, 2006; Ziburkus and Guido, 2006). After this point, inputs continue to strengthen and the number of innervating inputs declines until approximately P32 (Figure 1.2). While this latter phase of refinement was also thought to rely exclusively on spontaneous activity, recent research has uncovered a role for visual experience in maintaining the mature circuitry (Hooks and Chen, 2008). Visual deprivation via late dark rearing after P16 can increase RGC input number and weaken synaptic strength, however, this change is plastic and can be reversed by re-exposure to light. Ongoing study continues to enhance our understanding of synaptic refinement in this system, and the body of work performed to characterize this circuit enables investigation of the molecules that direct and regulate the refinement process.

Mechanisms underlying retinogeniculate refinement

While the developmental timing and connectivity changes that occur during retinogeniculate pruning are fairly well understood, knowledge of the molecular mechanisms underlying this process is limited. Given the clear importance of retinal activity for proper refinement, some of the first molecules implicated in retinogeniculate pruning were those required for normal retinal waves, such as the β2 subunit of the nicotinic acetylcholine receptor (Grubb et al., 2003; Muir-Robinson et al., 2002; Rossi et al., 2001). The no b-wave (nob) mouse, a mouse with prolonged retinal wave activity, also displays pruning defects, although, in this case, eye-specific territories initially develop only to desegregate as waves persist abnormally beyond eye opening (Demas et al., 2006). Additionally, disruption of axon guidance cues known to direct retinotopy impairs eye-specific segregation and causes other wiring defects (Pfeiffenberger et al., 2005;

Pfeiffenberger et al., 2006). The identification of these molecules as critical for retinogeniculate refinement aligns with electrophysiological and axon guidance studies of the retinogeniculate system.

More recently, an unexpected class of molecules has been implicated in synaptic refinement in the retinogeniculate system. A number of immune molecules and their CNS homologs have been found to be required for proper pruning in the dLGN, including class I major histocompatibility complex (MHCI) molecules, complement cascade components C1q and C3, and neuronal pentraxins 1 and 2 (NP1, NP2), CNS relatives of immune molecule long pentraxin 3 (PTX3) (Hong and Chen, 2011; Huberman et al., 2008). In the immune system, these molecules help phagocytes identify pathogens or debris in need of removal, and it appears that they may perform a similar function in the brain, in this case, denoting synapses in need of pruning. Mice deficient in MHCI molecules or complement cascade components C1q or C3 do not properly form eye-specific territories; instead, relay neurons in knockout mice receive input from both eyes, indicating a failure to prune overlapping connections (Datwani et al., 2009; Huh et al., 2000; Stevens et al., 2007). Double NP1/2 knockouts experience delayed pruning, as eye-specific segregation takes longer to reach completion in these animals (Bjartmar et al., 2006). The discovery that these immune molecules regulate retinogeniculate refinement is quite intriguing, and begs the question of how exactly they execute this function. If immune molecules normally signal to phagocytic cells in the immune system, could they be doing something similar in the brain?

Microglia play critical roles in the developing brain

Microglia are the resident phagocytes and immune cells of the CNS. Due to their macrophage-like properties, much of the work on microglia has focused on their reactive

role in CNS diseases, however, recent studies indicate that microglia regulate a variety of processes that occur throughout the life of an organism (Cronk and Kipnis, 2013; Eyo and Wu, 2013; Hanisch and Kettenmann, 2007). These multifunctional cells may even act prenatally, as fate mapping has shown that microglia enter the brain as early as embryonic day 9 (E9) (Ginhoux et al., 2010). Microglia are therefore present as the brain develops, and are important cellular mediators, and possibly instigators, of programmed cell death (PCD) (Marin-Teva et al., 2004; Wakselman et al., 2008). Microglia may also regulate cell numbers in the developing brain via effects on neurogenesis and neural progenitor cells, although more work is necessary to confirm these findings (Morgan et al., 2004; Sierra et al., 2010; Sultan et al., 2013). Importantly, as we learn more about microglial function in the developing and adult CNS, it becomes increasingly clear how many parallels exist between their function in the brain and macrophage function in the immune system. This suggests that many molecular mechanisms may be shared between the CNS and immune system, and that a great deal can be learned by taking advantage of this conservation.

Microglia: specialized CNS "macrophages"

A key to understanding microglia may be their high degree of similarity to macrophages. Microglia can be considered the tissue-resident macrophages of the CNS and they share a common origin with other tissue-resident macrophages: the embryonic yolk sac (Cronk and Kipnis, 2013). Microglia are also functionally similar to macrophages, as they play an important role in the development and maintenance of healthy tissue, but have a reactive phenotype involving phagocytosis and cytokine secretion during disease or injury (Prinz et al., 2014; Ransohoff and Cardona, 2010). These cells can even be recognized by many of the same markers (Saijo and Glass, 2011), which is sometimes

problematic as commonly used microglial markers, such as Iba-1, cannot distinguish between resident microglia and infiltrating peripheral immune cells during disease.

Due to these many commonalities, it is unsurprising that mechanisms regulating microglia and functions executed by microglia are comparable to those involving macrophages in the immune system. A number of molecules and signaling pathways have already been found to behave analogously in these two systems. For instance, CX3CL1, or fractalkine, regulates monocyte chemoattraction in the immune system via CX3CR1 (Ravichandran, 2011), and mice lacking this receptor exhibit fewer microglia during brain development and a decrease in synaptic pruning (Paolicelli et al., 2011). In the immune system, toll-like receptors on macrophages initiate an inflammatory response, and their microglial counterparts perform the same function, particularly during neurodegenerative disease (Ransohoff and Brown, 2012; Wyss-Coray and Rogers, 2012). Complement molecules tag pathogens or debris for removal by macrophages in the immune system, and they also appear to tag debris, such as amyloid plaques and apoptotic cellular material for microglial engulfment during neurodegenerative disease or injury (Afagh et al., 1996; Fonseca et al., 2004; Lambris and Tsokos, 1986; van Lookeren Campagne et al., 2007). Microglia also secrete many of the same pro-inflammatory cytokines as macrophages, such as TNF-α and interleukin family members, that may play a role in neurodegenerative diseases or other instances of neuroinflammation (Perry et al., 2010; Ransohoff and Perry, 2009; Wyss-Coray and Rogers, 2012).

However, microglia are specialized cells and exhibit notable differences from macrophages. Unlike macrophages, microglia appear to be a self-renewing population, with little contribution from bone marrow-derived monocytes except after irradiation

(Prinz et al., 2014). They have also recently been found to carry a unique genetic signature, for, although they share many common genes and signaling pathways with macrophages, a recent profiling study found that resident microglia express genes not found in immune cells or tissue resident macrophages, including *Fcrls*, *Olfml3*, *Tmem119*, *P2ry12*, *Hexb* and *Tgfbr1* among others (Butovsky et al., 2014). These genes can be used to differentiate resident microglia from peripheral cells that enter the brain during disease, a property of tremendous utility to the field. Furthermore, as microglia participate in a variety of processes in the CNS, they may use some of their shared signaling molecules in a way that does not mimic macrophage function. One example of this is the ability of pro-inflammatory molecule TNF-α to regulate homeostatic synaptic scaling (Beattie et al., 2002; Stellwagen and Malenka, 2006). Yet, the overwhelming similarities between microglia and macrophages allow the immune system to serve as a foundation for understanding potential microglial functions and signaling pathways.

Microglia influence synapse development and function

Part of the reason that microglia in the healthy brain went overlooked for so long may have to do with terminology and perception. Microglia in the healthy, adult brain are often referred to as "resting" cells and possess a small cell body and numerous fine, branched processes (Ransohoff and Perry, 2009). During CNS disease, microglia retract their processes, adopt an ameboid morphology, and become highly phagocytic, a state that is controversially and ambiguously referred to as "activated." Advances in live imaging technologies have enabled improved visualization of resting microglia, and exciting new data obtained using two-photon live imaging challenge the existing nomenclature. Resting microglia are actually quite dynamic, continuously extending and retracting processes to

sample the brain parenchyma (Davalos et al., 2005; Nimmerjahn et al., 2005). While this surveillance behavior allows them to rapidly detect and respond to injury, process motility may have other functions, as it changes in response to neuronal activity (Li et al., 2012; Wake et al., 2009). Intriguingly, resting microglia also make contact with dendritic spines, raising the question of what microglia-synapse contact might signify (Tremblay et al., 2010; Wake et al., 2009).

Recent work has unveiled numerous types of microglia-synapse interactions in the CNS. In the developing brain, microglia phagocytose synaptic material, participating in synaptic refinement and shaping circuit function. Mice deficient in fractalkine receptor CX3CR1, a microglia-specific receptor in the healthy CNS, have fewer microglia during CNS development as well as reduced synaptic pruning and functionally immature synapses in the hippocampus (Paolicelli et al., 2011). These defects resolve as microglia numbers normalize during adulthood. Microglia-synapse interactions also appear to be experience-dependent, as changes in light exposure alter microglia process motility, spine contact, and phagocytosis in visual cortex (Tremblay et al., 2010). Some of the effects of microglia on synapse development may even occur prenatally, as loss of DAP12, a microglial signaling adaptor, impairs synapse maturation *in vivo* and in cultured neurons isolated from P0 DAP12-deficient pups (Roumier et al., 2004; Roumier et al., 2008). Neuronal activity also influences microglia-synapse interactions, likely due to the many neurotransmitter receptors expressed by microglia (Kettenmann et al., 2011). While early studies are contradictory, as some demonstrate increased process motility and synapse contact with increased activity and others show the reverse, it is clear that microglia can sense and respond to changes in activity (Eyo and Wu, 2013).

Given increasing evidence that microglia shape synaptic connectivity and function, recent work has begun to focus on the behavioral consequences of disrupting microglia-synapse interactions. Multiple studies using CX3CR1-deficient mice indicate that loss of this microglia-specific gene may have behavioral consequences. Heterozygous mice display deficits in memory, motor learning, and contextual fear conditioning, as well as impaired LTP induction (Rogers et al., 2011). Homozygotes exhibit reduced functional brain connectivity by functional magnetic resonance imaging (fMRI) and decreased social interaction and increased grooming behaviors (Zhan et al., 2014). Pathological grooming in the *hoxb8* mutant mouse has also been linked to microglial defects, as the *hoxb8* mutant phenotype can be rescued by bone marrow transplant, which allows wild type microglia to infiltrate into the mutant brain (Chen et al., 2010). Another study used bone marrow chimerism to introduce wild type microglia into a mouse model for Rett syndrome, which seemed to rescue some of the more severe deficits (Derecki et al., 2012). Additionally, recent work demonstrates that mice lacking microglia exhibit impaired motor learning as well as spine alterations, suggesting that microglia can influence synaptic changes that affect circuit function (Parkhurst et al., 2013).

Microglia in CNS disease

While much of this work has been done in mouse, it appears that microglia dysfunction can produce similar behavioral deficits to those recognized as hallmarks of neurodevelopmental and neuropsychiatric disorders, including autism spectrum disorder (ASD, impairments in social behavior) and obsessive compulsive disorder (OCD, pathological grooming). Various genome wide association studies (GWAS) conducted on human patients with these disorders have begun to identify immune molecules known to

regulate microglia directly or to regulate microglia-associated signaling cascades as potential risk factors (Voineagu et al., 2011). Furthermore, mouse models for psychiatric disorders based on maternal immune activation (MIA) often exhibit synaptic and/or microglial abnormalities (Giovanoli et al., 2013). The recent studies implicating microglia in synaptic circuit development and function could indicate a role for microglia in multiple neurodevelopmental and neuropsychiatric disorders, as defects in developmental circuit formation and wiring in humans are becoming increasingly associated with neurodevelopmental and neuropsychiatric impairments (Belmonte et al., 2004; Courchesne et al., 2007; Keshavan et al., 1994; Tye and Bolton, 2013). Moreover, dysregulated microglia and immune molecules are beginning to be observed in human patients with these disorders (Frick et al., 2013; Morgan et al., 2010; Tetreault et al., 2012; Vargas et al., 2005).

Many CNS neurodegenerative diseases are also associated with microglial dysfunction (Wyss-Coray and Rogers, 2012). Postmortem analysis of human tissue often reveals morphologically abnormal cells; however, it remains to be determined whether microglial dysfunction plays a causal role in pathogenesis or is a consequence of the neuroinflammation that occurs during disease progression (Napoli and Neumann, 2009; Schafer and Stevens, 2010; Tansey et al., 2008). Current studies on neurodegenerative diseases, including Alzheimer's disease (AD) and Huntington's disease (HD), indicate that synapse loss or dysfunction begins long before symptom onset (DiProspero et al., 2004; Scheff and Price, 2003; Selkoe, 2002), and one hypothesis is that microglia may be involved (Stephan et al., 2012). This appears to be a possibility in a mouse model of tauopathy (P301S), in which hippocampal synapse loss and microglial activation can be observed as

early as 3 months, while significant atrophy does not occur until at least 9-12 months of age (Yoshiyama et al., 2007). Although data are only correlative at this stage, aberrant re-activation of developmental microglial pruning programs may be one mechanism underlying neurodegenerative disease; and the implication of microglia in both neurodevelopmental and neurodegenerative diseases underscores their importance for normal brain development and function.

Tools for studying microglia

One reason the study of microglial function has lagged behind the study of other CNS cell types is due to issues of accessibility. To assay normal microglial behavior and function, work must be performed *in vivo* or *ex vivo*, as cultured microglia typically transform into an activated state and behave as they would during injury or disease. For this reason, most early studies relied on inferences made based on observations of microglial cell numbers, morphology, and markers of activation in immunostained, fixed tissue. As high-resolution *in vivo* imaging technologies have improved, this has begun to change. When used with a microglial reporter line, the CX3CR1-GFP mouse (Jung et al., 2000), microglia can be imaged through cranial windows or thin skull preparations in anesthetized or awake mice. This approach can be used to visualize microglial movement and dynamics basally and in response to various stimuli, and if mice are crossed to other reporter lines, this approach can be applied to examine microglial interactions with other cell types (Davalos et al., 2005; Nimmerjahn et al., 2005; Tremblay et al., 2010; Wake et al., 2009). Studies using these approaches have greatly enhanced our understanding of how microglia behave in the healthy brain, although they are limited to imaging brain regions near the skull.

New tools have also vastly improved our ability to regulate microglial function. In the past, to determine the effects of altering microglial function, broad anti-inflammatory agents such as minocycline were used, which would affect microglia in addition to a variety of other cells and inflammatory signaling cascades throughout the animal. Knockout mice could be used, but, to claim microglia-specific effects, the gene in question could not be expressed by other cell types. Recently, mice have been generated that will enable microglia-specific genetic deletion. Two mice make use of the CD11b promoter, which is specific to microglia in the brain, to drive the expression of the herpes simplex virus thymidine kinase (HSVTK) or the diptheria toxin receptor (DTR), which will lead to microglial death when mice are treated with ganciclovir or diptheria toxin, respectively (Duffield et al., 2005; Heppner et al., 2005). There are also mice that completely lack microglia, including the PU.1 null mice and CSF1R knockouts, although neither line lives to adulthood (Erblich et al., 2011; McKercher et al., 1996). New cre lines, the CX3CR1-Cre and –CreER mice, use the fractalkine receptor promoter to excise floxed genes of interest from microglia to assay cell-specific effects (Parkhurst et al., 2013; Yona et al., 2013). Additionally, these lines can be used to ablate microglia, as new work indicates that driving microglia-specific expression of the diptheria toxin receptor in CX3CR1-CreER mice leads to microglial ablation when diptheria toxin is administered (Parkhurst et al., 2013). Finally, new tools are also being used to deplete microglia, such as delivery of clodronate liposomes, although these will affect other CNS phagocytes as well (Faustino et al., 2011). These and other technical advances have already contributed greatly to our understanding of microglia, and will facilitate and quicken the pace of knowledge acquisition regarding microglia-specific functions in the years to come.

“Eat me” and “Don't eat me” signals regulate engulfment by phagocytes

Microglia are clearly integral to normal CNS development and function. Although the molecular mechanisms underlying their myriad roles in the brain are still being explored, one of their cellular properties features prominently during both CNS development and disease, that of phagocytosis (Prinz et al., 2014). This is an ability they share with their immune system counterpart, the macrophage, so understanding how phagocytosis is regulated in macrophages may provide insight into genes necessary for normal microglial function. The engulfment of apoptotic cells, debris, and pathogens is a key function of macrophages, and microglia similarly engulf material in the CNS (Ransohoff and Cardona, 2010; Ransohoff and Perry, 2009). To effectively perform this function, macrophages must differentiate between foreign or harmful material and healthy “self” cells that must remain in the body. They make this distinction via extracellular signals found on self and non-self material (Figure 1.3), commonly known as “eat me” and “don’t eat me” signals (Griffiths et al., 2007).

“Eat me” and “don’t eat me” signals regulate macrophage phagocytosis

“Eat me signals” are molecules or modifications on apoptotic or infected cells that allow for identification by phagocytes. There are many such signals, and they can take the form of previously internalized molecules that become exposed on the cell surface, modifications to existing surface molecules, or the binding of new molecules to the cell (Elward and Gasque, 2003; Gardai et al., 2006; Grimsley and Ravichandran, 2003; Lauber et al., 2004). Some classic examples of “eat me signals” are the phospholipid phosphatidylserine, which translocates from the inner to the outer surface of apoptotic cells, and members of the classical complement cascade, which can bind to or opsonize

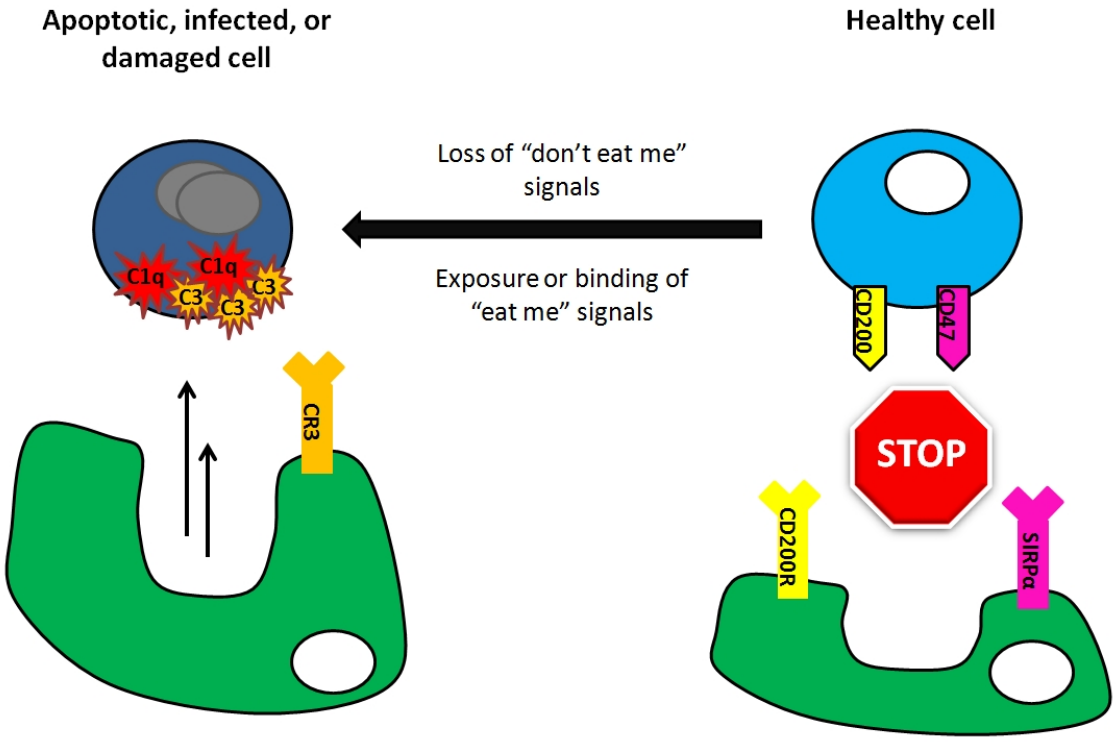

Figure 1.3. "Eat me" and "don't eat me" signals regulate phagocytosis in the immune system. Normal, healthy self cells (light blue, right) express "don't eat me" signals, such as CD47 and CD200, to instruct phagocytes (green) carrying receptors for these molecules, such as SIRPα and CD200R, not to engulf them. However, when cells become injured, infected, or apoptotic (dark blue, left), they downregulate or re-localize "don't eat me" signals, and express or are bound by "eat me" signals. Complement molecules such as C1q and C3 are classic "eat me" signals that encourage phagocytosis by signaling to receptors, such as CR3, on macrophages.

dying cells and debris. Phagocytes express a variety of cell surface receptors to detect these signals and may only need a subset of these receptors to function efficiently.

While it is important that phagocytes engulf apoptotic cells or pathogenic material to prevent harm to the organism, uncontrolled engulfment or an overactive immune response can be harmful. Therefore, mechanisms exist to protect "self" cells and inhibit phagocytic activity. Just as phagocytes are instructed to remove specific cells or material via "eat me" signals, they must be told which cells to avoid by "don't eat me" signals. "Don't eat me" signals are membrane bound or secreted molecules normally expressed by "self" cells and not by pathogens that are recognized by receptors on phagocytes (Elward and Gasque, 2003; Griffiths et al., 2007; Grimsley and Ravichandran, 2003). Many of these act as negative regulators of phagocyte function and can inhibit phagocytic activity. Classic examples of "don't eat me" signals are CD200 and CD47, which interact with receptors CD200R and SIRPα respectively to directly or indirectly inhibit engulfment by phagocytes.

My dissertation research investigates the role of CD47 and CD200 in CNS synaptic refinement (Chapter 4). In the immune system, CD47 directly protects self cells from inappropriate removal, as binding to its receptor, SIRPα, on phagocytes causes receptor tyrosine phosphorylation, which recruits SHP1 or SHP2 to inhibit phagocytosis (Barclay and Van den Berg, 2014; Matozaki et al., 2009). Red blood cells (RBCs) lacking CD47 exhibit a markedly increased rate of clearance compared to WT RBCs, and mice lacking the cytoplasmic tail of SIRPα also display increased RBC clearance, indicating that the CD47-SIRPα interaction is critical for inhibiting excess phagocytosis (Ishikawa-Sekigami et al., 2006; Oldenborg et al., 2000). Additionally, red blood cells opsonized with "eat me" signals,

such as C3b, are cleared much more effectively when they lack protective signal CD47 (Oldenborg et al., 2001).

CD200 plays a slightly different protective role. Its ligation to CD200R on phagocytes leads to receptor interaction with Dok2 and activation of RasGAP, which decreases synthesis of many pro-inflammatory cytokines including TNF-α, IFN-γ, and IL-1, and promotes synthesis of anti-inflammatory cytokines such as IL-10 and TGF-β (Barclay et al., 2002; Minas and Liversidge, 2006; Walker and Lue, 2013). CD200 is therefore thought to be primarily responsible for preventing macrophage activation and inflammatory signaling. CD200 deficient mice exhibit an increased number of activated myeloid cells and increased susceptibility to autoimmune disease development (Hoek et al., 2000; Wright et al., 2000). As microglial phagocytosis appears to be in important mechanism for sculpting developing synaptic circuits (Schafer and Stevens, 2013), I chose to focus on these two "don't eat me" signals to determine whether removing inhibition of phagocytosis or inflammation would have consequences for developmental synaptic refinement.

"Eat me" and "don't eat me" signals in the brain

Although "eat me" and "don't eat me" signals have been studied primarily in the immune system, it is becoming increasingly clear that they are utilized in the CNS as well (Figure 1.4) (Elward and Gasque, 2003). "Eat me" signals are often exposed on or bound to cells in the immune system in response to pathogens and inflammation, and they have been observed in the CNS under similar circumstances. Diseases characterized by inflammation, such as Alzheimer's disease, exhibit increased complement activation in both human tissue and mouse models, as well as complement localization to plaques and to the neuropil (Afagh et al., 1996; Fonseca et al., 2004; Wyss-Coray and Rogers, 2012). Whether this is

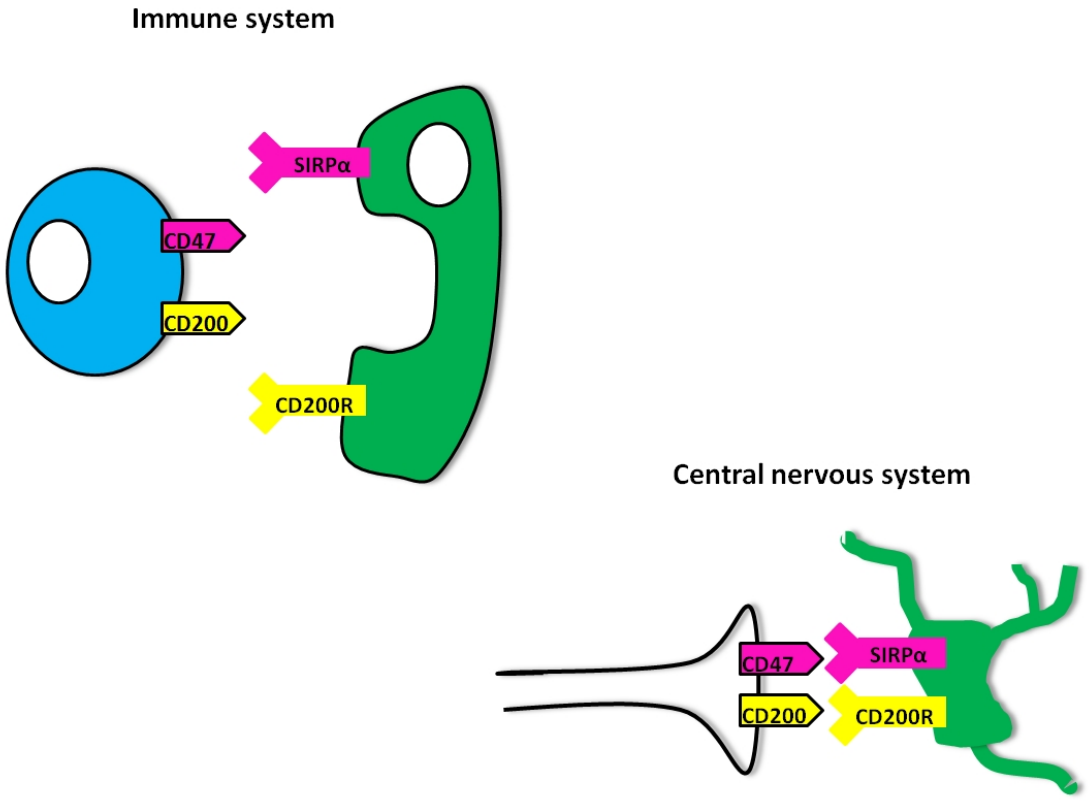

Figure 1.4. "Don't eat me" signals communicate with microglia in the CNS. In the immune system (left), "don't eat me" signals are expressed by healthy self cells (blue) and communicate with receptors on phagocytes (green). The same molecules have a similar distribution in the CNS (right), where "don't eat me" signals are expressed by neurons in addition to other self cells and communicate with receptors on microglia (green).

harmful, beneficial, or simply a hallmark of inflammation remains to be determined, as some argue that complement activation may lead to increased neurodegeneration, while others suggest that complement deposition on plaques enhances clearance by microglia. Complement activation has also been observed following acute injury, such as in cerebral ischemia, head trauma, and stroke, and in other chronic CNS diseases such as Amyotrophic Lateral Sclerosis (ALS) and Huntington's disease (HD) (Chiu et al., 2009; Gasque et al., 2000; van Beek et al., 2003; Yanamadala and Friedlander, 2010).

Just as "eat me" signals are increased during CNS disease and injury, "don't eat me" signals may be necessary to help protect the brain from excessive harm. Profiling data from human multiple sclerosis (MS) lesions indicate that CD47 is downregulated in diseased tissue (Koning et al., 2007). In mouse models for MS, known as experimental autoimmune encephalomyelitis (EAE), blocking CD47 with an antibody during the height of the disease worsens disease progression (Han et al., 2012). CD200-deficiency leads to a worsening of EAE symptoms in mouse models (Wright et al., 2000), and antibody blockade of this molecule promotes microglial activation and dopaminergic neuron loss in a rat model of Parkinson's disease (PD) (Zhang et al., 2011). A reduction in CD200 has also been observed in affected areas of human AD brains (Walker et al., 2009), and the CD200-CD200R interaction appears dysregulated in cells from PD patients (Walker and Lue, 2013). These data suggest that "don't eat me" signals play an important role in dampening inflammation and microglial activation and phagocytosis during CNS disease.

The involvement of "eat me" and "don't eat me" signals in CNS disease mirrors their function in the immune system. Unexpectedly, some of these same signals have been found to be critical for normal CNS development. Recent work has identified a role for the classic

"eat me" signals of the complement cascade, C1q and C3, in nervous system development. Mice deficient in either molecule fail to complete proper retinogeniculate pruning and exhibit overlapping ipsilateral and contralateral territories and multiply innervated relay neurons into adulthood (Stevens et al., 2007). There is also some evidence that these pruning defects occur throughout the brain, as juvenile C1q knockout mice have a greater density of axonal boutons on layer V neocortical pyramidal neurons as well as hyper-excitability and behavioral seizure activity (Chu et al., 2010). Defects in pruning may actually be beneficial once mice age, as C3KO mice exhibit enhanced LTP and cognition compared to aged WT mice, and aged C1qKO mice exhibit less cognitive memory decline (Shi et al., 2012; Stephan et al., 2013). The role of "don't eat me" signals in brain development is relatively unexplored, however, a recent study suggests that loss of the CD47 receptor, SIRPα, during a specific developmental time window influences synaptic maturation (Toth et al., 2013). Whether this is due to changes in "don't eat me" signaling or microglial phagocytosis is unknown.

Significance

While scientists have known about the existence of synaptic refinement for over 20 years, there is still little known about the molecular mechanisms that direct this process. Recent studies have begun to identify key molecules, and, intriguingly, many of them are shared with the immune system (Boulanger, 2009; Huberman et al., 2008; Shatz, 2009). Discovering additional molecules that regulate pruning and the cellular interactions underlying this process will greatly contribute to understanding a fundamental aspect of brain development. While we use the retinogeniculate system as a model, many of the molecules identified in this system have been found to regulate pruning in other areas and

to be involved in a variety of CNS diseases (Stephan et al., 2012). This highlights the power of the retinogeniculate system and the likelihood that new molecular discoveries will be applicable across brain regions and potentially disease relevant.

Another conceptual advance that could arise from this work is the notion that immune molecules and microglia are critical for the development of a normal, healthy brain. For many years, these molecules and cells were thought to be active only during injury or disease, but this idea is becoming outdated (Ransohoff and Perry, 2009). Recent work has shown that microglia survey their environment, contact spines, and shape synaptic circuits and behavior in healthy animals, and immune molecules have been implicated in synaptic refinement and circuit formation as well (Eyo and Wu, 2013; Huberman et al., 2008; Saijo and Glass, 2011). The work discussed in this dissertation ascribes a new function to microglia in developmental synaptic refinement and further challenges the idea that microglia are quiescent when not reacting to an insult. Additionally, this work is the first to describe an analogous immune-like function for "don't eat me" signals in the developing CNS, and demonstrates that immune molecules in the developing brain serve a regulatory role, keeping microglial phagocytic activity within an appropriate range.

Finally, identifying molecules and cells that play a critical role in brain development provides candidates to examine in a variety of CNS disorders and diseases. Many molecules previously shown to regulate nervous system development, including synapse formation and pruning, have since been implicated in various neurodevelopmental and neuropsychiatric disorders (van Beek et al., 2003). For instance, a number of molecules required for synapse formation, including Neuroligins and Shanks, have also been

implicated in autism and the generation of autistic-like behaviors (Kumar and Christian, 2009). Some of the same immune molecules that have been linked to developmental synaptic pruning have appeared, along with their regulators, in Genome Wide Association Studies (GWAS) of autism and schizophrenia and many of these molecules are also aberrantly expressed in neurodegenerative disease (Gasque et al., 2000; Voineagu et al., 2011). Microglial abnormalities have been associated with neurodevelopmental and neurodegenerative disease as well, making it likely that molecules that affect microglial function will be disease relevant (Dheen et al., 2007; Frick et al., 2013; Morgan et al., 2010). The molecules described in this work regulate synaptic refinement and microglial phagocytosis during development, and alterations in these molecules have already been observed in neurodegenerative disease. As defects in synaptic connectivity and brain wiring appear to be a common thread in a variety of CNS diseases, these molecules are likely to be implicated in additional neurodevelopmental and neurodegenerative diseases once their function in synaptic refinement in the healthy brain is revealed.

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Chapter 2:

Materials and methods

Mice

C57BL/6 mice were obtained from Charles River. CX3CR1::EGFP, C3KO, CR3 KO, Chx10-Cre, Rosa26-STOP-tdTomato, and CD47KO mice were obtained from Jackson Labs. CD200 knockout mice were generously provided by Dr. Agnes Vignery. SIRPα; Actin-CreER mice were provided by Dr. Hisashi Umemori. CX3CR1+/GFP;CCR2+/RFP tissue was obtained from Dr. Richard Ransohoff. For engulfment experiments, CR3 KO or C3 KO mice were crossed with CX3CR1::EGFP. All experiments using CX3CR1::EGFP, Chx10-cre, or Rosa26-STOP-tdTomato mice were performed with heterozygotes. For minocycline experiments, C57BL/6 or CX3CR1::EGFP mice were injected daily with minocycline (Sigma; 75mg/kg) or vehicle (saline) subcutaneously from P4-P8. Experiments were approved by the institutional care and use committee of Boston Children's Hospital in accordance with NIH guidelines for the humane treatment of animals.

Immunohistochemistry

Brains and eyes were harvested from mice following transcardial perfusion with PBS and 4% paraformaldehyde (PFA). Tissue was postfixed in 4% PFA for two hours after perfusion, and then washed 3X with phosphate buffered saline (PBS) and transferred to 30% sucrose for cryoprotection. For whole mount retinas, eyes were transferred to PBS following postfix and PBS wash.

If used for cryosectioning, tissue was embedded in a 2:1 mixture of 20% sucrose: OCT and stored at -80° C until use. Fourteen micron sections were collected, and then dried, washed with PBS, and blocked with a 5% bovine serum albumin (BSA) + .2% Triton-X 100 solution

for 1 or 2 hours (2hrs if sections were to be used for SIM imaging). Primary antibodies diluted in antibody buffer were applied to sections for overnight (O/N) incubation at 4° C, with the exception of Iba-1, in which slides were incubated O/N at room temperature (RT). After 3 PBS washes, secondary antibodies diluted 1:250 in antibody buffer were added to slides and incubated for 2hrs at RT. Slides were then washed 3X in PBS and mounted with Vectashield + DAPI (Vector Labs).

If used to make sliding microtome sections, brains were flash frozen and then mounted in OCT on the freezing stage of a sliding microtome. Forty micron floating sections were collected from the microtome blade using a paintbrush and placed in 24-well plates containing PBS. For staining, sections were washed with PBS, blocked with a 10% normal goat serum solution (NGS), and incubated O/N with primary antibodies diluted in 10% NGS +.3% Triton-X 100. The next day, sections were washed 3X in PBS, incubated for 2hrs at RT with secondary antibodies diluted 1:250 in the antibody diluent, and then washed 3X in PBS and applied to slides using a paintbrush. Sections were allowed to dry and then mounted with Vectashield + DAPI. For retina whole mount staining, retinas were dissected and incubated O/N at 4° C in primary antibody diluted in 1% NGS and 2% Triton-X 100. All other steps are identical to those described above.

All images were acquired using either an UltraView Vox spinning disk confocal microscope equipped with diode lasers (405nm, 445nm, 488nm, 514nm, 561nm, and 640nm) and Volocity image acquisition software (Perkin Elmer), an Imager.M2 microscope equipped with diode lasers (405, 488, 555 and 639nm) and Zen 2009 image acquisition software

(Carl Zeiss), or an Imager.Z1 microscope equipped with Axiovision software (Carl Zeiss).

Antibody dilutions used: CD47 (BD Pharmingen, 1:500), SIRPα (BD Pharmingen, 1:500), CD200 (R+D systems, 1:250), Homer (Synaptic systems, 1:200), Vglut2 (Millipore, 1:2000 IHC; 1:1000 ImmunoEM, 1:200 AT), Iba-1 (Wako, 1:400 O/N at room temperature IHC, 1:1000 ImmunoEM), SV2 (DSHB, 1:20), CD68 (Serotec, 1:200, clone FA-11), CR3 (CD11b, Serotec, 1:200, clone 5C6), DsRed2 (Clontech), C3 (MP Biomedicals, 1:200), TUJ1 (Covance, 1:400), GFAP (Sigma, 1:1000), Cleaved caspase (Asp175, Cell Signaling, 1:200), GluR1 (Millipore, 1:100 AT), GFP (rabbit, Millipore, 1:500), GFP (chicken, Abcam, 1:500)

Eye-specific segregation analysis

Mice were anesthetized with inhalant isofluorane and given intraocular injections of cholera toxin-beta subunit (CTB) conjugated to Alexa 488 (green) in the right eye and Alexa 594 (red) in the left eye as described in (Bjartmar et al., 2006). Mice were sacrificed the day after injection and tissue was processed and analyzed as previously described (Jaubert-Miazza et al., 2005, Stevens et al., 2007). Images were selected for analysis and thresholded blind to genotype, and only age-matched littermate controls were used.

Engulfment analysis

Mice were injected with anterograde tracers (CTB-594 and CTB-647) at P4 and perfused 24 hours later using the same methods as for immunohistochemistry. Brains were sectioned on the sliding microtome and 40μm sections were stained for Iba-1 if not already labeled by CX3CR1::EGFP. For each animal, two sections of medial dLGN were imaged and only

dLGNs with good dye fills were used for analysis. Images were acquired on a spinning disk confocal microscope at 63x with .2μm z-steps. For each dLGN, at least 4 cells were imaged in the ipsilateral territory and at least 4 cells were imaged in the contralateral territory (minimum 8 cells per dLGN, 16 cells per animal). For P5 regional analysis, optic tract microglia were imaged as well (2 fields per dLGN). Images were processed using ImageJ (NIH) and Imaris (Bitplane) software. ImageJ was used to subtract background from z-stacks of CTB channels (rolling ball radius = 10) and the microglia channel (rolling ball radius = 50) and a mean filter of 1.5 was applied to the microglia channel. Images were cropped such that only one microglia cell would be in each image to prepare for surface rendering. Imaris software was used to surface render each channel and obtain volume measurements. Internalized material was visualized by masking CTB channels with the microglia channel to subtract all material not contained within the microglia cell volume. The remaining engulfed input fluorescence was surface rendered using parameters previously determined for total RGC inputs and total volume of engulfed inputs was calculated. To determine % engulfment, the following calculation was used: Volume of internalized RGC inputs (μm^3)/Volume microglial cell (μm^3). For experiments involving normal development n=3 mice per condition. For experiments involving pharmacological manipulation of neuronal activity n=4 (TTX) or 5 (forskolin). For experiments involving KO or minocycline-treated mice n= 3 (CR3 KO) or 4 (C3 KO and minocycline) mice per genotype/condition. For all C3 and CR3KO engulfment experiments, littermates were also sex matched. For CD47KO and CD200KO experiments, percent engulfment was further normalized to ensure that variations in axon outgrowth did not affect engulfment calculations by dividing % engulfment by the input density for each field (input density =

total volume CTB-labeled inputs/total volume field of view). All experiments involving knockout animals were performed blind to genotype.

Quantitative PCR (qPCR)

Whole brains and retinas from PBS perfused mice were flash frozen on dry ice. RGCs and microglia were collected as described below. Whole brain tissue was manually homogenized and RNA was isolated using phenol chloroform extraction, while other cell and tissue RNA was collected after lysis in RLT buffer. The RNeasy mini kit (QIAGEN) was used to isolate and purify all RNA samples. After cDNA synthesis, qPCR was performed using the Applied Biosystems Cells to Ct Power SYBR green kit. QPCR reactions were assembled for the genes of interest (cd47, sirpα) using .5µl of cDNA per reaction and samples were run on the Rotogene qPCR machine (QIAGEN). Expression levels were compared using the ddCt method normalized to GAPDH. All samples were normalized to P5 and only males were used for whole brain, retina, and microglia isolation.

Microglia activation analysis

Mice were perfused as described for immunohistochemistry and 2-4 40µm free floating sections containing dLGN were stained from each brain (for Iba-1 and CD68). Sections were mounted as described above, and then imaged using a spinning disk confocal microscope. Two medial dLGNs per animal were imaged, and two 20x z-stacks were acquired to fully capture each dLGN using 2µm z-steps. Maximum intensity projections were created, and each microglia cell was given a score of 0-5 based on morphology and CD68 abundance. The activation state of microglia was categorized from 0 (lowest

activation) to 5 (highest activation) depending on the following criteria. First, the processes morphology was scored as 0 (>15 thin processes with multiple branches), 1 (5-15 thick processes with branches), 2 (1-5 thick processes with few branches), 3 (no clear processes). Second, the expression pattern of CD68 was analyzed and scored as 0 (no/scarce expression), 1(punctate expression), 2 (aggregated expression or punctate expression all over the cell). Finally, for each cell analyzed, morphology and CD68 scores were summed and a final score of microglia activation state (0–5) was assigned. For each condition/age dLGN from 3 mice were analyzed (n=3). All analyses were performed blind and littermates were sex matched whenever possible.

Microglia density quantification

The same sections used for activation analysis were also used for cell 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.

Structured Illumination Microscopy (SIM)

For synaptic colocalization, 14μm sections were imaged at 100x magnification using the Zeiss Elyra S.1 system. A 3nm z stack with an interval of 0.101nm was captured with brightfield illumination and Zen software (Zeiss) was used to generate the structured illumination microscopy image. Three dimensional reconstructions of these images were

subsequently generated and annotated with Zen and Photoshop software (Adobe). For microglial engulfment of Vglut2, 16μm cryosections of CX3CR1+/EGFP dLGN were immunolabeled. Images were acquired with an Elyra PS.1 Combi with LSM 780 Confocal on an Axio Observer inverted microscope equipped with a 63x Plan Apo objective (Zeiss). Z-stacks were collected using 5 rotations and 5 phase shifts of a grid specific to the objective with a 1K X 1K EMCCD camera. Laser lines included on the system were 405, 488, 561 and 642. ZEN 2010 software (Zeiss) was used for acquisition and image processing for super resolution.

Western blot analysis

Frozen brain samples from PBS perfused mice were transferred into lysis buffer containing SDS (25mM Hepes pH 7.5, 95mM NaCl, 10mM EDTA, 2% SDS and protease inhibitor cocktail (Roche 05 892 970 001)). Samples were homogenized using the Tissue Lyser II system (Qiagen) for 2 minutes at speed setting 20 1/s. Subsequently, samples were centrifuged for 2 minutes at 10000rpm at 4° C. Supernatants were extracted and centrifuged again for a further 2 minutes at the same speed after which the resulting supernatants were collected. Twenty milligrams of each protein sample were loaded and separated by SDS-PAGE on 10% tris-glycine gels under denaturing conditions with the mini Protean II gel electrophoresis apparatus (Biorad, USA). Kaleidoscope pre-stained standard molecular weight markers (Biorad, USA) were run alongside samples to enable identification of band sizes. Separated proteins were then transferred to a nitrocellulose membrane, Hybond-ECL (Amersham RPN 3032D), in a mini gel tank (Biorad, USA) at 4° C.

Membranes were incubated in 5% fat free milk powder in tris-buffered saline (TBS) for 1 hour to block non-specific binding sites. Primary antibodies diluted in 5% fat free milk powder in TBS were then added and membranes incubated O/N at 4° C on a slow rotator. Membranes were washed 3 times every 15 minutes for 30 minutes at RT with TBS and subsequently incubated with horse radish peroxidase (HRP) conjugated secondary antibody diluted in 5% milk in TBS for 1 hour at RT and again washed in TBS 3 times every 15 minutes for 1 hour. Membranes were then treated with ECL reagents from the Super Signal detection kit (Perbio). Specific protein complexes were revealed by placing membranes in autoradiography cassettes (Appligene, USA) and developed by brief exposure (5 seconds to 10 minutes) to Thermo Scientific CL-XPosure film. Quantification was performed using the gel function in ImageJ (NIH).

Antibodies used: Vglut 2 (Millipore) and Homer (Synaptic systems) 1:20000, Actin 1:1000.

In situ hybridization

Cryosections were collected as described for immunohistochemistry (14μm) and dried at 65° C for 30 minutes. Tissue was subsequently fixed in methanol at -20° C for 20 minutes before being washed 3X with PBS. Sections were then incubated in a proteinase K solution (proteinase K 1μg/ml in 50mM Tris pH 7.5 and 5mM EDTA) for 10 minutes at RT before carrying out another PBS wash. Tissue was then re-fixed in 4% PFA for 5 minutes at RT before again washing with PBS. An acetylation step was subsequently carried out by incubating the sections in a solution containing acetic anhydride (triethanolamine 0.09M, HCl 0.09M, 0.2% acetic anhydride) for 10 minutes at RT before a further washing step.

Endogenous peroxidase activity was then blocked by incubating in a 0.3% hydrogen peroxide solution before a final wash in PBS.

Before continuing, a hybridization chamber was humidified by adding 5XSSC to the reservoir chamber. Slides were subsequently placed into the chamber and hybridization solution (1x pre-hybridization solution (Sigma P-1415), 25mg yeast tRNA (Roche), and 50% formamide (American Bioanalytical)) was applied to the sections for 1hr at RT. Relevant probes were then diluted in hybridization solution and heat denatured at 80° C for 15 minutes before being applied to the sections. A glass coverslip was mounted on top of the sections, which then incubated O/N at 62° C.

Coverslips were removed by washing in 2XSSC (Sigma) at 62° C and sections were placed in a fresh 2XSSC solution for a further 5 minutes. Sections were subsequently transferred to a 0.2XSSC solution at 55° C before being washed with the same solution over a 3hr period. Sections were then placed at RT and washed a further two times with TBS. Antibody blocking solution was applied to sections for 1hr before adding Sheep anti-DIG:POD (Roche) at 1:2000 in blocking solution alongside relevant primary antibodies for IHC and incubating O/N at 4° C.

Sections were washed 3X for 10 minutes with TBS and then a further 2X with TBST (TBS and 0.3%Triton X-100). TSA staining solution with an appropriate fluorophore was then applied to sections according to the manufacturers instructions (PerkinElmer) for 1hr at RT before again washing with TBS. For double labeling with two in situ probes, sections

were first incubated in 0.3% hydrogen peroxide to kill the 1st POD before repeating the staining with a sheep anti-fluorescein POD.

For double in-situ staining alongside an antibody for IHC, sections were washed again in TBS before incubating with a conjugated secondary antibody against the species in which the primary antibody was made for 2hrs at RT. After washing again with TBS, coverslips were mounted on top of the sections with Vectashield +DAPI (Vector labs).
The CD47 probe was designed using an Open Biosystems plasmid, NSE was obtained from the Greenberg lab (HMS) and SIRPα plasmid was provided by Hisashi Umemori.

Microglia isolation

Microglia were isolated from male, C57BL/6 mice as described in (Pino and Cardona 2011). Briefly, mice were transcardially perfused with cold HBSS and whole brains were manually homogenized in RPMI. Samples were applied to a percoll gradient, and after a 30min spin at 500G, cells were collected from the 30%-70% interphase, pelleted and washed. Microglia isolation was followed immediately by RNA isolation using the RNeasy mini kit (QIAGEN) and cDNA synthesis.

Neuron cultures

Retinal ganglion cells (RGCs) were acutely isolated from P5 C57BL/6 mice after serial immunopanning steps to yield >99.5% purity as described in (Barres et al., 1998). Cells were lysed at the end of immunopaning and RNA and cDNA were isolated using the Applied Biosystems Cells to Ct kit.

Synaptosome fractionation

The fractionation protocol was adapted from a previous report (Hahn et al., 2009) . Cortex (300–350mg) from P21 mice was homogenized in 1.5ml of homogenization buffer (0.32M sucrose, 1mM NaHCO3, 1mM MgCl2, 0.5mM CaCl2, protease inhibitor). Homogenate was then adjusted to 1.25M sucrose and 0.1mM CaCl2 in a total of 5ml. Homogenate was overlaid on 5ml of 1M sucrose and spun at 100,000G (SW41-Ti rotor, Beckman) for 3hrs at 4° C. Interface was collected and designated as synaptic membrane fraction (SPM). SPM (500µl) was then added to 2ml of 0.1mM CaCl2 and 2.5ml of 40mM Tris, pH 6, with 2% Triton X-100, and placed on rocking platform for 20 min at 4° C. The sample was then spun at 35,000G (SS-34 rotor, Sorvall) for 20 min at 4° C and supernatant was collected as the extrajunctional fraction. The pellet was air-dried and resuspended in 1ml of 0.1mM CaCl2 and 1ml of 40mM Tris, pH 8, with 2% Triton X-100, and placed on rocking platform for 60 min. Resuspended pellets were then spun at 140,000G (SW41-Ti rotor, Beckman) for 30 min at 4° C, and supernatant was collected as presynaptic fraction. The insoluble fraction was resuspended in 1ml of 20mM Tris pH 7.4 with 1% SDS and designated as the postsynaptic fraction. Extrajunctional and presynaptic fractions were acetone-precipitated and resuspended in 1ml of 2 mM Tris, pH 7.4, with 1% SDS. Synaptic membrane fraction and equivalent volumes of extrajunctional, presynaptic and postsynaptic membrane fractions were then transferred to PVDF membrane and probed with CD47 (BD Pharmingen 1:500).

Minocycline injection

WT or CX3CR1::EGFP heterozygote mice were injected daily with minocycline (Sigma;

75mg/kg) or vehicle (saline) subcutaneously from P4-P8. The dose, route, and frequency of injections were determined based on earlier work (Buller et al., 2009) and on our own observations. All subsequent analyses were performed blind to treatment condition.

Intraocular injection of TTX or forskolin

P4 CX3CR1::EGFP heterozygotes were anesthetized with isofluorane and given an intraocular injection of drug (0.5μM TTX or 10mM forskolin) and vehicle (saline or DMSO) into the left and right eyes, respectively. Injection volume was approximately 200 nL. 4-5hrs after first injection, mice received a second intraocular injection of CTB-594 and 647 into the left and right eyes, respectively. Mice were sacrificed at P5 for analysis.

Cell death quantification

For experiments to quantify cell death, P4 WT mice were given an intraocular injection of drug (0.5μM TTX or 10μM forskolin) and vehicle (saline or DMSO) into the left and right eyes, respectively. 4-5hrs after first injection, mice received a second intraocular injection of CTB 594 and 647 into the left and right eyes, respectively. Mice were subsequently sacrificed at P5. Only retinas from those mice in which dye tracing resulted in complete fills of RGC axons were analyzed. Retina were subsequently embedded, cryosectioned (14μm), and immunolabeled for cleaved caspase-3 (Asp175) and total nuclei were stained with DAPI. The percent of cleaved-caspase-positive cells was calculated from the RGC layer. For each condition (2 animals per condition), four retinas were quantified, two 20x fields of view per retina. n=16 fields of view/condition.

Retinal ganglion cell quantification

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 at 63x. 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.

Electron Microscopy

EM was performed in collaboration with the J. Lichtman laboratory. For EM, 30nm sections were sliced using a Diatome 45 diamond knife and a Leica UM-6 ultramicrotome. The sections were collected on kapton tape as described by Hayworth et al. (2006). After collection, the tapes were adhered to silicon wafers using double-sided carbon tape. The sections were then post stained with uranyl acetate and lead citrate for 3 minutes. The entire wafer assembly was then coated with 10nm of carbon. The sections were then imaged using a Zeiss Sigma field emission scanning electron microscope (FESEM) operating in backscattered electron mode.

For double immunoEM experiments, primary antibodies and secondary antibodies were incubated together. For immunogold labeling, tissue was prepared identically with minor modifications. 1.4nm nanogold conjugated to appropriate species-specific antibodies was used for secondary labeling (Nanoprobes). Following all secondary antibody incubation steps, tissue was processed to increase the size of the nanogold with HQ silver

enhancement for 5 min (Nanoprobes). Tissue was then post-fixed in 1% osmium tetroxide and embedded as previously described (Tremblay et al., 2010). Images were acquired on a JEOL 1200EX-80kV.

Quantification of EM

Four fields of view (10,000x) of dLGN containing microglia (4 independent experiments) were imaged. Statistics were calculated per cell, n=20. The % frequency of engulfed material was subsequently calculated per dLGN. To calculate cumulative probability of VGlut2 localization, 4 fields of view (10,000x) of dLGN containing microglia and/or RGC terminals (2 independent experiments) were imaged. Neuropil measurements were performed from views containing either microglia or RGC terminals. A total of 10 measurements were made for each structure (microglia, RGC terminal, or neuropil, n=10). The total area of VGlut2 versus neuropil, microglia, or RGC terminal was subsequently calculated by measuring the area of the neuropil, microglia, or RGC terminal followed by manual thresholding of VGlut2 immunoreactivity and measurement of the thresholded area with ImageJ software.

Array Tomography

Array Tomography was performed as previously described with minor modifications (Greer et al., 2010; Margolis et al., 2010; Micheva and Smith, 2007; Ross et al., 2010; Stevens et al., 2007). Briefly, acute dLGN slices (300μm thick) were fixed in 4% paraformaldyhde for 1.5hrs at RT and embedded in LR White resin using the benchtop protocol. Ribbons of between 20-30 serial 100nm thick sections of both WT and KO

littermates were mounted side by side on subbed glass coverslips and immunostained with anti-VGlut2 and anti-GluR1. Serial sections were imaged using a Zeiss Imager.Z1 microscope, 63x objective and subsequent volumes were aligned using ImageJ (NIH) with the multistackreg plugin (Brad Busse). Fields of view (3-4 per animal, 3 animals per genotype) were analyzed in Bitplane Imaris and custom software to count synapses. A synapse was counted if the distance between the center point of a VGlut2 puncta and a GluR1 puncta was equal to or less than the sum of the radii of the two puncta plus an empirically determined scaling factor of 0.1μm. All data were subsequently normalized to WT values. All experiments were carried out and analyzed blind to genotype.

Statistical Analysis

For all statistical analyses, GraphPad Prism 5 software (La Jolla, CA) was used. Analyses used include one-way ANOVA, unpaired or paired, two-tailed or one-tailed, Student's t-test, 95% confidence or Mann-Whitney U test. All p values are indicated in figure legends.

Chapter 3:

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

Contributions: All experiments were designed by Dorothy Schafer and Beth Stevens. Dorothy Schafer and Emily Kate Lehrman developed engulfment analysis. All experiments and data analysis were performed by Dorothy Schafer with the exception of the following: wild type developmental engulfment analysis, CR3KO engulfment analysis, minocycline engulfment analysis, regional engulfment analysis, and microglia cell density counts were performed and analyzed by Emily Kate Lehrman, microglia activation assay was performed by Ryuta Koyama, array tomography was performed by Amanda Kautzman.

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Introduction

Early in development neurons make far more synaptic connections than are maintained in the mature brain. Synaptic pruning is an activity-dependent developmental program in which a large number of synapses that form in early development are eliminated while a subset of synapses is maintained and strengthened (Hua and Smith, 2004; Katz and Shatz, 1996; Sanes and Lichtman, 1999). While it is clear that neuronal activity plays a role, the precise cellular and molecular mechanisms underlying this developmental process remain to be elucidated.

Microglia are the resident CNS immune cells which have long been recognized as rapid responders to injury and disease, playing a role in a broad range of processes such as tissue inflammation and clearance of cellular debris (Hanisch and Kettenmann, 2007; Kreutzberg, 1996; Ransohoff and Perry, 2009). In contrast to disease pathology, the function of microglia in the normal, healthy brain is far less understood. However, recent studies suggest that microglia may play a role in synaptic remodeling and plasticity in the healthy brain (Davalos et al., 2005; Nimmerjahn et al., 2005; Paolicelli et al., 2011; Tremblay et al., 2010a; Wake et al., 2009). For example, microglia within the juvenile visual cortex modify their association with dendritic spines in response to changes in visual sensory experience (Tremblay et al., 2010a). A more recent study provides evidence that disruptions in microglia function result in delayed maturation of hippocampal synaptic circuits (Paolicelli et al., 2011). Moreover, data from these studies suggest that microglia may be phagocytosing dendritic spines. These intriguing studies raise several interesting and important questions. The precise function of microglia at synaptic sites, the molecular

mechanism(s) underlying microglia-mediated synaptic engulfment, and the long term consequence(s) of disrupting microglia function on synaptic circuits remain a mystery.

A candidate mechanism by which microglia could be interacting with developing synapses is the classical complement cascade. Complement cascade components C1q and C3 localize to immature synapses and are necessary for the developmental pruning of retinogeniculate synapses (Stevens et al., 2007). While provocative, the mechanism by which complement mediates synaptic pruning has remained completely unknown. Complement components function in the immune system by binding and targeting unwanted cells and cellular debris for rapid elimination through several different pathways. These molecules are classic "eat me" signals, which are known to communicate with receptors on immune cells (Elward and Gasque, 2003; Lauber et al., 2004). Among the many mechanisms by which complement may mediate synaptic pruning is phagocytosis, which makes microglia, the resident CNS phagocyte, a candidate.

Given the questions that have now emerged regarding the role of microglia at CNS synapses, we sought to address precisely how microglia are interacting with developing synaptic circuits and determine the long-term consequences of disrupting microglia function on neural circuit development. In the current study, we demonstrate that microglia engulf presynaptic retinal inputs undergoing synaptic pruning in the postnatal brain and determine that this process is regulated by neuronal activity. Furthermore, we identify signaling through a phagocytic receptor, complement receptor 3 (CR3/CD11b-CD18/Mac-1), expressed on the surface of microglia and its ligand, complement component C3 localized to synaptically-enriched regions, as a key molecular mechanism underlying engulfment of developing synapses. Importantly, disruption of CR3/C3 signaling was

specific to microglia in the CNS and resulted in sustained deficits in brain wiring. Taken together, these observations provide a role for microglia in the healthy, developing brain, and provide a cellular and molecular mechanism by which microglia are physically interacting with synaptic elements.

Results

Microglia engulf RGC inputs during a period of active synaptic pruning

To investigate the functional role of microglia in developmental synaptic remodeling, we used the mouse retinogeniculate system, a classic model for studying activity-dependent developmental synaptic pruning (Feller, 1999; Huberman et al., 2008; Shatz and Kirkwood, 1984). Early in development, retinal ganglion cells (RGCs) form exuberant synaptic connections with relay neurons throughout the dorsal lateral geniculate nucleus (dLGN) of the thalamus. During the pruning period, RGC synaptic inputs originating from the same eye as well as between eyes compete for territory throughout the dLGN (Chen and Regehr, 2000; Hooks and Chen, 2006; Jaubert-Miazza et al., 2005; Ziburkus and Guido, 2006). Spontaneous retinal activity plays critical role in this refinement process, however the underlying cellular and molecular mechanisms remain poorly understood. (Del Rio and Feller, 2006; Feller, 1999; Penn et al., 1998; Shatz, 1990; Torborg and Feller, 2005).

During this robust pruning period (P5 in mouse), we used high resolution confocal imaging to assess the interactions between microglia and synaptic inputs throughout the dLGN. Contralateral and ipsilateral presynaptic inputs from RGCs were visualized in the dLGN by intraocular injection of anterograde tracers, cholera toxin β subunit conjugated to Alexa 594 (CTB-594) and Alexa 647 (CTB-647), respectively (Figure 3.1A). Microglia were

labeled using the CX3CR1+/GFP mouse line in which all microglia express EGFP under the control of fractalkine receptor, CX3CR1, expression (Figure 3.1, Figure 3.2, Figure 3.11) (Cardona et al., 2006; Jung et al., 2000; Saederup et al., 2010).

At an age consistent with robust synaptic pruning (P5), microglial processes were in close association with RGC presynaptic inputs (Figure 3.1B, Figure 3.3A). Upon closer examination, we detected numerous fluorescently labeled RGC inputs within the processes and soma of microglia (Figure 3.1B). Internalization was further confirmed by assessing confocal z-stacks through individual microglia. This specific example is a microglia sampled from a region containing similar densities of overlapping ipsilateral (blue) and contralateral (red) RGC inputs (Figure 3.1A) which are undergoing active synaptic remodeling to establish non-overlapping eye specific territories (Figure 3.4A) (Godement et al., 1984; Guido, 2008; Huberman et al., 2008; Sretavan and Shatz, 1986; Ziburkus and Guido, 2006). Consistent with simultaneous pruning of inputs from both eyes, contralateral (red) and ipsilateral (blue) RGC inputs were engulfed and localized within the microglia (Figure 3.1B). In addition, consistent with widespread pruning of RGC inputs throughout the P5 dLGN, we observed engulfment of RGC inputs in all synaptic regions (monocular and binocular). These data suggest that microglia engulf RGC inputs undergoing active synaptic remodeling.

To confirm that inputs are phagocytosed by microglia, RGC inputs from both eyes were labeled with CTB-594 and colocalization with CD68, a marker of lysosomes specific to microglia, was assessed in P5 dLGN. As suggested by previous dye-labeling experiments,

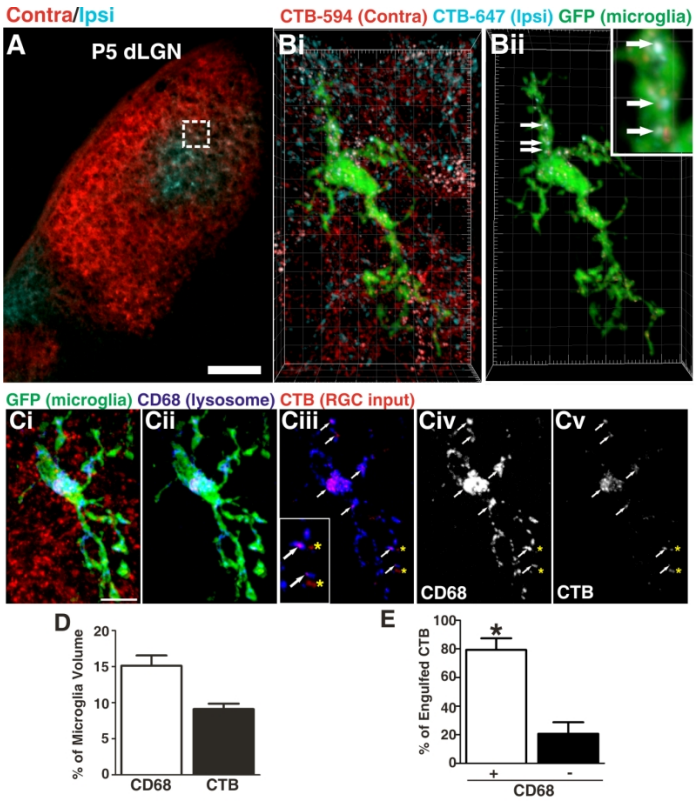

Figure 3.1. Microglia engulf RGC inputs undergoing active synaptic pruning in the dLGN. A, A representative low magnification image of P5 dLGN. Ipsilateral inputs are labeled with CTB-647 (blue) and contralateral inputs are labeled with CTB-594 (red). Scale bar = 100 µm. Bi, A microglia (EGFP, green) sampled from the border region of ipsilateral (blue) and contralateral (red) projections (inset in A). Bii, All CTB fluorescence outside the microglial volume has been subtracted revealing RGC inputs (red and blue) that have been engulfed (arrows, enlarged in inset). Grid line increments = 5 µm. Ci, A representative microglia (green, EGFP) from P5 dLGN. RGC inputs from both eyes are labeled with CTB-594 (red) and lysosomes are labeled with anti-CD68 (blue). Cii, The same microglia in which all CTB fluorescence outside the microglia volume has been removed revealing lysosomes (blue) and engulfed RGC inputs (red). Ciii, The same cell in which only the lysosomes (blue) and RGC inputs (red) are visualized in which most inputs (red) are localized within CD68-positive lysosomes (blue; white arrows). There are few instances in which CTB is not localized to lysosomes (yellow asterisks). Inset is enlarged region of Ciii. Civ-v, The CD68 (Civ) and CTB (Cv) channels alone. Scale bar = 10 µm. D, Quantification of % volume of microglia occupied by CD68-positive lysosomes (white bar) and RGC inputs (black bar), n=3 P5 mice. E, There are significantly more engulfed inputs localized to lysosomal compartments (white bars) versus non-lysosomal compartments (black bars). *P<0.001 by Student's t-test, n=3 P5 mice. All error bars represent s.e.m.

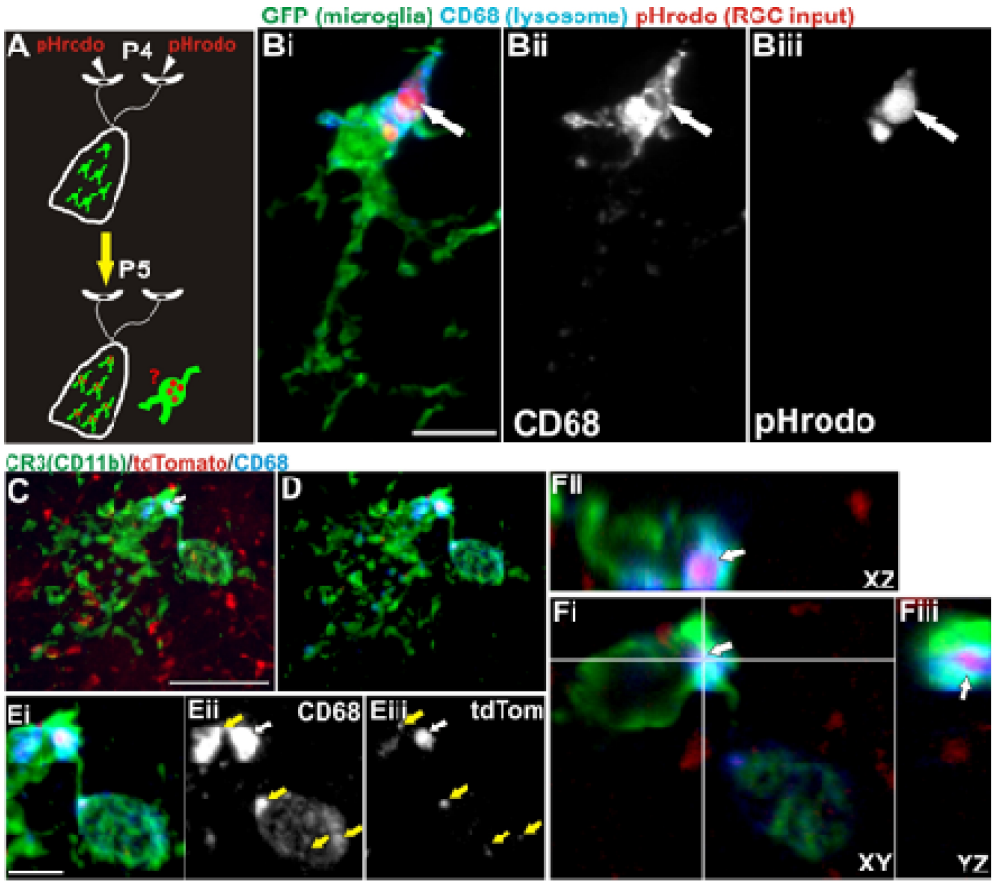

Figure 3.2. Microglia engulf RGC synaptic inputs within the P5 dLGN into lysosomal compartments. A, Schematic of strategy used to further assess phagocytosis using pHrodo. Bi, Microglia (green) within the dLGN engulf RGC inputs (pHrodo, red, Biii) into lysosomal compartments (CD68, blue, Bii) during peak retinogeniculate pruning (P5). Scale bar = 10 μm. .C-F, The reporter line Rosa 26-Stop-tdTomato was crossed with Chx10-cre to specifically label RGC inputs in the dLGN (CHX10-cre::tdTomato). dLGN from P5 tdTomato-expressing mice were immunolabeled for CR3 alpha subunit, CD11b (green) to label microglia, CD68 (blue) to label lysosomes within microglia, and dsRed2 (red) to amplify the tdTomato expressed in RGC inputs. C, CR3/CD68-positive microglia that has engulfed RGC inputs (arrow). D, The same cell in which any RGC inputs that are not within the microglia have been subtracted. Ei-iii, An enlarged region of D. Similar to CTB-labeled inputs, tdTomato-labeled RGC inputs are within lysosomal compartments of microglia (arrows; white arrow denotes region shown orthogonally in F). C,D Scale bar = 15 μm. Ei-iii, Scale bar = 5 μm. F, Orthogonal views of engulfed RGC inputs designated by the white arrow in E. Any uncolocalized tdTomato labeling are inputs that have not been engulfed and are outside of the microglia volume (see C).

the majority of engulfed RGC inputs were completely colocalized within lysosomal compartments (Figure 3.1C-E). There were rare instances in which engulfed RGC inputs did not colocalize (Figure 3.1Ciii and E) and we suspect that these inputs are either in the process of being phagocytosed or are in phagosomal or endosomal compartments prior to lysosomal degradation. To further validate that microglia phagocytose RGC inputs, pHrodo-dextran, an anterograde tracer and pH-sensitive dye, was used to label RGC inputs (Figure 3.2 A-B) (Deriy et al., 2009; Miksa et al., 2009). Because pHrodo only fluoresces once it enters acidic compartments of lysosomes, any pHrodo-positive fluorescence within a microglia confirms phagocytosis of RGC inputs. Similar to previous experiments, pHrodo-positive RGC inputs were localized within microglia (Figure 3.2A-B). Furthermore, in addition to anterograde tracing with CTB and pHrodo, RGC input engulfment was also assessed within the P5 dLGN using a genetic approach, double transgenic mice expressing tdTomato under the control of Chx10, a transcription factor expressed by RGCs (Chx10-cre/Rosa26-STOP-tdTomato) (Figure 3.2C-F). Similar to CTB experiments, we observed tdTomato-labeled RGC inputs within lysosomal compartments of microglia. Importantly, these experiments exclude the possibility that engulfment is due to injury secondary to ocular injections. Together, we demonstrate that microglia phagocytose RGC inputs during a peak period of synaptic pruning in the dLGN.

Microglia-mediated engulfment of RGC inputs is developmentally regulated

To begin to address whether microglia-mediated engulfment of RGC inputs contributes to the normal process of synaptic pruning, we assessed the developmental regulation of microglia phagocytic capacity. We first characterized microglia activation state through development and observed a unique class of microglia in the early postnatal

dLGN as compared to older ages (P30) (Figure 3.3). Microglia within the early postnatal dLGN had characteristic features of more 'activated' cells traditionally associated with disease including increased phagocytic capacity (assessed by morphology and CD68 immunoreactivity; Figure 3.3C,D). Interestingly, early postnatal microglia also had processes, a morphological characteristic of 'resting' microglia which are resident in the healthy adult brain (Figure 3.3B) (Lynch, 2009; Ransohoff and Perry, 2009).

To address whether engulfment of RGC inputs was developmentally regulated, we developed an *in vivo* phagocytosis assay (Figure 3.4A). Using high resolution confocal microscopy followed by 3D reconstruction and surface rendering (Figure 3.4D), internalization of ipsilateral (CTB-647; blue) and contralateral (CTB-594; red) RGC inputs was quantified within the volume of each microglia (CX3CR1+/EGFP) throughout the dLGN. To control for variation in microglia volume, the following calculation was used: % Engulfment = Volume of internalized RGC inputs (μm^3)/Volume of microglia (μm^3). Consistent with microglial involvement in normal developmental synaptic pruning, engulfment of RGC inputs was developmentally regulated. During a developmental period of robust pruning (P5), engulfment was high (Figure 3.4B,Di). As few as 4 days later (P9), when much of the pruning is nearly complete, engulfment of RGC inputs was significantly reduced (Figure 3.4B,Dii). Thus, microglia-mediated engulfment of RGC inputs is temporally correlated with a period of robust synaptic pruning within the developing dLGN. Importantly, similar to P5 dLGN, microglia within the P9 dLGN still retained phagocytic capacity as assessed by morphology and CD68 expression (Figure 3.3C,D).

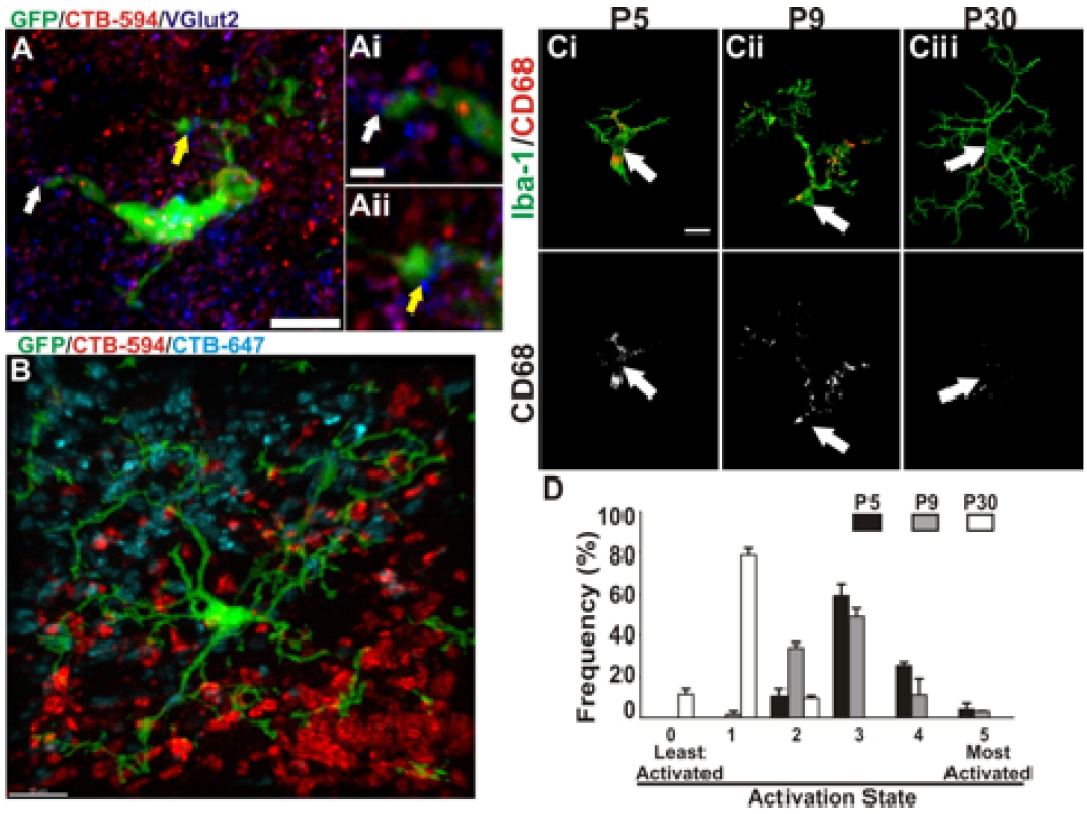

Figure 3.3. 'Activated,' process-bearing microglia interact with RGC synaptic inputs within the P5 dLGN. A, Representative image of a microglia (green, CX3CR1::EGFP) from a P5 dLGN. RGC inputs are labeled with the anterograde tracer CTB-594 (red) and RGC terminals are labeled with anti-VGlut2 (blue). Ai-ii, Enlarged regions from A demonstrating processes closely associated with and contacting synaptic endings of RGCs (white arrow in Ai and yellow arrow in Aii). B, In P30 dLGN, resting microglia (green) processes associate but do not overlap with CTB-labeled ipsilateral (CTB-647, blue) and contralateral (CTB-594, red) RGC inputs. A,B, Scale bars = 10 μm. Ai-ii, Scale bar = 2 μm. C, Confocal images of microglia immunolabeled for Iba-1 (green) and CD68 (red). The CD68 channel is visualized alone in lower panel images. Arrows indicate position of cell soma. Scale bar = 10 μm. D, Quantification of microglia activation state in the developing dLGN. Microglia activation was assessed blind on a scale of 0-5 based on morphology and CD68 immunoreactivity. Microglia in P5 (black bars) and P9 (grey bars) dLGN were consistently shifted toward increased activation state whereas cells sampled from older mice (P30, white bars) were consistently less activated (n=3 mice/age). Error bars represent s.e.m.

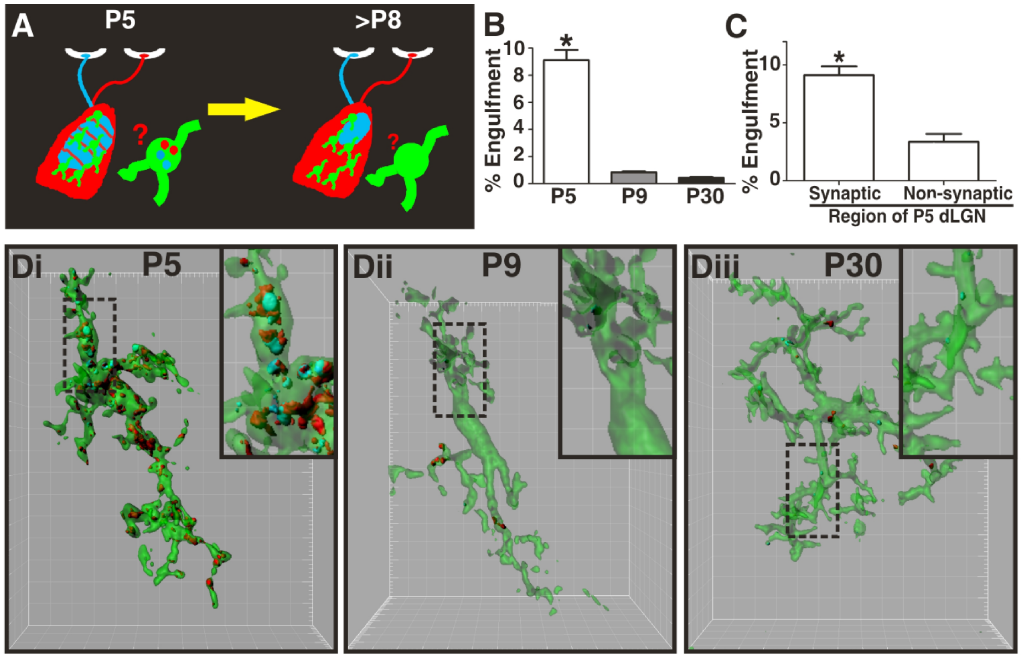

Figure 3.4. Microglia-mediated engulfment of RGC inputs is developmentally regulated. A, Schematic of retinogeniculate pruning and strategy used for assessing engulfment. Contralateral (red) and ipsilateral (blue) inputs overlap at early postnatal ages (P5). Inputs from both eyes prune throughout the dLGN during the first postnatal week and this is largely complete by P9/10. Engulfment was analyzed throughout the dLGN. B, Engulfment of RGC inputs is significantly increased during peak pruning in the dLGN (P5). *P<0.001 by one-way ANOVA, n=3 mice/age. C, Engulfment in P5 dLGN occurs most significantly in synapse-enriched (contralateral and ipsilateral dLGN) versus non-synaptic (optic tract) regions. *P<0.01 by Student's t-test, n=3 P5 mice. All error bars represent s.e.m. D, Representative surface rendered microglia from P5 (fluorescent image is shown in 1B), P9, and P30 mouse dLGN. Engulfment of RGC inputs occurs during peak pruning (P5) versus older ages (P9 and P30). Enlarged insets denoted with a black dotted line. Grid line increments = 5 µm.

These data suggest a more specific mechanism is driving engulfment specifically during the peak pruning period in the P5 dLGN.

Microglia-mediated engulfment of RGC inputs is regulated by neural activity

Synaptic pruning is thought to result from competition between neighboring axons for postsynaptic territory based on differences in patterns or levels of activity (Hua and Smith, 2004; Katz and Shatz, 1996; Sanes and Lichtman, 1999). In the dLGN, it is thought that RGC inputs compete for territory such that those inputs which are less active or 'weaker' are pruned and lose territory as compared to those inputs that are 'stronger' or more active, which elaborate and strengthen (Del Rio and Feller, 2006; Dhande et al., 2011; Huberman et al., 2008; Penn et al., 1998; Shatz, 1990; Torborg and Feller, 2005). This competition can occur between inputs from the same eye as well as between inputs from both eyes (Chen and Regehr, 2000; Hooks and Chen, 2006; Jaubert-Miazza et al., 2005; Ziburkus and Guido, 2006). To determine whether microglia-mediated engulfment of RGC inputs is regulated by neural activity, P4 CX3CR1+/EGFP mice were injected with TTX (0.5 μM) to block RGC activity or forskolin to increase activity (10 mM) (Cook et al., 1999; Dunn et al., 2006; Shatz and Stryker, 1988; Stellwagen and Shatz, 2002; Stellwagen et al., 1999) in the left eye and vehicle (saline or DMSO, respectively) in the right eye. In order to distinguish inputs from each eye, RGC inputs were anterogradely labeled with CTB-594 (TTX or forskolin inputs) and CTB 647 (vehicle inputs) following drug injection (Figure 3.5A,D). At P5, mice were sacrificed and engulfment was assessed in a region with a similar proportion of ipsilateral and contralateral eye inputs.

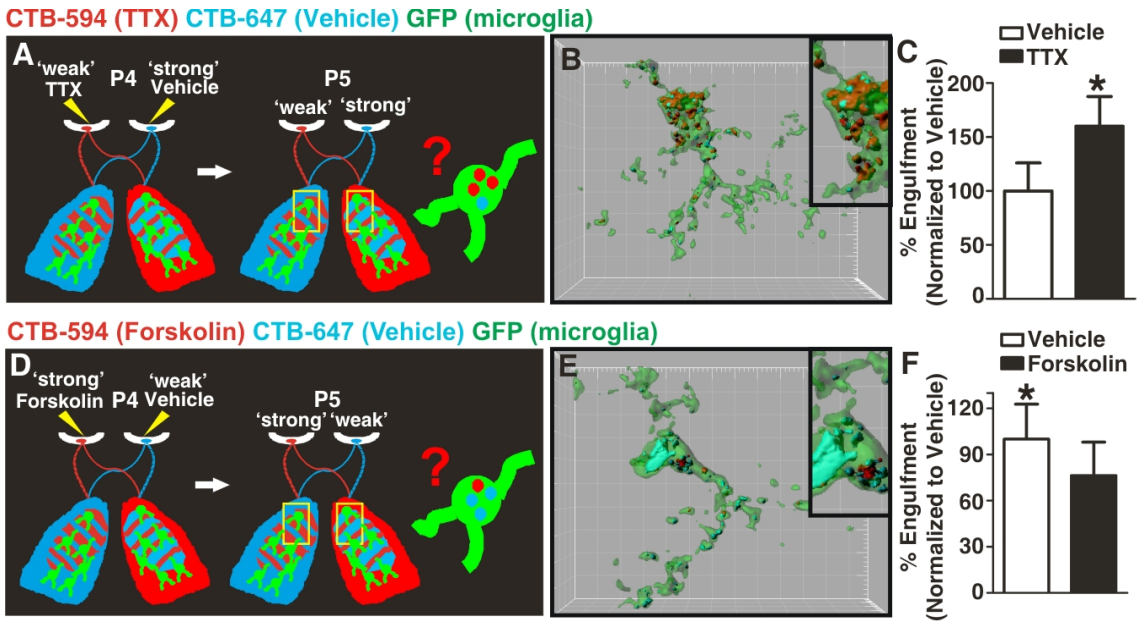

Figure 3.5. Microglia-mediated engulfment of RGC inputs is regulated by neural activity. A, D Schematic of strategies used for assessing microglia engulfment following disruption of dLGN pruning by manipulation of neuronal activity. B, Representative P5 microglia (green) surface rendered from the border region of ipsilateral and contralateral projections in which left and right eyes were treated with TTX (red) and vehicle (blue), respectively. Inset is an enlarged region demonstrating the increase in engulfment of inputs from the 'weaker', TTX-treated eye (red) as compared to those inputs derived from the 'stronger' vehicle-treated eye (blue). Grid line increments = 5 µm. C, Significantly more TTX-treated inputs (black bar) are engulfed as compared to vehicle-treated inputs (white bar). *P<0.04 by Student's t-test, n=4 mice/treatment. E. Representative P5 microglia (green) surface rendered from the border region of ipsilateral and contralateral projections in which left and right eyes were treated with forskolin (red) and vehicle (blue), respectively. Inset is an enlarged region demonstrating an increase in engulfment of inputs from the 'weaker', vehicle-treated eye (blue) as compared to those inputs derived from the 'stronger' forskolin-treated eye (red). Grid line increments = 5 µm. F, Significantly more vehicle-treated inputs (white bar) are engulfed as compared to forskolin-treated inputs (black bar) within the same dLGN. *P<0.04 by Student's t-test, n= 5 mice/treatment. All error bars represent s.e.m.

When mice were injected with TTX and vehicle in the left and right eyes, respectively, microglia phagocytosed significantly more inputs from the less active TTX-treated eye (CTB-594, red) as compared to the vehicle-treated eye (CTB-647, blue) (Figure 3.5B,C). Likewise, mice injected with forskolin and vehicle engulfed significantly more inputs from the vehicle-treated eye (CTB-647, blue) as compared to the more active forskolin-treated eye (CTB-594, red) (Figure 3.5E,F). Importantly, this effect occurred in the absence of any significant increase in RGC death (Figure 3.6). Taken together, these data demonstrate that microglia-mediated engulfment of RGC inputs is regulated by activity such that microglia preferentially engulf inputs from the 'weaker' eye and suggest that microglia are active participants in synaptic pruning.

Microglia engulf presynaptic elements specific to RGCs

While it is clear that microglia engulf RGC inputs in a developmental and activity-dependent manner, it is unclear whether engulfed material is axonal and/or synaptic. Consistent with synaptic engulfment, significantly more RGC inputs were engulfed within synaptic-enriched of the P5 dLGN compared to a non-synaptic region, the optic tract (Figure 3.4C). To better determine the identity of engulfed material, electron microscopy was performed.

Microglia were identified by EM using criteria previously described including a small, irregular-shaped nucleus containing substantial amounts of coarse chromatin and a cytoplasm rich in free ribosomes, vacuoles, and lysosomes (Mori and Leblond, 1969; Sturrock, 1981). Consistent with our confocal data, we observed several inclusions completely within the microglia cytoplasm including several double membrane-bound

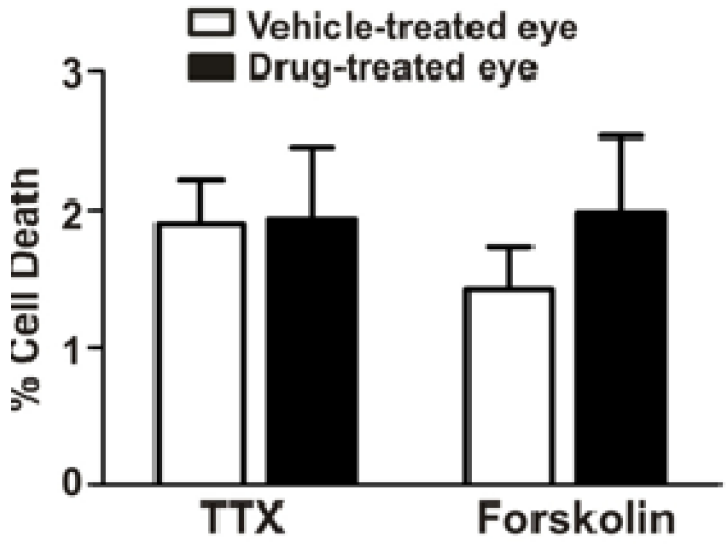

Figure 3.6. RGC death is not affected by TTX or forskolin treatment. WT mice were treated with TTX (0.5 μm) or forskolin (10 mM) in the left eye and vehicle (saline or DMSO, respectively) into the right eye. RGC death was quantified by measuring the % of cleaved caspase-positive cells out of total DAPI-positive cells within the RGC layer of the retina. RGC death was not significantly different between drug-treated (black bars) and vehicle-treated (white bars) eyes. Error bars represent s.e.m. n = 16 fields of view.

structures which contained 40 nm vesicles, data consistent with engulfment of presynaptic terminals (Figure 3.7A,B and Figure 3.8). In a few instances, structures reminiscent of juxtaposed pre and postsynaptic structures were observed (Figure 3.7Aii).

To further confirm microglia-mediated phagocytosis of synaptic elements, immunohistochemical electron microscopy (immunoEM) for the microglia marker Iba-1 was performed and quantified in the P5 dLGN (Figure 3.7C) (Tremblay et al., 2010b). Consistent with EM data described above, we observed membrane-bound structures containing 40 nm presynaptic vesicles that were completely surrounded (Figure 3.7D) or enwrapped (Figure 3.7E) by DAB-positive microglial cytoplasm. To further support that microglia engulf material specific to presynaptic terminals, 40 nm vesicles were enriched in presynaptic terminals (Figure 3.7Bii,F) and very rarely visualized in cross or longitudinal sections of axons (Figure 3.7G). Indeed, presynaptic elements were observed within 35% of the microglia sampled (Figure 3.7I). Interestingly, several intact presynaptic terminals (Figure 3.7F) and all engulfed or enwrapped presynaptic inputs (Figure 3.7A,B,D,E) lacked mitochondria, a characteristic feature of presynaptic terminals. Previous work has suggested that sensory deprivation or pharmacological blockade of neuronal activity (i.e., TTX) results in reduced mitochondria in presynaptic terminals known to undergo subsequent elimination (Hevner and Wong-Riley, 1993; Tieman, 1984). Thus, we suspect that these terminals deficient in mitochondria may be those destined for elimination.

In addition to presynaptic element engulfment, 63% of the sampled cells contained structurally unidentifiable membrane-bound inclusions within microglial lysosomal

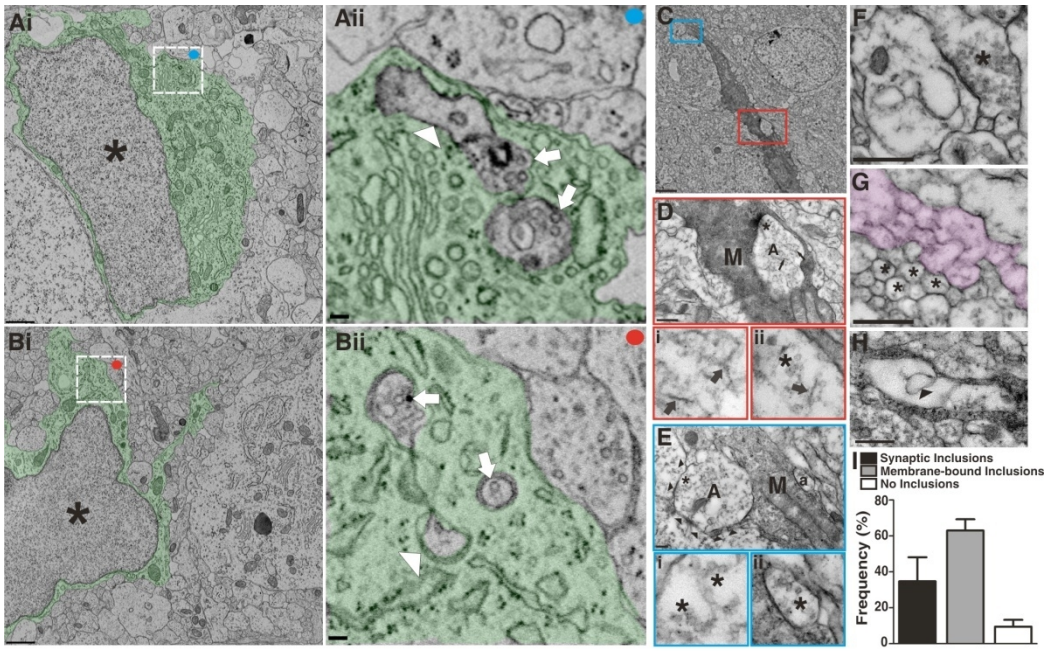

Figure 3.7. Microglia engulf presynaptic elements undergoing active synaptic pruning. Ai, Bi. Low magnification EM of microglia. Asterisks denote the nucleus and the cytoplasm is pseudocolored green. Scale bar = 1 µm. Aii, Bii. Magnified regions of Ai and Bi (white boxes) demonstrating membrane-bound elements engulfed by microglia. Arrows designate elements containing presynaptic machinery (40 nm vesicles). The arrowhead in Aii designates engulfed material resembling juxtaposed postsynaptic elements. Scale bar = 100 nm. C, Low magnification EM of a microglia immunolabeled for Iba-1 in P5 dLGN (DAB-positive cell). Red and blue boxes indicate enlarged regions in D and E, respectively. Scale bar = 2 µm. D, RGC input (**A**) localized within the Iba-1-positive microglia (**M**). Within the engulfed input, neurofilaments (arrows, enlarged in Di and Dii) and 40 nm vesicles (asterisks, enlarged in Dii) are indicative of presynaptic machinery. D, Scale bar = 500 nm. E, RGC input (**A**) enwrapped by a microglial process (**M,** arrowheads denote microglial process). 40 nm Vesicles are also visible (asterisks, enlarged region in Ei). Another presynaptic element (**a**) containing 40 nm vesicles is surrounded by microglia cytoplasm (enlarged region in Eii). Scale bars = 100 nm. F, An intact excitatory synapse in P5 dLGN in which the presynaptic terminal (asterisk) contains 40 nm vesicles. Scale bar = 500 nm. G, Cross (asterisks) or longitudinal sections (pseudocolor) through axons are relatively void of vesicles. Scale bars = 500 nm. H, A membrane-bound structure (arrowhead) completely within a microglial (**M**) lysosome (**L**). Scale bar = 500 nm. I, The frequency at which engulfed material was observed in microglia from P5 dLGN, n=20 cells. Error bars represent s.e.m.

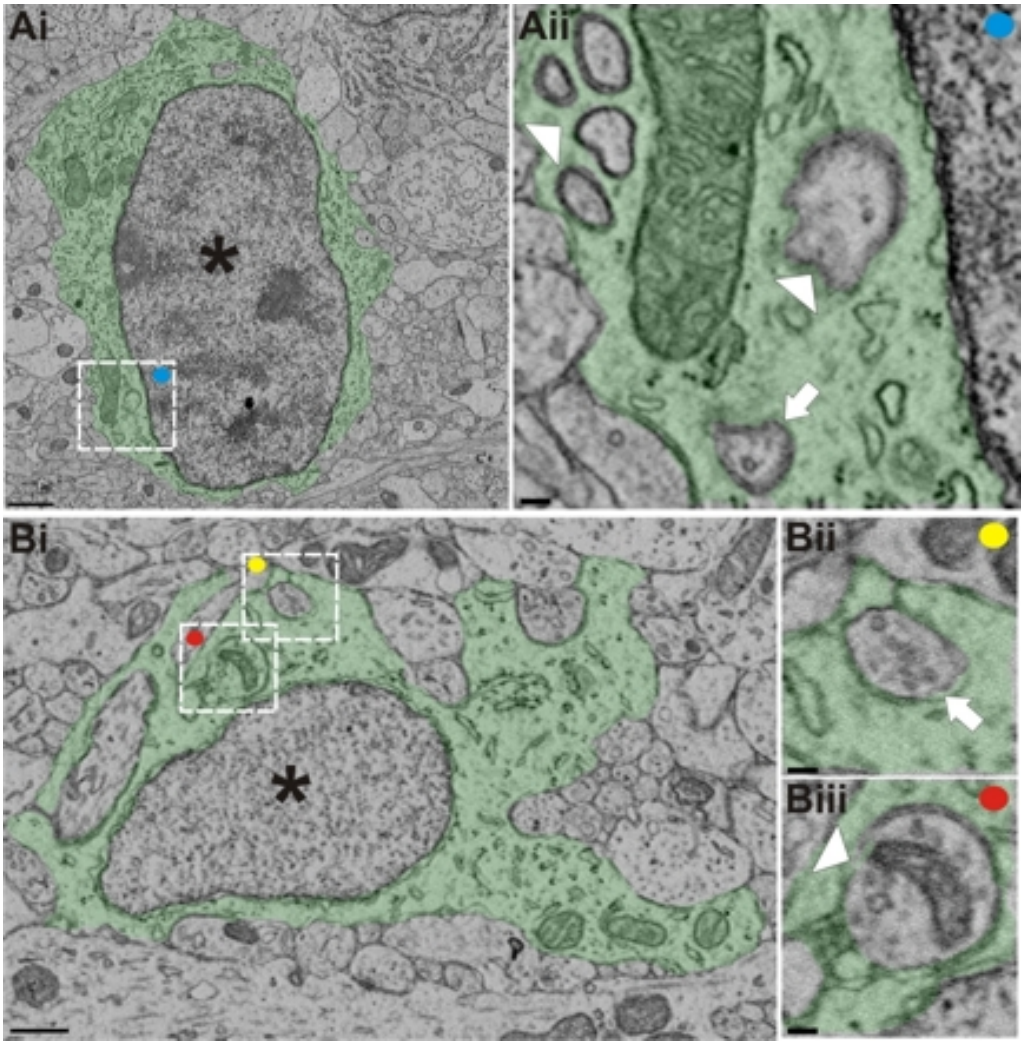

Figure 3.8. Microglia engulf presynaptic elements within the P5 dLGN. Ai, Bi, Representative EM images of microglia. Asterisks denote the nucleus and the cytoplasm is pseudocolored green. Aii, Magnified region of Ai (white dotted line) demonstrating membrane-bound elements engulfed by microglia. The arrow designates elements containing presynaptic machinery (40 nm vesicle) and the arrowhead designates engulfed material that is not distinguishable by ultrastructure. Bii-iii, Magnified region of Bi (white dotted line) demonstrating membrane-bound elements enwrapped (Bii) or engulfed (Biii) by microglia. The arrow designates elements containing presynaptic machinery (40 nm vesicles) and the arrowhead designates engulfed material that is not distinguishable by ultrastructure. Ai, Scale bar = 1 µm. Bi, Scale bar = 500 nm. Aii, Bii-iii Scale bar = 100 nm.

compartments (Figure 3.7H). We suspect that this membranous cellular material is synaptic material rapidly degraded in lysosomal compartments thereby rendering it undistinguishable by ultrastructure. Unlike presynaptic elements, engulfed material resembling postsynaptic elements was very rarely observed (Figure 3.7Aii). However, rapid degradation of structural elements may preclude visualization of the postsynaptic density. Importantly, there were rare instances in which no engulfed material was observed within microglia (Figure 3.7I, no inclusions, 10% of sampled cells).

To directly address whether microglia are engulfing RGC presynaptic terminals, immunohistochemistry in P5 dLGN for presynaptic machinery specific to RGCs (i.e., VGlut2) followed by high resolution imaging was performed. 3D structural illumination microscopy (3D-SIM), a technique enabling 2X the resolution of light microscopy (Gustafsson, 2000), was used to assess the P5 dLGN of CX3CR1+/EGFP mice immunolabeled for VGlut2. 3D-SIM data revealed VGlut2 immunoreactivity within the EGFP-positive cytoplasm of microglial cells (Figure 3.9A-D). Consistent with previous confocal and ultrastructural data (Figures 3.1-3.8), these data suggest that microglia are engulfing RGC presynaptic terminals.

To further confirm that microglia were engulfing RGC presynaptic terminals, double immunoEM in P5 dLGN for iba-1 (DAB) and a presynaptic marker specific to RGC terminals, VGlut2 (immunogold; Figure 3.9E-G) was performed. Consistent with 3D-SIM data previously described, we observed immunogold labeling for VGlut2 within the microglia cytoplasm and lysosomes (Figure 3.9F,G). Because immunogold was overexposed in order to gain contrast against the DAB reactivity, vesicle membranes surrounding the VGlut2 labeling were not observed within intact presynaptic terminals (Figure 3.9E) or microglia

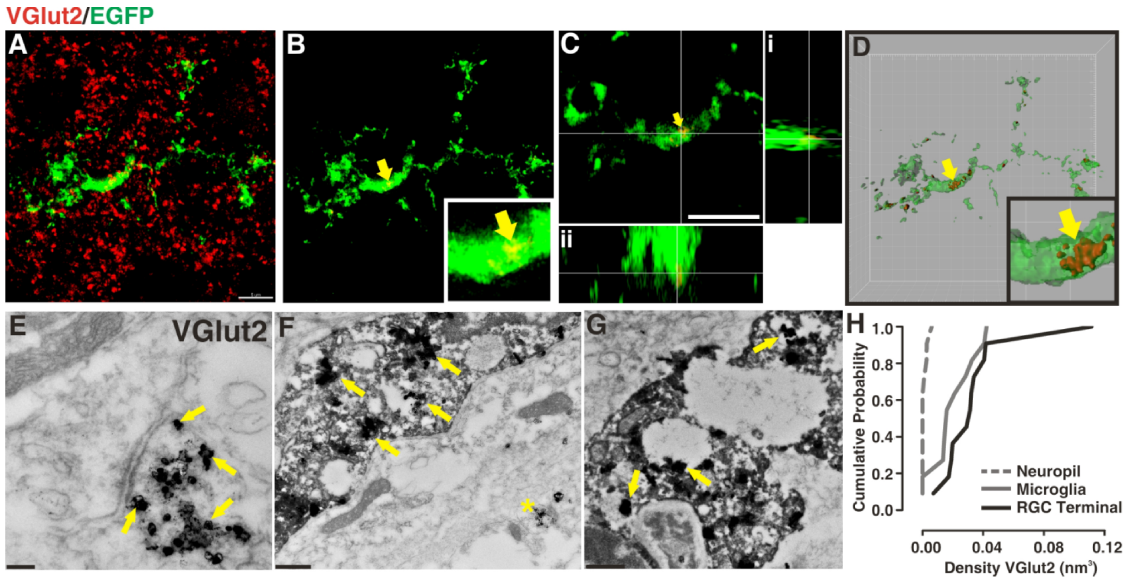

Figure 3.9. Microglia engulf presynaptic terminals specific to RGCs. A-D, 3D-SIM in P5 CX3CR1+/EGFP dLGN in which microglia are labeled with EGFP (green) and RGC presynaptic terminals are immunolabeled with anti-VGlut2 (red). A, Maximum intensity projection (MIP) of microglia and VGlut2 immunostaining in P5 dLGN. B, MIP in which all VGlut2 fluorescence (red) that is not within the microglia (green) has been subtracted. Yellow arrow designates examples of engulfed VGlut2-positive elements, enlarged in inset. C,D Orthogonal views (C) and surface rendering (D) of region in B (yellow arrow and inset). A-D, Scale bar = 5 µm. D, grid line increments = 2 µm. E-G, Double immunoEM in P5 dLGN for iba-1 (DAB) and VGlut2 (immunogold). E, RGC presynaptic terminals are enriched with VGlut2 immunoreactivity (immunogold, yellow arrows). F,G, Similar to RGC terminals (E) , microglial cytoplasm (DAB) and lysosomes contain VGlut2 immunogold labeling (yellow arrows). Asterisk in F denotes a VGlut2-positive presynaptic terminal within the same field of view as the microglia. Scale bars = 100nm. H, Cumulative probability demonstrates that there is increased probability of VGlut2 localization to a RGC terminal (black solid line) or microglia (grey solid line) versus random occurrence throughout the neuropil (grey dotted line). For each structure, n=10.

(Figure 3.9F,G). In addition, cumulative probability calculations demonstrated an increased probability of VGlut2 localized to an RGC terminal or microglia as compared to random immunoreactivity throughout the neuropil (Figure 3.9H). Similar to results from confocal microscopy experiments (Figures 3.1-3.5), these ultrastructural data reveal that microglia engulf presynaptic terminals specific to RGCs.

Deletion of the CR3/C3-dependent phagocytic signaling decreases the capacity of microglia to engulf RGC inputs

What molecular mechanism(s) underlies microglia-mediated engulfment of synaptic inputs? In the peripheral immune system, phagocytic cells can interact with several different immune-related signaling pathways to mediate clearance of cellular material. Included among these pathways are proteins belonging to the classical complement cascade, which bind surface receptors expressed by phagocytic cells. Given previous work demonstrating that complement component C3 is enriched at synapses and is necessary for pruning of retinogeniculate synapses (Stevens et al., 2007), we hypothesized that C3 ligand-receptor signaling may be one molecular mechanism by which microglia interact with and engulf RGC synaptic inputs. Consistent with this hypothesis, CR3, a high affinity receptor for activated C3 (Akiyama and McGeer, 1990; Perry et al., 1985), was specifically upregulated in microglia in the P5 dLGN and downregulated at later developmental time points (Figure 3.10A). Importantly, other cell types known to express the surface receptor CR3 and/or have phagocytic capacity (i.e., infiltrating monocytes, macrophages, etc.) were completely absent from the P5 dLGN and surrounding brain tissue (Figure 3.11)

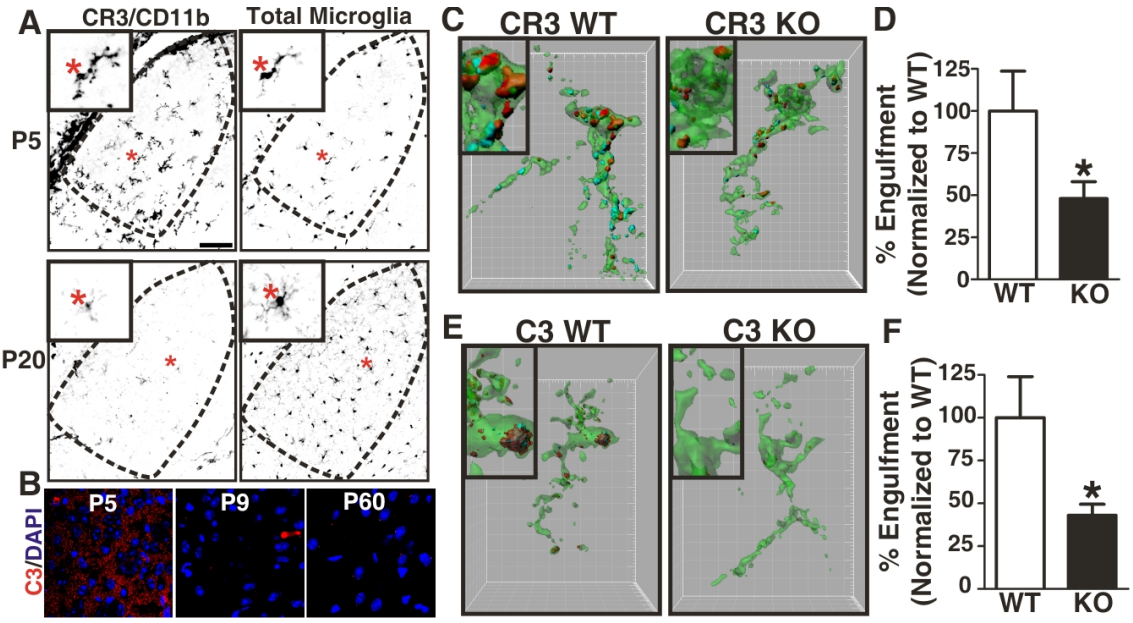

Figure 3.10. CR3/C3-dependent signaling regulates engulfment of synaptic inputs by microglia. A, Immunohistochemistry for the alpha subunit of CR3 (CD11b) reveals that microglia express high levels of CR3/CD11b (left column) in the P5 dLGN (top panels) versus older ages (P20, bottom panels). Total microglia are visualized with GFP (CX3CR1+/EGFP, right column). Insets are magnified regions (red asterisks). Scale bar = 100 µm. B, Immunohistochemistry in the developing dLGN for C3 (red). A single plane confocal image reveals that C3 levels are increased in the P5 dLGN versus older ages (P9, P60). Scale bar = 10 µm. C,E, Representative surface rendered microglia (green) from P5 dLGN of WT (left) or KO (right) littermates in which RGC inputs were labeled with CTB-594 (red, contralateral) and CTB-647 (blue, ipsilateral). Insets are enlarged regions demonstrating reduced RGC input engulfment (red and blue) in CR3 (C) and C3 (E) KO mice. Grid line increments = 5 µm. D,F, P5 CR3 KO (D) and C3 KO (F) mice (black bars) engulf significantly fewer RGC inputs as compared to WT littermates (white bars). All data are normalized to WT control values. D, **P*<0.04 by Student's *t*-test, n=3 mice/genotype. E, **P*<0.01 Student's *t*-test, n=4 mice/genotype. All error bars represent s.e.m.

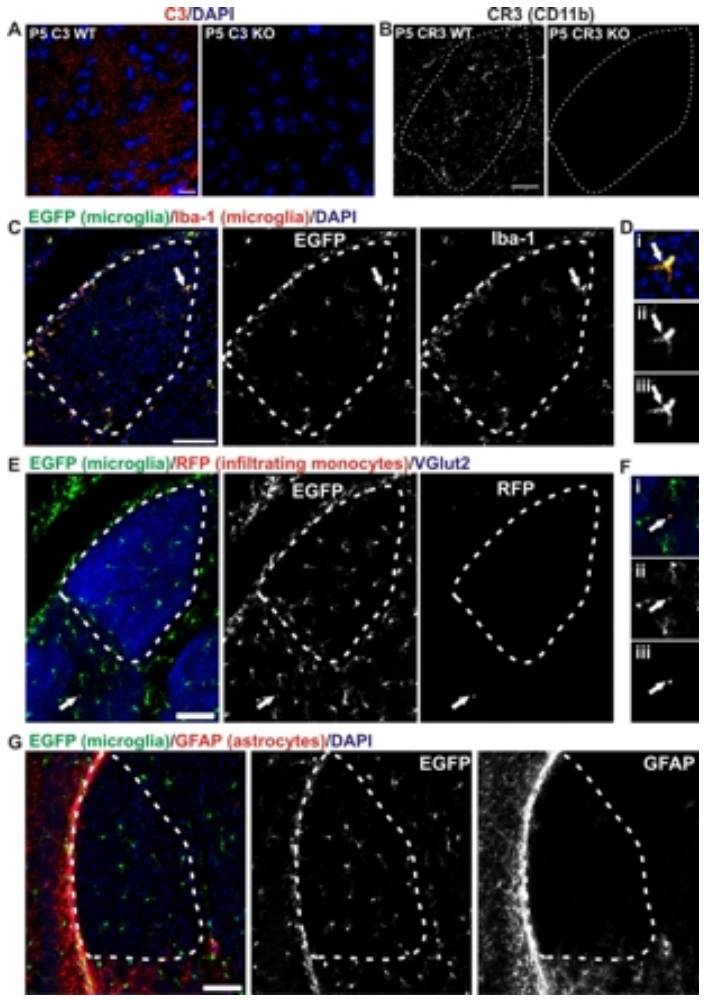

Figure 3.11. CR3 expression is high and restricted to microglia in the P5 dLGN. A, Single plane confocal images of dLGN immunostained for C3 (red) from P5 C3 WT (left) and KO (right) littermates. Signal for C3 is completely absent in C3 KO dLGN. Scale bar = 10 µm. B, Immunohistochemistry for CR3/CD11b in P5 CR3 WT (top panel) and KO (bottom panel) dLGN (outlined with white dotted line). The protein expression of the alpha subunit of CR3 is completely absent in the dLGN of CR3 KO mice. Scale bars = 100 µm. C, dLGN from P5 CX3CR1+/EGFP mice demonstrating complete colocalization of EGFP (green; middle) with the microglia marker, Iba-1 (red; right). Di-ii, Single cell magnified from C (arrows). E, P5 CX3CR1+/EGFP CCR2+/RFP mice in which EGFP is expressed under the control of the fractalkine receptor, CX3CR1 to label microglia and RFP is expressed under the control of the chemokine receptor, CCR2 to label infiltrating monocytes/macrophages. All mice were heterozygous for CX3CR1 and CCR2. There is a complete absence of infiltrating monocytes/macrophages (red) within the P5 dLGN. The arrows denote a GFP/RFP-positive cell near the vLGN enlarged in F. Scale bar = 100 µm. F, Enlarged region from E denoted with arrows as either merged channels (i), EGFP alone (ii), or RFP alone (iii). G. dLGN from a P5 CX3CR1-EGFP mouse in which resident microglia are labeled with EGFP (green), astrocytes are labeled with an anti-GFAP antibody (red), and total nuclei are labeled with DAPI (blue). At P5, astrocytes were frequent in vLGN but virtually absent from the dLGN.

(Akiyama and McGeer, 1990; Perry et al., 1985). As a result, in the context of the P5 brain, genetic manipulation of CR3 is specific to microglial cells. Similar to CR3 and consistent with our previous work, immunohistochemistry for CR3 ligand, C3 was enriched in synaptic regions of P5 dLGN and downregulated by P9, an age when pruning is largely complete (Figure 3.10B) (Stevens et al., 2007). These data demonstrate that CR3 and its ligand, C3, are expressed at an appropriate age and location to mediate RGC input engulfment.

Using the *in vivo* phagocytosis assay previously described (Figure 3.4), engulfment was assessed in P5 mice lacking functional CR3 (CR3 KO) due to a genetic deletion of the alpha subunit, CD11b (Figure 3.11B) (Coxon et al., 1996) or mice deficient in CR3 ligand, C3 (C3 KO) (Figure 3.11A). Microglia sampled from P5 CR3 or C3 KO mice had a statistically significant decrease in capacity to engulf RGC inputs as compared to WT littermate controls (Figure 3.10C-F). Taken together, these data demonstrate that phagocytic signaling through CR3 and its ligand C3 is one molecular mechanism by which microglia engulf RGC inputs.

Disruption of CR3 signaling in microglia results in sustained deficits in structural remodeling of RGC inputs

During the first postnatal week, overlapping inputs from both eyes segregate into eye specific territories (i.e., eye-specific segregation) resulting in the termination of ipsilateral and contralateral inputs in distinct non-overlapping domains in the mature dLGN (see Figure 3.4A) (Godement et al., 1984; Guido, 2008; Huberman et al., 2008; Jaubert-Miazza et al., 2005; Sretavan and Shatz, 1986; Ziburkus and Guido, 2006). Consistent with our hypothesis that microglia play a role in synaptic pruning, C3 KO mice have previously been shown to have deficits in eye-specific segregation (Stevens et al.,

2007). To determine whether microglia are mediators of C3-dependent synaptic refinement in the CNS, we quantified eye-specific segregation in CR3 KO mice. Ipsilateral and contralateral RGC inputs were labeled by intraocular injection of CTB-594 (red) and CTB-488 (green), respectively. Animals were subsequently sacrificed within 24 hrs of the initial dye injection and overlap (yellow) between contralateral and ipsilateral RGC projection territories was quantified. In this experimental paradigm, an increase in the % overlap between the ipsilateral and contralateral projections within the dLGN is indicative of a deficit in synaptic pruning (Bjartmar et al., 2006; Huh et al., 2000; Pham et al., 2001; Ravary et al., 2003; Stevens et al., 2007).

Consistent with the hypothesis that microglia mediate complement-dependent synaptic pruning, a statistically significant increase in ipsilateral and contralateral input overlap was observed in P10 and P30 CR3 KOs as compared to WT littermate controls (Figure 3.12A-C). This increase in overlap was attributed to a significantly broader ipsilateral projection territory (Figure 3.12D) and a small, but not significant, increase in the contralateral projection territory (Figure 3.12E). Furthermore, at higher magnification we detected aberrant ipsilateral and contralateral RGC inputs within the inappropriate monocular region (contralateral and ipsilateral, respectively) in mature CR3 KO dLGN (P30; Figure 3.12F,G). In addition to genetic manipulation of CR3, microglia involvement in eye-specific segregation was further validated by manipulating microglia function pharmacologically using minocycline, an established inhibitor of microglial 'activation' (Buller et al., 2009) (Figure 3.13A-E). Similar to CR3 KO data, minocycline (P4-P8; 75 mg/kg) treatment during the peak of the pruning period resulted in reduced

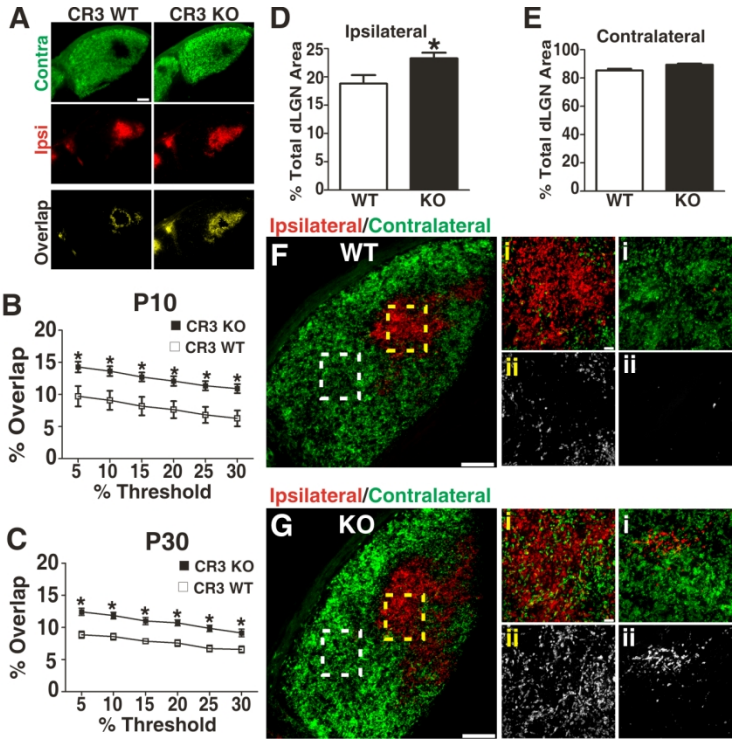

Figure 3.12. CR3 KO mice have sustained deficits in eye-specific segregation. A, Representative image of a P30 WT (left) demonstrates minimal overlap (yellow) between ipsilateral (red) and contralateral (green) RGC inputs. Indicative of a synaptic pruning deficit, CR3 KO mice (right) had increased overlap (yellow) of ipsilateral (red) and contralateral (green) RGC inputs. Scale bar = 100 µm. B-C, P10 (B) and P30 (C) CR3 KO mice had statistically significant, threshold-independent deficits in retinogeniculate pruning . D, The percentage of ipsilateral territory is significantly increased in P30 CR3 KO mice as compared to WT littermate controls. E, Although trending toward an increase, there is no statistically significant difference in percentage of contralateral territory. F,G dLGN from P30 CR3 WT (F) or KO (G) mice, dotted line boxes in lower magnification image (left panels) correspond to ipsilateral region magnified in middle panels (yellow i-ii) or contralateral region magnified in far right panels (white i-ii). Bottom panels in F,G (ii) are contralateral (CTB-488, green, left panel) channel or ipsilateral (CTB-594, red, right panel) alone. G, There were increased aberrant contralateral projections (middle panel; i, green and ii) within the ipsilateral territory in P30 CR3 KO mice as compared to WT littermates (F, middle panel). Similarly, there were aberrant ipsilateral projections (right panel; i, red and ii) within contralateral regions of the dLGN in CR3 KO mice as compared to WT littermates (F, right panel). Left panels, scale bar = 100 µm. Middle and right panels, scale bar = 10 µm. B-C, **P*<0.0001 by Student's *t*-test, n=6 (P10) or 4 (P30) mice/genotype. D, **P*<0.03 by Student's *t*-test, n=4 mice/genotype. All error bars represent s.e.m.

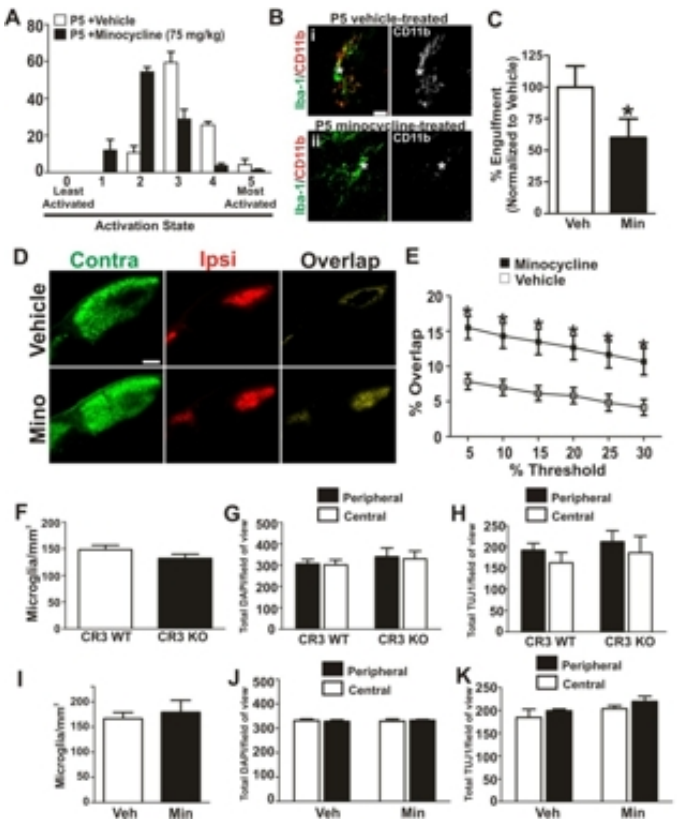

Figure 3.13. Genetic or pharmacological disruption of microglial function results in abnormal synaptic remodeling in the absence of RGC death or altered microglial density. A, Quantification of microglia activation state in the developing dLGN following daily systemic treatment with minocycline during the peak of the pruning period. Microglia in P5 dLGN of vehicle-treated mice (white bars) were consistently shifted toward increased activation state as compared to minocycline-treated mice (black bars). B, Confocal images of microglia in P5 dLGN immunolabeled for Iba-1 (green) and phagocytic receptor CR3 alpha subunit CD11b (red). CD11b immunoreactivity is decreased following minocycline treatment (ii) compared to vehicle-treated littermates (i). Asterisks indicate position of cell soma. Scale bar = 10 μm. C, Microglia from P5 minocycline-treated mice (Min) engulf significantly fewer RGC inputs as compared to vehicle-treated littermates (Veh). Data are normalized to control values. *P<0.04 by paired Student's t-test. D, Representative images of a P10 vehicle or minocycline-treated mouse. Indicative of a synaptic pruning deficit, there was increased overlap (yellow) of ipsilateral (red) and contralateral (green) territories. Scale bar = 100 μm. E, Deficits in eye-specific segregation in minocycline-treated mice were statistically significant and threshold independent. *P<0.0001 by Student's t-test. F, I, There is no significant difference between density (mm^2) of microglia in the dLGN of P5 CR3 KO (F) or minocycline-treated (I) mice. G,H,J,K, RGC cell number (G,J TUJ1-positive) and total DAPI-positive cells (H,K) are not significantly different between P10 CR3 KO versus WT mice (G,H) or minocycline versus vehicle-treated mice (J,K). For all experiments, n=3 mice/genotype or treatment. All error bars represent s.e.m.

microglial phagocytic function (i.e., reduced RGC input engulfment) at P5 and a statistically significant deficit in eye-specific segregation at P10 (Figure 3.13C-E). Importantly, prior to any analyses we confirmed that any phenotype in KO or drug-treated mice was not due to differences in total RGC number within the retina and/or density of microglia within the dLGN (Figure 3.13F-K).

Taken together, disruption in microglia function by pharmacological (minocycline) or more specific genetic strategies (CR3 or C3 KOs) results in sustained deficits in eye-specific segregation within the dLGN. Furthermore, given that microglia are the only CNS cell that express CR3 in the postnatal dLGN, these data suggest that microglia are mediators of synaptic remodeling in the retinogeniculate system and represent a key cellular mechanism underlying complement-dependent synaptic pruning (Stevens et al., 2007).

Disruption in CR3/C3-dependent signaling in microglia results in sustained deficits in synaptic connectivity

If CR3/C3-dependent signaling in microglia is a mechanism underlying developmental synaptic pruning, then a sustained increase in synapse density would be expected in the absence of these molecules. To test this possibility, retinogeniculate synapse density was quantified in adult CR3 KOs (P32-35) using array tomography (AT), a powerful tool for high resolution imaging and quantification of synaptic density *in vivo* (Greer et al., 2010; Margolis et al., 2010; Micheva and Smith, 2007; Ross et al., 2010). RGC presynaptic terminals within the dLGN were labeled with an antibody directed against VGlut2 and postsynaptic excitatory sites were labeled with anti-GluR1. As suggested by the eye-specific segregation assay, there was a statistically significant increase (1.3-fold increase) in RGC synapse density (i.e., juxtaposed GluR1 and VGlut2 puncta) in adult CR3

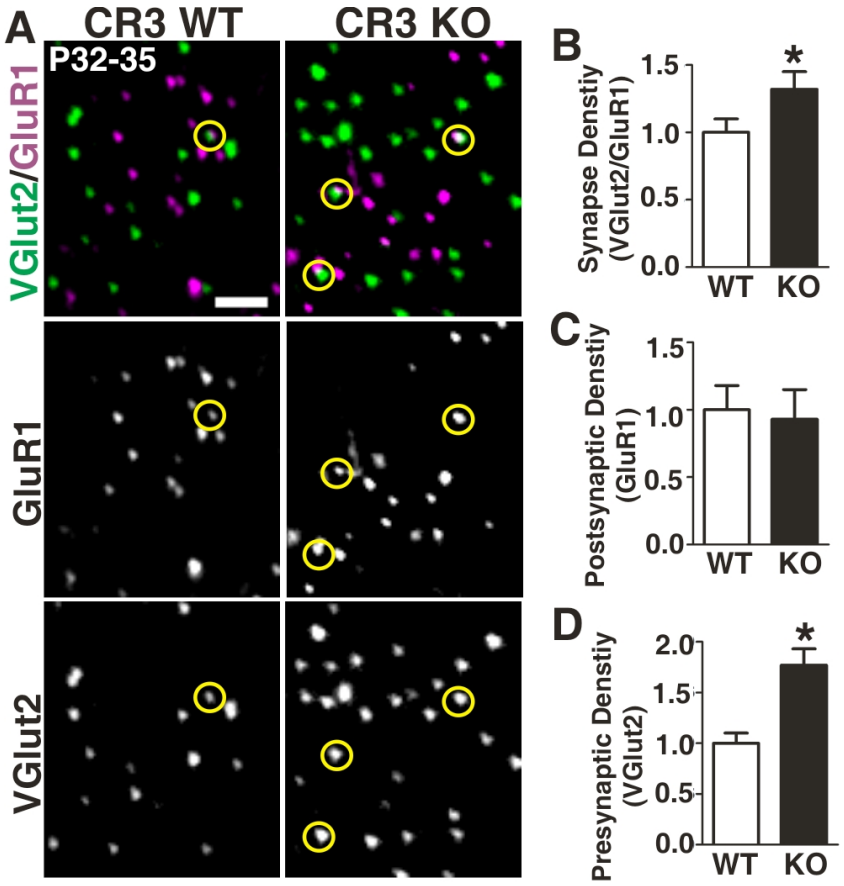

Figure 3.14. CR3 KO mice have a sustained increase in synapse density. A, Single plane array tomography images for VGlut2 (green) to label RGC terminals and GluR1 (purple) to label postsynaptic excitatory sites in P32-35 dLGN of CR3 KO (right) and WT littermate controls (left). Yellow circles indicate synapses defined by VGlut2 and GluR1 immunoreactivity. Scale bar = 2 µm. B-D, Quantification of retinogeniculate synapse (B, VGlut2/GluR1-positive), postsynaptic (C, GluR1), and presynaptic/RGC terminal (D, VGlut2) density indicates that there is a statistically significant increase in retinogeniculate synapse density and total RGC terminal density in CR3 KOs as compared to WT littermates. *P<0.03 Man-Whitney U test, n=3 mice/genotype. Error bars represent s.e.m.

KOs as compared to WT littermates (Figure 3.14A,B). Consistent with our previously published work (Stevens et al., 2007), adult C3 KO mice had an identical 1.3-fold increase in VGlut2-containing synapses as compared to WT littermate controls (Figure 3.15). Interestingly, there was also a significant increase in the density of total (both synapse associated and non-associated) VGlut2-positive puncta in CR3 KOs (1.8-fold increase) as compared to WT littermates (Figure 3.14D). We hypothesize that these excess VGlut2-positive puncta represent residual immature synapses as well as retracted or unopposed immature presynaptic terminals that were not eliminated by phagocytic microglia. Taken together, these data implicate CR3/C3 signaling as a mechanism regulating synaptic connectivity.

Because microglia are the only cell type within the P5 dLGN and surrounding brain tissue to express CR3 (Figure 3.10; Figure 3.11) (Akiyama and McGeer, 1990), our data directly implicate microglia as mediators of anatomical pruning and identify CR3/C3-dependent signaling as an underlying molecular mechanism.

Discussion

In this study, we demonstrate that microglia are mediators of synaptic pruning in the normal, developing brain and identify neural activity and CR3/C3-dependent signaling as underlying mechanisms. Specifically, we demonstrate that: 1) Microglia in the postnatal dLGN engulf RGC presynaptic terminals during active synaptic remodeling. 2) Engulfment of RGC inputs is regulated by neuronal activity. 3) Engulfment of RGC inputs is regulated by CR3/C3-dependent phagocytic signaling specific to microglia. 4) Genetic (CR3 and C3 KO) or pharmacological perturbations that disrupt microglia function result in deficits in structural remodeling of synapses. 5) Defects in synaptic circuitry are sustained into

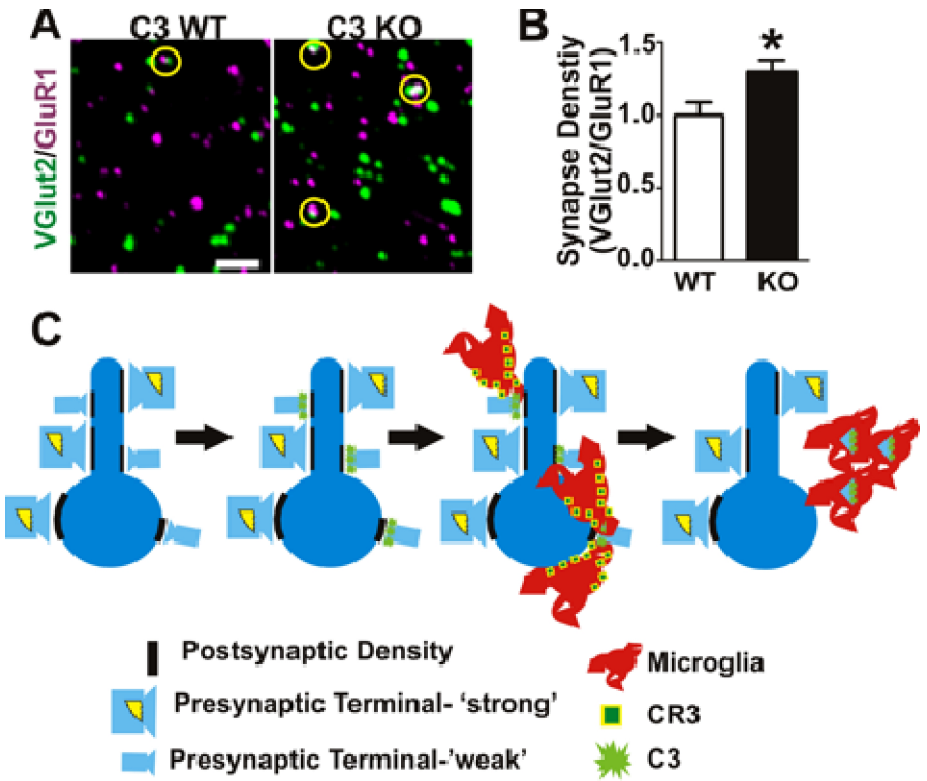

Figure 3.15. Array tomography in adult C3 KO dLGN and model of microglia-mediated synaptic pruning. A, Single plane array tomography images for VGlut2 (green) to label RGC terminals and GluR1 (purple) to label postsynaptic excitatory sites in P32-35 C3 KO and WT littermate controls. Yellow circles indicate bona fide synapses defined by VGlut2 and GluR1 immunoreactivity. Scale bar = 2 μm. B, Quantification of synapse density indicates that there is a statistically significant increase in RGC synapses (VGlut2/GluR1-positive) as compared to WT littermates. All data are normalized to WT control values. *P<0.03 by Mann-Whitney U test, n=3 mice/genotype. Error bars represent s.e.m. C, Model of microglia-mediated synaptic pruning illustrates that early in development the postsynaptic cell (dark blue) is innervated by exuberant presynaptic terminals. We propose that the activated form of C3 (iC3b) "tags" weak or inappropriate synapses for selective removal by phagocytic microglia expressing the C3 surface receptor, CR3. As a result, only appropriate synapses remain and become strengthened.

adulthood in CR3 and C3 KO mice. We propose a model in which neural activity and complement work cooperatively to mediate engulfment of RGC inputs, a process that may underlie synaptic pruning in the developing CNS (Figure 3.15).

Microglia engulf RGC presynaptic inputs during peak synaptic pruning

One question arising is whether engulfment of RGC inputs by microglia is an active process. Particularly during CNS disease, microglia are known scavengers that phagocytose cellular debris (Hanisch and Kettenmann, 2007; Napoli and Neumann, 2009; Ransohoff and Perry, 2009). Furthermore, glia are known to engulf axonal material during large-scale developmental pruning of axons in Drosophila and synaptic pruning at the mammalian neuromuscular junction (Bishop et al., 2004; Freeman, 2006; Rochefort et al., 2002). While our results do not rule out the possibility that axonal material may also be engulfed, our data suggest that microglia play an active role in the removal of transient, intact presynaptic elements. Indeed, in comparison to large scale developmental axonal pruning, there is no evidence that local CNS synaptic pruning, such as in the case of the retinogeniculate system, involves classic axonal or synaptic degeneration (Dhande et al., 2011; Hahm et al., 1999; Snider et al., 1999; Sretavan and Shatz, 1984). Earlier EM work in the developing mammalian dLGN demonstrated that RGCs transiently synapse within the inappropriate region of the dLGN (Campbell and Shatz, 1992; Campbell et al., 1984). These transient synapses contained presynaptic machinery including a high density of vesicles, but were subsequently eliminated by an undetermined mechanism. Given our high resolution light microscopy and ultrastructure data, we suggest that microglia are actively pruning these transient synaptic connections via a phagocytic mechanism (Figure 3.15).

We provide several lines of evidence implicating microglia in the local pruning of transient, intact retinogeniculate synapses in the absence of axon debris or degeneration. First, in experiments involving anterograde tracing of RGCs (engulfment and eye-segregation assays), intraocular injections of dye occur less than 24 hrs prior to tissue harvesting and fixation. If neurons or axons were degenerating, we would not expect effective dye uptake and tracing of the entire RGC projection. Furthermore, previous work has demonstrated that RGC normal programmed cell death is essentially complete by P4/P5 (Farah and Easter, 2005). Taken together, any CTB labeling observed within the dLGN is, more likely, originating from a healthy RGC cell body and axon. Second, previous work using dye tracing or fluorescent protein to label small subsets of RGC afferents in the dLGN demonstrate that RGC axons and arbors within the dLGN undergoing active pruning remain intact and unfragmented (Dhande et al., 2011; Hahm et al., 1999; Snider et al., 1999; Sretavan and Shatz, 1984). Consistent with these data, our EM experiments demonstrated that engulfed material as well as surrounding dLGN neuropil did not appear to have classic signs of axonal or synaptic degeneration such as multilamellar bodies, electron-dense cytoplasm, lack of synaptic vesicles within presynaptic terminals, etc. (Hoopfer et al., 2006; Perry and O'Connor, 2010). Lastly, we observed sustained increases in the number of intact, structural synapses by eye specific segregation and array tomography analyses in mice with disrupted microglia function (C3 KO, CR3 KO, and minocycline-treated mice). If synapses degenerated prior to engulfment, we would not expect to observe increased numbers of healthy, intact synapses in KO mice. Taken together, our data suggest that engulfed presynaptic elements were healthy, intact, and specifically engulfed by microglia.

Engulfment of RGC inputs by microglia is an activity-dependent process

Previous work has demonstrated that microglia have the capacity to interact with synaptic elements in response to neurotransmitter release and/or sensory experience (Biber et al., 2007; Fontainhas et al., 2011; Nimmerjahn et al., 2005; Ransohoff and Perry, 2009; Tremblay et al., 2010a; Wake et al., 2009). Furthermore, microglia can contribute to synaptic plasticity in the adult CNS and, more recently, in the context of the normal developing hippocampus (Paolicelli et al., 2011; Pascual et al., 2011; Roumier et al., 2008). Our data provide insight into mechanisms by which microglia may interact with synapses and contribute to activity-dependent synaptic plasticity. When competition between inputs from the two eyes was enhanced by pharmacological manipulation (i.e., TTX or forskolin), we found that microglia preferentially engulfed inputs from the eye in which neuronal activity was decreased or 'weaker'. Although it is not yet known whether or how microglia target specific 'weaker' synapses, these data are consistent with previous work demonstrating that such a competition results in decreased territory of the 'weaker' inputs and increased territory of 'stronger' inputs within the dLGN (Del Rio and Feller, 2006; Huberman et al., 2008; McLaughlin et al., 2003; Penn et al., 1998; Shatz, 1990; Shatz and Stryker, 1988; Stellwagen and Shatz, 2002; Stellwagen et al., 1999; Torborg and Feller, 2005).

In the retina, spontaneous, correlated neuronal activity from both eyes (i.e., retinal waves) drives the elimination of synapses and segregation of inputs into eye-specific territories in the dLGN (Del Rio and Feller, 2006; Feller, 1999; Huberman et al., 2008; McLaughlin et al., 2003; Penn et al., 1998; Stellwagen and Shatz, 2002; Torborg and Feller, 2005). Interestingly, complement and complement receptor-deficient mice have similar pruning deficits to mice in which this correlated firing has been disrupted (e.g., cAMP-

analog injection, *β2nAChR*$^{-/-}$ mice, etc.) (Stevens et al., 2007), suggesting the intriguing possibility that complement cascade activation and function is regulated by neural activity. Neural activity could also directly regulate microglia function (i.e., activation, recruitment, phagocytic capacity) through complement-independent mechanisms. Alternatively, neural activity may drive the elimination of synapses by other mechanisms which ultimately lead to complement activation and/or microglia-mediated engulfment. Future studies will aim to address how neural activity, complement, and microglia may interact to contribute to developmental synaptic pruning (Figure 3.15).

CR3/C3-dependent signaling: A molecular pathway underlying microglia-mediated synaptic pruning

Synaptic pruning likely involves several mechanisms that cooperatively interact to establish precise synaptic circuits. We suggest that microglia may be a common link and identify CR3/C3 signaling as one pathway underlying microglia-synapse interactions and microglia-dependent pruning in the developing CNS. One of the major questions raised by these findings is precisely how secreted complement proteins mediate the selective elimination of synapses by microglia. In the immune system, C3 is cleaved into an activated form, iC3b, which covalently binds to the surface of cells or debris and targets them for elimination by macrophages via specific phagocytic receptor signaling (e.g., CR3) (Lambris and Tsokos, 1986; van Lookeren Campagne et al., 2007). Similar to the immune system, we propose that activated C3 (iC3b/C3b) could selectively 'tag' weak synapses (Figure 3.15). Consistent with C3 "tagging" subsets of RGC terminals, previous confocal analysis revealed colocalization of C3 with pre and postsynaptic markers in the developing dLGN (Stevens et al., 2007). Furthermore, mice deficient in CR3, C3, and C1q, the initiating protein of the

classical complement cascade, exhibit strikingly similar defects in developmental synaptic pruning (Figure 3.12, 3.14 and Figure 3.15). Alternatively, other complement-dependent and/or independent mechanisms may be involved. For example, C3 could bind all synapses and only those synapses that are 'stronger' or more active are selectively protected by membrane-bound complement regulatory molecules (Kim and Song, 2006; Song, 2006). Another possibility is that ubiquitously expressed protective molecules, such as "don't eat me" signals (Griffiths et al., 2007), could be lost from weaker synapses, enabling C3 to promote their removal. In contrast, selective, activity-dependent elimination of synapses could be driven by a complement-independent mechanism which subsequently results in complement binding and/or microglia-mediated engulfment. For example, MHC class I molecules, another class of immune molecules demonstrated to play a role in retinogeniculate pruning, have been shown to be activity-dependent, localized to synapses, and co-localized with C1q leaving the possibility that MHC class I molecules may play an upstream role in microglia-mediated pruning of synapses (Corriveau et al., 1998; Datwani et al., 2009; Goddard et al., 2007; Huh et al., 2000).

While our data demonstrate that CR3/C3 signaling specific to microglia is involved in the pruning of developing circuits and suggest that engulfment is the underlying mechanism, CR3 and C3 may be acting through other pathways independent of phagocytosis or may be downstream of other signaling pathways to mediate pruning. In addition, engulfment deficits in CR3 and C3 KO mice were reduced to approximately 50% of WT littermate control values suggesting that other complement receptor-dependent (e.g., CR4, CRig, etc.) and independent phagocytic mechanisms may also be involved. Moreover, these data may indicate that activated microglia engulf inputs in the absence of

"eat me" signals, suggesting a baseline level of phagocytic activity in need of regulation. Future studies will aim to address whether and how specific synapses are eliminated by complement and other microglia-dependent mechanisms and how neural activity plays a role in this process.

Complement-dependent engulfment of synaptic inputs: A more global mechanism underlying remodeling of neural circuits in the healthy and diseased CNS?

Our data raise the question as to whether complement and/or microglia-dependent engulfment of synaptic inputs represents a more global mechanism underlying CNS neural circuit plasticity. While in at least one other developing system local axonal retraction and synapse elimination appear to occur independent of microglia (Cheng et al., 2010), recent work describes a role for microglia at developing hippocampal synapses (Paolicelli et al., 2011). In addition, *in vivo* imaging studies in the cortex revealed that microglia dynamics and interactions with neuronal compartments change in response to neural activity and experience (Davalos et al., 2005; Nimmerjahn et al., 2005; Tremblay et al., 2010a; Wake et al., 2009). While these studies describe microglia dynamics at synapses, a precise function and molecular mechanism(s) underlying microglia-synapse interactions in these brain regions was unknown. Our study provides mechanistic insight on the dynamic between microglia and developing synapses and provides complement-dependent signaling as a potential mechanism in other brain regions. Consistent with this idea, deficits in complement component C1q results in an increase in the number of presynaptic boutons and exuberant excitatory connectivity in the cortex (Chu et al., 2010). Future studies will aim to test the role of complement in microglia-synapse interactions in other CNS regions known to undergo activity-dependent synaptic remodeling.

In addition to relevance in global remodeling of circuits in the healthy brain, our findings have important implications for understanding mechanisms underlying synapse elimination in the diseased brain. Consistent with this idea, abnormal microglia function and complement cascade activation have been associated with neurodegeneration of the CNS (Alexander et al., 2008; Beggs and Salter, 2010; Rosen and Stevens, 2010; Schafer and Stevens, 2010). Indeed, in a mouse model of glaucoma, a neurodegenerative disease associated with RGC loss and gliosis, C1q and C3 are highly upregulated and deposited on retinal synapses and C1q deficiency or microglial 'inactivation' with minocycline provide significant neuroprotection (Howell et al., 2011; Steele et al., 2006; Stevens et al., 2007). In addition to diseases associated with neurodegeneration, recent data from genome-wide association studies and analyses of postmortem human brain tissue have suggested that microglia and/or the complement cascade may also be involved in the development and pathogenesis of neurodevelopmental and psychiatric disorders (e.g., autism, obsessive compulsive disorder, schizophrenia, etc.) (Chen et al., 2010; Havik et al., 2011; Monji et al., 2009; Pardo et al., 2005; Vargas et al., 2005). Thus, an intriguing possibility remains that microglia and/or complement dysfunction may be directly involved in diseases associated with synapse loss, dysfunction, and/or development.

Together, our data offer insight into mechanisms underlying activity-dependent synaptic pruning in the developing CNS, provide a role for microglia in the healthy brain, and provide important mechanistic insight into microglia-synapse interactions in the healthy and diseased CNS.

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Chapter 4:

"Don't Eat Me" Signals Prevent Excess Microglia-Mediated Retinogeniculate Pruning

Contributions: All experiments were designed by Emily Kate Lehrman and Beth Stevens. All experiments and data analysis were performed by Emily Kate Lehrman with the exception of the following: SIM imaging and analysis and CD47 western blot analysis were performed by Daniel Wilton, in situ hybridization was performed by Arnaud Frouin, and CD200 engulfment analysis, CD200 and CD47 microglia activation assays, and CD200 and CD47 microglia cell density counts were performed by Stephen Chang. CD47 synaptosome fractionation data provided by Hisashi Umemori. RGCs provided by Allison Bialas.

Introduction

Over the past decade, work from our lab and others has revealed that microglia are essential for normal brain development and function. We identified microglia as the cellular effectors mediating developmental retinogeniculate refinement and found that they remove synaptic inputs via complement-dependent phagocytosis (Schafer et al., 2012). However, despite this progress, we do not yet know how microglia target specific synapses for removal. Microglia must determine which inputs to engulf and which to avoid to create mature synaptic circuits, a crucial decision with potentially far-reaching functional and behavioral consequences. One possible mechanistic insight comes from a common feature shared by molecules previously shown to regulate mouse retinogeniculate refinement. These molecules, including class I major histocompatibility complex (MHCI) molecules, complement cascade components C1q and C3, and neuronal pentraxins 1 and 2 (NP1, NP2), are all either immune molecules or homologs of immune molecules (Bjartmar et al., 2006; Datwani et al., 2009; Huh et al., 2000; Stevens et al., 2007). While the initial discovery of immune molecules as regulators of retinogeniculate refinement came as a surprise, it fits nicely with the knowledge that microglia, the brain's immune cells, phagocytose synaptic inputs. Taken together, these data suggest that microglia may determine which synapses to engulf using mechanisms borrowed from the immune system.

Indeed, some of the immune molecules required for proper refinement are best known for regulating phagocytosis of pathogens or debris, such as classical complement cascade proteins C1q and C3. These molecules are canonical "eat me" signals, which instruct macrophages to engulf and clear apoptotic or non-self material by signaling to receptors on phagocytes (Elward and Gasque, 2003; Grimsley and Ravichandran, 2003).

We found that C3 functions similarly during brain development, directing microglia to phagocytose synaptic inputs during the retinogeniculate pruning period by engaging microglial C3 receptor CR3 (Chapter 3, Schafer et al., 2012). Mice deficient in C1q, C3, or the other aforementioned immune molecules exhibit a similar loss of function phenotype: failure to complete retinogeniculate refinement and, in most cases, maintenance of many weak, supernumerary presynaptic inputs. These phenotypes indicate an inability to remove unnecessary connections during development, and suggest that these molecules work to promote the pruning process, potentially by identifying synaptic inputs in need of removal. Our data highlighting the importance of the C3-CR3 interaction for microglia-mediated pruning indicate that the signaling pathways and functions of these molecules may be conserved between the central nervous system (CNS) and the immune system. Yet, this does not address the question of how secreted molecules like C1q and C3 are capable of directing microglia to remove specific synapses.

C1q and C3 can diffuse locally throughout the neuropil, and as we do not yet know of a receptor or adaptor that recruits them to specific sites, it is possible that these molecules may bind to a variety of different synapses. Perhaps there is a yet-to-be-determined receptor that is specifically expressed by weak or inappropriately targeted synapses, but, even so, given that microglial phagocytosis still occurs in the absence of these molecules and that excess pruning may be linked to disorders such as schizophrenia (Arnold, 1999; Keshavan et al., 1994; Woo and Crowell, 2005), it seems likely that a failsafe would be in place to prevent the inappropriate removal of necessary connections. Another key feature of microglia-mediated retinogeniculate pruning is that it appears to involve the exclusive removal of presynaptic inputs, while axons and other cellular compartments

remain intact (Chapter 3, Schafer et al., 2012). This also hints at the existence of a protective signal or inhibitory factor that would restrict microglial engulfment activity to synaptic terminals. One such safeguard exists in the immune system to prevent phagocytosis of "self" material by macrophages, which is especially important when these cells are working to remove pathogenic substances during inflammation or injury. The molecules that perform this protective function in the immune system are called "don't eat me" signals, and serve as identifiers of "self" that inhibit engulfment, either directly or indirectly, by engaging receptors on phagocytic cells (Gardai et al., 2006; Griffiths et al., 2007; Grimsley and Ravichandran, 2003). These molecules include complement inhibitors, such as factor H and Decay accelerating factor (DAF/CD55), sialic acids, and cluster of differentiation (CD) molecules CD31, CD47, and CD200. I hypothesize that "don't eat me" signals serve a similar protective function during periods of heightened microglial phagocytosis and act to prevent the removal of excess synaptic connections during synaptic refinement. Given that synaptic refinement must be tightly regulated to enable the removal of supernumerary inputs without the elimination of necessary connections, I predict that the CNS, like the immune system, would employ more than one of these protective signals to ensure that bystander damage in the form of inappropriate pruning does not occur.

"Don't eat me" signals, including DAF, sialic acids, CD47 and CD200 are indeed present in the developing CNS (Mi et al., 2000; Schnaar et al., 2014; Webb and Barclay, 1984; Zhang et al., 1998), although their function during this period has not been well explored. To address whether these signals function analogously in the brain and protect retinogeniculate synapses from excess microglia-mediated pruning, I focused on two "don't eat me" signals, CD47 and CD200. Although these molecules have not been well studied

during brain development, they are both expressed in the developing CNS by neurons in addition to other cell types and are both Ig superfamily proteins with microglial receptors, signal regulatory protein–alpha (SIRPα) and CD200R, respectively (Matozaki et al., 2009; Walker and Lue, 2013). In the immune system, CD47 has been found to directly inhibit macrophage phagocytosis via binding to SIRPα (Okazawa et al., 2005; Oldenborg et al., 2001). Conversely, CD200 may affect phagocytosis indirectly via its inhibition of macrophage activation and inflammatory signaling (Minas and Liversidge, 2006), and mice lacking CD200 have more activated microglia displaying increased Cd11b and CD45 (Hoek et al., 2000). Microglia activation is a broad term used to indicate a state in which microglia have a more reactive phenotype, often characterized by an amoeboid morphology, increased phagocytic capacity, upregulation of specific cell surface markers, and secretion of pro-inflammatory cytokines (Ransohoff and Perry, 2009). CD200 may also inhibit excess microglial proliferation or infiltration into the CNS, as adult CD200KO brains and retinas have been reported to have increased numbers of these cells (Hoek et al., 2000). Both molecules have also been implicated in CNS disease, where the absence or blockade of these signals may lead to a worsening of disease severity and progression for a variety of neurodegenerative diseases in humans and mouse models, including Multiple Sclerosis (MS), Alzheimer's Disease (AD), and Parkinson's Disease (PD) (Koning et al., 2007; Walker et al., 2009; Wright et al., 2000; Zhang et al., 2011). These data suggest that "don't eat me" signals serve a protective function in the CNS, and that loss of these signals during inflammatory diseases leads to the inappropriate removal of "self" material. This could involve a variety of targets, including myelin or synaptic connections, as increased myelin engulfment occurs with CD47 blockade in a model of traumatic axonal injury, and CD200

downregulation has been proposed as a cause of chronic inflammation and microglial activation in Alzheimer's disease (AD), a disease in which inappropriate synapse loss is known to occur (Gitik et al., 2011; Knobloch and Mansuy, 2008; Walker et al., 2009). These disease studies bolster my hypothesis, and support the idea that these molecules function similarly in the CNS to their immune system counterparts.

Here, I demonstrate for the first time that protective signals are required to prevent excess microglia-mediated pruning during development. I show that CD47 and its receptor, SIRPα, are expressed in the right time and in the correct cell types and locations to serve this protective function during retinogeniculate refinement. I find that mice deficient in either CD47 or CD200 exhibit decreased overlap between eye-specific territories in the dorsal lateral geniculate nucleus of the thalamus (dLGN), a phenotype that could indicate increased pruning as territories cease to overlap when redundant left and right eye inputs are removed to ensure that each dLGN relay neuron receives input from only one eye. This similar phenotype may arise in the two knockout mice via different mechanisms, as CD47KO microglia exhibit increased engulfment of synaptic inputs, which could lead to increased or accelerated pruning, while CD200KO microglia have no engulfment defect, indicating that the CD200KO overpruning phenotype may have a separate cause. My results also indicate that microglial and neuronal SIRPα may be differentially regulated to perform specific functions in different cell types and brain regions during development. Taken together, these data suggest that "don't eat me" signals serve an important regulatory function during microglia-mediated pruning and that their absence alters the course of normal retinogeniculate refinement.

Results

CD47 is enriched in the dLGN during peak pruning

To determine whether CD47 could regulate microglia-mediated pruning during development, I first examined its localization and expression within the dLGN. Immunohistochemistry (IHC) for CD47 reveals punctate staining throughout the neuropil in all brain regions examined, as expected for a ubiquitous protective signal (Figure 4.1A, Figure 4.2, Figure 4.3A). However, CD47 is highly localized to the dLGN during peak pruning (P5-P10), displaying a similar enrichment to that observed with vesicular glutamate transporter 2 (Vglut2), a marker of retinogeniculate synapses (Figure 4.1A-B). This enrichment is most noticeable at postnatal day (P5), which corresponds to the period of peak microglial engulfment of synaptic inputs, and can still be observed at P10, when the formation of eye-specific territories is nearing completion (Guido, 2008; Hong and Chen, 2011; Jaubert-Miazza et al., 2005). CD47 levels increase in other brain regions during development and decline slightly in the dLGN after P5 to reach a relatively uniform expression pattern throughout the brain by P30, at which point dLGN pruning is nearing completion (Figure 4.1A, Figure 4.2A). High-resolution imaging using structured illumination microscopy (SIM) reveals that CD47 colocalizes with both pre- and postsynaptic markers in the dLGN (Figure 4.1C). Moreover, western blot analysis of synaptosome fractions from P21 mouse cortex confirms this synaptic localization and also demonstrates that much of the CD47 protein in synaptosomes may be perisynaptic (Figure 4.1D), similar to synaptic regulatory proteins such as cadherins and fibroblast growth factors (FGFs). The unusual abundance of CD47 in the dLGN during the pruning period and its localization to synapses makes it well suited to regulate the pruning process. CD47 may

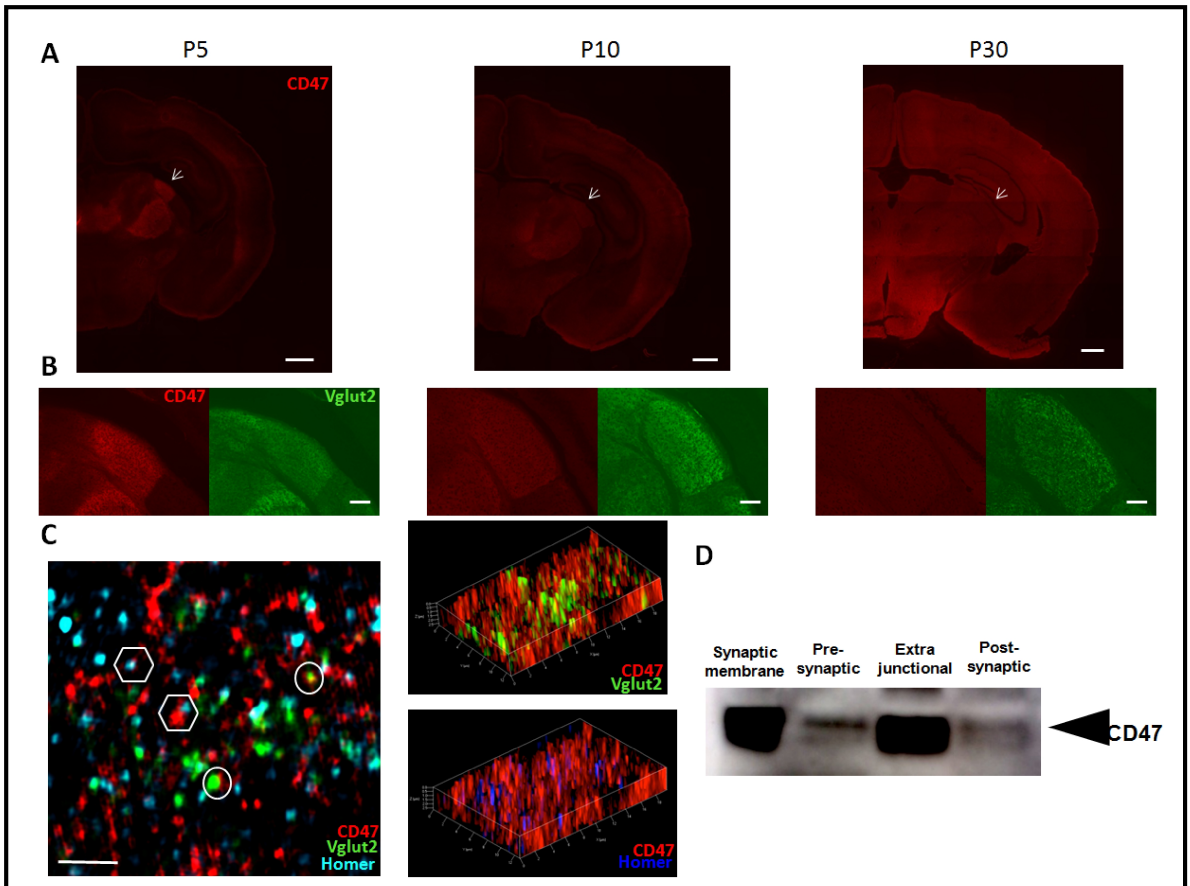

Figure 4.1. CD47 is developmentally regulated and synaptically localized in the dLGN during the pruning period. A, Representative low magnification mosaics of P5, P10, and P30 WT coronal sections. CD47 is enriched in the dLGN (arrow) during peak pruning at P5 and P10, but evenly distributed throughout the brain at P30. Scale bar = 500μm. B, CD47 (red) is highly enriched in the dLGN during pruning, similar to Vglut2 (green), a marker of retinogeniculate synapses. Scale bar = 50μm. C, SIM image of P5 dLGN (left), demonstrating that CD47 (red) colocalizes with both presynaptic (Vglut2, green, circles) and postsynaptic (Homer, blue, hexagons) puncta during peak pruning. Three-dimensional views of the entire z-stack (right) are provided for better visualization of colocalization of CD47 with synaptic markers. Scale bar = 2μm. D, Western blot analysis of synaptosome fractions from P21 mouse cortex indicates that CD47 is enriched in the synaptic membrane and extra junctional fractions, but also present in the pre- and post-synaptic fractions.

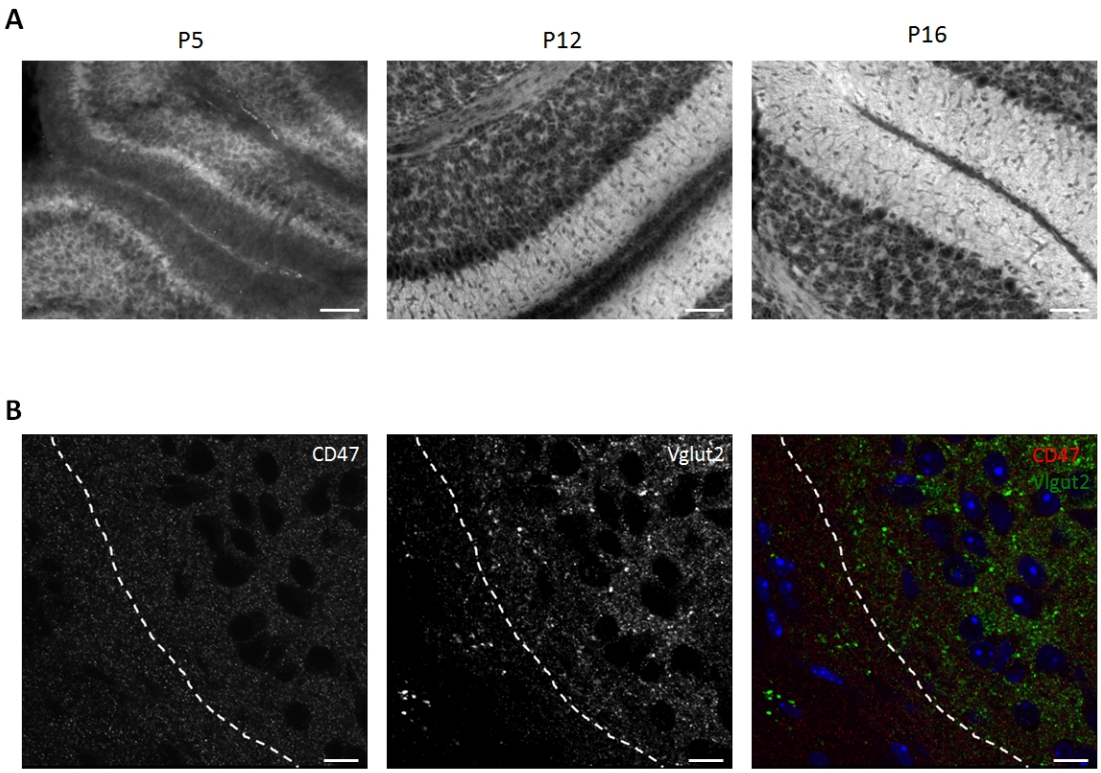

Figure 4.2. CD47 is developmentally regulated in the cerebellum and localized to the optic tract. A, Images of CD47 staining in the cerebellum of WT mice. At P5, CD47 is localized to the purkinje cell layer, which is where climbing fiber synapses are located at that developmental time point. By P12 and P16, CD47 moves to the molecular layer, coincident with the movement of climbing fiber synapses up purkinje cell dendrites that occurs during cerebellar pruning. Scale bar = 50μm. B, Confocal images of CD47 and Vglut2 in the P5 dLGN, dashed line indicates the border of the dLGN and optic tract. Vglut2 (green and middle panel) is confined to the dLGN, whereas CD47 (red and left panel) stains both regions.

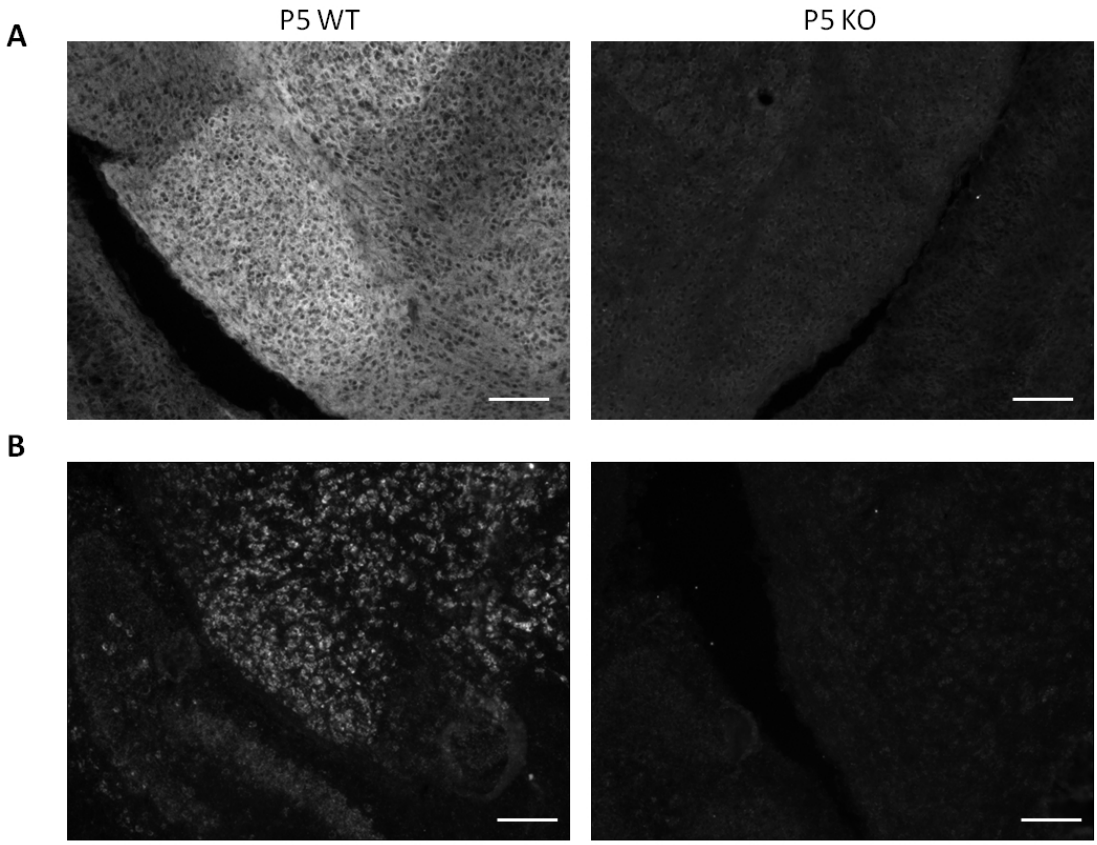

Figure 4.3. The CD47 antibody and in situ probe are specific. A, Representative 10X images of dLGN from P5 CD47KO (right) and WT (left) littermates demonstrate that the antibody does not stain knockout tissue. B, Representative 10X images of dLGN from P5 CD47KO (right) and WT (left) littermates demonstrate that the CD47 in situ probe does not label knockout tissue. Scale bars = 50μm

also be upregulated in other brain regions, such as hippocampus and cerebellum, coincident with the onset of pruning in those regions (Figure 4.1A, Figure 4.2A).

CD47 mRNA displays a similar pattern of expression, with heightened expression in the dLGN during pruning prior to upregulation in other brain regions (Figure 4.4D, Figure 4.3B). Double fluorescent in situ hybridization (FISH) indicates that CD47 is produced by neurons in the dLGN at P5 and that this expression persists throughout the pruning period and begins to decline at P30, while other regions initiate CD47 expression later (Figure 4.4D). Quantitative PCR (qPCR) using whole brain lysates demonstrates that CD47 expression is relatively stable throughout development (Figure 4.4B), although this obscures region-specific variability. Consistent with presynaptic localization, CD47 is also produced in the retina and message appears to be highest at P5, which may correspond to the high level of CD47 protein in the P5 dLGN (Figure 4.4A). To determine whether CD47 mRNA is made by retinal ganglion cells (RGCs), which serve as the object of retinogeniculate refinement, I performed qPCR on acutely isolated RGCs from P5 mice and found that they indeed make CD47, and that CD47 is more highly expressed in RGCs than other cell types, such as acutely isolated P5 microglia (Figure 4.4C). These data indicate that CD47 is well positioned to regulate microglia-mediated pruning in the dLGN.

CD47-deficient microglia exhibit increased engulfment of RGC inputs

To test the hypothesis that CD47 acts as a "don't eat me" signal to inhibit excess microglial phagocytosis of synaptic inputs during development, I turned to our *in vivo* microglia engulfment assay (Chapter 3, Schafer et al., 2012). For these experiments, intraocular injection of anterograde tracers (cholera toxin β-subunit (CTB) conjugated to Alexa dyes) is performed to label presynaptic RGC inputs and microglia are visualized using

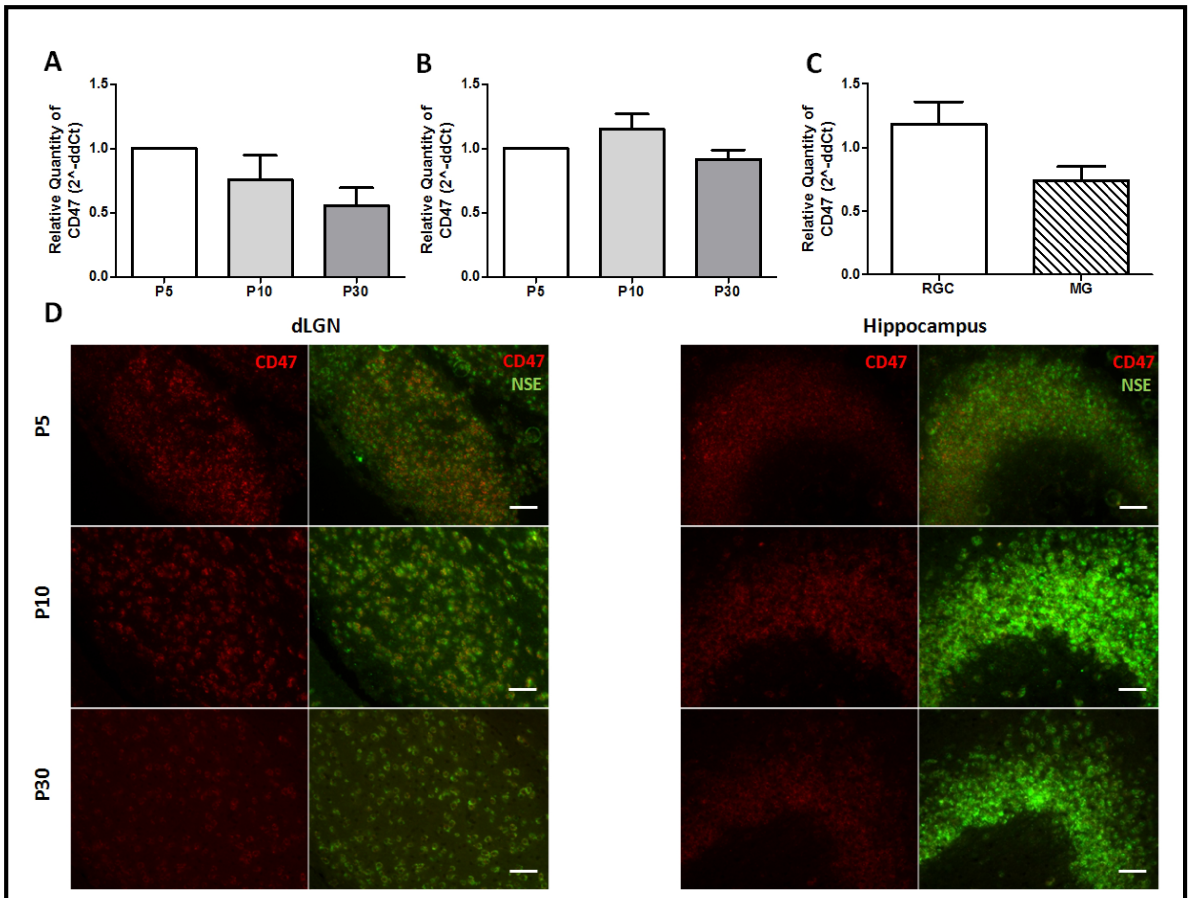

Figure 4.4. CD47 is expressed more highly in the dLGN and retina during the pruning period. A, CD47 is most highly expressed in the retina during peak pruning (P5) as measured by qPCR, n=3 mice per age. B, CD47 expression in whole brain is relatively constant throughout development, n=3 mice per age. C, CD47 is more highly expressed in acutely isolated P5 RGCs than acutely isolated P5 microglia (MG) as measured by qPCR, n=2 RGC samples, 3 microglia samples. D, Representative double FISH images of dLGN (left) and hippocampus (right) from WT mice. CD47 (red) colocalizes with neuronal marker NSE (green) in both regions, but appears more highly expressed in the dLGN than hippocampus during peak retinogeniculate pruning (P5) and declines in the dLGN after the pruning period (P30). Scale bar = 50μm. All error bars represent s.e.m.

IHC for Iba-1. Then, high-resolution imaging and three-dimensional (3D) reconstruction of z-stack images are used to determine the volume of engulfed RGC inputs. I examined CD47KO and WT littermates during peak pruning in the dLGN (P5) and found that CD47KO microglia engulfed approximately 1.5X the volume of inputs compared to that engulfed by their WT littermates (Figure 4.5A-B, $p<.07$ using Student's t-test, n=6 WT, 6 CD47KO, analysis of additional litters in progress). This phenotype was not due to a uniform increase in engulfment; instead, about half the cells in a CD47KO animal display wild type levels of engulfment (inputs occupying 0-10% of the microglia cell volume), while the rest of the cells engulf increased inputs. Most of the remaining KO cells engulfed about 2X the wild type volume of inputs, and one or two cells per animal could typically be observed engulfing up to 3-4X wild type levels (inputs occupying 30-40% of the microglia cell volume), something never observed in WT animals (Figure 4.5C). The variability in the knockout suggests that other factors may also work to prevent excess engulfment, and that microglia may be a heterogeneous population of cells.

Further examination of microglia in CD47KO and WT littermates yielded no additional abnormalities in morphology or markers representing phagocytic capacity. I used our microglia activation assay (Figure 3.3), in which scores are assigned based on lysosome abundance, as measured by CD68 immunoreactivity, and cell morphology, as determined by counting branches on Iba-1 stained microglial cells, to assess these two properties in fixed tissue. CD47KO microglia exhibited a comparable level of activation in the dLGN to their wild type littermates at P5 and P10, with a similar percentage of cells from each animal being assigned a particular score (Figure 4.6A-B). The numbers of microglia within the dLGN were also similar between CD47KO and WT littermates (Figure

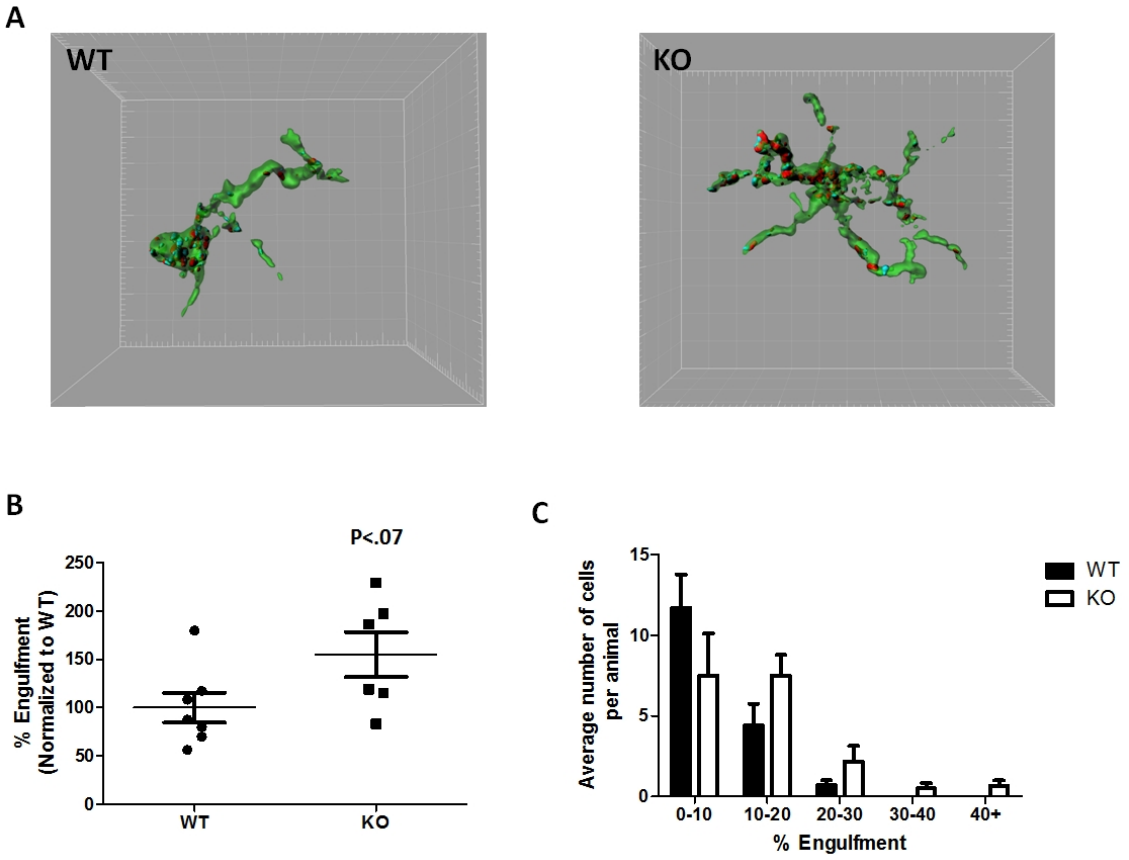

Figure 4.5. CD47-deficient microglia exhibit increased engulfment. A, Representative images of CD47KO (right) and WT (left) littermate microglia (green) with internalized CTB-labeled inputs (red and blue). Grid line increments = 5μm. B, CD47KO microglia engulf more CTB-labeled inputs than WT littermate microglia, n=6 mice per genotype, p<.07 using Student's t-test. C, Binning cells by percent engulfment shows that the CD47 distribution is shifted to the right, indicating that, although some cells still exhibit WT levels of engulfment, there are many cells that engulf at higher than WT levels. All error bars represent s.e.m.

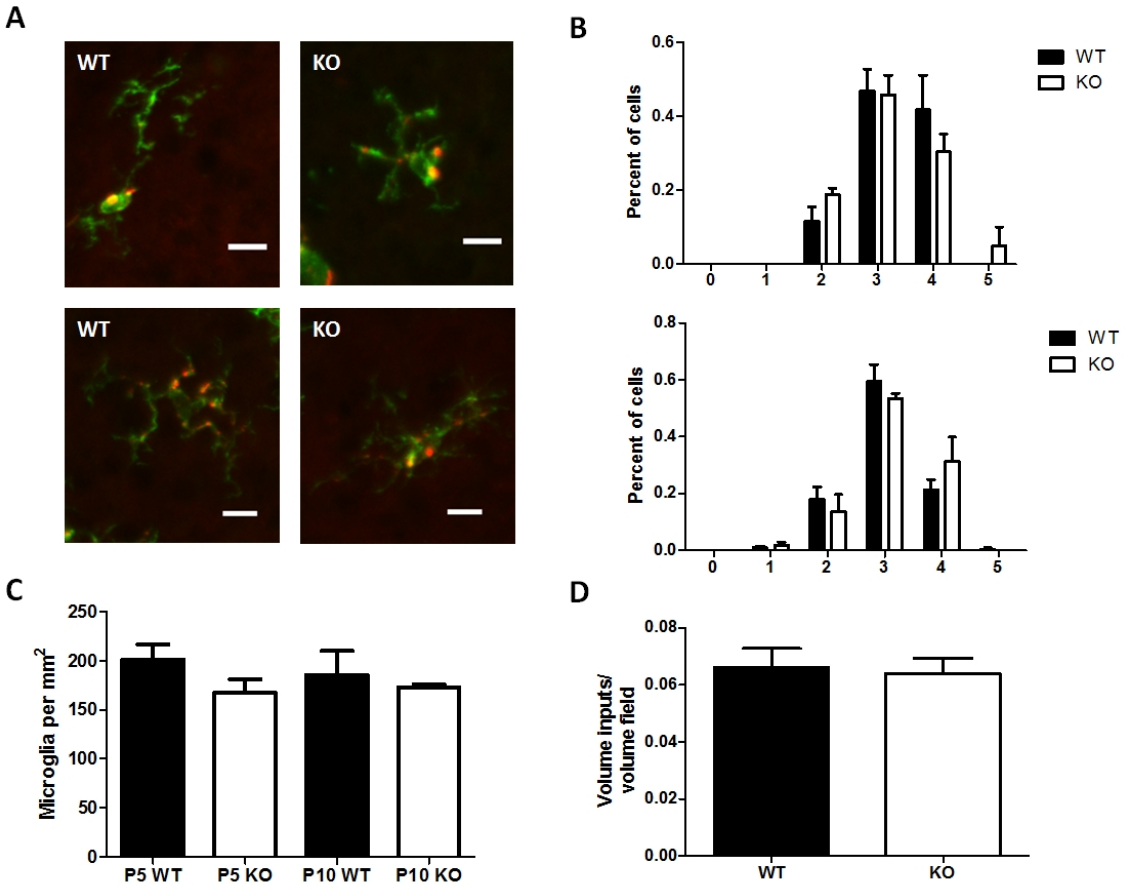

Figure 4.6. CD47KO mice do not exhibit alterations in microglia activation, cell density, or input innervation. A, Representative images of P5 (top) and P10 (bottom) CD47KO and WT littermate dLGN microglia stained for Iba-1 (green) and CD68 (red) to label microglia and lysosomes, respectively. Scale bars = 10µm. B, Results of the microglia activation assay for P5 (top) and P10 (bottom) CD47KO and WT littermates. Microglia activation is similar for the two genotypes, n=3 mice per age per genotype. C, Quantification of microglia cell density in CD47KO and WT littermates indicates that there is no difference in cell density, n=3mice per age per genotype. D, Quantification of the volume of CTB-labeled inputs within the P5 dLGN as measured using Imaris software (volume of inputs/volume of dLGN field analyzed). CD47KO mice do not appear to have reduced innervation into the dLGN, n=6 mice per genotype. All error bars represent s.e.m.

4.6C), and KO and WT microglia were indistinguishable based on their average three-dimensional cell volume (data not shown). These data indicate that CD47KO microglia engulf more retinogeniculate inputs during peak pruning, despite cell-to-cell variability, and that this phenotype is not due to changes in microglia activation. This suggests that CD47 is required to inhibit excess microglia-mediated pruning, and that once the "brakes" on pruning are removed, microglia can consume additional synapses.

CD47KO mice display increased or accelerated pruning

To address the functional consequences of excess microglial engulfment of RGC inputs, I investigated whether this increased pruning could impact retinogeniculate refinement. During typical refinement, there is a great deal of overlap between inputs from the left and right eyes when pruning is initiated at P5, yet distinct eye-specific territories can be observed with minimal overlap at the border of the ipsilateral and contralateral regions only five days later at P10 (Guido, 2008; Jaubert-Miazza et al., 2005; Sretavan and Shatz, 1986; Ziburkus and Guido, 2006). Given our findings that decreased microglial engulfment during peak pruning led to decreased retinogeniculate refinement in CR3 knockout mice (Chapter 3, Schafer et al., 2012), I hypothesized that increased engulfment would produce the opposite result, or overpruning. Performing eye-specific segregation analysis on P10 CD47KO and WT littermates revealed significantly less overlap between eye-specific territories in KOs compared to WT littermate controls (Figure 4.7A-C). This suggests that increased microglial engulfment of RGC inputs leads to overpruning in the dLGN. Retinal neuron numbers were comparable between KOs and WTs, indicating that the decrease in overlap does not result from loss of retinal neurons in CD47KOs (Figure 4.8C). This phenotype could also result if outgrowth is impaired in CD47KO animals,

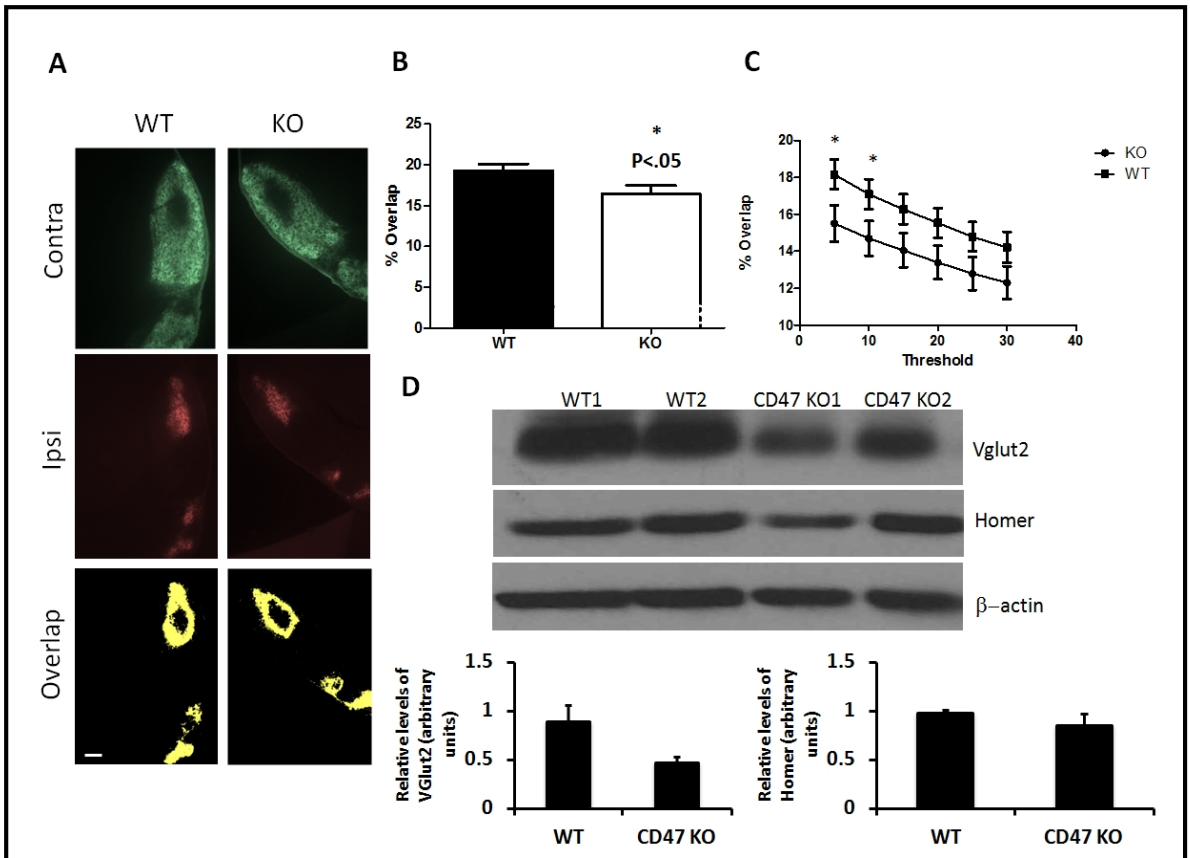

Figure 4.7. CD47KO mice have an overpruning defect. A, Representative images of CD47KO (right) and WT littermate (left) P10 dLGN contralateral (green) and ipsilateral (red) territories. CD47KOs exhibit less overlap (yellow) between the two territories, indicating increased pruning, n=8KO, 7WT, Scale bar = 10μm. B, CD47KOs have a significant reduction in overlap, *p<.05, Student's t-test. C, The difference in overlap diminishes as thresholding becomes more conservative, *p<.05, Student's t-test. D, Representative western blot for synaptic markers from 3 month-old striatum. CD47KO mice have lower levels of presynaptic marker Vglut2, but postsynaptic Homer appears unchanged. Quantification below. All error bars represent s.e.m.

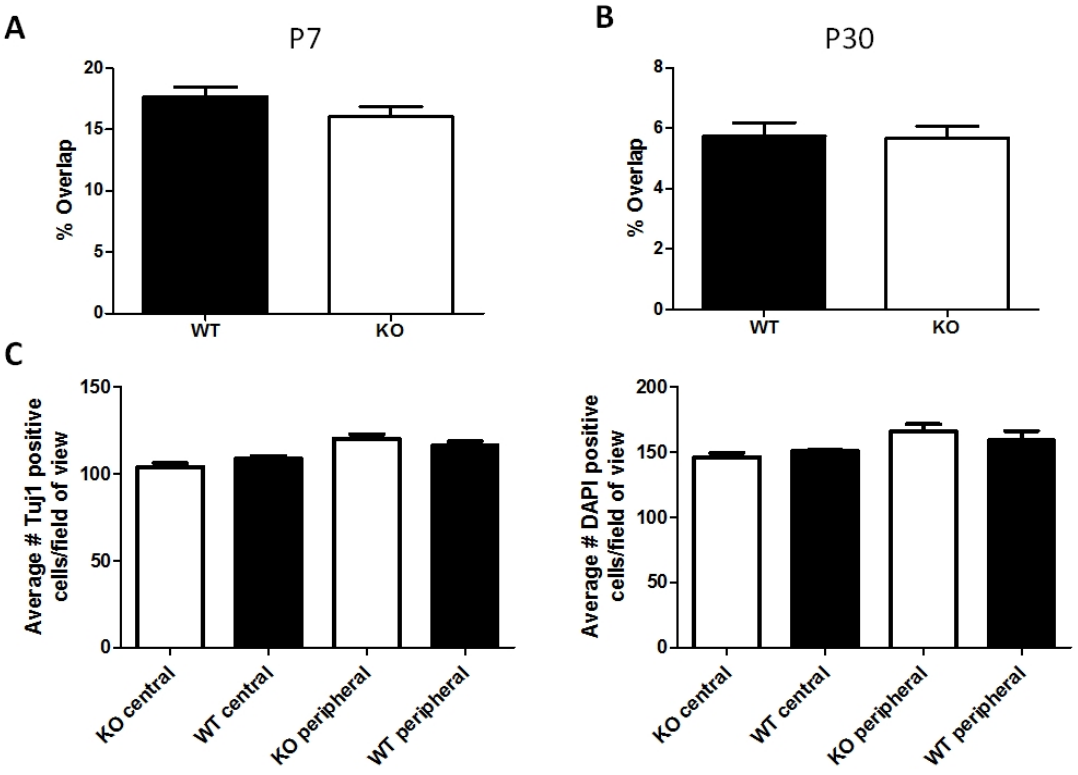

Figure 4.8. CD47KO mice exhibit accelerated pruning that is not caused by loss of retinal cells. A, Quantification of P7 eye-specific segregation data. CD47KO and WT littermates do not have a statistically significant difference in overlap, but there is a trend toward increased pruning in P7 CD47KOs, n=9WT, 7KO. B, Quantification of P30 eye-specific segregation data. CD47KO and WT littermates do not exhibit differences in percent overlap at this age, n=3WT, 6KO. C, Quantification of cell numbers in the retina. CD47KO and WT littermates do not exhibit alterations in the number of total cells (DAPI, right) or the number of retinal neurons (Tuj1, left) at P10, n=3 mice per genotype. All error bars represent s.e.m.

a phenotype that has been described in cultured hippocampal neurons (Murata et al., 2006). I used 3D reconstruction of CTB-labeled inputs in the dLGN to assess outgrowth while performing microglia engulfment analysis, and found equivalent innervation in KO and WT littermates (Figure 4.6D), suggesting that this is not responsible for the phenotype. As this period of refinement involves both a reduction in inappropriately targeted inputs and the arborization of those inputs that remain (Dhande et al., 2011; Snider et al., 1999), it is possible that decreased overlap could result from a failure to properly elaborate arbors. To address this, I am performing single RGC arbor tracing experiments in CD47KO and WT littermates carrying an RGC-specific tamoxifen inducible Cre and a Thy1-STOP-YFP transgene. Low doses of tamoxifen will allow me to visualize individual YFP-labeled arbors in sagittal dLGN sections that I can reconstruct and trace using Imaris software to determine whether arbor sizes are reduced in CD47KOs.

One question raised by this phenotype is whether the pruning process begins earlier in CD47KO mice or is accelerated compared to the normal timeline. When I imaged dLGNs at P5 for the microglia engulfment assay, CD47KO and WT littermates appeared indistinguishable, and pruning did not appear to have been initiated. However, by P7, the decrease in overlap between eye-specific territories in CD47KO versus WT littermates can be visualized, although this is variable and not seen in every litter examined (Figure 4.8A). This suggests that increased microglial phagocytosis may accelerate the pruning process, and it is possible that the variability in engulfment at P5 could lead to the variable levels of overlap observed at P7.

Another important question is whether this increase in pruning is sustained into adulthood. To address questions of phenotype maintenance in this system, we typically

perform the eye-specific segregation assay again at P30, which is near the end of the retinogeniculate refinement period (Hong and Chen, 2011; Huberman et al., 2008). At this time point, CD47KO and WT littermates display similar levels of overlap between eye-specific territories (Figure 4.8B), however, it is somewhat difficult to interpret this result as this assay is not designed to discern overpruning (for further explanation, see discussion). To better address this question, I am performing quantification of structural synapses in P60 CD47KO and WT littermates, an age chosen to ensure that pruning and arborization changes should be completed.

Intriguingly, this overpruning may also occur in other brain regions, as western blot analysis of synaptic proteins in the striatum revealed lower Vglut2 levels in three month-old CD47KO mice, while levels of postsynaptic marker Homer were unchanged (Figure 4.7D). While these data are preliminary, they suggest that overpruning may lead to a reduction in synapse numbers that persists into adulthood, and that CD47 may be utilized as a protective signal throughout the brain.

CD47 receptor SIRPα is developmentally regulated in microglia during the pruning period

To determine whether the protective function of CD47 in the developing dLGN is in fact due to direct inhibition of microglial phagocytosis, I sought to examine the expression and localization of its known microglial receptor, SIRPα. SIRPα (SHPS-1/CD172a/P84/BIT/ MyD-1) is an Ig superfamily protein expressed by microglia and other cell types in the brain, including neurons (Adams et al., 1998; Chuang and Lagenaur, 1990; Comu et al., 1997; Ohnishi et al., 2005). Work in the immune system has shown that binding of CD47 to SIRPα on macrophages induces receptor tyrosine phosphorylation, followed by binding of SHP2 or SHP1 to the SIRPα cytoplasmic tail to inhibit phagocytosis

(Barclay and Van den Berg, 2014; Matozaki et al., 2009). Although microglial SIRPα has not been studied in the context of normal brain development, the CD47-SIRPα interaction has been shown to play a role in phagocytosis of myelin after traumatic axonal injury, with blockade of SIRPα preventing binding to CD47 on myelin and enabling increased microglial phagocytosis (Gitik et al., 2011). This interaction mirrors CD47-SIRPα function in the immune system, and represents the inhibitory interaction between presynaptic CD47 and microglial SIRPα that I hypothesize is a mechanism for synaptic protection during developmental microglia-mediated pruning in the dLGN.

For SIRPα to regulate retinogeniculate refinement, it would have to be present on microglia in the dLGN during the pruning period. To assess SIRPα localization, I performed immunohistochemical analysis on the developing brain. SIRPα appears broadly expressed throughout the brain and increases during development (Figure 4.9A). Higher magnification images reveal that SIRPα staining exhibits a punctate pattern, similar to that of synaptic staining. Intriguingly, neuropil staining for SIRPα is lower in the dLGN during the pruning period, and higher magnification images of P5 dLGN reveal SIRPα-stained microglia (Figure 4.9B). Microglia staining can be observed in other brain regions as well at P5, but is no longer visible in older mice, although this could be due to either a decrease in microglial SIRPα or an increase in neuronal SIRPα that occludes microglial staining.

To visualize microglial and neuronal SIRPα expression throughout development, we used double fluorescence in situ hybridization (Figure 4.10A). These data mimic the IHC data in that neuronal SIRPα expression is lower in dLGN than in other brain regions, but reveal a microglia-specific expression pattern in the dLGN during peak pruning. At

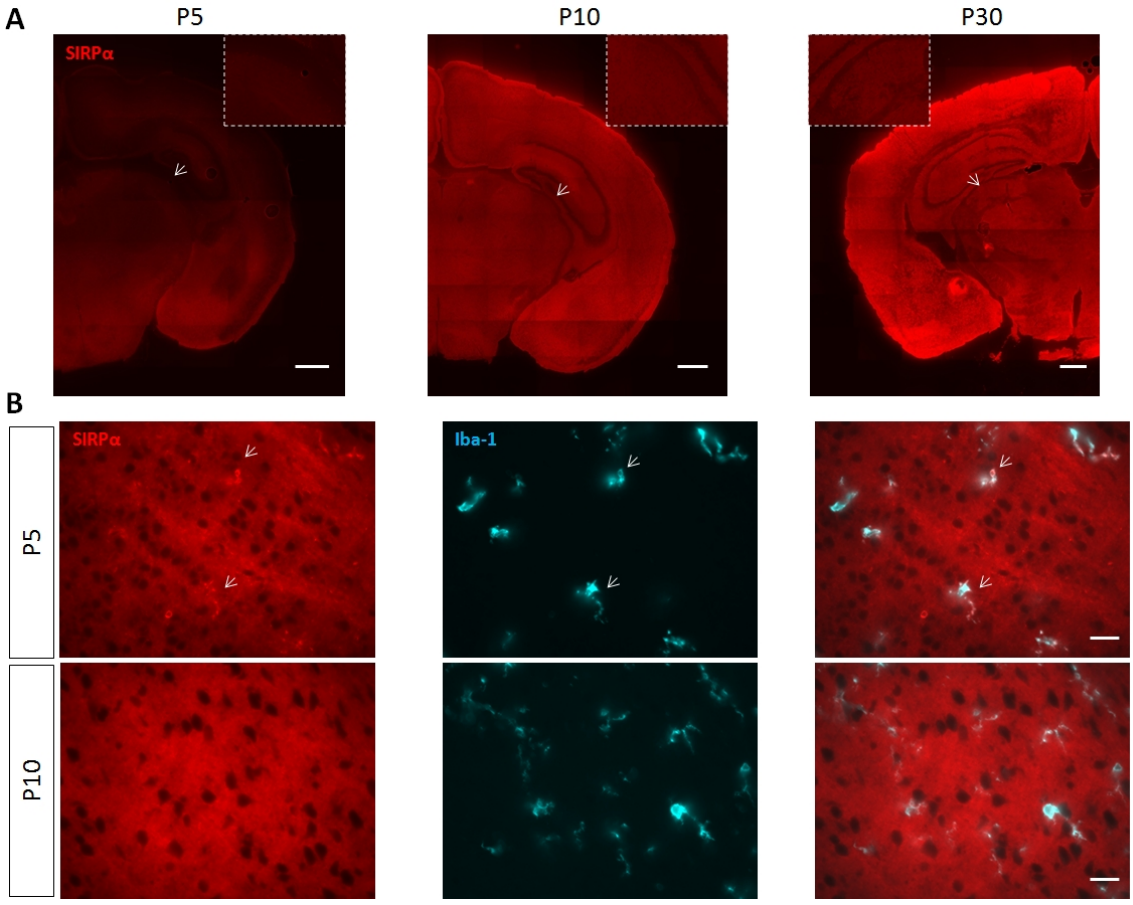

Figure 4.9. SIRP~ is more highly localized to microglia than neurons during peak pruning. A, Representative low magnification mosaics of P5, P10, and P30 WT coronal sections. SIRPα is slightly lower in the dLGN (arrow) versus other regions at P5, and SIRPα levels increase with age. Inset is a higher magnification image of the dLGN from each mosaic. Scale bar = 500μm. B, SIRPα (red) is highly colocalized to Iba-1 positive microglia (blue) in the P5 dLGN, but this is not observed at P10. Scale bar = 10μm.

P5 and P10, SIRPα expression appears to be primarily microglial in the dLGN and co-localizes with IHC for microglia marker Iba-1. There is some co-localization between SIRPα and Iba-1 in other brain regions at P5, but, unlike in the dLGN, SIRPα colocalizes primarily with neuronal marker NSE in all other regions examined at P10. By P30, SIRPα appears only to be made by neurons in the dLGN and throughout the brain. I have also observed the P5 microglial enrichment and the downregulation of microglial SIRPα after completion of the anatomical pruning period using acutely isolated microglia from whole brain (Pino and Cardona, 2011). Compared to acutely isolated RGCs, SIRPα is expressed approximately six-fold more by microglia at P5 as measured by qPCR (Figure 4.10B). Microglial SIRPα is expressed at similar levels in acutely isolated microglia from P5 and P10 brains, but declines dramatically by P20 (Figure 4.10C). In contrast, SIRPα qPCR performed on whole brain samples indicates that global SIRPα levels are stable and may increase slightly over development, which parallels the neuronal staining results (Figure 4.10D). Taken together, these data indicate that microglial and neuronal SIRPα are differentially regulated, and the high levels of microglial SIRPα expression during early development suggest a need for the inhibition of phagocytosis during this period. The unique expression pattern of SIRPα in the dLGN during the pruning period supports the hypothesis that the CD47-SIRPα interaction serves to inhibit excess pruning during retinogeniculate refinement.

To investigate the role of SIRPα in microglia-mediated pruning, we are collaborating with Dr. Hisashi Umemori and obtained his conditional SIRPα mice. His lab recently used these mice to demonstrate that SIRPα regulates synaptic maturation in the hippocampus, as a decrease in presynaptic marker staining intensity is only observed if SIRPα is knocked

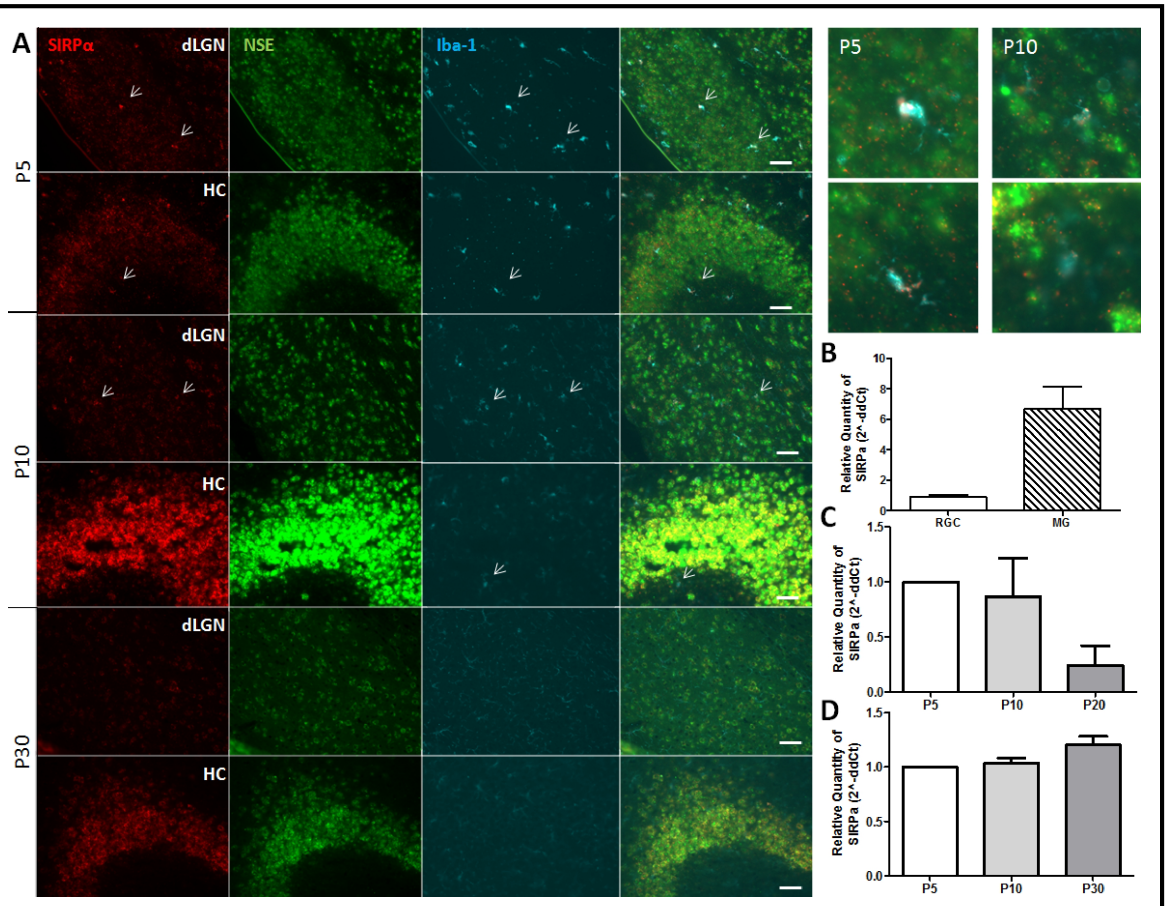

Figure 4.10. SIRP~ is highly expressed by microglia in the dLGN during the pruning period. A, Representative 20X images of FISH (SIRPα, red and neuronal marker NSE, green) combined with IHC (microglial marker Iba-1, blue) in the dLGN and hippocampus. SIRPα is primarily expressed by microglia in both regions at P5, but is expressed by neurons in the P10 hippocampus while still being expressed by microglia in the P10 dLGN. SIRPα is expressed by neurons in both regions at P30. Arrows indicate microglia shown in enlarged images on the right to better display colocalization. Scale bar = 50μm. B, Acutely isolated microglia (MG) express approximately 6-fold more SIRPα than acutely isolated RGCs as measured by qPCR, n=2 RGC samples, 3 microglia samples. C, SIRPα declines in acutely isolated microglia as the pruning period nears completion (P20), n=3 samples per age. D, SIRPα slightly increases throughout development in whole brain lysates as measured by qPCR, n=3 samples per age. All error bars represent s.e.m.

down starting at P15, coincident with the period of hippocampal synaptic refinement (Toth et al., 2013). Although this study employed a global actin-CreER driver to excise floxed SIRPα, the observed synapse loss occurs concurrently with the hippocampal period of microglia-dependent maturation and engulfment (Paolicelli et al., 2011), raising the intriguing possibility that microglial SIRPα may be involved in mediating the observed synaptic changes. I have used tamoxifen delivery with this driver line to create a germline knockout and am currently breeding up a colony to investigate whether microglia-mediated pruning is uninhibited in SIRPαKO mice. Unfortunately, this experiment will not rule out the possibility that neuronal SIRPα also participates in pruning, however, the expression pattern of SIRPα in the P5 and P10 dLGN increases the likelihood that a dLGN phenotype will be linked to loss of microglial, and not neuronal, SIRPα. Microglial SIRPα function can also be studied by isolating microglia from global SIRPα knockout mice and examining their phagocytic behavior in culture, which is an experiment I plan to conduct to support *in vivo* results obtained using the germline KO.

CD200-deficient mice exhibit alterations in retinogeniculate refinement

In the immune system, a variety of "don't eat me" signals are employed to confer "self" protection, although they achieve this protective effect via different mechanisms (Barclay et al., 2002; Griffiths et al., 2007). Given the many apparent similarities between retinogeniculate refinement and self versus non-self discrimination in the immune system, I hypothesized that this could be the case in the developing CNS as well. In this way, rather than functioning redundantly, "don't eat me" signals could work together to promote synaptic protection by independently inhibiting different aspects of microglial behavior. CD200 is another well known "don't eat me" signal expressed in the developing brain, and

its mediation of self protection in the immune system is thought to be due to regulation of macrophage activation, inflammatory signaling, and cell numbers (Hoek et al., 2000; Minas and Liversidge, 2006; Walker and Lue, 2013; Wright et al., 2000). As these properties are also likely to affect CNS pruning, CD200 may be a "don't eat me" signal that works in a different, but complementary, manner to CD47.

To determine whether CD200 could serve as a "don't eat me" signal to regulate microglia-mediated pruning during development, I examined its localization in the developing brain. Immunohistochemistry for CD200 reveals punctate staining similar to that of CD47, although CD200 is broadly expressed throughout the brain and does not appear to be enriched in any particular region at P5. Interestingly, CD200 protein appears to decrease with age in the dLGN and is nearly absent by P30 when retinogeniculate refinement is nearly complete (Figure 4.11A). High-resolution SIM images demonstrate that CD200 also colocalizes with pre- and postsynaptic markers, however, unlike CD47, CD200 appears to be more highly localized to postsynaptic Homer-positive puncta in the dLGN (Figure 4.11B).

To determine whether CD200 also acts as a "don't eat me" signal to prevent overpruning in the retinogeniculate system, I examined overlap between ipsilateral and contralateral territories in the dLGN using the eye-specific segregation assay described earlier. Similar to CD47KO mice, CD200KO mice exhibit significantly decreased overlap between emerging eye-specific territories in the dLGN at P10 (Figure 4.12A-B). I am currently in the process of quantifying retinal cell numbers to confirm that this decrease in overlap is not due to a reduction in the number of healthy retinal neurons, and will assess earlier time points to investigate whether pruning is accelerated in CD200KOs as well.

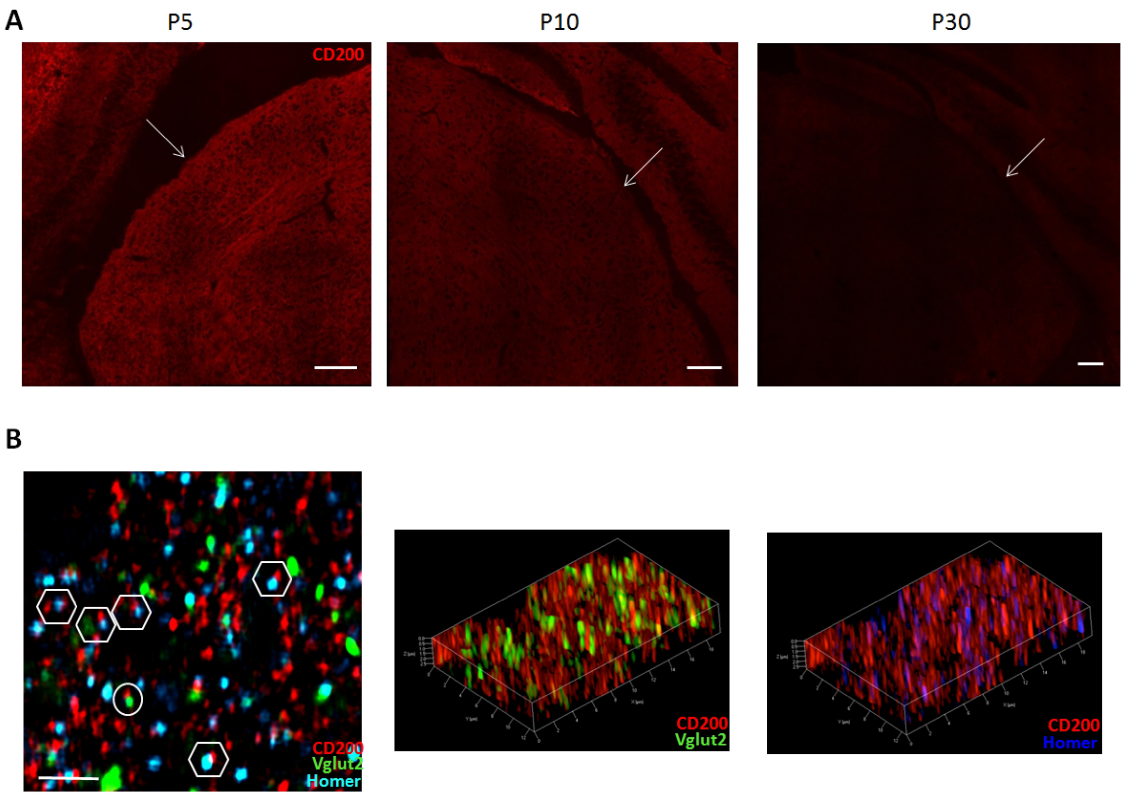

Figure 4.11. CD200 is localized throughout the brain during peak pruning before declining in the dLGN. A, Representative 10X images of CD200 in WT dLGN (arrow). CD200 is localized to the dLGN at P5, but begins to decline relative to other areas at P10 and is nearly absent by P30. Scale bar = 50μm. B, SIM images of CD200 (red) indicating that it primarily colocalizes with postsynaptic Homer (blue, hexagons) rather than presynaptic Vglut2 (green, circles). Three-dimensional images of the z-stack are provided for another view of colocalization (right). Scale bar = 2μm.

Given the similarities of this phenotype to that of the CD47KO mice (Figure 4.7A-C), I next used our engulfment assay to evaluate whether it might be caused by increased phagocytosis of RGC inputs during peak pruning. However, unlike CD47KO microglia, CD200KO microglia do not engulf more inputs in the P5 dLGN than WT littermate microglia (Figure 4.12C). At the time of engulfment analysis (P5), microglia cell density and activation state also do not appear to be different in CD200KO and WT littermates (Figure 4.12D-E). This suggests that CD200 deficiency may give rise to increased or accelerated retinogeniculate pruning via a different mechanism than CD47, which is consistent with what is known about these molecules from the immune system (Barclay et al., 2002; Griffiths et al., 2007). Whether this could be caused by increased microglia numbers after P5 or increased inflammatory signaling, two commonly described features of CD200KO mice (Broderick et al., 2002; Hoek et al., 2000; Walker and Lue, 2013), is a matter I am currently investigating by assessing microglia numbers at the time of the eye-specific segregation analysis (P10) and measuring cytokine levels in CD200KO mice. This phenotype could also occur if CD200KO microglia do not exhibit the normal developmental decrease in engulfment, and I will address this possibility by conducting engulfment analysis after peak pruning (P9) in CD200KO mice.

Discussion

My results demonstrate for the first time that protective signals are required to prevent excess microglia-mediated pruning during development. I identified CD47 and CD200, two classical "don't eat me" signals, as regulators of retinogeniculate refinement in the developing dLGN. Both molecules are expressed in the dLGN during the pruning period

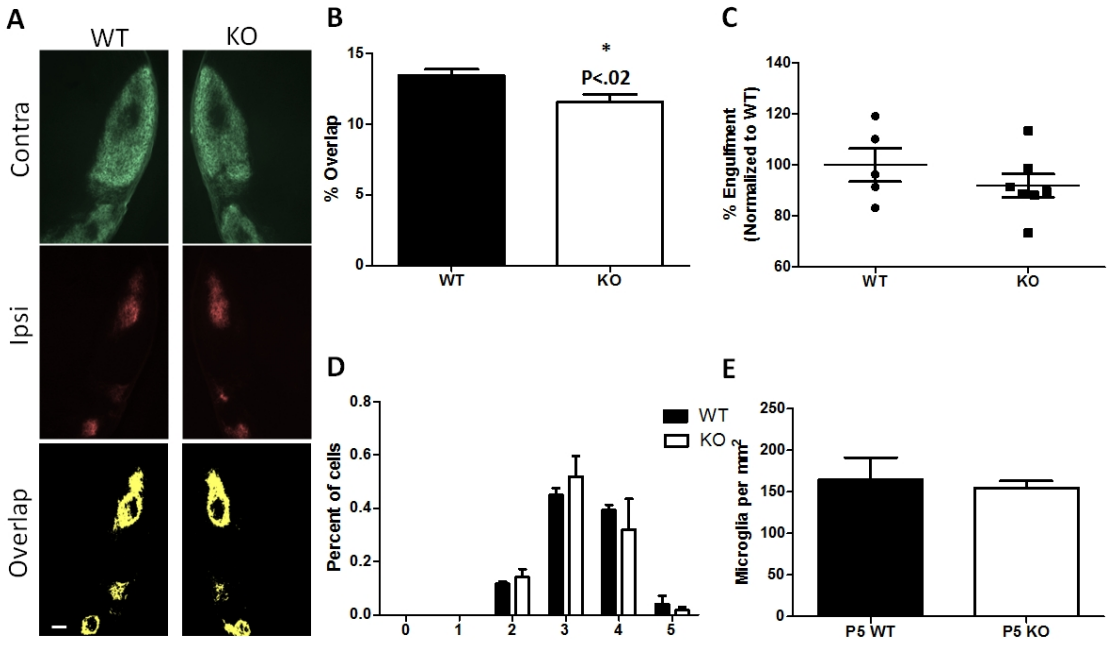

Figure 4.12. CD200-deficient mice exhibit overpruning at P10 without changes in microglial engulfment, activation, or density. A, Representative images of CD200KO (right) and WT littermate (left) P10 dLGN contralateral (green) and ipsilateral (red) territories. CD200KOs exhibit less overlap (yellow) between the two territories, indicating increased pruning, n=3KO, 4WT, Scale bar = 10μm. B, CD200KOs have a significant reduction in overlap, *p<.02, Student's t-test. C, CD200KO microglia engulf approximately the same amount of inputs as WT littermate microglia, n=7KO, 5WT. D, CD200KO microglia activation is not different from WT at P5, n=3 mice per genotype. E, CD200KO microglia cell density is not different from WT at P5, n=3 mice per genotype. All error bars represent s.e.m.

(Figure 4.1, 4.11), however, CD47 exhibits striking regional specificity during this time. Loss of CD47 leads to increased microglial engulfment of retinogeniculate inputs during peak pruning as well as decreased overlap between eye-specific territories in the dLGN at P10 (Figure 4.5, 4.7). This could indicate increased pruning or an acceleration of the normal pruning process. Preliminary data from adult mice demonstrating a reduction in synaptic markers suggest that this is, in fact, an overpruning phenotype that leads to a prolonged decrease in synapses (Figure 4.7). I also found that CD47 receptor SIRPα is primarily expressed by microglia in the dLGN during the pruning period, and that microglial SIRPα expression declines with age while neuronal expression is relatively constant (Figure 4.10). CD200 deficiency also leads to a decrease in overlap between eye-specific territories in the dLGN at P10, but does not appear to alter microglial engulfment at P5 (Figure 4.12). Together, these data reveal that mechanisms used to identify and protect self cells in the immune system are similarly utilized to protect necessary synaptic connections during CNS development, and future experiments will further elucidate the mechanisms underlying this function.

My results raise several important questions that I am currently investigating. First, is whether the CD47KO overpruning phenotype extends into adulthood or has behavioral consequences. Second, is if the CD47KO phenotype is mediated by its interaction with microglial SIRPα. Next, I plan to further investigate the CD200 overpruning phenotype to determine its underlying cause. I will also examine whether loss of both CD47 and CD200 has a synergistic effect on pruning. Finally, I would like to investigate whether loss of protective signals allows microglia to engulf non-synaptic material or other cellular compartments, such as axons. Answers to these questions will provide a more complete

understanding of CD47 and CD200 function and the role of protective signals in retinogeniculate pruning.

A major open question that has been difficult to resolve is whether the CD47 and CD200 knockout eye-segregation phenotypes represent a true overpruning or an acceleration of development that simply reaches its normal endpoint earlier. One reason this has been challenging is due to limitations of the eye-specific segregation assay. While the eye-specific segregation assay provides information about synaptic refinement and the removal of inappropriately targeted inputs (Huberman et al., 2008; Jaubert-Miazza et al., 2005), it may not be sensitive enough to detect overpruning or excess elimination of inputs once eye-specific territories have formed. As shown in Figure 4.7, I found that CD47KO mice were hyper-refined, or had less overlap between left and right eye inputs at P10 as compared to littermate controls. This result, paired with the increased engulfment exhibited by CD47KO microglia (Figure 4.5), strongly suggests overpruning. However, the major type of pruning that occurs after P10 involves the removal of supernumerary inputs from monocularly innervated relay neurons, which is not something the eye-specific segregation assay is designed to measure (Chen and Regehr, 2000; Hong and Chen, 2011; Hooks and Chen, 2006; Ziburkus and Guido, 2006). To address whether there is overpruning in the adult, I am instead conducting structural synapse quantification in the dLGN using high-resolution microscopy and performing western blot analysis for synaptic proteins from whole brain and microdissected regions of interest. In future studies, we plan to use electrophysiology to address whether there is enhanced functional elimination of retinogeniculate inputs in CD47 KOs in the postnatal and adult brain.

Regardless of the answer to the above question, I still plan to investigate whether the observed changes in pruning have behavioral consequences. Visual system development is a precisely timed process, and an acceleration of dLGN pruning is likely to have consequences that affect other visual areas. I plan to perform behavioral assays to evaluate visual function, such as the optomotor task and the visual water maze, to determine whether increased pruning or accelerated development affects normal visual processing. If future experiments substantiate preliminary data that CD47KOs have reduced synapse numbers in non-visual areas, such as striatum, other behavioral functions relevant to those regions can be assessed as well. Interestingly, a recent report demonstrated that CD47KOs have defects in behavioral assays related to schizophrenia, including prepulse inhibition and social interactions (Koshimizu et al., 2014). As schizophrenia is a disorder characterized by circuit disconnectivity (Schmitt et al., 2011), these data suggest that CD47 deficiency may lead to overpruning in brain regions and circuits beyond the retinogeniculate system. I am planning to conduct further behavioral analysis and examine pruning in other brain regions with the help of collaborators, which I will discuss in more detail in the next chapter.

As mentioned in the main text, I am pursuing the hypothesis that the CD47KO overpruning phenotype is due to impairment of CD47 signaling to microglial SIRPα. This interaction has been shown to underlie phagocytosis in both the immune and nervous systems (Gitik et al., 2011; Okazawa et al., 2005; Oldenborg et al., 2001), however, CD47 does have other binding partners, such as Thrombospondin-1 and Integrin$\alpha v\beta 3$, as well as neuronal SIRPα (Brown and Frazier, 2001; Matozaki et al., 2009). I will perform engulfment and eye-specific segregation analyses using global SIRPα knockout mice to

determine whether they phenocopy the CD47KO defects. While neuronal SIRPα could still be playing a role, the in situ hybridization data suggest that a P5 or P10 dLGN phenotype could be attributed to microglial SIRPα (Figure 4.10). Experiments addressing phagocytosis in cultured microglia isolated from SIRPαKO mice can also be performed to supplement *in vivo* data.

One additional question is whether this has implications beyond the synapse. Our previous work demonstrated that microglia perform most of their engulfment in synaptic regions, and our electron microscopy analysis of the material engulfed in the dLGN by wild type microglia indicated that it most likely represents presynaptic terminals (Chapter 3, Schafer et al., 2012). However, we do not know what keeps microglial engulfment confined to the synapse and prevents engulfment of other parts of the cell. It could be due to specific synaptic localization of "eat me" signals, but it is possible that "don't eat me" signals could play a role in restricting microglia to synapses as well. CD47 appears to be expressed in both synaptic regions and non-synaptic regions like the optic tract (Figure 4.2B), and live immunostaining of cultured hippocampal neurons reveals it to be on the surface of the entire cell (data not shown). One possibility is that the broad localization of CD47 throughout the neuron prevents microglial engulfment of other parts of the cell. In order to test whether CD47 prevents engulfment of axons or other cellular compartments in addition to synapses, I am examining engulfment in the optic tract and plan to conduct EM on CD47KO microglia to visualize the ultrastructure of the material they engulf. If CD47KO microglia are also engulfing axons, this could have far-reaching implications for regional connectivity and circuit development.

Immune molecules tightly regulate retinogeniculate refinement

Previous work from our lab and others has implicated a number of immune molecules and their homologs in retinogeniculate refinement (Boulanger, 2009; Huberman et al., 2008; Shatz, 2009). All of these molecules have been found to promote pruning, and loss of these signals produces a similar under-pruning phenotype, or a lack of normal refinement and formation of eye-specific territories (Huberman et al., 2008). CD47 and CD200 are the first immune molecules shown to yield the opposite phenotype, that of overpruning, or more "mature" eye-specific territories. Mice carrying a mutation in the Down Syndrome critical region (DSCR) are the only other mice known to have an excessively refined dLGN, although, due to the roles of DSCAM in cell spacing and fasciculation, this most likely represents anatomical abnormalities and not overpruning (Blank et al., 2011). The identification of CD47 and CD200 as molecules critical for developmental synaptic pruning further promotes the idea that CNS refinement is analogous to the immune system process of self versus non-self recognition. Complement molecules C1q and C3 are well known "eat me" signals that opsonize material in need of removal from the body. "Don't eat me" signals, such as CD47 and CD200, keep the immune system from generating too strong a reaction and removing self cells. Using what is known from the immune system, one can imagine that CD47 and CD200 are present on synapses that form appropriate connections, while C1q and C3 identify synapses in need of removal. Both sets of molecules signal to microglial receptors, much like they do to macrophages in the immune system, and regulate their phagocytic behavior. This model is supported by *in vitro* data demonstrating that sialic acid, another immune "don't eat me" signal, prevents complement binding and microglial removal of neurites in culture (Linnartz et al., 2012). It is not yet clear whether neuronal pentraxins and MHCI molecules are repurposing their

immune system functions to promote the pruning of unneeded synaptic connections, but as NP1 and NP2 are homologs of PTX3, a C1q regulatory protein, this may be a possibility.

Potential mechanisms underlying activity-dependent synaptic pruning by microglia

Another open question raised by this work is why specific synapses would be targeted for protection or removal. The answer to this mystery of specific synaptic targeting may lie in neuronal activity. Studies of synapse elimination performed at the neuromuscular junction indicate that stronger synaptic inputs "win" the competition for postsynaptic territory, and many studies in the retinogeniculate system demonstrate that spontaneous activity is necessary for the formation of eye-specific territories (McLaughlin et al., 2003; Penn et al., 1998; Sanes and Lichtman, 1999; Shatz and Stryker, 1988; Stellwagen and Shatz, 2002; Torborg and Feller, 2005). We found that microglia preferentially engulf weaker inputs in the dLGN, suggesting that there must be a neuronal activity signal that they can identify (Chapter 3, Schafer et al., 2012). While CD47 and CD200 have never been linked to neuronal activity, unpublished work from our lab indicates that C1q undergoes activity-dependent transcription (Rosen and Stevens, 2011). If C1q is secreted from strong synapses, it may be able to diffuse to and "opsonize" nearby weaker synapses.

There are then two possible ways in which the system can achieve specificity (Figure 4.13). One model relies on C1q and C3 for specificity, in that CD47 and CD200 would be ubiquitously expressed while C1q and C3 would recognize and bind to weak synapses that would then be eliminated by microglia. There is some indication that this may be a prospective mechanism, as data from our lab demonstrate that C1q localizes to less active retinogeniculate synapses in an *in vitro* system for studying activity-dependent

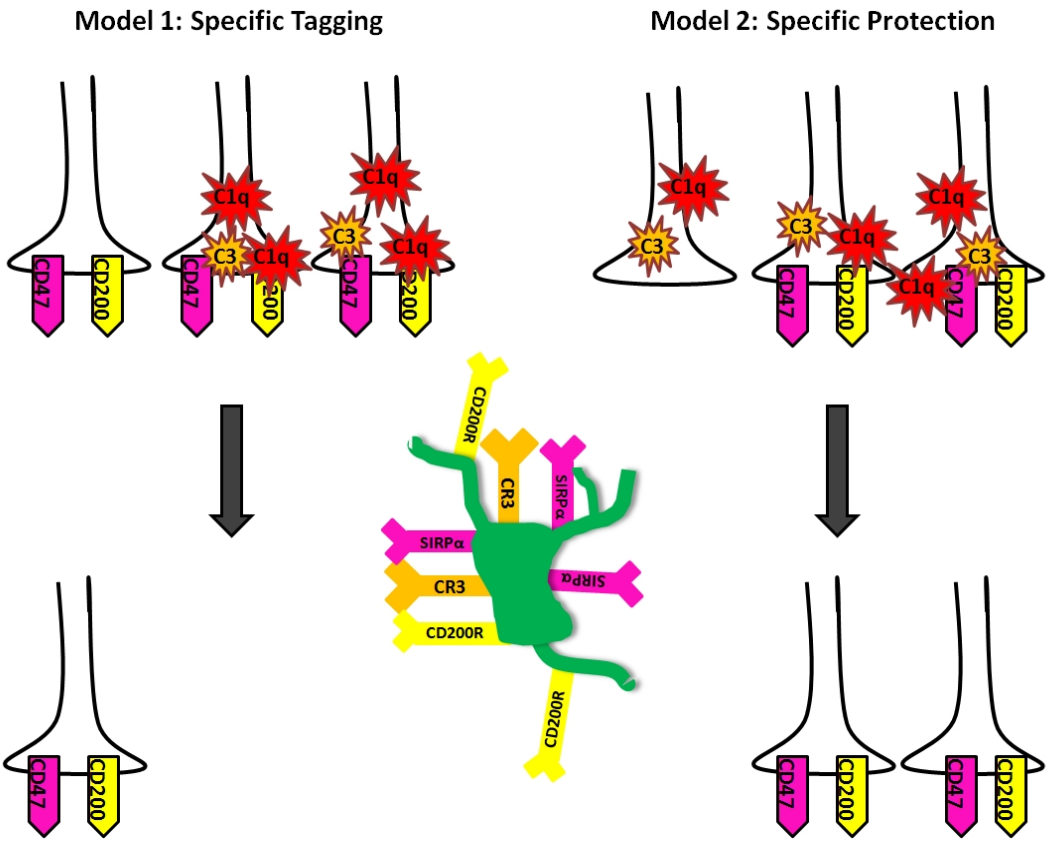

Figure 4.13. Two possible models for specific synapse removal. In the specific tagging model (left), complement (red and orange) opsonizes only those synapses in need of removal, while "don't eat me signals" (pink and yellow) are ubiquitously expressed. Microglia (green) would evaluate levels of complement and "don't eat me signals" using receptors for both types of molecules in deciding which synapses to engulf. In the specific protection model (right) complement contacts many synapses, and only those that do not have a "don't eat me" signal are engulfed. In this model, microglial phagocytosis is inhibited by the presence of "don't eat me" signals.

retinogeniculate refinement (Koyama et al., 2013). This model would require the existence of a receptor or adaptor on weak synapses to localize C1q and C3, and microglia would then have to read out the relative abundance of "eat me" and "don't eat me" signals to determine which synapses to engulf. The other model is based on the idea that, as secreted proteins, C1q and C3 diffuse broadly throughout the neuropil, which would require specific protection of the synapses that must be maintained. How "don't eat me" signals would achieve synaptic specificity is unknown, but as CD47 is lost from membrane subdomains during apoptosis (Gardai et al., 2005), similar signaling mechanisms at weak or inappropriate synapses could lead to selective downregulation or re-localization of CD47. Furthermore, these models are not mutually exclusive, and it is possible that both specific opsonization and specific protection may occur.

Microglial phagocytosis is increased in the absence of molecular "brakes"

Previous work from our lab identified C1q and C3 as positive regulators of microglial phagocytosis in the developing brain, as loss of these molecules reduces microglial engulfment, consistent with their function as "eat me" signals (Bialas and Stevens, 2013; Schafer et al., 2012). However, C1q and C3-deficient microglia still engulf some RGC inputs during the pruning period, indicating that they are not solely responsible for directing microglial phagocytic activity. Continued engulfment in knockouts could suggest the existence of other unidentified "eat me" signals, or indicate that, during this period of development in which microglia are more activated, some element of phagocytosis is constitutive. If activated microglia exhibit a high basal level of non-specific phagocytic activity, mechanisms would be required to ensure they do not blindly engulf

material in their environment, but such mechanisms have not been established during CNS development.

I propose that CD47 serves as a "brake" on excessive phagocytosis. In the absence of CD47, microglia engulf increased RGC inputs during peak pruning (Figure 4.5). Previous studies have shown that CD47KO mice exhibit increased microglial engulfment of material in the CNS, but this has always been observed in the context of disease or injury, in which microglia are known to behave pathologically in other ways (Gitik et al., 2011; Koenigsknecht and Landreth, 2004). This is the first indication that removing the brakes on microglial phagocytosis can alter engulfment in the healthy brain.

CD200 also appears to be a "break" on some aspects of microglial behavior. While loss of CD200 does not produce increased engulfment at P5 (Figure 4.12), it likely regulates microglia in other ways that produce excess pruning, as CD200KOs are known to have increased microglia numbers, microglia activation, and pro-inflammatory signaling (Hoek et al., 2000; Walker and Lue, 2013; Wright et al., 2000). Alterations in these properties could also be pathological during brain development, and future experiments will determine if these abnormalities are observed in CD200KO mice during the pruning period. Microglia are known to have both beneficial and harmful effects during disease, and my data suggest that, while microglia are required for normal CNS development, they can cause harm if their phagocytic behavior is not properly controlled by endogenous inhibitory signals.

CD47 and CD200 may yield the same phenotype via non-redundant mechanisms

CD47 and CD200 are often grouped together as "don't eat me" signals and regarded as functionally similar. However, based on their use of different receptors and the immune

system abnormalities observed in knockout animals, they likely arrive at a similar phenotype via different routes. CD47 is a classic example of a "don't eat me" signal, as it directly inhibits macrophage phagocytosis upon binding to SIRPα (Okazawa et al., 2005; Oldenborg et al., 2001). CD47KO RBCs are rapidly cleared as macrophages no longer identify them as "self" and attempt to remove them from the body (Oldenborg et al., 2000). Unchallenged knockout mice are healthy, and do not exhibit gross alterations in inflammatory or other immune signaling molecules. Alterations in macrophage or microglia number or activation state have not been reported in these mice. In contrast, CD200KO mice exhibit increased expression of pro-inflammatory cytokines as well as increased numbers and activation of microglia (Hoek et al., 2000). Unlike CD47 mice, which are refractory to EAE (Han et al., 2012), CD200KO mice exhibit increased disease severity (Wright et al., 2000). Increased phagocytosis in CD200KO mice has not often been reported, and is likely to be an indirect effect of increased inflammation, rather than direct regulation of microglia.

These differences may influence how CD47 and CD200 regulate synaptic refinement during brain development. The enrichment of CD47 in the dLGN during the pruning period is suggestive of a direct role in phagocytosis (Figure 4.1), indicating that it may bind SIRPα and inhibit engulfment in that specific region during that specific time period. CD200 does not display enrichment, and is instead similarly abundant throughout the brain during the pruning period (Figure 4.11). Additionally, while a deficiency in either molecule produces a similar decrease in overlap between eye-specific territories (Figure 4.7, 4.12), their microglia do not appear to behave in the same way. This suggests that CD200 likely regulates synaptic refinement though a different mechanism than CD47, as microglial

engulfment of RGC inputs was not altered in P5 CD200KO mice (Figure 4.12), in contrast to the increase in engulfment observed in CD47KO microglia (Figure 4.5).

There are several potential mechanisms that could underlie the CD200KO overpruning phenotype and must be explored. First, it will be important to assess cell numbers in these mice, as loss of "don't eat me" signals could lead to excessive self cell removal (Griffiths et al., 2009). If retinal numbers are normal, CD200KO microglia will have to be analyzed closer to when the overpruning phenotype is observed at P10 to determine if engulfment exhibits the expected developmental downregulation at this time and if microglia numbers or activation have changed. CD200 is known to inhibit microglia-derived inflammatory signaling via its binding to CD200R, which promotes CD200R interaction with Dok2, activation of RasGap, and inhibition of downstream Ras activation and inflammatory signaling (Walker and Lue, 2013). I will therefore examine the levels of pro-inflammatory cytokines and chemokines in CD200KO and WT littermates to assess inflammatory signaling in these mice. Recent work indicates that astrocytes are also capable of engulfing RGC inputs during the pruning period in the dLGN (Chung et al., 2013), so I can also investigate whether dysregulated signaling in CD200KOs affects astrocyte-mediated pruning. Regardless of the outcome, it is clear that CD47 and CD200 have different mechanisms of action, and I am currently examining double knockout mice to determine whether the overpruning phenotype is exacerbated by loss of multiple non-redundant protective mechanisms. If loss of both genes has a synergistic effect, it could support the idea that more than one protective mechanism is used to prevent major disruptions to circuit remodeling, and that combined immune dysregulation and increased engulfment produces a more severe phenotype. My work promotes the idea that

protective molecules regulate the removal of "self" material, however, it challenges the notion that these molecules perform this function redundantly.

The CD47-SIRPα interaction may prevent excess engulfment during development

The binding of CD47 to SIRPα on macrophages in the immune system is known to inhibit phagocytosis (Okazawa et al., 2005). Given the similar functions of immune molecules in retinogeniculate refinement and self versus non-self discrimination, I anticipate that CD47 binding to microglial SIRPα inhibits the removal of necessary connections during development. My data indicate that SIRPα is highly expressed in microglia during the pruning period in the dLGN and that microglial SIRPα levels decline as pruning nears completion (Figure 4.10). These results suggest that microglial SIRPα is expressed at the right time and place to inhibit excessive pruning, and future engulfment and eye-segregation experiments with the germline SIRPα knockout mouse will be used to investigate this possibility.

One factor that may complicate these findings is that SIRPα is also expressed by neurons. Previous studies on SIRPα in the CNS have shown that it has the capacity to regulate behavior, in that SIRPα tyrosine phosphorylation affects body temperature regulation and circadian patterns of locomotion in mice (Maruyama et al., 2012; Nakahata et al., 2003). Recent work by our collaborator, Dr. Hisashi Umemori, indicates that SIRPα regulates synaptic maturation in the hippocampus and that impairments occur when it is removed from the brain starting at P15 (Toth et al., 2013). All of these findings have relied on whole brain samples or genetic mouse models in which both microglial and neuronal SIRPα were affected. These studies have made the assumption that neuronal SIRPα is responsible for these effects, however, that is not a foregone conclusion given the role of

microglia in hippocampal engulfment at P15 and the recent studies showing that changes in microglial genes can affect behavior (Chen et al., 2010; Paolicelli et al., 2011; Parkhurst et al., 2013; Rogers et al., 2011; Zhan et al., 2014).

Unfortunately, creating a microglia-specific SIRPα knockout mouse has proven challenging. I obtained the conditional SIRPα floxed mouse from Dr. Umemori and planned to use the recently published CX3CR1-CreER line to excise SIRPα specifically in microglia during the pruning period. Based on his suggestions, I administered tamoxifen to P0 pups, but found that this did not effectively eliminate SIRPα from microglia by P5, and that tamoxifen negatively affected microglia cell health and integrity. More recently, we learned that CX3CR1 is also expressed in a subset of neurons during brain development, and while this is still under investigation, it makes the CX3CR1-CreER line unsuitable for investigating microglia-specific SIRPα function.

To avoid having to use tamoxifen in my experiments, I administered tamoxifen to SIRPα conditional mice crossed to the actin-CreER driver line to remove SIRPα globally and create a germline knockout. While experiments conducted with these mice cannot be used to prove that findings are due to microglial SIRPα, the microglial expression and localization of SIRPα in the dLGN during the time I assay engulfment and eye-specific segregation make it a more likely possibility (Figure 4.9, 4.10). I will also examine microglia in SIRPα KO mice for changes in activation state or cell numbers, which could also affect pruning. Additionally, I can isolate microglia from the global SIRPαKO and examine their phagocytic behavior in culture. Microglial SIRPα has never been studied in the developing brain. Determining that the CD47-SIRPα interaction regulates microglia-

mediated pruning would not only be a novel finding, but would strengthen the ties between immune and nervous system protective mechanisms.

My work demonstrates that immune protective signals are required to prevent excess pruning in the developing retinogeniculate system. Further experimentation is necessary to understand the overpruning phenotypes in mice deficient in "don't eat me" signals CD47 and CD200, however, the identification of these molecules as critical regulators of synaptic refinement and microglial function represents a paradigm shift and may have broad implications. These data indicate that the developing CNS utilizes immune mechanisms to shape neural circuits, and therefore may provide a hint as to other candidate molecules to investigate for involvement in pruning. Additionally, understanding how these and other immune mechanisms shape neural circuit refinement may yield insight into the circuit alterations that occur in disorders characterized by immune dysregulation, such as ASD and schizophrenia. Therefore, future work investigating these and other immune protective signals in the developing CNS may offer not only a more complete picture of synaptic refinement but also therapeutic potential.

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Chapter 5:

Discussion

Synaptic refinement has long been recognized as a critical developmental process required for the removal of unnecessary or inappropriate connections and the establishment of mature neural circuitry. In the past decade, molecules required for refinement have begun to be identified and, surprisingly, given the immune privileged status of the brain, many of them are immune or immune-related molecules (Boulanger, 2009; Hong and Chen, 2011; Shatz, 2009). To determine how molecules involved in innate and adaptive immunity could regulate synaptic circuit development, we turned to the brain's immune cells: microglia. Study of these cells had been restricted to disease or injury models for many years; however, new findings were beginning to demonstrate that microglia play an important role in the development and function of the healthy brain (Hanisch and Kettenmann, 2007; Paolicelli et al., 2011; Ransohoff and Perry, 2009). My findings further emphasize the necessity of microglia for normal brain development and provide a more complete picture of synaptic refinement as well as a new way of conceptualizing brain development.

The data presented in this dissertation indicate that microglial engulfment of synaptic inputs is essential for normal refinement in the retinogeniculate system. Immune molecules communicate with microglia to regulate this process, and deficiencies in microglia-mediated pruning lead to lasting defects in the formation of eye-specific territories and the removal of excess synapses. Importantly, microglial phagocytosis is both developmentally regulated and activity-dependent, which is consistent with the current understanding of pruning (Hua and Smith, 2004; Huberman et al., 2008; Katz and Shatz, 1996; Sanes and Lichtman, 1999). My findings also demonstrate that "don't eat me" signals are required to protect the developing CNS from excess or accelerated pruning, and

indicate that these molecular brakes restrict microglial engulfment and may prevent their behavior from becoming pathological. My work in this area is still ongoing, and I plan to conduct additional experiments to better understand the mechanisms by which "don't eat me" signals impact refinement as well as if the overpruning that results from loss of these signals has behavioral consequences. Many open questions remain, including whether "eat me" and "don't eat me" signals direct pruning throughout the brain and if they are relevant to the immune dysfunction observed in neurodevelopmental or neuropsychiatric diseases. Although we do not yet have all the answers, the data presented in this dissertation indicate that brain development involves a delicate balance between synaptic protection and synaptic pruning, and demonstrate that it shares many similarities with self versus non-self discrimination in the immune system.

Microglia shape synaptic circuits in the normal, healthy brain

Our work defines a critical role for microglia in synaptic circuit refinement in the developing visual system. I found that microglial engulfment of retinal ganglion cell (RGC) inputs is developmentally regulated and occurs during the peak of the pruning period in the dorsal lateral geniculate nucleus of the thalamus (dLGN). Engulfment tapers off as the formation of eye-specific territories nears completion at P9, even though at that time microglia are still relatively activated and capable of phagocytosis. I also observed that microglia preferentially engulf inputs in synaptic regions versus regions containing axon tracts, which suggests that the CTB-labeled material they engulf represents synapses, and that they must be capable of discerning synapses from axons when deciding what to engulf. We also found that microglia are capable of distinguishing weak inputs from strong, and although they will engulf both types of inputs, they display a significant preference for

weak synapses. When microglia are quieted by administration of anti-inflammatory antibiotic minocycline, engulfment is reduced and eye-specific territories fail to form, indicating the importance of microglia-mediated pruning for retinogeniculate refinement.

These studies also identified immune molecules as key regulators of microglial engulfment during development. We identified "eat me" signal C3, a member of the classical complement cascade, and its microglia-specific receptor, CR3, as molecules that promote microglial phagocytosis of synaptic inputs in the dLGN. C3 is localized to the dLGN during peak pruning and reduced by P9, and microglial CR3 is also expressed during early postnatal development and greatly reduced by P20. Mice lacking these molecules exhibit decreased engulfment as well as increased overlap between eye-specific territories in the dLGN and increased numbers of synapses, suggesting that loss of signals that promote phagocytosis leads to incomplete synaptic pruning. I then identified molecules required to protect against excess pruning, "don't eat me" signals CD47 and CD200. In mice deficient in these molecules, eye-specific territories exhibit less overlap than what is normally observed during developmental retinogeniculate refinement, suggesting increased or accelerated pruning. This phenotype appears to result from increased microglial engulfment in CD47KO mice; however, more work is necessary to determine the underlying cause of the phenotype in CD200 deficient animals. The CD47 receptor, SIRPα , is developmentally regulated in microglia in the dLGN during peak pruning, and my future work will investigate whether inhibition of phagocytosis via SIRPα signaling is part of the synaptic protection mechanism. This is the first demonstration that protective signals are required for proper circuit refinement, and together, these studies provide a wealth of new knowledge about microglial function and regulation in the healthy, developing brain.

Do immune molecules and microglia regulate pruning throughout the brain?

While our work identifies microglia as critical cellular mediators of synaptic circuit refinement in the retinogeniculate system, we do not yet know if this extends to pruning in other brain regions. Interestingly, recent studies from other groups suggest that circuit refinement by microglia may indeed be a broadly utilized strategy for circuit maturation and modification. Microglia have been directly observed engulfing synaptic material in the hippocampus during the time associated with refinement and maturation in this region, and mice lacking microglia-specific fractalkine receptor CX3CR1 have fewer microglia in the hippocampus during development and delayed synaptic maturation (Paolicelli et al., 2011). The mechanism underlying hippocampal synaptic engulfment is not yet known, and one possibility is that complement and fractalkine work together, with fractalkine recruiting microglia through CX3CR1 and complement binding to synapses in need of removal. SIRPα was also shown to be required for hippocampal synapse maturation during this same developmental window (Toth et al., 2013). Although this defect is assumed to result from loss of neuronal SIRPα, contributions from microglia cannot be ruled out since a global actin-CreER driver line was used to remove this gene. If my experiments using the global SIRPα knockout mouse reveal increased phagocytosis of RGC inputs during peak pruning, future collaborative work with Dr. Umemori can focus on separating the effects of neuronal and microglial SIRPα in the hippocampus.

The hippocampus is not the only other region in which microglia regulate synapse maturation. Defects in synapse maturation in the CX3CR1 knockout mouse have also been observed in the barrel cortex (Hoshiko et al., 2012). Similar to observations in the hippocampus, loss of CX3CR1 delays microglial recruitment to this region, which in turn,

seems to impair functional maturation of postsynaptic glutamate receptors. Whether or not this phenotype is related to alterations in phagocytosis remains to be explored. Another stereotyped pruning process occurs in the cerebellum at the climbing fiber-purkinje cell synapse (Hashimoto and Kano, 2005). Microglia have not yet been implicated in pruning in this region, but the striking, developmentally regulated localization of CD47 in the cerebellum, which parallels the movement of climbing fiber synapses as they travel from purkinje cell bodies to dendrites, suggests that this may be a possibility (Figure4.2A). I am currently examining cerebellar pruning in CD47KO animals. If CD47 appears to regulate pruning in this region, it would indicate that microglial phagocytosis and protective signals may be hallmarks of normal brain development.

Microglia as activity sensors

We now know that microglia are capable of sensing and responding to neural activity during development, in that they display preferential engulfment of weak synapses during peak pruning. However, neural activity underlies a multitude of circuit-level changes that occur throughout the brain, including synaptic plasticity and homeostasis (Greer and Greenberg, 2008). The ability of microglia to alter their behavior in response to neural activity could place them at the center of a variety of processes, and it is clear that microglia retain their sensor ability in the adult brain, where they continuously monitor their environment with active filopodial movement that is reduced by TTX application and lowered body temperature (Davalos et al., 2005; Nimmerjahn et al., 2005; Wake et al., 2009). Microglia also transiently contact spines and synapses, and although the function of this spine contact is not yet clear, it does appear to be activity dependent. If young, light reared mice are placed into the dark during the visual critical period, microglial contact

with spines is altered in V1, in that microglia appear to contact larger spines and contact interfaces cover more area (Tremblay et al., 2010). If mice are then re-exposed to light for two days, microglia exhibit more phagocytic inclusions, which may represent engulfment of synaptic material. This indicates that microglia, and potentially microglial engulfment, may play a role in activity dependent synaptic remodeling throughout the brain.

The activity sensor properties of microglia may not always lead to engulfment. As immune cells, microglia are also capable of secreting cytokines and chemokines and initiating an inflammatory response (Ben Achour and Pascual, 2010; Wyss-Coray and Rogers, 2012). Glial-derived TNF-α has been shown to be required for homeostatic synaptic scaling, although this cytokine can be secreted from both astrocytes and microglia and more needs to be done to identify the source (Beattie et al., 2002; Stellwagen and Malenka, 2006). Work using cultured microglia has demonstrated that they can affect neural activity, and that they do so by secreting glycine and L-serine into the culture medium (Hayashi et al., 2006; Moriguchi et al., 2003). Additionally, activation of cultured microglia with lipopolysaccharide (LPS) has been shown to indirectly increase AMPAR-mediated spontaneous EPSCs in the CA1 of hippocampal acute slice via astrocyte intermediaries (Pascual et al., 2011), although it is unclear if this occurs in a non-inflammatory context.

Microglia clearly sense and respond to neural activity, however, the mechanisms employed to communicate activity-related signals to microglia are not yet known. For surveillance functions or the modulation of neural activity via secreted signals, microglia may make use of the many receptors they express to respond directly to neurotransmitters or neuromodulators (Kettenmann et al., 2011). Recent work from our lab indicates that

visually-evoked activity can also regulate microglial gene expression, including genes involved in motility and the cellular mechanics of engulfment (Schafer et al., 2013). However, for functions involving phagocytosis, their more macrophage-like behaviors would need to be engaged, suggesting that some pro- or anti-phagocytosis cues must be activity regulated to direct activity-dependent engulfment behavior, such as the engulfment of weaker synapses during retinogeniculate refinement. One possibility is that pro-phagocytosis signals like "eat me" signals might specifically bind to weak synapses. Alternatively, anti-phagocytosis "don't eat me" signals could be specifically downregulated by or re-localized away from weak synapses, or upregulated by or more highly concentrated on strong synapses. Then again, local activity changes could regulate receptors on nearby microglial processes, and thereby affect phagocytosis. The regulation of these molecules could resemble that described in the punishment model (Figure 1.1, Lichtman and Colman, 2000), with complement molecules serving as the secreted punishment signals that bind weak or asynchronously firing synapses. "Don't eat me" signals could then act as the local protective mechanism, and could either be lost from weak synapses or increased on strong synapses to regulate refinement.

Future work can be directed toward determining whether the "eat me" and "don't eat me" signals identified as regulators of microglial engulfment during development instruct microglia to prune weaker synapses. The *in vivo* competition assay we used to determine that microglia preferentially phagocytose weaker synapses in the dLGN can be performed in knockout mice to investigate whether microglia retain that preference. If they do not, the missing signal may be critical for microglia to discern neural activity levels. Our lab has also developed a co-culture model of the retinogeniculate system to better

visualize and study the molecular mechanisms regulating activity-dependent synaptic refinement (Koyama et al., 2013). I plan to use this system to determine whether CD47 is specifically down- or upregulated on individual synapses known to be weak or strong, respectively. Microglia-spine contact can also be assessed after dark adaptation in mice deficient in "eat me" or "don't eat me" signals to determine whether the presumably activity-dependent contacts reported in the cortex are altered in the absence of these molecules (Tremblay et al., 2010). Identifying the molecules that transmit phagocytosis-related neural activity information to microglia will provide additional insight into mechanisms underlying microglia-mediated pruning during development, and into conditions in which pathological neural activity may be involved, such as neurodegenerative disease.

Microglia and astrocytes: partners in pruning?

Recent work indicates that microglia are not the only cells capable of engulfing RGC inputs during the retinogeniculate pruning period. A new study from the Barres lab demonstrates that immature astrocytes also engulf synaptic inputs in an activity-dependent manner, and that genetic disruption of this function has lasting consequences for retinogeniculate refinement (Chung et al., 2013). Astrocytes are not motile and do not engulf as much material as microglia when examined on a cell-to-cell level, but as there are more immature astrocytes than microglia during the pruning period, they may still make a substantial contribution to pruning. Interestingly, astrocytes rely on conserved phagocytic receptors to engulf RGC inputs, MERTK and MEGF10, and orthologs of MEGF10 have been shown to regulate axon pruning by *Drosophila* glial cells (Draper) and phagocytosis of apoptotic cells in *C. elegans* (CED-1) (MacDonald et al., 2006; Zhou et al., 2001; Ziegenfuss

et al., 2008). This mechanism is complement independent, suggesting that distinct mechanisms regulate pruning in astrocytes and microglia. However, what is not yet established is whether microglia and astrocytes work together to shape synaptic circuits and whether one type of cell can compensate if pruning by the other is impaired.

The astrocyte pruning study provides a small hint that pruning between the two cells may be coordinated, in that microglial pruning is transiently increased in P5 *Mertk-/-* mice that have reduced astrocyte engulfment (Chung et al., 2013). MERTK is expressed by both cell types, and how MERTK-deficiency could produce different effects in these two types of cells is still unknown. Although this observation requires further substantiation, coordinated pruning between microglia and astrocytes could be responsible for the relatively small magnitude of the overpruning defect in mice deficient in "don't eat me signals." An intriguing question for future investigation is whether astrocyte pruning is reduced when microglial pruning is increased in CD47KO mice. Additionally, given that astrocyte phagocytosis has been reported to increase with inflammatory stimuli or in inflammatory diseases (Kalmar et al., 2001; Morcos et al., 2003), it is also possible that astrocyte engulfment may be altered in CD200KO mice if they have enhanced pro-inflammatory signaling, which is one of the mechanisms I plan to explore as a possible cause of CD200KO overpruning.

Parallels between nervous system and immune system function

The vast majority of molecules recently identified as regulators of developmental synaptic pruning have been immune or immune-related, including members of the classical complement cascade (Boulanger and Shatz, 2004; Hong and Chen, 2011; Shatz, 2009). All of these molecules have a similar role in promoting normal pruning, and loss of these

molecules impedes removal of overlapping and supernumerary connections in the dLGN (Bjartmar et al., 2006; Datwani et al., 2009; Huberman et al., 2008; Huh et al., 2000; Stevens et al., 2007). In this study, I have, for the first time, identified immune molecules required to inhibit pruning, "don't eat me signals" CD47 and CD200, that appear to serve as molecular brakes to prevent pruning from proceeding too quickly or removing too many inputs. This discovery enhances our understanding of brain development, and suggests that protective mechanisms are required to ensure that circuits form normally. These molecules, as well as "eat me" signals C1q and C3, regulate microglia, the myeloid-derived immune cells of the brain, by binding microglial receptors, including SIRPα and CR3 (Griffiths et al., 2007). Taken together, these findings present a new model of synaptic refinement, one that shares many common mechanisms with self versus non-self discrimination in the immune system.

The major task facing the immune system is the removal or destruction of apoptotic cells and pathogenic or non-self material to prevent harm to the body. However, the immune system has to complete this task while avoiding two potential negative consequences: the accidental removal of self material and an excessive inflammatory response (Elward and Gasque, 2003; Grimsley and Ravichandran, 2003). To successfully achieve this balance, two types of molecular cues are needed. "Eat me" signals are expressed by or bound to only the cells or debris in need of removal. Phagocytes possess receptors for these signals, which can be membrane bound or secreted, and use them to identify material that must be engulfed (Hochreiter-Hufford and Ravichandran, 2013). "Don't eat me" signals are markers of self, and are ubiquitously expressed by self cells and not pathogens, and downregulated or lost from cells once they become apoptotic or

unhealthy (Gardai et al., 2006; Griffiths et al., 2007). Not only do these signals inhibit inappropriate removal by communicating with receptors on phagocytes, some of them also negatively regulate the inflammatory response (Walker and Lue, 2013). Failure of "eat me" signals can lead to illness, while deficiencies in "don't eat me" signals can cause increased susceptibility to autoimmune diseases (Wright et al., 2000).

We have now identified classic "eat me" and "don't eat me" signals as key regulators of microglia-mediated pruning. Their function appears to be highly conserved, and if we consider inappropriate connections analogous to non-self and necessary connections similar to self, we see that microglia are performing the same discrimination task as their immune system counterparts. My discovery of the involvement of "don't eat me" signals provides a key missing piece necessary to complete this analogy, and paves the way for further exploration of the role of immune molecules in synaptic refinement. The molecules we have identified are only a subset of those involved in the self versus non-self discrimination task in the immune system (Griffiths et al., 2007; Hochreiter-Hufford and Ravichandran, 2013), and a major open question is whether other immune molecules also regulate microglia-mediated pruning.

Do other immune and immune regulatory molecules influence pruning?

The immune system employs a large number of cellular signaling mechanisms to ensure that the removal of harmful material occurs with minimal non-specific removal of self cells (Elward and Gasque, 2003). Given the striking similarities between this process and microglia-mediated synaptic refinement in the dLGN, it seems likely that other immune molecules may also regulate pruning during brain development. Identifying such molecules may seem like a daunting task in light of the complexity of the immune system,

but candidates can be chosen based on the "eat me" and "don't eat me" model of microglia regulation. To qualify as a good candidate, an immune molecule must function within the context of the signals and cells we have already identified as mediators of phagocytosis. Two classes of molecules emerge as sources of candidates: complement regulators and "find me" signals.

The complement cascade functions as part of the innate immune system to target pathogens or debris for cell lysis or removal by phagocytes. A variety of molecules act to either enhance or inhibit the complement cascade during apoptosis and the elimination of non-self material (Kim and Song, 2006). Of these, a subset are expressed in the CNS and may serve as candidates to regulate microglia-mediated pruning. One such candidate is CD55 or Decay accelerating factor (DAF), a GPI-linked complement inhibitor that acts at the level of C3. Expressed by neurons, DAF has been shown to be upregulated in diseases associated with complement activation, such as experimental autoimmune encephalomyelitis (EAE), and can protect against complement opsonization when ectopically expressed (van Beek et al., 2005). DAF upregulation has also been observed in neural crest-derived cell lines during pathfinding and axon outgrowth and shown to protect against complement-mediated lysis (Zhang et al., 1998). While it has not been studied *in vivo* during brain development, its neuronal localization and inhibition of C3 make it an interesting candidate. Another interesting complement regulatory molecule is the recently identified CSMD1 (Lau and Scholnick, 2003). CSMD1 is highly expressed in the developing CNS and appears to inhibit the classical complement cascade by preventing C3 deposition and causing degradation of its proteolytic product, C3b. This makes it an interesting candidate protective signal as it could prevent "eat me signal" opsonization

(Escudero-Esparza et al., 2013; Kraus et al., 2006). Finally, there is some evidence to suggest that well-known neural adhesion and recognition molecules may also behave as immune regulatory molecules (Tian et al., 2009). For instance, Sema-7A has been shown to play an important role in the negative regulation of T cell activation (Czopik et al., 2006), further supporting the idea that the CNS and immune system share molecules and suggesting that perhaps canonical neuronal signaling mechanisms can have immune-like functions. The fact that semaphorins have been implicated in axonal pruning in the hippocampus (Bagri et al., 2003), a region in which microglial engulfment has also been demonstrated (Paolicelli et al., 2011), makes this an intriguing possibility.

Another interesting, but poorly understood, class of molecules is the "find me" or "come get me" signals. These molecules are thought to be secreted from apoptotic cells to attract macrophages (Grimsley and Ravichandran, 2003; Ravichandran, 2011), and given the similarities of macrophage engulfment of dying cells to microglial engulfment of inappropriate synapses, it would be interesting to see if these molecules function analogously in the developing CNS. Thus far, only a handful of molecules have been described as "find me" signals in mammalian cells: lysophosphatidylcholine (LPC), sphingosine 1-phosphate (S1P), a fragment of fractalkine, and ATP, all of which are found in the CNS. Interestingly, fractalkine and ATP may exert some "find me" like effects on microglia, as fractalkine receptor knockouts have fewer microglia during development and ATP is necessary for microglia to extend processes and move toward sites of injury (Davalos et al., 2005; Hoshiko et al., 2012; Paolicelli et al., 2011). Additionally, an S1P receptor modulator that reduces microglial activation is used as a treatment for relapsing

Multiple Sclerosis (MS), although it is unclear if its effects are due to blockade of S1P "find me" signaling (Jackson et al., 2011; Noda et al., 2013).

If we extend the analogy of microglia-mediated pruning of inappropriate connections to the removal of apoptotic cells, another interesting candidate comes to the fore. Phosphatidylserine (PS) is exposed on the outer surface of apoptotic cells, and allows for identification by phagocytes (Grimsley and Ravichandran, 2003). In the immune system, CD47 normally covers the entire cell surface, but, during apoptosis, its expression is reduced or re-localized into patches, revealing PS in CD47-negative regions (Gardai et al., 2005). However, the mechanism underlying this interesting re-localization behavior is not yet known. One area for future study in our system is whether PS exposure is increased in CD47KOs, or whether weaker synapses in either our *in vitro* or *in vivo* competition assay lose CD47 and are associated with PS externalization (Koyama et al., 2013; Schafer et al., 2012). A better understanding of the interaction between these two molecules could shed light on CD47 function and regulation during the pruning period.

Consequences of microglial dysfunction in the normal and inflamed brain

As interest has grown in understanding microglial function in the healthy brain, microglia have been implicated in what seems like an ever-increasing number of processes. Therefore, it is unsurprising that disruption of these cells, which we have already shown has consequences for circuit refinement, also produces behavioral alterations. Furthermore, there is a growing body of evidence linking microglia to both neurodegenerative diseases and neurodevelopmental disorders, and microglial abnormalities may be related to the inflammation or other immune-related changes that occur in the brain during these diseases (Frick et al., 2013; Wyss-Coray and Rogers, 2012).

Thus, as we learn more about the immune molecules regulating microglia-mediated pruning, we are likely to uncover molecular mechanisms that are also disrupted in disease.

Defects in microglial function influence behavior

Given that microglia are now recognized as participants in neural circuit formation and modulation, it follows that mice harboring microglial alterations display abnormal behavior. Normal microglial function may be required for proper learning, as mice carrying only a single copy of microglial receptor CX3CR1 exhibit deficiencies in memory, motor learning, and contextual fear conditioning (Rogers et al., 2011). However, this may vary based on experimental paradigm as improved learning has also been observed using this strain (Maggi et al., 2011). Mice in which microglia have been completely eliminated using genetic ablation also display defects in motor learning (Parkhurst et al., 2013). Excessive grooming has been observed in mice carrying microglia-specific genetic lesions and mice missing both copies of CX3CR1 display reductions in functional connectivity between brain regions and impaired social interactions (Chen et al., 2010; Zhan et al., 2014). Microglia may also play a role in establishing baseline sex-specific behavior, as microglia in male and female rats display different levels of activation, and altering activation levels pharmacologically produces behavior more typical of the opposite sex (Lenz et al., 2013; Schwarz et al., 2012). Of the microglial genes that have been linked to behavior, CX3CR1 has direct ties to engulfment, suggesting that behavioral changes in knockout mice may result from reduced microglia-mediated refinement or maturation.

There may even be some evidence linking "eat me" and "don't eat me" signals to behavioral changes. C1q knockout mice have been found to have seizures, a result that may imply the presence of excess synapses in other brain regions (Chu et al., 2010). This result

suggests that “eat me” signals could be required to promote microglial engulfment of unnecessary synaptic connections throughout the brain. Moreover, aged C1q and C3 knockouts perform better than aged WT mice in certain cognitive and memory tasks, a possible indication that reduced engulfment could lead to less synapse loss or neurocognitive decline later in life (Shi et al., 2012; Stephan et al., 2013). Additionally, “don’t eat me” signal CD47 may be required for normal learning. A series of studies indicated that CD47 is upregulated in the hippocampus during memory consolidation, and that blocking CD47 with antibody or complimentary antisense oligonucleotide injection into the dentate gyrus impairs memory retention in a one-way inhibitory avoidance learning task (Chang et al., 2001; Huang et al., 1998; Lee et al., 2000). While these results do not necessarily implicate microglia in this process, especially since CD47 is known to bind neuronal SIRPα as well as other ligands (Brown and Frazier, 2001), it is possible to speculate that the CD47 upregulation that occurs during memory consolidation helps protect newly formed connections from microglial removal.

To better understand how loss of CD47-dependent synaptic protection affects behavior, studies would have to be performed either very early, at a time point in which SIRPα is primarily microglial, or based on visual system function, as this is a system in which CD47 signaling to microglia appears critical. I plan to perform behavioral analysis on CD47KO mice, and appropriate tests given the aforementioned stipulations include measuring ultrasonic vocalizations (USVs) in young pups and examining visual function via the optomotor and visual water maze assays in adults. While defects in these behavioral assays would not conclusively prove that it is the protective function of CD47 that is required for normal behavior, they could suggest that excessive or accelerated pruning

influences behavioral output. This possibility could be investigated by examining the same behaviors in other mice with similar overpruning phenotypes, like CD200KOs, and potentially the global SIRPα knockout mouse. Given the behavioral changes observed in mice lacking other microglia-pruning related genes, it seems likely that the excess pruning observed in CD47KO mice would have behavioral consequences.

What are the consequences of an overpruned brain?

Most of the studies described in the previous section depict behavioral alterations that occur when microglia are not doing their job, either due to their absence or due to loss of function in genes known to drive normal microglial function. One major open question is what happens when microglia are doing their job a little too well? This appears to be the case in CD47KO mice, as microglial engulfment of synaptic inputs is increased in the absence of the necessary molecular "brakes". This indicates that continuous inhibitory "self" signaling is required to ensure proper circuit formation, and that, in its absence, microglia may not be able to correctly differentiate appropriate versus inappropriate synaptic connections. Additionally, as CD47 appears to be localized along the entire cell membrane, loss of these signals may lead to the engulfment of more than just synapses, such as microglial phagocytosis of axons. I am currently examining phagocytosis in the CD47 knockout optic tract, a non-synaptic region bordering the dLGN, to determine whether microglia engulf more material in this region. Our data demonstrate that microglia phagocytose far less material in this region in wild type mice (Chapter 3, Schafer et al., 2012), however, my preliminary observations indicate that this may not hold true in CD47 knockouts. Inappropriate engulfment of axons could have far-reaching effects on neural circuitry, particularly if it occurs in a relay center like the thalamus.

My results indicate that loss of protective signals leads to increased pruning or accelerated development in the retinogeniculate system, and future experiments will determine whether this applies more globally. If these protective mechanisms are broadly employed by a variety of brain regions to prevent excess synapse removal, as our preliminary data from the striatum suggest, then loss of these molecules could have extensive consequences for circuit formation. Furthermore, many events in circuit development are precisely timed with critical developmental windows (Hensch, 2004; LeBlanc and Fagiolini, 2011), so if increased pruning causes a circuit to reach its mature state prematurely, this could affect function and influence the development of connected circuits in other brain regions. Whether loss of protective signals affects regional connectivity or overall brain function is a major open question for future study.

While the effects of loss of "don't eat me" signals on brain function remain to be explored, studies in mouse models and human patients indicate that altered connectivity may play a role in neurodevelopmental and neuropsychiatric disorders. Studies on autism spectrum disorder (ASD) have yielded mixed findings, with some indicating hypo-connectivity in fMRI studies or mouse models, while others suggest hyper-connectivity (Belmonte et al., 2004; Tye and Bolton, 2013). Still other studies propose an initial overgrowth of connections followed by a period of pruning back (Courchesne et al., 2007). The data on schizophrenia are more cohesive, with many groups characterizing the disease as one of disconnectivity (Keshavan et al., 1994; Schmitt et al., 2011). I plan to determine whether increased pruning could lead to autistic or schizophrenic-like behaviors in CD47 or CD200 knockout mice by performing behavioral testing. Interestingly, a very recent study characterized CD47KO mouse behavior and demonstrated that knockouts display

defects associated with schizophrenia, including impaired prepulse inhibition and social interactions (Koshimizu et al., 2014). While the authors do not propose a mechanism to explain these defects, it is possible that loss of protective signals and increased pruning could be an underlying cause. Further behavioral analysis of these mice and mice with related overpruning defects is required to determine whether loss of "don't eat me" signals could be a possible mechanism underlying ASD or schizophrenia.

Immune dysregulation and neurodevelopmental disorders

In support of a potential role for "don't eat me signals" in these disorders, recent studies indicate that immune dysregulation may be a key feature of autism and schizophrenia (Goines and Van de Water, 2010; Vargas et al., 2005). Microarray analyses of postmortem ASD tissue samples revealed an enrichment of genes related to immunity and inflammation as well as to microglia activation (Voineagu et al., 2011). Additionally, unpublished data from the same group indicate that "don't eat me" signals, including CD200 and its receptor CD200R, are significantly downregulated in ASD brains (Dan Geschwind, personal communication) and that "eat me" signals, such as C1q, are upregulated. We plan to collaborate with the Geschwind lab to examine CD47 and CD200 localization in control and ASD human tissue. Many studies of schizophrenia have also revealed an increase in inflammatory cytokines as well as microglial activation, and some antipsychotics have been suggested in *in vitro* studies to have anti-inflammatory effects (Frick et al., 2013; Kneeland and Fatemi, 2013; Monji et al., 2013). Minocycline, a broad anti-inflammatory agent, has even shown some success as a treatment for schizophrenia (Dean et al., 2012). Intriguingly, CD200 and CD47 were altered in a recent study that used the maternal immune challenge model (MIA) of schizophrenia, in which pregnant dams are

prenatally injected with Poly IC, a synthetic double stranded RNA analog that mimics viral infection, to produce offspring with ASD and schizophrenia-relevant behavioral phenotypes (Meyer, 2013). The data from this study suggest that CD47, CD200, and their receptors may be dsyregulated in Poly IC treated mice and that stress can further alter the expression of these molecules (Giovanoli et al., 2013). Prenatal inflammation alone appears to increase the expression of these genes, while the addition of peripubertal stress decreases the expression of CD47, CD200, and their receptors. While it is unclear what overexpression of "don't eat me" signals could represent, data from cancer studies indicate that ectopic expression of CD47 by cancer cells prevents removal by phagocytes (Jaiswal et al., 2009), so perhaps increased expression of "don't eat me" signals in the CNS could inhibit normal pruning.

This intriguing finding lends itself to the idea that a "double hit" of genetic susceptibility plus environmental insult may underlie the development of neurodevelopmental or neuropsychiatric disorders (Eapen, 2011; Goines and Ashwood, 2013). Using CD47 as an example, this model would suggest that loss of this molecule causes alterations in brain development that may not produce recognizable behavioral defects on their own, but that, combined with prenatal inflammation or postnatal stress, would synergize with the dysregulation of other immune molecules to produce more severe defects. This hypothesis is easily testable by performing MIA on CD47 or CD200KOs or exposing these animals to postnatal stress and assessing behavior compared to naïve knockout animals. If these treatments lead to behavioral defects, treated animals can be examined for changes in microglia-mediated pruning as well as for other microglial

alterations to determine whether immune dysregulation leads to additional engulfment or microglia activation in these "unprotected" mice.

However, it is also possible that loss of protective signals may lead to immune dysregulation directly, as these signals normally prevent the release of pro-inflammatory cytokines by microglia (Barclay et al., 2002; Walker and Lue, 2013). I plan to examine the levels of cytokines, complement, and other pro-inflammatory molecules in CD47 and CD200 knockout brains to determine whether inflammation is altered compared to wild type. This could underlie changes in microglial phagocytosis and could be related to the immune dysregulation often observed in autism and schizophrenia.

There is already some data to suggest that dysregulation of immune molecules regulating complement or microglia may lead to autistic or schizophrenic-like symptoms. Mice lacking CSMD1, a likely inhibitor of the classical complement cascade, display behavioral alterations consistent with neuropsychiatric disorders (Steen et al., 2013). As CSMD1 was identified as a susceptibility gene in a genome wide association study (GWAS) study of schizophrenic patients (Havik et al., 2011), these data suggest that loss of immune regulatory signals that inhibit "eat me signals" implicated in microglia-mediated pruning could lead to schizophrenia. Furthermore, mice lacking the fractalkine receptor, which have been previously found to have microglial pruning defects, also appear to exhibit changes in functional brain connectivity and autistic-like behaviors (Zhan et al., 2014). This study also implicates disrupted pruning in autism, and identifies the brain's immune cells, microglia, as key players. Taken together, these data suggest that immune dysregulation that affects microglial pruning may underlie the development of neurodevelopmental or neuropsychiatric disorders.

Microglia-mediated pruning may be aberrantly reactivated in neurodegenerative disease

One interesting commonality among the "eat me" and "don't eat me" signals we have identified as regulators of microglia-mediated pruning during development is the growing evidence of their involvement in neurodegenerative disease. "Eat me" signals, including C1q and C3, are known to be upregulated in the late stage of various diseases, including Alzheimer's disease (Afagh et al., 1996; Gasque et al., 2000; Stephan et al., 2012). Inflammation is also increased during these diseases, although it is currently unclear whether the increase in complement levels is a cause or consequence of inflammation. Conversely, "don't eat me" signals, including CD47 and CD200, are reduced in a variety of neurodegenerative diseases, including Multiple Sclerosis (MS) and Parkinson's disease (PD) (Koning et al., 2007; Zhang et al., 2011). CD200 is known for its role as an inhibitor of inflammation, so loss of this molecule may play a part in the initiation or continuation of inflammation in disease. Finally, microglial activation has been observed in these diseases as well (Dheen et al., 2007; Perry et al., 2010), meaning the cells are more phagocytic and likely secreting pro-inflammatory cytokines. The combined dysregulation of "eat me" and "don't eat me" signals would facilitate a favorable environment for phagocytosis.

The concomitant upregulation of "eat me" signals and downregulation of "don't eat me" signals alongside growing evidence that synapse loss is one of the earliest events in many neurodegenerative diseases, such as Alzheimer's (AD) and Huntington's disease (HD), leads to an interesting hypothesis (DiProspero et al., 2004; Scheff and Price, 2003). Perhaps a shared mechanism among these diseases is an aberrant reactivation of the developmental pruning program in the absence of molecular brakes (Stephan et al., 2012). It is unclear whether "eat me" signal upregulation or "don't eat me" signal downregulation

occurs first, but it would be possible to determine the order of these events by examining the expression and localization of these molecules in mouse models of AD and HD early in disease progression. Given that loss of "don't eat me" signals would remove the constitutive inhibition of inflammation, it seems reasonable that loss of these signals would occur first. Once this protective shield has been removed, inappropriate opsonization of neurons, myelin, or synapses by complement could lead to phagocytosis by microglia.

This model yields testable therapeutic possibilities. If loss of "don't eat me" signals appears to be one of the earliest pathological events in mouse models of AD or HD, we can attempt to restore these molecules via viral delivery and assess whether synapse loss or disease progression is improved. Alternatively, if "eat me" signal upregulation appears to be the initiating factor, we can attempt to lessen complement in disease models by crossing mice to complement knockouts or administering secreted complement inhibitory molecules, such as factor H. We can then perform similar analysis of synapse numbers and disease progression to determine whether decreasing "eat me" signals might have therapeutic potential. Finally, we can target microglia themselves using minocycline injection, which will reduce microglial activation, but can also have broad anti-inflammatory effects in the CNS and throughout the body. Interestingly, minocycline has been reported to have a beneficial effect on some neurodegenerative diseases, such as HD and AD, although it is unclear whether this is due to a decrease in microglial phagocytosis (Kim and Suh, 2009). Employing our engulfment assay to investigate the effects of minocycline on phagocytosis in mouse models of these diseases would be a good first step in determining whether inappropriate microglial "pruning" contributes to pathology,

although better tools need to be developed to more specifically inhibit microglial phagocytosis for future experiments.

Conclusion

My work adds to the growing body of evidence demonstrating that microglia dynamically regulate synaptic circuit formation in the developing brain (Schafer and Stevens, 2013; Wake et al., 2013). Their phagocytic function plays a critical role in synaptic refinement, indicating that engulfment of material in the CNS is a feature of normal brain development and not restricted to injury or disease, although it may bear some mechanistic similarities to more pathological phagocytosis. Importantly, my identification of "don't eat me" signals CD47 and CD200 as protective molecules that prevent excess pruning adds depth to our understanding of both retinogeniculate refinement and microglial regulation. These studies highlight the notion that synapse refinement is not only about the removal of inappropriate connections, but also about the protection of necessary connections that are to be maintained. This appears to be accomplished by a system of molecular checks and balances in which well-known immune molecules guide microglial engulfment. In this way, synaptic refinement bears a striking resemblance to self versus non-self discrimination by phagocytes in the immune system, and conceptualizing this developmental process as an immune system analog may lead to the identification of additional immune regulatory molecules involved in pruning.

These molecules converge on microglia to either encourage or discourage their engulfment of synaptic material. While this is essential for proper pruning, my results demonstrate that, if left unchecked, phagocytosis during development can easily become pathological. Therefore, "don't eat me" signals serve as necessary molecular brakes to

prevent uncontrolled microglial engulfment, similar to the prevention of bystander damage in the immune system. As these signals are also known for their inhibition of microglial inflammatory signaling, it is possible that they also prevent cytokine release during brain development, and my future studies will examine whether knockout brains exhibit increased inflammation. This could potentially create a positive feedback loop that shifts the balance in favor of phagocytosis if "eat me" signals are among the molecules upregulated when "don't eat me" signals are lost. Investigating the relationship between these signals will enhance our understanding of the regulation of microglia-mediated pruning.

A major open question left unanswered by this work is how "eat me" and "don't eat me" signals correctly identify synapses in need of removal and those that must be maintained. This is a challenging question, and one that hinges upon determining whether "eat me" or "don't eat me" signals are activity regulated, and thereby capable of being localized to or lost from weak synapses. Another important issue in need of resolution to answer this question is whether microglial phagocytosis is based on the relative abundance of "eat me" and "don't eat me" signals expressed on a cellular target, or whether the presence of any "don't eat me" signals serves as a molecular override. Studies using cultured cells and phagocytes indicate that the former may be the case (Oldenborg et al., 2001), which suggests that microglia may be capable of making fairly complex decisions. Given the repeated appearance of microglia and "eat me" and "don't eat me" signals in numerous disease models and patient samples, determining the answers to these questions will greatly enhance our understanding of CNS development and may have therapeutic

potential for a variety of neurodevelopmental, neuropsychiatric, and neurodegenerative diseases.

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